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T-cell Co-regulatory Molecule Expression in Renal Angiomyolipoma and Pulmonary Lymphangioleiomyomatosis

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Abstract

OBJECTIVES—To investigate the expression of B7-H3 and B7-H1 in renal angiomyolipoma (AML) tumors and the related, devastating syndrome of pulmonary lymphangioleiomyomatosis (LAM). We recently reported the high expression of T-cell co-regulatory B7-H ligands in renal cell carcinoma tumor vasculature and tumor cells. AML is a highly vascular tumor that most frequently emanates from the kidney. Events leading to its pathogenesis remain enigmatic and understudied.

METHODS—Immunohistochemical methods were used to assess the tumor expression of B7-H1 and B7-H3 in paraffin-embedded tissues from 110 patients who had undergone partial or radical nephrectomy for renal AML and from 7 patients with LAM who had undergone lung biopsy.

RESULTS—B7-H3 was expressed by 100% of the AML and LAM specimens, and B7-H1 expression was detected in only 2.7% of the specimens studied. Both membranous and cytoplasmic B7-H3 expression was noted in the smooth muscle, blood vessel, and lipoid cell components of the tumors; however, no expression was detected in the adjacent, normal parenchyma tissue. B7-H3 staining was noted in a median of 90% (range 20%–100%) of cells from renal AMLs and was independent of patient age ($P = .43$), sex ($P = .27$), tumor size ($P = .21$), and symptomatic presentation ($P = .35$).

CONCLUSIONS—B7-H3 was expressed at high levels in renal AMLs and pulmonary LAM, and B7-H1 was infrequently expressed in these tumors. Additional studies are needed to evaluate the utility of B7-H3 as a diagnostic marker or immune/angiogenic target to improve the management of AML and the potentially devastating condition of LAM, for which effective treatment is lacking.

T-cell co-regulatory molecules are constitutively present on antigen-presenting cells and function to regulate intracellular signaling pathways mediating antigen-specific T-cell responses. Although expression of these co-regulatory molecules is typically limited to lymphoid cells, aberrant co-regulator expression has been reported in association with a number of human malignancies.^{1–7} Because T cells play an integral role in mediating antitumor immunity, aberrant expression of co-regulatory molecules has been cited as a potential mechanism by which tumors might evade host immune surveillance, for example, by inducing T-cell anergy or apoptosis.^{3,8}

One particular group of co-regulators whose expression has been demonstrated in various cancers is the B7 family of ligands. We have reported that B7-H1 and B7-H4 are aberrantly

expressed by renal cell carcinoma (RCC) cells and that the expression of these ligands correlated with advanced tumor pathologic features and adverse clinical outcomes.^{1,4,5} Moreover, we recently reported evidence of abundant B7-H3 and B7-H4 expression in the vasculature of RCC and demonstrated associations of B7-H vascular expression with aggressive RCC pathologic features and decreased cancer-specific survival.^{1,9}

Angiomyolipoma (AML) is a tumor of the kidney consisting of fat, blood vessels, and smooth muscle that, for the most part, is considered benign. These lesions have a propensity to grow locally,¹⁰ however, resulting in complications such as flank pain, hematuria, hypertension, and retroperitoneal hemorrhage. Although AMLs are classically associated with the tuberous sclerosis (TS) complex, approximately 70%–80% of renal AMLs occur sporadically,¹¹ predominantly in women in their fourth to seventh decade.

The etiology of AML tumors remains poorly understood, because although linkage analysis has identified 2 genes associated with the TS complex, TSC1 on chromosome 9q34¹² and TSC2 on chromosome 16p13,^{13,14} the function of these gene products in the development of renal AML has not been well defined. AML has also been observed in association with other lesions that exhibit malignant-type behavior. Thus, it has been proposed that the perivascular epithelioid cell (PEC), a histologically and immunohistochemically distinctive cell type, might be a progenitor cell for AMLs and a family of related lesions collectively known as PEComas,^{15,16} which includes pulmonary lymphangioleiomyomatosis (LAM).

