Low AZGP1 Expression Predicts for Recurrence in Margin-Positive, Localized Prostate Cancer

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BACKGROUND. Men with positive margins after radical prostatectomy (RP) for localized prostate cancer (PC) have a 40–50% biochemical relapse rate at 5 years. Adjuvant radiotherapy improves biochemical progression-free and overall survival in men with positive margins, but is associated with increased toxicity. There is an urgent need to identify new prognostic markers to define the group of patients who would benefit from multimodality therapy.

METHODS. Nuclear β-catenin, membranous secreted frizzled-related protein 4 (sFRP4), zinc-alpha 2-glycoprotein (AZGP1), and macrophage inhibitory cytokine-1 (MIC-1) have previously been identified as molecular markers of outcome in localized PC. From these published studies, we identified a subset of patients with positive margins. The aim of this study was to assess the association between these four molecular markers and outcome in men with margin-positive, localized PC.

RESULTS. We identified 186 men with positive margins from 330 men with localized PC; 53% had preoperative PSA >10 ng/ml, 72% extraprostatic extension (EPE), 24% seminal vesicles involvement (SVI), and 57% RP Gleason score ≥ 7. AZGP1 ($P = 0.009$), membranous sFRP4 ($P = 0.03$) and MIC-1 ($P = 0.04$) expression predicted for biochemical relapse on univariate analysis. Only absent/low AZGP1 expression ($P = 0.01$) was an independent predictor of recurrence in margin-positive, localized PC when modeled with preoperative PSA ($P = 0.2$), EPE ($P = 0.2$), SVI ($P = 0.4$), Gleason score ≥ 7 ($P = 0.5$) and adjuvant treatment ($P = 0.4$). Furthermore, there was an association between absent/low AZGP1 expression and clinical recurrence ($P = 0.007$).

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CONCLUSIONS. AZGP1 is a potential molecular marker for biochemical relapse in men with margin-positive, localized PC. Routine assessment of this biomarker may lead to better selection of patients who will benefit from post-RP radiotherapy. *Prostate 71: 1638–1645, 2011.* © 2011 Wiley-Liss, Inc.

KEY WORDS: **AZGP1; margins; prostate cancer**

INTRODUCTION

Prostate cancer (PC) is the fifth most commonly diagnosed cancer worldwide in men [1] and the most commonly diagnosed cancer in men in developed countries. In 2005, 2,949 Australian men died due to PC, the second leading cause of cancer mortality (13.4%) [2]. Assessment of prognosis remains one of the most important issues in localized PC as it forms the basis of clinical decision-making as to whether or not to proceed to surgery and/or radiotherapy.

A positive surgical margin, as defined by malignant cells at the inked margin [3] is known to confer a poorer prognosis following radical prostatectomy (RP). Positive surgical margins are reported in 11–37% of patients treated by RP [4–7]. Men with positive surgical margins have a twofold increase in the risk of biochemical recurrence compared with those with a negative surgical margin [4,5,7]. However, not all patients with a positive surgical margin will recur, with most reports suggesting a 30–35% recurrence rate [4,5,7]. Furthermore, the 10-year disease-specific mortality with a positive margin remains very low at $<10\%$ [3–7]. Adjuvant radiotherapy (within 16 weeks of surgery) reduces the rate of biochemical relapse and metastasis, and improves overall survival in men with positive surgical margins and/or pT3 tumors [8–10]. Given that there are twice as many grade III toxicities when adjuvant radiotherapy is added to surgery [8,9], there is an urgent need to identify which patients are most likely to benefit from adjuvant treatment.

A number of independent prognostic markers for biochemical relapse have been identified in men with localized PC including membranous secreted frizzled-related protein 4 (sFRP4) [11], nuclear β-catenin [12], macrophage inhibitory cytokine-1 (MIC-1) [13] and zinc-alpha 2-glycoprotein (AZGP1) [14], which is also a predictor of metastatic disease. While these studies assessed the utility of these molecules as prognostic markers in the surgical setting, there is now more routine use of adjuvant radiotherapy for high risk localized PC, in particular those with positive surgical margins. This raises the questions of [1] which men with positive surgical margins are at greatest risk of recurrence and should receive post-operative radiotherapy and [2] which men do not need further therapy. Therefore, our aim was to examine the association between the patterns of these molecular markers and relapse in men with positive margins post-RP.

