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Expression of Semenogelins I and II and Its Prognostic Significance in Human Prostate Cancer

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BACKGROUND. Little is known about the role of semenogelins, seminal plasma proteins that play critical roles in semen clotting and subsequent liquefaction in the presence of zinc and prostate-specific antigen, in human malignancies.

METHODS. We investigated the expression of semenogelins in four human prostate cancer lines by RT-PCR and Western blotting as well as in 70 radical prostatectomy specimens by immunohistochemistry. Effects of semenogelin overexpression on prostate cancer cell proliferation were also assessed.

RESULTS. mRNA/protein signals for semenogelins I (SgI) and II (SgII) were detected only in androgen-sensitive LNCaP cells cultured with zinc. Transfection of SgI/SgII increased/decreased cell growth of androgen receptor (AR)-positive/semenogelin-negative CWR22Rv1 in the presence of zinc, whereas it showed marginal effects in AR-negative/semenogelin-negative PC-3 and DU145. Immunohistochemical studies showed that SgI and SgII stain positively in 55 (79%) and 31 (44%) cancer tissues, respectively, which was significantly higher than in corresponding benign tissues [SgI-positive in 13 (19%) cases ($P < 0.0001$) and SgII-positive in 15 (21%) cases ($P = 0.0066$)]. Among the histopathological parameters available for our patient cohort, there was an inverse association only between Gleason score (GS) and SgII expression ($GS \leq 7$ vs. $GS \geq 8$: $P = 0.0150$; GS7 vs. $GS \geq 8$: $P = 0.0111$). Kaplan–Meier and log-rank tests further revealed that patients with SgI-positive/SgII-negative tumor have the highest risk for biochemical recurrence ($P = 0.0242$).

CONCLUSIONS. These results suggest the involvement of semenogelins in prostate cancer and their prognostic values in predicting cancer progression after radical prostatectomy. Additional functional analyses of semenogelins are necessary to determine their biological significance in prostate cancer. *Prostate 71: 1108–1114, 2011.* © 2010 Wiley-Liss, Inc.

KEY WORDS: **seminal plasma proteins; immunohistochemistry; prostate-specific antigen; zinc; biochemical recurrence**

INTRODUCTION

Prostate cancer has been a leading cause of cancer-related death among men [1,2]. Although radical prostatectomy can offer the possibility of cure of localized prostate cancer, a substantial number of patients will develop recurrent disease following the surgery [3–5]. Clinical outcomes in prostate cancer have been strongly correlated with histopathological factors (e.g., Gleason grade, stage, surgical margin status) as well as numerous biomolecules [4,5]. Nonetheless, these markers remain insufficient to precisely predict the potential for recurrence or metastasis. Further controversy includes the selection of appropriate

Abbreviations: SgI, semenogelin I; SgII, semenogelin II; PSA, prostate-specific antigen; FBS, fetal bovine serum; RT, reverse transcription; PCR, polymerase chain reaction; TMA, tissue microarray; AR, androgen receptor; GS, Gleason score.

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patients who will benefit from hormonal therapy immediately after a definitive treatment [2,6].

Semenogelins, seminal plasma motility inhibitors predominantly secreted by the seminal vesicle, are the main structural components of human semen coagulum. Semenogelin I (SgI), a ~50 kDa protein, and semenogelin II (SgII), a ~63 kDa protein, are encoded by two homologous genes located 11.5 kb apart in the chromosome 20 q12–q13.1 regions, and as such, share 78% of their amino acid sequence [7–9]. Semen spontaneously coagulates upon ejaculation thereby trapping sperm. Semenogelins inhibit sperm motility; however, within minutes, the coagulum liquefies thus releasing motile sperm. This process is hastened by kallikrein-related peptidase 3, also known as prostate-specific antigen (PSA), which targets and degrades semenogelins into lower molecular mass (5–20 kDa) fragments [9–11]. Semenogelins regulate the activity of PSA in that they initiate their own degradation by chelating Zn^{2+}, which normally acts to inhibit the protease activity of PSA [12]. Thus, physiological functions of semenogelins in male reproductive organs, in conjunction with zinc and PSA, have been thoroughly studied.

