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**14. ABSTRACT** I received approval of the DOD-HSRRB in April, 2005 and began the study shortly after receiving approval. We are enclosing copies of published and accepted papers in the Anticancer Research. These papers address parts of both Specific Aims outlined in the project. I submitted an abstract of our accepted paper, "Cathepsin B Expression Indicates that Prostate Cancer is Similar in African-American and Caucasian Men" to the Department of Defense Prostate cancer research program meeting: Innovative Minds in Prostate Cancer Today (IMPACT). Our abstract was selected and accepted for presentation on September 5-6 meetings in Atlanta, GA. This is significant achievement for our work that started in April 2005. Our paper, "Heterogeneity of Cathepsin B and Stefin A Expression in Gleason Pattern 3+3 (score 6) Prostate Cancer Needle Biopsies" was published in Anticancer Research 27: 1407-1414, 2007. We showed that small foci of Gleason pattern 3+3 (histological score 6) tumors in needle biopsies have heterogeneous cathepsin B and stefin A immunostaining. We have shown that score 6 tumors are heterogeneous and some of the tumors had micrometastases. We have suggested that stratification of these tumors by cathepsin B and stefin A in relation to clinical data may assist in identification of aggressive cancer and treatment selection.

**15. SUBJECT TERMS**
Cathepsin B, stefin A, Immunohistochemistry, Elisa assay, Prostate biopsy, Prostatectomy, Caucasian and African-American Prostate Cancers, Heterogeneity in Gleason score tumors, Treatment selection, Biomarkers for aggressive prostate cancer

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**Revised Final Report, submitted August 28, 2008**
Reporting periods March 2004 - March 2008
**Award Number W81XWH-04-1-0245**

Title: "Prediction of Aggressive Human Prostate Cancer by Cathepsin B"

**Introduction:**
Cathepsin B (CB) is required for degradation of basement membrane and extracellular matrix proteins, including prostate cancer (PC) cell invasion to the subjacent stroma and extracellular matrix proteins followed by metastasis to distant organs. Activities of CB are regulated by a highly specific endogenous inhibitor stefin A (SA). Our rationale for studying immunohistochemical localization of CB and SA in prostatic biopsy and radical prostatectomy samples was to determine whether the ratio of CB: SA could identify aggressiveness of PC in biopsy and prostatectomy specimens from Gleason score 5 to 7 tumors. Since African-American (black) men have more aggressive PC than Caucasian (white) men, we rationalized that localization of CB and SA could provide a new group of biomarkers for distinguishing aggressiveness of this cancer in biopsy and prostatectomy specimens. Localization was expected to show a higher ratio of CB: SA in black men who hhave more aggressive PC than white men. Our rationale led us to test our hypothesis using two Specific Aims:

We found that small foci of Gleason histological score 6 tumors in needle biopsies were heterogeneous as shown by CB to SA localization. Most of the score 6 tumors were not aggressive, but PC biopsies should be evaluated for identifying those which were aggressive. Biopsies of score 7 and higher should be routinely evaluated by CB and SA.

**Aim 1:** *Determine the relationship between quantitative immunohistochemical data on CB and stefin A and Elisa assay in laser capture microdissection (LCM)-microdissected and un-dissected serial sections from biopsy and prostatectomy samples.*

Our working hypothesis for Specific Aim 1 was that localization of CB and SA in laser capture microscope (LCM) dissected prostate sections would show minimum heterogeneity than un-dissected tissue sections. Dissected sections would be also used for measurement of CB and SA by Elisa assay. Our studies were conducted by localization of CB/stefin A in relation to score 6 (3+3 patterns) tumors and then in score 5 (2+3 and 3+2) and score 7 (3+4 and 4+3 patterns) tumors.

**Aim 2:** *Establish in a retrospective study whether the ratio of CB to stefin A in biopsy samples reliably reflect the ratio in prostatectomy tissue samples of the same white and African-American patient.*

Our working hypothesis for Specific Aim 2 was that localization patterns of CB to SA evaluated in Aim 1 can be further tested in a large number of biopsy and prostatectomy samples. Analysis of localization data would provide an index that could be reliably applied in determination of aggressiveness of PC was more aggressive in black than white men.

**Body of the report according to the tasks:**
Collection of Common Information: The tasks in Specific Aims 1 and 2 require common information, such as clinical data, Gleason grade/score and tumor patterns, prostate biopsy and prostatectomy tissue samples. About 85% of PC patients have Gleason score 5, 6, or 7 tumors and we focused on these scores. We also recorded races of men, including age, clinical stages, pre-

and post-surgery PSA, tumor margins, lymph node metastasis or lack thereof, mortality/survival data) from the medical records of the patients who were biopsied and also had prostatectomy surgery at the Minneapolis VAMC and/or the Virginia Urology Center.

Since tissue sections and clinical data were used for both Specific Aims, studies on these aims progressed concurrently. The statement of work section, therefore, did not include any specific time frame for each task. The utilization of tissue samples and clinical data overlapped in both Aims.

Task 1: We selected biopsy and prostatectomy tissue samples of prostate cancer patients with Gleason score 5, 6, and 7 tumors from the Minneapolis VA Medical Center and the Virginia Urology Center. Drs. Ewing and Ramnani graded prostate biopsy and prostatectomy tissue sections and provided paraffin tissue sections for immunohistochemical study. For control studies, benign prostatic hyperplasia (BPH) sections were used. These sections were used to localize CB and stefin A reaction product patterns in malignant and non-malignant areas.

Task 2: Our goal was to dissect biopsy and prostatectomy tissue sections showing Gleason score 5 to 7 tumors by laser capture microdissection (LCM) techniques for localization of CB and SA and measuring them by Elisa assay. We conducted LCM dissection of prostate biopsies and prostatectomy tissue samples. We found that cancerous areas usually occupied 5 to 10% of a biopsy core. This amount of cancerous area was inadequate for conducting both localization and Elisa assay. In view of this finding, we de-emphasized LCM study. Our limited evaluation of the LCM-dissected Gleason score 6 (3+3 patterns) tumors showed heterogenous immunostaining suggesting that prostate cancer was heterogeneous even in small dissected sections.

Task 3: Our finding in task 2 led us to de-emphasize LCM dissection and their evaluation for studying racial differences in the distribution of CB and SA. We obtained 4 to 6 micrometer serial sections from biopsy as well as prostatectomy tissue samples. In general, we obtained at least 4 biopsy sections (some of the biopsy specimens had inadequate amount of cancerous tissue). Prostatectomy tissue samples provided the required number of tissue sections for our study. Hematoxylin and eosin stained sections were graded by Drs. Ewing and Ramnani.

Task 4: We determined prostate specific antigens (PSA) localization patterns in selected number of cases. We used prostate tissue sections for localization of CB and stefin A reaction products. We acquired images of CB and SA reaction products directly from microscope slides to a computer-based image analysis system. About 15 images of CB and stefin A were acquired for each prostatectomy specimens, but only four images of biopsy sections. We determined the ratios of CB to stefin A and related them to the clinical data.

Task 5: Quantitative immunohistochemical and Elisa assay data were related to the Gleason scores. Localization of CB and SA allowed determination of reaction product ratios, namely, CB>stefin A, CB<stefin A, and CB=stefin A in scores 5 to 7. We related localization and clinical data (such as pre-and post- surgery PSA levels, clinical stages of pelvic lymph node metastases, and mortality/survival of patients. Post-prostatectomy elevation (increase) in serum total PSA would be indicative of the recurrence of disease. We have reported our findings in the enclosed papers and unfinished manuscripts (attached with this report). Elisa assay study is still under progress and will be completed in due course.

Task 6: We had proposed to use several statistical methods for analysis of localization data and used them as needed in publications. Each statistical method is detailed with publications. We determined the statistical significance and published our findings in the peer-reviewed journals. Unpublished data have been also added in the detailed description of the text.

**Factors associated with our study:**

The actual start date of our study was delayed by a year primarily because of the delay in receiving approval of the DOD-HSRRB (Log No. A12517). The actual study began around 20th April, 2005 while the funding project funding period began in March of 2004. This was reported in my earlier reports. The funding of this grant ended on February 28, 2007. I was also granted one year NO COST extension until March 31, 2008. We have been studying the unfinished part of the project, but at a considerably reduced speed because there are no funds or technician in the laboratory. The remaining work will be finished in due course and the DOD's support will be acknowledged in publications. The agency will be provided a copy of our publications.

**Body of the report according to the publications and unpublished study in progress:**

1. A brief summary of our paper:

Background. The cysteine protease cathepsin B (CB) is involved in degradation of basement membrane and extracellular matrix proteins, cancer cell invasion, and its progression in other biological compartments. Activities of CB are usually regulated in part by its endogenous inhibitor stefin (cystatin) A (SA). Localization of CB and SA in formalin-fixed archival RP tissue samples has shown a significant relationship of a ratio of CB>SA with pelvic lymph node metastases. In this study, we evaluated CB and SA as biomarkers of PCa in prostate needle biopsy samples.

Patients and Methods. Immunostainings of cathepsin B and stefin A of 65 biopsy sections were imaged, quantified, and analyzed with Student's t-test ($p<0.05$).

Results. Patients had T1c to T3b clinical stages and pre-surgery total prostate specific antigen serum levels from 1.25 to 20.0ng/ml. Cathepsin B and stefin A reaction products were found in the cytoplasm of basal and columnar/cuboidal cells of benign prostatic hyperplasia (BPH), prostatic intraepithelial neoplasia (PIN), and neoplastic cells. Statistical analysis of immunostaining data showed that CB alone was not significantly different in benign prostatic hyperplasia (BPH), prostatic intraepithelial neoplasia (PIN) and prostate cancer (PCa), but SA alone was significantly lower in PCa ($p<0.001$) when compared to BPH and PIN glands. Ratios of CB to SA were significantly higher in PCa when compared to BPH and PIN glands ($p<0.001$) and also in PIN compared to BPH ($p=0.036$).

Conclusion. This is the first study to characterize small foci of Gleason pattern 3+3 PCa in needle biopsies by CB and SA. We found heterogeneous cathepsin B and stefin A immunostaining Gleason pattern 3+3 tumors in needle biopsies.

**Paper:** Sinha, A. A., Morgan, J. L., Wood, N., Betre, K., Reddy, A., Wilson, M. J., Ramnani, D.: Heterogeneity of Cathepsin B and Stefin A Expression in Gleason Pattern 3+3 (Score 6) Prostate Cancer in Needle Biopsies. Anticancer Research, 27: 1407-1414, 2007. A PDF is enclosed.

2. A brief summary of another paper:

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 (such as cathepsins, matix metalloproteinase), 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, would show that the African-American men have more aggressive PCa than white men.

Patients and Methods: Fifty Gleason histologic score 6/7 PCa cases were selected out of 130 patients using similar clinical criteria (such as Gleason grade/score, pre-RP serum total PSA levels, clinical stage and age). Benign prostatic hyperplasia (BPH) samples were used 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 node metastases. 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. In this study, we found that after prostatectomy AA patients did not regularly monitor PSA levels and/or consulted their physicians/surgeons as white patients. The follow-up was significantly lower in black patients than white patients ($p=0.0001$). As a result, white patients received four times more adjuvant therapies than black men.

**Paper:** Sinha, A. A., Morgan, J. L., Buus, R. J., Ewing, S. L., Fernandes, E. T., Le, C., Wilson, M. J.: Cathepsin B Expression is Similar in African-American and Caucasian Prostate Cancer Patients. Anticancer Research 27:3135-3142, 2007. The PDF file is enclosed.

IMPACT: Our study indicated that previous conclusion of differences in AA and Caucasian PCa patients requires re-evaluation with respect to the role of proteases (such as cathepsin B, matix metalloproteinase) in invasion and metastasis of cancer cells in these two races. We suggest that multiple factors are involved in generating more aggressive PC in AA men.

**Paradigm Shift:** The impact of the DOD funding has led us to show that aggressiveness (invasiveness) of prostate cancer (PCa) was similar in AA and Caucasian men. This finding has led to the paradigm shift from the previous paradigm stating that PCa was more aggressive (invasive) in AA than white patients.

**Abstract** of our study on black and white prostate cancer men was presented at the INNOVATIVE MINDS IN PROSTATE CANCER TODAY (IMPACT) meetings during the Department of Defense Prostate Cancer Research Program meetings of 2007 September 5-6 at Atlanta, GA, we had presented our study on 'Cathepsin B Expression Indicates that Prostate Cancer is Similar in African-American and Caucasian Men'. The authors were Akhouri A. Sinha; Jenifer L. Morgan; and Michael J. Wilson, POSTER # P4-6, page 111.

**Validation of our findings in another race and ethnic group of PCa patients:**

**Paper:** Sinha, A. A., Morgan, J. L., Betre, K., Wilson, M. J., Le, C. and Marks, L. S.: Cathepsin B expression in Native Japanese and Japanese-American prostate cancer patients: An immunohistochemical study. Anticancer Res., 28:2271-2278, 2008. A PDF file is enclosed.

The initial proposal to the DOD did not include this study because we had not anticipated that aggressiveness of PCa in black men will be similar to those in white men. In view of this new finding, we tested the above biomarkers in Japanese men with prostate cancer. Using the above biomarkers, we tested the existing concept that the Japanese and other Asian men have a lower rate of aggressive PCa than Japanese men living in the U.S.A.

This adjunct study was supported by the Research Service of the Minneapolis VAMC and in part by a grant from the Prostate Cancer Foundation (CaPCURE) to LSM. This work has been published in Anticancer Res., 28:2271-2278, 2008. We selected PCa cases prior to localization of CB and SA in Japanese population. We matched Japanese patients according to the Gleason grade/score, pre-RP serum total PSA levels, clinical stage and age prior to evaluation of immunostainings greatly minimized subjectivity associated with the evaluation of markers in this ethnic sub-population of PCa patients. We found similar CB and SA immunostaining in Japanese patients who have organ-confined and moderately-differentiated PCa. Analysis of the reaction product data provides indirect evidence that invasiveness of PCa is similar in the two Japanese patient populations. Thus, we have validated our study on black and white PCa patients by studing this group of patients. A pdf file of this adjunct study is enclosed.

Summary of validation study on Japanese men: Background: Invasiveness (or aggressiveness) of prostate cancer (PCa) varies significantly in patients. Metastasis is responsible for about 90% deaths in human carcinomas. Japanese-American (J-A) men who have immigrated to the U.S.A. and acquired the Western lifestyle usually have more invasive PCa than native Japanese (NJ) living in Japan. Many criteria (such as age, body weight, body fat, food habits, nutrition, hormone levels, medical care, pathological grade, tumor volume, nuclear size and shape, pre-biopsy serum total prostate specific antigen (PSA) levels, PSA density, pro-PSA expression, clinical stage and/or relationship of prostatic stroma and carcinomatous epithelia) have been used to show differences in the Japanese population. The specific reasons for these differences remain unknown. Selective utilization of criteria has provided inconsistent results. Objective of this study was to examine immunostainings of cathepsin B (CB) and its endogenous inhibitor stefin A (SA) in tissue microarray (TMA) and radical prostatectomy (RP) tissue sections in the hope of obtaining insights into the invasiveness of PCa in Japanese patients.

Patients and Methods: TMA and RP sections were evaluated in 50 men (25 NJ and 25 J-A) for CB and SA reaction products. The CB and SA immunostainings were imaged directly from microscope slides to a computer using a high performance Charge Coupled Device (CCD) digital camera, quantified using Metamorph software, analyzed using the two-sample *t*-test, and confirmed by multiple regression analysis.

Results: The CB and SA proteins were localized in the carcinomatous glands and isolated cancer cells in the TMA and RP sections. The Gleason scores and pre-surgery serum total prostate specific antigen (PSA) levels did not differ significantly in the NJ and J-A patients ($p$=0.14, $p$=0.16, respectively). The Chi-square analysis of clinical stage *versus* place of birth showed that the NJ patients had significantly more T2a and T2b clinical stages than the J-A patients who had more advanced T2c and T3a stages ($p$=0.003). The CB and SA immunostainings and their ratios in Gleason score 6 tumors did not show any difference, but the CB: SA ratios in score $\geq$7 tumors approached significance levels.

