Serum-Soluble B7x Is Elevated in Renal Cell Carcinoma Patients and Is Associated with Advanced Stage

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Abstract

B7x is the newest member of the B7-CD28 family and is thought to dampen immune responses via negative costimulation. Tumor expression of B7x was recently described in renal cell carcinoma (RCC) and was associated with poor outcome. We developed an assay to detect serum-soluble B7x (sB7x) and investigated 101 patients with clear cell RCC who underwent nephrectomy between 2003 and 2007. For controls, we obtained serum from 101 sex-matched blood donors within the same age range. Following an ELISA for sB7x, detectable levels (>0.1 ng/mL) of sB7x were observed in 53 RCC patients compared with 18 controls ($P < 0.001$). Median (range) concentrations of sB7x for RCC patients and controls were 14.4 ng/mL (0.1–56.9) and 2.7 ng/mL (0.2–37.1), respectively. For RCC patients with detectable sB7x, median levels were significantly higher for patients with a tumor thrombus (19.2 versus 6.6 ng/mL; $P = 0.007$), positive lymph nodes (41.3 versus 10.3 ng/mL; $P = 0.018$), and distant metastases at nephrectomy (43.3 versus 8.5 ng/mL; $P = 0.002$) and tended to be higher in patients with high-grade tumors (18.8 versus 8.5; $P = 0.090$). Additionally, median sB7x levels for tumor-node-metastasis stage I to IV RCC were 6.6, 10.3, 14.5, and 43.3 ng/mL, respectively ($P = 0.012$). In this first evaluation of sB7x in RCC, we show that RCC patients are more likely to have detectable sB7x compared with controls and higher sB7x levels correlate with advanced tumor stage. These early results merit further investigation of this serum marker for potential diagnostic and prognostic purposes. [Cancer Res 2008;68(15):6054–8]

Introduction

A plethora of histologic features and molecules have recently been implicated as predictors of renal cell carcinoma (RCC) patient outcome (1, 2). Some of these prognostic markers additionally represent attractive moieties for targeted therapy (3–5); however, nearly all features predictive of outcome require processing and analysis of RCC tumor specimens. Although alterations of various blood variables, such as platelet count, erythrocyte sedimentation rate (ESR), and lactate dehydrogenase, are associated with malignancy (1, 2), none remains specific or aids in the diagnosis of a renal mass. Unlike tumors of the ovary (CA-125), prostate (PSA), testis (AFP, b-HCG, and LDH), colon (CEA), pancreas (CA-19-9), and liver (AFP), a standard serum marker that is useful for diagnosis or identification of early recurrence for RCC patients is lacking. Although serum proteins that might prove useful to detect the presence of advanced or recurrent RCC have been reported (2), none has translated into standard of care management for RCC patients. As such, there is a pressing need for novel serum markers to improve on the management and therapy of RCC.

RCC is an immunogenic tumor. Related to this, several important immune costimulatory molecules, including B7-H1 (PD-L1), PD-1, and B7x, have been observed to be expressed by tumor cells or lymphocytes that infiltrate RCC (3–6). Thus, we and others have suggested that these costimulatory molecules may function to undermine host antitumor immune surveillance to facilitate cancer progression, a hypothesis that is supported by disease progression and diminished survival following surgical extirpation of RCC tumors that express these costimulatory molecules (3–6). Discovered in 2003, B7x (B7-H4, B7S1) represents the newest member of the B7-CD28 family of costimulatory ligands (7–9). Experimental evidence to date indicates that B7x down-regulates peripheral immune responses via negative T-cell costimulation (7–10). Additionally, B7x has recently been reported to be aberrantly expressed by RCC tumor cells and was associated with metastatic disease progression and poor survival (3). Prompted by these observations, we developed a serum assay that detects soluble B7x and investigated the serum of patients with clear cell RCC as well as the serum of matched blood donor controls. Herein, we show that serum-soluble (sB7x) is much more likely to be detectable in RCC patients compared with controls. In addition, our study indicates that higher levels of sB7x are associated with tumor extension such as tumor thrombus, nodal and distant metastases, and advanced tumor stage. These results provide a strong rationale for further investigation of sB7x for diagnostic, prognostic, and potentially therapeutic purposes in RCC.

