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Original Article

Bromodomain 4 protein is a predictor of survival for urothelial carcinoma of bladder

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Abstract: Background: Bromodomain 4 (BRD4) protein is a double bromodomain-containing protein that binds preferentially to acetylated chromatins. BRD4 is essential for cellular growth and has been implicated in cell cycle control, DNA replication and carcinogenesis. However, its expression profile and prognostic value in urothelial carcinoma of the bladder (UCB) have not been investigated. Methods: Real-time quantitative PCR (qRT-PCR) and Western blot were used to explore BRD4 expression in UCBs and normal bladder tissues. Moreover immunohistochemistry (ICH) was used to detect the expression of BRD4 in UCBs. Spearman's rank correlation, Kaplan-Meier plots and Cox proportional hazards regression model were used to analyze the data. Results: Up-regulated expression of BRD4 mRNA and protein was observed in the majority of UCBs by qRT-PCR and Western blot when compared with their paired normal bladder tissues. Clinicopathological analysis was showed a significant correlation existed between the higher expression of BRD4 protein with the histological grade, lymph node metastasis and distant metastasis ($P < 0.05$); Survival analysis by Kaplan-Meier survival curve and log-rank test demonstrated that elevated BRD4 expression in bladder cancer tissue predicted poorer overall survival (OS) compared with group in lower expression. Notably, multivariate analyses by Cox's proportional hazard model revealed that expression of BRD4 was an independent prognostic factor in UCB. Conclusions: These results suggest that the aberrant expression of BRD4 in human UCB is possibly involved in the tumorigenesis and development, and the BRD4 protein could act as a potential biomarker for prognosis assessment of bladder cancer. Further studies on the cellular functions of BRD4 need to address these issues.

Keywords: Urothelial carcinoma of the bladder, Bromodomain 4 protein, immunohistochemical, prognosis

Introduction

Bladder cancer is the second most common genitourinary tumor in human populations, and it was estimated in 2013 that 72,570 new cases of cancer of the urinary bladder were diagnosed in the United States and 15,210 deaths were attributable to bladder cancer [1]. Clinically, radical cystectomy (RC) remains the most common treatment for patients with muscle-invasive UCB or for patients with superficial disease that is at high risk of recurrence and progression [2]. Despite advances in surgical technique and an improved understanding of the role of pelvic lymphadenectomy, the 5-year cancer-speciic survival (CSS) remains at only 50-60% [3]. In addition, while providing important prognostic information on UCB, the currently clinical and pathological variables have a limited ability to predict tumor recurrence, progression, and patient survival. The most possibly underlying reason might be the heterogeneous biological properties of UCB. Therefore, the search for specific genes alterations which determine biological nature and behavior of UCB would be of utmost importance to optimize individual therapy. However, such reliable biomarkers are still substantially limited.

The BrD and extraterminal domain (BET) proteins bind to acetylated lysine residues in histones, recruit chromatin-modifying enzymes to target promoters, and function as coactivators or corepressors in a context-dependent manner [4]. The BET family consists of BRD2, BRD3, BRD4, and the testisspecific member BRDT,

Table 1. Correlation between Bromodomain 4 expression and clinicopathological characteristics of urothelial carcinoma of the bladder patients

Parameters	Group	Total	BRD4 expression		*P value*
			Low	High	
Gender	Male	41	23	18	0.170
	Female	47	33	14	
Age (years)	< 59	37	24	13	0.838
	≥ 59	51	32	19	
Histological grade	G_1	30	27	3	0.000
	G_2, G_3	58	29	29	
Tumor size (cm)	< 3.5 cm	43	28	15	0.778
	≥ 3.5 cm	45	28	17	
Tumor stage	T_a, T_1	57	37	20	0.736
	T_2, T_3, T_4	31	19	12	
Lymph nodes metastasis	N_0	75	54	21	0.000
	N_1, N_2	13	2	11	
Distant metastasis	No	78	55	23	0.000
	Yes	10	1	9	
Tumor multiplicity	Unifocal	46	27	19	0.313
	Multifocal	42	29	13	

