B7-H3 Over Expression in Prostate Cancer Promotes Tumor Cell Progression

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Purpose: We compared B7-H3 expression in benign prostatic hyperplasia and prostate cancer tissue specimens, and determined the effects of low B7-H3 expression on the PC-3 human prostate cancer cell line using RNA interference.

Materials and Methods: B7-H3 expression in prostate specimens was determined by enzyme-linked immunosorbent assay. A PC-3 cell line with low B7-H3 expression was established by RNA interference to investigate the effect of B7-H3 on cell proliferation, adhesion, migration and invasion in vitro.

Results: B7-H3 in tissue samples was significantly higher in the prostate cancer group than in the benign prostatic hyperplasia group (mean ± SEM 174.73 ± 56.80 vs 82.69 ± 46.19 ng/gm, p <0.001). B7-H3 expression down-regulated by small interfering RNA decreased cell adhesion to PC-3 fibronectin more than 30%, and migration and Matrigel™ invasion up to 50%. No apparent impact was observed on cell proliferation.

Conclusions: B7-H3 is aberrantly expressed in prostate cancer. In addition to modulating tumor immunity, B7-H3 may have a novel role in regulating PC-3 cell progression.

Key Words: prostate, prostatic neoplasms, neoplasm invasiveness, cell adhesion, cell migration inhibition

Prostate cancer is the second leading cause of cancer death in men. Although localized PCa is highly curable by surgery, metastatic PCa is relatively incurable. Thus, understanding the molecular mechanisms of PCa progression should be helpful to develop efficient treatments for the disease.

Roth et al reported that B7-H3 expression in neoplastic tissue is higher and correlates with worsening prostate cancer clinicopathological features. Zang et al also found that patients with PCa who had enhanced B7-H3 expression were more likely to have cancer cells spread to other parts of the body at surgery and they were at increased risk for clinical cancer recurrence and cancer specific death. Recently Parker et al reported that enhanced B7-H3 staining of primary PCa tissue was associated with an increased risk of biochemical recurrence after salvage radiation therapy. Those findings suggest that B7-H3 has an important role in PCa progression.

B7-H3, a member of the B7 immunoregulatory family, was identified in 2001 by database searches of a human dendritic cell derived cDNA library. Previous studies showed that B7-H3 protein can be expressed in dendritic cells, and the liver, lung and...
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MATERIALS AND METHODS

Patients
This study was approved by the ethics committee of Soochow University for Clinical Investigation. Included in the study were 13 patients with PCa who underwent radical retropubic prostatectomy. Patients were excluded from analysis if they received hormonal or radiation therapy before radical retropubic prostatectomy or underwent previous prostate surgery. A total of 19 patients with BPH served as controls. Tissue was collected, separated and divided into 2 groups during preparation and analysis.

Tissue Extracts and B7-H3 ELISA
Each tissue sample was collected and weighed in the same manner during preparation. The diagnosis of each tissue was confirmed by frozen section stained with hematoxylin and eosin. Extract preparation was done as recommended by the manufacturer (Cell Signaling Technology®). Briefly, each tissue sample was prepared with PBS and homogenized in cell lysis buffer containing Protease Inhibitor Cocktail Set I (Calbiochem®). After incubation on ice for 30 minutes the homogenate was centrifuged at 14,000 × gravity for 10 minutes at 4°C and supernatant was collected for ELISA assay. ELISA kits produced at our laboratory were used to measure B7-H3, as described previously.10 B7-H3 concentrations were determined using a standard curve with an 8-parameter curve fit analysis program. We calculated the B7-H3 level per gm of each tissue sample.

Immunohistochemistry and Staining
Surgical specimens were obtained from the Pathology Department at First Affiliated Hospital of Soochow University. Frozen tissues were cut at 5 μm and stained with mAb against B7-H3. Briefly, sections were fixed in cold acetone for 10 minutes. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 5 minutes, followed by incubation with normal goat serum for 30 minutes at room temperature. Sections were then incubated with anti-B7-H3 mAb (4H7 from our laboratory), diluted 1:50, for 30 minutes. Horseradish peroxidase conjugated antimouse IgG was used as the secondary antibody. Staining was developed using an ABC immunoperoxidase staining kit (Dako, Glostrup, Denmark) according to manufacturer instructions.

