Zinc-alpha2-glycoprotein Expression as a Predictor of Metastatic Prostate Cancer Following Radical Prostatectomy

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The risk of metastatic progression for prostate cancer patients who undergo radical prostatectomy is best estimated presently based on prostate-specific antigen (PSA) doubling time (PSADT). However, additional markers of risk are needed to identify patients who may benefit from aggressive salvage treatment. A decrease in zinc-alpha2-glycoprotein (AZGP1) mRNA levels in malignant prostate epithelium was previously shown to predict biochemical recurrence, as defined by rising levels of serum PSA after radical prostatectomy. We assessed the reliability with which AZPG1 expression could predict clinical recurrence and metastatic progression. Using immunohistochemical methods, we analyzed AZPG1 expression in malignant prostate epithelium in prostatectomy specimens from 228 prostate cancer patients. Low (i.e., absent or weak) AZGP1 expression was associated with clinical recurrence (defined as confirmed localized recurrence, metastasis, or death from prostate cancer; hazard ratio [HR] = 4.8, 95% confidence interval [CI] = 2.2 to 10.7, *P*<.001) and with bony metastases or death from prostate cancer (HR = 8.0, 95% CI = 2.6 to 24.3, *P*<.001). Among the 17 patients in the cohort in whom clinical recurrence was associated with short PSADT, absent or weak AZGP1 expression was observed in 13 patients. If these preliminary findings are validated in independent cohorts, the measurement of AZGP1 levels in radical prostatectomy specimens may permit an accurate and timely assessment of risk of metastatic progression after radical prostatectomy. [J Natl Cancer Inst 2006;98:1420–4]

Although many published studies have assessed the performance of candidate biomarkers in predicting time to relapse of prostate cancer following radical prostatectomy [reviewed in *(1)*], no molecular markers suitable for routine clinical practice that can identify those prostate cancer patients with a high risk of early clinical progression or prostate cancer–specific mortality have been found. Of the prognostic factors in current use for prostate cancer, prostate-specific antigen doubling time (PSADT), a measure of the rate of increase in serum prostate-specific antigen (PSA) levels, is the best predictor of both progression-free and cancer-specific survival following radical prostatectomy *(2–4)*. The identification of more reliable markers of clinical prostate cancer progression would assist clinicians in selecting patients who might benefit from earlier or more aggressive systemic treatment and give patients with a low risk of clinical progression the option of deferring treatments that have a negative impact on quality of life.

To identify clinically useful prognostic biomarkers of prostate cancer progression after radical prostatectomy, we performed a meta-analysis using a variation of Stouffer's method *(5,6)* of the three published gene expression datasets (including one from our group) that combined gene expression data derived from primary prostate cancer tissues with outcome data *(7–9)*. The goal of the meta-analysis was to identify expression signatures as well as individual genes that were common to all three datasets. Preliminary data (not shown) showed a strong association betweeen decreased levels of the mRNA encoding zinc-alpha2-glycoprotein (AZGP1 or ZAG) and biochemical recurrence in all three datasets. AZGP1 is a soluble 41-kDa protein present in most body fluids and in the secretory epithelial cells of many tissues, including those of the breast and prostate *(10)*. Absent or weak AZGP1 expression in malignant prostate epithelium has been associated with a higher Gleason score *(11)*. Absent or reduced AZGP1 mRNA or protein expression (relative to that in normal cells) has also been reported to be associated with a shorter time to recurrence after radical prostatectomy (recurrence was defined as the presence of either a rising PSA value after surgery or clinical metastases) *(9)*.

To determine whether reduced AZGP1 expression is a useful prognostic biomarker for prostate cancer, we tested its association with clinical recurrence, progression to metastatic disease, and prostate cancer–specific survival in a cohort of prostate cancer patients who were treated by radical prostatectomy for localized prostate cancer at St Vincent's Hospital, Sydney, Australia (Human Research Ethics Committee Approval H00/088). We previously studied 732 men with localized prostate cancer treated with radical prostatectomy and no preoperative therapy between 1986 and 1999 at our institution to determine the association of clinicopathologic features with disease-free survival *(12)*. The subgroup of patients assessed for AZGP1 expression in the current study are those patients of the 732 for which full clinicopathologic features and outcome data were available, and for which tissue blocks could be accessed from the radical prostatectomy specimens for use in tissue microarrays. There were no other selection criteria. This group of patients had radical prostatectomy performed between 1988 and 1998 (median of 1995). The median preoperative serum PSA level was 9.4 ng/mL (range = 1–280 ng/mL). The mean pathologic stage was pT2C (range = pT2A to pT4A). The mean Gleason score in the radical prostatectomy specimen was 6 (range = 3–10).

