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p300 (Histone Acetyltransferase) biomarker predicts prostate cancer biochemical recurrence and correlates with changes in epithelia nuclear size and shape

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Abstract

Background—*p300* impacts the transcription of several genes involved in key pathways critical to PCa progression. Therefore, we evaluated the prognostic value of *p300* expression and its correlation with nuclear alterations seen in tumor cells in men with long term follow-up after radical prostatectomy (RP).

Methods—NCI Cooperative Prostate Cancer Tissue Resource tissue microarray cores of 92 RP cases (56 non-recurrences and 36 PSA recurrences) were utilized for the study. *p300* expression was assessed by quantitative immunohistochemistry and nuclear alterations in Feulgen-stained nuclei were evaluated by digital image analysis using the AutoCyte™ Pathology Workstation. Cox proportional hazards regression, Spearman's rank correlation, and Kaplan-Meier plots were employed to analyze the data.

Results—*p300* expression significantly correlated with nuclear alterations seen in tumor cells; specifically with circular form factor (p=0.012) and minimum feret (p=0.048). *p300* expression in high grade tumors (Gleason score ≥7) was significantly higher compared to low grade tumors (Gleason score <7) [17.7% vs. 13.7%, respectively, p=0.03]. TNM stage, Gleason score, and *p300* expression were univariately significant in the prediction of PCa biochemical recurrence free survival ($p \leq 0.05$). *p300* expression remained significant in the multivariate model (p=0.03) while Gleason score showed a trend toward significance (p=0.06). Patients with a Gleason score ≥7 and *p300* expression >24% showed the highest risk for PCa biochemical recurrence (p=0.002).

Conclusions—*p300* expression correlates with nuclear alterations seen in tumor cells and has prognostic value in predicting long-term PCa biochemical recurrence free survival.

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INTRODUCTION

Prostate cancer (PCa) is the second leading cause of cancer death among men in the United States, with an anticipated 218,890 newly diagnosed cases and nearly 27,000 deaths in 2007 (1). In a series of nearly 2,000 patients treated with radical prostatectomy at Johns Hopkins Hospital, 304 men developed PSA recurrence (15%) and were monitored without hormone therapy until demonstration of metastasis (2). Of these men, 34% developed distant metastases over a median period of 8 years from the time of the first postoperative PSA elevation (2). Han et al. (3) updated this study cohort, reporting 360 recurrences (17%) in 2,091 men with PCa. They used three preoperative or postoperative variables to create nomograms to assess biochemical recurrence-free survival probabilities. This study demonstrated the overall actuarial PSA-free survival probabilities at 5, 10, and 15 years to be 84%, 72% and 61%, respectively.

Clearly, the accumulation of repeated insults to the prostate over time through diet, infection, inflammation and aging results in a cascade of biological and molecular events which can result in malignancy. Therefore, PCa is a heterogeneous malignant disease where its' development and progression depends upon the biology of inflammation of the prostate as well as hereditary (genetic susceptibility), epigenetic and somatic gene defects. Many of these alterations are permanent and reflect transition to malignancy and progression to metastasis.

In the search for new molecular biomarkers to predict biochemical recurrence free survival in men with PCa, several potential serologic and histological biomarkers have been evaluated (4-8). At the tissue level, Gleason score and pathological stage are significant predictors of biochemical recurrence and metastasis (9,10). Further, investigators have used nuclear structure alterations i.e. change in nuclear size, shape, DNA content and chromatin structure, to predict stage, biochemical recurrence and metastasis in men with PCa (11-15). Recently, Seligson et al. (16) showed that the levels of acetylated histones correlate with increasing tumor grade and global histone modification pattern is able to identify disease subtypes with distinct risks of tumor recurrence in men with PCa.

