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Expression of E6AP and PML predicts for prostate cancer progression and cancer-specific death

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Background: The promyelocytic leukemia (PML) tumor suppressor plays an important role in the response to a variety of cellular stressors and its expression is downregulated or lost in a range of human tumors. We have previously shown that the E3 ligase E6-associated protein (E6AP) is an important regulator of PML protein stability but the relationship and clinical impact of PML and E6AP expression in prostatic carcinoma is unknown.

Methods: E6AP and PML expression was assessed in tissue microarrays from a phase I discovery cohort of 170 patients treated by radical prostatectomy for localized prostate cancer (PC). Correlation analysis was carried out between PML and E6AP expression and clinicopathological variates including PSA as a surrogate of disease recurrence. The results were confirmed in a phase II validation cohort of 318 patients with associated clinical recurrence and survival data.

Results: Survival analysis of the phase I cohort revealed that patients whose tumors showed reduced PML and high E6AP expression had reduced time to PSA relapse ($P = 0.012$). This was confirmed in the phase II validation cohort where the expression profile of high E6AP/low PML was significantly associated with reduced time to PSA relapse ($P < 0.001$), clinical relapse ($P = 0.016$) and PC-specific death ($P = 0.014$). In multivariate analysis, this expression profile was an independent prognostic indicator of PSA relapse and clinical relapse independent of clinicopathologic factors predicting recurrence.

Conclusion: This study identifies E6AP and PML as potential prognostic markers in localized prostate carcinoma and supports a role for E6AP in driving the downregulation or loss of PML expression in prostate carcinomas.

Key words: E6AP, PML, prostate cancer, prognostic marker, cancer recurrence

introduction

Prostate cancer (PC) is the most common noncutaneous cancer in men. PC is also a leading cause of cancer deaths in males in developed Western countries and causes significant morbidity and mortality globally [1]. There are many novel approaches being tested to improve outcome of patients with disseminated PC including inhibition of proteasomal degradation. Indeed, the proteasome inhibitor Velcade (bortezomib) is already in clinical use for the treatment of several hematological diseases [2] and phase I/II clinical trials have shown promising results for the treatment of castrate-resistant PC (reviewed in [3]).

E6-associated protein (E6AP), an E3 ubiquitin ligase, and promyelocytic leukemia (PML), a tumor suppressor gene which plays a critical role in the cellular response to a variety of stress conditions, including genotoxic stress, have been implicated in prostate carcinogenesis *in vitro* and *in vivo*. E6AP plays a key role in the regulation of proteasomal degradation of PML [4–6]. PML-deficient cells have impaired ability to undergo apoptosis and cellular senescence [5, 7, 8]. PML knockout mice develop tumors when challenged with carcinogens [8], and have enhanced tumorigenesis in cooperation with oncogenic activation, such as PTEN and Ras [7, 9]. Specifically, one allelic loss of PML is sufficient to drive invasive prostate adenocarcinoma in a PTEN mouse model [9]. Downregulation or complete loss of PML protein has been observed in prostate adenocarcinomas, where the expression of PML was inversely correlated with the progression of disease from prostate intraepithelial neoplasia (PIN) to invasive carcinoma [10]. Furthermore, mice which overexpress E6AP develop dysplastic lesions resembling PIN [11]. Since we observed that PML levels are increased in the prostates of E6AP null mice [6], we hypothesized that deregulation of E6AP may be linked to PML loss in PC. Hence, the aim

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of this study was to define the expression of E6AP and PML in human PC samples and determine whether changes in expression were associated with disease outcome.

methods

patient population

The phase I discovery cohort was derived from a previously described group of 170 archival formalin-fixed, paraffin-embedded radical prostatectomy (RP) specimens collected from the Royal Berkshire Hospital, Reading, UK [12]. 136 patients had sufficient tumor in the TMA and full clinicopathological characteristics while 120 patients had available data for PSA relapse (supplementary Table S1, available at *Annals of Oncology* online). The phase II validation cohort ($n = 318$) was selected from a previously described group of consecutive 732 patients treated with RP for localized PC at St Vincent's Hospital (Sydney, Australia) based on tissue availability [13]. Patients who received neoadjuvant hormonal therapy were excluded. The flow of patients through the study according to the ReMARK criteria [14] is listed in supplementary Table S1, available at *Annals of Oncology* online. Patient follow-up is described in supplementary Methods, available at *Annals of Oncology* online. This study has Ethics Committee approvals (Discovery cohort 106/02L; Validation cohort H00/088).

