Loss of AZGP1 as a Superior Predictor of Relapse in Margin-Positive Localized Prostate Cancer

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BACKGROUND. Positive surgical margins (PSMs) in localized prostate cancer (PC) confer a two- to three-fold increased risk of biochemical relapse (BR). Absent/weak AZGP1 expression and Gleason grade $\geq$4 at the margin are each independent predictors of BR in patients with PSMs. Our study aimed to determine whether the biomarkers AZGP1 expression and Gleason grade at the site of a PSM are significant independent markers of biochemical and clinical relapse (CR) when modeled together and whether one of these biomarkers may be superior in its capacity to predict outcome.

METHODS. A cohort of 275 consecutive patients with margin-positive localized PC following surgery were assessed for Gleason grade and AZGP1 expression at the PSM. BR-free survival was the primary end-point, while CR-free survival and PC-specific death were secondary endpoints. Kaplan–Meier Analysis and Cox Proportional Hazards Modeling were performed.

RESULTS. Absent AZGP1 expression was significantly associated with increased risk of BR ($P = 0.001$) and PC-specific death ($P = 0.02$). Gleason grade $\geq$4 at PSM was associated with BR ($P = 0.02$), CR ($P = 0.003$), and PC-specific death ($P = 0.004$). On multivariable analysis, absent AZGP1 expression remained an independent predictor of BR (HR 2.4, 95%CI 1.5–3.9, $P < 0.001$) when modeled with Gleason grade at margin (HR 1.3, 95%CI 0.9–1.9, $P = 0.16$), preoperative PSA ($P = 0.002$), seminal vesicle involvement ($P = 0.002$), extraprostatic extension ($P = 0.001$), Gleason score ($P = 0.01$), adjuvant treatment ($P = 0.75$), linear length of the involved margin ($P = 0.001$) and margin number ($P = 0.09$).

CONCLUSION. Absent AZGP1 expression is an independent predictor of BR in margin-positive localized PC and is associated with increased PC-specific mortality in a Phase II study. Absent AZGP1 expression was superior to Gleason grade at PSM in predicting relapse

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and should be incorporated into subsequent clinical trials of post-operative radiotherapy in men with margin-positive PC. *Prostate 76:1491–1500, 2016.*

KEY WORDS: **AZGP1; Gleason grade; localized prostate cancer; positive surgical margin; radiotherapy**

INTRODUCTION

Prostate cancer (PC) is the most common non-cutaneous cancer in men and a leading cause of cancer deaths in males in developed Western countries, causing significant morbidity and mortality globally [1]. Despite this, only a minority of PCs have the capacity for metastasis and are potentially lethal [2]. This variation in outcome makes assessment of prognosis one of the most important management challenges.

A positive surgical margin (PSM) is regarded an adverse outcome following radical prostatectomy (RP) [3]. It is defined by the presence of cancer cells extending to the inked surface of the prostatic specimen, indicating that resection may be incomplete [3]. PSMs are reported to occur in 13–31% of men treated with RP [4–11], and confer a two- to three-fold increased rate of BR [4,6,7,9]. These patients derive the greatest biochemical recurrence-free survival benefit from adjuvant radiotherapy [12]. However, the majority of patients with PSMs will not manifest recurrence, with relapse rates less than 45% at 10 years [4,6,10]. Furthermore, there is inconsistent data regarding the increased risk of PC-specific mortality [5,6,8,11]. Adjuvant radiotherapy in this situation decreases the rates of biochemical and metastatic relapse [13–15]; however, a significant problem with a universal policy of adjuvant radiotherapy is the unnecessary exposure of patients who would never have experienced recurrent cancer to the morbidity associated with radiation.

Multiple pathologic variables have been proposed as possible prognostic factors in men with PSMs to select patients, who might benefit from adjuvant radiotherapy [3]. Chuang et al. [16,17] found that there was a significantly lower risk of recurrence in patients with a PSM due to capsular incision compared to that due to extracapsular extension, which is possibly a reflection of the increased aggressiveness and metastatic potential of cancer cells that have already advanced into extraprostatic tissue. Although there remains controversy over the significance of the anatomical location of the positive margin, namely apical versus non-apical, there is some consensus that the linear extent of a PSM is a predictor of relapse, and that patients with multiple PSMs have a poorer prognosis than those with single PSMs [6,9,18–21]. In our previous study, men with Gleason grade 4 or 5 at margin had a significantly increased risk of BR compared with men with Gleason grade 3 at the margin ($P = 0.003$) [22]. In fact, men with Gleason grade 3 at the margin had the same prognosis as those with negative margins [22]. Similarly, men with a Gleason score of 8–10 at the margin were more than twice as likely to experience BR than those with Gleason score 6 at the margin ($P = 0.03$), and that Gleason score at the margin was in fact more predictive of relapse than the Gleason score of the dominant nodule [23].