There are numerous transcriptional coactivators involved in transcription and chromatin remodeling in androgen dependent and independent PCa. *p300*, a transcriptional coactivator that acetylates histones found in the nucleosome, has been shown to be differentially expressed in a number of tumors (17-19). Debes et al. (20) demonstrated that *p300* is involved in the IL-6-mediated transactivation of the androgen receptor (AR) in the absence of androgens in PCa cells. Others have shown a similar role of *p300* in the presence of androgens (21). In addition, Debes et al. (22) showed that p300 plays a key role in PCa epithelial cell proliferation.

The National Cancer Institute (NCI) engaged multiple institutions to prepare the Cooperative Prostate Cancer Tissue Resource (CPCTR) tissue microarrays (TMAs). We obtained TMAs from this resource that included tumor tissue from a unique patient cohort of 92 men with long term follow-up to assess biochemical recurrence after surgical treatment for PCa. Using the TMAs from this patient cohort, we recently demonstrated the ability of nuclear morphometry determined by digital image analysis to predict biochemical recurrence with an AUC-ROC of 80% compared to pathology with an AUC-ROC of 67% (23). Using the same patient cohort, we asked if expression levels of *p300*, which acetylates core histone residues, could predict biochemical recurrence free survival in men with PCa. We also evaluated the association between *p300* expression, nuclear structure alterations, Gleason score and pathologic stage.

MATERIAL AND METHODS

Prostate Tissue Specimens Dataset

The CPCTR-TMA is the result of a project funded by NCI RFA released in April, 2000 and four academic institutions [George Washington University Medical Center (Washington DC); Medical College of Wisconsin (Milwaukee, WI); New York University School of Medicine (New York, NY); and the University of Pittsburgh (Pittsburgh, PA)] were funded to form a national prostate cancer tissue resource, CPCTR. The resource is entirely funded by an individual Cooperative Agreement Grant from the NCI to each of the four participating sites (24,25). The CPCTR resource functions as a "virtual tissue bank" with a central database with all four participating sites working jointly with the NCI. Additionally, the methods for TMA construction employ a standardized protocol, a database containing standardized common data elements, and a supporting bioinformatics database with outcome results are also provided in a manuscript (26). Information about the NCI-CPCTR project and how to obtain these bioreagents can be found on the web at http://cpctr.cancer.gov.

NCI-CPCTR Patient Cohort

Pathological material from a total of 299 PCa chronologically consecutive radical prostatectomy patients were arrayed over four blocks with a single focus of tumor from each patient tumor represented in duplicate 0.6mm core spots. For determination of PSA recurrence, an algorithm was defined where the PSA values needed to increase >0.4 ng/dl (single value) or a PSA values >0.2 ng/dl with additional subsequent increasing values (27). The date of initial PSA rise (either the date of the single value >0.4 ng/ml or the date of the PSA value >0.2 ng/ml, before subsequent rising PSA values) was subtracted from the date of initial PSA nadir to determine the months to PSA recurrence. A total of 92 PCa cases (n = 56 non-recurrence and n = 36 recurrence) contained complete information for the study (Table 1).

Measurement of *p300* protein expression

Immunohistochemistry for *p300* expression in PCa was performed on formalin-fixed paraffin biopsy sections using a DAKO AutoStainer. After dewaxing and dehydration, sections were placed in a rice steamer with citrate buffer (pH 6.0) for twenty minutes. The 6 micron sections were pretreated with 0.3% hydrogen peroxide for ten minutes, washed with deionized water and phosphate buffer (PBS, pH 7.4), and incubated with 0.5% Triton X-100 and 0.5% milk in PBS for 5 minutes at room temperature. The DAKO EnvisionPlus IHC kit was used for immunostaining. Briefly, the sections were blocked with 5% milk in PBS containing 0.1% Triton X-100 for 20 minutes and then incubated with the specific antibody for this protein (Santa Cruz Biotechnology, Santa Cruz, CA) at pre-determined dilutions with PBS containing 0.5% milk and 0.1% Triton X-100 at room temperature for one hour in a humidified chamber. After washing, the sections were sequentially incubated with biotinylated Envision secondary antibody, streptavidin-HRP, and freshly prepared DAB chromogen substrate. The *p300* immunohistochemistry (IHC) stained tissues were counterstained with hematoxylin for one minute and mounted (supplementary figure 1).