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Representative formalin-fixed, paraffin-embedded blocks of radical prostatectomy specimens were obtained from the archives of the Department of Anatomical Pathology at St Vincent's Hospital, Douglass Hanly Moir Pathology (Sydney, Australia), and Sugerman Hampson Macquarie Pathology (Sydney, Australia). Each patient was represented by a mean of three 1-mm tissue core biopsies taken from each of the 228 paraffin-embedded specimens in tissue microarrays. Mean and median follow-up times after surgery among the 228 patients were 89.4 and 88.9 months, respectively. Ninety-nine (43%) of the 228 patients developed biochemical recurrence, defined as a serum PSA concentration of ≥0.2 ng/mL after surgery followed by consecutive, further rises *(3)*. Mean and median times from radical prostatectomy to biochemical recurrence were 31.8 and 23.8 months, respectively.

AZGP1 expression in malignant prostate epithelium in tissue taken from formalin-fixed, paraffin-embedded radical prostatectomy specimens was measured by immunostaining using a commercially available goat polyclonal anti-human AZGP1 antibody (1 : 250 dilution; *sc-11238*, Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactivity was revealed by incubating the tissue microarray sections with biotinylated secondary anti-goat antibodies (1 : 200; Vector Laboratories, Burlingame, CA) followed by detection with Vector ABC Elite (Vector Laboratories). Expression was visualized using 3,3′-diaminobenzidine (DAB+, DAKO, Carpinteria, CA) as substrate and counterstained with Shandon's hematoxylin solution. Immunostaining was performed by using an automated platform (DAKO Autostainer, DAKO). Each core biopsy in the tissue microarrays was scored separately, and the highest intensity score present for an individual patient was recorded as the final score. The intensity of immunostaining in malignant prostate epithelium was assessed by a histopathologist (JGK) who was blinded to patient outcome. Staining intensity and hence AZGP1 expression were stratified in the categories of absent to weak staining (Fig. 1, A and B) and strong staining (2+ or 3+ staining intensity [Fig. 1, C and D]) as described previously *(9)*.

Variables were evaluated as predictors of disease relapse using the Kaplan–Meier method *(13)* and log-rank test and by univariate and multivariable analyses in Cox proportional hazards models *(14)*. The variables studied were Gleason score, preoperative serum PSA level, pathologic stage, and PSADT. All proportional hazards assumptions were verified graphically. All *P* values corresponded to two-sided tests, and $P<.05$ was considered statistically significant. No adjustments were made for multiple testing. Pathologic stage was entered as a categorical variable and stratified by pT2 versus pT3 and above. Preoperative PSA level was entered as a continuous variable. Gleason score was entered as a categorical variable and stratified as Gleason score 8–10 versus Gleason score ≤7 or Gleason score 7–10 versus Gleason score ≤6, as identified for each analysis. PSADT in months was calculated from a minimum of two PSA values and estimated by extrapolation if the interval between the first and last PSA measurement was less than 12 months as described previously *(15)*. Recurrence was defined as one of three types: biochemical recurrence, defined as a serum PSA concentration of ≥0.2 ng/mL after surgery followed by consecutive, further rises *(3)* (n = 99); clinical recurrence (total n = 29), defined as confirmed local recurrence (n = 9), bony metastasis (n = 11), or death from prostate cancer (n = 9); or distant metastatic progression (total n = 20), defined as bony metastasis (n = 11) or death from prostate cancer (n = 9). For clarity, these definitions are used consistently below.

The initial goal of this study was to confirm the association of AZGP1 expression with biochemical recurrence. Absent (n = 15) or weak (n = 51) AZGP1 expression in malignant prostate epithelium, as indicated by immunohistochemical analysis of radical prostatectomy specimens, was observed in 66 of the 228 patients (28.9%) and was an independent predictor of biochemical recurrence in a Cox proportional hazards analysis ($P<.001$, data not shown). The association of weak or absent AZGP1 expression with biochemical recurrence persisted after adjustment for Gleason grade ([Gleason score 7–10 versus Gleason score ≤6] $P = .04$), preoperative PSA ($P<.001$), and pathologic stage ($P = .0018$). These results are in agreement with the findings of Lapointe et al. *(9)* and add further support to the idea that absent or weak AZGP1 expression is an independent predictor of biochemical recurrence *(9)*.

