PD-1 Is Expressed by Tumor-Infiltrating Immune Cells and Is Associated with Poor Outcome for Patients with Renal Cell Carcinoma

R. Houston Thompson,[1] Haidong Dong,[2] Christine M. Lohse,[3] Bradley C. Leibovich,[1] Michael L. Blute,[1] John C. Cheville,[4] and Eugene D. Kwon[1,2]

Abstract

Purpose: B7-H1 is expressed by clinically aggressive forms of renal cell carcinoma (RCC) and predicts adverse outcome. B7-H1 is known to impair host immunity via interaction with the Programmed Death-1 (PD-1) receptor, which is expressed by activated T cells. Levels of immune cells expressing PD-1 (PD-1$^+$) in clinical RCC tumors have not been evaluated. Thus, we tested whether immune cell PD-1 expression is observed within aggressive RCC tumors.

Experimental Design: Between 2000 and 2003, 267 patients underwent nephrectomy at our institution for clear cell RCC and had fresh-frozen tissue available for review. These RCC specimens were immunostained using anti – PD-1 (clone MIH4) and outcome analyses were conducted.

Results: Mononuclear immune cell infiltration was observed in 136 (50.9%) specimens. PD-1$^+$ immune cells were present in 77 of these 136 (56.6%) tumors. In contrast, RCC tumor cells did not express PD-1. Patients with PD-1$^+$ immune cells were significantly more likely to harbor B7-H1$^+$ tumor cells ($P < 0.001$), larger tumors ($P = 0.001$), and tumors of higher nuclear grade ($P = 0.001$). Likewise, intratumoral PD-1$^+$ immune cells were associated with advanced tumor-node-metastasis stage ($P = 0.005$), coagulative tumor necrosis ($P = 0.027$), and sarcomatoid differentiation ($P = 0.008$). With a median follow-up of 2.9 years, 52 patients died from RCC. Univariately, patients with PD-1$^+$ immune cells were at significant risk of cancer-specific death compared with PD-1$^-$ patients (risk ratio, 2.24; $P = 0.004$).

Conclusions: Levels of immune cells expressing PD-1 were increased in patients with high-risk RCC tumors. Interactions between immune cell PD-1 and B7-H1 may promote cancer progression by contributing to immune dysfunction in patients with RCC.

Renal cell carcinoma (RCC) is an immunogenic tumor that characteristically harbors abundant infiltrating lymphocytes (1). Several reports, however, indicate that lymphocytes within RCC tumors are often rendered dysfunctional (2–4). Thus, it seems that RCC tumors possess a potent regional ability to impair host antitumor immunity. Elucidating mechanisms responsible for immune dysfunction within the tumor microenvironment may prove useful to improve immunotherapeutic approaches for the treatment of RCC.

The Programmed Death-1 (PD-1) receptor was first described in 1992 (5) as a member of the B7 family of costimulatory molecules that modulate T cell antigen-specific receptor signaling and govern T cell activation, inactivation, and survival (6). Membranous expression of the PD-1 glycoprotein has been described in association with activated T cells, B cells, and mature dendritic cells (7). Murine studies indicate that PD-1 functions as a negative regulator of immune responses, as is supported by the development of autoimmune glomerulonephritis, arthritis, and cardiomyopathy in PD-1 knockout mice (8).

One known ligand for PD-1, B7-H1 (PD-L1, CD274), has been extensively studied in patients with RCC. We have reported that B7-H1 is aberrantly expressed by both primary and metastatic RCC tumor cells (9, 10). When present in RCC tumors, B7-H1 portends adverse pathology, aggressive tumor behavior, and poor survival in patients with RCC with long-term follow-up (11). A putative mechanism whereby B7-H1 may worsen the clinical behavior RCC tumors may be via impairment of host antitumor immunity. Specifically, B7-H1 is known to down-regulate immune responses, at least in part, by interacting with the inhibitory PD-1 receptor that is expressed by activated T cells as well as other immune cells.