which share a common domain architecture [5]. BRD4 is a ubiquitously expressed nuclear protein of 200 kDa that contains two tandem bromo-domains (BDI and BDII) and an extraterminal (ET) domain [6]. Recent research showed BRD4 plays an imoprtannt role in the regulation of cell growth [7]. By photobleaching assays, Dey showed that BRD4 are mobile in the nucleus and transiently associate with acetylated chromatin, as has been reported for other non-histone nuclear proteins [8]. Studies have revealed important roles for BRD4 proteins in development and certain types of cancer. For example, recent study showed BRD4 are higher expression in melanomas compared with nevi and BRD4 knockdown is sufficient to recapitulate the antitumoral effect of BET inhibitors in melanoma cell [9]. Zuber showed that suppression of BRD4 using shRNAs or the small-molecule inhibitor JQ1 led to robust antileukaemic effects in vitro and in vivo [10]. So the function of BRD4 makes it a possible target in anticancer therapy. But there have been no reports about BRD4 in bladder cancer and the role of BRD4 in blader cancer is still unknown.

In the present study, we examined both BRD4 mRNA and protein expression by real-time quantitative PCR (qRT-PCR) and Western blot and investigate the expression of the human BRD4 proteins by Immunohistochemistry (IHC) and identify their potential roles in tumor occurrence, development and prognosis for patients with bladder cancer.

Materials and methods

Patients and specimens

For qRT-PCR and Western blot analysis, we collected 12 paired fresh UCBs and normal tissue samples from patients who underwent surgery between March 2013 and November 2013. In addition, a cohort of 88 formalin fixed, paraffin embedded tissues of UCBs diagnosed between January 2007 and December 2011 at the Department of Urology, Shanghai Tenth People's Hospital of Tongji University (Shanghai, China) was retrieved. The cases selected were based on distinctive pathologic diagnosis of UCBs, undergoing transurethral resection, partial cystectomy and radical cystectomy without preoperative chemotherapy or radiotherapy. The disease stage of each patient was classified or reclassified according to the 2002 AJCC staging system [11]. The patients were totally 41 men and 47 women, whose age range from 38 to 79 years (median: 59 years). Clinicopathological characteristics in our study are presented in **Table 1**. All patients were followed up until November 2013 with a median observation time of 38 months.

Patients were only included in the study if they had provided written consent to participate in the study after receiving oral and written information regarding its course and purpose. Approval for the study was received from the Ethics Committee of the host institution.

Real-time quantitative PCR

Total RNA was isolated tissue using TRIZOL reagent according to the manufacturer's protocol (Invitrogen). RNA was reverse transcribed using SuperScript First Strand cDNA System (Invitrogen) according to the manufacturer's

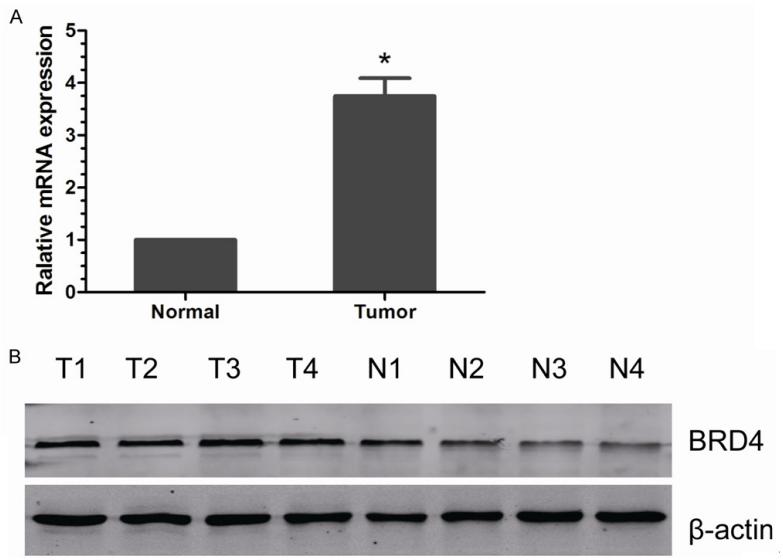

Figure 1. Expression of BRD4 mRNA and protein in the human UCB surgical specimens. A. Rrelative mRNA expression of BRD4 was higher in UCB tissues than in matched normal bladder tissues ($P < 0.05$). B. BRD4 protein expression was higher in the UCB tissues than in matched normal bladder tissues. N, normal bladder tissues; T, UCB tissues.