Of the 99 patients who experienced a biochemical recurrence, only 29 (29%) subsequently developed a clinical recurrence within 5 years. The median time to clinical recurrence after biochemical recurrence was 52.9 months. We initially sought to measure the association of absent or weak AZGP1 expression with early clinical recurrence in our group of 228 patients. Kaplan–Meier estimates of cumulative clinical recurrence–free survival, in which recurrence is defined as evidence of local recurrence, distant metastases, or death, were derived for prostate cancer patients whose radical prostatectomy specimens exhibited strong AZGP1 staining versus those whose specimens showed absent or weak staining (Fig. 1, E). Absent or weak AZGP1 expression, compared with strong staining, was strongly associated with clinical recurrence (hazard ratio [HR] = 4.8, 95% confidence interval [CI] = 2.2 to 10.7, $P<.001$) (Table 1). We performed similar statistical analyses for the outcome of distant metastatic progression. Despite the low number (n = 20) of patients experiencing this outcome, weak or absent AZGP1 expression was nevertheless associated with a higher risk of distant metastatic progression (HR = 8.0, 95% CI = 2.6 to 24.3, $P<.001$; Fig. 1, F).

The assessment of the risk of clinical metastatic progression of prostate cancer after prostatectomy is best predicted currently by PSADT following biochemical recurrence. Hence, we believed it was important to evaluate any potential relationship between a rapid PSADT and AZGP1 expression in prostatectomy specimens. Thus, we examined the relationships among absent or weak AZGP1 and PSADT and the risk of clinical recurrence. Because PSADT was not used in the clinical management of patients in the cohort to identify those at risk of clinical recurrence, PSADT data were available for only 68 of the 99 men who experienced biochemical recurrence. Analysis of Kaplan–Meier estimates of clinical recurrence–free survival (Fig. 1, G) showed that AZGP1 expression was also statistically significantly associated with this outcome in the subgroup of patients with PSADT data ($P = .009$, log-rank test). We divided patients into two groups according to whether PSADT was less than 12 months or greater than or equal to 12 months based on a published nomogram that was devised to predict