Authors' Affiliations: Departments of [1]Urology, [2]Immunology, [3]Health Sciences Research, and [4]Laboratory Medicine and Pathology, Mayo Medical School, Mayo Clinic, Rochester, Minnesota

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Note: R.H. Thompson and H. Dong contributed equally to this work.

Requests for reprints: Eugene D. Kwon, Departments of Urology and Immunology, Mayo Clinic, 200 First Street, SW Rochester, MN 55905. Phone: 507-284-8371; Fax: 507-284-4987; E-mail: kwon.eugene@mayo.edu.

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To date, however, levels of intratumoral PD-1 expression have not yet been systematically linked to pathologic features or outcome for patients with malignancy, particularly in patients with RCC. Because tumor cell B7-H1 expression portends a poor prognosis for patients with RCC and inhibits immune cell function by interacting with PD-1 (as well as a putative non–PD-1 receptor), we surmised that levels of immune cell–associated PD-1 may be increased in high risk RCC tumors. Consistent with this, we show that the presence of tumor-infiltrating PD-1$^+$ immune cells predict adverse pathology and outcome for patients with clear cell RCC tumors.

Materials and Methods

Patient selection. Upon approval from the institutional review board, we reviewed the Mayo Clinic Nephrectomy Registry to identify 267 patients treated with radical nephrectomy or nephron-sparing surgery for unilateral, sporadic, noncystic clear cell RCC between 2000 and 2003. In addition, patients were selected based on the availability of fresh-frozen tissue because the PD-1 antibody used in this study (clone MIH4) has only been optimized to stain fresh-frozen, not paraffin-fixed, tissue during immunohistochemical analysis. A registered nurse abstractor assigned to the Nephrectomy Registry used a variety of means to update disease status and patient vital status annually, including a review of the Mayo medical record, cross-links with the Mayo Tumor Registry, and letters to both the patient and local physician. For those patients who had died, the cause of death was determined using the underlying cause of death listed on the death certificate or, when necessary, from direct correspondence with the local physician. To date, <3% of patients in the Nephrectomy Registry have been lost to follow-up.

Clinical and pathologic features. The clinical features studied included age, sex, and symptoms. Patients with a palpable flank or abdominal mass, discomfort, gross hematuria, acute onset varicocele or constitutional symptoms were considered symptomatic at presentation. The pathologic features studied included histologic subtype, tumor size, the 2002 primary tumor classification, regional lymph node involvement, distant metastases at nephrectomy, the 2002 tumor-node-metastasis stage groupings, nuclear grade, coagulative tumor necrosis, sarcomatoid differentiation, mononuclear cell infiltration (recorded as absent, focal, moderate, or marked), and tumor B7-H1 expression (as previously reported; ref. 9). These features were obtained by a review of all microscopic slides from the nephrectomy specimens by a urologic pathologist (J.C. Cheville), without knowledge of patient outcome.

Immunohistochemistry and scoring. Immunohistochemistry was done on fresh-frozen tissue using a mouse anti-human PD-1 monoclonal antibody (clone MIH4) in a method previously described (12). The slides were then reviewed by a urologic pathologist (J.C. Cheville) who assessed the percentage of tumor cells or tumor-infiltrating mononuclear immune cells expressing PD-1 in 5% increments, while remaining blinded to tumor B7-H1 status and patient outcome.

Statistical methods. Comparisons among the clinical and pathologic features were evaluated using χ^2 and Fisher's exact tests. Overall and cancer-specific survival was estimated using the Kaplan-Meier method. The duration of follow-up was calculated from the date of surgery to the date of death or last known follow-up. The associations of immune cell PD-1 expression with outcome were evaluated using Cox proportional hazards regression models univariately, and after adjusting for mononuclear immune cell infiltration and the Mayo Clinic Stage, Size, Grade, and Necrosis Score—a prognostic composite score specifically developed for patients with the clear cell RCC subtype (13). These associations were summarized using risk ratios and 95% confidence intervals. Statistical analyses were done using the SAS software package (SAS Institute, Cary, NC). All tests were two-sided and $P < 0.05$ were considered statistically significant.