instructions. The BRD4 sense primer was 5'-CAAATCCAGCATCTCAGCAG-3', and the antisense primer was 5'-AGGAAGCACCCACAATCTACA-3'. For the GAPDH gene, the sense primer was 5'-TGCACCACCAACTGCTTAGC-3', and the antisense primer was 5'-GGCATGGACTGTGGTCATGAG-3'. The PCR amplification were performed for 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, on a Applied Biosystems 7900HT (Applied Biosystems) with 1.0 μl of cDNA and SYBR Green Real-time PCR Master Mix (Takara). Data was collected and analyzed by SDS2.3 Software (Applied Biosystems). The expression level of each candidate gene was internally normalized against that of the GAPDH. The relative quantitative value was expressed by the $2^{-\Delta\Delta Ct}$ method. Each experiment was performed in triplicates and repeated three times.

Western blot assay

Tissues were lysed in lysis buffer containing protease inhibitor cocktail. Protein concentration was determined using a Bio-Rad protein assay system (Bio-Rad). Equivalent amounts of proteins were separated by SDS-PAGE, and then transferred to polyvinylidene difluoride membranes (Bio-Rad). After being blocked in Tris-buffered saline (TBS) containing 5% nonfat milk, the membranes were incubated with primary antibodies against BRD4 (1:500; Abcam), β-actin (1:1000; Abcam) at 4°C for 12 hours, and then with anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase at room temperature for 2 hours. ECL detection reagent (Amersham Lifen Science, Piscataway, NJ) was used to demonstrate the results.

Immunohistochemistry staining

Paraffin sections (4 μm thick) were deparaffinized in xylene and rehydrated in grade alcohol, followed by boiling in 10 mmol/L of citrate buffer (pH 6.0) for antigen retrieval. After inhibition of endogenous peroxidase activities for 30 min with methanol containing 0.3% H_2O_2, the sections were blocked with 2% bovine serum albumin for 30 min and incubated overnight at 4°C with primary monoclonal rabbit anti-human BRD4 antibody (1:200; Abcam). After washing thrice with PBS, the slides were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG for 30 min, followed by reaction with diaminobenzidine and counterstaining with Mayer's hematoxylin. Negative control was done by omission of the primary antibody and substituting it with non-specific rabbit IgG.

Evaluation of immunohistochemical staining

The evaluation of the immunohistochemical staining was performed independently by two authors without knowledge of the clinicopathological information. The immunoreactive scores besides BRD4 were determined by the sum of extension and intensity as literature reported previously [12]. The intensity of the staining was scored using the following scale: 0, no staining of the tumor cells; +, mild staining; ++, moderate staining and +++, marked staining. The area of staining was evaluated and recorded as a percentage: 0, less than 5%; +, 5%-25%; ++, 26%-50%; 3+, 51%-75% and 4+, more than 75%. The combined scores were recorded and