LAM is a rare, aggressive disease seen almost exclusively among women of reproductive age in which diffuse, bilateral proliferation of abnormal smooth muscle cells occurs in the lungs and leads to progressive respiratory failure.^{17,18} The abnormal and highly proliferative smooth muscle cells of pulmonary LAM and renal AML have been found to be nearly identical.^{19,20} As such, it has been further postulated that LAM might encompass malignant-like metastases that emanate from AML tumors. Regardless, the pathogenesis of LAM remains poorly understood, and effective treatment of this condition is desperately lacking.^{19,20} Thus, additional studies of this aggressive disorder are needed to elucidate novel systemic therapies.

Given the common postulated origin of PEComa tumor cells, together with the high levels of B7-H ligand expression reported in RCC tumor vasculature,^{1,9} we investigated the expression of the T-cell co-regulatory molecules B7-H1 and B7-H3 in renal AML and pulmonary LAM.

MATERIAL AND METHODS

Patient Selection

On approval from the Mayo Clinic institutional review board, we reviewed our institutional Nephrectomy Registry to identify all patients treated with radical nephrectomy or nephron-sparing surgery for renal AML from 1970 to 2005. All specimens were pathologically confirmed as AML and were subtyped according to the histologic variant of AML²¹ on pathologic review by one of us (Y.S.). Seven patients with a diagnosis of LAM in whom lung tissue from a biopsy or resection was available were also identified.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissues were cut into 5- μ m sections, deparaffinized, and rehydrated in a graded series of ethanols. Staining of paraffin-embedded AML sections for B7-H1 was performed as previously described.⁵ For B7-H3, antigen retrieval was performed by heating the AML and LAM tissue sections in 1 mM ethylenediaminetetraacetic acid (pH 8) to 121°C using a Digital Decloaking Chamber (Biocare Medical, Walnut Creek, CA), cooling to 90°C, and incubating for 5 minutes. The sections were washed in Wash Buffer (Dako, Carpinteria, CA) before being placed onto the Autostainer Plus (Dako).

The sections were blocked for endogenous peroxidase for 5 minutes using endogenous Blocking Solution (Dako), washed twice, and incubated for 5 minutes in Serum-Free Protein Block (Dako) followed by incubation for 60 minutes in 100 $\mu\text{g/mL}$ purified goat anti-human B7-H3 antibody (R&D Systems, Minneapolis, MN) diluted 1:80 with da Vinci Green antibody diluent (Biocare Medical). The sections were incubated for 15 minutes in probe from the Goat HRP-Polymer Kit (Biocare Medical, No. GHP516L), washed, and incubated for 15 minutes with polymer from the Goat HRP-Polymer Kit. The sections were visualized by incubating in Betazoid DAB (Biocare Medical) for 5 minutes. Then they were counterstained with hematoxylin, dehydrated in ethanol, cleared in xylene, and coverslipped.

B7-H1 and B7-H3 Quantification

The percentages of tumor cells that stained positive for B7-H1 were quantified in 5% increments in accordance with previous studies of B7-H1 expression in RCC.⁵ AMLs were considered positive for B7-H1 if histologic evidence was present of cell-surface membrane staining in >5% of cells; cases with <5% tumor staining were considered negative. B7-H3 expression was quantified in 10% increments, consistent with our previous evaluation of B7-H3 expression in RCC,⁹ with cases demonstrating <10% tumor staining considered negative. Quantitation was done by a pathologist (Y.S.) without knowledge of the patient outcomes.

Statistical Analysis

The associations of B7-H3 staining with the clinicopathologic features were evaluated using Spearman rank correlation coefficients and Wilcoxon rank sum tests. Statistical analyses were performed using the Statistical Analysis Systems software package (SAS Institute, Cary, NC). All tests were 2-sided, and $P < .05$ was considered statistically significant.