tissue microarray construction, immunohistochemistry and assessment

For the phase I discovery cohort, each prostatectomy specimen was represented by two 1-mm-diameter tumor cores. For the phase II validation cohort, a mean of three biopsies (range 2–5) of PC representative of the primary, secondary, and, if present, tertiary Gleason grades and one biopsy of non-neoplastic prostate tissue from the same zone as the cancer were arrayed. Immunohistochemistry is described in the supplementary Methods, available at *Annals of Oncology* online. The primary and secondary cohorts were each assessed by a specialist histopathologist (PC and JGK) who were blinded to patient outcome. Nuclear expression of PML and E6AP were scored as the percentage of cancer cells stained.

statistical analysis

Disease-specific relapse was measured from the date of RP to the date of relapse (biochemical or clinical), last follow-up or death. Kaplan–Meier and log-rank analyses evaluating disease relapse were carried out by dichotomizing the raw PML and E6AP scores in a stepwise fashion (i.e. using a cutoff of 10%, then 10%, up to 90%) [15]. Assessment of these results revealed the natural split in the data and thus the cutoffs to define low/high expression were derived from the phase I discovery cohort. These cutoffs were then validated in the phase II cohort. The E6AP score for each case represented the maximum nuclear expression across all cores in order to reflect staining in the highest grade tumor. To test the relationship between PML and E6AP and known clinicopathologic variates (dichotomized according to standard criteria) [16], Pearson's χ^2 test was used. Where the minimum expected value in a cell in the contingency table was <5, Fisher's exact test was used. For these tests, PML and E6AP were dichotomized according to the findings above; high PML was defined as >90% average nuclear expression, low PML <90%. High E6AP defined as maximum nuclear expression across all cores ≥80% staining versus low E6AP maximum nuclear expression across all cores defined as <80%. For ordinal variates, groups were compared using Mann–Whitney *U*-tests. Further survival analysis was carried out using univariate and multivariate analyses in a Cox proportional hazards model for PML/E6AP status and other clinical and pathologic predictors of outcome as previously described [15]. The multivariate model was produced by assessing