There is a developing body of evidence demonstrating that absent/weak AZGP1 expression is a significant independent predictor for BR, irrespective of Gleason score or margin status [24–27]. Men with localized PC in whom AZGP1 expression is absent/weak have a significantly increased risk of BR ($P = 0.01$) and metastatic relapse compared to men with moderate/high AZGP1 expression ($P < 0.001$) [24]. In a subset of men with margin-positive disease in this study, men in whom AZGP1 expression is absent/weak had a significantly increased risk of BR compared to men with moderate/high AZGP1 expression ($P = 0.01$) [25].

The evidence for prognostic markers in margin-positive localized PC is based largely on biochemical rather than clinical endpoints and there is little data comparing the relative merits of the various markers. The aim of this study was to determine whether AZGP1 expression and Gleason grade at the site of PSM are independent markers of recurrent disease in margin-positive localized PC and whether one of these biomarkers may be superior in its capacity to predict outcome.

MATERIALS AND METHODS

A previously studied cohort of 285 consecutive patients with pathologic identified margin-positive localized PC, who underwent open RP between 1997 and 2003 at St Vincent's Hospital, was identified from our database following ethics approval (12/231) [22]. Patients were excluded from this cohort on the basis of inadequate follow-up data or neoadjuvant hormone therapy. Available formalin-fixed, paraffin-embedded specimens, and accompanying slides were obtained and independently reviewed for the presence of a PSM by one of two urological pathologists

(JGK, RG). A further 10 patients were excluded on the basis that a PSM could not be identified, either because not all tissue blocks were available or because the PSM may have been apparent in a plane of the specimen that had been cut out by previous sections. The final cohort consisted of 275 patients. Our previous AZGP1 study was performed in a separate cohort of patients treated between 1990 and 1995 [25]. The patient cohort used for this study had previously been investigated for the prognostic effect of the Gleason grade at the PSM [22], but had never been assessed for AZGP1 expression. Therefore, the patient cohort in this study was an independent phase II validation cohort for AZGP1 as a prognostic marker. In addition, this is the only study designed to compare the prognostic efficacy of Gleason grade at PSM and AZGP1 expression to assess if one is superior to the other.

Histopathologic review also included assessment of Gleason grade/pattern at margin and plane of involvement. It was thought appropriate to re-grade positive margin sites due to a shifting of Gleason grading in clinical practice since the 2005 ISUP consensus meeting. The highest grade present was documented for analysis, based on evidence that a higher Gleason grade at margin confers a poorer prognosis [22], and the observation that common crush and thermal artefacts at the margin can make accurate discrimination of both a primary and secondary grade difficult. The plane of each margin was characterized as either "extraprostatic extension" (EPE) or "capsular incision," except at the apex where the lack of distinctive histologic boundaries makes accurate distinction difficult.

Tissue microarrays containing 1 mm cores of margin-positive areas of cancer were constructed and immunohistochemistry performed using a goat polyclonal anti-human AZGP1 antibody (1:250 dilution, *sc-11238*, Santa Cruz Biotechnology) to visualize AZGP1 expression using the established protocol [24]. Core biopsies were separately scored for AZGP1 staining by two independent observers blinded to patient outcomes (JGK, HMB). Staining intensity was stratified into absent (0), weak (1+), moderate (2+), and strong (3+) AZGP1 expression (Fig. 1). The lowest intensity score per patient was documented as the final score for analysis based on findings that a lower intensity score confers a poorer prognosis [24,25]. All discrepancies in scoring were reviewed by both observers, with a consensus score established.

BR-free survival was the primary endpoint, measured from the date of RP until either the occurrence of BR or the date of last follow-up. BR was defined by a measured serum PSA concentration $\geq$0.2 ng/ml, with a consecutive further increase [24]. Secondary endpoints were clinical relapse (CR) as determined by confirmed local recurrence and/or a positive scan confirming bony or visceral metastasis, and PC-specific survival as measured from the date of RP to confirmed date of death due to PC.

