Original Article

B7-H4 expression is correlated with tumor progression and clinical outcome in urothelial cell carcinoma

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Abstract: Objective: To investigate the mRNA and protein levels of B7-H4, a B7 family molecule, in human urothelial cell carcinoma (UCC), to analyze the relationship between B7-H4 protein expression level and pathological stage of UCC, and to examine the potential of B7-H4 as a prognostic factor in UCC. Methods: mRNA and protein levels of B7-H4 were measured in pairs of tumor tissues and matched adjacent nontumor tissue obtained from patients with UCC by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and immunohistochemical staining, respectively. Association of the protein level of B7-H4 with pathological tumor stage and the overall survival of UCC patients were also analyzed. Results: B7-H4 mRNA and protein level were significantly higher in UCC tumor tissues compared with adjacent nontumor tissues as assessed by qRT-PCR and immunohistochemical staining, respectively. Higher B7-H4 protein levels were observed in patients with more advanced pathological stage of UCC and were also associated with decreased overall survival of patients with UCC. Conclusions: The findings from this study indicate that B7-H4 has the potential to be an independent prognostic indicator for UCC.

Keywords: Bladder cancer, B7 family, B7-H4, prognosis, gene expression

Introduction

Bladder cancer, with 383,000 new cases diagnosed worldwide in 2008 [World Cancer Research Fund International (WCRF International)], is the ninth most common cancer in the world and the second most common genitourinary malignancy [1, 2]. About 95% of bladder cancers are urothelial cell carcinoma [UCC, also known as transitional cell carcinoma (TCC)] [3]. UCC is a type of malignant tumour originating from the urothelium lining the urinary tract from the renal calyces to the ureteral orifice. UCC is a clinically heterogeneous disease, with 70% of total cases presenting non-muscle-invasive tumors and 30% presenting muscle-invasive tumors [4-6]. Muscle invasive tumors usually implies metastases and poor prognosis [4-6]. Surgery is the option for most people with UCC. However, a significant number of patients suffer from disease recurrence and progression after radical cystectomy [6]. For example, a group from the University of Texas MD Anderson Cancer Center found that metastases developed in 97 of the 382 patients (25%) with transitional cell carcinoma of the bladder a median of 12 months after cystectomy [7]. Therefore, there is an urgent need to identify prognostic biomarkers with high specificity and sensitivity for UCC in order to distinguish tumors with the potential to progress and metastasize.

T cell-mediated immunity depends on specific recognition of antigen-major histocompatibility complex (MHC) by T cell receptor (TCR) and co-regulatory signals [8]. The co-regulatory signals come from the B7 family molecules, which are a group of structurally related, peripheral membrane proteins mainly located on activated antigen presenting cells (APCs) [8]. B7 molecules function as co-regulatory ligands by binding to corresponding receptors on T cell surfaces, producing co-regulatory signals to either enhance or decrease T cell-mediated, antigen-specific immune responses [9]. The newly identified B7 family member, B7-H4 (also known as B7x or
B7-H4 has been reported to be highly expressed in different tumors, including ovarian, breast, non-small-cell lung cancers, etc, however, there is a little or no B7-H4 expression in normal tissues [12, 13]. It has also been reported that B7-H4 promotes malignant transformation and lymph node metastasis [12, 14, 15].

Up to date, however, no reports have investigated the clinical significance of B7-H4 expression in patients with bladder cancer. In this study, we analyzed B7-H4 expression in UCC tissues and normal urothelium tissues using both immunohistochemical method and real time RT-PCR. Additionally, we investigated the relationship between B7-H4 expression level and clinicopathological variables and evaluated the prognostic values of B7-H4 using log-rank survival analysis.

Materials and methods

Patient identification

The study protocol was approved by the ethics committee of the Third Affiliated Hospital of Soochow University, and all tissue samples were collected from patients and donors with appropriate informed consent. The criteria for study enrollment were histopathological diagnosis of UCC of the bladder, no history of other tumour, no chemotherapy before surgery, availability of sufficient tumour sample, and the potential to follow-up. By applying these criteria, sixty two patients who underwent surgeries for bladder cancer (between July 2006 and July 2012) at the Department of Urology, the Third Affiliated Hospital of Soochow, Changzhou, Jiangsu, China, were included in this study. The patients were followed for 3-6 months after surgery with cystoscopic examination at the outpatient clinic. The tumors were classified according to the 2010 Union for International Cancer Control (UICC) TNM classification for pathologic staging and the 2004 World Health Organization classification for the pathological grading based on the findings of clinical, radiological, or histological examinations [16, 17].

