Cathepsin B Expression is Similar in African-American and Caucasian Prostate Cancer Patients

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Abstract. *Background: Increased incidence and mortality of prostate cancer (PCa) suggest that U.S. African-American men have more invasive cancer than Caucasian men. Invasive PCa requires several proteases, including the cysteine protease cathepsin B (CB), for degradation of basement membrane and extracellular matrix proteins prior to cancer cell migration across biological compartments. Our objective was to determine whether CB immunostaining patterns, in relation to clinical data, could distinguish invasive PCa in African-American and Caucasian patients. Patients and Methods: Fifty Gleason score 6/7 PCa cases were selected for similar clinical data with benign prostatic hyperplasia (BPH) samples as controls. Immunostainings were imaged directly from microscope slides to a computer using a digital camera. Data were quantified using Metamorph software, analyzed using the two-sample t-test and confirmed by multiple regression. Results: Ratios of CB to its endogenous inhibitor stefin A (SA) immunostainings were greater in PCa than BPH, but were not significantly different in PCa of either race. The African-American patients did not show increased CB immunostaining, indicating that the contribution of this protease to invasiveness was similar in both races. Conclusion: When veterans received equal medical care at the Minneapolis Veterans Affairs Medical Center, African-American patients did not show increased PCa invasiveness. Our conclusion is supported by analysis of post-surgery serum total PSA levels and cancer cell invasion to margins/capsules, seminal vesicles and/or lymph nodes. Invasiveness of PCa does not appear to be race-dependent. The previous conclusion of race-based differences in PCa requires re-evaluation with respect to the role of proteases (such as CB, matrix metalloproteinase) in invasion and metastasis of cancer cells.*

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Many studies have shown that incidence, death rate, tumor volume, age, Gleason grade/score, and/or the serum total PSA level greatly influence the course of prostate cancer (PCa) in men of different races and ethnicities (such as Caucasian, African-American, Asian-Pacific Islander, Native-American and Hispanics) (1-5). The above parameters suggest that PCa in U.S. African-American men is more invasive (aggressive) than men of Caucasian and other races and ethnicities. A variety of biomarkers (such as PSA-density, caveolin-1, Bcl-2, p53, c-MYC, cell proliferation, apoptosis, Bcl-2 and BAX proteins) have been utilized to explain the increased aggressiveness of PCa in different races (6-8). In contrast to benign prostatic hyperplasia (BPH) tumors, invasive PCa cells degrade basement membrane (BM) and extracellular matrix (ECM) proteins prior to their migration and invasion to the subjacent stroma (9-11). Our review has shown that proteases are critical in the development of invasive PCa, but have not been utilized to assess invasive characteristics of PCa in African-American men in comparison to Caucasian patients (9-12).

Prostate cancer cells develop the ability to degrade BM and ECM proteins utilizing one or more proteases to invade subjacent prostatic stroma, margins/capsules, seminal vesicles, pelvic lymph nodes and/or other organs (9-11). Invasive PCa cells successfully invade beyond the prostatic margins/capsules to distant organs. The cysteine protease cathepsin B (CB) is involved in the degradation of BM and ECM proteins, and cancer cell invasion and progression in many solid organ cancers (such as breast, colon, brain, lung and melanoma) (10, 12). Sinha *et al.* have shown that CB activity was significantly increased in PCa when compared to the activities of

Table I. *Clinical data in African-American and Caucasian prostate cancer patients.*