Chi-square testing assessed the correlation between variables. The relationships between AZGP1 expression/Gleason grade at margin and BR-free survival, clinical relapse-free survival, and PC-specific mortality were examined using Kaplan–Meier analysis [28] and Cox Proportional Hazards Modeling [29]. Other clinicopathologic variables known to influence BR were modeled either as continuous, dichotomous, or trichotomous variables as appropriate and were selected for the multivariable model based on previous studies [22,24,25]. A P-value < 0.05 was required for significance and all reported P-values were two-sided. All statistical analyses were performed using SPSS.

RESULTS

The characteristics of the cohort of 275 patients with margin-positive localized PC are listed in Table I. At a median follow up of 12.1 years (0.6–17.5 years), 125 patients had experienced biochemical recurrence (46%). There were 24 clinical relapses (9%) and 9 PC deaths (3%). The 10-year BR rate was 43%.

This cohort was treated prior to the evidence of the benefit of adjuvant radiotherapy in margin-positive patients [12]; therefore, only 22% of patients had adjuvant radiotherapy, and an additional 29% any form of adjuvant therapy (Table I). A single focus of PSM was evident in 47% of patients. There was evidence of EPE at the PSM in 31% of patients, while the margin was associated with capsular incision in 22%. The remaining 47% of patients had margin involvement only at the apex.

Absent AZGP1 expression was found in 16% of patients (44/274), while 35% had weak expression (97/275), 31% moderate expression (85/275), and 18% strong expression (49/275) (Fig. 1). The initial analysis was done using the previously identified cut-off [24,25]; absent/weak AZGP1 expression was significantly associated with a poorer BR-free survival compared with moderate/strong expression (HR 1.5, 95%CI 1.1–2.2, $P = 0.02$) (Fig. 2). However, absent AZGP1 expression compared to the presence of any AZGP1 expression (weak/moderate/strong) was also significantly associated with a poorer BR-free survival (HR 2.1, 95%CI 1.4–3.1, $P = 0.001$) (Fig. 3A). Men with absent AZGP1 expression also had a trend toward a higher risk for CR (HR 2.3, 95%CI 1.0–5.6, $P = 0.06$), and a significant increase in PC-specific mortality (HR 4.3, 95%CI 1.2–16.1, $P = 0.02$) (Fig. 3B and C). Absent AZGP1 expression was significantly associated with

