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Expression of B7 Homolog 1 (B7H1) Is Associated with Clinicopathologic Features in Urothelial Bladder Cancer

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Data Interpretation D
Manuscript Preparation E
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Background: B7 homolog 1 (B7H1) plays an important role in regulating tumor immunity. The purpose of this study was to probe the relationship between B7H1 expression and clinical outcomes in urothelial bladder cancer.

Material/Methods: We investigated 110 urothelial bladder cancer cases. The expressions of B7H1 in tumors were analyzed by immunohistochemistry and RT-PCR. The correlation between B7H1 expression and survival rate was analyzed by log-rank test.

Results: B7H1 expression was significantly increased in cancerous tissues compared to normal tissues ($p<0.05$). B7H1 expression was not associated with sex, age, diameter, or the combination of these factors ($p>0.05$). The positive expression of B7-H1 was positively correlated with grade, stage, recurrence, and metastasis ($p<0.05$). The RT-PCR results were consistent with the immunohistochemistry outcomes. Furthermore, the expression of B7H1 in tumors was highly correlated with the survival rate ($p<0.05$).

Conclusions: Expression of B7H1 is correlated with clinicopathologic features in bladder cancer. Up-regulation of B7H1 can result in progression and recurrence of urothelial bladder cancer.

MeSH Keywords: Antigens, CD274 • Survival Analysis • Urinary Bladder Neoplasms

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Background

Urothelial bladder cancer (UBC) is a common disease, accounting for approximately 7% of all cancer cases [1,2]. Transurethral resection combined with intravesical chemotherapy is the standard treatment for Ta and T1 disease in urothelial bladder cancer, but the outcomes remain poor [3]. Some studies have shown that the clinical outcome of UBC is associated with immune responses in cancer patients with anti-immune cells [4,5]. Response to therapy can be predicted by the combination of clinical outcomes and tissue-based molecular markers, which could help in administering the optimal local and systemic therapy in UBC patients.

B7 homolog 1 (B7-H1), also known as programmed death ligand1 (PD-L1), is the third member of the B7 family [5]. T cells are immune cells that play a critical role in mediating anti-tumor immunity. A large numbers of T cells are present in bladder cancer, and the cells positively control the local anti-tumor responses, such as CD^{4+} T cells and CD86 [6]. B7-H1 can inhibit proliferation of T cells and is a negative stimulus in the process of activation of T cells through suppressing the secretion of some cytokines, for example, interferon-γ (IFN-γ), interleukin-10 (IL-10), IL-14, and IL-2 [7,8]. Expression of B7-H1 was reported to be higher in tumor tissues and the number of immune cells in peripheral blood was found to be related with tumor escape [9]. Up-regulation of B7-H1 has been reported to be correlated with progression of thyroid cancer, lung cancer, ovarian cancer, colon cancer, and melanoma [10–14]. A previous study showed that B7H1 expression is involved in the mitogen-activated protein kinase (MAPK) pathway, especially the extracellular signal-regulated kinas (ERK) pathway in bladder cancer [15]. In the *in vitro* lung cancer microenvironment, B7H1 expression was up-regulated, with increasing transforming growth factor β (TGF-β) and was associated with regulatory T cell generation [16]. Moreover, overall survival was correlated with B7H1 expression in patients with UBC. These findings suggest that B7H1 plays an integral role in the immunological escape mechanism in UBC.

Many studies showed that B7H1-positive expression was associated with rapid cancer progression. The expression of B7H1 is significantly associated with the clinicopathological variables and postoperative survival in esophageal cancer [17]. Patients with high B7H1 expression show shorter survival than those with low B7H1 expression.

In this study, we investigated the B7H1 expression in UBC patients and analyzed the relationship between expression of B7H1 and clinicopathological features. The results may contribute to clinical treatment of UBC.