	African-American	Caucasian	*p*-value
Number of patients	25	25	-
Age at RP, range (Mean±SEM)	47.87-73.99 (62.12±1.43)	53.82-73.57 (65.12±1.11)	0.1
Pre-prostatectomy PSA in ng/ml, range (Mean±SEM)	0.92-26.2 (7.24±1.47)	0.8-29.9 (11.32±2.36)	0.15
Number of years since RP, range (Mean±SEM)[a]	3.33-23.98 (11.25±1.38)	15.29-22.29 (17.61±0.44)	0.0001
Clinical stages[b]	T2a-T3a, T3c, N1	T2a-T3a, T3c, N1	-
Gleason grades	3 and 4	3 and 4	-
Number of patients with post-surgery PSA <0.2 ng/ml	15	7	-
PSA in ng/ml, range (Mean±SEM)	0.04-0.17 (0.07±0.01)	0.09-0.11 (0.10±0.002)	0.07
Number of patients with post-surgery PSA ≥0.2 ng/ml	10	18	-
PSA in ng/ml, range (Mean±SEM)	0.2-82.9 (12.11±7.98)	0.2-467.2 (30.03±25.80)	0.5

[a]Data updated as of June 1, 2006. [b]Stages C1, C2, and D1 of the Whitmore-Jewett stages were converted to T3a, T3c, and N1, according to the TNM classification. RP=radical prostatectomy, PSA=prostate-specific antigen, and SEM=standard error of the mean.

endogenous cysteine protease inhibitors, such as stefin (cystatin) A (SA) (13). Earlier, Kos *et al.* concluded that ratios of CB to SA were better prognosticators for cancer patient survival than were levels of CB or SA alone (14). In a recent study of formalin-fixed, archival radical prostatectomy (RP) tissue samples, Sinha *et al.* evaluated CB and SA immunostainings and showed that a ratio of CB>SA in PCa had a significant relationship with pelvic lymph node metastases (15). In contrast, a ratio of CB≤SA was associated with less aggressive (latent) PCa which was often confined to the gland (15). Our objective was to determine whether immunostaining patterns of CB and SA proteins, including their ratios, in relation to clinical data would differentiate PCa invasiveness in African-American and Caucasian patients.

Patients and Methods

Data collection. Fifty archival RP PCa cases were selected out of a total of 130 African-American and Caucasian patients according to the overall similarity of Gleason grade, age, pre-serum total PSA levels and clinical stage (Table I). Initially, 35 African-American cases were selected at the Minneapolis Veterans Affairs Medical Center, but 10 patients did not have complete clinical data and were not included in the study. The remaining 25 African-American PCa cases were evaluated by immunohistochemical (IHC) methods. From a collection of 95 PCa cases, 25 Caucasian RP patients who showed overall similarity in the selection parameters to African-American patients were selected. For controls, eight BPH cases without any evidence of malignancy in the pathology report were used. Tissue sections from the 58 cases were used for localization of CB and SA. Reaction products were analyzed in relation to clinical data (such as race, ethnicity, pre- and post-RP PSA levels, clinical stage, Gleason score, cancer cell invasion to margins/capsules, seminal vesicles and/or pelvic lymph nodes, and treatment follow-up). Follow-up medical records were updated as of June 2006. All samples and medical information were collected with approval of the Institutional Review Boards of the Veterans Affairs Medical Center and the University of Minnesota, Minneapolis, MN, USA.

Prostatectomy sample processing. Gleason grade/score is one of the most powerful independent prognostic factors in PCa (16). Radical prostatectomy tissue sections were selected showing primary (principal) and secondary Gleason patterns 3+3 (score 6), 3+4 (score 7) and 4+3 (score 7) tumors, as reported by Gleason (16) and modified by the 2005 International Society of Urologic Pathology Consensus Conference (17). Since there were few cases with patterns 3+4 or 4+3 (score 7) tumors, they were considered together. Formalin-fixed, paraffin-embedded archival blocks were stored in air conditioned rooms by the Surgical Pathology Service. Six to eight freshly cut serial sections (5 to 6 μm thick) for CB, SA and control studies by IHC methods were used. In addition, hematoxylin and eosin stained sections were used for pathological grading by one of us (SLE).