Materials and Methods

Patient selection. After obtaining Institutional Review Board approval, we identified 101 patients with clear cell RCC treated surgically at the Mayo Clinic between 2003 and 2007 who provided a preoperative serum sample. Preoperative collection of serum for consenting patients was initiated at the Mayo Clinic beginning in 2003. Each serum sample was stored at −80°C and 200 μL from each specimen were thawed, aliquoted, and sent overnight to Memorial Sloan-Kettering Cancer Center where a sandwich ELISA for the

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doi:10.1158/0008-5472.CAN-08-0869

detection of sB7x was performed, blinded to patient health information and pathologic features. In addition, sera from 101 sex-matched blood donors within the same age range were purchased from Innovative Research. ELISA for the detection of sB7x was also performed for these patients who served as controls for this study.

Sandwich ELISA detection for B7x. High-binding polystyrene plates (Corning, Inc.) were coated overnight at 4°C with 0.2 μg/well of anti-B7x (clone H74; eBioscience). The coating solution was tipped off and free binding sites were blocked with 200 μL/well of SuperBlock blocking buffer (Pierce Biotechnology) and 10% bovine serum for 1 h at room temperature. After washing thrice with PBS + 0.05% Tween 20, 25 μL were added to each well followed by 25 μL of standards and human serum in triplicate. B7x protein, produced as previously reported (7), was diluted in bovine serum to 100, 50, 25, 10, 2, 0.5, and 0.1 ng/mL and used as a standard. After 90 min of incubation at room temperature, the plates were washed thrice with PBS-Tween 20 and incubated with 50 μL (2 μg/mL) of biotinylated anti-B7x (R&D Systems) for 1 h at room temperature. After washing thrice with PBS-Tween 20, peroxidase streptavidin, diluted to 1:1,000 with bovine serum albumin-PBS + Tween 20, was added at 50 μL/well. After 30 min at room temperature, the plates were washed thrice with PBS-Tween 20 followed by one time with PBS. Then, ABTS substrate plus 1:1,000 30% H_2O_2 were added at 100 μL/well and allowed to develop at room temperature for 20 to 30 min. The plates were then read at 405 nm using a SpectraMax M2 fluorescence absorbance cuvette (Molecular Devices). The minimum detectable concentration was determined to be >0.1 ng/mL.

Laboratory, clinical, and pathologic features. Preoperative laboratory values, clinical features at presentation, and pathologic features of the RCC tumors were obtained from the Mayo Clinic Nephrectomy Registry. This registry is prospectively maintained with over 300 variables abstracted for each patient by a registered nurse abstractor. The laboratory values studied included serum creatinine, hemoglobin, and ESR. Clinical features included age, sex, local or systemic symptoms at presentation, microscopic or gross hematuria at presentation, and Eastern Cooperative Oncology Group (ECOG) performance status. Pathologic features of the RCC tumors included size, the 2002 primary tumor classification, presence of tumor thrombus, regional lymph node involvement, distant metastases, the 2002 tumor-node-metastasis (TNM) stage groupings, nuclear grade, coagulative tumor necrosis, sarcomatoid differentiation, and multifocality.

Statistical methods. Associations of the presence of and levels of sB7x with these features were evaluated using Spearman rank correlation coefficients and Kruskal-Wallis, Wilcoxon rank sum, Fisher's exact, and χ^2 tests. Because clinical follow-up for the patients included in this study is in its infancy (median follow-up is approximately 1 y because most of the serum samples came from patients treated in 2006 or 2007), associations with outcome were not evaluated. Statistical analyses were performed using the Statistical Analysis System software package (SAS Institute). All P values were two sided and $P < 0.05$ was considered statistically significant.