Table 1. The clinicopathologic parameters of the 98 cases.

	n		n
Age (y)		Metastatic tumor	
<65	46	Metastasis	11
≥65	52	Non-metastasis	87
Tumor grade		Tumor size (diameter)	
I	23	<3 cm	32
II	46	≥3 cm	66
III	29	Focus	
Tumor stage		Solitary	45
Ta–T1	25	Multiple	53
T2–T4	73		
Tumor type			
Primary	57		
Recurrence	41		

Material and Methods

Clinical study

We enrolled 98 patients (22 females and 76 males) who underwent bladder urothelial cancer surgery at Yantai Affiliated Hospital of Binzhou Medical University from 1 September 2009 to 1 October 2011. We obtained cancerous tissue samples from each patient, as well as 12 tissue samples adjacent to tumors. None of the patients in this study had received any radiation therapy or chemotherapy before the operation. All patients were diagnosed by the method of cystoscopy, ultrasound, computerized tomography urography (CTU), and pathology. Written permission was obtained from the patients prior to surgery. All patients were confirmed with pathological examination. All tissues were stored in a liquid nitrogen tank after surgical resection.

The tumors were classified according to the tumor-node-metastasis (TNM) staging system of the International Union Against Cancer (1997) and the World Health Organization (WHO). All clinicopathologic parameters are summarized in Table 1.

This study was approved by Medical Ethics Committee of Binzhou Medical University.

Immunohistochemistry

Bladder cancer tissues and tissue adjacent to bladder cancer were fixed with 4% paraformaldehyde (Beijing North of Conde Clinical Reagent Co., Beijing, China), embedded in paraffin, and

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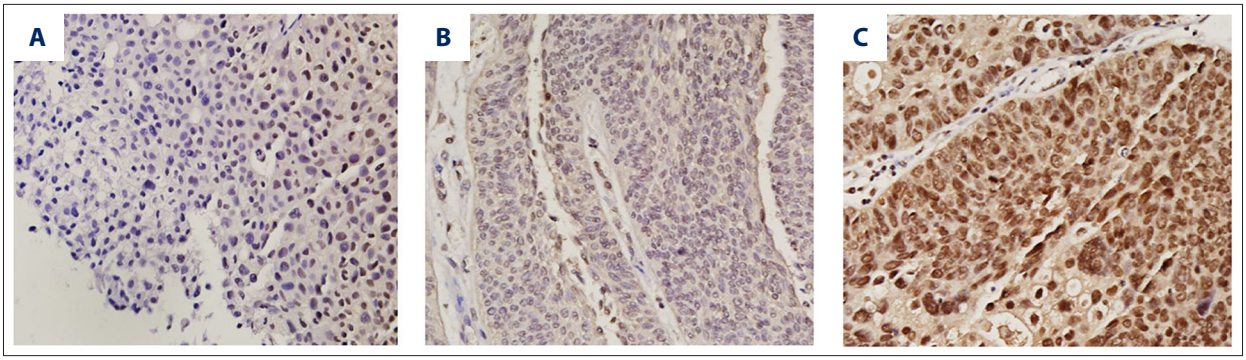

Figure 1. B7H1 protein expression in normal bladder tissue, UBC tissue, and positive tissue. Representative slides (×400) of B7H1 protein staining in normal bladder tissue (**A**), UBC tissue (**B**), and positive tissue (**C**).

then sliced into 4-μm sections. All sections were incubated for 4 h at room temperature with anti-B7H1 antibody (1: 50, Boster, China). Phosphate-buffered saline (PBS) was used as a blank control to replace the first antibody. The second antibody was diluted to 1: 500. Positivity of cell plasma or membranes was indicated by tan color. Three randomly selected fields were observed at high magnification (*400). Greater than 10% positive cells was regarded as positive. The results were analyzed using AlphaView SA software (Thermo Fisher Scientific, Waltham, MA, USA).

RT-PCR

Total RNA was extracted from fresh urothelial bladder cancer tissue. The mRNA was reverse-transcribed into first-strand cDNA using M-MLV reverse transcriptase (Promega, USA). PCR reactions were carried out using specific primers for B7H1 (sense: 5'-GCCGACTACAAGCGAATTAC-3', antisense: 5'-TCTCAGTGTG CTGGTCACAT-3', product length 233 bp). β-actin served as the internal control (sense: 5'-CGGGAAATCGTGCGTGAC-3'', antisense: 5'-TAGAAGCATTT GCGGTGG-3', product length511 bp).