Staining was assessed semiquantitatively, as previously described.20 Briefly, the expression level was assessed at the pathology laboratory at First Affiliated Hospital of Soochow University in blinded fashion by counting and calculating the percent of cells with positive staining.

PC-3 Cells
The PC-3 human prostate cancer cell line was maintained in F12 medium (Gibco® supplemented with 10% FBS (HyClone®). All culture media contained 1% penicillin/streptomycin. Culture was performed in a humidified 5% CO2 environment at 37°C. After cells attained 70% to 80% confluence they were harvested with 0.25% trypsin and split at a 1:3 ratio.

Gene Expression and Protein Secretion of B7-H3
Total RNA was extracted and reverse transcribed for cDNA. Reverse transcriptase-polymerase chain reaction was done using B7-H3 primer (forward 5'-GCCAGCTT-CACCTTGCTTGCAGTT-3' and reverse 5'-TTGCCGCAGCACGGCAGCTGATGTT-3'). The specificity of amplification products was confirmed by agarose gel electrophoresis. B7-H3 protein expression was analyzed by FCM using a Cytomics™Fc 500 device.

siRNA Transfection
We used human B7-H3 siRNA with green fluorescent protein expression. Its sequence had sense and antisense sequences, including B7-H3 sense 5'-CUAGCCUUAUAUCUGGCUUUUTT-3' and antisense 5'-AAAGGCGCAUUAAGGCUAGTT-3'. The negative control was 5'-sense UUCUCCGAACGUUGACGUTT-3' and antisense 5'-ACGUGACAGUUCGAGAATT-3'. PC-3 cells were seeded in 6-well plates and treated in F12 medium containing 10% FBS to 70% to 80% confluence. Before transfection the medium was discarded and replaced by serum/antibiotic-free F12 medium. Transfection was done using Lipofectamine™ 2000 reagent according to manufacturer instructions. Cells were transfected with siRNA for 6 hours. The final siRNA concentration used in the experiment was 100 nM. After transfection cells were allowed to recover in F12 medium containing 10% FBS for 24 hours before further experimentation.
mRNA and Protein Quantification

After 24-hour transfection total RNA was extracted and reverse transcribed for cDNA. B7-H3 gene levels were absolutely quantified with β-actin as the internal control. Gene expression was assessed as the fold change from that of the control. The β-actin primers were forward 5'-CTCTCATCCTGGGCCCTCGTGT-3' and reverse 5'-GCTGTCACCTTACCACGGTCC-3'. FCM was used to determine B7-H3 protein expression after transfection.

Cell Proliferation by MTT Assay

The MTT assay was used to study the effect of B7-H3 siRNA interference on PC-3 cell proliferation. B7-H3 or control siRNA transfected PC-3 cells were plated at 10,000 cells per well in a 96-well plate for 24, 48 or 72 hours. At each time point after discarding the medium 100 μl F12 containing 20 μl MTT (Sigma®) (5 mg/ml) were added to each well. After incubation at 37°C for 4 hours the MTT solution was removed. Dimethyl sulfoxide (100 μl) was added to each well and mixed to dissolve the dark blue formazan crystals that formed. The proportion of viable cells was determined by reading optical density using test wave length (570 nm) and reference wave length (630 nm) with a Multiskan™ Mk3 ELISA reader. The assay was done in quintuplicate for each group and repeated in triplicate.

FN Coating of High Bind Microplate

FN (Sigma) (10 μg/ml) solution composed of 10 μg/ml FN, 20 mmol/l tris-HCl (pH 7.4), 150 mmol/l NaCl, 1 mmol/l MgCl2, 1 mmol/l CaCl2 and 1 mmol/l MnCl2 was added to a 96-well high bind microplate (Corning®) at 100 μl per well and allowed to incubate at 4°C overnight. The plate was incubated with blocking buffer composed of 10 mmol/l HEPES (pH 7.4), 140 mmol/l NaCl, 5.4 mmol/l KCl, 5.56 mmol/l glucose, 3% bovine serum albumin, 1 mmol/l MgCl2, 2 mmol/l CaCl2 and 1 mmol/l MnCl2 at 37°C for 2 hours and air dried before analysis.