Results

Analysis of sB7x in RCC versus control patients. Among the 101 RCC and 101 control patient samples studied, sB7x was detected in 53 and 18 patients, respectively ($P < 0.001$). The average detectable (±SD) sB7x level for patients with RCC was 16.3 ± 16.4 ng/mL, with a median of 14.4 and a range of 0.12 to 56.9 ng/mL. In contrast, the average sB7x for controls was 6.8 ± 9.3 ng/mL, with a median of 2.7 and a range of 0.17 to 37.1 ng/mL.

Analysis of RCC patients. Among the 101 patients with RCC, the median age was 63 (range, 28–90) and median tumor size was 5.6 cm (range, 1.2–21.5). The median (range) preoperative laboratory features for RCC patients were 1.1 mg/dL (0.6 –2.8) for serum creatinine, 14.0 (8.9–17.2) for hemoglobin, and 11 (0–135) for ESR. The remaining clinical and pathologic features are summarized in Table 1. Associations of the presence of sB7x with laboratory, clinical, and pathologic features are summarized in Table 2. None of the features studied was statistically significantly associated with the presence versus absence of sB7x.

Analysis of RCC patients with detectable sB7x. Among the 53 cases with detectable sB7x, sB7x levels tended to increase as tumor extent increased (Table 3). For example, median sB7x levels for RCC patients with and without tumor thrombus were 19.2 and 6.6 ng/mL, respectively ($P = 0.007$). sB7x levels were also significantly higher for RCC tumors that extended into the regional lymph nodes ($P = 0.018$) or had metastasized by the time of nephrectomy ($P = 0.002$). When these features were combined together, sB7x levels increased with increasing 2002 TNM stage group ($P = 0.012$; Fig. 1). Additionally, patients with high-grade RCC were more likely to have higher sB7x levels (median, 18.8 ng/dL) compared with low-grade RCC (median, 8.5 ng/dL), although this difference did not reach statistical significance ($P = 0.090$). There were no statistically significant associations between the level of sB7x and age (Spearman rank correlation coefficient, −0.03; $P = 0.829$), serum creatinine (0.03; $P = 0.840$), hemoglobin (−0.10; $P = 0.464$), ESR (−0.07; $P = 0.641$), or tumor size (0.13; $P = 0.337$).

Table 1. Clinical and pathologic features for 101 RCC patients

Feature	*n*
Sex	
Male	65
Female	36
Local or systemic symptoms	46
Gross or microscopic hematuria ($n = 96$)	39
ECOG performance status ($n = 100$)	
0	90
1	3
2	5
3	2
2002 primary tumor classification	
pT1a	31
pT1b	26
pT2	11
pT3a	7
pT3b	24
pT3c	1
pT4	1
Tumor thrombus	26
Regional lymph node involvement	
pNX/pN0	94
pN1/pN2	7
Distant metastases	
pM0	89
pM1	12
2002 TNM stage grouping	
I	56
II	8
III	24
IV	13
Nuclear grade	
1	11
2	50
3	34
4	6
Coagulative tumor necrosis ($n = 100$)	27
Sarcomatoid differentiation ($n = 99$)	1
Multifocality	6