Results

Pathologic review. Among the 267 patients, mononuclear immune cell tumor infiltration was absent in 131 (49.1%) specimens, focally present in 58 (21.7%), moderately present in 59 (22.1%), and markedly present in 19 (7.1%) patients. Expression of PD-1 (PD-1$^+$) by immune cells was observed in 77

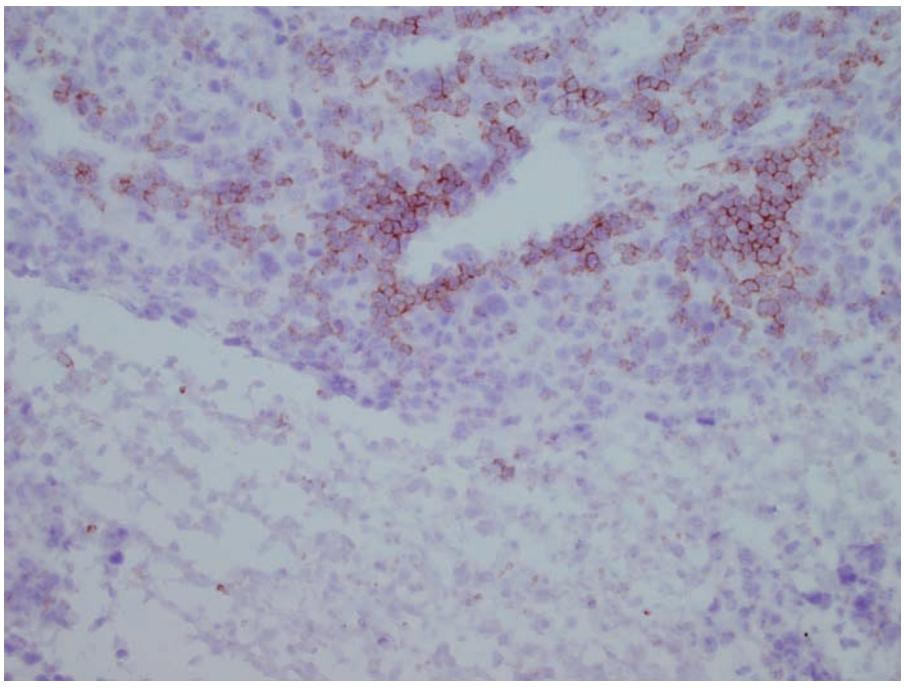

Fig. 1. Photomicrograph of clear cell RCC tumor-infiltrating mononuclear immune cells (original magnification, ×200). The cell surface brown reactivity represents expression of PD-1. *Bottom left,* coagulative tumor necrosis.

Table 1. Clinical and pathologic features by tumor-infiltrating immune cell PD-1 expression for 267 patients with clear cell RCC