Conclusion: The overall matching of specimens according to the Gleason grade/score, pre-RP serum total PSA levels, clinical stage and age prior to evaluation of immunostainings greatly minimizes subjectivity associated with the evaluation of markers in this ethnic sub-population of PCa patients. CB and SA immunostaining is similar in Japanese patients who have organ-confined and moderately-differentiated PCa. Analysis of the reaction product data provides indirect evidence that invasiveness of PCa is similar in the two Japanese patient populations.

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PDF file of this unpublished manuscript is enclosed. Unpublished manuscript was submitted for review and publication in August 2008.

# CHARACTERIZATION OF PROSTATE CANCER IN NEEDLE BIOPSY BY CATHEPSIN B, CELL PROLIFERATION AND DNA PLOIDY

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Running Title: Analysis of Biomarkers in Needle Biopsy and/or Prostatectomy Specimens

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**Abstract**

Background: More than 30% of prostate cancer patients have extra-prostatic invasions by cancer cells at radical retropubic prostatectomy. Approximately, 40% patients elect prostatectomy, but 60% select other treatments using initial diagnostic information. Prior to selecting any treatment these patients would benefit by assessment of the nature of cancer in biopsy specimens additional biomarkers. Our objective was to determine localization patterns of three distinct groups of biomarkers (cathepsin B, cell proliferation: MIB-1 and DNA ploidy) in the prostate needle biopsy sections in the hope of establishing localization similarities (or differences) in biopsy and RP specimens.

Materials and Methods: Prostate needle biopsy specimens and matched radical prostatectomies from 47 patients with cancer were evaluated; none had lymph node metastases. Only biopsy tissue sections were stained with rabbit anti-cathepsin B (CB) antibody or mouse anti-human stefin (cystatin) A (SA). The extent and intensity of staining were quantified using an image analysis system equipped with Metamorph software. The ratio of CB to SA was calculated for each biopsy cancer and matched benign prostatic hyperplasia (BPH) and benign prostatic acini. This ratio was correlated with serum PSA, biopsy Gleason score, biopsy DNA ploidy, biopsy MIB-1 (Ki-67) index, prostatectomy Gleason score, prostatectomy cancer volume, pathologic stage, and surgical margin status.

Results: Patients ranged in age from 48 to 74 years (mean, 65 years). Mean preoperative serum PSA was 9.1ng/ml (range, 3.6-28.2 ng/ml). At prostatectomy, 17 patients had Gleason score 6 and 30 had Gleason score 7 cancer. Mean cancer volume was 1.64 cc (range, 0.7-2.9). Four (8.5%) had extra-prostatic extension of cancer, 7 (14.9%) had positive surgical margins, 2 (4.3%) had seminal vesicle involvement, and none had lymph node metastasis. Preoperative serum PSA correlated with cancer volume at radical prostatectomy (P=0.038). There was no correlation of prostatectomy Gleason score with cancer volume or pathologic stage. Geometric mean of CB to SA was 1.45 (range 1.12-1.87) in benign prostatic hyperplasia and 2.99 in cancer specimens (range, 2.30-3.89) (P=0.0001). The ratio of CB to SA in prostate needle biopsy had no correlative association with preoperative serum PSA concentration, biopsy MIB-1 index, overall biopsy DNA ploidy status, prostatectomy cancer volume, Gleason score, positive surgical margin, or pathologic stage. There was no correlation of % S phase cells based on DNA and MIB-1, a marker for cell proliferation, Gleason score, pathology stage, or surgical margins at prostatectomy.

Conclusion: Our study has indicated that the ratio of CB to SA is significantly higher in biopsies PCa than in BPH, as it was in previously studied RP cases. Since CB is involved in invasiveness of cancer cells, it may be elevated in small and large tumors and thus no correlation with tumor volume. The percentage of S-phase cells and DNA ploidy in needle biopsies predicts cancer volume in RP. We have shown that localization of three distinct biomarkers in biopsies reliably reflects similarity of localization in RP specimens. These biomarkers clarify the nature of cancer in biopsied specimens and thus, allowing patients to select other treatments, including prostatecomy.

**Acknowledgment**

This research was supported by the Department of Defense Grant # W81XWH-04-1-0245, USPHS National Cancer Institute Grant # CA100203 to A. A. S. and in part by the Research Service of the Minneapolis Veterans Affairs Medical Center by providing laboratory and office space facilities to the senior author (AAS). Senior author and his laboratory personnel were provided demographic data only after the results of CB and SA image analysis were provided to the first author. Senior author localized CB and SA and thus, was blind for the study until completion of the work. They gratefully acknowledge the technical assistance of Mrs. Jenifer Morgan, of the Department of

Genetics, Cell Biology and Development, University of Minnesota, MN and the staff of Library Service and Research Services of the Minneapolis VAMC.

**Report on unpublished work in progress:**

The final form of this manuscript, including presentation of data and figures may change in the final manuscript.

# Characterization of prostate cancer by cathepsin B and stefin A by Immunohistochemistry and Elisa assay

Akhouri A. Sinha, Ph.D. [1, 3, 4, 5], Stephen L. Ewing, M.D. [2, 3, 5], Eduardo T. Fernandes, M.D. [3, 5], Michael J. Wilson, Ph.D. [2, 4, 5], Janko Kos, Ph.D. [6]

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## Introduction

Cysteine protease cathepsin B (CB), a lysosomal cysteine protease (molecular weight 25-30 kDa), is located on a single gene on chromosome 8p22 [1-3] and involved in cancer cell invasion and its progression across biological compartments in prostate cancer (PCa) [4,5] and many solid organ cancers (such as breast, colorectal, brain, melanoma, lung) ) [6-14]. CB probably acts in concert with serine

proteases and matrix metalloproteases [6] and is one of essential proteases required in degradation of basement membrane (BM) and extracellular matrix (ECM) proteins, and this degradation is a pre-requisite for cancer cell invasion allowing migration of cancer cells to the adjacent stroma and elsewhere [6,13,15-18]. Activities of CB are regulated by its endogenous inhibitor stefin (cystatin) A (SA) [5,19]. Friedrich et al. [20] reported lower activities of cathepsins B, H, and L in prostate tumor tissue homogenates than in adjacent normal tissues in comparison to cysteine protease inhibitors (CPIs). They showed that the primary cultures of prostate samples and prostate cell lines showed higher CB activities in tumor cells when compared to normal cells. They did not localize CB or SA by immunohistochemical (IHC) techniques or relate their study to clinical data of patients. We have shown that the activities of CB was significantly lower in relation to CPIs in benign prostatic hyperplasia (BPH) glands whereas CB activities were significantly elevated in PCa in relation to CPI acitivities [5]. Aggressive PCa had increased invasion to prostatic margins/capsules, microvessels, lymphatics, seminal vesicles, bladder neck and/or pelvic lymph nodes than less aggressive ones [19]. Kos et al. [21] had concluded that ratios of CB to SA were better prognosticators for survival than the levels of CB or SA alone. Our objective was to determine the relationship of CB and SA proteins by immunohistochemistry and their amounts in laser capture microdissected prostate cancer cells by Elisa assay.

**Materials and Methods**

We have studied 107 archival radical prostatectomy (RP) tissue samples of white PCa patients by antibodies against CB and SA and immunohistochemical methods and analyzed quantitative immunostaining data in relation to archival clinical data (pre- and post-RP PSA levels, clinical stages, Gleason scores, cancer cell invasion status to margins/capsules, seminal vesicles and/or pelvic lymph nodes, and treatment follow-up) at the Minneapolis Veterans Affairs Medical Center (VAMC) (Table 1). Most of the follow-up data from the medical records were updated as of June 30, 2007, but will be updated prior to publication.

We obtained formalin-fixed, paraffin-embedded RP tissue sections (about 5 m thick) for immunohistochemistry in addition to hematoxylin and eosin (H&E) stained sections for Gleason grades by one of us (SLE) [22,23]. Benign prostatic hyperplasia (BPH) or benign prostate glands were used as controls. All samples and medical information were collected according to the approval of the Institutional Review Boards (IRB) of the VAMC and the University of Minnesota, Minneapolis, MN.

**Antibodies Against Cathepsin B and Stefin A:** We localized CB and SA antibodies according to the methods described by us [5,19,24]. Mouse monoclonal anti-human liver CB (clone IM27L) immunoglobulin G (IgG) was obtained from Oncogene Research Products (Calbiochem, Cambridge, MA). Mouse monoclonal anti-human SA antibody IgG was purchased from KRKA (Novo Mesto, Slovenia) and polyclonal goat anti-human SA from R& D Systems (Minneapolis, MN). All antibodies in this study were affinity purified on immobilized protein A or human SA by the manufacturers. Phosphate buffered saline (PBS) and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO). We have already reported the molecular weights of CB (21 to 31 kDa) and SA (11 kDa) in prostatic tissues [4,19,25]. Our antibodies did not show any cross reactivity with other proteins in western blots [4,19,25].

**Immunohistochemical localization of cathepsin B and stefin A:** We localized CB and SA in RP tissue sections using IHC localization techniques [19,26,27]. Briefly, antigen retrieval was carried out in 10 mM citrate buffer (pH 6.0) using a Decloaking Chamber Pro machine (Biocare Medical, Walnut Creek, CA). Mouse anti-CB and mouse or goat anti-human SA antibodies were localized in adjacent sections. Negative control sections were incubated with pre-immune mouse or goat serum in lieu of

the primary antibodies. The reaction products were developed, usually less than 10 minutes, with fresh-filtered 3, 3'-diaminobenzidine (DAB) solution (0.25 mg/ml; Sigma) in PBS with 0.01% $H_2O_2$ as the substrate. Chromogenic reaction product was enhanced with diluted osmium tetroxide solution.

**Quantification of localization data using Metamorph image analysis system:** The immunostainings for CB and SA were quantified using a computer-based image analysis system equipped with Metamorph software (Universal Imaging Corp., West Chester, PA, USA), as we reported previously [5,19,24]. Briefly, the images of ten-to-15 different areas showing prostate cancer and invasive cells were acquired at 200X (10X ocular and 20X objective) magnification directly from microscope slides to a computer using a Zeiss (Carl Zeiss, Inc., Thornwood, NY, USA) microscope and a high performance Charge Coupled Device (CCD) digital camera (Photometrics, Tucson, AZ, USA) which is capable of 1317X1035 imaging array with 6.8X6.8 micrometer pixels and 12-bit digitization. On the basis of gray values ranging from 4,095 to 0, white to black respectively, threshold boundaries of the immunostainings were created [5,19,24]. The utilization of neutral and green filters provided optimum imaging of the reaction products. In the RP sections, benign ('normal') and/or BPH areas were imaged at least two microscope fields away from the carcinoma and used as controls. BPH glands were used as controls. The measurements of CB and SA are presented as range and mean with standard error of the mean (SEM).

**Laser Capture Microdissection:** Prostate tissue sections showing cancer and BPH areas were made from selected for isolation of cancerous and BPH glands using a laser capture microdissection (LCM) machine (Arcturus Engineering Inc., Mountain View, CA). Briefly, the technique involved the initial photographing of cancerous and BPH glands followed by covering sections with transparent 100 m-thick ethylene-vinyl acetate films, isolation of the selected areas, and their photography. Isolated glands and cells were homogenized for measuring CB and SA by an Elisa assay which is a quantitative sandwich enzyme immunoassay technique, also described by others [28-30]. Samples were added to a micro-titer plate that was pre-coated with a monoclonal antibody specific for CB and incubated overnight at 4$^{o}$C. The wells were washed with blocking solution, 5% BSA (bovine serum albumin) in PBS (phosphate buffer saline). Diluted experimental and control samples were added to the wells and incubated for 2 hours at room temperature. After washing away unbound substances, secondary antibody that is enzyme-linked polyclonal, rabbit antibody specific for CB was added to the wells. Following several washes to remove unbound antibody-enzyme reagent, a substrate solution HRP (horse-radish-peroxidase)-conjugated anti-rabbit IgG was added to the wells and color developed in proportion to the amount of CB bound in the initial step. This color development was stopped by 2 M H2SO4 and the intensity of the color was measured at 450 nm using a micro-plate reader (Model 450, Bio-Rad, USA). Turk and associates developed Elisa assay kits for stefin A were obtained from KRKA, Inc. (Novo Mesto, Slovania) [29]. Monoclonal murine anti-stefin A antibody was used in Elisa assay, as detailed by Strojan et al.[30].

**Data Analysis:** The immunostaining data were analyzed using the two-sample Student's *t*-test (significance of $p \leq 0.05$), Chi-square, and/or multiple regressions**.**

**Result and discussion:**

We have conducted a preliminary analysis of additional 107 radical prostatectomy specimens (36 Gleason score 6 and 71 score 7 tumors) from white PCa patients. We have partially analyzed clinical and CB and SA localization data. Age of our patient population ranged between 52 and 76 years with a mean of 66 years. Sixty seven patients had no pelvic lymph node metastasis whereas 30 had metastasis and the status was unknown in 10 patients. Forty six patients had invasion of cancer cells to margins/capsules and 60 did not. Clinical stages ranged from T1b to T3c and N1 and M1. These

patients had seminal vesicle invasion in 24 patients, but not in 82 patients with unknown status in 1 patient. Cathepsin B and stefin A immunostaining data are yet to be analyzed in relation to clinical parameters. This study is in progress. We will submit a copy of the published paper to the DOD.

The table 1 shows the number patients and their clinical distribution according to the Gleason grades, age, PSA levels, nodal status and other clinical parameters.

Table1 Distribution of Prostate Cancer Patients

| | |
|---|---|
| No. of Patients | 107 |
| Gleason 6 Cases | 36 |
| Gleason 7 Cases | 71 |
| Age at RP, Range (Mean±SEM) | 52.39-76.30 (66.00±0.49) |
| Pre-Prostatectomy PSA in ng/ml, Range (Mean±SEM) | 0.2-146 (14.34±2.76) |
| | |
| Post-Surgery Data | |
| Clinical Stage * | T1b, T1c, T2a, T2b, T2c, T3a, T3c, N1, M1 |
| Node Negative | 67 |
| Node Positive | 30 |
| Unknown node Status | 10 |
| Margin/Capsule Negative | 60 |
| Margin/Capsule Positive | 46 |
| Unknown Margin/Capsule Status | 1 |
| Seminal Vesicle Negative | 82 |
| Seminal Vesicle Positive | 24 |
| Unknown Seminal Vesicle Status | 1 |
| Confined to the Prostate | 72 |
| Not Confined to the Prostate | 33 |
| Confined Status Unknown | 2 |
| No. of years since RP, Range (Mean±SEM) ** | 4.24-26.29 (17.76±0.42) |
| No. of patients with post-surgery PSA <0.2ng/ml | 13 |
| PSA in ng/ml, Range (Mean±SEM) | 0.04-0.17 (0.10±0.01) |
| No. of patients with post-surgery PSA ≥0.2ng/ml | 69 |
| PSA in ng/ml, Range (Mean±SEM) | 0.2-6551 (219.94±108.02) |
| No. of patients with no post-surgery PSA levels | 25 |
| No. of patients with post-surgery treatment *** | 41 |
| No. of years since post-surgery treatment | 0.03-8.81 (1.67±0.36) |

| | |
|---|---|
| No. of patients with biochemical failure **** | 82 |

* Whitmore-Jewett stages C1, C2, D1, and D2 were converted to T3a, T3c, N1, and M1 according to the TNM classification.