Assays

Cell adhesion. B7-H3 (100 μl) or control siRNA transfected PC-3 cell suspension (1 x 10⁶/ml) was plated in each well of an FN coated 96-well high bind microplate. After incubation for 4 hours at 37°C with 5% CO₂ unattached cells were washed away with PBS. The attached cell number per well was measured by MTT assay done in quintuplicate for each group and repeated in triplicate.

In vitro wound scrape. B7-H3 or control siRNA transfected PC-3 cells were incubated in 6-well plates. A small wound area was made in the confluent monolayer with a 200 μl pipette tip in a lengthwise stripe. Cells were then washed twice with PBS and incubated in serum-free F12 medium at 37°C in a 5% CO₂ incubator for 24 hours. Photographs were taken at different times from 0 to 24 hours. Wound width was measured at 200× magnification using a BX50 microscope (Olympus®) with a calibrated eyepiece grid (1 mm/100 μm graduation). Ten measurements were made at random intervals along the wound length. Data were averaged and expressed as a percent of the original width. This experiment was done in triplicate.

Invasion. A co-culture system was used as an alternative method to evaluate PC-3 cell invasiveness. Briefly, the upper portion of Transwell® inserts with an 8 μm pore size and a 6.5 mm diameter was coated with 20 μl Matrigel diluted 1:3 in serum-free F12 and incubated at 37°C for 4 hours. The coated inserts were placed in the well of 24-well plate with 600 μl F12 containing 10% FBS in the bottom chamber. After 12 hours of serum starvation the trypsinized B7-H3 or control siRNA transfected PC-3 cells were harvested and diluted to a 5 x 10⁶/ml cell suspension with serum-free F12. Each cell suspension (100 μl) was added to the upper chambers. After incubation at 37°C for 24 hours in a 5% CO₂ atmosphere the noninvading cells and gel were removed from the upper chamber with cotton tipped swabs. The cells were rinsed with PBS and cells on the filters were fixed with methanol for 30 minutes and stained with crystal violet solution (Sigma). The number of invading cells on the filters was counted in 5 random fields per filter at 200× magnification in triplicate wells of each group.

Statistical Analysis

PCA and BPH B7-H3 expression on immunohistochemical staining was compared and assessed using the chi-square test.
Other data are shown as the mean and range or mean ± SEM of 3 independent experiments. Statistical comparisons were performed using Student’s t test. All p values were determined by 2-sided tests with significance considered at p < 0.05 using SPSS® 13.0.

RESULTS

Patient Demographics
The table lists the mean age, age distribution, preoperative PSA, pathological Gleason score and pathological stage of patients with PCa. There was no statistically significant difference in age between the 2 groups (p = 0.670). PSA was significantly higher in the PCa group (p < 0.001).

Tissue Samples and Immunohistochemical Staining
B7-H3 levels in the PCa group were significantly higher than in the BPH group (mean 174.73 ± 56.80 vs 82.69 ± 46.19 ng/gm, p < 0.001, fig. 1). Immunohistochemical staining revealed significantly over expressed B7-H3 in tumor tissue (chi-square 13.987, p < 0.001). B7-H3 expression was detected in more than 50% of cells in 7 of the 13 PCa specimens but none was detected in BPH specimens. In 4 of the 13 PCa specimens and 5 of the 19 BPH specimens 25% to 50% of cells stained positively. In 2 of the 13 PCa specimens and 14 of the 19 BPH specimens there was less than 25% positive staining (fig. 2).

Down-Regulation by siRNA in PC-3 Cells
To determine the efficiency of RNA interference we first analyzed the levels of B7-H3 protein transcription and expression in the B7-H3 and control siRNA, and untransfected groups. Figure 3, A and B shows B7-H3 mRNA expression in PC-3 cells. There was markedly decreased gene expression after transfection of B7-H3 siRNA for 24 hours compared with
transfection in the control siRNA and untransfected groups (mean 53.6% ± 6.5% and 55.7% ± 7.9%, respectively, p < 0.01). However, there was no significant difference between the control siRNA and untransfected groups (p = 0.657). A similar decrease was found in protein synthesis and secretion (mean 53.5% ± 2.9% and 56.3% ± 3.7%, respectively, p < 0.01, fig. 3, C). This indicates that the siRNA down-regulated B7-H3 gene was specific and efficient.

