Characterization of Prostate Cancer in Needle Biopsy by Cathepsin B, Cell Proliferation and DNA Ploidy

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Abstract. *Background: Our objective was to determine localization patterns of three distinct groups of biomarkers (cathepsin B, MIB-1 and DNA ploidy) in prostate needle biopsy sections to establish localization similarities (or differences) in biopsy and retropubic prostatectomy specimens (RPs). Materials and Methods: Prostate needle biopsy specimens and matched RPs from 47 patients with cancer were evaluated. Biopsy and RP sections were stained with anti-cathepsin B (CB) and anti-stefin (cystatin) A (SA) and for cell proliferation and DNA ploidy. The ratio of CB to SA in stained cells was calculated for each biopsy cancer and matched benign prostatic hyperplasia (BPH) sample. Results: The geometric mean of CB to SA was 1.45 in BPH and 2.99 in cancer specimens (p=0.0001). The percentage of S-phase cells and DNA ploidy status in needle biopsy was associated with cancer volume in RP cases (p=0.03). Conclusion: Our study has indicated that the ratio of CB to SA is significantly higher in prostate cancer biopsy specimens than in BPH. The percentage of S-phase cells and DNA ploidy in needle biopsies predicts cancer volume of RPs. We have shown that localization of three distinct biomarkers in biopsies reliably assesses the nature of prostate cancer in biopsy sections.*

About 190,000 men were expected to be diagnosed with prostate cancer (PCa) and 27,400 to die from it in 2009 (1). The initial prognosis of PCa in needle biopsy sections usually utilizes Gleason grading system, prostate pathology, serum total prostate-specific antigen (PSA) levels, clinical tumor stage and age (2-4). These data are often used in treatment selection and prediction of prognosis for an individual patient. About 40% patients elect radical prostatectomy (RP) (5-8). Surgery patients also have additional assessment of their cancer by definitive Gleason grades/scores, cancer cell invasion to prostatic capsules/margins, extra-prostatic areas and/or pelvic lymph node metastasis; all of these may be used in assessing the aggressiveness of PCa (9-13). In contrast, the remaining 60% patients may select an alternative treatment such as watchful waiting, hormonal therapy, radiation/brachytherapy, chemotherapy, immunotherapy or their combinations (6, 8, 14). These patients usually make treatment decisions using the biopsy-based assessment of their cancer (6, 14, 15). Since the biological behavior of individual cancer (16-19) and patient outcomes (20-24) are unknown at the time of the initial diagnosis and treatment, further assessment of needle biopsy sections by a set of biomarkers may provide additional useful data for making more informed decisions, especially for patients who do not select surgery.

The value of many biomarkers (such as cathepsin B (25-28), MIB-1 index (29-32), DNA ploidy (13, 33-35), microvessel density, *p53* gene mutation, *p27* deletion, *Bcl-2* and *Rb* (36, 37) is controversial (13, 19, 38, 39). Most of these biomarkers have been evaluated in biopsy and/or RP tissue sections (25-27, 40, 41), but not in matched biopsy and RP sections from the same patients. Our review has shown that a panel of biomarkers which can provide clues as to the nature of PCa may also assist patients and their physicians in treatment decisions. The invasion-associated cysteine protease cathepsin B (CB) is involved in degradation of basement membrane, extracellular matrix and adherent junction proteins (42, 43), as well as in progression of cancer cells to the

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prostatic stroma and other compartments (42, 44, 45). We selected CB because it is elevated in PCa and other solid tumor types (such as melanoma, bladder, lung, colorectal and breast cancer) when compared to benign counterparts (25-27, 42, 44, 45). Evaluation of stefin (cystatin) A (SA), an endogenous inhibitor of CB, provides an indirect assessment of CB activity in prostate and many solid tumors (42, 44, 45). We have shown a ratio where CB>SA is associated with pelvic lymph node metastasis, indicating the aggressive nature of PCa within individual Gleason scores (25-27); these ratios do not, however, discriminate aggressiveness of cancer between two different Gleason grades/scores (27, 41). Cell proliferation is significantly increased in PCa when compared to benign counterparts (29). The MIB-1 staining index has been associated with survival of PCa patients (30, 31, 36, 46). DNA ploidy adds prognostic information for some PCa patients (2, 13, 35, 47, 48). Furthermore, these biomarkers can be readily evaluated in formalin-fixed paraffin-embedded biopsy and RP tissue sections. Our objective was to assess PCa with the expectation that localization of CB, cell proliferation by MIB-1 and DNA ploidy in the matched biopsy and RP sections may predict the nature of this cancer, especially in patients who do want surgery.