Immunohistochemistry. Mouse monoclonal anti-human liver CB immunoglobulin G (IgG) was obtained from Oncogene Research Products (Calbiochem, Cambridge, MA, USA). Polyclonal goat anti-human SA was purchased from R&D Systems (Minneapolis, MN, USA) and mouse monoclonal anti-human SA antibody IgG from KRKA (Novo Mesto, Slovenia). These antibodies were affinity purified on immobilized protein A or human SA by the manufacturers. Bovine serum albumin was obtained from Sigma (St. Louis, MO, USA). Phosphate-buffered saline (PBS) was prepared in our laboratory using sodium chloride, potassium chloride, sodium phosphate dibasic, and potassium phosphate monobasic in double-distilled water (pH 7.35). Vectastain ABC-peroxidase secondary antibody kits were purchased from Vector (Burlingame, CA, USA). Earlier, we reported the molecular weights of CB (21 to 31 kDa) and SA (11 kDa) in prostatic tissues as determined by western blotting (15, 18, 19). In this study, a new set of antibody IgGs other than those used in earlier studies were employed (13, 15, 18, 20). The new set of antibodies against CB and SA showed the same molecular weights as the previous antibodies and did not show cross reactivity in western blots.

Cathepsin B and SA were localized in RP tissue sections using IHC localization techniques (13, 15, 18). Briefly, antigen retrieval was performed in 10 mM citrate buffer (pH 6.0) using a Decloaking Chamber Pro machine (Biocare Medical, Walnut Creek, CA, USA). Boiling of deparaffinized archival sections in citrate buffer unmasked antigenic sites as indicated by IHC

localization of CB and SA. Sections without citrate boil did not localize antibodies. Negative control sections were incubated with pre-immune mouse or goat serum in lieu of primary antibody. The reaction products were developed for 10 minutes with fresh-filtered 3,3'-diaminobenzidine solution (0.25 mg/ml; Sigma) in PBS with 0.01% hydrogen peroxide as the substrate and enhanced with osmium tetroxide solution.

Quantification of localization data by Metamorph Image Analysis System. Immunostainings of CB and SA were quantified using a computer-based image analysis system equipped with Metamorph software (Universal Imaging Corp., West Chester, PA, USA) (13, 15, 18). Briefly, images of CB and SA reaction products were acquired at x200 directly from the microscope slide to a computer using a digital camera (Photometrics, Tucson, AZ, USA) attached to a Zeiss microscope. Utilization of neutral and green filters optimized reaction product imaging. Based on gray values ranging from 4,095 to 0, white to black respectively, threshold boundaries of immunostaining were created and expressed as a percentage of the total field area under view. Measurements of CB and SA are presented as range and mean ± standard error of the mean.

Data analysis. Data were analyzed using the two-sample *t*-test and confirmed with multiple regression.

Table II. *Distribution of post-RP patients with biochemical failure.*

	African-American	Caucasian
Number of patients	10	18
PSA≥0.20 ng/ml	10	18
Margin/Capsule positive	4	10
Seminal vesicle invasion	5	2
Lymph node positive	1	2
Confined to prostate	1	7
Number of patients with treatment after RP	2	8

Biochemical failure is defined as post-RP serum PSA levels ≥0.20 ng/ml. Table shows the breakdown of African-American and Caucasian patients with biochemical failure and other parameters of invasiveness. Seminal vesicle invasion was more common in African-American men. Caucasian patients had more cancer cell-positive margins/capsules than African-American men with PCa. In the present subset of patients, Caucasian men with organ-confined PCa had more biochemical failure than African-American patients. Caucasian patients with biochemical failure had longer follow-up than African-American patients. Two Caucasian patients had invasions at two sites.