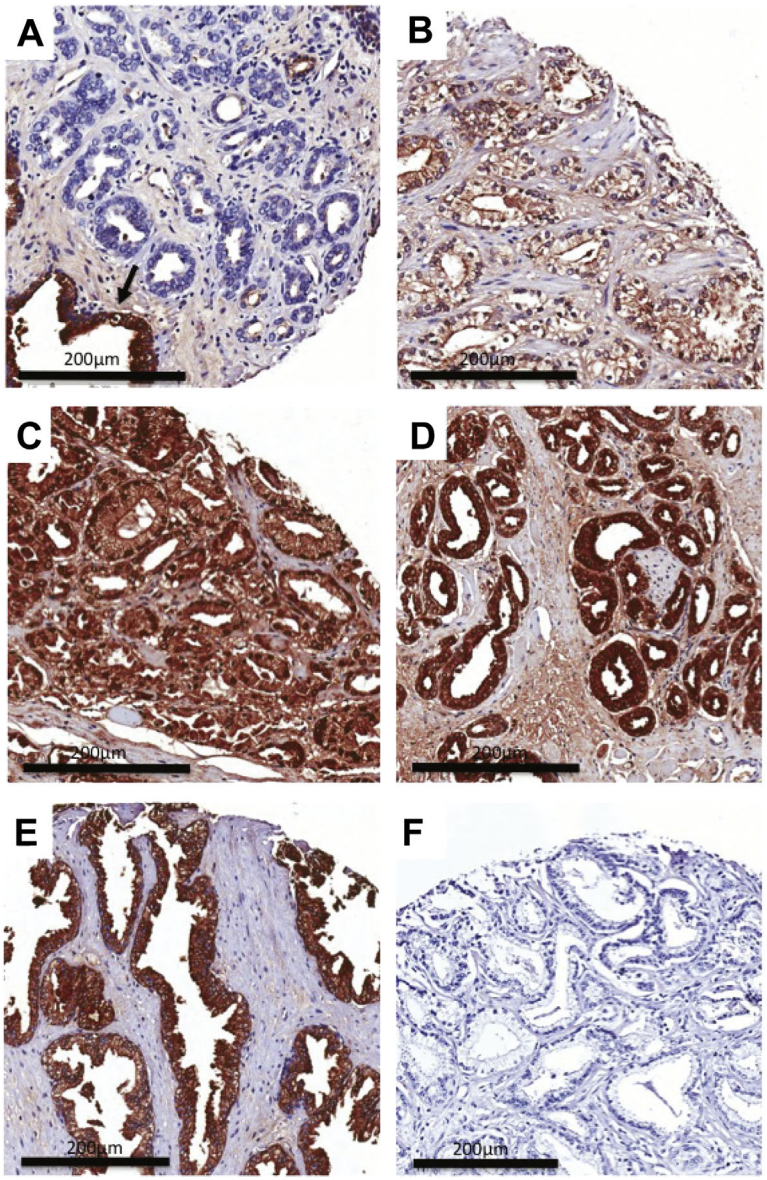

Fig. 1. Photomicrographs of paraffin-embedded prostate tissue immunostained for AZGP1, showing the spectrum of staining intensities observed in this study: (**A**) grade/pattern 3 cancer showing complete loss of AZGP1 immunostaining, with the black arrow indicating 3+ staining in an adjacent non-neoplastic gland; (**B**) grade 3 cancer showing 1+ immunostaining; (**C**) grade 4 (+secondary grade 3) cancer showing 2+ immunostaining; (**D**) grade 3 cancer at showing 3+ immunostaining; (**E**) non-neoplastic glands at the margin as a positive control; (**F**) grade 3 cancer at the margin stained with IgG as a negative control.

higher Gleason grade at margin ($P = 0.04$), higher pathologic T stage ($P < 0.001$), higher Gleason score of the dominant nodule ($P = 0.002$), EPE ($P < 0.001$), and the use of adjuvant therapy ($P = 0.009$) on χ^2 analysis, but not with pre-operative PSA ($P = 0.24$), seminal vesicle invasion (SVI) ($P = 0.20$), or the presence of multiple PSM ($P = 0.54$).

Gleason grade 3 was the highest grade at the margin in 46% of patients (126/275), while grade ≥ 4 was the highest Gleason grade in 54% (149/275). One patient had Gleason grade 5 at the margin (0.4%). Gleason grade at margin of ≥ 4 was significantly associated with a shorter BR-free (HR 1.5, 95%CI 1.1–2.2, $P = 0.02$) and clinical relapse-free (HR 4.5, 95%CI 1.5–13.0, $P = 0.003$) (Fig. 4A and B). All PC-specific deaths occurred in men with Gleason grade ≥ 4 at the margin ($P = 0.004$; Fig. 4C). Gleason grade of ≥ 4 at margin was found to be significantly associated with higher pre-operative PSA ($P = 0.04$), higher pathologic T stage ($P = 0.005$), higher Gleason score of the dominant nodule ($P < 0.001$), EPE ($P = 0.002$), and the use of adjuvant therapy ($P = 0.02$) on χ^2 analysis, but not

TABLE I. Clinicopathologic Characteristics of Cohort With Margin-Positive Localized Prostate Cancer Treated With Radical Prostatectomy

Characteristics	Number	(% or range)
Age, years	61	(46–80)
Preoperative PSA, ng/ml	Median 8.2	(3.1–63.0)
<10	178	(64%)
≥10	96	(35%)
Not reported	1	(1%)
Gleason score of dominant nodule		
≤6	74	(27%)
7	170	(62%)
≥8	31	(11%)
Pathologic stage		
pT2	102	(37%)
pT3	161	(59%)
pT4	12	(4%)
EPE		
Absent	104	(38%)
Present	171	(62%)
SVI		
Absent	236	(86%)
Present	39	(13%)
Not reported	1	(1%)
Node involvement		
Absent	229	(83%)
Present	3	(1%)
Not done	39	(14%)
Not reported	4	(2%)
Adjuvant therapy		
None	195	(71%)
Any adjuvant therapy	80	(29%)
Radiotherapy	60	(22%)
Number of margins per case		
Single	210	(76%)
Multiple	65	(24%)
Plane of involvement per case		
Apex only per case	128	47
Capsular incision	61	22
Any EPE present	86	31

with SVI ($P = 0.31$) or the presence of multiple margins ($P = 0.61$).