In addition to expression in the seminal vesicle and other male genital organs, immunoreactivity to semenogelins has been demonstrated in non-genital organs, such as the trachea, salivary gland, pancreas, kidney, and retina [13,14], suggesting a physiological role of these proteins as modulators of zinc-dependent proteases throughout the body [10,12]. Semenogelins have also been detected in human malignancies, including lung carcinomas [15], melanoma [15], and leukemias [16]. Expression of semenogelins in genitourinary cancers has not been extensively studied, but Lundwall et al. [13] detected mRNAs of SgI and SgII in an androgen-sensitive prostate cancer cell line LNCaP. This study also demonstrated semenogelin immunoreactivity in a single case of human prostate cancer, using an antibody that recognizes both SgI and SgII. The purpose of this study was to further elucidate semenogelin expression and its potential role as a biomarker in prostate cancer.

MATERIALS AND METHODS

Cell Culture

The human prostatic adenocarcinoma cell lines, LNCaP, CWR22Rv1, PC-3, and DU145, were purchased from the American Type Culture Collection (Manassas, VA). The cells were maintained in RPMI 1640 medium (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS) and cultured in the presence of zinc chloride (Sigma, St. Louis, MO) for 48 hr prior to isolation of RNA or protein.

Reverse Transcription (RT)-Polymerase Chain Reaction (PCR)

Total RNA was extracted, using Trizol reagent (Invitrogen, Carlsbad, CA), from the human seminal vesicle specimen and the prostate cancer cell lines cultured with 100 μM zinc for 48 hr. Isolated RNA was reverse transcribed to cDNA, using Omniscript (Qiagen, Valencia, CA) with oligo-dT-primer. Subsequent PCR was performed, using Advantage 2 PCR kit (Clontech, Mountain View, CA), as previously described [13]. PCR conditions included an initial denaturation at 95°C for 1 min, 40 cycles consisting of 95°C for 30 sec and 68°C for 1 min, and an extra incubation at 68°C for 1 min. The following primers were used: SgI, 5′-GCAGACACCAACATGGATCT-CA-3′ and 3′-CTGAGGTCAACTGACACCTTGA-5′; SgII, 5′-AGCATGAGGTTGCCCAAGATGA-3′ and 3′-GAGGTCGGGTGACACCTTGC-5′; and GAPDH, 5′-CTCCTCCACCTTTGACGCTG-3′ and 3′-CATACC-AGGAAATGAGCTTGACAA-5′. Reaction products were analyzed by electrophoresis in a 2.5% agarose gel containing ethidium bromide.

Western Blotting

To detect semenogelin proteins in prostate cancer cells cultured with 100 μM zinc for 48 hr, Western blotting was performed, as described previously [17] with some modifications. Briefly, equal amounts of protein (50 μg) obtained from cell extracts were separated in 10% sodium dodecyl sulfate–polyacrylamide gels and transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) by electroblotting, using a standard protocol. The membranes were blocked with 5% nonfat milk in Tris-buffered saline (Bio-Rad) for 1 hr at room temperature and then immunoblotted with each primary antibody [SgI, diluted 1:1,000, Abcam (Cambridge, MA); SgII, diluted 1:1,000, Abcam; or β-actin, diluted 1:1,000, Santa Cruz Biotechnology (Santa Cruz, CA)] for 2 hr at room temperature, followed by incubation with respective secondary antibodies for 1 hr at room temperature. Antibody–protein complexes were visualized using an enhanced chemiluminescence kit (Thermo Scientific, Rockford, IL) and Kodak X-OMAT film (Eastman Kodak, Rochester, NY). As positive controls, CWR22Rv1 cells transfected with semenogelin expression plasmids (pSG5-SgI and pSG5-SgII by inserting full-length SgI and SgII, respectively), according to GeneJuice transfection instructions (Novagen, Gibbstown, NJ), were used.