Evaluation of B7-H4 staining

The slides were examined by two pathologists, and the sections were evaluated according to the immunohistochemical scores (IHS) [18, 19]. The staining intensity the proportion of positive cells was semiquantitatively evaluated. The staining intensity was scored as 0, no staining; 1, weak staining; 2, moderate staining; and 3, intense staining. The proportion of positive cells was scored as 0 (< 5% positive cells), 1 (6-25% positive cells), 2 (26-50% positive cells), 3 (51-75% positive cells), and 4 (> 75% positive cells). The final B7-H4 staining score was calculated using the percent of positive cell score × staining intensity score ranging 0-12. In this study, the B7-H4 expression is defined as weak positive (lower expression) when score is less than 4, and positive (higher expression) when score is equal to or more than 4.
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Table 1. Primers and probes for human B7-H4 and β-actin

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers and probes</th>
<th>Sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>B7-H4</td>
<td>Forward primer</td>
<td>CACCAGGATAACATCCTCTCAGTGA</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>TGGCTTGCAAGGTAAGAATGA</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>FAM-AAGCTGAGATAATCCCTCAGGCTAMRA</td>
</tr>
<tr>
<td>β-Actin</td>
<td>Forward primer</td>
<td>GGAAGGTGAGGTCGGAGT</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>CGTCTCATCCGTGACGGT</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>FAM-TTGGTGTATTTGGCGGCTAMRA</td>
</tr>
</tbody>
</table>

Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Tumor tissues were frozen in liquid nitrogen immediately until RNA extraction. Total RNA was extracted from tissues using a total RNA purification kit (Shenergy Biocolor BioScience and Technology Co., Shanghai, China) according to the manufacturer’s instructions. One microgram of total RNA was reversely transcribed to cDNA with 100 units of Moloney murine leukemia virus (M-MLV) reverse transcriptase (USB, Cleveland, OH, USA) according to the manufacturer’s protocol. TaqMan® gene expression assays (Applied Biosystems, Foster City, CA, USA) were used to quantify mRNA expression of human B7-H4 and β-actin (internal control) genes. Primers and probes in the TaqMan assay are presented in Table 1. PCR reactions were performed on a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) in duplicate in a 10 µl volume containing 5 µl Universal PCR Master Mix (Applied Biosystems), 0.5 µl TaqMan® assay and 4.5 µl diluted cDNA (50 ng reverse-transcribed RNA). PCR cycling conditions were 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. PCR products were visualized on 1.2% agarose, purified and then verified by sequencing. The relative expression level of B7-H4 mRNA was normalized with β-actin expression level and calculated using the 2^{ΔΔCt} method [20].

Statistical analysis

Statistical analyses were performed using the GraphPad Prism version 5.0 software package (GraphPad Software, San Diego, CA, USA). Data are presented as the mean standard error (SE) from at least three independent experiments. Paired samples t-test was used to analyze significant differences between the UCC and adjacent nontumor tissue. The relationship between B7-H4 expression and clinical parameters was evaluated using Pearson χ² test. The overall survival rates were calculated by the Kaplan-Meier method, and the difference in survival was compared with the log-rank test. The Cox proportional hazards regression model was used for univariate and multivariate analyses to assess the effects of the clinicopathological variables and B7-H4 expression on overall survival. Two-tailed P values < 0.05 were considered to be statistically significant.

Results

B7-H4 expression in human UCC tissues

B7-H4 expression in 62 tissue specimens obtained from patients with bladder cancer was assessed by immunohistochemical staining. Interobserver agreement in the assessment of immunohistochemical findings was excellent. Positive B7-H4 immunohistochemical staining was predominantly observed on the membrane and in cytoplasm of the urothelial cancer cells (Figure 1), while weak staining was found in normal bladder tissues (Figure 1A). B7-H4 mRNA expression in tumour tissues and adjacent non-tumour tissues from 20 UCC patients was assessed by real-time RT-PCR. As shown in Figure 2, the mRNA levels of B7-H4 in UCC tumour samples was significantly higher than those in non-tumour tissue samples (P = 0.012).

Correlations of B7-H4 expression with clinicopathological parameters for UCC patients

The relationship between B7-H4 protein expression (immunohistochemical staining) and clinicopathologic features is summarized in Table...
Gender was not found to be significantly associated with B7-H4 expression ($P = 0.4299$). There was a significant association between B7-H4 expression and the cancer grade: [low grade (G1/2: 8 out of 19: 42.1%) and high grade (G3/4: 39 out of 43: 90.7%)]. In addition, B7-H4 expression was significantly higher in patients with muscle invasive tumors (43 out of 51: 84.3%) than in those with superficial tumors (4 out of 11: 36.4%). The rate of B7-H4 higher expressing specimens in patients with recurrence (41 out of 43: 95.4%) was also significantly higher than that for B7-H4 lower expressing patients without recurrence (6 out of 19: 31.6%).