On univariable analysis, preoperative PSA ($P < 0.001$), SVI ($P = 0.002$), Gleason score of the dominant nodule ($P < 0.001$), linear length of the involved margin ($P = 0.002$), and adjuvant therapy ($P = 0.02$) were also significant predictors of BR (Table II). Interestingly, patients who did not have adjuvant therapy had a more favorable outcome than those who had treatment, likely due to a selection bias for patients with poorer prognostic features for adjuvant therapy. The presence of EPE in the specimen as a whole did not significantly influence biochemical recurrence in our cohort ($P = 0.80$), which may have been the result of wider surgical margins being taken in instances where EPE was suspected prior to or during surgery. Pathologic stage ($P = 0.62$) and node involvement ($P = 0.09$) did not significantly influence prognosis. Of the margin-specific variables, multiple positive margins ($P = 0.03$), and EPE at the plane of the involved margin ($P = 0.03$) were each significantly associated with a poorer prognosis (Table II).

On multivariable analysis, absent AZGP1 expression remained an independent predictor of BR ($P < 0.001$), while Gleason grade at the margin was no longer significant ($P = 0.16$), when modeled with preoperative PSA ($P = 0.002$), SVI ($P = 0.002$), EPE ($P = 0.001$), Gleason score of the dominant nodule ($P = 0.01$), adjuvant treatment ($P = 0.75$), linear length of the involved margin ($P = 0.001$), and the presence of multiple positive margins ($P = 0.09$) (Table II). Node involvement was not included in the multivariable analysis because of the small number of positive cases (n = 3). Gleason grade ≥4 at the PSM was associated with higher Gleason score of the dominant nodule ($P < 0.001$) by χ^2 analysis, which may account for a lack of significance on multivariable analysis.

DISCUSSION

This study demonstrates that absent expression of AZGP1 is a significant independent predictor of biochemical recurrence in men with margin-positive localized PC in a phase II validation cohort and that it is a potentially a better predictor of outcome than the Gleason grade at the PSM. In addition, absent AZGP1 expression was associated with an increased risk of PC death. Gleason grade ≥4 at margin was associated with both clinical recurrence and PC-specific mortality, but was not significant on multivariable analysis as the number of CR events and PC deaths remains small. Further validation in a phase III study is warranted to confirm the association with clinical endpoints.

AZGP1 encodes the protein zinc-alpha 2-glycoprotein, a soluble 41 kDa polypeptide first isolated in human plasma in 1961 [30]. The AZGP1 protein is produced by epithelial cells of the prostate, breast, skin, salivary glands, liver, kidney, lungs, and gastrointestinal tract [31]. Its physiological role has yet to be defined, however, proposed functions include involvement in the immune response due to similarity of structure and sequence to Class I MHC molecules, protein transport and stimulation of lipolysis and therefore a role in cancer cachexia [32].

