B7-H3 Ligand Expression by Prostate Cancer: A Novel Marker of Prognosis and Potential Target for Therapy

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Abstract

B7 coregulatory ligands can be aberrantly expressed in human disease. In the context of cancer, these ligands may act as antigen-specific inhibitors of T-cell–mediated antitumoral immunity. We recently reported that B7-H1 expression by carcinomas of the kidney and bladder portends aggressive disease and diminished survival. The expression of these proteins in prostate cancer, however, has not been investigated. We evaluated B7-H3 and B7-H1 protein expression in the pathologic specimens of 338 men treated for clinically localized prostate cancer between 1995 and 1998 with radical retropubic prostatectomy. Expression levels of B7-H3 in prostate cancer were correlated with pathologic indicators of aggressive cancer as well as clinical outcome. We report that B7-H3 is uniformly and aberrantly expressed by adenocarcinomas of the prostate, high-grade prostatic intraepithelial neoplasia, and four prostate cancer cell lines, whereas B7-H1 is rarely expressed. B7-H3 is expressed by benign prostatic epithelia, although at a more reduced level relative to neoplastic tissue. Increasing levels of B7-H3 intensity correlate with worsening clinicopathologic features of prostate cancer. Marked B7-H3 intensity, present in 67 (19.8%) specimens, confers a >4-fold increased risk of cancer progression after surgery (risk ratio, 4.42; $P < 0.001$). A survey of normal tissues revealed that B7-H3 is expressed within the liver, urothelium, and fetal kidney. In summary, B7-H3 is aberrantly expressed in all prostate cancers and represents an independent predictor of cancer progression following surgery. Moreover, B7-H3 encompasses a novel diagnostic and potential therapeutic target for the clinical management of prostate cancer and, perhaps, other malignancies as well. [Cancer Res 2007;67(16):7893–900]

Introduction

The B7 family of proteins encompasses critical ligands that interact with known and unknown receptors to regulate T lymphocyte activation and function (1). It is well established that the B7-1 (CD80) and B7-2 (CD86) costimulatory ligands interact with CD28 to foster T-cell activation following coupling of the T-cell receptor (TCR) to cognate antigen-bearing MHC complexes. In contrast, owing to their relatively recent discovery, the precise role(s) of the other ligands in the B7 family (B7-H1, B7-H2, B7-H3, and B7-H4) is far less clear. Several of these B7-H molecules (e.g., B7-H1 and B7-H4) are expressed in human cancers and have been implicated as coregulatory inhibitors that may induce T-cell anergy or apoptosis on antigen recognition (1–3). We and others have shown that malignancies that express B7-H1 or B7-H4 are more likely to exhibit aggressive behavior and are associated with poor clinical outcome (4–6). Thus, it seems that human malignancies may exploit T-cell coinhibitory ligands to induce immunosuppression, thereby facilitating cancer progression.

B7-H3, first identified in 2001, is a member of the B7 ligand family and is thought to serve as an accessory coregulator of T-cell responses following initial antigen priming (7). B7-H3 mRNA has been identified in multiple tissues and tumor cell lines but not in quiescent peripheral blood monocytes. Protein expression of B7-H3 has been shown in placenta (8) and can be induced in activated dendritic cells, monocytes, and T cells (7).

At present, there is no consensus about the physiologic or pathophysiologic roles of B7-H3 because both immune stimulatory and inhibitory effects have been described for this ligand. *In vitro* studies show that B7-H3 promotes T-cell proliferation and IFN-γ expression (7). Further evidence in support of a stimulatory role for B7-H3 on T-cell function includes reduced rates of acute and chronic cardiac allograft rejection in B7-H3 knockout mice (9). In contrasting studies, B7-H3 has been shown to impair type 1 T-helper cell responses and inhibit cytokine production (10). *In vivo* antibody-mediated blockade of B7-H3 in mice has been reported to lead to elevations in T-cell activation and more severe forms of experimental autoimmune encephalitis (10, 11). Additionally, B7-H3 has been implicated to confer protection from natural killer (NK) cell–mediated cytolysis (12). The B7-H3 receptor, although not yet fully characterized, is thought to be expressed by activated T cells and is apparently distinct from other known T-cell costimulatory receptors, including CTLA-4, ICOS, and PD-1 (7). The presence of two distinct receptors has been postulated to explain the duality of stimulatory and inhibitory function imparted by B7-H3.

In the context of malignancy, the precise role of B7-H3 remains equally polarized. Murine studies have shown that B7-H3–transfected tumors regress rapidly, purportedly due to $CD8^+$ and NK cell–mediated antitumoral responses stimulated by B7-H3 (13–15). B7-H3 expression has been identified in gastric cancers, non–small cell lung cancers (NSCLC), and neuroblastomas; however, the clinical significance of B7-H3 within these tumors is conflicting (12, 16, 17). Finally, it has recently been reported that B7-H3, expressed by infiltrating T cells within colorectal tumors, may be critical in orchestrating antitumoral immune responses (18).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Although the incidence of almost all other forms of human malignancy has shown a recent decline, the incidence of prostate cancer continues to rise. In 2006 alone, it is estimated that >234,000 new cases of prostate cancer were diagnosed and that 27,350 men died from this disease (19). The mainstay of therapy for advanced prostate cancer, unchanged since 1941, continues to be palliative androgen deprivation. As such, understanding the role of B7-H family ligands may prove particularly critical to prostate cancer given that no curative therapy exists for patients with advanced forms of this disease. In light of this, various immunotherapeutic approaches to improve advanced prostate cancer treatment are being aggressively explored, including antibody-mediated blockade of the costimulatory receptor CTLA-4, the first down-regulatory TCR described (20). CTLA-4 blockade has been shown to augment T-cell–mediated antitumoral immunity in animal prostate cancer models and, consequently, has moved forth into several phase I and II clinical trials for patients with advanced prostate cancer (21). Analogous to CTLA-4, prostate tumor B7-H ligands may inhibit antitumoral T-cell responses. To date, however, the distribution and role of B7-H family ligands within prostate cancer has not yet been defined.