Table 2. Associations of presence of sB7x with laboratory, clinical, and pathologic features for 101 RCC patients

	sB7x		
	Absent (n = 48)	Present (n = 53)	
Feature	Median (range)		P
Serum creatinine	1.1 (0.8–2.6)	1.1 (0.6–2.8)	0.818
Hemoglobin	14.0 (10.2–17.2)	14.2 (8.9–16.6)	0.884
ESR	14 (0–116)	8 (1–135)	0.358
Age at surgery (y)	65.5 (28–83)	60 (41–90)	0.426
Tumor size (cm)	6.0 (1.3–21.5)	5.5 (1.2–19.6)	0.706
Feature	n (%)		P
Sex			
Male	32 (66.7)	33 (62.3)	0.645
Female	16 (33.3)	20 (37.7)	
Local or systemic symptoms	22 (45.8)	24 (45.3)	0.956
Gross or microscopic hematuria (n = 96)	20 (43.5)	19 (38.0)	0.585
ECOG performance status (n = 100)			
0	45 (93.8)	45 (86.5)	0.322
>0	3 (6.3)	7 (13.5)	
2002 primary tumor classification			
pT1a and pT1b	28 (58.3)	29 (54.7)	0.733
pT2	4 (8.3)	7 (13.2)	
pT3a, pT3b, pT3c, and pT4	16 (33.3)	17 (32.1)	
Tumor thrombus	12 (25.0)	14 (26.4)	0.871
Regional lymph node involvement			
pNX/pN0	45 (93.8)	49 (92.5)	1.00
pN1/pN2	3 (6.3)	4 (7.6)	
Distant metastases			
pM0	42 (87.5)	47 (88.7)	0.855
pM1	6 (12.5)	6 (11.3)	
2002 TNM stage grouping			
I	27 (56.3)	29 (54.7)	0.913
II	3 (6.3)	5 (9.4)	
III	11 (22.9)	13 (24.5)	
IV	7 (14.6)	6 (11.3)	
Nuclear grade			
1 and 2	28 (58.3)	33 (62.3)	0.687
3 and 4	20 (41.7)	20 (37.7)	
Coagulative tumor necrosis (n = 100)	13 (27.7)	14 (26.4)	0.889
Sarcomatoid differentiation (n = 99)	0	1 (1.9)	1.00
Multifocality	3 (6.3)	3 (5.7)	1.00

Discussion

Serum assays to aid in the assessment and, more importantly, treatment of patients with renal cancers are currently lacking. We describe an assay that detects a soluble form of B7x, a protein that when expressed by tumor cells is thought to dampen host immunity by triggering inhibitory T-cell signaling. In addition, we show that sB7x is more likely to be detected in the sera of RCC patients relative to healthy control patients. Our study further suggests that elevated levels of sB7x correlate with more aggressive tumor progression and advanced TNM stage. The present observations, combined with a previous demonstration that tumor cell expression of B7x predicts poor outcome (3), further suggest that B7x functions as a negative regulator of immunity in the clinical setting. This initial description of sB7x in RCC warrants further investigation of this assay for diagnostic and prognostic purposes, especially given the lack of clinically useful serum markers currently available for RCC patients.

Discovered in 2003, B7x is the newest member of the B7-CD28 family (7–9). To date, *in vitro* and *in vivo* studies consistently implicate B7x as a costimulatory inhibitor of $CD4^+$ and $CD8^+$ T-cell proliferation, cell cycle progression, interleukin-2 production, and antitumor immunity (7–11). Despite widespread B7x mRNA expression in various human tissues, the lack of immunohistochemical staining of B7x in most normal human organs indicates that expression of this protein is relatively restricted (12). B7x is a type I transmembrane protein; however, its cognate immune cell receptor has not yet been identified. Aberrant expression of B7x has been observed in multiple malignancies, including cancers of the breast (11, 13), lung (14), ovary (11, 15), uterus (16), prostate (17), and kidney (3). Thus, B7x has been proposed as an attractive

potential therapeutic target for human malignancies, including RCC.

Simon and colleagues (18, 19) recently used an ELISA method to establish that sB7x concentrations in ovarian cancer patients are significantly elevated relative to healthy blood donors. Additionally, this group concluded that sB7x is specific for ovarian cancer because elevated levels of sB7x could not be detected in the sera of patients with colon, breast, lung, and prostate cancer using their sB7x ELISA method (18). Our study, in which we used a distinct capture-detection antibody set, seems to refute the claim that sB7x is a marker limited to ovarian cancer. However, we surmise that detectable levels of sB7x and higher levels of sB7x will be more likely to occur in patients with tumors known to ectopically express B7x, including the ovary (11), kidney (3), and prostate (17). However, further studies will be needed to determine whether sB7x can, in fact, be observed in the sera of patients with any form of a B7x-expressing tumor.