Cell Proliferation Assay

We used the MTT (thiazolyl blue) assay to assess cell growth, as described previously [17] with minor modifications. Cells (3×10^3) seeded in 96-well tissue culture plates were first transfected with pSG5, pSG5-SgI, or pSG5-SgII, as described above, and then cultured in medium supplemented with 10% FBS in the presence or absence of 100 μM zinc. After 4 days of treatment, we added 10 μl of MTT (Sigma) stock solution (5 mg/ml) to each well with 0.1 ml of medium for 4 hr at 37°C. Then, we replaced the medium with 100 μl of DMSO, incubated for 5 min at room temperature, and measured the absorbance at a wavelength of 570 nm with background subtraction at 655 nm.

Tissue Samples and Immunohistochemical Staining

Appropriate approval from the Institutional Review Board of the University of Rochester Medical Center was obtained prior to construction and use of the tissue microarray (TMA). Prostate TMA was constructed from 70 formalin fixed paraffin embedded prostatectomy tissue specimens retrieved from the Surgical Pathology archives, using 1.0 mm cores of representative benign and tumor lesions. The mean age of the patients at presentation was 60.2 years (range: 42–78 years) and the mean follow-up after the surgery was 29.2 months. None of the patients had received therapy with hormonal reagents, radiation, or other anticancer drugs pre- or post-operatively prior to clinical or biochemical recurrence. Biochemical recurrence was defined as a single PSA level of ≥0.2 ng/ml. TMA sections (4 μm thick) were immunohistochemically labeled, using the same primary antibodies to SgI (diluted 1:1,000, Abcam) and SgII (diluted 1:1,000, Abcam) as utilized for Western blotting, performed on the automated staining system, as described previously [18]. Appropriate positive controls (human seminal vesicle tissue) were run concurrently. As a negative control, sections were treated in an identical fashion except for replacing the primary antibody with non-immune rabbit IgG. German Immunoreactive Score (0–12) was calculated, separately in benign and malignant glands, by multiplying the percentage of immunoreactive cells (0% = 0; 1–10% = 1; 11–50% = 2; 51–80% = 3; 81–100% = 4) by staining intensity (negative = 0; weak = 1; moderate = 2; strong = 3). Scores were considered negative (0–1), weakly positive (2–4), moderately positive (6–8), and strongly positive (9–12).

Statistical Analyses

Differences in cell growth in the two groups were analyzed by Student's *t*-test. Differences in semenogelin expression in prostate TMA were analyzed by Fisher's exact test or Chi-square test. Survival rates in patients were calculated by the Kaplan–Meier method and comparison was made by log-rank test. Multivariate analysis was then performed with the Cox proportional hazards regression model. *P* values less than 0.05 were considered to be statistically significant.

RESULTS

Expression in Prostate Cancer Cell Lines

We first examined the expression of SgI and SgII in human prostate cancer cells by RT-PCR. As depicted in Figure 1A, mRNA signals for SgI and SgII were detected in androgen receptor (AR)-positive LNCaP cells cultured in the presence of zinc. The levels of their expression were lower when no zinc was added in culture media (data not shown). No semenogelin transcripts were detected in other tested lines, including CWR22Rv1, PC-3, and DU145 cells. Western blot analysis was also performed to determine if these prostate cancer cell lines express semenogelin proteins.