RESULTS

A total of 124 patients underwent radical nephrectomy or nephron-sparing surgery for renal AML from 1970 to 2005 at our institution. Of these patients, 110 had tissue from the surgical specimen available for study. The patient demographics are listed in Table 1. The median patient age at surgery was 55.5 years (range 25–84), and the median size of the resected AMLs was 5.0 cm (range 0.3–44.0). Seven patients (6.4%) from our series had a diagnosis of TS.

We detected B7-H1 expression in 3 of 110 AMLs (2.7%); expression was detected in 5%, 10%, and 40% of the cells from these tumors. B7-H3 expression was found in the AMLs from all 110 patients, with a median staining level of 90% (range 20–100). B7-H3 immunoreactivity was noted on the cell membrane and in the cytoplasm of the tumor cells (Fig. 1A). Moreover, expression within the AMLs was diffuse: 20%–40% of tumor cells stained positive for B7-H3 in 13, 50%–70% of cells stained positive in 15, 80% stained positive in 15, 90% stained positive in 45, and 100% stained positive for B7-H3 in 16 specimens. Of the 110 specimens, 52 contained normal renal tissue adjacent to the AML, and no B7-H1 or B7-H3 expression was found in the uninvolved parenchyma in any of the samples (Fig. 1B).

Of the histologic components of the AML, intense membrane staining was noted diffusely throughout the myoid cells (Fig. 1C) and in the adventitia of the tumor blood vessels (Fig. 1D). Weak focal staining was seen in a few of the endothelial cells within the AMLs, but the blood vessels within the normal renal parenchyma showed no B7-H3 staining (Fig. 1E). Similarly, cytoplasmic B7-H3 staining was found in the lipid component of all the AMLs (Fig. 1F), and no expression was detected in normal perinephric adipose tissue (Fig. 1G).

B7-H3 expression in renal AMLs did not correlate with patient sex, as a median of 90% of cells from the tumors of the 20 men and 90 women in our series stained for B7-H3 ($P = .27$).

Likewise, no association was noted between B7-H3 expression and patient age ($P = .43$), tumor size ($P = .21$), or symptomatic presentation ($P = .35$).

We also compared B7-H3 expression among the various histologic subtypes of renal AML; in particular, we compared the expression in the 2 most prevalent variants from our series, triphasic and lipomatous. The median number of cells expressing B7-H3 from tumors with a triphasic histologic type was 90% (range 20%–100%) compared with 70% (range 20%–100%) for lipomatous tumors ($P < .001$). Also all 4 epithelioid AMLs evaluated contained intense staining of the cell membrane and cytoplasm (Fig. 1H). Also, B7-H3 expression did not differ significantly ($P = .31$) between the AMLs from the 7 patients in our series with TS (median 90% of cells staining positive, range 80%–100%) and the 103 patients with sporadic lesions (median 90% of cells positive, range 20%–100%).

Finally, we evaluated B7-H3 expression in the pulmonary tissue from 7 patients with a diagnosis of LAM who had undergone diagnostic biopsy or resection of radio-graphically noted lung lesions. All patients were women, with a median age of 34 years (range 30–52). B7-H3 was expressed in the LAM specimens from all 7 patients (Fig. 2), with a median of 90% (range 70–100) of cells demonstrating B7-H3 immunoreactivity.

COMMENT

To our knowledge, we report the first evaluation of T-cell co-regulatory molecule expression in renal AML and pulmonary LAM. We found that the T-cell co-regulator B7-H3 was expressed by 100% of the AML and LAM specimens evaluated, with expression in the smooth muscle, blood vessel, and lipid components of the tumors. In contrast, B7-H1 was infrequently expressed by AMLs, and no B7-H1 or B7-H3 staining was observed in the adjacent, normal renal parenchyma.