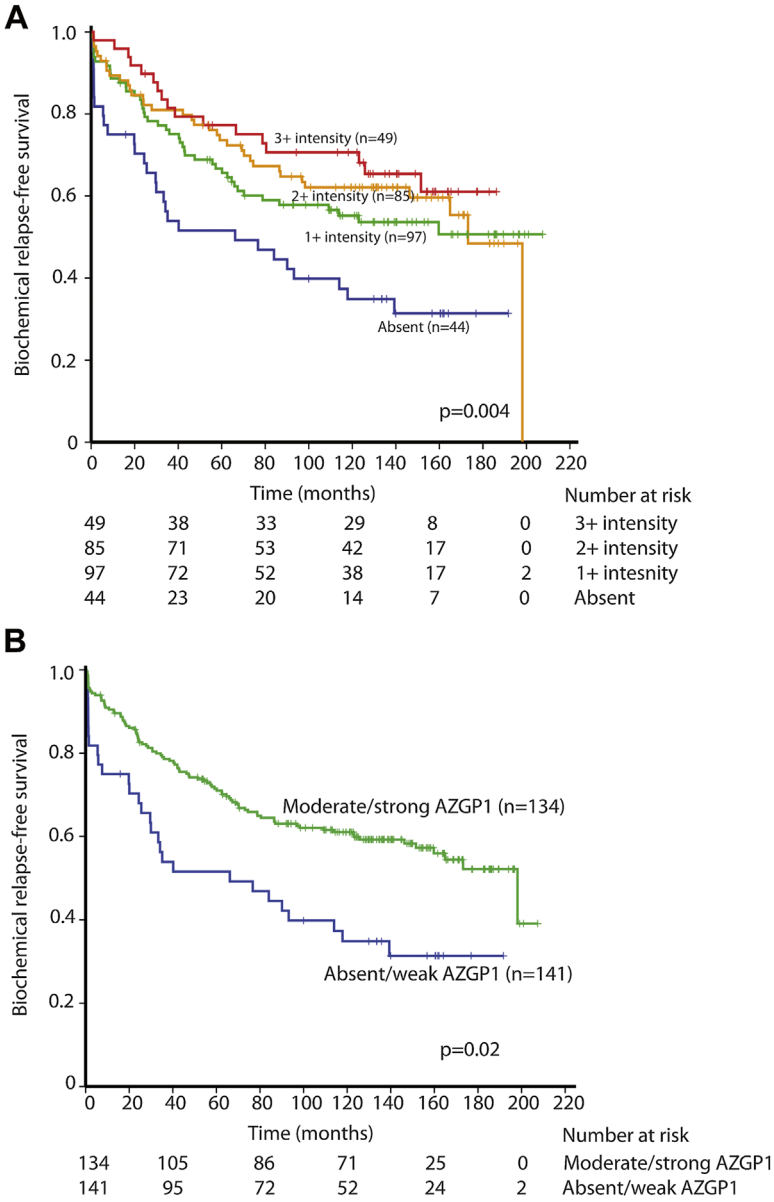

Fig. 2. Kaplan–Meier curves assessing the association between biochemical relapse-free survival and (**A**) AZGP1 expression stratified by each intensity score; (**B**) absent/weak (0/1+) versus moderate/strong (2+/3+) AZGP1 expression.

This study adds to the evolving body of evidence suggesting the relative loss or absence of AZGP1 contributes to PC progression from localized disease to BR and metastatic disease [24–27]. Its expression appears to be regulated by androgens, based on the observed increased expression of AZGP1 mRNA in the LNCaP prostate cell line under androgen exposure [33,34], and the presence of androgen response elements in the promoter region of the AZGP1 gene [34]. It is, therefore, possible that there is a relationship between the loss of AZGP1 expression and progression to androgen-independence in PC. AZGP1 mRNA expression is repressed in the presence of high levels of GATA-2, which is also associated with BR and distant metastatic progression. This suggests a potential link between high GATA-2 and low AZGP1 in the progression towards aggressive PC [33]. Our study found that absence of any expression of AZGP1 was more predictive of BR than dichotomizing patients according to absent/weak versus moderate/strong expression. This highlights the possible protective role of AZGP1 expression in PC progression, such that any AZGP1 expression, even when weak, confers a significantly better prognosis than total absence of expression.