Feature	PD-1^{-}* (*n* = 190)	PD-1^{+}* (*n* = 77)	*P*
Age at surgery (y)			
<65	102 (53.7)	36 (46.8)	0.305
≥65	88 (46.3)	41 (53.2)	
Sex			
Female	71 (37.4)	19 (24.7)	0.047
Male	119 (62.6)	58 (75.3)	
Symptoms at presentation	92 (48.4)	48 (62.3)	0.039
Primary tumor size (cm)			
<5	87 (45.8)	18 (23.4)	0.001
5 to <10	69 (36.3)	33 (42.9)	
≥10	34 (17.9)	26 (33.8)	
2002 Primary tumor classification			
pT_1	116 (61.0)	30 (39.0)	0.011
pT_2	22 (11.6)	17 (22.1)	
pT_3	51 (26.9)	29 (37.7)	
pT_4	1 (0.5)	1 (1.3)	
Regional lymph node involvement	8 (4.2)	7 (9.1)	0.143
Distant metastases at nephrectomy	28 (14.7)	14 (18.2)	0.484
2002 Tumor-node-metastasis stage groupings			
I	112 (59.0)	29 (37.7)	0.005
II	17 (9.0)	13 (16.9)	
III	33 (17.4)	20 (26.0)	
IV	28 (14.7)	15 (19.5)	
Nuclear grade			
1	12 (6.3)	2 (2.6)	0.001
2	73 (38.4)	17 (22.1)	
3	92 (48.4)	42 (54.6)	
4	13 (6.8)	16 (20.8)	
Coagulative tumor necrosis	46 (24.2)	29 (37.7)	0.027
Sarcomatoid differentiation	2 (1.0)	6 (7.8)	0.008
Positive tumor B7-H1 expression	83 (43.7)	59 (76.6)	<0.001

*Values expressed as *n* (%).

(56.6%) of the 136 patients whose tumors contained mononuclear immune cell infiltrates (Fig. 1), with a median expression level of 20% (range, 1-80). Tumor cell expression of PD-1 was not demonstrated. Among patients with focal, moderate, and marked mononuclear cell infiltration, PD-1^{+} immune cells were shown in 22 (37.9%), 38 (64.4%), and 17 (89.5%) specimens, respectively ($P < 0.001$). A comparison of clinical and pathologic features by PD-1 expression for all 267 patients is shown in Table 1. Patients with intratumoral PD-1^{+} immune cells were significantly more likely to have symptoms at presentation ($P = 0.039$) and larger tumors ($P = 0.001$). RCC tumors in those patients with PD-1^{+} immune cells were also observed to be of higher nuclear grade ($P = 0.001$), more advanced tumor-node-metastasis stage ($P = 0.005$), and exhibited B7-H1^{+}–expressing tumor cells ($P < 0.001$), the presence of coagulative necrosis ($P = 0.027$), and sarcomatoid differentiation ($P = 0.008$).

Clinical outcome. At last follow-up, 69 of the 267 patients studied had died, including 52 patients who died from RCC at a median of 1.3 years following surgery (range, 0-4.5). Among the 198 patients who were still alive at last follow-up, the median duration of follow-up was 2.9 years (range, 0-5.6). Overall survival rates (SE, number still at risk) at 1, 2, and 3 years following surgery were 89.8% (1.9%, 238), 80.3% (2.5%, 184), and 74.3% (2.9%, 101), respectively. Cancer-specific survival rates (SE, number still at risk) at the same time points were 91.6% (1.7%, 238), 83.8% (2.3%, 184), and 79.8% (2.7%, 101), respectively.

Univariately, patients with tumors infiltrated by PD-1^{+} immune cells were significantly more likely to die from RCC compared with patients without intratumoral PD-1^{+} immune cells (risk ratio, 2.24; 95% confidence interval, 1.30-3.86; $P = 0.004$; Fig. 2). In addition, patients with PD-1^{+} immune cells were significantly more likely to die from any cause compared with patients with tumors lacking PD-1^{+} immune cells (risk ratio, 1.81; 95% confidence interval, 1.12-2.92; $P = 0.015$). After adjusting for mononuclear immune cell infiltration, patients with tumors infiltrated by PD-1^{+} immune cells were 81% more likely to die from RCC, although this increased risk of death was not statistically significant (risk ratio, 1.81; 95% confidence interval, 0.89-3.69; $P = 0.100$). In addition, after adjusting for the Stage, Size, Grade, and Necrosis Score, the presence of PD-1^{+} immune cells was not statistically significantly associated with death from RCC (risk ratio, 1.66; 95% confidence interval, 0.96-2.88; $P = 0.071$).