The stained TMAs were scanned with a BLISS virtual slide scanner [Bacus Laboratories, Lombard, IL] at 40x magnification using the WebSlide® digital microscope slide format. This creates a database input file that lists information on every CPCTR-TMA core and provides an automatic link to the WebSlide® Net Viewer ActiveX Control (Bacus Labs, Lombard, IL) for a visual TMA core database. These BLISS virtual slide images were processed using a TMA score software program [Bacus Laboratories, Lombard, IL] that quantified *p300* expression by measuring percentage of tumor area positive for the *p300* antigen in each PCa case.

Measurement of Nuclear Alterations

Using ~5μm sections prepared from the TMA blocks, Feulgen DNA-staining was performed per the manufacturer's instructions (TriPath Imaging Inc, Burlington, NC). Next, a minimum of 125 intact, Feulgen-stained cancer nuclei were captured from the 0.6mm spots for each case using an AutoCyte Pathology Workstation (APW) [TriPath Imaging, Inc., Burlington, NC] and the QUIC-DNA software (11,12,28). The QUIC-DNA software calculated a total of 40 nuclear alterations [listed in Ref. (28)], including nuclear size, shape, DNA content and chromatin texture features (at a step size of one pixel), for each nuclei captured. For each case, the variance of each nuclear alteration was determined, thereby reducing the complexity of the nuclear alteration database to a single set of 40 variables for each case.

Statistical Methods

All data were analyzed using Stata™ v10.0 statistical analysis software (Stata Corporation, College Station, TX). A non-parametric k-sample chi-squared test for equality of medians was used to evaluate differences in the non-normally distributed ages. Wilcoxon's ranksum test was used to test for distribution differences and Fisher's exact test was used to test for differences in proportions between patients with and without biochemical recurrence. Correlations of *p300* expression with Gleason score, pathologic stage and nuclear alterations were evaluated using Spearman's rank correlation coefficients. Univariate Cox proportional hazards regression was used to identify significant prognostic factors for PCa biochemical recurrence. Ties were handled by the Breslow method, and the proportional hazard assumption was verified by examination of residual plots. We determined optimal cut-point for dichotomized *p300* expression data using classification and regression tree analysis. Kaplan-Meier survival plots were created to demonstrate the ability of the *p300* expression, pathologic stage and Gleason score to predict PSA recurrence free survival. Univariately significant variables were further considered in multivariate model. Statistical significance in this study was set as $p \leq 0.050$.

RESULTS

The demographic and pathologic information for the biochemical (PSA) recurrence and non-recurrence groups of PCa patients are shown in Table 1. This table shows that patients with biochemical recurrence tended to have higher Gleason scores and higher pathologic stages. The mean *p300* expression levels (% area positive for *p300* immunostaining) in the biochemical (PSA) recurrence and non-recurrence groups of men were 18.69% ± 9.03% and 14.40% ± 6.53%, respectively ($p = 0.009$).

The *p300* protein expression was significantly higher in high grade tumors (Gleason score ≥7: 17.70% ± 7.50%) compared to low grade tumors (Gleason score <7: 13.67% ± 7.83%) ($p = 0.03$). The mean *p300* expression in pathologic stage T2 and T3 patients was 15.48% ± 7.16% and 17.20% ± 9.01%, respectively ($p = 0.43$). We observed significant associations between *p300* protein expression and nuclear alterations seen in tumor cells in these CPCTR-TMA radical prostatectomy tissue samples. Of particular interest, it was noted that the circular form factor (rho = -0.26; $p = 0.012$) and minimum feret (rho = 0.21; $p = 0.048$) exhibited statistically significant correlations with *p300* protein expression. An assessment of other nuclear features, such as area (rho = 0.16; $p = 0.12$), excess of gray value (rho = 0.17; $p = 0.10$) and standard deviation of gray value (rho = -0.17; $p = 0.10$), showed a trend toward statistical significance for correlation with *p300* expression levels.