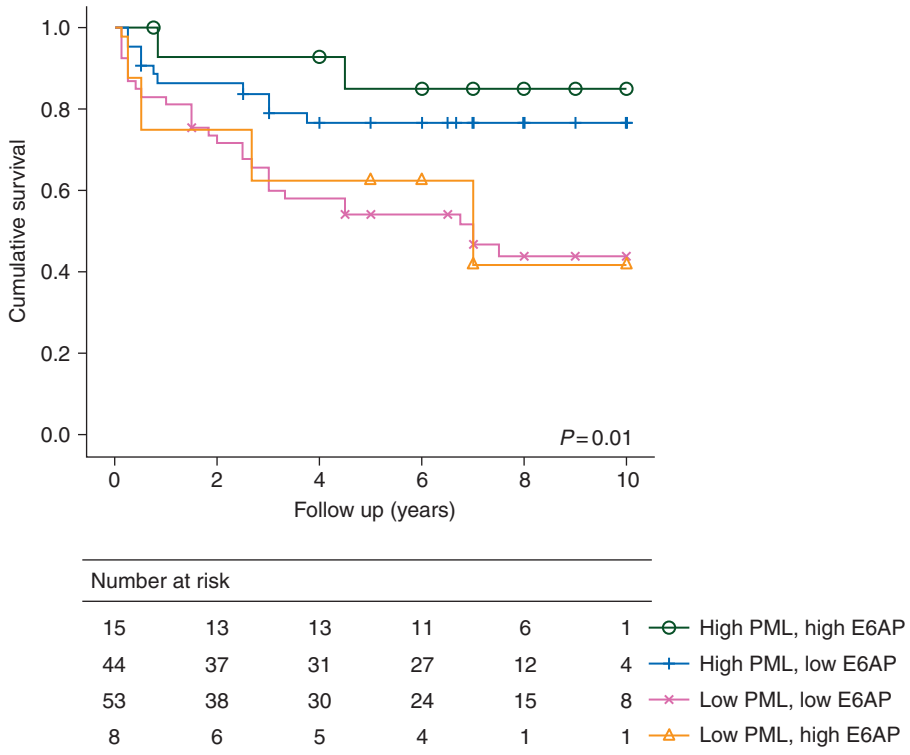

Figure 1. Kaplan–Meier analysis of PSA relapse for combined PML and E6AP expression in the phase I discovery cohort ($n = 136$). Stratified as high PML/high E6AP, high PML/low E6AP, low PML/high E6AP and low PML/low E6AP. High PML defined as >90% average nuclear expression, low PML <90%. High E6AP defined as ≥80% maximum nuclear expression across all cores, low E6AP <80% nuclear expression.

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PML/E6AP status with other baseline covariates of clinical relevance, such as Gleason score, pathological stage and preoperative PSA, which were modeled as dichotomous or continuous variates as appropriate. A value of $P < 0.05$ was required for significance. All reported P values were two-sided. All statistical analyses were carried out using SPSS.

results

The clinical and pathologic characteristics of the phase I discovery and phase II validation cohorts are summarized in supplementary Table S2, available at *Annals of Oncology* online. The phase I discovery cohort had a median follow-up of 7.9 years with 37% of patients having biochemical recurrence. The phase II validation cohort had a median follow-up of 15.2 years with a biochemical recurrence rate of 47%, clinical recurrence 12% and PC-specific death rate 6%. Data for clinical recurrence and PC-specific deaths were not available for the phase I discovery cohort due to the shorter follow-up period.

association between PML/E6AP and outcome in the phase I discovery cohort

PML was strongly expressed in 46% ($n = 63$) of PCs. Based on analysis of the phase I discovery cohort, high PML was defined as >90% average nuclear expression and low PML <90%. High E6AP was defined as ≥80% maximum nuclear expression across all cores and low E6AP <80% nuclear expression. Low PML expression was significantly associated with older age at diagnosis ($P = 0.04$), but there was no significant association between PML expression and preoperative serum PSA level, extraprostatic extension (EPE), surgical margin positivity, seminal vesicle invasion (SVI) or Gleason score (all $P > 0.05$). E6AP expression was high in 18% ($n = 25$) and low in 82% ($n = 111$) of PCs, but there were no significant associations between E6AP status and the clinicopathologic parameters (all $P > 0.05$).

Low PML expression was significantly associated with shorter biochemical relapse-free survival ($P = 0.001$). There was no significant difference in survival of patients stratified by E6AP expression. Using combined expression of PML and E6AP stratified as high PML/high E6AP, high PML/low E6AP, low PML/high E6AP and low PML/low E6AP showed that patients with low PML and high E6AP expression had significantly worse biochemical relapse-free survival ($P = 0.012$) (Figure 1).

association between PML/E6AP expression and outcome in the phase II validation cohort

The phase I discovery cohort was small with limited follow-up, therefore, the relationship between PML and E6AP expression using the phase I cutoffs and survival was further explored in