Results

Profile of prostate cancer patients. In the present study, invasive PCa was defined by elevated post-RP serum total PSA levels and cancer cell invasion to prostatic margins/capsules, seminal vesicles and/or pelvic lymph nodes. Our samples included 16 cases of Gleason score 6 and nine cases of score 7 for each race. The age and pre-surgery serum total PSA levels of African-American and Caucasian PCa patients were not significantly different at RP ($p=0.10$ and 0.15, respectively) (Table I). Post-RP PSA levels of <0.2 ng/ml were found in 15 African-American and seven Caucasian men and PSA levels of ≥0.2 ng/ml in 10 African-American and 18 Caucasian men (Table I). Post-RP PSA levels were not significantly different when both races were compared (Table I, Table II). Clinical stages for all cases were T2a to T2c (TNM classification) and C1, C2, D1 (Whitmore-Jewett classification); both systems were compared by Humphrey and Walther (21). The Whitmore-Jewett stages C1, C2 and D1 were converted to T3a, T3c, and N1, respectively according to the TNM classification. Clinical stages were essentially similar in both races. The time of post-RP follow-up of patients differed significantly ($p=0.0001$) in African-American and Caucasian patients (Table I). Lower incidence of biochemical failure in African-American patients is due to shorter follow-up time when compared to Caucasian patients.

Our analysis of CB and SA localization data by two-sample *t*-test showed that African-American and Caucasian PCa patients were similar and the results were not significantly different in the two races as expected (Table I). This was confirmed by multiple regression analysis which was also insignificant ($p=0.62$). Analysis of post-RP serum total PSA levels ≥0.2 ng/ml indicated that 10 African-American and 18 Caucasian patients had biochemical failures (Table II). A single African-American patient and seven Caucasian patients with organ-confined PCa had biochemical failure, while one Caucasian patient had follow-up treatment without elevated serum PSA levels. We found that two African-American and eight Caucasian men with extraprostatic cancer cell invasion received follow-up treatment between 0.06 and 4.87 years after RP. The remaining patients (23 African-American and 17 Caucasian men) did not receive further treatment or their status was unknown.

Cathepsin B and stefin A immunostaining in BPH glands. Immunostainings of CB and SA proteins were found predominantly in basal cells and some cuboidal/columnar cells of BPH glands in RP tissue sections (Figure 1a-b). Cathepsin B alone ranged from 1.79 to 3.47, SA alone from 2.81 to 5.02, and their ratios from 0.44 to 0.79 in BPH glands.

Cathepsin B and stefin A immunostainings in cancer. In Gleason grade 3 and 4 tumors (patterns 3 and 4 or score 6 and 7 tumors), CB and SA immunostainings were observed in cuboidal/columnar cancerous glands and isolated cells in RP sections of African-American (Figure 1c-d, g-h) and Caucasian patients (Figure 1e-f, i-j). In general, the distribution of reaction products for CB alone and SA alone showed variation between and within Gleason scores. Cathepsin B alone and SA alone reaction products were not