** Data updated as of June 1, 2006

*** Post-surgery treatments included hormone therapy, chemotherapy, and/or radiation.

**** Biochemical Recurrence defined as post-RP serum PSA levels ≥ 0.20 and/or post-surgery treatment

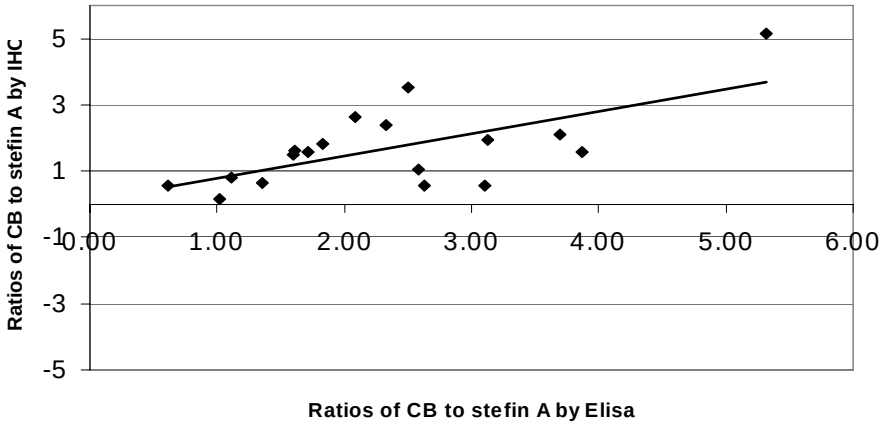
SEM=Standard Error of the Mean

In table 2, we have shown that immunohistochemical localization of CB and stefin A in microdissected prostate cancer cells. Statistical analysis showed that some Gleason score 6 tumors CB>SA ratios and others had CB<SA indicating heterogeneity of this score.

Table 2. Measurement of CB and SA in LCM-dissected prostate cells

| Gleason Score 6 Tumors | n | Ratio CB/SA | CB (ng/ml) | SA (ng/ml) |
|---|---|---|---|---|
| CB>SA | 12 | 3.06±0.3* | 48.0±4.6* | 20.5±0.8 |
| CB≤SA | 7 | 1.29±0.2 | 25.8±2.4 | 16.2±1.53 |

Graph showing relationship of immunostaining ratios of CB to SA and the ratios of CB to SA after




Elisa Assay. Cathepsin B and stefin A were measured in formalin-fixed, paraffin embedded Gleason score 6 tumors by Elisa assay. The LCM dissected glands were extracted by a RIPA buffer (100ml of 0.5M tris-HCL, pH 7.4, 1.5M Nacl, 2.5% deoxycholic acid, 10% NP-40, 10mM EDTA at room temperature) [31] and adjusted to a ml volume for uniformity between Elisa assays. Frozen prostate samples with Gleason score 6 tumors showed similar levels of CB and stefin A by Elisa assay (data not included). SEM= standard error of the mean. Asterisks indicate significance at 0.05 (Student t-

test). Linear regression analysis of CB to stefin A ratios by immunohistochemistry and Elisa assay techniques showed significant relationship (p=0.0033). IHC=immunohistochemistry.

**Acknowledgements**:

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Key Research Accomplishments:

Study #1

- Immunostainings of CB and SA and their ratios were heterogeneous even in Gleason score 6 prostate needle biopsy and radical prostatectomy tissue sections.
- Immunostainings of CB alone were not significantly different in BPH, PIN and PCa, but SA alone was significantly lower in PCa ($p<0.001$) when compared to BPH and PIN glands.
- Ratios of CB to SA were significantly higher in PCa when compared to BPH and PIN glands ($p<0.001$) and also in PIN compared to BPH ($p=0.036$).
- The average ratios of CB to SA showed an inverse relationship to T2a to T3b clinical stages.
- Ratios of CB to SA could be used to assess aggressiveness of prostate cancer in relation to biopsy-associated clinical data.
- Gleason score 6 (3+3 pattern) tumors had micro-metastases as indicated by recurrence of cancer.

Study #2

- Selection of black and white prostate cancer patients, according to the clinical criteria (such as Gleason grade, serum total PSA levels, clinical stage and age), provided that their cancer was similar at the initial diagnosis.
- Since the Veterans at the Minneapolis Veterans Affairs Medical Center received similar treatment, our PCa samples were comparable in African-American and Caucasian patients.
- 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
- 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.
- Aggressiveness of prostate cancers was similar in both races.
- Invasion and progression of cancer cells in black and white patients may not be race-dependent because the underlying chemistry is not race-dependent.

**Reportable Outcomes:** Our study has resulted in three papers published in a peer-reviewed journal and two manuscripts yet to be published.

Study #1. Sinha, AA, Morgan, JL, Wood, N., Betre, K., Reddy, A., Wilson, MJ, Ramnani DM.: Heterogeneity of Cathepsin B and Stefin A Expression in Gleason Pattern 3+3 (score 6) Prostate Cancer Needle Biopsies" in Anticancer Research 27:1407-1414, 2007. PDF file is enclosed.

Qian J., Bostwick DG, Iczkowski KA, Lang K, Betre K, Wilson MJ, Le C, Sinha AA: Characterization of Prostate Cancer in Needle Biopsy by Cathepsin B, Cell Proliferation and DNA ploidy. Submitted for review and publication in a journal, August 2008.

Study # 2. Sinha, A. A., Morgan, J. L., Buus, R. J., Ewing, S. L., Fernandes, E. T., Le, C. Wilson, M. J.: Cathepsin B Expression is Similar in African-American and Caucasian Prostate Cancer Patients. Anticancer Research 27:3135-3142, 2007. PDF file is enclosed.

Conclusion: We have made significant contributions:

1. We have shown that small foci of Gleason pattern 3+3 (histological score 6) prostate cancer in needle biopsies contain heterogeneous population of cancer cells. Some of these cells develop aggressive PCa. This indicates that there may be micro-metastastatic cells even in Gleason score 6 tumors. Our conclusion is based on utilization of biopsy tissue sections followed by localization of cathepsin B and stefin A as biomarkers. We suggest that the biopsies of the Gleason score 6 tumors should be evaluated for by these biomarkers prior to treatment selection.

2. Previous studies have shown that black men have higher incidences, increased tumor volumes, higher Gleason grades, clinical stages, and/or serum total PSA levels than white PCa patients. We expected to find increased CB immunostainings in AA than the white patients. Instead, we found similar CB and SA immunostainings in black and white PCa patients suggesting that cancer was similar in both races. When, we looked for the reasons these results, we found that the previous studies did not select patients according to the defined clinical criteria (Gleason grade/score, serum total PSA levels, clinical stage and age) prior to conducting studies and comparing results in the two races. Thus, the previous studies were conducted on unmatched PCa patients. We selected PCa patients for study using the above criteria at the VAMC. This approach greatly minimized selection biases and provided results without many complicating factors (such as socioeconomic status, education, access to healthcare, health insurance, cultural biases and/or distrust of white male/female physicians by African-American males) which had previously influenced the conclusions.

The DOD funding led to the shift in the previous paradigm that stated, "prostate cancer (PCa) was more aggressive (invasive) in black than white patients" to one that states that this cancer is similar in both races if studies were conducted in matched groups of patients. Our approach led to a finding indicating that the black patients were not monitoring their disease in spite of the fact that they had equal access and similar treatment at the VAMC. In contrast, the white patients followed-up their disease at the VAMC. This difference was significant ($p=0.0001$) between the races. The result was that the white patients received four times more additional treatments than black patients. This may be contributing factor in developing aggressive PCa in black patients.

Our finding drew attention of the general and African American media and this can be ascertained via Akhouri A Sinha black prostate using the internet search engines and profession publications. I also gave radio interview to a Philadelphia radio station that broadcasted our findings.

**Validation of our findings in another race and ethnic group of PCa patients:**

Since our study on black and white men with CB and SA immunostainings contradicted numerous previous studies showing differences in PCa of black and white men. We tested our findings in PCa of another race and ethnic group. This adjunct study was supported by the Research Service of the Minneapolis VAMC and in part by a grant from the Prostate Cancer Foundation (CaPCURE) to LSM. This work has been published in Anticancer Research 28:2271-2278, 2008. We selected PCa cases prior to conducting immunostainings and analyses of data. We matched Japanese patients according to the Gleason grade/score, pre-RP serum total PSA levels, clinical stage and age prior to evaluation of immunostainings data. This greatly minimized subjectivity associated with the evaluation of markers in this ethnic sub-population of PCa patients. We found similar CB and SA immunostaining in Japanese patients who have organ-confined and moderately-differentiated PCa. Thus, we have validated our study on black and white PCa patients by studying this group of patients.

Supporting Data:

Sinha, A. A., Morgan, J. L., Buus, R. J., Ewing, S. L., Fernandes, E. T., Le, C. Wilson, M. J.: Cathepsin B Expression is Similar in African-American and Caucasian Prostate Cancer Patients. Anticancer Research 27:3135-3142, 2007.

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J Qian J., Bostwick DG, Iczkowski KA, Lang K, Betre K, Wilson MJ, Le C, Sinha AA: Characterization of Prostate Cancer in Needle Biopsy by Cathepsin B, Cell Proliferation and DNA ploidy. Submitted for review and publication in August 2008.

Sinha AA, Ewing SL, Fernandes ET, Wilson MJ, and Kos J: Characterization of prostate cancer by cathepsin B and stefin A by Immunohistochemistry and Elisa assay. Manuscript under preparation.

Validation study paper:
Sinha, A. A., Morgan, J. L., Betre, K., Wilson, M. J., Le, C. and Marks, L. S.: Cathepsin B expression in Native Japanese and Japanese-American prostate cancer patients: An immunohistochemical study. Anticancer Res., 28:2271-2278, 2008.

We are enclosing ‘pdf’ files of the published papers, IMPACT abstracts. Unpublished manuscript and data together with figures and tables are in the body of the report.

# Heterogeneity of Cathepsin B and Stefin A Expression in Gleason Pattern 3+3 (Score 6) Prostate Cancer Needle Biopsies

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**Abstract.** *Background: There is a significant positive association of increased ratios of cathepsin B to its endogenous inhibitor stefin (cystatin) A in prostatectomy tumors with pelvic lymph node metastases. Needle biopsy diagnosis of prostate cancer is critical in initial treatment selection. The objective was to characterize cathepsin B and stefin A immunostaining patterns in needle biopsies of histologically similar Gleason pattern 3+3 (score 6) foci in relation to pretreatment clinical data. Materials and Methods: Immunostaining of cathepsin B and stefin A of 65 biopsy sections were imaged, quantified and analyzed with Student's t-test ($p<0.05$). Results: Patients had T1c to T3b clinical stages and pre-surgery total prostate-specific antigen serum levels from 1.25 to 20.0 ng/ml. Cathepsin B and stefin A reaction products were found in the cytoplasm of basal and columnar/cuboidal cells of benign prostatic hyperplasia (BPH), prostatic intraepithelial neoplasia (PIN) and neoplastic cells. Ratios of cathepsin B to stefin A were significantly higher in prostate cancer when compared to that in BPH and PIN glands. Conclusion: Small foci of Gleason pattern 3+3 tumors in needle biopsies have heterogeneous cathepsin B and stefin A immunostaining. Stratification of these tumors in relation to clinical stage by cathepsin B and stefin A may assist in treatment selection.*

The Gleason score of a prostate tumor is the most powerful predictor of future cancer progression. A number of molecular markers including DNA ploidy, chromosomal marker 8q24, cell proliferation, prostate stem cell antigen, TGF-B1, p53, Bcl-2, E-cadherin, Ki-67, cyclin D1, microvessel density, and prostate-specific antigen (PSA) have been studied in an attempt to subclassify prostate cancers (PCa) to account for differences in patient survival with a given Gleason score (aggressive *vs.* latent cancers). These markers were assessed primarily in radical prostatectomy (RP) specimens and in a limited number of biopsy samples (1-7), but are of limited value in diagnosis (8). Proteases degrade basement membrane (BM) and extracellular matrix (ECM) proteins, which is a prerequisite for cancer cell invasion and metastasis in many solid organ cancers (such as breast, bladder, lung, brain and melanoma) (9-20). Proteases appear to be likely biomarker candidates for assessing prostate biopsies for clues into the potential aggressiveness of PCa. Such information in relation to pretreatment clinical data could assist treatment selection (such as RP, chemotherapy, hormone therapy, brachytherapy/external beam radiation, immunotherapy, and/or watchful waiting) (6, 21-23).

The cysteine protease cathepsin B (CB) is involved in the degradation of BM and ECM proteins, cancer cell invasion and progression. Activities of CB are usually regulated in part by its endogenous inhibitor stefin (cystatin) A (SA) (9-12). Immunohistochemical localization of CB and SA in formalin-fixed archival RP tissue samples has shown a significant relationship of a ratio of CB>SA with pelvic lymph node metastases (11). That study confirmed the heterogeneity and invasiveness of PCa, which was recognized by Gleason in his grading of RP samples (24-26). The next step in evaluating CB and SA as biomarkers of PCa is to study them in prostate needle biopsy samples. We proposed that to examine possible heterogeneity in CB and SA expression in biopsy samples, PCa samples of a single Gleason grade should be studied because of the homogenous histological character. Thus, our objective was to characterize CB and SA immunostaining in needle biopsy sections of histologically and morphologically similar Gleason primary and secondary pattern 3+3 (score 6) tumors.

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*Key Words:* Prostate cancer, stefin A, biopsy, heterogeneity, cathepsin B.

Table I. *Distribution of prostate cancer patients with Gleason pattern 3+3 (score 6) tumors.*

| | |
|---|---|
| Number of biopsy samples | 65 |
| Caucasian | 54 |
| African-American | 11 |
| Pre-prostatectomy data | |
| Age at prostatectomy range (mean±SEM; years) | 47-73 (62.7±0.8) |
| Gleason score 6 tumors (number of cases) | 65 |
| Presurgery PSA range (mean±SEM; ng/ml) | 1.25-20 (6.7±0.5) |
| Clinical stage (number of cases) | |
| T1c | 1 |
| T2a | 14 |
| T2b | 13 |
| T2c | 34 |
| T3a | 2 |
| T3b | 1 |
| Post-prostatectomy data | |
| Range of number of years since RRP (mean±SEM)[1] | 5.85-9.12 (6.68±0.79) |
| Post-surgery PSA range (mean±SEM; ng/ml) | 0-0.62 (0.02±0.01) |
| Number of patients with PSA≤0.1 ng/ml | 62 |
| Range of PSA levels (mean±SEM; ng/ml) | 0-0.12 (0.003±0.02) |
| Number of patients with PSA>0.1 ng/ml | 3 |
| Range of PSA levels (mean±SEM; ng/ml) | 0.21-0.62 (0.42±0.29) |
| Lymph node negative (number of patients) | 59 |
| Unknown lymph node status (number of patients) | 6 |
| Positive capsule/margins (number of patients) | 2 |
| Negative capsule/margins (number of patients) | 63 |
| Distant metastasis negative (by bone scan) (number of patients) | 36 |
| Unknown distant metastasis status (clinically) (number of patients) | 29 |
| TNM | T1-3 N0-x M0-x |

[1]Used December 31, 2005 as the end date.

## Materials and Methods

*Data collection.* Biopsy samples were collected and fixed in formalin, then processed in Prefer fixative (Anatech Ltd, Battle Creek, MI, USA) in microwave processors. Date of surgery, pre- and post-RP PSA levels, clinical stage, tumor volume, margin/capsule status, lymph node status, and metastasis data were collected. All samples and data were collected after obtaining approval of the Virginia Urology Center (Richmond, VA, USA) Institutional Review Board (IRB). The senior author and his collaborators, including laboratory personnel, did not have access to HIPPA required patient information. Therefore, approval of the Minneapolis (VAMC and/or U of M) IRB was not required. The investigators had access to demographic data only after submission of immunostaining, image analysis and quantification data to the Virginia Urology Center.

*Sample selection and processing of samples.* Gleason grade/score is one of the most powerful independent prognostic factors in PCa (24, 25). Biopsy tissue sections showing primary (principal) and secondary Gleason patterns 3+3 (score 6), as reported by Gleason (24, 25) and modified by the 2005 International Society of Urologic Pathology (ISUP) Consensus Conference (26), were chosen to minimize the influence of Gleason patterns on immunostaining data (Table I) While primary and secondary patterns 2+4 and 4+2 can result in Gleason score 6 tumors, they are relatively rare and were not included in this study. We started with an initial sample size of 100 cases; however, the foci of cancer were exhausted in many paraffin blocks, decreasing the number of available cases. Our selection provided 65 Gleason score 6 (patterns 3+3) tumors as determined in needle biopsies and confirmed in RP specimens. Biopsy samples were collected from the Virginia Urology Center archives and sections were graded according to the Gleason grading system by DMR.