MATERIALS AND METHODS

Patient Population

A cohort of archival formalin-fixed, paraffin-embedded specimens with a positive margin ($n = 186$) was selected from a previously studied group of 330 patients [8–11]. Numerous molecular marker studies have been performed using this well characterized cohort, which consists of localized PCs treated with RP between 1989 and 1996 at a single tertiary hospital [8–11]. All surgery was performed by one of six specialist urologists. All studies were approved by the St Vincent's Hospital Human Research Ethics Committee (H00/088).

Patients were followed post-operatively by their surgeons on a monthly basis until satisfactory urinary continence was obtained and then at 3-month intervals until the end of the first year, at 6-monthly intervals to 5 years and yearly thereafter.

Relapse was defined by the following criteria: biochemical disease progression with a serum PSA concentration ≥ 0.2 ng/ml increasing over a 3-month period or local recurrence on digital rectal examination confirmed by biopsy or by a subsequent rise in PSA [11–14].

Molecular Markers

We have previously published several molecular marker studies using this cohort, but for this study we identified molecular markers that were independent predictors of biochemical and/or clinical relapse in localized PC on multivariate analysis. All the studies were performed by immunohistochemistry on tissue microarrays, which were constructed using the original archival formalin-fixed paraffin-embedded tissue blocks. Using these criteria, four molecular marker studies were identified: membranous sFRP4 [11], nuclear β-catenin [12], MIC-1[13], and AZGP1 [14]. The immunohistochemical techniques have been published previously [11–14].

In each study, the immunostaining was scored by two independent observers who were blinded to the patient outcomes. One of the observers was an anatomical pathologist (JGK, CSL). AZGP1 staining was

scored by intensity into the categories of absent (0), weak (1), moderate (2+) and strong (3+) [14]. Membranous sFRP4 was scored as percentage of the cancer cells with positive membranous staining [11]. MIC-1 was scored as the percentage of the cancer cells with positive cytoplasmic staining [13]. Nuclear β-catenin was scored as the percentage of the cancer cells with positive nuclear staining [12].

STATISTICAL ANALYSIS

Biochemical relapse free survival was measured from the date of RP to relapse or the date of last follow-up. Kaplan–Meier analyses were performed to examine the relationships between the four molecular markers and biochemical relapse-free survival [15]. Univariate and multivariate analysis were performed using a Cox proportional hazards model [16]. The variables examined were the four molecular markers: nuclear AZGP1, membranous sFRP4, β-catenin, and MIC-1 status, and clinicopathological predictors of outcome such as preoperative PSA, pathologic stage, seminal vesicle involvement (SVI), Gleason score and extraprostatic extension (EPE). The molecular markers were stratified according to the previously published cut-points: AZGP1 absent/weak versus moderate/strong [14], membranous sFRP4 $\leq$20% versus >20% [11], nuclear β-catenin <10% versus $\geq$10% [12] and MIC-1 <40% versus $\geq$40% [13]. Variables were included in the multivariate Cox proportional hazards model if they had a P-value <0.1 on univariate testing and a stepwise selection procedure was used to define the model. The predictive discrimination of the multivariate models was assessed by a Harrell's C statistic. All P-values corresponded to two-sided tests and $P < 0.05$ was considered statistically significant. All statistical analyses were performed using Statview 4.5 software (Abacus Systems, Berkeley, CA) and ACCorD (V. Gebski, NHMRC Clinical Trials Center, University of Sydney).

RESULTS

Among the published cohort of 330 men with localized PC, we identified 186 with positive margins post-RP. At a median follow-up of 109 months (13–217 months), 41% (77/186) patients had biochemical relapse and 6% (12/186) had a clinical relapse (four local recurrence, eight distant metastases/death from PC). Given the nature of the cohort, it was unsurprising that there was a relatively high rate of poor prognostic factors with 53% of men having a preoperative PSA >10 ng/ml, 72% EPE, 24% SVI, and 57% Gleason score $\geq$7 (Table I). This cohort was treated before there was evidence for a benefit from adjuvant radiotherapy so only 36% (67/186) men received adjuvant therapy as defined by the EORTC trial 22911 (Table I) [8].