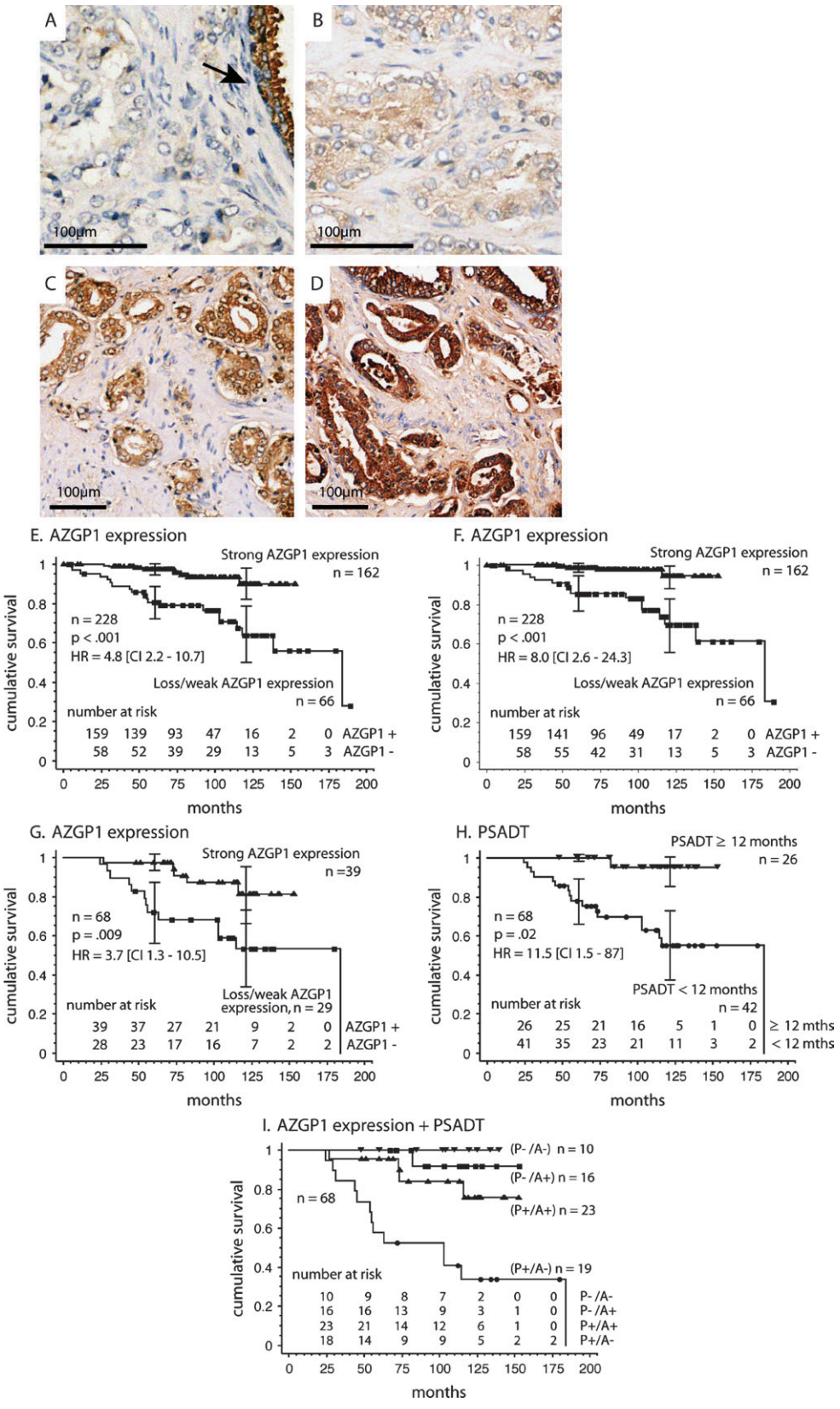

Fig. 1. Association of AZGP1 with prostate cancer outcome. (**Upper panel**) Photomicrographs of paraffin-embedded prostate tissue obtained during radical prostatectomy immunostained for AZGP1 showing the spectrum of staining intensities observed in this study: **A**) Gleason grade 4 prostate cancer showing complete loss of AZGP1 immunostaining. The **arrow** indicates strong AZGP1 immunostaining in the adjacent benign prostate epithelium; **B**) Gleason grade 3 prostate cancer showing weak immunostaining; **C**) Gleason grade 3 prostate cancer showing 2+ intensity immunostaining; and **D**) Gleason grade 3 prostate cancer showing 3+ intensity immunostaining. Original magnification ×200. (**Lower panel**) Kaplan–Meier survival curves showing recurrence-free survival. **E**) Clinical recurrence (confirmed local recurrence, bony metastasis, or death)–free survival in patients stratified by AZGP1 expression (absent or weak, designated as AZGP1–, versus strong [i.e., 2+ or 3+], designated as AZGP1+). Number of patients at risk and 95% confidence intervals (CIs) for the relapse-free survival estimates (shown as **error bars**) are indicated at 5 years ([AZGP1−, 0.73 to 0.92] and [AZGP1+, 0.95 to 1.0]) and 10 years ([AZGP1−, 0.50 to 0.80] and [AZGP1+, 0.83 to 0.98]). **F**) Distant metastatic progression (bony metastases or death from prostate cancer)–free survival in patients stratified for AZGP1 expression (absent or weak versus strong). Number of patients at risk and 95% confidence interval for the relapse-free survival estimates (shown as **error bars**) are indicated at 5 years ([AZGP1−, 0.77 to 0.94] and [AZGP1+, 0.97 to 1.0]) and 10 years ([AZGP1−, 0.55 to 0.84] and [AZGP1+, 0.88 to 1.0]). **G**) Clinical recurrence (confirmed local recurrence, bony metastasis, or death)–free survival in patients for whom prostate-specific antigen doubling time (PSADT) data was available stratified for AZGP1 expression (absent or weak versus strong). Number of patients at risk and 95% CI for the relapse-free survival estimates (shown as **error bars**) are indicated at 5 years ([AZGP1-, 0.55 to 0.84] and [AZGP1+, 0.93 to 1.0]) and 10 years ([AZGP1-, 0.33 to 0.74] and [AZGP1+, 0.66 to 0.97]). **H**) Clinical recurrence (confirmed local recurrence, bony metastasis, or death)–free survival in patients for whom PSADT data were available stratified by PSADT. Number of patients at risk and 95% confidence interval for the relapse-free survival estimates (shown as **error bars**) are indicated at 5 years ([<12 months, 0.66 to 0.91] and [≥12 months, 1.0 to 1.0]) and 10 years ([<12 months, 0.38 to 0.73] and [≥12 months, 0.85 to 1.0]). **I**) Clinical recurrence (confirmed local recurrence, bony metastasis, or death)–free survival in patients for whom PSADT data were available stratified by both PSADT and AZGP1 expression (P+, PSADT < 12 months; P−, PSADT ≥ 12 months; A−, absent or weak AZGP expression; A+, strong AZGP1 expression).