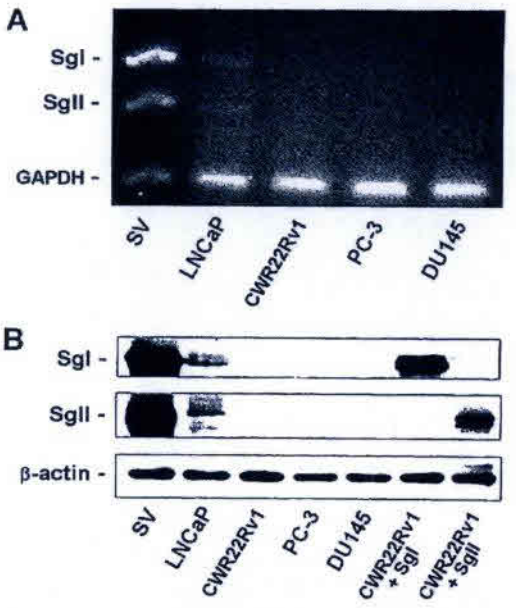

Fig. 1. Expression of SgI and SgII in human prostate cancer cell lines. **A**: Transcripts were amplified by RT-PCR and separated by electrophoresis for SgI (180 bp) and SgII (130 bp). Human seminal vesicle (SV) served as the positive control. PCR products derived from GAPDH mRNA (71 bp) served as the internal control. **B**: Cell extracts were analyzed on Western blots, using an antibody to SgI (upper), SgII (middle), or β-actin (lower). Human seminal vesicle (SV) and CWR22Rv1 cell line transfected with a semenogelin expression plasmid (pSG5-SgI or pSG5-SgII) served as the positive controls. The 52 kDa (for SgI) and 65 kDa (for SgII) proteins were detected, as indicated. β-Actin expression (43 kDa) served as the internal control.

Correlating with their mRNA expression, SgI and SgII proteins were detected only in LNCaP cells (Fig. 1B).

Effects on Prostate Cancer Cell Proliferation

We next performed the MTT assay to evaluate the effects of semenogelins on cell growth of prostate cancer lines. SgI or SgII was expressed in CWR22Rv1, PC-3, and DU145 that were then cultured for 4 days in the presence or absence of additional zinc. As expected, 100 μM of zinc significantly inhibited the growth of AR-positive CWR22Rv1 cells (Fig. 2; $P < 0.0001$). In the presence of zinc, transfection of SgI significantly increased the growth of CWR22Rv1 cells by 12% ($P = 0.0137$), whereas SgII overexpression decreased it by 14% ($P = 0.1696$). In contrast, SgI and SgII only marginally affected the growth of CWR22Rv1 cells in the absence of additional zinc. In addition, overexpression of semenogelins, with or without zinc, showed marginal effects (i.e., <5%) on the growth of AR-negative PC-3 and DU145 cells, although zinc significantly inhibited the growth of these cells (figure not shown).

Immunoreactivity in Prostate Cancer Tissue Samples

We then performed immunohistochemical stains for SgI and SgII in 70 radical prostatectomy specimens. Positive signals were detected predominantly in nuclei of epithelial cells (Fig. 3). Cytoplasmic or luminal staining is also seen in some cases of carcinoma glands. The results of semenogelin expression in tissue samples are summarized in Table I.

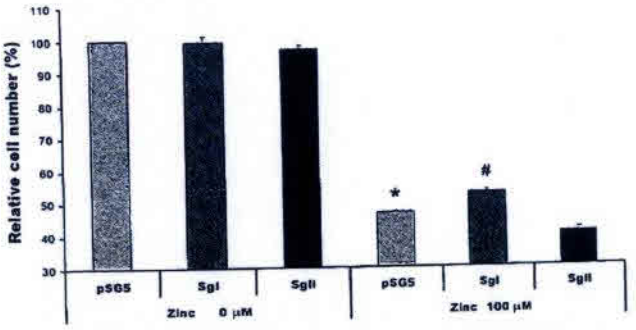

Fig. 2. Cell growth of the prostate cancer line overexpressing SgI or SgII. CWR22Rv1 cells transfected with either pSG5 (vector only), pSG5-SgI, or pSG5-SgII were cultured in medium supplemented with 10% FBS in the presence or absence of 100 μM zinc for 4 days, as indicated. Proliferation was assayed with MTT, and growth induction or suppression is presented relative to cell number with pSG5 vector transfection/no zinc treatment (first lane; set as 100%). Each value represents the mean + SD of at least three determinations. *$P < 0.0001$ (vs. pSG5 vector transfection/0 μM zinc). #$P = 0.0137$ (vs. pSG5 vector transfection/100 μM zinc).