We have previously reported that aberrant expression of coregulatory T-cell inhibitory ligands in renal cell carcinoma portends more aggressive disease and increased risk of cancer-specific death (4, 6). In this current study, we examined B7-H1 and B7-H3 protein expression in >300 men with prostate cancer treated exclusively with radical retropubic prostatectomy (RRP). We report that B7-H1 is seldom expressed in benign or malignant prostate tissue. In contrast, B7-H3 is expressed in normal prostatic epithelium and is expressed even more intensely by malignant prostate tumor cells both within patient specimens as well as by prostate cancer cell lines *in vitro* (i.e., DU145, LNCaP, PC-3, and 22Rv1). Moreover, we show that increasing intensity of prostate cancer B7-H3 expression correlates with adverse clinicopathologic features of disease. Lastly, we report that B7-H3 performs as an independent predictor of prostate cancer progression and may prove useful both as a diagnostic or prognostic marker to assess prostate cancer patients and, perhaps, as a target to facilitate immunotherapeutic responses.

Materials and Methods

Patient selection. Following Institutional Review Board approval, we identified 435 consecutive patients with regionally localized prostate adenocarcinoma who were treated exclusively with RRP between 1995 and 1998 (22).

Clinical and RRP pathologic features. The clinical and RRP pathologic features evaluated included preoperative serum prostate-specific antigen (PSA), tumor volume, extraprostatic extension, Gleason score, seminal vesicle involvement, surgical margins, and lymphocytic infiltration (none, focal, moderate, or marked). The prostate glands were evaluated at the time of surgery by a standardized, limited sampling protocol using frozen section technique followed by reevaluation the following day with H&E-stained permanent sections. The surgically excised prostate was examined in the fresh state. The prostate was inked and the prostatic apex, bladder base, and distal urethral margins were examined microscopically as described previously (23). The prostate was then serially section from apex to base, and at least eight standard sections through the peripheral zone of the right and left sides and one each of the right and left seminal vesicles were obtained for microscopic evaluation. The number of sections examined was contingent on tumor volume, averaging 14 sections (range, 13–63). Estimates of tumor volume in cubic centimeters (cm^3) were calculated using the three-dimensional measurements of the tumor at the time of initial evaluation. The combination of the gross and microscopic observations served as the framework for the tumor volume estimates. Two urologic pathologists (T.J.S. and J.C.C.) reviewed the RRP specimens, achieving consensus for extraprostatic extension, Gleason score, seminal vesicle involvement, and surgical margins. Extraprostatic extension was defined as seminal vesicle involvement or malignant cell invasion outside the prostatic capsule into adipose tissues or tumor surrounding large nerves/ganglia beyond the prostatic capsule. These clinical and pathologic features were also combined into the GPSM score, which takes into consideration preoperative serum PSA and RRP Gleason score, seminal vesicle involvement, and positive surgical margins (24).

Patient outcome. Digital rectal exams were done and serum PSAs were obtained every 3 or 4 months for the first 2 years following RRP, every 6 months for the next 3 years, and annually thereafter (23). Systemic progression was determined radiographically. Local recurrences were diagnosed by clinical examination or needle biopsy. Cancer progression was defined as a postoperative PSA level of $\geq$0.4 ng/mL, local recurrence, or radiographic evidence of systemic progression.

B7-H3 and B7-H1 immunohistochemistry. Formalin-fixed, paraffin-embedded tissues were cut into 5-μm sections, deparaffinized, and rehydrated in a graded series of ethanols. Antigen retrieval was done by heating tissue sections in 1 mmol/L EDTA (pH 8) to 121°C using a Digital Decloaking Chamber (Biocare Medical), cooling to 90°C, and incubating for 5 min. Sections were washed in wash buffer (DAKO) before being placed onto the Autostainer Plus (DAKO) to conduct the following protocol. Sections were blocked for endogenous peroxidase for 5 min using endogenous blocking solution (DAKO), washed twice, and then incubated for 5 min in serum-free protein block (DAKO) followed by incubation for 60 min in purified goat anti-human B7-H3 antibody (100 μg/mL; R&D Systems) diluted 1:80 with DaVinci Green antibody diluent (Biocare Medical). Sections were incubated for 15 min in probe from Goat HRP-Polymer kit (Biocare Medical), washed, and incubated for 15 min with polymer from Goat HRP-Polymer kit. Sections were visualized by incubating in Betazoid 3,3′-diaminobenzidine (Biocare Medical) for 5 min. Sections were counterstained with hematoxylin, dehydrated in ethanol, cleared in xylene, and coverslipped. Staining of paraffin-embedded prostate tissue sections for B7-H1 was done as described previously (25).

Anti-B7-H3 competition assay. Goat anti-human B7-H3 antibody was combined at 1:30 with either recombinant human B7-H3-Fc fusion protein (R&D Systems) or, as an additional control, P-Selectin-Fc fusion protein (BD Biosciences) and incubated at room temperature for 30 min. Immunohistochemical staining, in conditions identical to those stated above for B7-H3, was then done with paraffin-embedded tissue sections.

Quantification of B7-H1 and B7-H3 expression. Percentages of tumor cells that stained positively for B7-H1 were quantified in 5% increments by a urologic pathologist (T.J.S.) without knowledge of patient outcome. The tumor was considered positive for B7-H1 if there was histologic evidence of cell surface membrane staining. Cases with <5% tumor staining were considered negative. The percentages of tumor and adjacent nontumor cells that stained positive for B7-H3 were quantified in 10% increments by a urologic pathologist (Y.S.) without knowledge of patient outcome. The intensity of B7-H3 expression was recorded as absent, weak (partial membrane staining), moderate (partial membrane and cytoplasmic staining), or marked (complete circumferential membrane and cytoplasmic staining). In addition, one quarter of the B7-H3–stained RRP specimens was independently reviewed by a second urologic pathologist (T.J.S.), yielding substantial agreement with respect to scoring tissues for marked B7-H3 as opposed to moderate or weak B7-H3 intensity (κ statistic, 0.73). As such, scoring of prostate tumors for marked B7-H3 expression was determined to be readily discernible and reproducible.

Flow cytometric analyses of B7-H3 expression by human prostate cancer cell lines. Four commonly studied human prostate cancer cell lines (DU145, LNCaP, PC-3, and 22Rv1) were examined for B7-H3 expression using the same B7-H3 affinity-purified goat polyclonal antibody that we used for tissue immunohistochemistry as well as a murine monoclonal anti-human B7-H3 antibody (R&D Systems). Goat polyclonal anti-human B7-H3 antibody was biotinylated using a solid-phase biotinylation kit (Pierce).