patient-specific probabilities of metastasis-free survival at 1 and 2 years after local therapy with PSA rise as the only evidence of disease *(4)*. This model includes PSADT as a predictor of metastatic progression, with an incremental increase in risk of metastatic progression associated with a decreasing PSADT from 12 to 0 months. *(4)*. Analysis of

Table 1. Cox proportional hazards analyses of the association of AZGP1 immunostaining, Gleason score, and PSADT with clinical recurrence after radical prostatectomy*

	Univariate analyses		Multivariable analysis	
Risk factor for recurrence†	HR (95% CI)	*P* value‡	HR (95% CI)	*P* value‡
Gleason score§ (8–10 versus ≤7)	1.34 (0.8 to 3.2)	.51	0.62 (0.2 to 2.2)	.46
PSADT‖ (<12 months versus ≥12 months)	11.5 (1.5 to 86.9)	.02	11.98 (1.6 to 90.6)	.02
Absent or weak AZGP1¶	4.8 (2.2 to 10.7)	<.001	3.3 (1.2 to 9.5)	.03

*HR = hazard ratio, CI = confidence interval, PSADT = prostate-specific antigen doubling time, AZGP1 = zinc-alpha2-glycoprotein.

†Recurrence was defined as the presence of confirmed local recurrence, bony metastases, or death from prostate cancer.

‡Calculated from a Cox proportional hazards model.

§Gleason score was entered as a categorical variable and stratified as Gleason score 8–10 versus Gleason score ≤7.

‖PSADT was modeled as a categorical variable and stratified as PSADT <12 months versus ≥12 months.

¶AZGP1 staining was entered as a categorical variable and stratified as absent or weak versus strong immunostaining.

Kaplan–Meier estimates (Fig. 1, H) of clinical recurrence–free survival in patients stratified according to this cut point indicated that PSADT was associated with clinical recurrence (P = .02, log-rank test).

In Cox proportional hazards models constrained by the lower number of subjects with an available PSADT, PSADT was an independent predictor of clinical recurrence in univariate analyses (P = .02) and in multivariable models incorporating AZGP1 expression and Gleason score (P = .02) (Table 1). Among the 68 patients for whom PSADT was available, absent or weak AZGP1 expression was present in 13 of the 17 subjects who had short PSADT–associated clinical recurrences (one recurrence was experienced by a patient with a long PSADT). Importantly, patients with both short PSADT and absent or weak AZGP1 expression (n = 19) constituted a poorer prognosis group (Fig. 1, I) compared with those patients with high AZGP1 expression and a rapid PSADT (n = 23), those with high AZGP1 expression and a slow PSADT (n = 16), and those with a slow PSADT and absent or weak AZGP1 (n = 10). The association of weak or absent AZGP1 expression and clinical recurrence was of borderline statistical significance in a multivariable analysis (HR = 3.3, 95% CI = 1.2 to 9.5, P = .03) reflecting some lack of concordance between levels of AZGP1 expression and PSADT.

Although the function of AZGP1 in prostate cancer is unknown, AZGP1 RNA expression is increased in the prostate cancer cell line LNCaP and the breast cancer cell line T-47D in the presence of androgen *(16,17)*. Hence, loss of AZGP1 expression may be associated with the progression to an aggressive, androgen-independent phenotype.

Our data provide independent validation of the association between the loss of AZGP1 expression and biochemical recurrence of prostate cancer and support a link between absent or weak AZGP1 expression in radical prostatectomy specimens and clinical recurrence after prostatectomy. Given that only one-third of prostate cancer patients progress from biochemical recurrence to clinical disease, the decision to expose patients to the morbidity of therapy should be considered carefully in the context of the risk of metastatic progression. If these data are validated, clinical assessment of AZGP1 expression immediately following radical prostatectomy has the potential to identify patients at high risk of metastatic progression and may assist in the selection of patients for more aggressive treatment trials.