On univariate analysis, pre-operative PSA > 10 ng/ml, T3 pathologic stage, SVI, Gleason score $\geq$7 and EPE were significant predictors of biochemical relapse, while adjuvant treatment did not appear to significantly influence outcome ($P = 0.05$) (Table II). Using the previously described cutpoints, the four molecular markers were assessed for the ability to predict biochemical relapse. Kaplan–Meier curves demonstrated that absent/low AZGP1 ($P = 0.007$), membranous sFRP4 $\leq$20% ($P = 0.03$) and MIC-1 <40% ($P = 0.04$) were each associated with a worse prognosis (Fig. 1, Table II). The level of nuclear β-catenin in the cancers was not significantly associated with outcome ($P = 0.07$) (Fig. 1, Table II).

Cox Proportional Hazards modeling was used to identify independent predictors of outcome in this cohort. An analysis of the clinicopathologic factors, demonstrated that SVI (HR 1.8, 95% CI 1.1–3.2, $P = 0.03$) was an independent clinicopathologic factor that predicts biochemical recurrence when modeled with pre-operative PSA (HR 1.5, 95% CI 1.0–2.6, $P = 0.1$), EPE (HR 1.9, 95% CI 1.0–3.7, $P = 0.07$) and Gleason score (HR 1.5, 95% CI 0.9–2.8, $P = 0.1$). An analysis of the molecular factors showed that AZGP1 (HR 6.1, 95%CI 2.0–19.0, $P = 0.002$) was an independent predictor of biochemical recurrence when modeled with membranous sFRP4 (HR 2.1, 95%CI 0.8–5.3, $P = 0.2$), MIC-1 (HR 1.2, 95%CI 0.4–3.4, $P = 0.8$) and nuclear β-catenin (HR 1.2, 95%CI 0.4–3.4, $P = 0.7$). A combined multivariate analysis demonstrated that AZGP1 ($P = 0.01$) was the only independent predictor of biochemical relapse in men with positive margins when modeled with preoperative PSA ($P = 0.1$), EPE ($P = 0.2$), SVI ($P = 0.3$) and Gleason score ($P = 0.5$) (Table III). The predictive discrimination of the multivariate model including AZGP1, pre-operative PSA, EPE, SVI, and Gleason score was higher (Harrell's C statistic 0.668) than in the same model excluding AZGP1 (Harrell's C statistic 0.649). This suggests a modest improvement in the discriminative capacity of the model with the addition of AZGP1. Given that 21 patients had received adjuvant radiotherapy, the analysis was also undertaken excluding those patients. This demonstrated that AZGP1 (HR 2.1, 95%CI 1.1–4.0; $P = 0.02$) was still the only independent predictor of outcome when modeled with preoperative PSA (HR 1.5, 95%CI 0.8–2.9; $P = 0.2$), EPE (HR 1.7, 95%CI 0.8–3.7; $P = 0.2$), SVI (HR 1.4, 95%CI 0.7–3.0; $P = 0.4$) and Gleason score (HR 1.2, 95%CI 0.5–2.7; $P = 0.6$).

Although there were only a small number of clinical recurrences in our cohort, a Kaplan–Meier analysis demonstrated an association between AZGP1

TABLE I. Clinicopathologic and Molecular Characteristics for 186 Patients With Margin-Positive Localized PC Treated With RP

Characteristic	Number (%)
Mean age	63 (range 47–75)
Median follow-up (months)	109 (range 13–217)
Number of cases with available data on expression of molecular markers	
Membranous sFRP4	123 (87)
Nuclear β-catenin	121 (86)
MIC-1	100 (71)
AZGP1	112 (60)
Adjuvant treatment post-RP (n = 67)	
Endocrine treatment	46 (25)
Radiotherapy alone	15 (8)
Radiotherapy + endocrine treatment	6 (3)
Pathologic stage	
pT2A	2 (1)
pT2B	3 (1)
pT2C	45 (24)
pT3A	74 (40)
pT3B	28 (15)
pT3C	26 (14)
pT4A	8 (4)
Pre-operative PSA (n = 174)	
Mean (ng/ml)	18 (1–191)
PSA > 10	92 (53)
Surgical margin involvement	
Apical only	22 (12)
Two positive margins	48 (26)
Multiple	116 (62)
Extraprostatic extension	134 (72)
Lymph node involvement	5 (3)
SVI	44 (24)
Gleason score	
≤6	79 (42)
7	71 (38)
≥8	36 (19)

and clinical recurrence. Absent/low AZGP1 predicted for a shorter clinical relapse-free survival (Fig. 2, $P = 0.007$).