Subsequently, 1 μg of biotinylated polyclonal goat anti-human B7-H3 or monoclonal mouse anti-human B7-H3 antibody was incubated with 1×10^6 cells. After 30 min of incubation, cells were washed in fluorescence-activated cell sorting buffer (1% PBS, 3% fetal bovine serum, 3 mmol/L EDTA) and stained with streptavidin (BD PharMingen) or goat anti-mouse antibody (Biosource), both R-phycoerythrin tagged. For blocking studies, either 10 μg of recombinant B7-H3-Fc fusion protein or an irrelevant control fusion protein (P-Selectin-Fc, BD PharMingen) was combined with primary antibody before cell staining. Surface expression of B7-H3 protein was quantified using a FACSCalibur fluorescence-activated cell sorter (Becton Dickson) and analyzed with FlowJo software (Tree Star).

Normal tissue microarray evaluation. Slides containing normal tissue present in a tissue microarray (TMA; Cybrdi, Inc.) were stained with goat anti-human B7-H3 antibody as described above. B7-H3 expression was evaluated by a urologic pathologist (Y.S.) and quantified in fashion similar to that for RRP specimens.

Statistical methods. Cancer progression following RRP was estimated using the Kaplan-Meier method. The duration of follow-up was calculated from the date of RRP to the date of cancer progression, last follow-up, or the last postoperative serum PSA measurement. Comparisons of cancer progression between patients with and without archived tissue available for study were evaluated using a log-rank test. Tumor and nontumor B7-H3 expression were compared using the signed rank test. Associations of B7-H3 intensity with clinical and RRP pathologic features were evaluated using Kruskal-Wallis and χ^2 tests, whereas associations of B7-H3 intensity with cancer progression following RRP were evaluated using Cox proportional hazards regression models. Statistical analyses were done using the Statistical Analysis System software package (SAS Institute). All tests were two sided and P values of <0.05 were considered statistically significant.

Results

Comparison of patients with and without tissue. There were 338 (78%) of the 435 eligible patients with archived paraffin-embedded tissue available for study. There was no statistically significant difference in cancer progression following RRP between patients with and without tissue available for study ($P = 0.289$).

Clinical and RRP pathologic features and patient outcome. The clinical and pathologic features studied are summarized in Table 1. At last follow-up, 93 of the 338 patients studied experienced cancer progression at a median of 3.9 years following RRP (range, 0.1–9.7). Among the 245 patients who did not progress, the median duration of follow-up was 9.1 years (range, 0.1–11.3). The estimated cancer progression-free survival rates (SE, number still at risk) at 1, 3, 5, and 7 years following RRP were 95.0% (1.2%, 319), 88.0% (1.8%, 286), 81.1% (2.2%, 256), and 75.6% (2.4%, 220), respectively.

B7-H1 expression in prostate cancer. Only three (0.8%) cases had histologic evidence of B7-H1 cell surface membrane staining: one case had 5% B7-H1–positive tumor cells and two cases had 20% B7-H1–positive tumor cells (data provided in Supplementary Fig. S1). There were so few cases exhibiting B7-H1–positive tumors that the evaluation of associations of tumor B7-H1 expression with pathologic features at RRP or cancer progression following RRP could not be done.

B7-H3 expression in prostate cancer. Strong B7-H3 expression was apparent in RRP tissue specimens stained with anti-B7-H3 antibody. Blocking studies done in the presence of competing human B7-H3-Fc fusion protein revealed essentially no anti-B7-H3 antibody staining. In contrast, blocking of anti-B7-H3 using a P-Selectin-Fc fusion protein did not alter B7-H3 staining of prostate tumor specimens. As such, the specificity of the affinity-purified polyclonal antibody that we used to immunohistochemically stain patient RRP specimens for tumor B7-H3 expression was confirmed (data provided in Supplementary Fig. S2*A*–*C*).

Table 1. Summary of clinical and RRP pathologic features for 338 patients with prostate adenocarcinoma

Feature	Median (range)
Preoperative serum PSA, ng/mL (n = 324)	6.3 (0.6–112.0)
Tumor volume, cm^3 (n = 334)	2.7 (0.0005–67.5)
GPSM score (n = 324)	8 (6–16)
Feature	***n* (%)**
Gleason score	
5	6 (1.8)
6	158 (46.8)
7	141 (41.7)
8	18 (5.3)
9	15 (4.4)
Seminal vesicle involvement	
Absent	303 (89.6)
Present	35 (10.4)
Surgical margins	
Negative	197 (58.3)
Positive	141 (41.7)
Extraprostatic extension	
Absent	268 (79.3)
Present	70 (20.7)
Lymphocytic infiltration	
Absent	95 (28.1)
Focal	182 (53.8)
Moderate	57 (16.9)
Marked	4 (1.2)

We found that all 338 prostate cancer cases studied had positive tumor B7-H3 expression, ranging from 40% to 100% (Fig. 1*A*–*C*). In fact, 282 (83.4%) of the cases had 100% tumor B7-H3 expression; however, the intensity of expression varied. There were 65 (19.2%) cases with weak tumor B7-H3 intensity, 206 (61.0%) with moderate, and 67 (19.8%) with marked intensity. Marked tumor B7-H3 intensity was seen mostly in large neoplastic glands, with staining that was primarily circumferential cell membranous as well as cytoplasmic. Those cases with <100% B7-H3–positive tumor cells were generally seen in small neoplastic foci of low Gleason grade. To extend our observations further, we tested four human prostate cancer cell lines (DU145, LNCaP, PC-3, and 22Rv1) for B7-H3 expression using flow cytometric analysis. Consistent with our observations in formalin-fixed, paraffin-embedded prostate tissues, all four of these human prostate cancer cell lines exhibited expression of B7-H3 (data provided in Supplementary Figs. S3 and S4).

All but two of the 338 RRP cases examined had areas of normal, atrophic, or hyperplastic prostatic epithelia for evaluation and these areas showed B7-H3 expression ranging from 20% to 100% (Fig. 2*A*). The intensity of nontumor B7-H3 expression was weak in most cases (224; 66.7%), whereas 111 (33.0%) cases had moderate intensity and only 1 (0.3%) case had marked nontumor B7-H3 intensity. The intensity of tumor B7-H3 expression was significantly higher than nontumor B7-H3 expression ($P < 0.001$).