Several limitations to our present study merit discussion. We recognize that the overall number of patient serum specimens that we studied is relatively small. Nevertheless, that we observed statistically significant correlations between sB7x and tumor thrombus, nodal and distant metastases, and tumor stage indicates promise for sB7x as a serum marker for RCC. Larger studies are clearly warranted and may help to elucidate important potential cut points to translate sB7x into the clinical arena as a serum tumor marker. In addition, studies involving longer follow-up will be required to establish if sB7x is associated with other clinical outcomes, including RCC progression-free and cancer-specific survival. Likewise, separate investigations will be needed to determine if sB7x is a general marker for all renal masses, including benign tumors, papillary, and chromophobe RCC, or if it is a relatively specific marker for clear cell RCC (a tumor that is known to aberrantly express B7x). It should also be noted that the overlapping ranges we observed with sB7x suggest that it is unlikely

Table 3. Associations of sB7x levels with laboratory, clinical, and pathologic features for 53 RCC patients with detectable sB7x

Feature	Median (range) sB7x	*P*
Sex		
Male	14.8 (0.1–56.9)	0.762
Female	11.9 (0.3–45.3)	
Local or systemic symptoms		
No	14.4 (0.1–56.9)	0.950
Yes	12.4 (0.1–55.6)	
Gross or microscopic hematuria (n = 50)		
No	14.4 (0.2–56.9)	0.719
Yes	10.3 (0.1–55.6)	
ECOG performance status (n = 52)		
0	14.4 (0.1–56.9)	0.688
>0	1.5 (1.0–41.3)	
2002 primary tumor classification		
pT1a and pT1b	6.6 (0.1–37.9)	0.128
pT2	16.2 (0.3–47.6)	
pT3a, pT3b, pT3c, and pT4	18.4 (0.3–56.9)	
Tumor thrombus		
No	6.6 (0.1–47.6)	0.007
Yes	19.2 (1.5–56.9)	
Regional lymph node involvement		
pNX/pN0	10.3 (0.1–56.9)	0.018
pN1/pN2	41.3 (18.4–45.3)	
Distant metastases		
pM0	8.5 (0.1–55.6)	0.002
pM1	43.3 (18.4–56.9)	
2002 TNM stage grouping		
I	6.6 (0.1–37.9)	0.012
II	10.3 (0.3–19.3)	
III	14.5 (0.3–55.6)	
IV	43.3 (18.4–56.9)	
Nuclear grade		
1 and 2	8.5 (0.1–42.7)	0.090
3 and 4	18.8 (0.3–56.9)	
Coagulative tumor necrosis		
No	13.6 (0.1–55.6)	0.300
Yes	16.5 (1.0–56.9)	
Multifocality		
No	14.0 (0.1–56.9)	0.802
Yes	14.9 (1.4–34.6)	