DISCUSSIONS

This study shows that absent/weak AZGP1 expression is an independent predictor of biochemical recurrence in men with margin-positive, localized PC following RP. Furthermore, low AZGP1 expression is associated with clinical relapse. These data provide strong evidence that AZGP1 may identify which men with positive surgical margins have poorer prognostic disease and should be considered for adjuvant radiotherapy.

Recent studies have sought to more accurately define the risk of a positive surgical margin by studying the impact of factors such as the location and number of positive margins, the linear extent, and the plane of the involved margin [4,5]. For instance, a margin involving the bladder neck or posterolateral surface of the prostate may have a more significant adverse impact on prognosis than an involved apical or anterior margin [17,18]. In contrast, other investigators have found no association between the location of positive surgical margins and recurrence rates [19,20]. Likewise, initial reports suggested that when the positive surgical margin occurred at a site of capsular incision it did not have adverse prognostic significance, while more recent series have demonstrated a significantly poorer prognosis for patients with capsular incision compared to those with negative margins [21,22]. Studies assessing the association between the extent of the positive margin and the risk of disease progression have yielded varying results. Babaian et al. [23] found that patients with postive margins of greater than 3 mm in linear extent had a significantly greater PSA-recurrence risk, as did Chuang et al. [21] in their analysis of a cohort with

TABLE II. Univariate Analysis of the Relationship Between Clinicopathologic, Molecular Markers and Biochemical Recurrence After RP in Margin-Positive, Localized PC

	HR (95% CI)	*P*-value
Pre-operative PSA ≥10 vs. <10 ng/ml	1.9 (1.1–3.2)	0.01
Pathologic stage pT3 vs. pT2	2.2 (1.2–4.1)	0.01
SVI present vs. absent	2.6 (1.6–4.2)	<0.0001
Gleason score ≥7 vs. ≤6	2.0 (1.2–3.3)	0.006
Extraprostatic extension present vs. absent	2.4 (1.3–4.5)	0.005
AZGP1 0 or 1+ vs. 2–3+	2.1 (1.2–3.8)	0.009
Membranous sFRP4 ≤20% vs. >20%	1.9 (1.1–3.5)	0.03
MIC-1 <40% vs. ≥40%	2.0 (1.1–3.8)	0.04
Nuclear β-catenin <10% vs. ≥10%	1.7 (0.9–3.2)	0.07
Adjuvant treatment no vs. yes	1.6 (1.1–2.5)	0.05