**Correlation between B7-H4 expression and bladder cancer recurrence-free rate**

The recurrence-free rate of UCC was analyzed by the Kaplan-Meier method. A time period of 60 months was defined to investigate the recurrence-free rate. The recurrence-free rate was determined from the date of the operation to the time of the detection of bladder cancer recurrence or the last follow-up. The impacts of B7-H4 staining, tumour stage, and cancer grade on the recurrence-free rate were investigated. A log-rank test revealed that positive B7-H4 expression was significantly associated...
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Table 2. Correlation between B7-H4 protein expression and clinicopathologic parameters of the patients with urothelial cell carcinoma (UCC) (n = 62)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Number of patients</th>
<th>B7-H4 expression Negative (%)</th>
<th>B7-H4 expression Positive (%)</th>
<th>P value (χ² test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td>0.4299</td>
</tr>
<tr>
<td>Male</td>
<td>48</td>
<td>10 (20.8)</td>
<td>38 (79.2)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>14</td>
<td>5 (35.7)</td>
<td>9 (64.3)</td>
<td></td>
</tr>
<tr>
<td>TNM stage</td>
<td></td>
<td></td>
<td></td>
<td>0.0029</td>
</tr>
<tr>
<td>Superficial</td>
<td>11</td>
<td>7 (63.6)</td>
<td>4 (36.4)</td>
<td></td>
</tr>
<tr>
<td>Invasive</td>
<td>51</td>
<td>8 (15.7)</td>
<td>43 (84.3)</td>
<td></td>
</tr>
<tr>
<td>Grade</td>
<td></td>
<td></td>
<td></td>
<td>0.0001</td>
</tr>
<tr>
<td>Low</td>
<td>19</td>
<td>11 (57.9)</td>
<td>8 (42.1)</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>43</td>
<td>4 (9.3)</td>
<td>39 (90.7)</td>
<td></td>
</tr>
<tr>
<td>Recurrence</td>
<td></td>
<td></td>
<td></td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Negative</td>
<td>19</td>
<td>13 (68.4)</td>
<td>6 (31.6)</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>43</td>
<td>2 (4.6)</td>
<td>41 (95.4)</td>
<td></td>
</tr>
</tbody>
</table>

An association between tumour-associated B7-H4 expression and clinicopathological features has been recently found in prostate, renal cell, and esophageal cancers. In prostate cancer, strong expression of B7-H4 is positively correlated with extra capsular extension, seminal vesicle invasion, and distant metastasis [21]. In clear-cell renal cell cancer, patients with B7-H4-positive tumors showed a poorer survival rate than those with B7-H4-negative tumors [22]. Higher B7-H4 expression was found to be significantly associated with poor prognosis of the patients suffering from gastric cancer [23]. B7-H4 expression in human esophageal squamous cell cancer was shown to be associated with cancer progression, reduced tumour immune surveillance and worse patient outcomes [24]. In this study, B7-H4 mRNA and protein level were found to be significantly higher in UCC tumour tissues compared with adjacent nontumor tissues as assessed by qRT-PCR and immunohistochemical staining, respectively. Higher B7-H4 protein levels were observed in patients with more advanced pathological stage of UCC and associated with decreased overall survival of patients with UCC. Thus, these previous studies along with our findings suggest that B7-H4 expression may serve as a universal prognostic indicator for various cancers.

Discussion

The co-regulatory B7 family members are cell-surface protein ligands, binding to receptors on lymphocytes to regulate immune responses [9]. They can provide either positive or negative signal to stimulate or inhibit T-cell activation [9]. B7-H4 is a recently identified member of the B7 family [10]. In this study, we investigated the mRNA and protein levels of B7-H4 in human UCC and analyzed the relationship between B7-H4 protein expression level and clinicopathological parameters of UCC.
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Figure 3. Kaplan-Meir curves demonstrating overall survival of 62 patients with urothelial cell carcinoma following surgery according to mRNA levels of B7-H4 (A), tumour stage (B), and tumour grade (C). P-value was determined by paired samples t-test.

Table 3. Univariate and multivariate analyses of different clinicopathological variables and B7-H4 expression status as predictors for overall survival of urothelial cell carcinoma (UCC)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>P-value</td>
</tr>
<tr>
<td>Sex (male vs. female)</td>
<td>1.341 (0.689-2.678)</td>
<td>0.354</td>
</tr>
<tr>
<td>TNM stage (high vs. low)</td>
<td>2.818 (1.545-5.032)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Grade (G3/4 vs. G1/2)</td>
<td>2.215 (1.310-4.925)</td>
<td>0.013</td>
</tr>
<tr>
<td>B7-H4 expression (high vs. low)</td>
<td>1.561 (1.125-2.358)</td>
<td>0.006</td>
</tr>
</tbody>
</table>

CI, confidence interval; HR, hazard ratio.

Table 3. Univariate and multivariate analyses of different clinicopathological variables and B7-H4 expression status as predictors for overall survival of urothelial cell carcinoma (UCC)

In conclusion, the present study has shown for the first time that B7-H4 mRNA and protein are increased in UCC tissues and that higher B7-H4 levels are associated with advanced clinical tumour stage and shorter overall survival. The precise role of B7-H4 in UCC development and progression, however, remains to be elucidated and further investigations in cell and animal models are in progress.

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Disclosure of conflict of interest

None.

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References