*Immunohistochemistry.* Formalin-fixed, paraffin-embedded needle biopsy blocks were sectioned at 5 to 6 μm (11,12, 27). Briefly, mouse anti-human CB IgG was obtained from Oncogene Research Products (Calbiochem, Cambridge, MA, USA). Mouse monoclonal anti-human SA IgG was purchased from KRKA (Novo Mesto, Slovenia) and goat anti-human SA IgG from R&D Systems (Minneapolis, MN, USA). Antibodies were affinity purified using immobilized protein A or human SA by the manufacturer. Antibodies used for this study had not been used in our past research, and, therefore, are a new set of IgGs. Bovine serum albumin (BSA) was obtained from Sigma (St. Louis, MO, USA).

The molecular weights of CB (21 to 31 kDa) and SA (11 kDa) in prostatic tissues have been published (11, 12, 27). Antibodies did not show any cross-reactivity with other proteins in western blots (11, 12). Antigen retrieval was carried out in 10 mM citrate buffer (pH 6.0) using a Decloaking Chamber Pro machine (Biocare Medical, Walnut Creek, CA, USA). Mouse anti-CB IgG and mouse or goat anti-human SA IgG localized in adjacent sections. Since the number of biopsy sections was limited, prostatectomy sections were used for negative controls and were incubated with pre-immune mouse or goat serum in lieu of primary antibody. The reaction products were developed, usually less than 10 minutes, with fresh-filtered 3, 3'-diaminobenzidine (DAB) solution (0.25 mg/ml; Sigma) in phosphate-buffered saline with 0.01% hydrogen peroxide as the substrate. Chromogenic development was viewed through a light microscope and reaction product was enhanced with osmium tetroxide.

*Quantification of localization data using the Metamorph image analysis system.* Immunostaining was quantified using a computer-based image analysis system equipped with Metamorph software (Universal Imaging Corp., West Chester, PA, USA), as reported previously (11, 12, 27). Briefly, images of reaction products for CB and SA were acquired at a magnification of x400 directly from the microscope slides to a computer using a digital camera (Photometrics, Tucson, AZ, USA) attached to a Zeiss microscope with neutral filters. On the basis of gray values ranging from 0 to 4095, black to white, respectively, threshold boundaries of immunostaining were created. All immunostained objects were included within the designated gray value range, except for biopsy edges which demonstrated more intense immunostaining indicating cut surface effects. Immunostainings were expressed as a percentage of the total field area under view at the selected magnification. Data are presented as mean±standard error of the mean (SEM).

*Statistical analysis.* Data were analyzed using univariate techniques. Statistical significance was determined using Student's *t*-test ($p<0.05$).

## Results

*Profile of prostate cancer patients.* The age of PCa patients at initial diagnosis ranged from 47 to 73 years (mean 62.7 years ± 0.8 year) with a mean follow-up period after surgery of 6.68 years (Table I). Bone scan (36/65 cases, 55.4%) and/or clinical data (29/65 cases, 44.6%) did not provide any evidence of distant metastasis. The regional pelvic lymph nodes were negative for cancer cells in 59 patients (59/65, 90.8%) and unknown in six cases (6/65, 9.2%). The clinical stages ranged from T1c to T3b with the majority of patients showing stage T2c (34/65, 52.3%) (Table I). Pre-surgery PSA ranged from 1.25 to 20.0 ng/ml (6.7±0.5), whereas, post-RP surgery PSA levels ranged from 0 to 0.62 ng/ml (0.02±0.01) (Table I). Three patients with pre-surgery PSA levels of 2.8, 5.9, and 3.64 ng/ml showed evidence of biochemical recurrence of PCa after 4.35, 5.03, and 4.00 years, respectively, as indicated by elevated post-surgery PSA levels (>0.1 ng/ml) (Table II). Two of these three cases had positive resection margins in RP specimens. Two of the three cases were given external beam radiation and had undetectable PSA at last follow-up. The third patient moved and was lost to further follow-up. PSA levels had not increased in the remaining 62 patients.

Table II. *Distribution of cathepsin B (CB) to stefin A (SA) ratios in patients with biochemical recurrence.*

| Patients with biochemical recurrence | Patient 1 | Patient 2 | Patient 3 |
|---|---|---|---|
| CB to SA ratio in PCa | 2.66 | 4.92 | 11.46 |
| Pre-surgery PSA | 5.9 | 2.8 | 3.64 |
| TNM stage | T2c N0 M0 | T2a N0 M0 | T2c N0 M0 |
| Margin status | Positive | Negative | Positive |
| Race | Caucasian | African-American | Caucasian |
| Post-surgery PSA | 0.62 | 0.21 | 0.12 |
| Additional treatment | EBR | EBR | Lost to follow-up |
| Current PSA | Undetectable | Undetectable | Lost to follow-up |

PCa: prostate cancer; PSA: prostate-specific antigen; EBR: external beam radiation.

*Immunohistochemical analysis.*

*a) Cathepsin B and stefin A in benign prostatic hyperplasia glands.* The immunostaining pattern of CB and SA in BPH glands in biopsy sections was used as a control. CB and SA immunostaining was present predominantly in the cytoplasm of basal cells and some cuboidal/columnar cells of BPH glands (Figure 1 A, B). Immunostaining of CB ranged from 1.48 to 5.43 (3.14±0.13) (Table III). Likewise, immunostaining of SA ranged from 1.09 to 4.41 (2.70±0.09). The ratios of CB to SA ranged from 0.62 to 2.94 (1.21±0.05) (Table III). We found that Prefer fixation of biopsy sections gave more intense CB reaction products than formalin fixation alone. Utilization of a Decloaking Chamber Pro provided more uniform antigen retrieval than in previous studies performed using a hot plate (9-11).

*b) Cathepsin B and stefin A in PIN glands.* In PIN glands, the two markers localized strongest in the basal cells (Figure 1 C, D). Immunostaining of CB ranged from 1.39 to 6.40 (3.34±0.23). Likewise, SA localization ranged from 1.03 to 3.96 (2.39±0.16). The ratios of CB to SA ranged from 0.47 to 4.5 (1.65±0.1) (Table III).

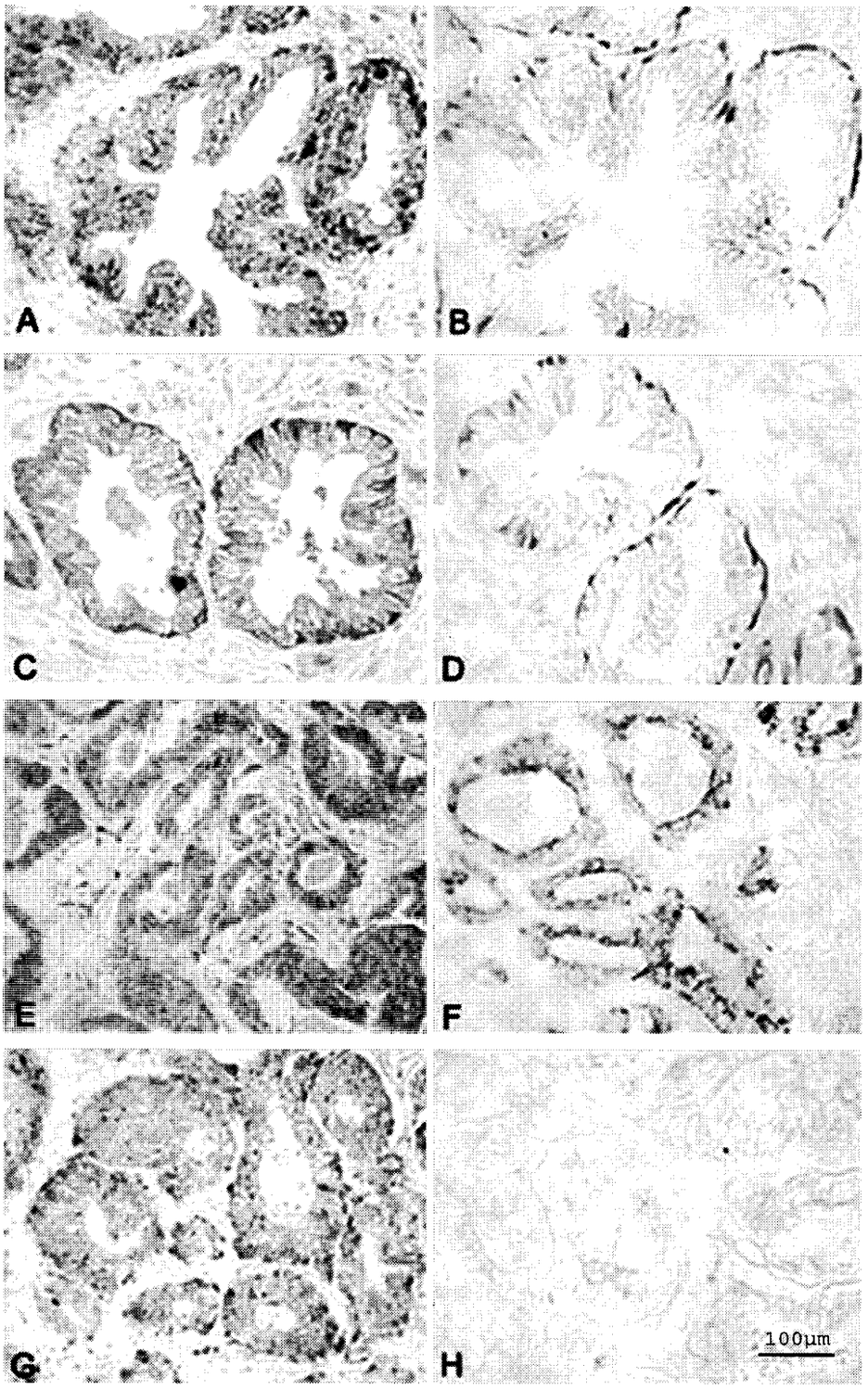


Figure 1. *Comparison of (A) cathepsin B and (B) stefin A immunohistochemical localization in BPH (CB to SA ratio=1.48); (C) CB and (D) SA in PIN glands (CB to SA ratio=1.35); (E) CB and increased (F) SA staining in PCa (CB to SA ratio=0.93); and (G) CB and (H) decreased SA staining of PCa (CB to SA ratio=20.5). (Immunoperoxidase, magnifications x400). Bar in H illustrates magnification for all figures.*

Table III. *Immunostainings of cathepsin B (CB), stefin A (SA), and cathepsin B to stefin A ratios in Gleason pattern 3+3 (score 6) tumors.*

| Protein localization | BPH | PIN | PCa |
|---|---|---|---|
| CB range (Mean±SEM) | 1.48-5.43 (3.14±0.13) | 1.39-6.40 (3.34±0.23) | 1.43-5.81 (3.26±0.12) |
| SA range (Mean±SEM) | 1.09-4.41 (2.70±0.09) | 1.03-3.96 (2.39±0.16) | 0.12-3.11 (1.02±0.09) |
| CB/SA ratio range (Mean±SEM)[1] | 0.62-2.94 (1.21±0.05) | 0.47-4.5 (1.65±0.19) | 0.85-19.54 (4.89±0.48) |

[1]The overall mean ratios of CB to SA were obtained from the ratio of each individual case. BPH: benign prostatic hyperplasia; PIN: prostatic intraepithelial neoplasia; PCa: prostate cancer; SEM: standard error of the mean; statistical significance was determined using Student's *t*-test ($p<0.05$). CB to SA ratios were significant when BPH was compared to PIN ($p=0.036$) and cancer ($p<0.0001$).

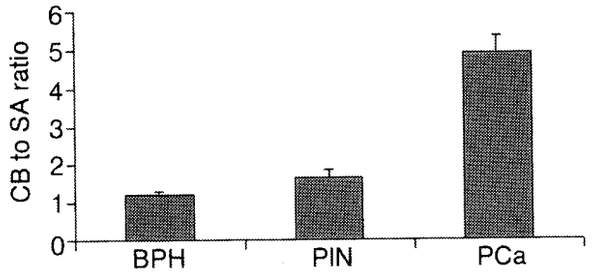


Figure 2. *Distribution of CB to SA ratios in prostate tissues. Ratios were significantly higher in PCa than in BPH or PIN. The ratios were significantly higher in PIN (p=0.036) and PCa (p<0.001) when compared to BPH. Prostate cancer had significantly higher ratios than PIN (p<0.001). Error bar=SEM.*

*c) Cathepsin B and stefin A in prostate cancer.* Cathepsin B and SA localized in cancerous cells of biopsies (Figure 1 E-H). The distributions of CB and SA protein reaction products showed considerable variation in Gleason score 6 tumors, much as we found in RP cases (11). Immunostaining of CB ranged from 1.43 to 5.81 (3.26±0.12). SA localization ranged from 0.12 to 3.11 (1.02±0.09). The considerable heterogeneity in the expressions of CB and SA was reflected in their ratios, which ranged from 0.85 to 19.54 (4.89±0.48). Immunostainings of CB alone were not significantly different in BPH, PIN and PCa, but SA alone was significantly lower in PCa ($p<0.001$) when compared to BPH and PIN glands (Table III). Ratios of CB to SA were significantly higher in PCa when compared to BPH and PIN glands ($p<0.001$) and also in PIN compared to BPH ($p=0.036$) (Table III, Figure 1 E-H, Figure 2).

*Relationship of cathepsin B and stefin A, clinical stages, and serum PSA levels.* Our data showed that higher ratios of CB to SA (>10) were predominantly associated with T2a, T2b and T2c clinical stages. There was no association with T1c, T3a and T3b stages, possibly due to a limited number of patients (Table III, Figure 3). The average ratios of CB to SA showed an inverse relationship to T2a to T3b clinical stages, except in a single case with T1c stage (Figure 4). Statistical analysis of CB to SA ratios in relation to each clinical stage was not significant.

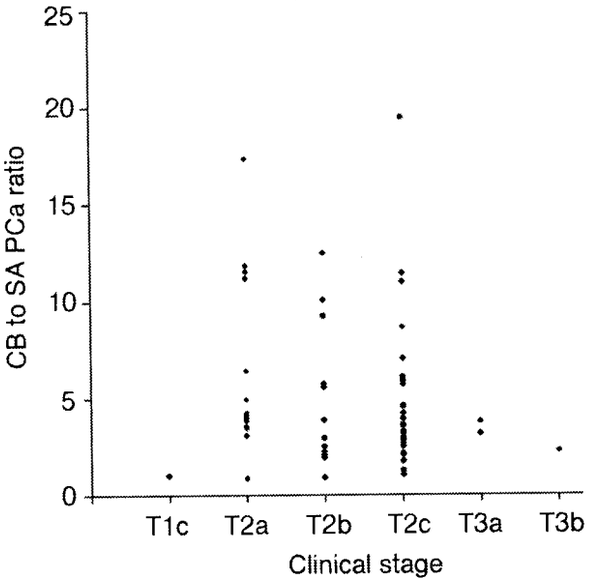


Figure 3. *Distribution of CB to SA ratios in relation to clinical stages. Ratios were higher in patients with T2a, T2b and T2c clinical stages than in a limited number of other stages.*

Pre-RP serum total PSA levels ranged from 1.25 to 20 ng/ml (6.56±0.47) (Table I). Tumors in nine patients (9/65, 13.8%) showing serum total PSA levels ≥10 ng/ml were associated with T2b, T2c, and T3a clinical stages (Figure 5). Fifty-five (55/65, 84.6%) patients had pre-RP serum PSA levels <10 ng/ml and the status of PSA levels was unknown in the remaining patients (Table I). PSA levels of <10 ng/ml did not show a relationship with clinical stages. Three patients with post-surgical rising PSA levels (biochemical recurrence) had clinical stages of T2c, T2c, and T2a and CB to SA ratios of 2.66, 4.92, and 11.46 respectively (Table II).