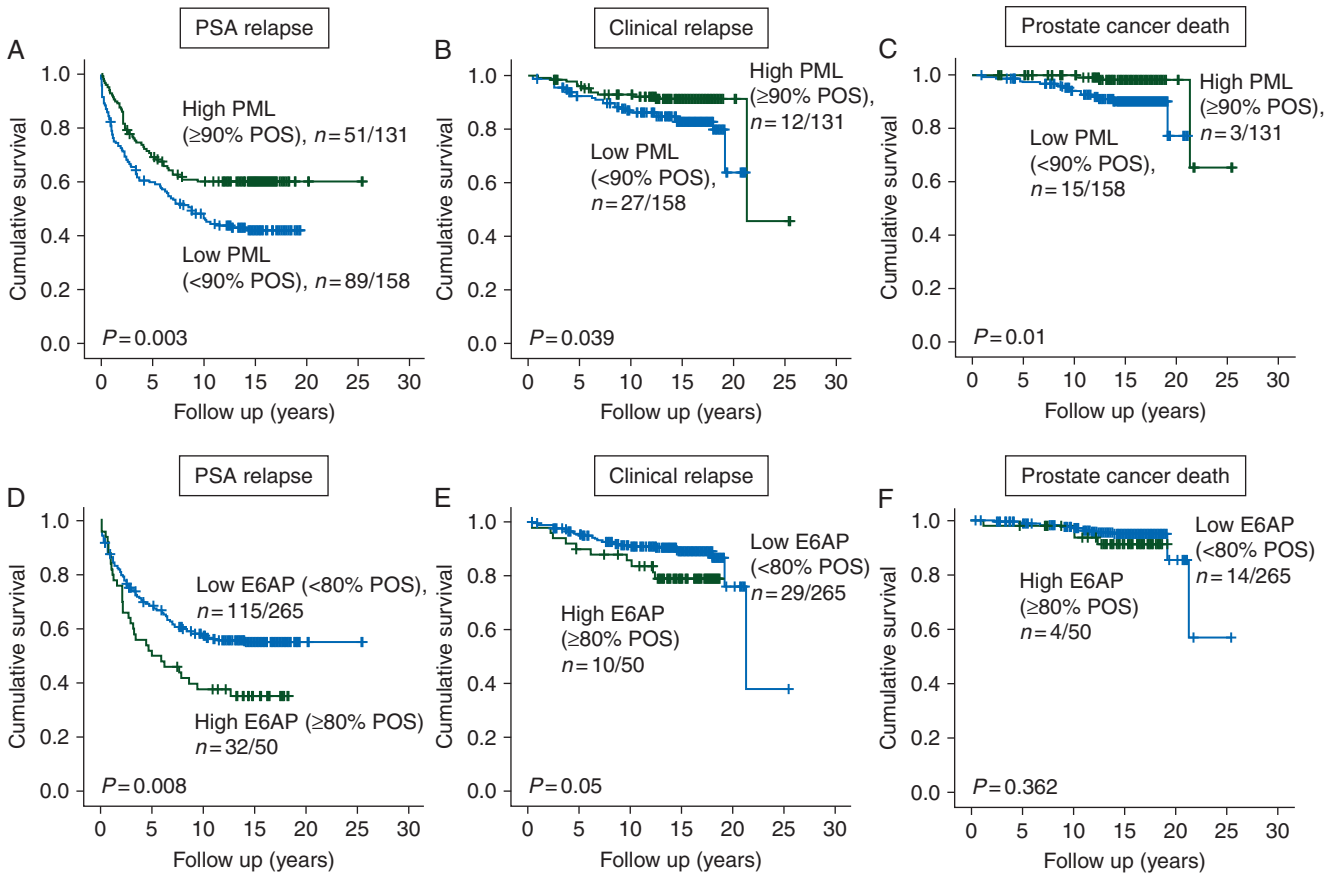

Figure 2. Kaplan–Meier analysis of PC progression for PML ($n = 298$) and E6AP ($n = 315$) expression in the phase II validation cohort. (A) PSA relapse high versus low PML expression. (B) Clinical relapse high versus low PML expression. (C). Prostate cancer-specific death high versus low PML expression. (D) PSA relapse high versus low ESAP expression. (E) Clinical relapse high versus low E6AP expression. (F) Prostate cancer-specific death high versus low E6AP expression. High PML defined as >90% average nuclear expression, low PML <90%. High E6AP defined as maximum nuclear expression across all cores ≥80% staining versus low E6AP maximum nuclear expression across all cores defined as <80%.

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a larger, independent phase II validation cohort with longer follow-up, which included clinical recurrence and disease -specific survival.

In the phase II validation cohort, PML was strongly expressed in 45% ($n = 131$) of PCs. E6AP expression was high in 16% ($n = 50$). Univariate analysis using a Cox proportional hazards model showed that EPE, Gleason score, pathologic stage and preoperative serum PSA levels were all significant predictors of biochemical and clinical relapse as well as PC specific death (supplementary Table S3, available at *Annals of Oncology* online).