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 Institutional Review Board (IRB) approval. Patients had not been treated before undergoing prostatectomy. The number of biopsy cores ranged 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 (IHC) localization 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 (49). For each block, a single 5 μm section was cut, stained with H&E, and examined independently by two pathologists (DGB and KAI).

Immunohistochemical localization of CB, SA, and MIB-1 expression. Rabbit anti-CB antibody (Oncogene Research Products, Calbiochem, Cambridge, MA, USA), mouse anti-human SA antibody (KRKA Novo Mesto, Slovenia), and mouse anti-human MIB-1 (Ki-67) antibody (DAKO, Carpinteria, CA, USA) were used to localize CB, SA, and Ki-67 antigen, respectively, in tissue sections using the avidin-biotin complex (ABC) method as reported before (25-27). Reaction products were developed with fresh-filtered 3,3-diaminobenzidine (DAB) solution (0.25 mg/ml; Sigma, USA) 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. Localization of CB and SA in benign prostatic hyperplasia (BPH) within two microscopic views at a magnification of ×200 was used as a control. Negative control sections were incubated with pre-immune rabbit or mouse serum in lieu of primary antibody.

Quantification of immunostaining using an 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, USA), as detailed in our recent paper (40). A total of 4-6 randomly selected images with CB and SA staining in each biopsy section were acquired at a magnification of ×400 directly from the microscope slides to a computer using a digital camera (Photometrics, Tucson, AZ, USA) 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 ×400. Since they were evaluated in several previous studies (25, 26, 40, 41), RP sections were not stained for 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 ×200 field (0.754 mm^2). Three fields in each section were randomly selected for study. The MIB-1 index was expressed as the percentage of 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 a 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, USA) 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 four 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 being non-diploid.

Statistical analysis. The difference in staining intensity between BPH and PCa in biopsy was analyzed using the geometric mean. The geometric mean and confidence intervals (CI) of the CB/SA ratio were calculated on a 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 pathological findings in RP was determined using Student's *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). The mean preoperative serum PSA was 9.1 ng/ml (range, 3.6-28.2 ng/ml). The mean Gleason score was 6.5 in 47 sets of prostate needle biopsies. Seventeen RP