Atrophic prostatic ducts and acini showed weak or no B7-H3 staining. Hyperplastic glands showed weak to moderate partial membranous staining, with positive basal and lateral surfaces and

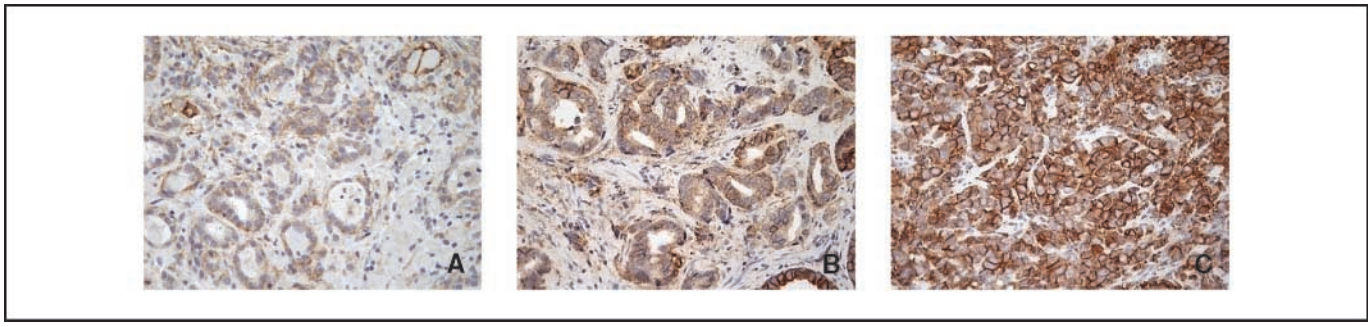

Figure 1. *A* to *C,* B7-H3 expression is observed in prostate cancer tumors and increases with Gleason score: 19.2% of cases showed weak (*A*) intensity typically in Gleason pattern 3 + 3 tumors, 61.0% of cases showed moderate (*B*) tumor B7-H3 intensity typically in Gleason 3 + 4 tumors, and 19.8% of cases showed marked (*C*) tumor B7-H3 intensity in large neoplastic glands typically of Gleason 4 + 4 tumors.

negative apical surfaces. This distribution corresponded to the apocrine compartment of prostatic epithelium and represented a consistently observable difference between benign and malignant epithelium. High-grade prostatic intraepithelial neoplasia (PIN) tended to have marked membranous and cytoplasmic staining (Fig. 2*B*), whereas seminal vesicles were completely negative (Fig. 2*C*). Lastly, no B7-H3 staining was seen in prostatic stroma or tumor-associated blood vessels.

Associations with B7-H3 expression. A comparison of tumor B7-H3 intensity with the clinical and RRP pathologic features studied is shown in Table 2. Tumor B7-H3 intensity was significantly associated with larger tumor volume, extraprostatic extension, higher composite GPSM score, as well as three of the four components comprising the GPSM score, including higher Gleason score, seminal vesicle involvement, and positive surgical margins. For example, none of the tumors with weak B7-H3 intensity had Gleason scores of 8 or 9 compared with 10 (4.9%) and 23 (34.3%) of the tumors with moderate and marked B7-H3 intensity, respectively ($P < 0.001$).

Univariately, patients with tumors of moderate B7-H3 intensity were 35% more likely to experience cancer progression following RRP compared with patients with tumors of weak intensity, but this difference did not achieve statistical significance [risk ratio, 1.35; 95% confidence interval (95% CI), 0.70–2.61; $P = 0.369$]. However, patients with tumors of marked B7-H3 intensity were over four times more likely to progress compared with patients with tumors of weak intensity (risk ratio, 4.42; 95% CI, 2.24–8.72; $P < 0.001$). The estimated cancer progression-free survival rates (SE, number still at risk) at 5 years following RRP were 92.1% (3.4%, 57), 86.0% (2.4%, 166), and 55.0% (6.2%, 33) for patients with tumors of weak, moderate, and marked B7-H3 intensity, respectively (Fig. 3).

The associations of tumor B7-H3 intensity with cancer progression after adjusting for each of the clinical and RRP pathologic features studied are shown in Table 3. Marked tumor B7-H3 intensity was significantly associated with cancer progression even after multivariate adjustment. For example, after accounting for the association of GPSM score with cancer progression, patients with tumors of marked B7-H3 intensity were still over twice as likely to progress compared with patients with tumors of weak B7-H3 intensity (risk ratio, 2.20; 95% CI, 1.03–4.70; $P = 0.042$).

Survey of B7-H3 expression in normal tissues. Immunohistochemical staining of B7-H3 in the normal tissues TMA is illustrated in Supplementary Fig. S5. For each normal tissue, ~30 separate organ tissue types from 278 different individuals were examined. B7-H3 expression was identified in liver, spleen, bladder epithelium, testis (Leydig cells), fetal kidney (medullary stroma), endometrium, placenta, adrenal gland, lymph node, thymus, and lung histiocytes. Normal prostate tissue showed weak to moderate membrane staining mainly in the basal and lateral surfaces of secretory epithelium. Both breast acini and ducts were B7-H3 positive. In colon and small intestine, epithelial cells were consistently negative; however, B7-H3 expression was identified in histiocytes and lymphocytes within the lamina propria in both tissues. B7-H3 expression was not identified in thyroid, adult kidney, cervix, ovary, skeletal muscle, cardiac muscle, salivary