χ^2 analysis of the association between PML expression and clinicopathologic parameters revealed significant associations between low PML expression and the use of adjuvant therapy ($P = 0.046$), higher pathologic stage ($P = 0.03$), higher preoperative PSA ($P = 0.01$) and SVI ($P = 0.01$), but not with EPE, higher Gleason score and margin positivity (all $P > 0.05$). Patients whose cancers had low PML expression had significantly poorer prognosis compared with those with high PML expression, as assessed by time to PSA relapse [HR 1.7, 95% confidence interval (CI) 1.18–2.35, $P = 0.004$], clinical relapse (HR 2.1, 95% CI 1.02–4.16, $P = 0.04$) and PC death (HR 6.4, 95% CI 1.47–28.13, $P = 0.01$) (supplementary Table S3, available at *Annals of Oncology* online; Figure 2A–C).

High E6AP expression was significantly correlated with higher Gleason score ($P = 0.01$) and SVI ($P = 0.03$), but not with use of adjuvant therapy, EPE, margin positivity, higher pathologic stage and higher preoperative PSA (all $P > 0.05$). Patients whose cancers had high nuclear expression of E6AP had significantly shorter time to PSA relapse (HR 1.7, 95% CI 1.14–2.49, $P = 0.009$) than those with low E6AP expression with a trend toward increased clinical relapse rate (HR 2.0, 95% CI 0.99–4.21, $P = 0.06$) (supplementary Table S3, available at *Annals of Oncology* online; Figure 2D and E). There was no association between E6AP expression and PC-specific death (HR 1.7, 95% CI 0.55–5.15, $P = 0.3$) (Figure 2F).

There was no significant interaction between PML and E6AP expression either with regards to biochemical relapse ($P = 0.997$) or clinical relapse ($P = 0.579$).

association between prognosis and combination model of E6AP/PML in the phase II validation cohort

To assess the prognostic significance of the combined PML and E6AP expression profile in the phase II validation cohort, cases were again stratified as high PML/high E6AP, high PML/low E6AP, low PML/high E6AP and low PML/low E6AP as in the phase I discovery cohort. Consistent with the findings in the phase I cohort, patients with low PML and high E6AP expression had the shortest biochemical relapse-free survival, ($P < 0.001$) (Figure 3A). The combination of low PML and high E6AP was a significant predictor of PSA relapse by univariate analysis (HR 4.5, 95% CI 2.2–9.0, $P < 0.001$) (supplementary Table S3, available at *Annals of Oncology* online). In multivariate analysis, low PML/high E6AP conferred a significant threefold risk of PSA relapse (HR 3.0, 95% CI 1.4–6.3, $P = 0.01$) (Table 1) when modeled with established clinical prognostic variates of adjuvant therapy ($P = 0.27$), EPE ($P = 0.39$), Gleason score ($P = 0.002$), margin positivity ($P = 0.81$), pathologic stage ($P = 0.14$), preoperative PSA ($P = 0.02$) and SVI ($P = 0.28$) (Table 1). The addition of PML and E6AP status substantially improved the multivariate model ($P < 0.0001$ for reduction in the log likelihood ratio).

