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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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).

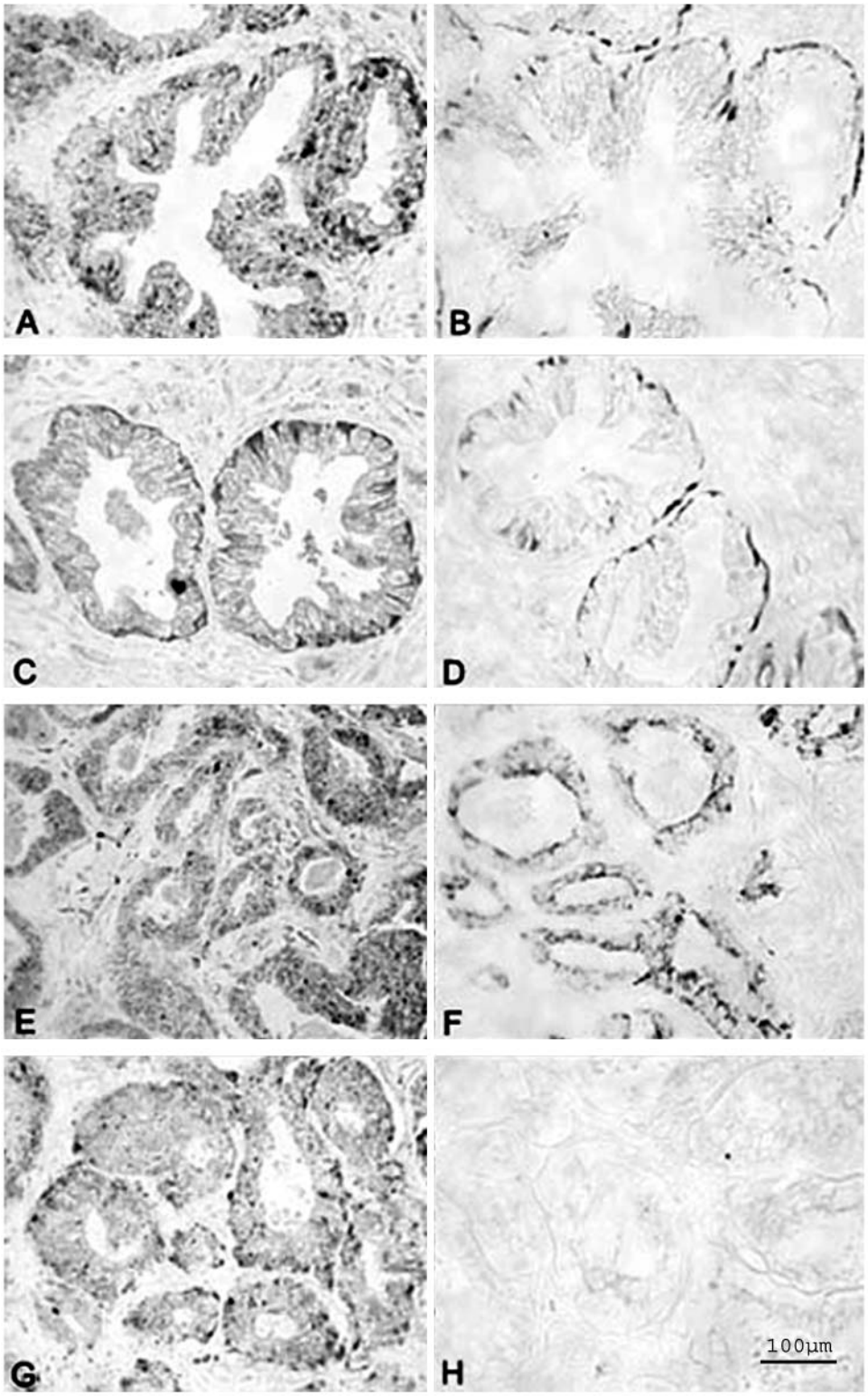

Figure 1. *Comparison of (A) cathepsin B and (B) stefin A immunohistochemical localization in BPH (CB to SA ratio=1.48); (C) CB and (D) SA in PIN glands (CB to SA ratio=1.35); (E) CB and increased (F) SA staining in PCa (CB to SA ratio=0.93); and (G) CB and (H) decreased SA staining of PCa (CB to SA 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 ($p<0.0001$).