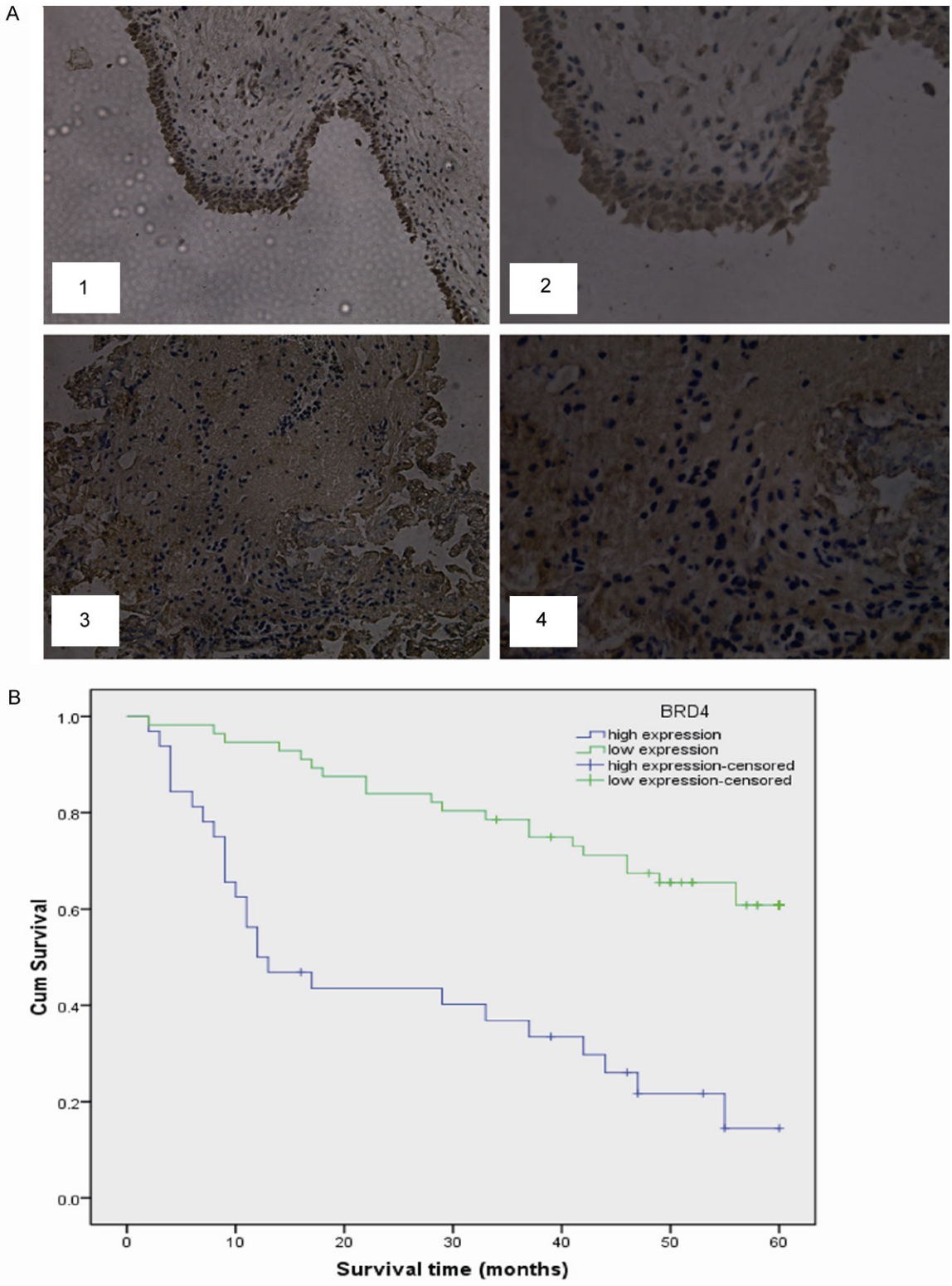

Figure 2. BRD4 protein expression in UBC surgical specimens and patient survival. A. ICH analysis of BRD4 protein expression in 88 cases of UCB tissues. 1. ICH expression of BRD4 in normal bladder tissues (× 100). 2. ICH expression of BRD4 in normal bladder tissues (× 200). 3. ICH expression of BRD4 in UCB tissues (× 100). 4. ICH expression of BRD4 in UCB tissues (× 200). B. The survival analysis of BRD4. Patients with higher BRD4 expression in tumor tissue were closely correlated with poorer overall survival than patients with tumor with lower expression ($P < 0.05$, respectively).

Table 2. Prognostic factors in Cox proportional hazards model

Variable	Univariate analysis			Multivariate analysis		
	Risk ratio	95% CI	*P*	Risk ratio	95% CI	*P*
Gender Male vs Female	1.097	0.746-1.827	0.563			
Age (years) ≥ 59 vs < 59	1.327	0.575-2.318	0.464			
Tumor multiplicity Unifocal vs Multifocal	1.216	0.897-2.351	0.714			
Tumor stage ≥ T1 vs < T1	3.783	2.964-4.587	0.107			
Histological grade G_2, G_3 vs G_1	3.741	2.363-6.946	< 0.001	3.417	2.411-6.637	< 0.001
Lymph node N_{1-2} vs N_0	4.472	3.267-7.616	< 0.001	3.324	2.079-5.745	0.012
Distant metastasis M_1 vs M_0	6.737	3.894-16.144	< 0.001	4.013	2.368-7.436	0.014
Bromodomain 4 high vs low	3.677	2.149-6.936	0.016	3.372	1.874-6.017	< 0.001

graded as follows: -, 0; +, 1-2; ++, 3-5; +++, 6-7. Additionally, for statistical analysis, the − and 1+ cases were pooled into the low-expression group, and the 2+ and 3+ cases were pooled into the high-expression group.