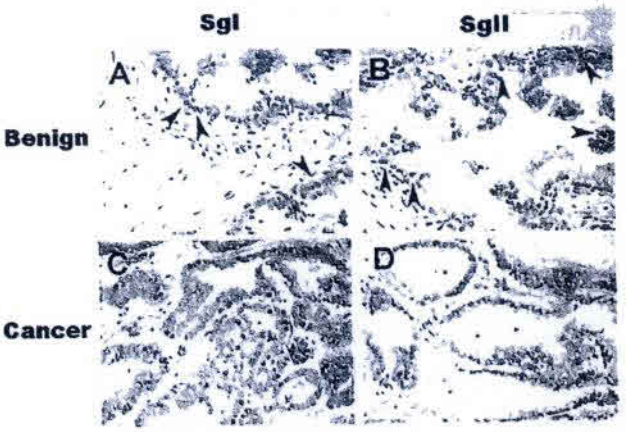

Fig. 3. Immunohistochemistry of SgI and SgII in benign and malignant prostate tissue. Occasional positive signals (arrowheads) of SgI (**A**) or SgII (**B**) were observed in benign epithelial cells but stronger, more diffuse staining of SgI (**C**) and SgII (**D**) was seen in carcinoma cells. Original magnification 200×.

Overall, SgI was positive in 13 (19%; all weak) benign tissues and in 55 (79%; 53 weak and 2 moderate) cancer tissues. Similarly, SgII was positive in 15 (21%; all weak) benign tissues and in 31 (44%; all weak) cancer tissues. Thus, the expression of SgI ($P < 0.0001$) or SgII ($P = 0.0066$) was significantly higher in carcinoma cells than in non-malignant epithelial cells. Higher scores of SgI and SgII were seen in two and nine cases, respectively, in benign glands, compared to cancer glands. Seventy cases included 28 SgI-positive/SgII-positive tumors, 27 SgI-positive/SgII-negative tumors, 3 SgI-negative/SgII-positive tumors, and 12 SgI-negative/SgII-negative tumors. Associations between SgI expression and SgII expression in prostate cancer were statistically significant ($P = 0.0234$). Among 55 SgI-positive cases, 28 (51%) were also SgII-positive, whereas 28 (90%) of 31 SgII-positive cases were SgI-positive.

Semenogelin Expression and Clinicopathologic Features

We evaluated the associations between the expression of semenogelin and histopathological features available for our patient cohort. No significant correlation between SgI expression and Gleason score (GS) was observed. In contrast, it was noted that SgII expression negatively correlates with GS (≤7 vs. ≥8: $P = 0.0150$; 7 vs. ≥8: $P = 0.0111$; ≤6 vs. ≥8: $P = 0.0674$; ≤6 vs. ≥7: $P = 0.8095$). There were no statistically significant correlations between staining and other histopathological parameters analyzed. In 14 patients with extraprostatic extension, SgI and SgII were positive in 12 (86%; all weak) and 7 (50%; all weak)

TABLE I. Expression of SgI and SgII in 70 Tumor and Benign Prostate Tissue Microarrays

	SgI				SgII			
	Negative (0–1)	Weak (2–4)	Moderate/ strong (6–12)		Negative (0–1)	Weak (2–4)	Moderate/ strong (6–12)	
Cancer (n = 70)	15	53	2	$P < 0.0001$	39	31	0	$P = 0.0066$
Benign (n = 70)	57	13	0		55	15	0	
Gleason score				$P > 0.10$				$P < 0.05$
≤6 (n = 26)	4	20	2		15	11	0	
7 (n = 37)	10	27	0		17	20	0	
≥8 (n = 7)	1	6	0		7	0	0	
Extraprostatic extension (pT3a)				$P > 0.10$				$P > 0.10$
Negative (n = 56)	13	41	2		33	23	0	
Positive (n = 14)	2	12	0		7	7	0	
Seminal vesicle involvement (pT3b)				$P > 0.10$				$P > 0.10$
Negative (n = 66)	14	50	2		36	30	0	
Positive (n = 4)	1	3	0		3	1	0	
Surgical margin status				$P > 0.10$				$P > 0.10$
Negative (n = 61)	13	46	2		33	28	0	
Positive (n = 9)	2	7	0		6	3	0	
Lymph node metastases				$P > 0.10$				$P > 0.10$
Negative (n = 45)	8	37	0		22	23	0	
Positive (n = 2)	0	2	0		0	2	0	

tumors, respectively. In four patients with seminal vesicle involvement, SgI and SgII were positive in three (75%; all weak) and one (25%; weak) tumors, respectively. In nine patients with positive surgical margins, SgI and SgII were positive in seven (78%; all weak) and three (33%; all weak) tumors, respectively. Additionally, both SgI and SgII were weakly positive in two patients with lymph node metastases (out of 47 cases with pelvic lymph node dissection).