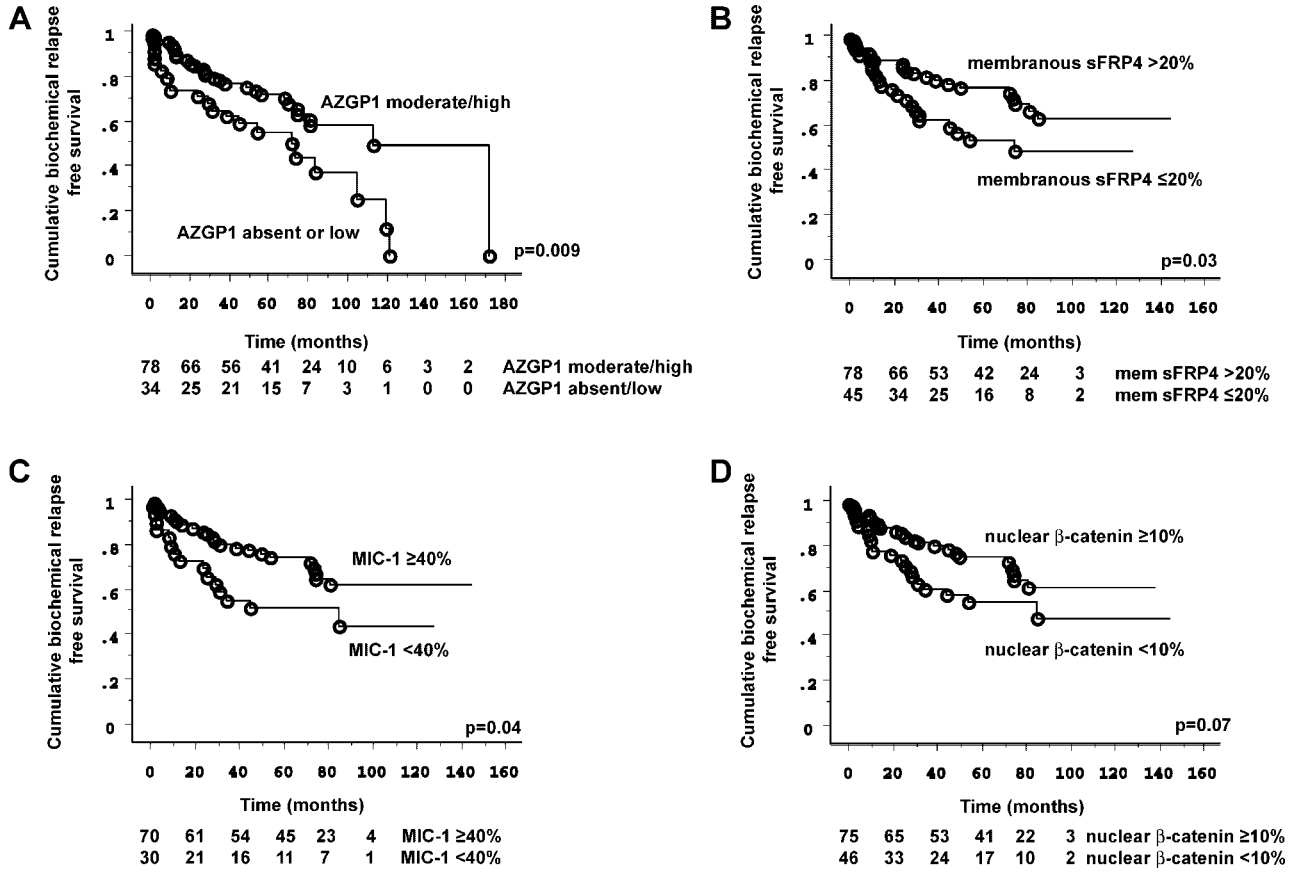

Fig. 1. Kaplan–Meier curves demonstrating the association between biochemical relapse-free survival and the molecular markers (**A**) AZGPI, (**B**) sFRP4, (**C**) MIC-I, and (**D**) β-catenin in men with margin-positive localized PC.

capsular incision. In contrast, Marks et al. [24] found no significant association between the extent of the involved margin and biochemical recurrence, possibly related to differences in the pathological interpretation of positive margin and the method of assessing the linear extent of margin involvement when multiple margins were involved. Most studies have described the extent of involvement subjectively as ''focal'' or ''extensive'' [8,25]. More recently, Gleason grade at the site of the positive margin has been identified as a potential prognostic marker [26]. The recent International Society of Urological Pathology (ISUP) consensus meeting recommended routine assessment of location and extent of positive margins in RP specimens [27].

Adjuvant radiotherapy post-RP for patients with high risk localized PC improves biochemical progression-free, metastasis-free and overall survival in men with one or more of the high risk features; positive surgical margin, SVI or EPE [8–10]. At a median follow-up of 5 years, the irradiated group in the EORTC 22911 trial had a biochemical relapse-free survival of 74% compared to 53% in the surgery alone group ($P < 0.0001$) [8]. Men with positive surgical margins had a 48% 5-year biochemical relapse free rate without radiotherapy compared to 76% in the

TABLE III. Multivariate Analysis of the Clinicopathological Factors and AZGPI Predicting Biochemical Recurrence of Margin Positive, Localized PC

	HR (95% CI)	*P*-value
Pre-operative PSA ≥10 vs. <10 ng/ml	1.6 (0.9–3.1)	0.1
Extraprostatic extension Present vs. absent	1.6 (0.7–3.4)	0.2
SVI present vs. absent	1.5 (0.7–3.1)	0.3
Gleason score ≥7 vs. ≤6	1.3 (0.6–2.9)	0.5
AZGP1 0 or 1+ vs. 2–3+	2.2 (1.2–4.0)	0.01