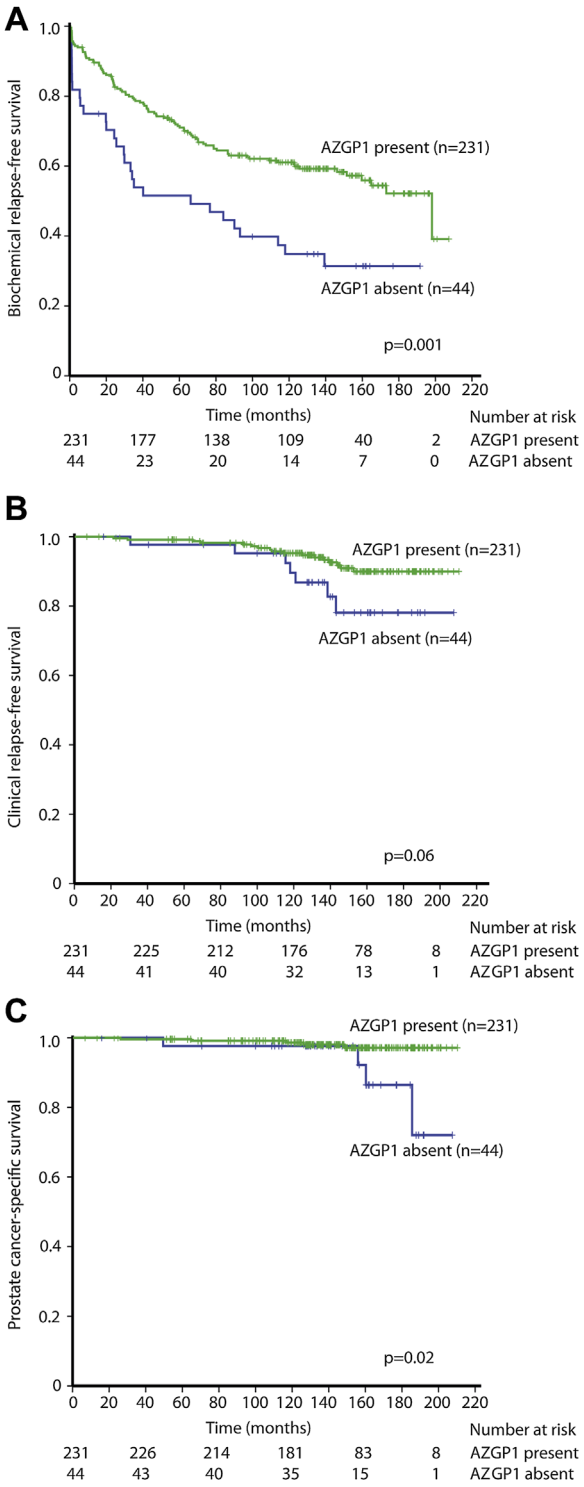

Fig. 3. Kaplan–Meier curves assessing the association between (**A**) biochemical relapse-free survival and absent versus present AZGP1 expression at margin; (**B**) clinical relapse-free survival and absent versus present AZGP1 expression at margin; (**C**) PC-specific survival and absent versus present AZGP1 expression at margin.

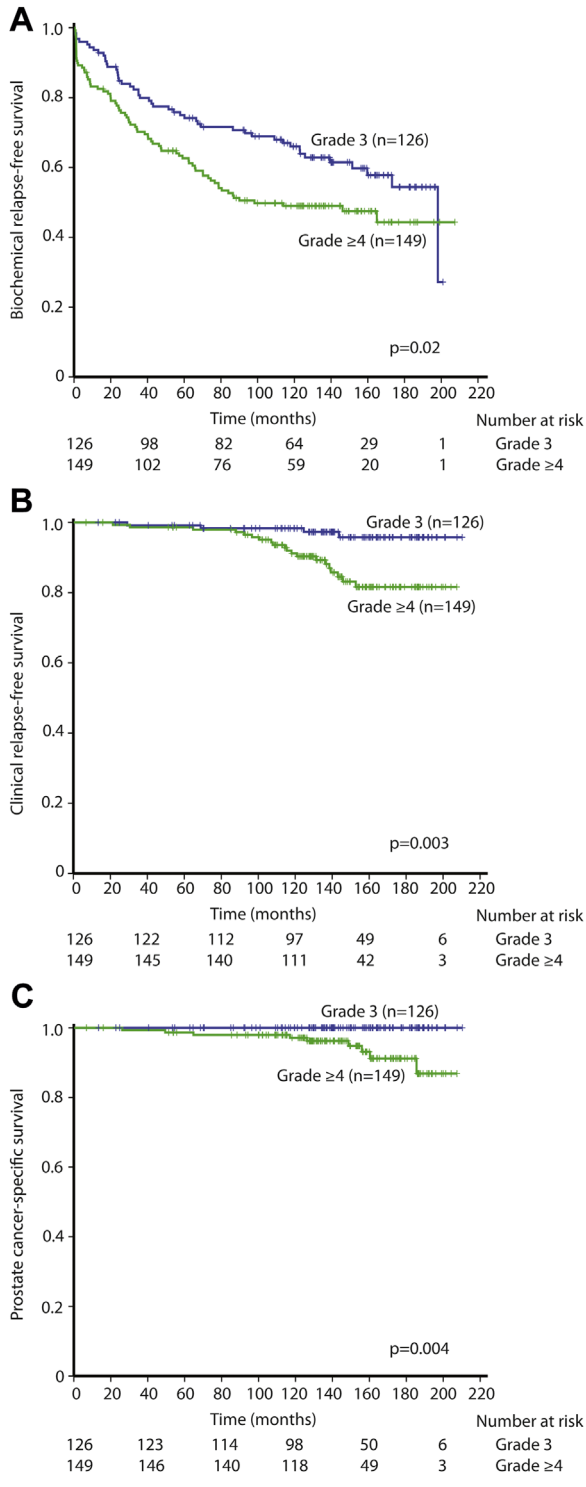

Fig. 4. Kaplan–Meier curves assessing the association between (**A**) biochemical relapse-free survival and Gleason grade at margin, (**B**) clinical relapse-free survival and Gleason grade at margin, and (**C**) PC specific-survival and Gleason grade at margin.