To assess possible associations between semenogelin staining and disease recurrence, we then performed Kaplan–Meier analysis coupled with log-rank test. Of the 70 patients with a mean follow-up of 29.2 months, 6 (8.6%) had a clinical or biochemical recurrence after radical prostatectomy. Of these, five (83%) cases exhibited GS ≥ 8, extraprostatic extension, seminal vesicle involvement, positive surgical margins, and/or lymph node metastasis. SgI alone (Fig. 4A; $P = 0.5409$) or SgII alone (Fig. 4B; $P = 0.2378$) showed no strong correlation with recurrence. Nonetheless, there were trends to weakly associate between SgI positivity or SgII negativity and a risk of recurrence. Interestingly, patients with SgI-positive/SgII-negative tumor had a significantly higher risk of recurrence (Fig. 4C), compared to those with SgI-positive/SgII-positive or SgI-negative tumor ($P = 0.0242$), SgI-positive/SgII-positive tumor ($P = 0.1087$), or SgI-negative tumor ($P = 0.2102$). Multivariate analysis revealed that semenogelin

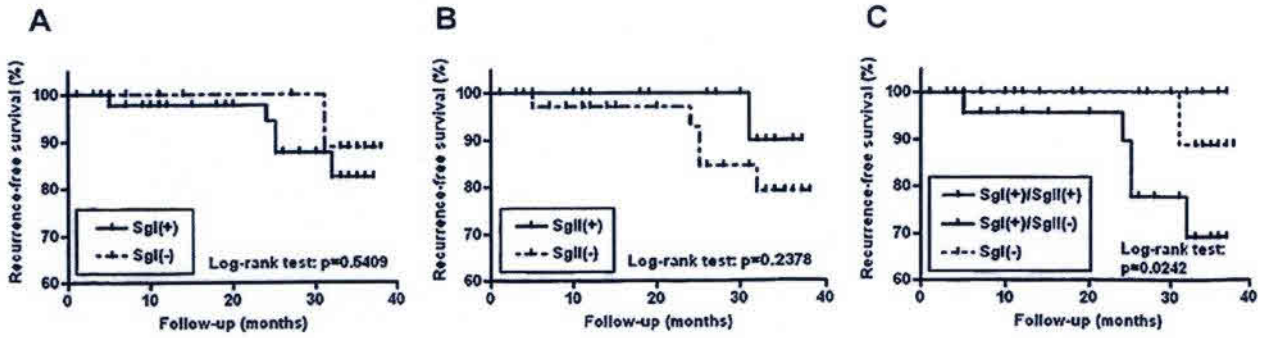

Fig. 4. Kaplan–Meier analysis of recurrence-free survival according to SgI expression (**A**), SgII expression (**B**), or both (**C**). Biochemical recurrence was defined as a single PSA level of ≥0.2 ng/ml.

expression, as well as each analyzed variable, is not an independent prognostic factor ($P > 0.05$) in our cohort.