**Effect of B7-H3 siRNA Interference on PC-3 Cells Proliferation.** To characterize the role of B7-H3 in PC-3 cell growth we measured the cell proliferation rate in vitro by MTT assay. There was no statistical significance in cellular proliferation between the control and experimental groups (fig. 4).

**Adhesion to FN.** Assay for tumor cell adhesion to FN is a commonly used method to study tumor cell metastasis. Figure 5 shows the inhibitory effect of B7-H3 siRNA interference on PC-3 cell adhesion to FN. Results revealed a greater than 30% decrease in cellular adhesion in B7-H3 siRNA transfected cells.

**Migration on wound scrape assay.** To determine whether B7-H3 acts as a tumor migration regulator we used the wound scrape assay to evaluate cell motility. siRNA inhibition of B7-H3 significantly decreased PC-3 cell migration in the wound scrape model. Time course analysis of wound closure showed that a monolayer was reestablished within a significantly shorter period in the control siRNA transfected group than in the B7-H3 siRNA transfected group (fig. 6).

**Invasion ability.** After down-regulating, the gene expression of B7-H3 by siRNA in vitro assay on Matrigel filters revealed that the number of invaded PC-3 cells was decreased up to 50% (fig. 7).

**DISCUSSION**

Recent accumulated evidence indicates that B7-H3 has a critical role in tumor progression and metastasis. Screening specimens from patients with NSCLC indicated that high B7-H3 expression significantly correlates with lymph node metastasis. Wu et al reported that B7-H3 expression is related to survival time and tumor infiltration depth in gastric carcinoma cases. Our previous study indicated...
that circulating B7-H3 in serum is a highly sensitive biomarker for NSCLC and increased circulating B7-H3 suggests a poor clinical prognosis for NSCLC. B7-H3 overexpression also reportedly correlates with tumor aggressiveness and clinical outcome, suggesting that B7-H3 has an important role in tumor cell progression.

Since we realized the limitation of using immunohistochemical methods for semiquantitative analysis, we also used ELISA. Our results showed aberrant B7-H3 expression in tumor, in accord with findings by Roth et al. However, why is B7-H3 overexpressed in neoplastic tissue and why does it correlate with pathological indicators of aggressive cancer and clinical outcome? Does it have a role in tumor progression? To seek an answer we further investigated the effects of low B7-H3 expression on PC-3 biological features.

Carcinogenesis is a multiple step process in which cancer cells lose proliferation control, disseminate from a localized primary tumor mass to invading adnexa and metastasize to distant organs. Limitless cell growth is an important alteration in cancer cell phenotypes. We first determined the effects of B7-H3 depletion on PC-3 cell growth. The proliferation of B7-H3 siRNA transfected cells was the same as that of controls and there was no obvious difference in colony formation, cell cycle distributions or apoptosis (data not shown). We then performed adhesion, migration and invasion assays to examine whether B7-H3 depletion affects tumor dissemination ability.

The adhesive ability of tumor cells is associated with tumor recurrence and metastasis. FN is an important cell adhesion molecule in the extracellular matrix. This molecule mediates cell adhesion and migration, and has a significant role in tumor invasion and metastasis. We noted a distinctive inhibitory effect of B7-H3 siRNA interference on PC-3 cell adhesion to FN. This indicates that B7-H3 may be a potential target to block interaction between tumor cell adhesion molecules and their ligands to prevent cancer metastasis.

To further investigate whether B7-H3 contributes to tumor metastasis we performed a wound scrape assay to evaluate cell motility and an invasion assay to assess tumor cell invasiveness in vitro to determine 2 aspects of the mechanisms of cell metastasis toward distant tissue. Results indicate that cell migration explained most wound closure enhancement while evidence in support of cell proliferation was not observed. This suggests that B7-H3 has a putatively important role in tumor migration and invasiveness, indicating higher aggressiveness and poor clinical outcome. Nevertheless, underlying molecu-
lar mechanisms of the actions of B7-H3 still require further research in vitro and in vivo.

CONCLUSIONS
B7-H3 is aberrantly expressed in prostate cancer. Our study indicates that B7-H3 may regulate tumor progression in a novel way, in addition to acting as an immunoregulatory protein, and it may serve as a potential molecule target for tumor therapy. Although preliminary data are interesting, further studies are needed to determine the potential target for treatment.

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REFERENCES