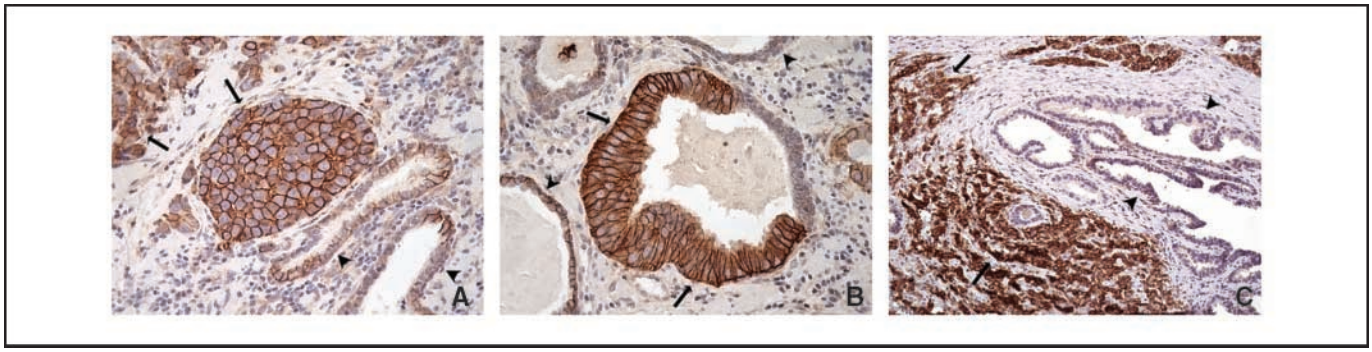

Figure 2. *A* to *C,* B7-H3 expression is identified in normal prostate epithelium (*A, arrowheads*); however, expression is significantly less than that in malignant tissue (*A, arrows*). Marked B7-H3 staining is observed in areas of high-grade PIN (*B, arrows*), a premalignant lesion associated with prostate cancer. B7-H3 expression in benign prostate epithelium (*B, arrowheads*) is significantly weaker. B7-H3 expression is absent within seminal vesicle (*C, arrowheads*) yet is observed in malignant tissue (*C, arrows*).

Table 2. Comparison of clinical and RRP pathologic features by tumor B7-H3 intensity

Feature	Tumor B7-H3 intensity			*P*
	Weak (*n* = 65)	Moderate (*n* = 206)	Marked (*n* = 67)	
		Median (range)		
Preoperative serum PSA (ng/mL)	5.5 (1.1–22.7)	6.1 (0.6–39.7)	7.3 (1.0–112.0)	0.056
Tumor volume (cm^3)	1.0 (0.0005–26.3)	2.6 (0.004–67.5)	6.1 (0.024–60.0)	<0.001
GPSM score	7 (6–12)	8 (6–15)	10 (6–16)	<0.001
Feature		*n* (%)		*P*
Gleason score				
5 or 6	49 (75.4)	106 (51.5)	9 (13.4)	<0.001
7	16 (24.6)	90 (43.7)	35 (52.2)	
8 or 9	0 (0.0)	10 (4.9)	23 (34.3)	
Seminal vesicle involvement				
Absent	62 (95.4)	191 (92.7)	50 (74.6)	<0.001
Present	3 (4.6)	15 (7.3)	17 (25.4)	
Surgical margins				
Negative	50 (76.9)	118 (57.3)	29 (43.3)	<0.001
Positive	15 (23.1)	88 (42.7)	38 (56.7)	
Extraprostatic extension				
Absent	61 (93.9)	171 (83.0)	36 (53.7)	<0.001
Present	4 (6.1)	35 (17.0)	31 (46.3)	
Lymphocytic infiltration				
Absent	21 (32.3)	59 (28.6)	15 (22.4)	0.100
Focal	37 (56.9)	110 (53.4)	35 (52.2)	
Moderate	5 (7.7)	35 (17.0)	17 (25.4)	
Marked	2 (3.1)	2 (1.0)	0	

gland, pancreas, stomach, esophagus, cerebrum, cerebellum, peripheral nerve, spinal cord, or lung parenchyma.

Discussion

We show that the T-cell coregulatory ligand B7-H3 is expressed by nearly every normal and pathologic prostate cell of epithelial origin. Whereas expression of B7-H3 is typically low within normal, atrophic, and hyperplastic prostate glands, levels of B7-H3 expression increase significantly within malignant prostate tumor cells. All prostate tumor specimens showed some degree of B7-H3 expression, as did four of the most commonly studied human prostate cancer cell lines. Moreover, 83% of these cancer specimens exhibited 100% tumor cell B7-H3 expression. Intense B7-H3 staining was observed in a subset of ~20% tumor specimens and was significantly associated with multiple adverse prognostic features of prostate cancer, including larger tumor volume, extraprostatic extension, higher Gleason score, seminal vesicle involvement, and positive surgical margins. Even after multivariate adjustment for these prognostic factors, or adjustment for the multiple-variable GPSM composite score, marked B7-H3 intensity persisted as a statistically significant predictor of prostate cancer progression following surgery. As an aside, lymphocytic infiltration was observed in many of the RRP specimens studied and was present at higher rates in tumors with marked B7-H3 intensity. This association, however, did not quite achieve statistical significance. Collectively, these results show that B7-H3 is a novel and independent prognostic marker for the assessment of prostate cancer patients. Thus, B7-H3 may prove useful for the clinical evaluation of patients who have, or are to undergo, surgical treatment for their cancer, especially to identify high-risk patients who are at increased risk of cancer progression and, therefore, most likely to benefit from early and aggressive adjunctive therapy.

In contrast, our present study failed to reveal significant levels of B7-H1 expression by either normal or neoplastic tissues within the prostate. In fact, <1% of prostate tumors showed any B7-H1 staining, which, when present, tended to be weak. Like B7-H3,

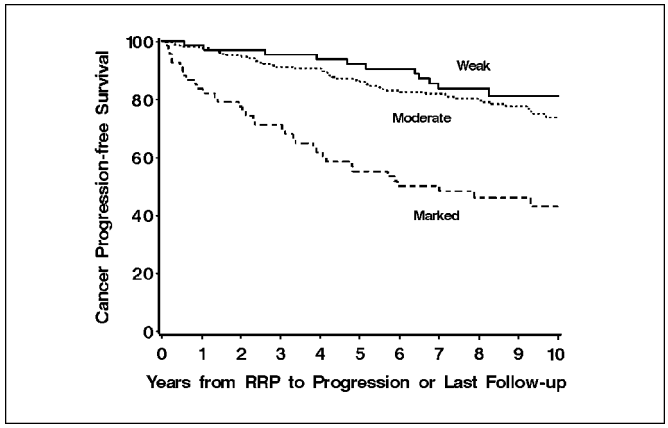

Figure 3. Cancer progression-free survival following RRP by tumor B7-H3 intensity. The estimated cancer progression-free survival rates (SE, number still at risk) at 5 y following RRP were 92.1% (3.4%, 57), 86.0% (2.4%, 166), and 55.0% (6.2%, 33) for patients with tumors that had weak, moderate, and marked B7-H3 intensity, respectively.