DISCUSSION

Compared with well-recognized physiological functions in male reproductive system [7–13], the role of semenogelins in human malignancies is poorly understood. To our knowledge, there have been only a few studies published, showing the expression of semenogelins in lung carcinomas [15], melanoma [15], and leukemias [16]. In prostate cancer, another study demonstrated the expression of SgI and SgII in an androgen-sensitive cell line LNCaP, but not in androgen-insensitive cell lines DU145 (with a faint SgII signal) and PC-3, by RT-PCR, as well as in a single case of tissue specimen by immunohistochemistry [13]. In the current study, we confirmed these findings in prostate cancer cell lines both in mRNA and protein levels. We additionally showed that another AR-positive prostate cancer CWR22Rv1 cells are negative for SgI and SgII. Interestingly, the addition of zinc in culture medium increases mRNA expression of SgI and SgII in LNCaP cells. The prostate contains the highest level of zinc of any soft tissue and its concentrations in prostate cancer, although a significant decrease is seen, remain much higher than those in other tissue or blood plasma [19,20]. Nonetheless, experimental evidence suggests that high zinc levels prevent prostate carcinogenesis, although it is controversial whether zinc supplements indeed decrease the risk of prostate cancer [20–22]. Furthermore, zinc shows an inhibitory effect on cell growth of prostate cancer [23,24]. Thus, zinc may exhibit contradictory effects on prostate cancer. Semenogelins have been known to bind Zn^{2+}, abundant in semen, to function as a regulator of PSA activity [12]. Accordingly, functional analysis of semenogelins in prostate cancer may provide not only an explanation for the conflicting results on zinc and prostate cancer but also potential therapeutic targets.

A pilot experiment was performed to see if semenogelins affect prostate cancer cell proliferation. Semenogelins showed marginal effects on the growth of AR-negative/semenogelin-negative PC-3 and DU145 cells in the presence or absence of additional zinc. Remarkably, co-expression of SgI and SgII resulted in an increase and a decrease, respectively, in the growth of AR-positive/semenogelin-negative CWR22Rv1 cells only in the presence of a high level of zinc. These results suggest that semenogelins may require both zinc and AR to function as modulators of prostate cancer cell proliferation. In these assays, however, we used a transient transfection method to co-express respective semenogelins, and generally low transfection efficiency might have obscured the effect of semenogelins on cell growth. As discussed above [23,24], zinc was confirmed to inhibit the growth of all the three prostate cancer lines examined. Further studies of semenogelins, in conjunction with zinc, androgen, AR, and PSA, each of which is deeply involved in prostate carcinogenesis and cancer progression, remain necessary.

As noted, Lundwall et al. [13] included only one case of prostate cancer in their immunohistochemical analysis. This is the first study that extensively elucidates semenogelin immunoreactivity in prostate cancer. There were highly significant differences in the expression of SgI and SgII between carcinoma and corresponding benign tissues. Most of SgII-positive tumors were SgI-positive, whereas roughly half of SgI-positive tumors were SgII-positive. Thus, strong correlations between expressions of SgI and SgII in prostate cancer were observed. These results suggest the involvement of semenogelins in prostate cancer development.

Among the histopathological parameters available for our patient cohort, GS showed an inverse association with SgII expression, but not with SgI expression. There were no significant associations between semenogelin expression and other parameters, including extraprostatic extension, seminal vesicle invasion, surgical margin status, and lymph node metastasis. The present study also analyzed and compared the prognostic value of semenogelin expression, using Kaplan–Meier survival curves and log-rank test. The expression status of either SgI alone or SgII alone did not strongly correlate with recurrence. It is noteworthy that a combination of semenogelins showed statistically significant differences: patients with SgI-positive/SgII-negative tumor had the highest risk of recurrence. However, the significance of this result may need to be taken into consideration with low recurrence rate ($n = 6$), possibly due to the relatively short follow-up duration (mean of 29.2 months) in our cohort of radical prostatectomy patients. This data, along with the results of our cell proliferation assay, suggests that SgI may promote prostate cancer progression and SgII may protect against it. In spite of numerous attempts, no reliable biomarkers for accurate prediction of prostate cancer recurrence besides preoperative PSA value and GS have been identified [4,5]. SgI and SgII could be such markers, yet further combinations with other potential markers may lead to identifying more independent prognostic predictors.

CONCLUSIONS

The expression and prognostic significance of SgI and SgII in prostate cancer were investigated. Our results may indicate that both SgI and SgII contribute to

prostate cancer development. Moreover, SgI and SgII are suggested to have contradictory effects on prostate cancer progression. Further studies including larger patient cohorts with longer follow-up are needed to validate these initial results. Additional functional analyses of semenogelins in prostate cancer are also necessary to determine their biological significance.

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