Differential expression of B7-H family members has been previously reported in various malignancies. Similar to our findings, high levels of B7-H3 expression relative to B7-H1 have been noted in prostate cancer⁶ and bladder cancer.⁷ Given its relatively recent discovery in 2001, the understanding of the mechanisms regulating B7-H3 expression has continued to evolve. In mice, B7-H3 is constitutively expressed by resting and activated B cells, macrophages, dendritic cells, and minor subsets of CD4+ and CD8+ T cells.²² In humans, B7-H3 mRNA expression can be detected in multiple tissues and cell lines; however, protein expression on human dendritic cells and monocytes has only been demonstrated in response to treatment with phorbol 12-myristate 13-acetate and ionomycin. Moreover, it appears that B7-H3 expression may be regulated by host factors or the tumor microenvironment, which has been supported by the differential protein expression of B7-H3 based on tumor type or even location within a given tumor.

Although the precise function of the B7 family of co-regulators are the subject of continued investigation, several of these molecules have been implicated as inhibitors of immune system surveillance.³ Aberrant expression of these molecules, therefore, may serve as a mechanism by which tumors are able to evade recognition and destruction by the host immune system. We have previously demonstrated expression of the T-cell co-regulatory molecule B7-H1 in RCC and found that expression correlated with advanced tumor stage and an increased risk of disease recurrence and cancer-specific death.⁵ In addition, we noted B7-H3 expression in the tumor vasculature from approximately 95% of RCC specimens and that such expression was associated with advanced tumor stage and decreased cancer-specific survival.⁹ Similarly, we have reported that the T-cell co-regulator B7-H4 is expressed by tumor vasculature in >80% of RCC specimens.¹ Given the proposed classification of AML and LAM as PEComas,¹⁶

which, as is RCC, are highly vascular tumors, together with the pseudomalignant and highly aggressive behavior of LAM, we sought to investigate B7-H ligand expression in these lesions.

In particular, we found high levels of B7-H3 expression primarily on the cell membrane but also in the cytoplasm from renal AML and pulmonary LAM specimens. Although cell-surface expression of the B7-H family of T-cell co-regulatory molecules is well established, cytoplasmic staining of B7-H3 has been previously demonstrated in RCC,⁹ prostate cancer,⁶ and bladder cancer.⁷ Moreover, a soluble form of B7-H3 was recently described²³ and was noted to be released by B7-H3-positive tumor cells but not by B7-H3-negative cells. As such, the cytoplasmic, as well as membrane, expression of B7-H3 by renal AMLs and pulmonary LAM reported in the present study is consistent with previous results in other cell types. The functional significance of nonmembrane-bound B7-H3 remains to be determined, because soluble B7-H3 was found to interact with the B7-H3 receptor on activated T cells, indicating a functionally active form of the molecule.²³

Renal AML, although not typically considered a malignant lesion, can nevertheless cause significant local complications. In addition to the risk of spontaneous retroperitoneal hemorrhage, AML expansion can encroach on the adjacent functioning parenchyma of the kidney to affect the patient's renal function. Given the benign nature of AMLs, renal-preserving treatments are preferred, including selective angioembolization and nephron-sparing surgery.¹¹ However, patients treated initially with angioembolization not infrequently require re-embolization or subsequent surgical intervention for recurrent bleeding, persistent symptoms, or a lack of regression of the lesion. In addition, preserving renal function with surgical resection of the multifocal, bilateral AMLs that occur in the setting of TS can be challenging. Therefore, pending the results of future investigations demonstrating a role for T-cell co-regulatory molecules in the pathogenesis of renal AML, molecules such as B7-H3 could serve as targets for novel therapeutic approaches to these patients. This strategy is currently being explored for patients with RCC.⁴

Given the high levels of B7-H3 expression by pulmonary LAM specimens, B7-H3 may also serve as a potential target for the treatment of this rare, but devastating, lung disease, which has been suggested to represent a pulmonary, and perhaps even metastatic, manifestation of AML.^{24,25} Up to 63% of women with LAM also have renal AML,^{19,26-28} and the abnormal smooth muscle proliferation of both conditions has been found to be nearly identical pathologically.^{19,20} Given the female sex predilection of these conditions, as well as the demonstration of sex-steroid receptor expression for both LAM^{24,29} and AML,³⁰ the possibility of affecting the growth of these lesions with hormonal therapy has also been investigated.^{19,20} However, the clinical results of hormonal therapy in patients with LAM have failed to show a consistent, durable response.¹⁹ Therefore, the need remains for the development of alternative therapies for these patients.