## Discussion

We have shown that immunostaining of CB and SA and their ratios are heterogeneous in small tumor foci of prostate needle biopsies, even though RP specimens from

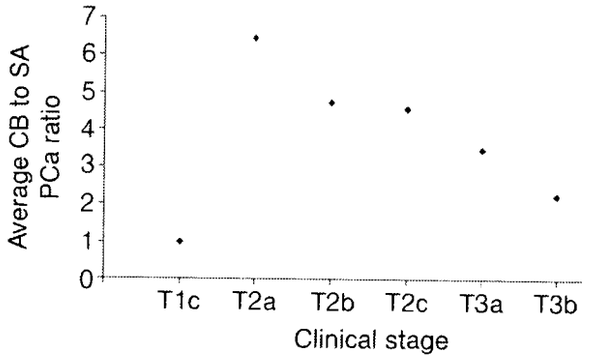


Figure 4. *Inverse relation of CB to SA ratios to clinical stages in T2a-T3b, except in a single case showing T1c stage.*

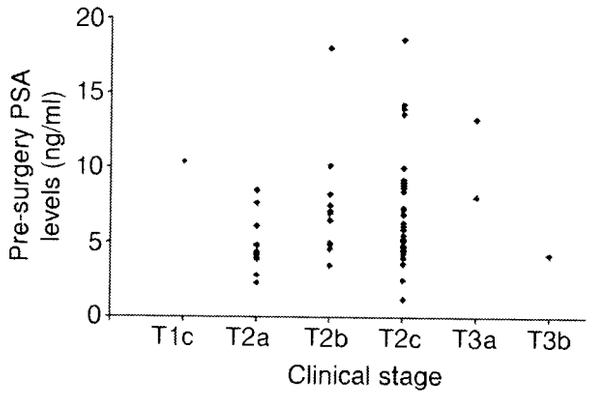


Figure 5. *Relationship of pre-prostatectomy PSA levels to clinical stages in prostate needle biopsies.*

the same patients were of the same Gleason pattern. Selection of needle biopsy cases showing Gleason pattern 3+3 (score 6) tumors, which are considered similar histologically and morphologically, provided a reasonable assurance that differences in CB and SA immunostainings were not due to PCa heterogeneity described by Gleason (24, 25). Our analysis of CB and SA immunostainings showed that ratios of CB to SA were significantly higher in malignant glands when compared to BPH and PIN glands. We found that 9 of 65 (13.8%) cases had CB to SA ratios greater than 10 whereas 56 (86.2%) cases had ratios lower than 10. We have shown that the distribution of CB and SA categorizes heterogeneity in small biopsy samples which are histologically and morphologically uniform. Earlier, Sinha *et al.* showed heterogeneity of CB and SA immunostaining in RP tissue sections showing Gleason score 6 tumors (11).

An ideal marker should distinguish clinically insignificant, organ-confined PCa from clinically significant cancer in which cancer cells invade prostatic margins/capsules and extraprostatic sites (namely, seminal vesicles and lymph nodes). Most of the existing biomarkers described and cited in the introduction section, including CB and SA, have not proven to be ideal. This indicates that a panel of biomarkers, which can be used on formalin-fixed paraffin-embedded sections, have the potential of characterizing aggressive and latent tumors in tissue sections containing small foci of PCa. Cathepsin B degrades BM and ECM proteins and facilitates cancer cell invasion and progression. The distribution of CB and SA provides an assessment of their role in archival formalin-fixed tissue samples. Additional support for studying CB and SA ratios comes from the earlier study of Sinha *et al.* showing relationships of the above biomarkers in RP tissue sections with metastases in pelvic lymph nodes (11).

Initial treatment decisions after PCa diagnosis utilize a variety of clinical data, namely, Gleason patterns/scores/ grades, serum total and/or free PSA levels, and clinical stages. The utility of any biomarker is greatly enhanced when it is related to the existing clinical data. We stratified this needle biopsy study of Gleason pattern 3+3 tumors according to ratios of CB to SA, serum total PSA levels and T2a, T2b and T2c clinical stages. We found that the average ratios of CB to SA showed an inverse relationship with T2a to T3b clinical stages. This led us to postulate that the inverse relationship of relatively high levels of CB to SA ratios to T2a clinical stage may be indicative of an early invasive stage of PCa.

Analysis of clinical data in the present study indicated biochemical failure in three patients as shown by post-surgery PSA levels of >0.1 ng/ml after about 5 years of follow-up. While we monitor these patients, we expect to see additional biochemical failures within 10 years of RP treatment. Recognizing limitations in our study due to small sample size, single Gleason grade/score and limited follow-up data, we suggest that some PCa patients would benefit from CB and SA immunostainings prior to treatment selection.

## Conclusion

CB and SA immunostainings have shown heterogeneity of PCa in small foci of needle biopsy sections with Gleason pattern 3+3 (score 6) tumors. This is the first study to characterize small foci of Gleason pattern 3+3 PCa in needle biopsies by CB and SA. Cathepsin B is an important biomarker due to its involvement in degradation of BM and ECM proteins and facilitation of cancer cell invasion and progression to adjacent and distant organ sites. Cathepsin B and SA can stratify small foci of PCa in needle biopsy sections, but the relationship of the CB:SA ratio to tumor aggressiveness needs to be examined in a larger number of patients in which post-surgery clinical outcomes over a longer period are known.

## Acknowledgements

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POSTER # P4-6 HEALTH DISPARITIES

# CATHEPSIN B EXPRESSION INDICATES THAT PROSTATE CANCER IS SIMILAR IN AFRICAN-AMERICAN AND CAUCASIAN MEN

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Incidence, age, tumor volume, death rate, Gleason grades/scores, clinical stages and/or serum total prostate specific antigen (PSA) levels have been used to suggest that prostate cancer (PCa) in U.S. African-American (AA) men is more aggressive than men of other races. Earlier studies had not investigated proteases which are essential for degradation of basement membrane (BM) and extracellular matrix (ECM) proteins supporting the aggressive (invasive) behavior of prostate cancer cells. Proteases facilitate the migration of cancer cells across the BM to the subjacent prostatic stroma and across ECM to other biological compartments. The Department of Defense Prostate Cancer Research Program Fiscal Year 2004 Idea Development Award has allowed us to investigate the expression of the cysteine protease cathepsin B in PCa of AA and Caucasian men. Our objective was to identify mechanistic factors that might distinguish differences in PCa of two groups of men in relation to clinical data (such as clinical stages, serum total PSA levels, and Gleason histological scores). We studied cathepsin B and its endogenous inhibitor stefin (cystatin) A in radical prostatectomy tissue sections from fifty Gleason score 6 and 7 tumors together with eight BPH control samples using immunohistochemistry. All samples selected for this study were from men who showed overall similarities in relation to Gleason grade/score, age, clinical stage and/or pre-surgery prostate specific antigen (PSA) levels. Clinical data indicated that Minneapolis veterans received equal medical care. Immunohistochemical reaction products were imaged directly from microscope slides to a computer using a digital camera. Data were quantified using Metamorph software and analyzed using the two-sample t-test and confirmed by multiple regression. The relative tissue areas of CB and SA immunostainings were essentially similar in AA and Caucasian patients indicating that invasiveness of cancer cells was similar in men of both races. We conclude that the role of cathepsin B, including the regulatory role of stefin A for this protease, does not appear to be race-dependent. Our conclusion, however, is tentative because of the small sample size and limited post-RP follow-up data.

IMPACT: Our study indicates that previous conclusion of differences in AA and Caucasian PCa patients requires re-evaluation with respect to the role of proteases (such as cathepsin B, matix metalloproteinase) in invasion and metastasis of cancer cells to other organs. We suggest that multiple factors are involved in generating more aggressive PC in AA men and they ought to be examined with a variety of molecular markers. These biomarkers are expected to distinguish aggressive prostate cancer in men of different races. Supported by DOD Grant: W81XWH-04-1-0245

THE NO-COST EXTENSION STUDY ENDS ON MARCH 31, 2008.

# 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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*Key Words:* Prostate, prostatectomy, protease cathepsin B, inhibitor stefin A, race, Gleason grade.

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

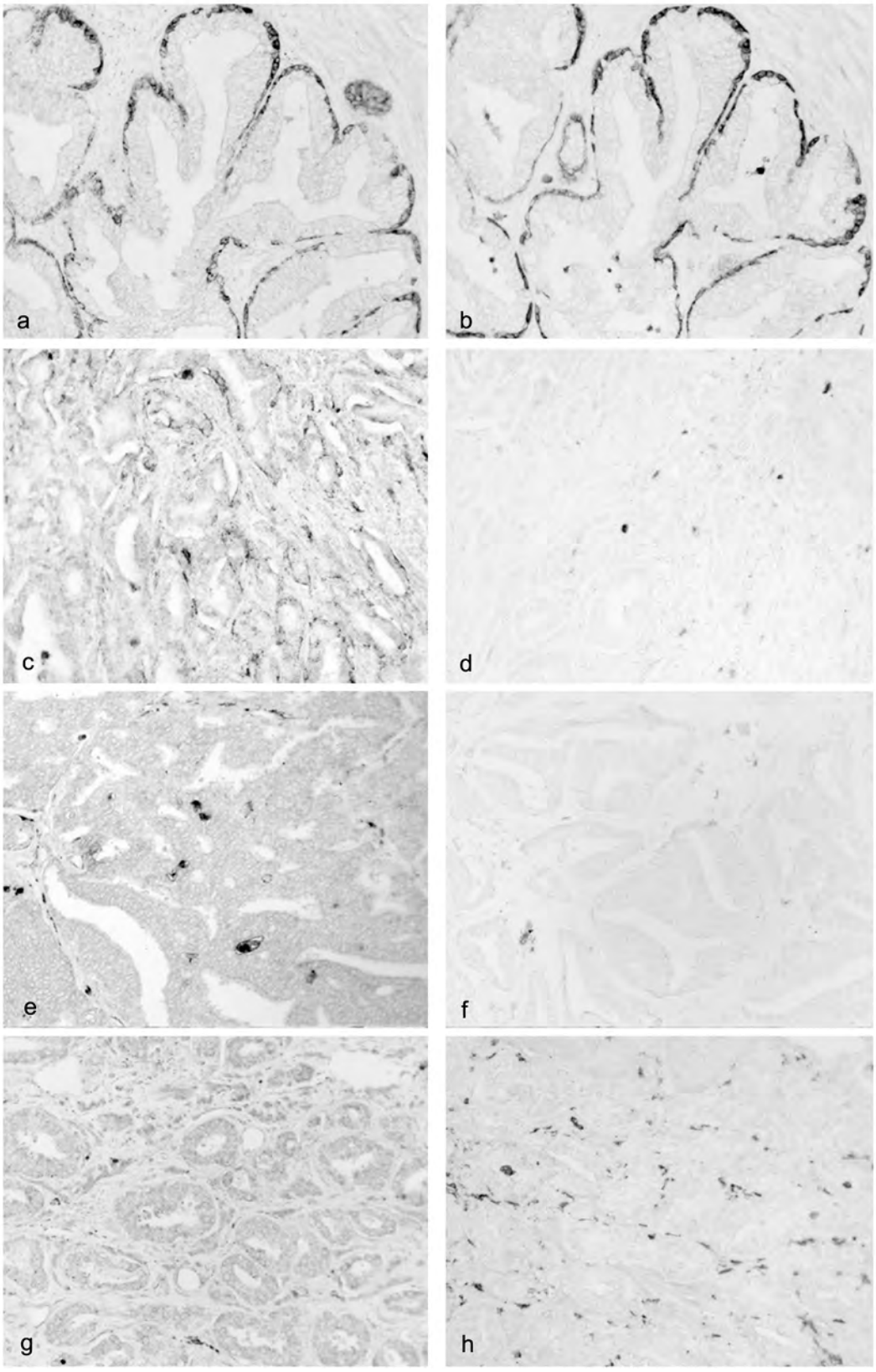


Figure 1. *continued*

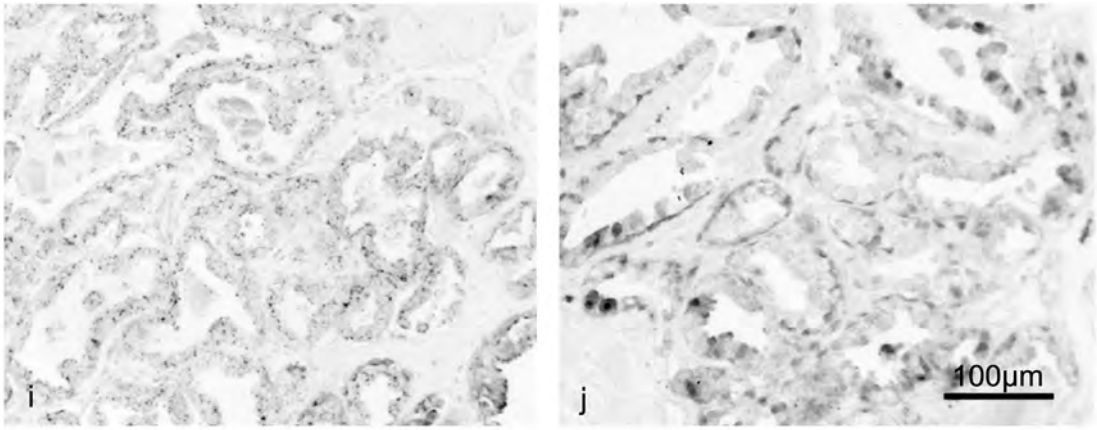


Figure 1. *Cathepsin B and stefin A immunostaining in study patients. The bar in (j) illustrates magnification for all figures. The African-American and Caucasian patients were matched for clinical data (see text). CB reaction products were found predominantly in basal cells of BPH glands of an African-American 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

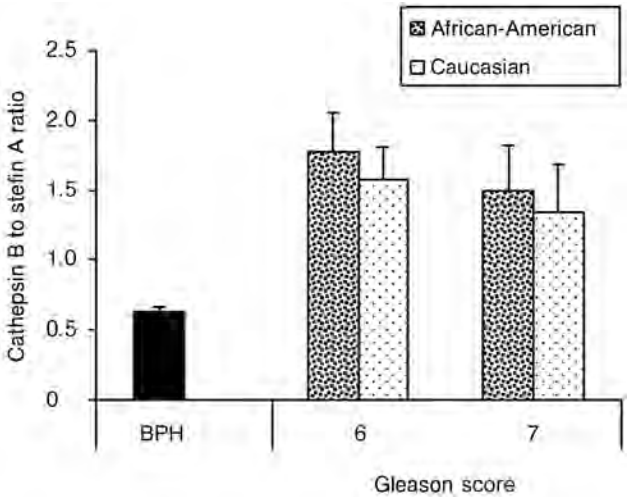


Figure 2. *Cathepsin B to stefin A ratios in African-American and Caucasian Gleason score 6/7 tumors. When the races were compared, ratios of CB to SA immunostaining were not significantly different in African-American and Caucasian men with BPH (p=0.19), Gleason score 6 (p=0.59) and 7 tumors (p=0.77). 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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# CHARACTERIZATION OF PROSTATE CANCER IN NEEDLE BIOPSY BY CATHEPSIN B, CELL PROLIFERATION AND DNA PLOIDY

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Running Title: Analysis of Biomarkers in Needle Biopsy and/or Prostatectomy Specimens

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**Abstract**

Background: More than 30% of prostate cancer patients have extra-prostatic invasions by cancer cells at radical retropubic prostatectomy. Approximately, 40% patients elect prostatectomy, but 60% select other treatments using initial diagnostic information. Prior to selecting any treatment these patients would benefit by assessment of the nature of cancer in biopsy specimens additional biomarkers. Our objective was to determine localization patterns of three distinct groups of biomarkers (cathepsin B, cell proliferation: MIB-1 and DNA ploidy) in the prostate needle biopsy sections in the hope of establishing localization similarities (or differences) in biopsy and RP specimens.

Materials and Methods: Prostate needle biopsy specimens and matched radical prostatectomies from 47 patients with cancer were evaluated; none had lymph node metastases. Only biopsy tissue sections were stained with rabbit anti-cathepsin B (CB) antibody or mouse anti-human stefin (cystatin) A (SA). The extent and intensity of staining were quantified using an image analysis system equipped with Metamorph software. The ratio of CB to SA was calculated for each biopsy cancer and matched benign prostatic hyperplasia (BPH) and benign prostatic acini. This ratio was correlated with serum PSA, biopsy Gleason score, biopsy DNA ploidy, biopsy MIB-1 (Ki-67) index, prostatectomy Gleason score, prostatectomy cancer volume, pathologic stage, and surgical margin status.