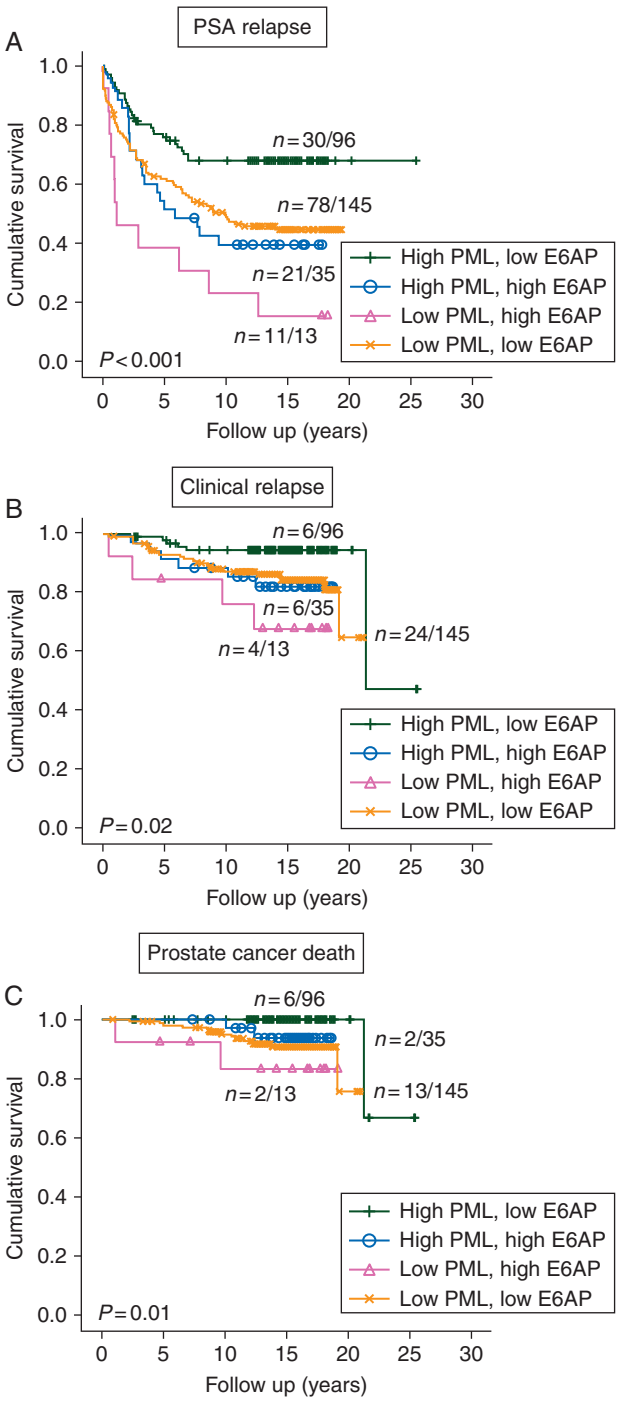

Figure 3. Kaplan–Meier analysis of PC progression for combined PML and E6AP expression in the phase II validation cohort ($n = 289$). (A) PSA relapse. (B) Clinical relapse. (C). Prostate cancer-specific death, stratified as high PML/high E6AP, high PML/low E6AP, low PML/high E6AP and low PML/low E6AP. High PML defined as >90% average nuclear expression, low PML <90%. High E6AP defined as maximum nuclear expression across all cores ≥80% staining versus low E6AP maximum nuclear expression across all cores defined as <80%.

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Table 1. Multivariate analysis using Cox proportional hazards model of PSA relapse, clinical relapse and prostate cancer-specific death of all prostate cancers in the validation cohort ($N = 318$)

Multivariate analysis		PSA relapse			Clinical relapse		
	Patient numbers	HR	95% CI	*P* value	HR	95% CI	*P* value
Combined PML and E6AP	289			**0.03**			0.22
High PML, low E6AP	96	–	–		–	–	
High PML, high E6AP	35	1.8	1.00–3.28	**0.049**	2.2	0.67–7.56	0.19
Low PML, low E6AP	145	1.6	1.02–2.48	**0.04**	2.3	0.87–6.18	0.09
Low PML, high E6AP	13	3.0	1.39–6.26	**0.01**	4.0	1.03–15.52	**0.045**
Adjuvant therapy (yes versus no)	318	1.3	0.82–2.02	0.27			
Extraprostatic extension (present versus absent)	318	0.7	0.31–1.59	0.39			
Gleason sum	315			**0.002**			0.86
7 versus ≤6		1.9	1.29–2.93	**0.002**	2.3	1.10–4.97	**0.03**
≥8 versus ≤6		2.1	1.29–3.49	**0.003**	1.8	0.71–4.35	0.22
Margin (positive versus negative)	316	1.2	0.69–1.61	0.81			
Pathologic stage (≥ pT3 versus ≤pT2)	318	2.0	0.81–4.70	0.14	2.5	1.16–5.20	**0.02**
Preoperative PSA (≤10 versus >10 ng/ml)	318	1.5	1.06–2.17	**0.02**			
Seminal vesicle invasion (present versus absent)	317	1.3	0.80–2.17	0.28			

Bold indicates a p value <0.05.

Patients with low PML/high E6AP had significantly shorter clinical relapse-free survival ($P = 0.016$) (Figure 3B). The combination of low PML and high E6AP was a significant predictor of clinical relapse (HR 6.9, 95% CI 1.86–25.86, $P = 0.004$) by univariate analysis (supplementary Table S3, available at *Annals of Oncology* online) and by multivariate analysis (HR 4.0, 95% CI 1.03–15.52, $P = 0.045$) when modeled with Gleason score ($P = 0.03$) and pathologic stage ($P = 0.02$) (Table 1). The addition of PML and E6AP status substantially improved the multivariate model ($P = 0.004$ for reduction in the log likelihood ratio). Only a limited multivariate analysis was possible due to the relatively small number of events.