The cycling parameters were: 94°C for 3 min, 90°C for 30 s, and 55°C for 30 s. The extension was 1 min at 72°C for 35 cycles. The PCR products were analyzed by Quantityone software (BioRad, USA). The change in mRNA expression was calculated by the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

SPSS 22.0 statistical analysis software was used to analyze the data. Data are expressed as mean ± standard deviation (SD). Independent samples were analyzed by χ^2 test and comparisons between groups were assessed by the *t* test. Survival rate was calculated with Kaplan-Meier analysis. Comparison of survival was made using the Mantel-Cox test. $p<0.05$ was considered statistically significant.

Results

B7H1 overexpression in urothelial bladder cancer tissue

The expression of B7-H1 protein was analyzed by immunohistochemistry (Figure 1). We found that the B7H1 expression was negative in normal bladder tissue. However, the rate of positive expression in urothelial bladder cancer tissue was 57.14% (56/98).

The correlation between B7H1 expression and clinicopathological parameters of UBC cases

We analyzed the correlation between the percentage of B7H1 expression and clinicopathological parameters (Table 2). The results showed that there was no association between B7H1-positive expression and either sex or age ($p>0.05$). Furthermore, B7H1 expression was not found to be associated with tumor metastasis, tumor size, or focus ($p>0.05$). According to the 2004 WHO criteria (low malignancy of urinary tract epithelial papilloma, low-grade Papillary urothelial carcinoma, high-grade papillary urothelial carcinoma), 98 specimens were classified as grade group and stage group. Interestingly, we found that B7H1 expression was increased in the high-grade group and in the stage group. In the grade groups, the percentages of B7H1 expression were 34.78%, 50.00%, and 86.20%, respectively. Similarly, the Ta-T1 group had 36% B7H1-positive expression and the T2-T4 group had 64.39% B7H1-positive expression. Tumor cases showed significantly different expression between B7H1 expression and grade group or stage group ($p<0.05$). Moreover, B7H1-positive expression was remarkably associated with tumor type ($p=0.00$). Twenty-two of 57 patients with primary tumors and 34 of 41 patients with tumor recurrence had B7H1-positive expression.

Association of B7H1 mRNA expression with patients' clinical characteristics

The B7H1 mRNA levels were measured by RT-PCR (Figure 2A). The results were similar to the protein expression. The levels

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Table 2. Connection between B7H1 expression and the clinicopathologic parameters of 98 UBC cases.

Parameters	B7H1 positive expression (%)		p	Parameters	B7H1 positive expression (%)		p
Sex			0.517	Tumor type			0.000
Female	59.09	(13/22)		Primary	38.59	(22/57)	
Male	56.58	(43/76)		Recurrence	82.93	(34/41)	
Age (y)			0.535	Metastatic tumor			0.198
<65	56.52	(26/46)		Metastasis	72.73	(8/11)	
≥65	57.69	(30/52)		Non-metastasis	55.17	(47/87)	
Tumor grade			0.000	Tumor size (diameter)			0.536
I	34.78	(8/23)		<3 cm	56.25	(18/32)	
II	50.00	(23/46)		≥3 cm	57.58	(38/66)	
III	86.20	(25/29)		Focus			0.465
Tumor stage			0.013	Solitary	55.56	(25/45)	
Ta–T1	36.00	(9/25)		Multiple	58.49	(31/53)	
T2–T4	64.39	(47/73)					