Results: Patients ranged in age from 48 to 74 years (mean, 65 years). Mean preoperative serum PSA was 9.1ng/ml (range, 3.6-28.2 ng/ml). At prostatectomy, 17 patients had Gleason score 6 and 30 had Gleason score 7 cancer. Mean cancer volume was 1.64 cc (range, 0.7-2.9). Four (8.5%)

had extra-prostatic extension of cancer, 7 (14.9%) had positive surgical margins, 2 (4.3%) had seminal vesicle involvement, and none had lymph node metastasis. Preoperative serum PSA correlated with cancer volume at radical prostatectomy (P=0.038). There was no correlation of prostatectomy Gleason score with cancer volume or pathologic stage. Geometric mean of CB to SA was 1.45 (range 1.12-1.87) in benign prostatic hyperplasia and 2.99 in cancer specimens (range, 2.30-3.89) (P=0.0001). The ratio of CB to SA in prostate needle biopsy had no correlative association with preoperative serum PSA concentration, biopsy MIB-1 index, overall biopsy DNA ploidy status, prostatectomy cancer volume, Gleason score, positive surgical margin, or pathologic stage. There was no correlation of % S phase cells based on DNA and MIB-1, a marker for cell proliferation, Gleason score, pathology stage, or surgical margins at prostatectomy.

Conclusion: Our study has indicated that the ratio of CB to SA is significantly higher in biopsies PCa than in BPH, as it was in previously studied RP cases. Since CB is involved in invasiveness of cancer cells, it may be elevated in small and large tumors and thus no correlation with tumor volume. The percentage of S-phase cells and DNA ploidy in needle biopsies predicts cancer volume in RP. We have shown that localization of three distinct biomarkers in biopsies reliably reflects similarity of localization in RP specimens. These biomarkers clarify the nature of cancer in biopsied specimens and thus, allowing patients to select other treatments, including prostatecomy.

Key Words: Prostate cancer, Needle biopsy, Radical prostatectomy, Cathepsin B, Stefin A, MIB-1, DNA ploidy,

## Introduction

About 186,320 men will be diagnosed with prostate cancer (PCa) and 28,660 will die from it in 2008 (1). Approximately, 40% of the newly diagnosed PCa patients elect to have retropubic radical prostatectomy (RP) (2-5). The diagnostic and prognostic information gained from the biopsy-associated clinical data (such as the Gleason score, serum total prostate specific antigen (PSA) levels, clinical tumor stage and age) cannot reliably predict the biological behavior of individual cancer (6-9) and patient outcomes (10-14). The definitive prostate cancer diagnosis, including grades/scores, cancer cell invasion to prostatic capsules, extra-prostatic areas and/or pelvic lymph node metastasis, benefits RP patients (15-19). However, about 60% of the patients make decisions for other treatments (such as watchful waiting, hormonal therapy, radiation/brachytherapy, chemotherapy, immunotherapy or their combinations) using the initial diagnostic information (3,20,21).

Various biomarkers, including cathepsin B (22-25), MIB-1 index (26-29), DNA ploidy (19,30-32), microvessel density, p53 gene mutation, p27 deletion, Bcl-2, and Rb, have been used to predict the aggressiveness of PCa (33,34), but the value of these biomarkers is controversial (9,19,35,36). For example, the cysteine protease cathepsin B (CB) is elevated in PCa and other solid organ cancers (such as melanoma, bladder, lung, colorectal, and breast cancer) (22-24,37-39). Stefin (cystatin) A (SA), an endogenous inhibitor, regulates enzymatic activities of CB in prostate and many solid organ cancers (37-39). Immunostaining ratios of CB and SA have been used discriminate more aggressive cancer within each Gleason score, as evidenced by pelvic lymph node metastasis (22-24), but not between the two Gleason scores/grades (24,40). Aggressive PCa was identified by the increased ratio of CB: SA together with pelvic lymph node metastasis (23). The MIB-1 staining

index has been associated with PCa patient survival (27,28,33,41). DNA ploidy adds useful prognostic information for some PCa patients (19,32,42-44). Our objective was to determine localization patterns of three distinct groups of biomarkers (cathepsin B, cell proliferation: MIB-1 and DNA ploidy) in the prostate needle biopsy sections in the hope of establishing localization similarities (or differences) in biopsy and RP specimens.

**Materials and Methods**

Matched prostatic needle biopsy and RP specimens from 47 PCa patients were collected from the Gainesville, Florida, Veterans Affairs Medical Center, with IRB approval. Patients had not been treated before undergoing prostatectomy. The number of biopsy cores varied from 6 to 19 (mean 11). Formalin-fixed, paraffin-embedded biopsy tissue sections were stained with hematoxylin and eosin (H&E) for diagnosis, and adjacent sections were stained for immunohistochemical study and DNA ploidy analysis. The thickness of tissue sections for DNA ploidy analysis was 6 microns. All RP specimens were formalin-fixed and serially sliced at *5* mm intervals perpendicular to the posterior aspect of the gland as reported previously (45). All tissue was submitted for histologic examination. For each block, a single 5 µm section was cut, stained with H&E, and examined independently by 2 pathologists (DGB and KAI).

Immunohistochemical Localization of CB, Stefin A, and MIB-1 expression: Rabbit anti-CB antibody (Oncogene Research Products, Calbiochem, Cambridge, MA), mouse anti-human SA antibody (KRKA Novo Mesto, Slovania), and mouse anti-human MIB-1 (Ki-67) antibody (DAKO, Carpinteria, CA) were used to localized CB, SA, and Ki-67

antigen, respectively, in tissue sections using the avidin-biotin complex (ABC) method as reported before (22-24). Reaction products were developed with fresh-filtered 3, 3-diaminobenzidine (DAB) solution (0.25 mg/mL; Sigma) in PBS with 0.01% $H_2O_2$ as the substrate. Chromogenic development was viewed under a light microscope. Reaction products usually developed in less than 10 minutes. Negative control sections were incubated with pre-immune rabbit or mouse serum in lieu of primary antibody.

Quantification of Immunostaining Using Image Analysis System: Immunostaining of CB and SA was quantified using a computer-based image analysis system equipped with Metamorph software (Universal Imaging, West Chester, PA) using previously described methods (22-24,40). A total of 4-6 randomly selected images with CB and SA staining in each biopsy section were acquired at a magnification of 400X directly from the microscope slides to a computer using a digital camera (Photometrics, Tucson, AZ) attached to a Zeiss Axioplan microscope. On the basis of gray values ranging from 4095 to 0, white to black, respectively, threshold boundaries of immunostaining were created. All immunostained objects included within the designated gray value range were expressed as a percentage of the total field area under view at the magnification of 400X. Radical prostatectomy sections were not used for localization of CB and SA.

Cell Proliferation Analysis: Immunostaining of MIB-1 (Ki-67) was reviewed by two investigators using a double-head microscope simultaneously without knowledge of the clinical status of the patients. Cancer foci with maximal MIB-1 expression were identified by scanning with light microscopy at low power. Cells with MIB-1 staining

were counted in a 200x field (0.754 $mm^2$). Three fields in each section were randomly selected for study. MIB-1 index was expressed as the percent nuclear area positive for MIB-1. The mean MIB-1 value (%) from each patient was used for statistical analysis.

DNA Ploidy Analysis: A representative paraffin tissue block from each biopsy was sectioned at 6 μm and stained with Feulgen dye following standard protocol. The nuclear DNA content, in the presence of concentrated hydrochloric acid, was hydrolyzed into its constituent nucleic acids. Feulgen dye then stoichiometrically bound to nucleic acids. Rat hepatocyte nuclear DNA was used as a standard external control of known DNA content. The CAS 200 imaging system (Bacus Lab, Lombard, IL) was used to measure staining intensity. Between 150 and 200 cancer cells were analyzed for each case. DNA ploidy status was assigned to the cancer cells based upon evaluation of the DNA histogram generated by the Quantitative DNA Analysis program. The percentage of nuclei in 4 categories, classified by the DNA index, was used for ploidy interpretation. These categories identified nuclei with DNA indexes between 0.90 and 1.10 (diploid), 1.11 and 1.79 (S-phase or aneuploid), 1.80 and 2.20 (tetraploid), or >2.20 (hypertetraploid). All cases with aneuploidy, tetraploidy and hypertetraploidy were defined as non-diploid.

Statistical Analysis: The difference in staining intensity between benign prostatic hyperplasia (BPH) tissue and PCa in biopsy was analyzed using the geometric mean method. The geometric mean and confidence intervals (CI) of CB/SA ratio were calculated on the log scale and then returned to the original scale of measurement by taking the antilog. The relationship of biopsy CB/SA ratio, MIB-1 index, and DNA

ploidy with pathologic findings in RP was determined using the Student *t* test, Chi-square, or Pearson Spearman correlation coefficient testing ($P < 0.05$).

**Results**

Patient profile: Patients ranged in age from 48 to 74 years (mean, 65 years). Mean preoperative serum PSA was 9.1ng/ml (range, 3.6-28.2 ng/ml). The mean Gleason scores in 47 sets of prostate needle biopsies was 6.5 (range 6-8). All patients had moderately differentiated, Gleason score 6 and 7 tumors without any pelvic lymph node metastasis. Seventeen RP patients had Gleason score 6 (3+3) and 30 had score 7 (3+4 and 4+3) (mean score, 6.8). Mean cancer volume in RP was 1.64 cc (range, 0.7-2.9). Four specimens (8.5%) had extraprostatic extension of cancer, 7 (14.9%) had positive surgical margins, and 2 (4.3%) had seminal vesicle involvement. Cancer volume in RP was associated with preoperative serum PSA (P=0.038).

Cathepsin B and stefin A: Cathepsin B and SA immunoreactivity was observed predominantly in basal cells and some cuboidal/columnar cells in BPH whereas PCa cells showed variable cytoplasmic staining (Fig. 1, 2). Table 1 shows the geometric mean distribution of ratios of CB to SA in BPH and PCa and their comparisons (Table 1). Ratio of CB: SA in the combined score 6 and 7 cancers was significantly higher than BPH (p=0.0001), with no difference between Gleason score 6 and 7 tumors (Table 1). Ratio of CB: SA was significantly higher for Gleason scores 6 and 7 tumors than BPH (P=0.0003, 0.0052, respectively) (Table 1). The ratio of CB to SA in prostate needle biopsy had no correlative association with preoperative serum PSA concentration, biopsy

MIB-1 index, overall biopsy DNA ploidy status, prostatectomy cancer volume, Gleason score, positive surgical margin, or pathologic stage (Table 2).

MIB-1 staining: The mean biopsy MIB-1 index was 7.7% (range 3.8-12.5%). The percentage of MIB-1 positive cells (MIB-1 index) in biopsy was not associated with biopsy CB/SA ratio, biopsy DNA ploidy, cancer volume, or Gleason score at radical prostatectomy (Table 2). There was no correlation of % S phase cells based on DNA and MIB-1, a marker for cell proliferation.

DNA ploidy analysis: Twenty-nine percent of cases were non-diploid. Table 3 shows that the incidence of non-diploid cancer in needle biopsy was associated with cancer volume in RP (p=0.03) (Table 3). The percentage of cells in S-phase was associated with the cancer volume in RP (P=0.03). DNA index was associated with preoperative PSA (P=0.02) and cancer volume at RP (P=0.02). There was no association of overall biopsy DNA ploidy status with CB/SA ratio, percentage of MIB-1 positive cells (MIB-1 index), or Gleason score.

**Discussion**

Our quantification of reaction products for CB and SA showed that their ratios were significantly higher in biopsies of PCa than in BPH areas. This finding is consistent with our previous reports on primary PCa (25). The ratio of CB and SA was not significantly different between Gleason score 6 and 7 tumors, this is also consistent with our study of RP specimens (24,25). In invasive PCa, CB is involved in degradation of basement

membrane, extracellular matrix and adherent junction proteins (37,46) as well as progression of cancer cells to the prostatic stroma (37-39). Our finding that there was no relationship between the extent of CB localization and tumor volume is also consistent with the previous reports showing the presence of invasive cancer cells in small and large PCa. In the present study, we did not find aggressive PCa, as indicated by pelvic lymph node metastases, primarily due to the fact the RP patients had moderately-differentiated tumors without any identifiable cancer cell invasions to the pelvic lymph nodes (22-24,47). Earlier, we had shown that the increased ratio of CB to SA was associated with PCa with lymph node metastasis (23). Since localization of CB was similar in biopsy sections from the present cases to the previously studied RP sections, it is reasonable to postulate that ratio of CB: SA in biopsies reliably reflects the status of PCa in RP specimens.

The MIB-1 staining index has been associated with PCa patient survival (27,28,33,41). In our present study, we found no correlation of MIB-1 index in prostate needle biopsies with pathologic findings in RP tissue sections. However, we studied moderately-differentiated Gleason 6 and 7 tumors without involvement of lymph node metastases. Ojea Calvo et al. reported similar findings (48). This lack of concordance between the biopsy and the RP might be caused by sampling variation (11,33).

The incidence of non-diploid cancer, DNA index and number of S-phase cancer cells in needle biopsy correlated with cancer volume at RP. These findings are consistent with previous reports that DNA ploidy adds useful prognostic information for some cancer

patients (19,32,42,43). The number of S-Phase cancer cells was reported as an independent predictor of prostate cancer outcome (26). Our findings indicate that the percentage of cells in S-phase, and DNA ploidy in needle biopsies predicts cancer volume in radical prostatectomy.

Conclusion: Our study has indicated that the ratio of CB to SA is significantly higher in prostate cancer biopsies than in BPH, as it was in previously studied RP cases (24). Since CB is involved in invasiveness of cancer cells, it may be elevated in small and large tumors provided there was lymph node metastasis. The percentage of S-phase cells and DNA ploidy in needle biopsies predicts cancer volume in RP. Our study has shown that localization of three distinct biomarkers in biopsies reliably reflect similar nature of prostate cancer patients who elect to have RP surgery or other treatments.