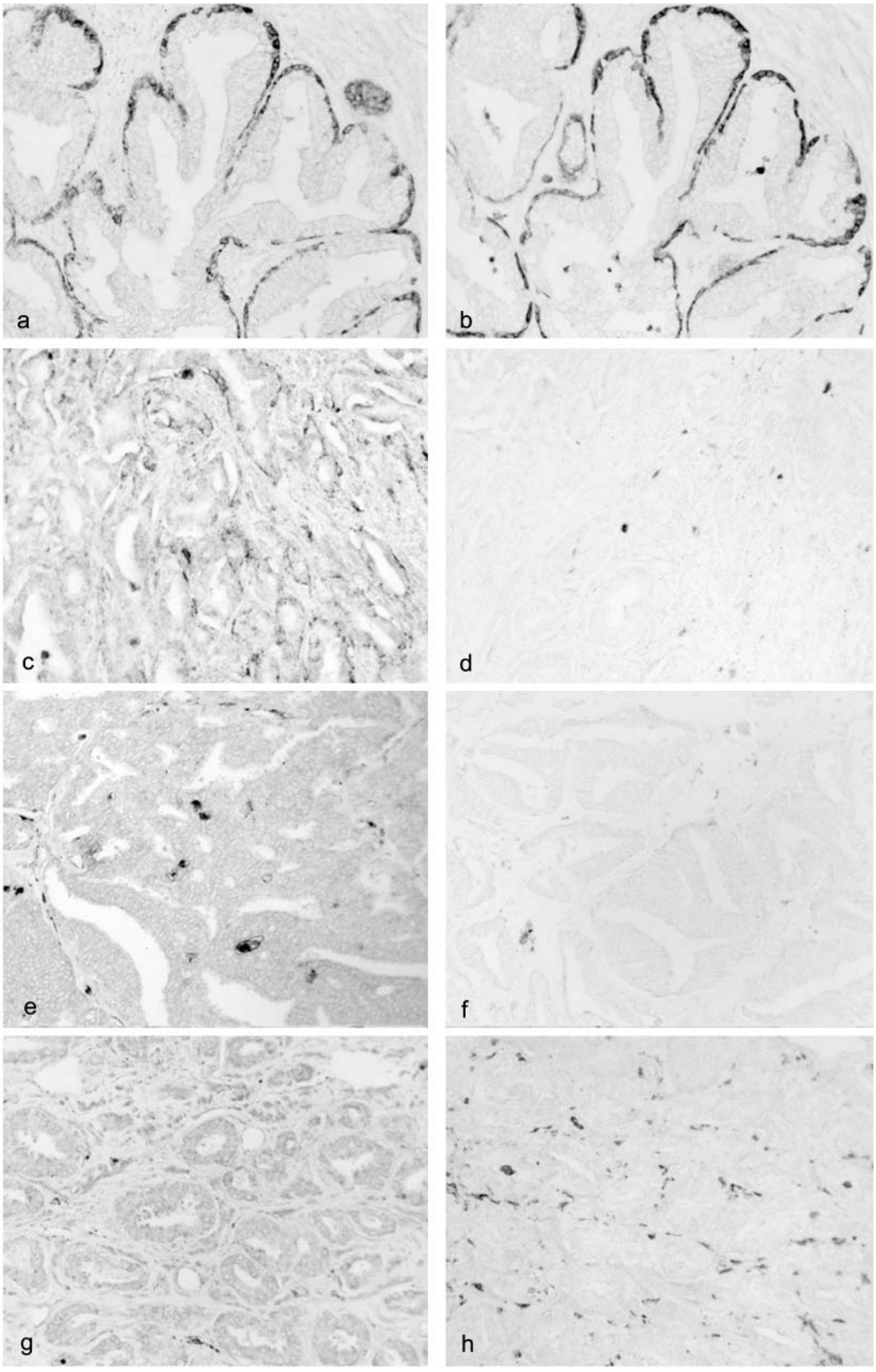

Figure 1. *continued*

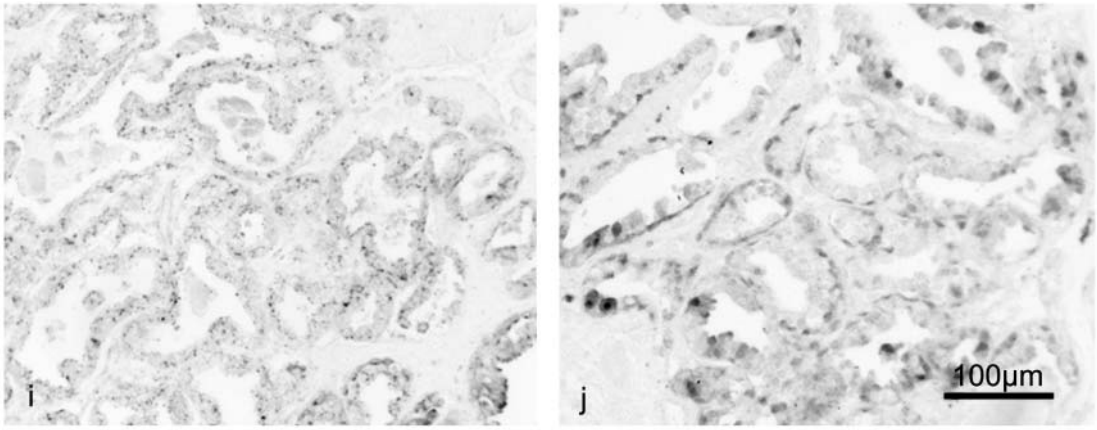

Figure 1. *Cathepsin B and stefin A immunostaining in study patients. The bar in (j) illustrates magnification for all figures. The African-American and Caucasian patients were matched for clinical data (see text). CB reaction products were found predominantly in basal cells of BPH glands of an African-American patient, with some localization in columnar cells (a). Immunostaining of SA was found in basal and some columnar cells of BPH glands from the same patient (b). The ratio of CB (a) to SA (b) reaction products was 0.82. Images are from Gleason score 6 (pattern 3+3) tumors. CB reaction products in a Gleason score 6 tumor of an African-American patient (c). Fewer SA reaction products were found in an adjacent section of the same tumor (d). The CB to SA ratio of (c) and (d) was 6.92, which is significantly higher than that of BPH. CB reaction products in a Gleason score 6 tumor of a Caucasian patient (e). SA immunostaining was found in an adjacent section of the same tumor (f). The CB to SA ratio of (e) and (f) was 9.00, which is significantly higher than that of BPH. Micrograph illustrates immunostaining for CB in a Gleason score 6 tumor of an African-American patient (g). An adjacent section (h) illustrates markedly more immunostaining for SA than in (d). Comparison of (g) and (h) shows a CB to SA ratio of 0.17, which is significantly lower than that of BPH. CB reaction products in a Gleason score 6 tumor of a Caucasian patient (i). SA immunostaining in the same tumor (j). The CB to SA ratio in (i) and (j) was 0.45, which is significantly lower than that of BPH. Heterogeneity in CB and SA immunostainings in Gleason score 6 tumors is illustrated. Similar heterogeneity found in score 7 tumors is not illustrated.*

significantly different ($p=0.37$, $p=0.20$, respectively) in Gleason score 6 tumors of African-American and Caucasian patients (Table III). Likewise, CB alone and SA alone reaction products in Gleason score 7 tumors of African-American and Caucasian patients were not significantly different ($p=0.22$, $p=0.15$, respectively) (Table III). Ratios of CB to SA were not significantly different in Gleason score 6 and 7 tumors of African-American and Caucasian patients ($p=0.59$, $p=0.77$, respectively) (Figure 2, Table III). The differences between the two groups of patients were not statistically significant. The means of the parameters were similar, therefore, a larger sample size would not show meaningful differences in African-American *versus* Caucasian patients (Table III).

Comparison of immunostainings in BPH and cancer. In African-American and Caucasian patients, reaction products for CB alone ($p=0.000014$ and $p=0.000052$, respectively), SA alone ($p=0.0000012$ and $p=0.0000021$, respectively) and their ratios ($p=0.001$ and $p=0.0007$, respectively) in Gleason score 6 tumors were significantly different from BPH (Figure 2). Likewise, reaction products for CB alone ($p=0.000019$ and $p=0.000035$, respectively) and SA alone ($p=0.00000099$ and