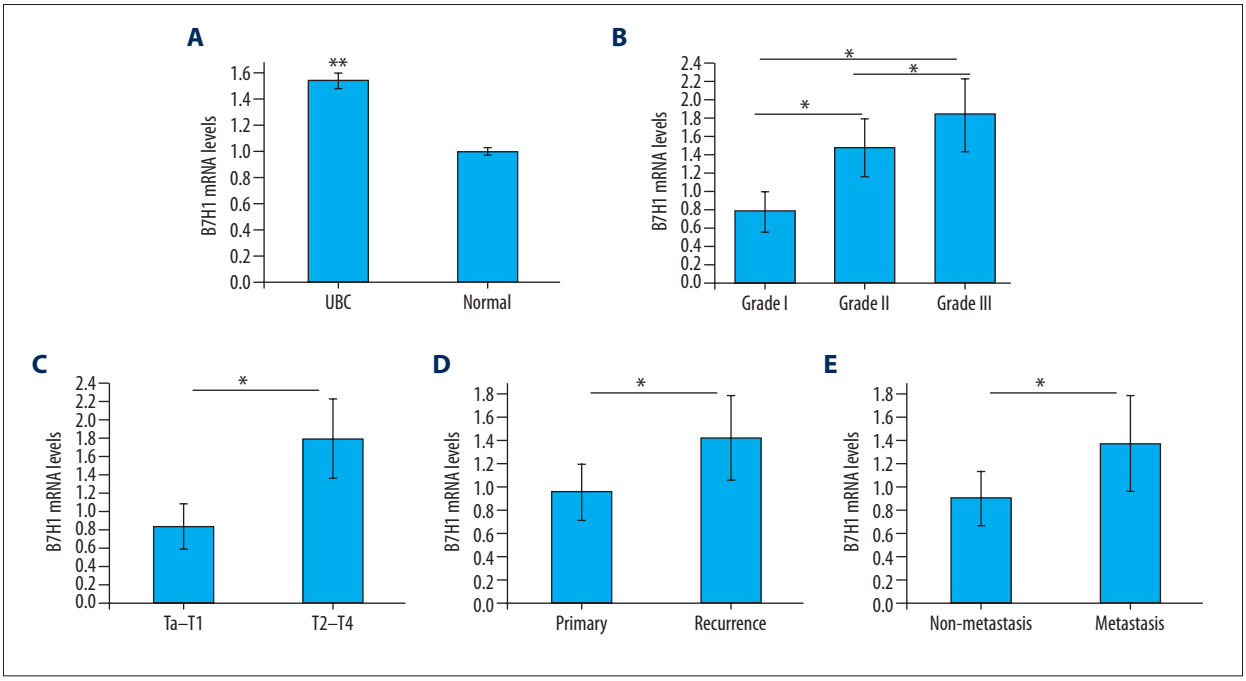

Figure 2. (**A–E**) The levels of B7H1 mRNA in specimens and the association between the B7H1 mRNA expression and clinical characteristics of UBC. * p<0.01, ** p<0.01.

of B7H1 mRNA were obviously increased in UBC tissue compared to normal bladder tissue. Furthermore, we analyzed the relationship between B7H1 mRNA expression and clinical characteristics of UBC. A significant association was found between B7H1 mRNA expression and grade group or stage group (p<0.05, Figure 2B, 2C). In high-grade or stage group, the B7H1 mRNA levels were more pronounced. Moreover, the B7H1 mRNA expression in UBC patients had a significant correlation with tumor recurrence and metastasis (p<0.05, Figure 2D, 2E).

Relationship of the expression of B7H1 to the rate of survival

None of the included patients received radiation therapy or chemotherapy before or after the operation. Patients were

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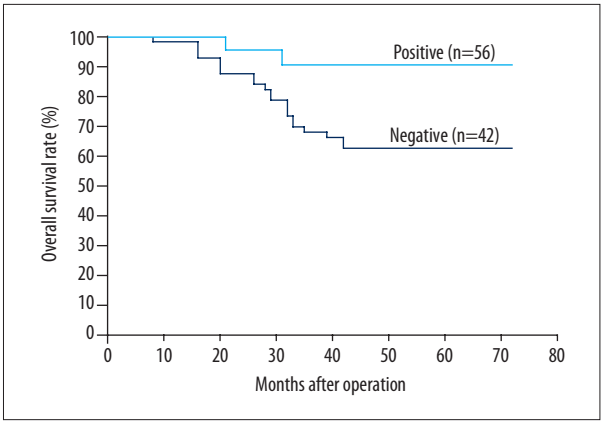

Figure 3. Overall 6-year survival rate in 98 patients. The patients with positive B7H1 expression (n=56) had a poorer prognosis than those with negative B7H1 expression (n=42). *P* value was determined by the log-rank test (χ^2=6.32, p=0.001).

followed up for 6 years from the day of the operation. During that time, 31 patients died due to recurrence of bladder cancer. The average survival time in patients with positive expression of B7H1 was 45 months, but it was 65 months in patients with negative expression of B7H1. The overall survival was significantly lower in patients with positive B7H1 expression than in those with negative B7H1 expression (62.50% *vs.* 90.48%, p=0.001).