Table 3. Associations of B7-H3 intensity with cancer progression following RRP

Feature	Risk ratio (95% CI)	*P*
Preoperative serum PSA*	1.44 (1.12–1.86)	0.005
B7-H3 intensity		
Weak	1.0 (reference)	
Moderate	1.24 (0.64–2.39)	0.530
Marked	3.60 (1.80–7.20)	<0.001
Tumor volume*	1.26 (1.08–1.45)	0.002
B7-H3 intensity		
Weak	1.0 (reference)	
Moderate	1.11 (0.57–2.15)	0.757
Marked	2.92 (1.43–5.94)	0.003
GPSM score	1.23 (1.13–1.35)	<0.001
B7-H3 intensity		
Weak	1.0 (reference)	
Moderate	1.07 (0.55–2.08)	0.848
Marked	2.20 (1.03–4.70)	0.042
Gleason score		
5 or 6	1.0 (reference)	
7	2.32 (1.39–3.86)	0.001
8 or 9	3.97 (1.96–8.05)	<0.001
B7-H3 intensity		
Weak	1.0 (reference)	
Moderate	1.09 (0.56–2.12)	0.801
Marked	2.25 (1.05–4.82)	0.036
Seminal vesicle involvement		
Absent	1.0 (reference)	
Present	2.00 (1.17–3.42)	0.011
B7-H3 intensity		
Weak	1.0 (reference)	
Moderate	1.33 (0.69–2.57)	0.390
Marked	3.64 (1.81–7.35)	<0.001
Surgical margins		
Negative	1.0 (reference)	
Positive	1.70 (1.12–2.60)	0.013
B7-H3 intensity		
Weak	1.0 (reference)	
Moderate	1.21 (0.62–2.35)	0.571
Marked	3.66 (1.83–7.33)	<0.001
Extraprostatic extension		
Absent	1.0 (reference)	
Present	2.14 (1.36–3.36)	0.001
B7-H3 intensity		
Weak	1.0 (reference)	
Moderate	1.23 (0.64–2.39)	0.532
Marked	3.21 (1.57–6.55)	0.001
Lymphocytic infiltration		
Absent	1.0 (reference)	
Focal	0.96 (0.59–1.56)	0.876
Moderate or marked	0.91 (0.50–1.67)	0.769
B7-H3 intensity		
Weak	1.0 (reference)	
Moderate	1.36 (0.70–2.62)	0.364
Marked	4.48 (2.26–8.87)	<0.001

*Analyzed on the natural log scale. As such, the risk ratio represents a 1-unit increase in the feature listed on the natural log scale, not the original scale.

B7-H1 encompasses another member of the B7 family of T-cell coregulatory molecules, sharing ~25% sequence homology with B7-H3 (26). The published data support the concept that B7-H1 is a coregulatory ligand that imparts inhibitory signals to T cells to down-regulate or terminate antigen-specific responses (27). Several investigators have shown that tumor cell expression of B7-H1 is strongly predictive of aggressive cancer behavior and of poor outcome for several human malignancies, including clear cell carcinoma of the kidney and transitional cell cancer of the bladder, supporting the hypothesis that expression of B7-H1 protein in cancers has a negative immunomodulatory effect and enhances tumor aggressiveness and progression (6, 28).

As opposed to B7-H1, the physiologic and pathologic role of B7-H3 is far less well understood. In the setting of experimental malignancy, some evidence indicates that B7-H3 acts as an immunostimulatory molecule. Specifically, transfection of B7-H3 into murine tumor lines of lymphoma, mastocytoma, and colon cancer has been reported to accelerate tumor rejection *in vivo* (13–15). In a clinical study of gastric carcinoma, B7-H3$^+$ tumor cells were noted in 58.5% of 102 specimens examined, and the presence of B7-H3$^+$ tumor cells was associated with improved patient survival (17). However, correlations between protein levels of tumor cell B7-H3 expression and clinical outcomes were not reported and B7-H3 protein expression was not shown as an independent predictor of disease progression in multivariate analysis.

Evidence also exists that B7-H3 may serve as an immunoinhibitory ligand that promotes tumor progression. For instance, B7-H3 ligand was shown on cell membranes of tumors obtained from children with advanced stage IV, but not early stage I, neuroblastoma (12). In a study of 70 patients with NSCLC, B7-H3 protein expression by tumors was associated with an increased risk for tumor metastases (16). However, outcome information pertaining to these patients, as well as the effect of intensity of primary tumor B7-H3 staining on risk for metastases, was not reported (12, 16). Hence, our current study adds significantly to the existing literature in that it illustrates that B7-H3 is uniformly overexpressed by adenocarcinomas of the prostate as well as almost every malignant cell comprising a given prostate tumor. Moreover, we show that B7-H3 expression acts as an independent predictive variable for rendering prognostic risk for prostate cancer progression following

surgery. To our knowledge, such associations between tumor cell overexpression of B7-H3 with aggressive cancer behavior and poor clinical outcome have not previously been reported.

The physiologic and pathologic roles of B7-H3 are not firmly established and further investigations are clearly warranted to elucidate its function. Our study indicates that B7-H3, expressed by adenocarcinoma of the prostate, potentially serves as an inhibitory T-cell coregulator similar to its homologues B7-H1 (2, 29–31) and, perhaps, B7-H4 (32). These related ligands can be aberrantly expressed by various forms of malignancy and may promote cancer progression by disarming localized T-cell–mediated antitumoral immunity. Alternatively, in light of the controversial role of B7-H3 in immunoregulation, it may be possible that B7-H3 is up-regulated in high-risk prostate tumors as a stress-induced molecule or to promote T-cell–mediated antitumoral responses against aggressive forms of this cancer. Based on the strong correlation between B7-H3 intensity and adverse clinicopathologic features of prostate cancer, we surmise that B7-H3 functions as an inhibitor of T-cell–mediated immunity that promotes aggressive clinical behavior by adenocarcinomas of the prostate.