While low PML expression was associated with shorter PC-specific survival (HR 6.4, 95% CI 1.47–28.13, $P = 0.01$), high E6AP expression was not (HR 1.7, 95% CI 0.55–5.15, $P = 0.3$). However, the combined expression profile of low PML/high E6AP was a significant predictor of PC-specific death ($P = 0.014$) (Figure 3C). Bivariate analysis demonstrated that PML expression was an independent predictor of PC-specific death (HR 5.2, 95% CI 1.2–22.76, $P = 0.03$) when modeled with Gleason score (Gleason 7 versus <6, HR 6.2, 95% CI 1.67–23.27, $P = 0.01$; Gleason score >8 versus 6, HR 5.9, 95% CI 1.41–25.01, $P = 0.02$). Multivariate analysis was not possible due to the small number of events.

discussion

This study demonstrates that low PML and high E6AP expression in localized PC is significantly associated with both biochemical and clinical relapse and is an independent predictor of poor prognosis on multivariate analysis. Furthermore, the association with biochemical relapse has been validated in an independent phase II cohort. In addition, low PML expression is associated with an increased rate of PC-specific death. The inclusion of disease-specific death and clinical recurrence end points is a major strength of this study.

Downregulation or loss of PML protein expression has been observed in a number of human cancers, including prostate carcinoma, consistent with PML being a cell growth and tumor suppressor [8, 10, 17]. In addition, adenovirus mediated overexpression of PML suppresses growth of PC cells *in vitro* and inhibits tumorigenicity in nude mice [18]. Although we did not observe complete loss of PML expression in our cohort, decreased PML expression was sufficient to predict for PC-specific death consistent with the findings that a single allelic loss of PML is sufficient to drive prostatic adenocarcinoma in a PTEN mouse model [9].

PML is essential for the formation and stability of the PML nuclear body (PML-NB) where PML serves as an essential scaffold and site of post-translational modifications of partner proteins [19]. PML and E6AP and were found to co-localize within PML-NBs, and loss of E6AP was associated with increased numbers of PML-NBs [6, 20]. Several ubiquitin ligases are responsible for PML downregulation in human cancers [reviewed in [21]], including E6AP, which is a direct E3 ligase of PML [6]. Cell lysates from prostate tissue of E6AP knockout mice showed elevated PML, while downregulation of E6AP using shRNA reduced PML ubiquitination [6].

As PML is vital to the integrity and function of the PML-NB, targeted degradation of PML by E6AP is likely to affect other known signaling pathways important in prostate carcinogenesis by disruption of PML-NBs such as p53-dependent induction of apoptosis. PML and p53 co-localize in PML-NBs, in a PML-dependent manner and PML is necessary for p53-dependent induction of DNA damage-induced apoptosis [5]. Prostate glands from E6AP null mice showed higher p53 expression, suggesting that p53 is a target of E6AP in the prostate gland [22]. Therefore, overexpression of E6AP may contribute to prostate carcinogenesis by regulation of p53 induced apoptotic pathways both by altering its protein expression and by proteasomal degradation of PML and disruption of PML-NBs (supplementary Figure S1, available at *Annals of Oncology* online).

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The present study is consistent with our previous biological data demonstrating that E6AP is an important regulator of PML protein stability [6], and strongly supports the hypothesis that downregulation of PML in PC is linked to deregulation of E6AP. E6AP levels were inversely correlated with PML and the combined profile of high E6AP/low PML was an independent predictor not only of PC progression, but also PC-specific death. Together these findings support the notion that pathways associated with PML destruction may contribute to PC progression and potentially a lethal phenotype. These findings should be assessed in a multicentre phase III cohort to further test their clinical utility as biomarkers of PC progression. In addition, E6AP may be a therapeutic target in PC, whose effects might be mitigated by restoration of PML protein levels.

funding

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disclosure

The authors have declared no conflicts of interest.

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