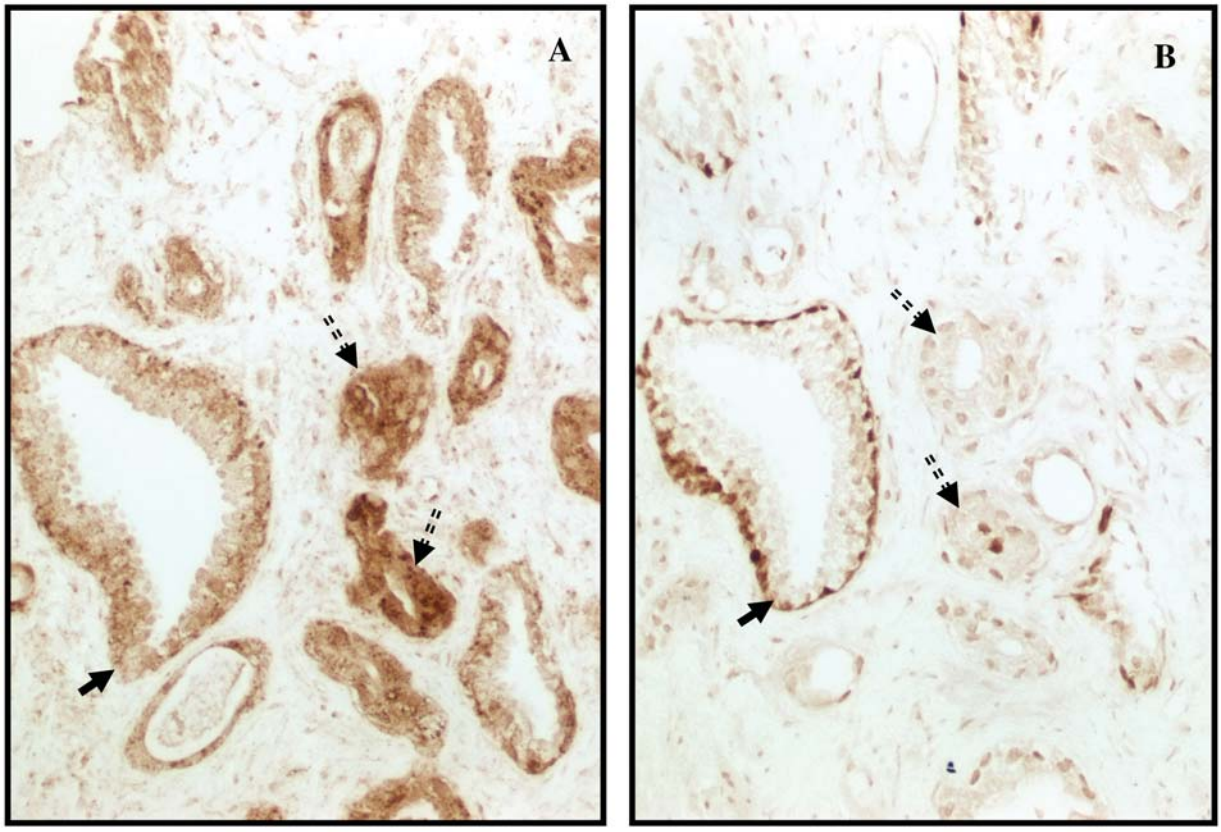

Figure 1: *A, Cathepsin B staining in a benign prostatic acinus (solid arrow) and two cancer acini (dotted arrow). B: Stefin A staining in the same focus (×200).*

patients had a Gleason score of 6 (3+3) and 30 had a score of 7 (3+4 and 4+3) (mean score, 6.8). The mean cancer volume in RP was 1.64 cm^3 (range, 0.7-2.9 cm^3). Four specimens (8.5%) had extraprostatic extension of cancer, 7 (14.9%) had positive surgical margins, and 2 (4.3%) had seminal vesicle involvement. These patients did not show any pelvic lymph node metastasis. Cancer volume in RP was associated with preoperative serum PSA (p=0.038).

Cathepsin B and stefin A. CB and SA immunoreactivity were observed predominantly in basal cells and some cuboidal/columnar cells in BPH, whereas PCa cells showed variable cytoplasmic staining (Figure 1). Table I shows the geometric mean distribution of the ratio of CB to SA in BPH and PCa and their comparisons. The ratio of CB:SA in the combined score 6 and 7 tumors was significantly higher than in BPH (p=0.0001; Table I). The ratio of CB:SA was also significantly higher for separate Gleason scores 6 and 7 tumors than for BPH (p=0.0003, p=0.0052, respectively; Table I). There were no differences in the ratios of CB:SA between Gleason score 6 and 7 tumors (Table I). 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 pathological stage (Table II).

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 the biopsy was not associated with biopsy CB/SA ratio, biopsy DNA ploidy, cancer volume, or Gleason score at RP (Table II). There was no correlation of the percentage 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 III shows that the incidence of non-diploid cancer in needle biopsy was associated with cancer volume in RP (p=0.03). The percentage of cells in the 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.

Table I. *Distribution of geometric mean and confidence intervals (CI) for cathepsin B (CB) and stefin A (SA) in Gleason score 6 and 7 prostate tumors and benign prostate hyperplasia (BPH).*

Prostate	n	CB:SA	
		Geometric mean	95% CI for the mean
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)
Compared to Cancer*			*p*-Value
BPH *vs*. Cancer			0.0001
BPH *vs*. Gleason 6 cancer			0.0003
BPH *vs*. Gleason 7 cancer			0.0052

*Two sample *t*-test was used for comparing values between BPH and cancer. The *p*-values were not adjusted for multiple comparisons.