Aberrant up-regulation of B7-H3 may occur as a relatively early event during malignant transformation, as B7-H3 overexpression is apparent in high-grade PIN, the putative precursor of prostate cancer (Fig. 2*B*). As such, B7-H3 may permit cells within high-grade PIN lesions to undermine host immunity, ultimately fostering invasion by inducing antitumoral immune tolerance or impairment. Additional studies to elucidate which factors regulate B7-H3 expression, particularly with respect to prostate neoplasia, are warranted. Preliminary studies done by our group further indicate that B7-H3 expression is retained by prostate tumor cells despite initiation of complete androgen ablative therapy (data provided in Supplementary Fig. S6). At present, however, detailed information pertaining to prostate cancer B7-H3 expression in subjects receiving (or who have failed) androgen ablative therapy is not available and remains a subject for future investigation by our group.

Beyond its potential function as an inhibitor of antigen-specific T-cell responses, B7-H3 may contribute to another unique characteristic that pertains to disseminated prostate cancer. Specifically, B7-H3 knockout mice exhibit diminished cortical bone density and increased long bone fracture susceptibility compared with wild-type mice. Based on this observation, it has been suggested that B7-H3 may be required for late-phase osteoblast differentiation and bone mineralization (33). Whether B7-H3 stimulates osteoblast signaling (or inhibits osteoclast activity) within prostate cancer metastases, resulting in the "signature" osteoblastic lesions associated with metastases to the bone, remains to be determined. Clearly, further dissection of B7-H3 signaling pathways, particularly the elucidation of B7-H3 receptor(s), as well as characterization of the role of B7-H3 in other malignancies will be critical to resolve such issues.

The introduction of aggressive PSA screening has catalyzed improvements in the early diagnosis of localized prostate cancer, ultimately culminating in downward stage migration and improved patient survival (34). However, PSA screening has also increased the detection of indolent or clinically insignificant cancers that do not affect patient mortality nor warrant aggressive treatment in the form of prostatectomy or radiotherapy. Current variables used to predict individual risk associated with prostate cancer, to assign rational and optimal treatment, include the nomograms that incorporate PSA level, clinical stage, biopsy Gleason score, and percentage of positive biopsy cores (35). Immunohistochemical assessment of B7-H3 expression in prostate biopsy specimens may prove useful to refine the precision of existing prognostic algorithms. Specifically, our data indicate that men with marked tumor B7-H3 expression in their prostatectomy specimens (along with other poor prognostic clinicopathologic features) encompass a high-risk population for disease progression and, therefore, a subset of patients who might benefit from more aggressive treatment or surveillance. In contrast, patients with cancers that exhibit weak B7-H3 expression may be better suited for less intensive forms of management. Such determinations, however, will require further investigation, including the evaluation of B7-H3 expression in diagnostic prostate needle biopsy specimens.

Distinct from PSA, which is not membrane bound but rather secreted by normal and malignant prostate cancer cells, B7-H3 is localized to the cytoplasmic membrane of prostate cancer cells, making B7-H3 a potential diagnostic and therapeutic target. Analogous to prostate-specific membrane antigen (PSMA), B7-H3 is expressed on the surfaces of both benign and malignant prostate cells, and expression within RRP specimens is predictive of cancer progression (36). Because PSMA is expressed in both primary prostate tumors and metastatic prostate cancer lesions, it has been used as a target for the treatment for prostate cancer. Based on our immunohistochemical analysis, we believe that B7-H3 may also prove to be a useful molecular target for treating prostate cancer. A mechanistic role for B7-H3 in promoting prostate cancer progression, based on its potential ability to impair T-cell–mediated immunity, can be envisioned, and as such, targeting B7-H3 to treat prostate cancer may prove more advantageous and mechanistically rational than the experience with targeting PSMA.

As we observed in our TMA analyses, B7-H3 expression is not restricted to prostate tissues. Specifically, B7-H3 is expressed in other normal adult tissues, most notably within the liver. B7-H3 mRNA expression has been reported in both lymphoid and nonlymphoid tissues (7). Our immunohistochemical survey of normal tissues indicates that the B7-H3 protein is also expressed on urothelial cells of the bladder and ureter, as well as stromal cells of the fetal kidney, whereas epithelial cells of the kidney do not express B7-H3 (Supplementary Fig. S5). In light of these observations, B7-H3 expression in hepatic and bladder carcinoma and various pediatric malignancies (i.e., Wilms' tumor) may be of particular interest in future studies.

The current study indicates that B7-H3 is a potentially useful diagnostic and prognostic marker for the assessment of prostate cancer. B7-H3 may provide a target to improve prostate cancer therapy. However, many questions pertaining to B7-H3 will still need to be addressed in future mechanistic and prospective clinical studies, including (*a*) strategies to preempt toxicity to the liver and other normal tissues that might occur consequent to the targeting of B7-H3 protein (which may differ in malignant versus normal tissues) and (*b*) assessment of B7-H3 expression by androgen-insensitive forms of disease that typically prove uniformly lethal for patients with advanced prostate cancer.

Conclusion

B7-H3 protein expression by prostate tumor cells encompasses an independent marker predictive of prostate cancer progression following RRP. In contrast, B7-H1 is infrequently expressed in prostate cancer, suggesting that human malignancies may use

one of multiple overlapping mechanisms to impair costimulatory T-cell activation to undermine host antitumoral immunity. Our study supports B7-H3 as a promising marker to improve prostate cancer diagnosis, prognostic assessment, and targeted treatment. Future studies, facilitated by the expression of B7-H3 on four of the most commonly studied human prostate cancer cell lines, will be required to understand mechanisms whereby B7-H3 may promote cancer progression and to ascertain the full utility of B7-H3 as a clinical marker of disease and target for therapy.

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Some of the authors have filed for exploitation of B7-H3 as a prognostic marker for cancer and, thus, disclose a potential for conflict of interest.