TABLE II. Cox Proportional Hazards Analysis of the Clinicopathologic Features Predicting Biochemical Relapse-Free Survival in Men With Margin-Positive Localized Prostate Cancer

	Univariable analysis		Multivariable analysis	
Variable	HR (95%CI)	*P*-value	HR (95%CI)	*P*-value
Preoperative PSA				
Continuous (per 1 ng/ml)	1.04 (1.0–1. 1)	<0.001	1.04 (1.0–1.1)	0.001
Pathologic stage				
≥pT3 vs. pT2	1.1 (0.8–1.6)	0.62	–	
SVI				
Yes vs. no	3.0 (2.0–4.5)	0.002	2.7 (1.7–4.5)	<0.001
EPE				
Yes vs. no	1.1 (0.7–1.5)	0.80	0.5 (0.3–0.8)	0.004
Gleason score of dominant nodule				
6	1		1	
7	1.4 (0.9–2.2)	0.12	1.2 (0.7–2.0)	0.42
≥8	3.9 (2.2–6.8)	<0.001	2.3 (1.2–4.5)	0.01
Node involvement				
Positive vs. negative	3.4 (0.8–13.8)	0.09	–	
Adjuvant treatment				
No vs. yes	0.6 (0.4–0.9)	0.02	1.2 (0.8–1.8)	0.45
Highest Gleason grade at PSM				
≥4 vs. 3	1.5 (1.1–2.2)	0.02	1.4 (0.9–2.0)	0.13
AZGP1 expression				
Absent vs. present	2.1 (1.4–3.1)	0.001	2.5 (1.6–4.0)	<0.001
Number of margins				
Multiple vs. single	1.5 (1.1–2.3)	0.03	1.3 (0.8–2.0)	0.2
Plane of involvement of margin[a]				
Any EPE vs. CI	1.8 (1.1–2.9)	0.03	–	
Linear length of the involved margin				
<3 vs. ≥3 mm	1.8 (1.2–2.5)	0.002	1.9 (1.3–2.7)	0.001

[a]Cases with only apical margins excluded.

A 2011 review reporting the findings of the 2009 ISUP consensus meeting on surgical margins highlighted the need for further study of the prognostic significance of the Gleason pattern specifically at margin [3]. Since then, Gleason score [23,35] and Gleason grade [22] at the PSM have been identified as significant predictors of biochemical recurrence. Though Gleason grade was not found to be a significant predictor of BR on multivariable analysis when modeled with AZGP1, the association demonstrated between Gleason grade ≥4 at margin and CR suggested that these men were significantly more likely to experience a recurrence of clinically significant disease. Notably, there were no PC-specific deaths recorded in men with Gleason grade 3 at margin in our cohort. The discrepancy between the Gleason grading data in our study and that by Savdie et al. [22] of the same cohort can be in part explained by the change in recommendations of the criteria for Gleason patterns 3 and 4 following the 2005 ISUP consensus meeting, a degree of inter- and intra-observer variability, and deeper sectioning of blocks leading to the review of a different plane of tissue.

Our findings are significant in the context of the known morbidity associated with adjuvant radiotherapy following RP. While adjuvant radiotherapy improves biochemical and metastasis-free survival in men with margin-positive localized PC, patients receiving radiotherapy consistently experience poorer bowel and urinary function, with almost twice the risk of urinary incontinence [36,37]. The incidence of Grade III severe toxicity also doubles when radiotherapy is added to surgery [14]. Gleason grade at the PSM has been proposed as part of the synoptic reporting for localized PC, however, these data suggest that AZGP1 expression may be a more effective discriminator of outcome and could allow more accurate identification of patients most likely to benefit from adjuvant radiotherapy. AZGP1 expression has been included in the randomized Phase III multicenter RAVES trial comparing adjuvant radiotherapy

to early-salvage treatment in patients with pT3 disease and/or PSMs [38].

CONCLUSION

In conclusion, absent AZGP1 expression is an independent predictor of BR in margin-positive localized PC and is associated with increased PC-specific mortality. Moreover, these patients with absent AZGP1 expression may potentially gain the greatest benefit from adjuvant radiotherapy, and these data provide the rationale for incorporating AZGP1 expression into prospective studies of post-RP radiotherapy such the RAVES trial.

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DISCLOSURE

None of the authors have any affiliations that they consider to be relevant or important with any organization that to any author's knowledge has a direct interest, particularly a financial interest, in the subject matter discussed.

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