Discussion

We show that PD-1 is expressed by mononuclear immune cells that infiltrate the RCC tumor microenvironment. Another novel observation reported in this study is that RCC tumors infiltrated by PD-1^{+} immune cells are significantly more likely to exhibit adverse pathologic features including increased tumor size, higher nuclear grade, and advanced tumor-node-metastasis stage. Moreover, despite the relatively short duration of follow-up for the patients studied, we observed that patients with RCC tumors infiltrated by PD-1^{+} immune cells were at significantly increased risk of cancer-specific death and overall mortality in univariate analyses. With longer follow-up, intratumoral immune cell PD-1 expression may prove to be an independent predictor of survival for patients with RCC tumors. In previous studies, we showed that expression of B7-H1 by RCC tumor cells was independently associated with poor outcome, including progression to metastases, cancer-specific death, and overall mortality (9–11). Because B7-H1 is one of the known ligands for PD-1, our work provides further evidence that the B7-H1/PD-1 pathway may function at the clinical level to impair immune surveillance, and thus, foster tumor progression.

Discovered in 1992 by Ishida et al., PD-1 is a cell surface glycoprotein within the B7 family of T cell costimulatory molecules (5). In contrast to CTLA-4, CD-28, and ICOS—which are all obligate disulfide-linked homodimers—PD-1 is monomeric, both in solution and on the cell surface (14). The cytoplasmic region for PD-1 contains an immunoreceptor tyrosine–based inhibitory motif and an immunoreceptor tyrosine–based switch motif, indicating a negative regulatory function (14). Cell surface expression of PD-1 has been observed on activated T cells, stimulated macrophages, and mature dendritic cells (7). PD-1 protein can also accumulate within the cytoplasm of natural regulatory T cells, becoming up-regulated to the cell surface following TCR activation (15). The known ligands for PD-1 include B7-H1 (PD-L1, CD274) and B7-DC (PD-L2, CD273; ref. 6). The broad distribution of these ligands suggests that the PD-1/B7-H1/B7-DC family

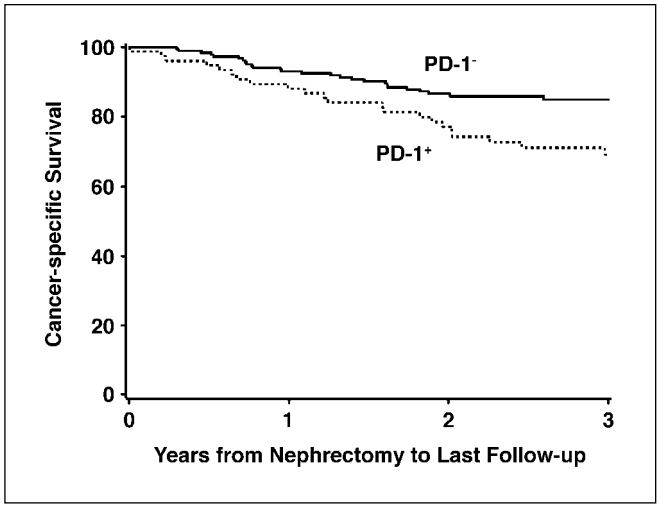

Fig. 2. Association of PD-1 expression with cancer-specific survival for 267 patients with clear cell RCC. The cancer-specific survival rates (SE, number still at risk) at 1, 2, and 3 yrs following nephrectomy were 93.0% (1.9%, 172), 86.6% (2.6%, 130), and 84.9% (2.8%, 66), respectively, for patients with PD-1$^-$ tumor-infiltrating mononuclear cells compared with 88.1% (3.7%, 66), 77.0% (4.9%, 54), and 69.7% (5.6%, 35), respectively, for patients with PD-1$^+$ tumor-infiltrating mononuclear cells ($P = 0.004$).

may regulate immune responses in both lymphoid and nonlymphoid organs (6). In addition, compared with other B7 costimulatory members, PD-1 is expressed at a relatively late phase following T cell activation, suggesting that the PD-1 pathway may primarily function at sites of inflammation in peripheral organs (14).