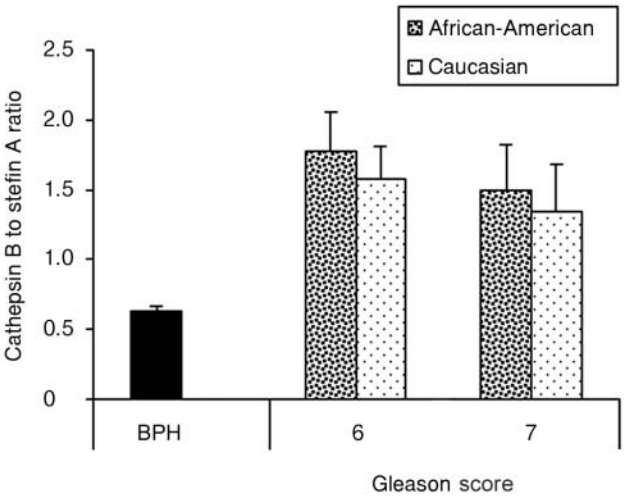

Figure 2. *Cathepsin B to stefin A ratios in African-American and Caucasian Gleason score 6/7 tumors. When the races were compared, ratios of CB to SA immunostaining were not significantly different in African-American and Caucasian men with BPH (p=0.19), Gleason score 6 (p=0.59) and 7 tumors (p=0.77). Similar results were found in Gleason score 6 and 7 tumors for CB alone (p=0.37 and 0.22, respectively) and SA alone (p=0.20 and p=0.15, respectively). Cathepsin B to stefin A ratios were significantly higher in Gleason score 6 and 7 tumors when compared to BPH controls. Data are presented as means ± standard error of the means.*

Table III. *Comparison of cathepsin B and stefin A immunostaining in Gleason score 6 and 7 tumors in African-American and Caucasian patients.*

Protein	Gleason Score 6			Gleason Score 7		
	African-American (n=16)	Caucasian (n=16)	*p*-value	African-American (n=9)	Caucasian (n=9)	*p*-value
Cathepsin B Range (Mean±SEM)	0.23-2.66 (0.75±0.15)	0.22-2.28 (0.93±0.13)	0.37	0.25-1.03 (0.60±0.08)	0.16-1.69 (0.84±0.16)	0.22
Stefin A Range (Mean±SEM)	0.10-1.60 (0.53±0.10)	0.20-1.71 (0.72±0.11)	0.20	0.20-0.91 (0.51±0.09)	0.21-1.95 (0.91±0.24)	0.15
Cathepsin B/Stefin A Ratio Range (Mean±SEM)	0.53-4.71 (1.78±0.29)	0.47-3.13 (1.59±0.22)	0.59	0.59-3.36 (1.49±0.34)	0.34-3.26 (1.35±0.34)	0.77

SEM=Standard error of the mean.

$p=0.00000079$, respectively) were significantly different in Gleason score 7 tumors when compared to BPH (Figure 2). Cathepsin B to SA ratios were significantly greater in Gleason score 7 tumors when compared to BPH in African-American patients ($p=0.036$), but were not in Caucasian patients although they did approach a significant level ($p=0.072$).

Discussion

Earlier studies have shown that CB, regulated by its endogenous inhibitor SA, is involved in cancer cell invasion and progression in PCa (13, 15, 18) and many other types of solid organ cancer (10, 12). Analysis of CB and SA immunostainings, including their ratios, in Gleason score 6 and 7 tumors did not differ between African-American and Caucasian patients, indicating a similar role of CB in the mechanism for PCa invasiveness of both races. Analysis of immunostaining data in relation to pre-RP serum total PSA levels, Gleason scores, age and/or cancer cell invasion to margins/capsules, seminal vesicles and/or pelvic lymph nodes did not show any difference. Our finding is consistent with that of Witte *et al.* who concluded that the biology of PCa was similar in African-American and Caucasian men (22). Our data are consistent with the idea that invasiveness and migration of PCa cells are mediated by proteases since the level of CB expression was greater in PCa than BPH (9-11). We postulate that degradation of BM and ECM proteins, including invasion and progression of cancer cells to other organs, may not be race-dependent.

Previous studies postulated differences in PCa of African-American patients from Caucasian and men of other races using incidence, death rate, tumor volume, age, Gleason grades/scores, and/or serum total PSA levels (1-5). Many factors, such as level of medical care, economic status, access to medical care, nutrition, in addition to the above parameters undoubtedly imparted differences in the earlier conclusion *versus* the present study. Our selection of patients, who received equal medical care at the Minneapolis Veterans Affairs Medical Center, minimized differences in PCa of African-American and Caucasian patients. Furthermore, previous studies did not include proteases as a factor to distinguish between African-American and Caucasian men, and, therefore, did not provide clues as to the biological basis of invasiveness and progression of PCa. Our conclusion of similar aggressiveness of PCa in African-American and Caucasian men based on the similar CB expression, elevated post-RP serum PSA levels and incidence of local metastasis is tentative and needs to be evaluated in a clinical trial.

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