Table II. *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 (ng/ml)			
<10	28	0.97	0.59
>10	13	0.95	
DNA ploidy			
Diploid	27	0.96	0.19
Nondiploid	11	1.02	
MIB-1 (%)			
<7.5	17	0.98	0.61
>7.5	16	0.99	
Tumor 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	

Discussion

Analysis of CB and SA immunostaining data in biopsy sections has shown that where CB<SA, this was associated with less aggressive PCa which did not have lymph node metastases, as reported in our earlier paper (26). Earlier, we had shown that a ratio where CB>SA in RP sections was associated with lymph node metastasis (26). Since matched biopsy and RP sections did not have lymph node metastases, CB and SA immunostaining was consistent in biopsy sections. In addition, the ratio of CB and SA between the Gleason scores 6 and 7 tumors was not significantly different in biopsy sections; this is also consistent with our previous study (27, 28). Since CB is involved in promoting cancer cell invasion in small and large tumors, lack of correlation between CB immunostaining and tumor volume is also consistent with the earlier results (26-28, 40). Thus, CB and SA immunostaining data in biopsy sections reliably reflects those reported in RP sections. We suggest that the ratios of CB: SA can assess the presence (or lack thereof) of aggressive PCa in the initial needle biopsy sections. Our study indicates that patients can use results of CB and SA immunostaining in their biopsy sections in the decision to select surgical or other treatments.

The MIB-1 staining index has been associated with PCa patient survival (30, 31, 36, 46). The number of S-phase cancer cells was reported as an independent predictor of prostate cancer outcome (29). In our present study, we found no correlation of MIB-1 index in prostate needle biopsies with pathological findings in RP tissue sections. Ojea Calvo *et al*. reported similar findings (50). This lack of concordance between the biopsy and the RP specimens might be caused by sampling variation (21, 36). 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 (2, 13, 35, 47). Our findings indicate that the percentage of cells in S-phase and DNA ploidy in needle biopsies predict cancer volume in PR.

Conclusion

CB and SA immunostaining in the present set of biopsies are consistent with less aggressive PCa, as shown by CB<SA, and without lymph node metastasis. The percentage of S-phase cells and DNA ploidy in needle biopsies predicts cancer volume in RP in matched biopsy and RP patients. In this study, we have shown that a panel of three distinct groups of biomarkers can clarify the nature of PCa in the needle biopsies. These biomarkers are associated with tumor invasiveness, cell proliferation and DNA ploidy and they provide an additional set of criteria for selecting surgery or other treatments and prediction of prognosis. Since our study is based on a relatively small number of cases, it should be confirmed by an expanded study.

Table III. *Significance of correlations (p-values) for tumor characteristics assessed by CB/SA ratio, MIB-1, DNA ploidy in biopsy and prostatectomy specimens.*

Variable	Tissue marker expression in needle biopsy			
	DNA index	% S-phase cells	CB/SA ratio	MIB-1 index
Preoperative PSA	**0.02**	>0.05	>0.05	>0.05
Biopsy Gleason Score	>0.05	>0.05	>0.05	>0.05
Biopsy CB/SA ratio:				
Tumors compared to BPH	>0.05	>0.05	**<0.0001**	>0.05
Gleason 6 tumors compared to BPH			**0.0003**	
Gleason 7 tumors compared to BPH			**0.005**	
Biopsy DNA ploidy	**<0.0001**	**<0.0001**	>0.05	>0.05
Biopsy % S-phase cells	**<0.0001**		>0.05	>0.05
Biopsy MIB-1 index	>0.05	>0.05	>0.05	
Prostatectomy Gleason score	>0.05	>0.05	>0.05	>0.05
Prostatectomy tumor volume	**0.02**	**0.03**	>0.05	>0.05
Positive surgical margins	>0.05	>0.05	>0.05	>0.05
Pathological stage	>0.05	>0.05	>0.05	>0.05

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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