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Table1. Distribution of geometric mean and confidence intervals (CI) for CB and stefin A in Gleason score 6 and 7 prostate cancers and BPH glands

| Stain | Prostate | n | Geometric Mean | 95% CI for the mean |
|---|---|---|---|---|
| CB & Stefin A | BPH | 51 | 1.448 | (1.120, 1.872) |
| | cancer | 47 | 2.990 | (2.297, 3.891) |
| | Gleason 6 cancer | 17 | 3.452 | (2.399, 4.969) |
| | Gleason 7 cancer | 30 | 2.756 | (1.905, 3.987) |
| Comparing to cancer* | | | | P=value |
| CB & stefin A | BPH vs. cancer | | | 0.0001 |
| | BPH vs. Gleason 6 cancer | | | 0.0003 |
| | BPH vs. Gleason 7 cancer | | | 0.0052 |

* The two sample t-test was used for comparing values between BPH and cancer. The P values were not adjusted for multiple comparisons. The CI indicates that the ratio of CB to SA will be between 2.23 and 3.99 for all cancer cases, between 2.4 and 4.97 for Gleason score 6, between 1.90 and 3.99 for score 7 tumors and between 1.12 and 1.90 for BPH in 95% of cases. Ratios of CB to SA were not significantly different between Gleason score 6 and 7 tumors (P=0.3727). Statistical analysis by the Student's t-test, $P<0.05$. Abbreviations: CB=Cathepsin B, SA=Stefin A, BPH=Benign Prostatic Hyperplasia, PCa=Prostate Cancer, CI=Confidence Intervals

Table2. Correlation of CB/SA Ratio with other Parameters

| | N | CB/SA Ratio Mean | P Value |
|---|---|---|---|
| Overall | 47 | 0.97 | |
| Gleason Score | | | |
| 6 | 17 | 0.98 | 0.42 |
| 7 | 30 | 0.95 | |
| Pretreatment PSA | | | |
| < 10 | 28 | 0.97 | 0.59 |
| ≥10 | 13 | 0.95 | |
| DNA Ploidy | | | |
| Diploiod | 27 | 0.96 | 0.19 |
| Nondiploid | 11 | 1.02 | |
| MIB-1 (%) | | | |
| <7.5 | 17 | 0.98 | 0.61 |
| ≥7.5 | 16 | 0.99 | |
| Cancer size (cm) | | | |
| <1.0 | 9 | 0.97 | 0.99 |
| ≥1.0 | 20 | 0.97 | |
| S-Phase Cells (%) | | | |
| <25 | 9 | 0.94 | 0.26 |
| ≥25 | 28 | 1.02 | |

Table3. Pre-and post-prostatectomy tumor characteristics assessed by CB/SA ratio, MIB-1, DNA ploidy in biopsy and/or prostatectomy specimens

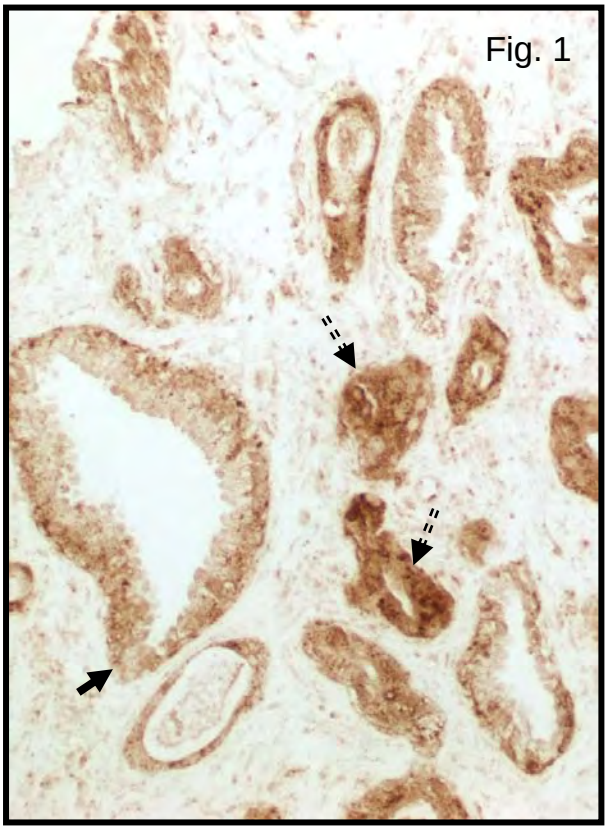
| Variable | Tissue Marker Expression in Needle Biopsy | | | |
|---|---|---|---|---|
| | DNA Index | % S Phase Cells | CB/SA Ratio | MIB-1 Index |
| Preoperative PSA | 0.02* | NS | NS | NS |
| Biopsy Gleason Score | NS** | NS | NS | NS |
| Biopsy CB/SA Ratio in all cancer tumors when compared to BPH | NS | NS | <0.0001 | NS |
| Biopsy CB/SA Ratio in score 6 tumors when compared to BPH | | | <0.0003 | |
| Biopsy CB/SA Ratio in score 7 tumors when compared to BPH | | | <0.0052 | |
| Biopsy DNA Ploidy | <0.0001 | <0.0001 | NS | NS |
| Biopsy % S-Phase Cells | <0.0001 | -- | NS | NS |
| Biopsy MIB-1 Index | NS | NS | NS | -- |
| Prostatectomy Gleason Score | NS | NS | NS | NS |
| Prostatectomy Cancer Volume | 0.02 | 0.03 | NS | NS |
| Positive Surgical Margins | NS | NS | NS | NS |
| Pathologic Stage | NS | NS | NS | NS |

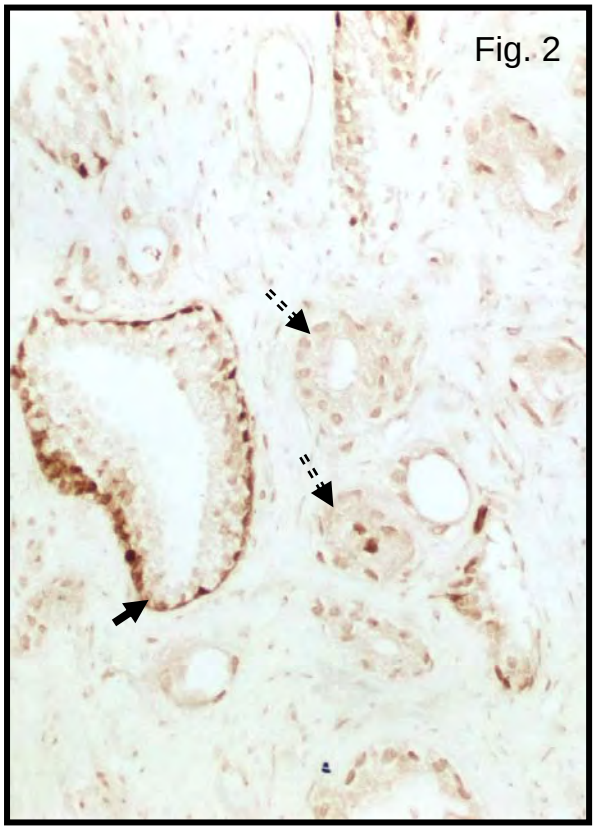
* The figures presented are P values
**NS: Not significant

**Figure Legends:**

Figure 1. Cathepsin B staining in one benign prostatic acinus (solid arrow) and two cancer acini (dotted arrow).

Figure 2. Stefin A staining in the same focus.



# Cathepsin B Expression in Prostate Cancer of Native Japanese and Japanese-American Patients: An Immunohistochemical Study

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**Abstract.** *Background: Japanese-American (J-A) men who have immigrated to the U.S.A. and acquired the Western lifestyle usually have more invasive prostate cancer (PCa) than native Japanese (NJ) living in Japan. The specific reasons for these differences remain unknown. The objective of this study was to examine immunostainings of cathepsin B (CB) and its endogenous inhibitor stefin A (SA) in tissue microarray (TMA) and radical prostatectomy (RP) tissue sections in the hope of obtaining insights into the invasiveness of PCa in Japanese patients. Patients and Methods: TMA and RP sections were evaluated in 50 men (25 NJ and 25 J-A) for CB and SA reaction products. The CB and SA immunostainings were imaged directly from microscope slides to a computer using a high performance charge coupled device (CCD) digital camera, quantified using Metamorph software, analyzed using the two-sample t-test, and confirmed by multiple regression analysis. Results: The CB and SA proteins were localized in the carcinomatous glands and isolated cancer cells in the TMA and RP sections. The Gleason scores and pre-surgery serum total prostate-specific antigen (PSA) levels did not differ significantly in the NJ and J-A patients ($p=0.14$, $p=0.16$, respectively). The Chi-square analysis of clinical stage versus place of birth showed that the NJ patients had significantly more T2a and T2b clinical stages than the J-A patients who had more advanced T2c and T3a stages ($p=0.003$). The CB and SA immunostainings and their ratios in Gleason score 6 tumors did not show any difference, but the CB:SA ratios in score ≥7 tumors approached significance levels. Conclusion: The overall matching of specimens according to the Gleason grade/score, pre-RP serum total PSA levels, clinical stage and age prior to evaluation of immunostainings greatly minimizes subjectivity associated with the evaluation of markers in this ethnic sub-population of PCa patients. CB and SA immunostaining is similar in Japanese patients who have organ-confined and moderately-differentiated PCa. Analysis of the reaction product data provides indirect evidence that invasiveness of PCa is similar in the two Japanese patient populations.*

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*Key Words:* Prostate cancer, Gleason grade, tissue microarray, cathepsin B, inhibitor stefin A.

Invasiveness (or aggressiveness) of prostate cancer (PCa) varies significantly in patients (1, 2), but metastasis is responsible for about 90% deaths (3-5). Japanese-American (J-A) men who have immigrated to the U.S.A. and acquired the Western lifestyle usually have more invasive PCa than native Japanese (NJ) living in Japan (6-12). Many criteria (such as age, body weight, body fat, food habits, nutrition, hormone levels, medical care, pathological grade, tumor volume, nuclear size and shape, pre-biopsy serum total prostate-specific antigen (PSA) levels, PSA density, pro-PSA expression, clinical stage and/or relationship of prostatic stroma and carcinomatous epithelia) have been used to show differences in the Japanese population. The specific reasons for these differences remain unknown. Selective utilization of criteria has provided inconsistent results. For example, Shiraishi *et al.* compared prostate autopsy samples of NJ, J-A, and Caucasian patients using tenascin, ras p21, and lectin-binding Helix Pomatia antigen (HPA) by immunohistochemical (IHC) techniques (13). They found that tenascin expression and HPA-positive cases were more

common in J-A than NJ, whereas ras p21 expression was lower in J-A than NJ. They concluded that PCa was more aggressive biologically in NJ than in J-A, but immunostained micrographs were not published. Fukagai *et al.* suggested that PCa in NJ was more advanced than in J-A living in Hawaii, presumably due to late PCa screening (14). Other studies have shown that the incidence and age-dependence of PCa were usually lower in NJ than J-A men (9, 15). Recently, Marks *et al.* concluded that the mechanism of carcinogenesis differed in NJ and J-A patients, possibly due to interactions of chromatin/genetic materials and nutrients (10). Veltri *et al.* have shown that ratios of epithelium and stroma in tissue micro-array (TMA) sections could distinguish the two groups of patients (11). Subsequently, Veltri *et al.* showed that nuclear size and shape and pro-PSA expression were significantly different in NJ and J-A PCa patients whereas pre-operative PSA levels, PSA density, and DNA were not (11, 12).

The above group of criteria did not include proteases (such as cysteine proteases, matrix metalloproteases, and/or serine proteases), alone or together, which are required for the degradation of basement membrane (BM) and extracellular matrix (ECM) proteins, and cancer cell invasion to other compartments (16-19). Proteases have been used to determine invasiveness of many solid organ carcinomas (prostate, breast, colon, brain and lung carcinomas and melanoma) in Caucasian and African-American men (16, 17, 19-28). Cancer cells also utilize proteases to degrade membrane proteins in the prostatic margins/capsules, seminal vesicles, bladder neck, lymph nodes and/or other organs before invading them (1, 16, 19, 21, 25). In contrast, benign prostatic hyperplasia (BPH) and benign ('normal') prostate cells do not degrade or invade the BM and ECM compartments (18). Since cathepsin B (CB) is one of the proteases involved in degradation of BM and ECM, cancer cell invasion and progression in many solid organ carcinomas, it was selected for evaluation in PCa of Japanese patients (16, 17, 21, 27). Our objective was to identify differences in the reaction products of CB and its endogenous inhibitor stefin A (SA) in tissue microarray (TMA) and radical prostatectomy (RP) tissue sections by IHC techniques in the hope of obtaining new insights into invasive PCa in Japanese patients using the group of patients earlier evaluated by Marks *et al.* (10) and Veltri *et al.* (11, 12).

## Patients and Methods

*Patient population.* In a retrospective study of 68 Japanese patients, Marks *et al.* (10) selected RP tissue samples of 50 (25 NJ and 25 J-A) PCa patients who had not received any treatment prior to surgery between 1994 and 2001. The Japanese men had organ-confined moderately differentiated PCa (10). The samples showed overall matching according to the Gleason grade/score, PSA levels, clinical stage and age at biopsy and/or RP.

*Tissue preparation and array construction.* The formalin-fixed, paraffin-embedded sections were evaluated for pathology and Gleason grades/scores by a single pathologist using hematoxylin and eosin (H&E) stained sections as described elsewhere (10, 11). Briefly, TMA blocks were constructed by Dr. Angelo De Marzo, as reported (10-12). Core tissue cylinders (diameter 0.6mm) were removed from pre-selected regions of benign ('normal') appearing glands and carcinomatous areas of the individual donor paraffin-embedded blocks. The cores were arrayed into new recipient blocks according to the methods of Kononen *et al.* (29). Eight core sections (4 benign and 4 PCa) per case were examined in this study. In addition to the TMA sections, paraffin-embedded RP tissue sections (5 to 6 μm) were obtained from each patient and evaluated at the Minneapolis Veterans Affairs Medical Center (VAMC). In the RP sections, BPH and benign glands at least two microscope fields away from the carcinomatous areas were selected for evaluation as controls. The Minneapolis investigators provided CB and SA immunostaining results to co-author Dr. Leonard S. Marks (LSM) prior to receiving any clinical data (Tables I and II). All the samples and medical information were collected by LSM according to the approved protocol by the Institutional Review Board (IRB) of the University of California Los Angeles, Los Angeles, CA. The Minneapolis investigators did not have access to the Health Insurance Portability & Accouutability Act of 1996 (HIPAA)-regulated patient identifying data.

*Immunohistochemical localization of cathepsin B and stefin A antibodies.* Since the CB and SA antibodies used in the previous study were unavailable (30-32), new antibodies were used in the present study. Briefly, mouse monoclonal anti-human liver CB immunoglobulin G (IgG) was obtained from Oncogene Research Products (Calbiochem, Cambridge, MA, USA) and polyclonal goat anti-human SA IgG from R&D Systems (Minneapolis, MN, USA). The molecular weights of CB and SA, their purities, and IHC localization techniques were reported earlier (30-32) and the immunoblot studies on the new antibodies were similar to those previously reported by us (30). Data using the new antibodies were evaluated and published previously (33, 34).

*Immunohistochemical localization of cathepsin B and stefin A.* Antigen retrieval was carried out in 10 mM citrate buffer (pH 6.0) using a Decloaking Chamber Pro machine (Biocare Medical, Walnut Creek, CA, USA) (30, 33, 34). The mouse anti-CB and goat anti-SA antibodies were localized in TMA and RP tissue sections using IHC localization techniques (33, 34). The reaction products were developed for 10 minutes with fresh-filtered 3, 3'-diaminobenzidine (DAB) and enhanced with dilute osmium tetroxide solution as reported (33, 34). The negative control sections were incubated with pre-immune mouse or goat serum *in lieu* of the primary antibodies and processed.

*Quantification of localization data using Metamorph image analysis system.* The immunostainings for CB and SA were quantified using a computer-based image analysis system equipped with Metamorph software (Universal Imaging Corp., West Chester, PA, USA), as we reported previously (30, 33, 34). Briefly, the images of the CB and SA reaction products were acquired at x200 (x10 ocular and x20 objective) directly from slides to a computer using a Zeiss (Carl Zeiss, Inc., Thornwood, NY, USA) microscope

Table I. *Distribution of biopsy data in Japanese prostate cancer patients.*

| | Native Japanese | Japanese-American | *p*-value |
|---|---|---|---|
| Number of prostate samples | 25 | 25 | - |
| Data by primary criteria | | | |
| Gleason score ≤6 | 5 | 15 | - |
| Gleasou score ≥7 | 2 | 5 | - |
| Pathology not in database | 18 | 5 | - |
| Gleason score (Mean±SD) | 4.9±1.9 | 5.7±1.4 | 0.07 |
| Pre-surgery PSA levels, range (Mean±SD) | 0.7-40.1 (11.7±9.5) | 0.7-26 (8.5±5.7) | 0.16 |
| Clinical stage at biopsy | N/A | N/A | - |
| Age at diagnosis, range (Mean±SD) | 58-75 (65.4±5.0) | 58-77 (69.7±4.9) | 0.004 |

N/A=not available; PSA=prostate-specific antigen, ng/ml; SD=standard deviation; Student's *t*-test, significant *p*-value≤0.05.