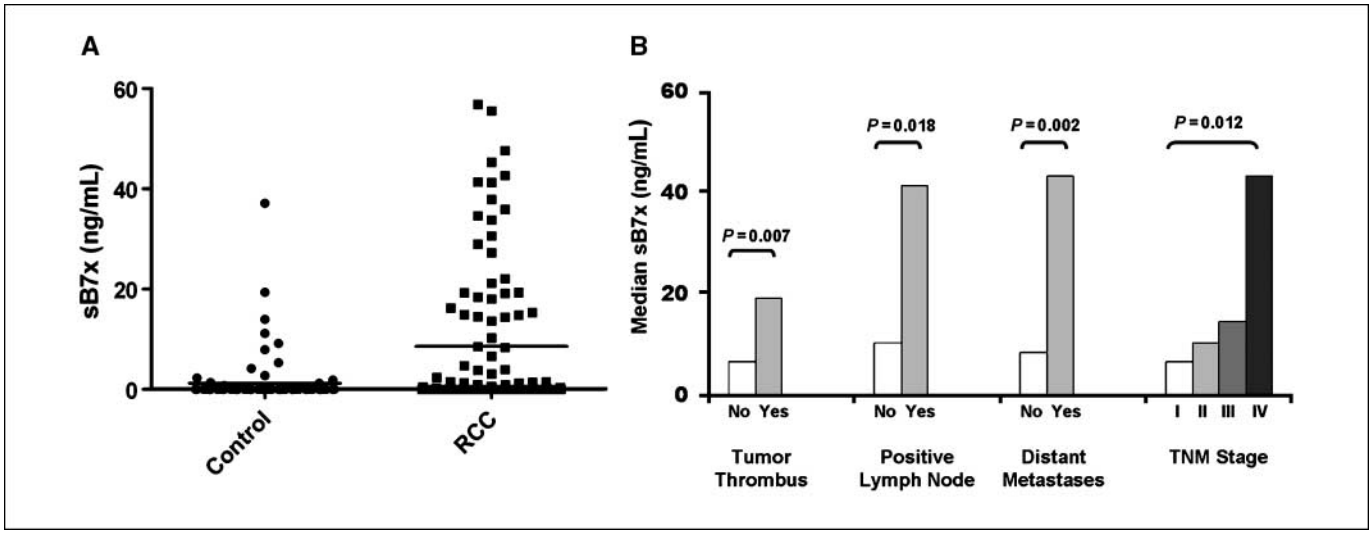

Figure 1. *A,* comparison of sB7x levels in controls and patients with RCC. The scatter plot displays the mean sB7x as horizontal lines, which are 1.2 and 8.6 ng/mL for controls and RCC patients, respectively ($P < 0.001$). *B,* comparison of sB7x concentration stratified by tumor thrombus, positive lymph nodes, distant metastases, and TNM stage grouping for 53 RCC patients with detectable levels of sB7x.

that sB7x will serve as an effective screening tool for a large population. Given that more than half of all contemporary patients now present with incidentally discovered renal masses, and that the management of incidentally found small renal tumors can be controversial, a serum marker that aids in discriminating between benign versus malignant renal tumors would have tremendous clinical utility.

In a prior study of 259 RCC patients, Krambeck and colleagues (3) reported that tumor expression of B7x correlates with multiple adverse pathologic features and diminished cancer-specific survival. Interestingly, B7x was also found to be consistently expressed on the endothelium of tumor vasculature, suggesting a potential role for B7x in tumor angiogenesis or, perhaps, neutralization of circulating antitumoral immune cells (3). Whether B7x expression by RCC vasculature facilitates systemic dissemination of sB7x remains unknown. Regardless, our present study, combined with the tissue-based study by Krambeck and colleagues (3), is consistent with the notion that B7x may function to impair host immunity, thereby facilitating tumor progression. Thus, novel approaches to target B7x in an effort to improve RCC treatment seem warranted.

Disclosure of Potential Conflicts of Interest

E.D. Kwon: patent filed for B7x (B7-H4) as prognostic marker for RCC. The other authors disclosed no potential conflicts of interest.

Acknowledgments

Received 3/6/2008; revised 5/8/2008; accepted 5/21/2008.

Grant support: NIH grant T32-CA82088.

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We thank Kim Rauen and Debra Head (Mayo Clinic, Rochester, MN) for identifying, thawing, and aliquoting the serum; the Stephen Hanson Family Fellowship for their generous support; and The Richard M. Schulze Family Foundation and The Helen and Martin Kimmel Foundation for their generous support of portions of this study.

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