We have also reported high levels of B7-H4 ligand expression by the tumor vasculature of RCC.¹ However, we have not optimized our B7-H4 immunohistochemical methods for staining paraffin-embedded, formalin-fixed tissues. As such, we recognize the lack of investigation of B7-H4 in AML and LAM as a significant limitation of our study. Also, because the present series has provided only an observational association of B7-H3 expression in renal AML and pulmonary LAM, the clinical significance of T-cell co-regulatory molecule expression in these conditions requires additional study. The physiologic and pathologic roles of T-cell co-regulators, in particular B7-H3, have not been well established. Nevertheless, because the adjacent, normal renal and lung parenchyma typically do not express immune cell coregulatory ligands, the high and consistent expression of B7-H3 by AML and LAM could represent a selective promising therapy for patients with these and other PEComa-related tumors.

CONCLUSIONS

We found high levels of expression of the T-cell coregulatory molecule B7-H3 in renal AML and pulmonary LAM. In contrast, B7-H1 was infrequently expressed by renal AMLs, suggesting that overlapping mechanisms may exist to allow these lesions to impair T-cell activation and undermine host antitumor immunity. These results support the potential use of B7-H3 as a marker of PEComa tumors and as a possible future therapeutic target, for example, in patients with TS-associated multiple, bilateral AMLs or in patients with LAM for whom effective treatments are lacking.

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Figure 1.

Immunostaining for B7-H3 in renal angiomyolipoma (AML). (A) Triphasic AML demonstrating intense, diffuse staining in all histologic components. (B) AML stained positive for B7-H3 (**Lower left**), and adjacent, uninvolved renal parenchyma did not (**Upper right**). Note, entrapped, normal renal tubules (arrows) did not express B7-H3. (C) Myoid cells within AML showing strong diffuse staining. (D) Thick-walled blood vessel of AML with B7-H3 expression in adventitial layer cells (arrows) vs (E) no staining in tissue section of normal renal artery. (F) Lipoid component of AML expressed B7-H3, but (G) normal adipose tissue did not. (H) Intense staining of cell membrane for B7-H3 seen in epithelioid variant of AML. (I) Triphasic AML negative for B7-H1 expression. Figs. A-G and I: original magnification $\times 200$; Fig. H: original magnification $\times 400$.

Figure 2. Immunohistochemical staining for B7-H3 in pulmonary lymphangioleiomyomatosis (LAM). Representative slide showing intense B7-H3 staining in lung biopsy specimen from patient diagnosed with LAM. Original magnification $\times 200$.

Table 1

Clinical characteristics of 110 patients with surgically resected renal AML

Characteristic	n (%)
Sex	
Female	90 (81.8)
Male	20 (18.2)
Symptoms at presentation	
Palpable flank/abdominal mass	3 (2.7)
Discomfort ipsilateral/contralateral side	48 (43.6)
Rash, sweats, weight loss, fatigue, early satiety	15 (13.6)
Gross hematuria	8 (7.3)
Any symptoms at presentation	56 (50.9)
Ipsilateral AMLs (n)	
1	97 (88.2)
2	8 (7.3)
3	2 (1.8)
4	2 (1.8)
5	1 (0.9)
Bilateral AMLs	5 (4.6)
Histologic subtype	
Triphasic	79 (71.8)
Lipomatous	20 (18.2)
Leiomyomatous	7 (6.4)
Ethelioid	4 (3.6)

AML = angiomyolipoma.