In vitro and *in vivo* studies show that engagement of PD-1 by B7-H1 inhibits T cell proliferation, survival, or function, whereas blockade of PD-1 promotes the generation of autoreactive T cells as well as greater autoantibody production (14). In addition, PD-1–deficient mice develop a lupus-like proliferative arthritis and glomerulonephritis that is accompanied by dilated cardiomyopathy secondary to T and B cell dysregulation (14). Additional studies show that patients with rheumatoid arthritis or Sjögren's syndrome harbor higher percentages of PD-1$^+$ lymphocytes relative to healthy subjects (14). More recently, Barber et al. reported that in mice, chronic lymphocytic choriomeningitis infection produces "exhausted" viral-specific CD8+ T cells that bear up-regulated levels of PD-1 (16). Interestingly, we observed that patients with PD-1$^+$ lymphocytes were significantly more likely to have microscopic coagulative tumor necrosis, raising the possibility that exhausted PD-1$^+$ immune cells and necrosis are governed by similar inflammatory mediators (Fig. 1). In other studies, PD-1 has been reported to be up-regulated in HIV-specific CD8+ T cells, and blockade of the PD-1/B7-H1 pathway has been shown to enhance the capacity for these cells to proliferate and survive (17–19). Collectively, the aforementioned studies support that PD-1 functions as a negative regulator of immune responses (particularly, T cell–mediated immunity), and that blockade of PD-1 may enhance antitumor and antiviral immunity.

RCC is an immunogenic tumor associated with high levels of infiltrating mononuclear immune cells comprised by a predominance of T cells. Paradoxically, however, increased T cell infiltration of RCC tumors confers a poor prognosis (1). Related to this, infiltrating lymphocytes within RCC tumors are often impaired and incapable of mediating complete tumor rejection (2–4). Taken together, these observations suggest that RCC tumors possess local mechanisms to undermine antitumor immunity. We have recently reported that B7-H1 is expressed by RCC tumor cells and is independently associated with aggressive RCC biology and poor cancer-specific survival (11). In the current study, we show that PD-1 is expressed by infiltrating mononuclear immune cells within RCC tumors. Additionally, we show that patients with RCC tumors infiltrated by PD-1$^+$ immune cells are significantly more likely to also harbor B7-H1$^+$ tumor cells. In fact, 95% of patients whose tumors were infiltrated by PD-1$^+$ immune cells also contained B7-H1$^+$ tumor cells or immune cells (data not shown). These results are consistent with the notion that the PD-1/B7-H1 pathway may, at least in part, contribute to the profile of immunosuppression observed in patients with RCC. These findings also confirm that at least one immunoinhibitory target for tumor cell B7-H1, i.e., PD-1, is readily expressed by infiltrating mononuclear immune cells within RCC tumors. Taken together, the results of the current study suggest that the PD-1/B7-H1 pathway may be operational to promote aggressive RCC progression in the clinical setting.

One limitation of this study warrants discussion. The PD-1 antibody employed in our present study has only been optimized for the immunohistochemical staining of fresh-frozen tissues. For RCC, such tissues have only been available within our institution since early 2000. Therefore, the duration of follow-up for patients in the current study is relatively short. Nevertheless, we observed a statistically significant association between tumor-infiltrating immune cell PD-1 expression and patient survival in univariate analyses, although not in a multivariate setting. Longer follow-up will be required to determine whether PD-1 represents an independent prognostic feature for patients with RCC.

Conclusion

Patients with RCC tumor-infiltrating PD-1$^+$ immune cells harbor aggressive tumors associated with unfavorable pathology and are at an increased risk of poorer survival. The PD-1/B7-H1 pathway may be operational to impair antitumor immunity, thereby promoting aggressive RCC progression in the clinical setting. Blockade of B7-H1 or PD-1 may facilitate antitumor immunotherapeutic responses to improve overall treatment for clinical RCC.

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