A limitation of this study is the relatively small number of clinical events in the patient group. Also, the lack of PSADT data for all 99 patients who experienced a biochemical recurrence is a disadvantage. Hence, the association between AZGP1 expression and prostate cancer progression must now be tested and validated for locally advanced prostate cancer in independent cohorts, including high-risk cohorts such as the one described by Daneshmand et al. *(18)*, as well as in clinical trial settings. Nonetheless, the data reported here support the further testing of a simple automated immunohistochemical assay for detecting AZGP1 levels that could eventually be used in the clinic to improve the prediction of metastatic spread of prostate cancer. The prognostic value that AZPG1 expression adds to that provided by PSADT alone is that AZGP1 levels can be measured in the radical prostatectomy specimen significantly sooner than a rising PSA can be detected, thereby providing timely information to guide adjuvant intervention.

References

(1) Quinn DI, Henshall S, Sutherland RL. Molecular markers of prostate cancer outcome. Eur J Cancer 2005;41:858–87.

(2) D'Amico A, Moul JW, Carroll P, Sun L, Lubeck D, Chen M-H. Surrogate end point for prostate cancer-specific mortality after radical prostatectomy or radiation therapy. J Natl Cancer Inst 2003;95:1376–83.

(3) Freedland S, Humphreys E, Mangold L, Eisenberger M, Dorey F, Walsh P, et al. Risk of prostate cancer-specific mortality following biochemical recurrence after radical prostatectomy. JAMA 2005;294:433–9.

(4) Slovin S, Wilton A, Heller G, Scher H. Time to detectable metastatic disease in patients with rising prostate-specific antigen values following surgery or radiation therapy. Clin Cancer Res 2005;11:8669–73.

(5) Stouffer S, Suchman E, DeVinney L, Star S, Williams RM Jr. The American soldier: adjustment during army life. Princeton (NJ): Princeton University Press; 1949.

(6) Becker B. Combining significance levels. In: Cooper H, Hedges L, editors. The handbook of research synthesis. New York (NY): Russell Sage Foundation; 1994. pp. 215–30.

(7) Singh D, Febbo PG, Ross K, Jackson DG, Manola J, Ladd C, et al. Gene expression correlates of clinical prostate cancer behavior. Cancer Cell 2002;1:203–9.

(8) Henshall SM, Afar DE, Hiller J, Horvath LG, Quinn DI, Rasiah KK, et al. Survival analysis of genome-wide gene expression profiles of prostate cancers identifies new prognostic targets of disease relapse. Cancer Res 2003;63:4196–203.

(9) Lapointe J, Lid C, Higgins J, van de Rijna M, Baire E, Montgomerya K, et al. Gene expression profiling identifies clinically relevant subtypes of prostate cancer. Proc Natl Acad Sci U S A 2004;101:811–6.

(10) Sanchez L, Lopez-Otin C, Bjorkman P. Biochemical characterization and crystalization of human Zn-alpha2-glycoprotein, a soluble class I major histocompatibility complex homolog. Proc Natl Acad Sci U S A 1997;94:4626–30.

(11) Hale L, Price D, Sanchez L, Demark-Wahnefried W, Madden J. Zinc alpha-2-glycoprotein is

expressed by malignant prostatic epithelium and may serve as a potential serum marker for prostate cancer. Clin Cancer Res 2001;7:846–53.

(12) Quinn DI, Henshall SM, Haynes A-M, Brenner PC, Kooner R, Golovsky D, et al. Prognostic significance of pathological features in localized prostate cancer treated with radical prostatectomy: implications for staging systems and predictive models. J Clin Oncol 2001;19:3692–705.

(13) Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. J Am Stat Assoc 1958;53:457–81.

(14) Cox DR. Regression models and life tables (life tables). J R Stat Soc 1972;34:187–9.

(15) Cannon G, Walsh P, Partin A, Pound C. Prostate-specific antigen doubling time in the identification of patients at risk for progression after treatment and biochemical recurrence for prostate cancer. J Urol 2003;62:2–8.

(16) Nelson P, Clegg N, Arnold H, Ferguson C, Bonham M, White J, et al. The program of androgen-responsive genes in neoplastic prostate epithelium. Proc Natl Acad Sci U S A 2002;99:11890–5.

(17) Chalbos D, Haagensen D, Parish T, Rochefort H. Identification and androgen regulation of two proteins released by T47D human breast cancer cells. Cancer Res 1987;47:2787–92.

(18) Daneshmand S, Quek M, Stein J, Liekovsky G, Cai J, Pinski J, et al. Prognosis of patients with lymph node positive prostate cancer following radical prostatectomy: long-term results. J Urol 2004;172:2252–5.

Notes

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