Statistical analysis

Computerized statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS), version 18.0. The *t*-test was used to analyze the data from qRT-PCR and Western blot in the tissues. Clinical and histopathologic information and the results from the ICH studies were entered into a database. The significance of BRD4 expression for tumor was analyzed by the Kaplan-Meier method, and the differences were evaluated by the log-rank test. Multivariable recurrence-free survival analyses were performed with the Cox proportional hazards model. Differences were considered significant if the *P*-value from a two-tailed test was < 0.05.

Results

Expression of BRD4 mRNA and protein in UCB tissues

We first examined BRD4 mRNA expression in 12 fresh urothelial carcinoma of bladder and paired adjacent normal bladder tissues by real-time quantitative PCR. It showed that the increasing BRD4 mRNA expression could be detected in bladder cancer samples in comparison with the normal bladder samples ($P < 0.05$, **Figure 1A**). To investigate whether BRD4 was also elevated at the protein level, Western blot was performed. We found that the protein level of BRD4 in tumor samples was significantly higher than that in normal bladder samples (**Figure 1B**).

Immunohistochemical analysis of BRD4 expression in urothelial carcinoma of bladder samples

We further analyzed BRD4 protein level in UCBs tissues and normal bladder tissues using an immunohistochemical approach. The tumorous or non-tumor staining was semiquantitatively scored by the intensity and the percentage of positive staining. ICH staining showed that the BRD4 protein was mainly accumulated in the nuclei of malignant cells. And the expression of BRD4 in UCBs was significantly higher than in normal bladder tissues (**Figure 2A**).

Relationship between BRD4 expression and UCB patients' clinicopathologic variables

In our UCB cohort, the relationship between the expression of BRD4 and patient clinical charac-

teristics was shown in **Table 1**. High expression of BRD4 was found to significantly correlate with higher histological grade ($P < 0.001$), lymph node metastasis ($P < 0.001$) and distant metastasis ($P < 0.001$). No significant difference in BRD4 expression was observed with gender, age, tumor size, tumor stage and tumor multiplicity ($P > 0.05$).

Relationship between clinicopathologic features, BRD4 expression, and UCB patients' survival: univariate survival analysis

In univariate survival analyses, cumulative survival curves were calculated according to the Kaplan-Meier method. Differences in survival times were assessed using the log-rank test. First, to confirm the representativeness of the UCBs in our study, we analyzed established prognostic predictors of patient survival. Kaplan-Meier analysis demonstrated a significant impact of well-known clinical pathological prognostic parameters, such as histological grade, lymph node status and distant metastasis status on patient survival ($P < 0.05$, **Table 2**). Assessment of survival in UCBs patients revealed that higher expression of BRD4 was correlated with adverse survival of UCB patients ($P = 0.016$, **Table 2**, **Figure 2B**).

Independent prognostic factors for UCB: multivariate cox regression analysis

Since variables observed to have a prognostic influence by univariate analysis may covariate, the expression of BRD4 and those clinicopathological parameters that were significant in univariate analysis (histological grade, lymph node status and distant metastasis status) were further examined in multivariate analysis. The results showed that the expression of BRD4 was an independent prognostic factor for overall patient survival (relative risk: 3.372, CI: 1.874-6.017, $P < 0.001$, **Table 2**). With regard to other parameters, histological grade, lymph node status and distant metastasis status were also shown to be an independent prognostic factor for overall survival ($P < 0.05$, **Table 2**).