Table II. *Distribution of radical prostatectomy data in Japanese prostate cancer patients.*

| | Native Japanese | Japanese-American | *p*-value |
|---|---|---|---|
| Gleason score 6 tumors after RP[1] | 4 | 5 | - |
| Gleason score >7 tumors after RP[1] | 16 | 18 | - |
| Unknown pathology | 5 | 2 | - |
| Mean Gleason score after RP (Mean±SD) | 7.2±0.8 | 6.9±0.5 | 0.14 |
| Pathological stage after RP[1] | | | |
| pT2a | 9 | 3 | - |
| pT2b | 12 | 7 | - |
| pT2c | 0 | 9 | - |
| pT3a | 4 | 6 | - |
| TNM clinical stages[2] | T2a, T2b, T3a | T2a, T2b, T2c, T3a | 0.003 |
| Prostate weight (grams), range (Mean±SD)[3] | 10-62 (34.8±13.6) | 21-105 (38.5±17.9) * | 0.43 |
| Number of years after RP, range (Mean±SD)[4] | 5.2-10.7 (7.4±1.7) | 5.6-14.4 (9.7±2.6) | 0.001 |
| Patients with unknown post-surgery PSA levels | 25 | 1 | - |
| Patients with PSA>0.2 ng/ml[5] | N/A | 7 | - |

[1]Data from Veltri *et al.* (12); [2]Chi-square test, stage *versus* place of birth; [3]data from Veltri *et al.* (11); [4]data calculated with January 1, 2007 as the end date; [5]17 Japanese-American patients had PSA<0.2 ng/ml; *n=22; RP=radical prostatectomy; PSA=protate-specific antigen; N/A=not available; TNM: T=primary tumor, N=regional lymph nodes, M=distant metastases; SD=standard deviation; Student's *t*-test, significant *p*-value≤0.05.

and a high performance Charge Coupled Device (CCD) (Photometrics, Tucson, AZ, USA) digital camera which uses a 1317x1035 imaging array with 6.8x6.8 micrometer pixels and 12-bit digitization. On the basis of gray values ranging from 4,095 to 0, white to black respectively, threshold boundaries of the immunostainings were created (30, 33, 34). The utilization of neutral and green filters provided optimum imaging of the reaction products. In the RP sections, benign ('normal') and/or BPH areas were imaged at least two microscope fields away from the carcinoma and used as controls. Since the proximity of the benign or BPH glands to the cancer was unknown in the TMA sections, immunostainings of BPH glands from the RP sections were used as controls. The measurements of CB and SA are presented as range and mean with standard error of the mean (SEM).

*Data analysis.* The immunostaining data were analyzed using the two-sample Student's *t*-test (significance of $p \leq 0.05$), Chi-square, and/or multiple regressions.

## Results

*Profile of prostate cancer patients.* Several studies on the same group of Japanese PCa patients have been published (10-12) and the relevant biopsy-and RP-associated data is presented in Tables I and II. The biopsy-associated data on the Gleason score and clinical stage were incomplete (Table I). After biopsy, the pathological diagnosis was known in 7 NJ and 20 A-J patients, but prostatectomy specimens revealed the pathological diagnosis in 20 (5 unknown) NJ and 23 (2 unknown) J-A patients (Tables I and II). The Gleason scores in the RP ($p$=0.14) and pre-surgery serum total PSA levels ($p$=0.16) did not differ significantly between the NJ and J-A patients (Tables I and II). The prostate weight did not differ in these Japanese patients ($p$=0.43) (Table II). Earlier studies had shown that prostate weight,

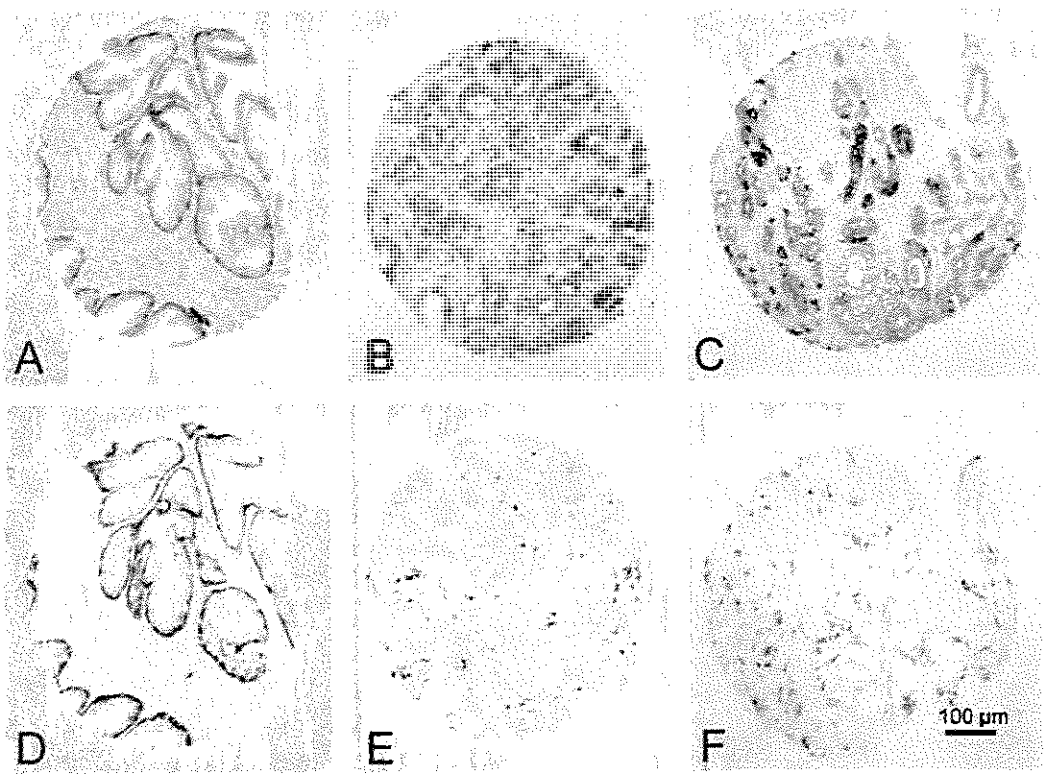


Figure 1. *Cathepsin B (CB) and stefin A (SA) immunostainings in tissue microarray (TMA) sections obtained from Japanese prostatectomy patients. CB reaction products (A) and SA (D) in benign prostatic hyperplasia glands. Reaction products of CB in Gleason score 7 NJ (B) and J-A (C), and of SA in adjacent sections of NJ (E) and J-A (F) tumors.*

serum PSA levels, pathological tumor stages and Gleason grades were similar in these NJ and J-A patients (10-12). The Chi-square analysis of clinical stage *versus* place of birth showed that the NJ patients had significantly more T2a and T2b clinical stages than the J-A patients who had more advanced T2c and T3a stages ($p$=0.003) (Table II). Therefore, the age at diagnosis, which was significantly different between the two groups, was utilized ($p$=0.004) (Table I). The follow-up after RP was significantly shorter in the NJ than the J-A men ($p$=0.001) (Table II). Marks *et al.* also showed that the J-A men had undergone RP surgery about 28 months earlier than the NJ patients and this was significantly different (10). Post-RP serum total PSA levels of ≥0.2 ng/ml indicated recurrence of PCa in seven of the JA patients, but similar data were not available for the NJ patients (Table II). The PSA level of ≥0.2 ng/ml is most commonly used in clinical practice (35). In 17 of the J-A patients, the PSA levels were <0.2 ng/ml and the PSA level was not known in one patient (Table II).

*Cathepsin B and stefin A in BPH glands.* In the TMA sections showing BPH glands, CB and SA proteins were localized in the basal and some cuboidal/columnar cells (Figure 1A, D). Similar localization was found in BPH glands in the RP sections. The immunostainings of CB alone and SA alone in BPH glands were different in the TMA sections compared to the RP sections ($p$=0.002 and $p$=0.024, respectively) (Figure 2). The ratios were not significantly different ($p$=0.13).

*Cathepsin B and stefin A in prostate cancer.* In general, the new antibodies against CB and SA produced lower levels of immunostainings in the TMA and RP sections than those evaluated earlier (30, 31). The CB and SA proteins were localized in the cuboidal/columnar carcinomatous glands and isolated cells in the TMA and RP sections of the NJ and J-A patients (Figure 1B, C, E, F). The CB alone and SA alone reaction products showed variations within and between the Gleason scores of both groups of patients.

*Cathepsin B and stefin A in Gleason score 6 and 7-9 tumors.* Comparative CB and SA reaction product data in the TMA and RP sections from the NJ and J-A patients are shown in Tables III and IV. The statistical analysis of CB and SA immunostainings in the Gleason score 6 tumors did not show any difference between the two groups (Table III). In the NJ

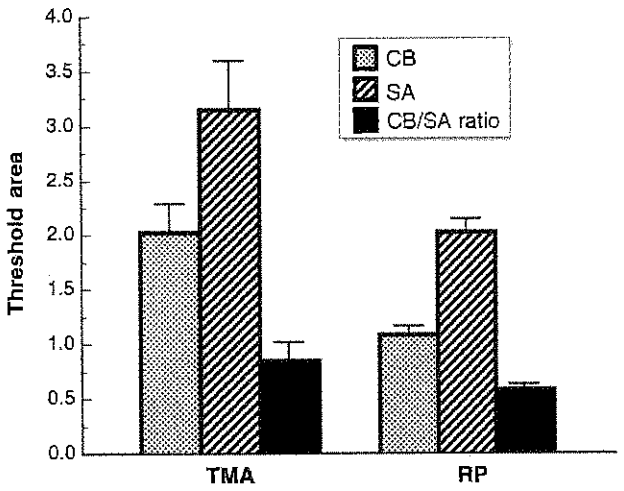


Figure 2. *The IHC reaction products for CB alone and SA alone in BPH glands were significantly higher (p=0.002 and p=0.024, respectively) in the TMA sections when compared to the RP tissue sections, but the CB to SA ratios were not (p=0.13).*

and J-A patients with a Gleason score ≥7, the CB alone immunostainings were not significantly different between the TMA and RP sections ($p$=0.49 and $p$=0.1, respectively) (Table IV). However, SA alone was significantly higher in the TMA sections of the NJ than the J-A patients ($p$=0.02), but not in the RP sections ($p$=0.98) (Table IV). In the J-A patients, the CB to SA ratios were higher in the TMA than the RP sections ($p$=0.08, marginally significant) (Table IV). The CB to SA ratios in the RP sections from the NJ patients were higher than the J-A patients ($p$=0.07, marginally significant) (Table IV).

For the multiple regression analyses, the immunostaining data in the TMA and RP sections were adjusted for tumor weight, PSA levels, clinical stage, age, and follow-up time for evaluation of the NJ and J-A patients. In general, the NJ patients showed slightly higher CB immunostainings in the TMA sections than those in the J-A patients (marginally significant, $p$=0.095). Similar analysis of CB immunostainings in the RP sections did not show any difference ($p$=0.28). The analysis of SA and CB: SA ratios did not show any difference in the TMA sections ($p$=0.21, $p$=0.35, respectively) or the RP sections ($p$=0.78, $p$=0.46, respectively) of the NJ and J-A patients.

## Discussion

Studies of Marks *et al.* (10) and Veltri *et al.* (11, 12) allowed us to categorize organ-confined and moderately differentiated PCa according to Gleason grade/ score, pre-biopsy serum total PSA levels, clinical stage, and age at biopsy and/or prostatectomy. These criteria are usually used in the initial diagnosis and prediction of prognosis for the PCa patients. The utilization of these criteria allowed overall matching of the Japanese patients prior to evaluation of the CB and SA immunostainings. The image analysis indicated similar CB and SA immunostaining, suggesting that the invasiveness of the cancer was similar in both groups of Japanese patients. The finding was consistent with our study of the same biomarkers in African-American and Caucasian PCa patients who were overall matched according to the above criteria (34). Further analysis indicated that the ratios of CB≤SA in the NJ and J-A patients were similar to the CB≤SA ratios in Caucasian patients who had organ-confined and less aggressive (or indolent) PCa (30). Thus, our finding of similar invasiveness of PCa was consistent with the dataset of Japanese patients who did not have lymph node metastases. In our earlier study of a sub-population of 97 Caucasian PCa patients, the ratios of CB>SA were associated with increased pelvic lymph node metastases ($p$=0.006) (30). Earlier, we had reported on increased cysteine protease CB activities in PCa in relation to its endogenous cysteine protease inhibitors (30, 31, 34), much as others have reported for many other solid organ carcinomas (16, 21, 36).

Our analysis of the above criteria, in addition to tumor weight and PSA density, indicated that the sub-populations of NJ and J-A patients were not significantly different (10-12). In contrast, significant differences of PCa in Japanese patients were found when a mixture of criteria (such as socioeconomic status, age, body weight, body fat, hormone levels, nutrition, medical care, tumor volume, nuclear size and shape, prostatic stroma and carcinomatous epithelia relationships), alone or together, were used in the study (10-12). Therefore, it is proposed that PCa patients should be matched according to the Gleason grade/score, pre-prostatectomy serum total PSA levels, clinical stage and age at diagnosis for comparison of this cancer in races and ethnic groups. These criteria greatly minimized subjectivity associated with the selection of samples and evaluation of biomarkers in the Japanese and other racial groups of patients. Since a small number of PCa cases were studied, the proposed criteria need to be validated in a large number of Japanese patients.

Image analysis of CB alone and SA alone indicated that the reaction products were significantly higher in BPH glands from the TMA sections than BPH glands in the RP sections ($p$=0.002 and $p$=0.024, respectively). The specific reasons for these differences are unknown, but it could be due to increased CB and SA immunostaining associated with a higher ratio of cut edges to surface areas in the TMA sections in comparison to the RP sections, sampling bias associated with selection of site-specific pathological grade in the TMA cores in comparison to the RP sections, or the cancer 'field effect' on normal-

Table III. *Immunostaining of Gleason score 6 tumors in the TMA and RP sections.*

| | TMA | | | RP | | |
|---|---|---|---|---|---|---|
| | NJ | J-A | *p*-value | NJ | J-A | *p*-value |
| CB | 0.46-1.82 (1.10±0.39) | 0.23-1.04 (0.58±0.19) | 0.32 | 0.21-0.56 (0.33±0.11) | 0.20-0.70 (0.52±0.12) | 0.31 |
| SA | 0.27-0.84 (0.52±0.17) | 0.16-1.91 (0.79±0.39) | 0.56 | 0.53-1.12 (0.82±0.17) | 0.27-1.71 (0.86±0.33) | 0.93 |
| CB/SA Ratio | 1.23-4.12 (2.34±0.90) | 0.38-2.04 (1.12±0.38) | 0.30 | 0.28-0.5 (0.39±0.06) | 0.19-1.82 (1.05±0.44) | 0.23 |

RP=radical prostatectomy, TMA= tissue microarray; CB=cathepsin B; SA=stefin A; Mean±SEM, standard error of the mean; Student's *t*-test, significant $p$-value≤0.05.

Table IV. *Immunostaining in Gleason score ≥7 tumors in the TMA and RP sections.*

| | TMA | | | RP | | |
|---|---|---|---|---|---|---|
| | NJ | J-A | *p*-value | NJ | J-A | *p*-value |
| CB | 0.26-1.41 (0.89±0.13) | 0.22-2.61 (1.10±0.27) | 0.49 | 0.16-1.32 (0.53±0.08) | 0.21-4.36 (0.96±0.23) | 0.10 |
| SA | 0.58-3.81 (2.00±0.33) | 0.22-1.87 (0.97±0.18) | 0.02 | 0.11-2.33 (0.89±0.17) | 0.12-4.35 (0.90±0.25) | 0.98 |
| CB/SA Ratio | 0.21-2.34 (0.63±0.22) | 0.33-3.72 (1.37±0.32) | 0.08 | 0.28-4.12 (0.95±0.28) | 0.23-9.55 (2.26±0.63) | 0.07 |

RP=radical prostatectomy, TMA= tissue microarray; CB=cathepsin B; SA=stefin A; Mean±SEM, standard error of the mean; Student's *t*-test, significant $p$-value≤0.05.

appearing prostate and/or BPH glands in TMA sections. Our limited study indicated that TMA sections are best suited for concurrent screening of many biomarkers, including CB and SA, but large RP tissue sections allow in-depth analysis of carcinomatous areas showing two or more Gleason grades/scores in relation to the stroma and invasive tumor edges.

In conclusion, the overall matching of specimens according to the Gleason grade/score, pre-RP serum total PSA levels, clinical stage and age prior to evaluation of immunostainings greatly minimizes subjectivity associated with the evaluation of CB and SA immunostaining in this ethnic population of PCa patients. CB and SA immunostaining is similar in sub-populations of Japanese PCa patients who have organ-confined and moderately-differentiated disease. Analysis of the reaction product data provides indirect evidence that invasiveness of PCa is similar in the NJ and the J-A patients.

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