References

1. Greenwald RJ, Freeman GJ, Sharpe AH. The B7 family revisited. Annu Rev Immunol 2005;23:515–48.
2. Dong H, Strome SE, Salomao DR, et al. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. Nat Med 2002;8:793–800.
3. Zang X, Loke P, Kim J, et al. B7x: a widely expressed B7 family member that inhibits T cell activation. Proc Natl Acad Sci U S A 2003;100:10388–92.
4. Krambeck AE, Thompson RH, Dong H, et al. B7-H4 expression in renal cell carcinoma and tumor vasculature: associations with cancer progression and survival. Proc Natl Acad Sci U S A 2006;103:10391–6.
5. Thompson RH, Gillett MD, Cheville JC, et al. Costimulatory molecule B7-H1 in primary and metastatic clear cell renal cell carcinoma. Cancer 2005;104:2084–91.
6. Thompson RH, Gillett MD, Cheville JC, et al. Costimulatory B7-H1 in renal cell carcinoma patients: indicator of tumor aggressiveness and potential therapeutic target. Proc Natl Acad Sci U S A 2004;101:17174–9.
7. Chapoval AI, Ni J, Lau JS, et al. B7-H3: a costimulatory molecule for T cell activation and IFN-γ production. Nat Immunol 2001;2:269–74.
8. Petroff MG, Kharatyan E, Torry DS, Holets L. The immunomodulatory proteins B7-DC, B7-H2, and B7-H3 are differentially expressed across gestation in the human placenta. Am J Pathol 2005;167:465–73.
9. Wang LQ, Fraser CC, Kikly K, et al. B7-H3 promotes acute and chronic allograft rejection. Eur J Immunol 2005;35:428–38.
10. Suh WK, Gajewska BU, Okada H, et al. The B7 family member B7-H3 preferentially down-regulates T helper type 1-mediated immune responses. Nat Immunol 2003;4:899–906.
11. Prasad DVR, Nguyen T, Li ZX, et al. Murine B7-H3 is a negative regulator of T cells. J Immunol 2004;173:2500–6.
12. Castriconi R, Dondero A, Augugliaro R, et al. Identification of 4Ig-B7-H3 as a neuroblastoma-associated molecule that exerts a protective role from an NK cell-mediated lysis. Proc Natl Acad Sci U S A 2004;101:12640–5.
13. Luo LQ, Chapoval AI, Flies DB, et al. B7-H3 enhances tumor immunity *in vivo* by costimulating rapid clonal expansion of antigen-specific CD8(+) cytolytic T cells. J Immunol 2004;173:5445–50.
14. Lupu CM, Eisenbach C, Kuefner MA, et al. An orthotopic colon cancer model for studying the B7-H3 antitumor effect *in vivo*. J Gastrointest Surg 2006;10:635–45.
15. Sun X, Vale M, Leung E, et al. Mouse B7-H3 induces antitumor immunity. Gene Ther 2003;10:1728–34.
16. Sun YP, Wang YS, Zhao JQ, et al. B7-H3 and B7-H4 expression in non-small-cell lung cancer. Lung Cancer 2006;53:143–51.
17. Wu CP, Jiang JT, Tan M, et al. Relationship between co-stimulatory molecule B7-H3 expression and gastric carcinoma histology and prognosis. World J Gastroenterol 2006;12:457–9.
18. Galon J, Costes A, Sanchez-Cabo F, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science 2006;313:1960–4.
19. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2006. CA Cancer J Clin 2006;56:106–30.
20. Leach DR, Krummel MF, Allison JP. Enhancement of antitumor immunity by CTLA-4 blockade. Science 1996;271:1734–6.
21. Hurwitz AA, Foster BA, Kwon ED, et al. Combination immunotherapy of primary prostate cancer in a transgenic mouse model using CTLA-4 blockade. Cancer Res 2000;60:2444–8.
22. Sebo TJ, Cheville JC, Riehle DL, et al. Predicting prostate carcinoma volume and stage at radical prostatectomy by assessing needle biopsy specimens for percent surface area and cores positive for carcinoma, perineural invasion, Gleason score, DNA ploidy and proliferation, and preoperative serum prostate specific antigen: a report of 454 cases. Cancer 2001;91:2196–204.
23. Sebo TJ, Cheville JC, Riehle DL, et al. Perineural invasion and MIB-1 positivity in addition to Gleason score are significant preoperative predictors of progression after radical retropubic prostatectomy for prostate cancer. Am J Surg Pathol 2002;26:431–9.
24. Blute ML, Bergstralh EJ, Iocca A, Scherer B, Zincke H. Use of Gleason score, prostate specific antigen, seminal vesicle and margin status to predict biochemical failure after radical prostatectomy. J Urol 2001;165:119–25.
25. Thompson RH, Kuntz SM, Leibovich BC, et al. Tumor B7-H1 is associated with poor prognosis in renal cell carcinoma patients with long-term follow-up. Cancer Res 2006;66:3381–5.
26. Ling V, Wu PW, Spaulding V, et al. Duplication of primate and rodent B7-H3 immunoglobulin V- and C-like domains: divergent history of functional redundancy and exon loss. Genomics 2003;82:365–77.
27. Dong H, Chen L. B7-H1 pathway and its role in the evasion of tumor immunity. J Mol Med 2003;81:281–7.
28. Inman BA, Sebo TJ, Frigola X, et al. PD-L1 (B7-H1) expression by urothelial carcinoma of the bladder and BCG-induced granulomata: associations with localized stage progression. Cancer 2007;109:1499–505.
29. Blank C, Brown I, Peterson AC, et al. PD-L1/B7H-1 inhibits the effector phase of tumor rejection by T cell receptor (TCR) transgenic $CD8^+$ T cells. Cancer Res 2004;64:1140–5.
30. Iwai Y, Ishida M, Tanaka Y, et al. Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. Proc Natl Acad Sci U S A 2002;99:12293–7.
31. Wintterle S, Schreiner B, Mitsdoerffer M, et al. Expression of the B7-related molecule B7-H1 by glioma cells: a potential mechanism of immune paralysis. Cancer Res 2003;63:7462–7.
32. Tringler B, Zhuo S, Pilkington G, et al. B7-H4 is highly expressed in ductal and lobular breast cancer. Clin Cancer Res 2005;11:1842–8.
33. Suh WK, Wang SX, Jheon AH, et al. The immune regulatory protein B7-H3 promotes osteoblast differentiation and bone mineralization. Proc Natl Acad Sci U S A 2004;101:12969–73.
34. Smith RA, von Eschenbach AC, Wender R, et al. American Cancer Society guidelines for the early detection of cancer: update of early detection guidelines for prostate, colorectal, and endometrial cancers. Also: update 2001—testing for early lung cancer detection. CA Cancer J Clin 2001;51:38–75; quiz 7–80.
35. Ross PL, Scardino PT, Kattan MW. A catalog of prostate cancer nomograms. J Urol 2001;165:1562–8.
36. Ross JS, Sheehan CE, Fisher HA, et al. Correlation of primary tumor prostate-specific membrane antigen expression with disease recurrence in prostate cancer. Clin Cancer Res 2003;9:6357–62.