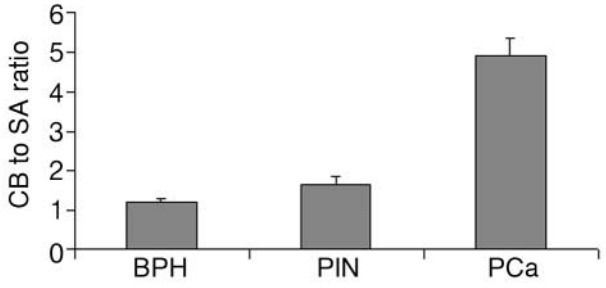

Figure 2. *Distribution of CB to SA ratios in prostate tissues. Ratios were significantly higher in PCa than in BPH or PIN. The ratios were significantly higher in PIN (p=0.036) and PCa (p<0.001) when compared to BPH. Prostate cancer had significantly higher ratios than PIN (p<0.001). Error bar=SEM.*

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.

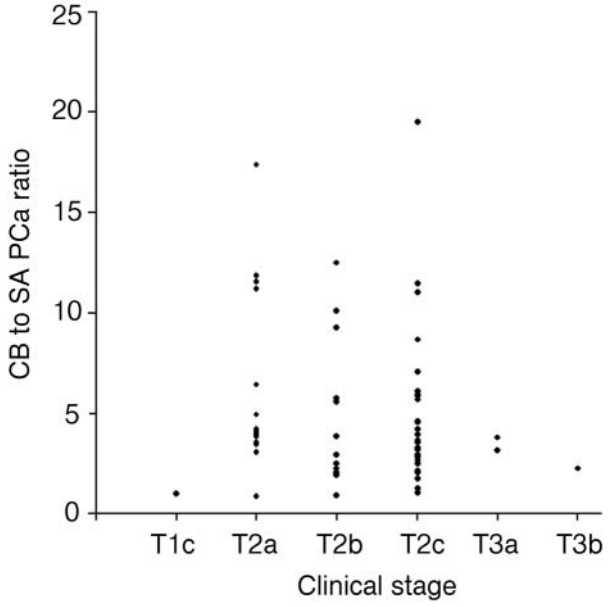

Figure 3. *Distribution of CB to SA ratios in relation to clinical stages. Ratios were higher in patients with T2a, T2b and T2c clinical stages than in a limited number of other stages.*

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

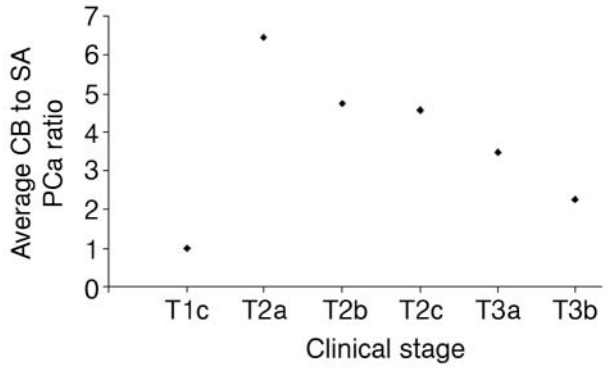

Figure 4. *Inverse relation of CB to SA ratios to clinical stages in T2a-T3b, except in a single case showing T1c stage.*

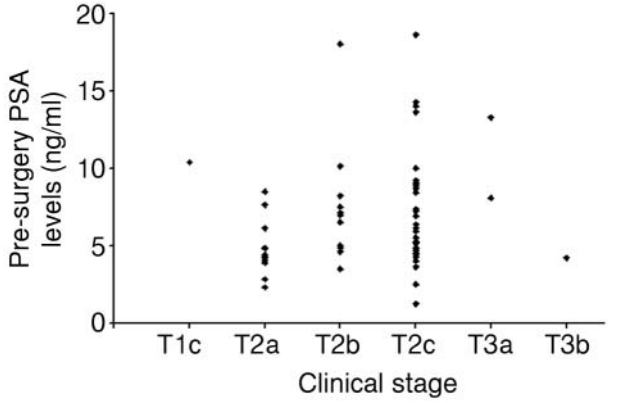

Figure 5. *Relationship of pre-prostatectomy PSA levels to clinical stages in prostate needle biopsies.*

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.

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