Discussion

Urothelial bladder cancer is the second most common malignant GU tumor. There are 330 000 new cases each year worldwide and more than 50% of patients relapse [18], eventually evolving into invasive bladder cancer and developing into the muscular layer. The 5-year survival rate is far below 50% [19]. In the present study, we found that B7H1 expression in UBC was associated with tumor grade, stage, and type. Furthermore, the levels of B7H1 were associated with the postoperative prognosis. These results are consistent with previous studies [5,20].

The occurrence and development of bladder cancer is a multi-step and multi-stage process involving the activation of oncogenes, inactivation of tumor-suppressor genes, and many signaling pathways. It was reported that toll-like receptor (TLR4) expression was decreased in UBC patients [4], which could activate transcription by taking part in the MyD88/MEK/STAT1 signaling pathway [21]. TLR4 is a critical molecule in the regulation of immune response and it participates in the immune escape process of UBC through up-regulation of B7H1 expression [4]. Our study also shows that B7H1 is correlated with UBC tissue specimens. B7H1 is highly expressed in isolated cancer tissues [22,23]. In laryngeal cancer, the expression of B7H1 was associated with the cancer grade accompanied by high expressions of CD83+CD200+ cells [23]. In urothelial cancer, B7H1 was expressed at high levels on major populations of $CD4^+$ and $CD8^+$ tumor-infiltrating lymphocytes (TILs) [5]. T cells and several cytokines have been implicated as regulators of B7H1 expression in various cancers. Cytokines produced by the host are important in immune inhibition, including TNF-α and INF-γ [24, 25]. It was demonstrated that TNF-α and INF-γ induced PD-L1 expression by the Janus kinase/signal transducer and activator of transcription 3 (JAK/STAT3) pathway and the phosphatidylinositol 3-kinase (PI3K)/AKT pathway in lung cancer [25]. These finding suggest the B7H1 is an important factor in tumor immunological escape.

The results of our clinicopathological analyses support that B7H1 is a tumor-influencing factor in UBC. We observed that positive B7H1 expression was strongly associated with WHO grade, and higher grade was associated with higher expression. This result is similar to that reported by Nakanishi [5]. In addition, we found there was a strong association between B7H1 positive expression and tumor recurrence or metastasis (Table 2, Figure 2). However, research has also found no association between B7H1-positive expression and clinical features such as stage and grade [20]. This result may be correlated with the number of participants or other factors in certain groups of patients. Nevertheless, it is clearly established that the positive expression of B7H1 predicts poor prognosis [5,20]. Our study also showed higher levels of B7H1 and lower survival in positive cases than in negative cases (Figure 3).

Research on the role of B7H1 expression in various types of cancer has been performed in many medical centers with many different sources of support [26–28]. Furthermore, many institutions have performed clinical studies on cancer treatment using B7H1 [29,30]. For example, in patients with metastatic melanoma treated with concurrent ipilimumab (anti-CTLA-4) and nivolumab (anti-B7H1), 17% of patients achieved a CR, with an overall survival (OS) rate for all patients of 79% at 2 years [31]. Many studies on bladder cancer have assessed the expression of B7H1 and its role in prognosis and targeted treatment. It is recommended that the expression of B7H1 should be assessed in bladder cancer patients after surgery, and it may be used as a target treatment in the future in bladder cancer [20,32,33].

Conclusions

In general, the expression of B7H1 is correlated with biological behavior in bladder urothelial cancer. Up-regulation of B7H1 can result in development of bladder cancer. B7H1 could be as a possible marker for urothelial carcinoma.

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