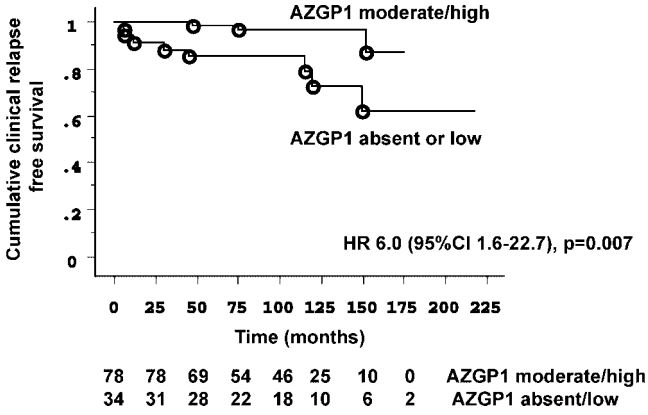

Fig. 2. Kaplan–Meier curve demonstrating the association between AZGP1 expression and clinical relapse-free survival in men with margin-positive localized PC.

irradiated group (<0.0001) [8]. Furthermore, the SWOG8794 trial with 20 years follow-up has now demonstrated a significant improvement in overall survival after adjuvant radiotherapy (10-year overall survival 74% vs. 66%, $P = 0.02$) [10]. On the other hand, there are significantly more Grade 2 and 3 late effects in the radiation treatment groups with complications twice as likely in those men who received radiotherapy [8,9]. In addition, surgical margin status is more predictive than EPE and SVI of a treatment effect with adjuvant radiotherapy [28]. Irrespective of other clinicopathologic features, patients with negative margins do not benefit from post-operative radiotherapy. Based on these data, for every 1,000 patients with positive margins, adjuvant radiotherapy would prevent biochemical relapse in 291 patients by year 5 ($P < 0.01$) [28].

AZGP1 is a 41 kDa soluble protein with a major histocompatibility complex-1 (MHC-1)-like fold in its structure [29]. AZGP1 protein is ubiquitous in normal prostate, breast, skin, salivary gland, liver, kidneys, respiratory, and the gastrointestinal tract [30]. It is synthesized by epithelial cells of many tissues including the prostate gland and is present in most body fluids [30]. Although yet to be fully defined, there have been a number of proposed functions for this protein including; lipid metabolism and hence the role in cancer cachexia; immunoregulation because of the MHC-1-like fold structure; protein transport; regulation of melanin production; and prevention of tumor proliferation through a role in tumor differentiation [31,32]. An earlier study demonstrated that AZGP1 has the same structure as the urinary lipid-mobilizing factor identified in patients with cancer cachexia [32].

AZGP1 expression in localized PC is predictive of biochemical and clinical recurrence and absent or weak AZGP1 immunostaining intensity is associated with high grade PC [33]. Three studies including our own have demonstrated that low AZGP1 expression in localized PCs is an independent predictor for biochemical recurrence [14,34,35]. Furthermore, our previous study demonstrated that absent or weak AZGP1 expression is associated with early clinical recurrence (HR 4.8, 95% CI 2.2–10.7, $P < 0.001$) and with bony metastases or death from PC (HR 8.0, 95% CI 2.6–24.3, $P < 0.001$) [14]. Our current study is the first to assess the role of AZGP1 expression in the high-risk group of men with margin-positive PC and to identify AZGP1 expression as a potential predictive biomarker to assess who should be offered adjuvant radiotherapy.

Although the function of AZGP1 in prostate carcinogenesis is unclear, AZGP1 expression appears to be androgen-regulated [36]. Our group recently demonstrated that while AZGP1 mRNA expression increased with androgen stimulation in LNCaP cells, expression is repressed by GATA-2 [37]. Strong expression of GATA-2 is also associated with biochemical recurrence (HR 1.69; 95%CI 1.02–2.8; $P = 0.043$) and progression to distant metastases (HR 3.00; 95%CI 1.00–8.94; $P = 0.0493$) in PC [37]. This suggests a previously unrecognized relationship between GATA-2, AZGP1 and androgen receptor signaling, which may have functional roles in the transition of PC cells to a more aggressive phenotype, potentially through dedifferentiation of PC cells.

CONCLUSIONS

This study demonstrates that absent or weak AZGP1 expression predicts for a poorer prognosis in men with margin-positive, localized PC. These data strongly suggest that AZGP1 may be a potentially useful molecular marker for identifying PC patients who will benefit from adjuvant radiotherapy.

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