Discussion

As a highly conserved class of epigenome readers, the bromodomain (BrD)-containing proteins have been shown to exert key roles at the interface between chromatin remodeling and transcriptional regulation. A left-handed four-helix bundle characterizes the three-dimensional structure of the BrD, which consists of a hydrophobic cleft between two conserved loops that interact with acetylated lysine residues [13]. In humans, there are estimated to be 61 BrDs encoded in 46 proteins, including chromatin regulators of the SWI/SNF superfamily of DNA helicases, histone acetyltransferases, as well as the BrD and extraterminal domain (BET) family of transcriptional regulators [14-16]. The BET family consists of BRD2, BRD3, BRD4, and the testis-specific member BRDT, which share a common domain architecture [17]. BET proteins bind to acetylated lysine residues in histones, recruit chromatin-modifying enzymes to target promoters, and function as coactivators or corepressors in a context-dependent manner [18]. Recent studies have revealed important roles for BET proteins in development, inflammation, and certain types of cancer. For example, high BRD2 levels have been found in a subset of human leukemia, and BRD2 overexpression in the lymphoid lineage triggers the development of B-cell lymphoma suggesting a prooncogenic function for this protein [19]. Previous studies have reported that BRD4 sustains melanoma proliferation and suppression of BRD4 using shRNAs or the small-molecule inhibitor JQ1 led to robust anti-leukaemic effects [9, 10].

Bladder carcinogenesis is characterized by distinct morphological, genetic and cellular events. Development and progression of bladder cancer to metastasis and lethal state are believed to be driven by multiple genetic alterations, the nature of which has remained poorly understood. In the present study, we sought to determine whether there was any difference in BRD4 expression between UCBs and normal tissue samples which had not been studied previously. This study demonstrated that BRD4 proteins were up-regulated in the UCBs, and explored available evidence of close correlation of BRD4 expression and the total patients' survival during a five-year follow-up survey.

To directly address the potential roles for BRD4 protein in the occurrence and development of bladder cancer, an elaborate experiment was conducted and a rigorous analysis was performed of human BRD4 mRNA and proteins on

a bladder cancer samples. Our results revealed that the BRD4 expression in bladder cancer tissues was remarkably higher than that in normal bladder tissues ($P < 0.05$). Miguel's study also indicated that the abnormal expression of BRD4 might be correlated with Melanoma oncogenic event [9].

In the present study, we found the expression level of BRD4 in nucleus was significantly associated with histological grade ($P < 0.001$), lymph node metastasis ($P < 0.001$) and distant metastasis ($P < 0.001$). It is suggested that BRD4 are associated with tumor development and progression and may promote tumor invasion. We further imagined that the BRD4 proteins may interfere with the activation of cellular signal transduction pathway, cell division cycle and tumor angiogenesis to influence biological behavior of tumor, and this had just been unraveled in the latest relevant researches. The role of DNA histone modification and related epigenetic mechanisms in carcinogenesis is now widely appreciated, as evidenced by the development of novel histone deacetylase inhibitors [20]. Recent study showed that Both Brd2 and Brd4 bind acetylated histones and mobilize chromatin modification to control cell cycle [21]. Brd4 thus plays a fundamental role in cell cycle and transcriptional programs that are important in cancer and viral transformation [22]. The findings suggest a novel role of BRD4 in carcinogenesis.

Ultimately, a total of 88 patients histologically proven bladder cancer with follow-up information were conducted a systematically analysis to confirm the relationship of the BRD4 proteins and outcome of patient initially. Our finding demonstrated that patients with lower expression of BRD4 in tumor tissue had a better overall survival than patients with higher expression ($P = 0.016$, respectively), providing an evidence that elevated expression of BRD4 in bladder cancer might facilitate an increased malignant and worse prognostic phenotype. It is noteworthy that by multivariate Cox analysis combining expression of BRD4 proteins with other parameters, BRD4 was found as an independent prognostic factor for patient survival ($P < 0.001$). The aberrant expression of BRD4 protein linked to a poor prognosis of patients has never been investigated in bladder cancer before.

In conclusion, we reported for the first time that BRD4 expression was upregulated in clinical UCB tissues, and high expression of BRD4 was associated closely with a more malignant clinical feature and poor prognosis of UCB patients. Our results suggest that BRD4 over-expression might be useful as a prognostic factor for UCB patients. Apparently, a further understanding of the molecular mechanism by BRD4 in human UCB would help in the discovery of novel targeted agents and might also lead to the development of new approaches for effective therapy of human UCB.

Disclosure of conflict of interest

None.

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