Gleason score showed significant correlation with several nuclear alterations seen in the tumor cells, including skewness of OD (rho = 0.24; $p = 0.0212$), excess of OD (rho = 0.22; p

= 0.0337), DNA ploidy (rho = 0.25; p = 0.0155), variance (rho = -0.20; 0.049), sum average-AC (rho = -0.23; p = 0.0280), sum variance-AC (rho = -0.31; p = 0.0023), cluster shade (rho = -0.28; p = 0.0064) and second diagonal moment (rho = -0.27; p = 0.0084). The pathologic stage also showed significant correlation with several nuclear alterations seen in tumor cells including skewness of gray value (rho = 0.22; p = 0.0323), DNA ploidy (rho = 0.28; p = 0.0078), variance (rho = -0.22; p = 0.0397), cluster shade (rho = -0.23; p = 0.0249), and second diagonal moment (rho = -0.21; p = 0.0440).

Upon univariate analyses, *p300* expression as a continuous variable was a significant prognosticator (p = 0.021) for PCa biochemical recurrence. A dichotomized population for *p300* expression was then defined with an optimal cutoff of 24% (85^{th} percentile), specifically patients were categorized as having either low (≤24.0%) or high (>24.0%) *p300* expression. Dichotomized pathologic stage, Gleason score and *p300* expression were univariately significant (Table 2) for prediction of biochemical recurrence. However, when these three variables were considered together in a multivariate Cox proportional hazards model, only *p300* expression was significant (Table 2). Figures 1A, 1B, and 1C show Kaplan-Meier survival curves for prediction of PCa biochemical recurrence free survival using pathologic stage, Gleason score, and *p300* expression, respectively.

Additionally, we stratified the NCI-CPCTR patients based upon Gleason score and *p300* expression status. Table 2 and Figure 1D show the ability of Gleason score and *p300* expression status combined to predict PCa biochemical recurrence free survival. Because there were only 4 patients with a Gleason score <7 & high *p300* expression, this subcategory was merged with cases having Gleason score ≥7 & low *p300* expression for these analyses. Patients with Gleason score ≥7 & high *p300* protein expression had a significantly higher risk of PCa biochemical recurrence (p = 0.002) (Table 2 & Figure 1D).

DISCUSSION

The nucleosome, i.e. the fundamental unit of chromatin organization, is composed of 146 base pairs of DNA wrapped in 1.65 turns around an octamer of the four core histones, H2A, H2B, H3, and H4 (29). Chromatin remodeling directly influences the activity of DNA as it relates to transcription, replication, and recombination and is regulated by two highly conserved mechanisms, post-translational modifications of histone residues (e.g. acetylation, methylation) and ATP-dependent nucleosome position reorganization.

Seligson et al. (16) showed that PCa cells have global level modifications in individual histones and that altered patterns of these modifications are predictive of clinical outcome. Polycomb group protein EZH2 causes methylation of histone H3 lysine 9 and histone H3 lysine 27 and its overexpression is associated with poor prognosis (30-32). The *p300*/CBP histone acetyltransferase (HAT) causes acetylation of all four core histone residues of the nucleosome. Hence, modifications of the nucleosome's net charge by neutralizing the positive charge of lysine ε-amino group alters DNA-histone interactions (cross-talk), which then modify transcriptional activity of the cell (33). Also, other nucleosome assembly proteins functionally interact and augment the activity of *p300*/CBP, and the presence of core histones appears to regulate the interaction between *p300* and key nucleosome assembly proteins that establish various chromatin organization states, impacting nuclear structure (nuclear importins and Lamins A & C) and functions (i.e. cell proliferation, DNA repair etc.) (34).

The *p300* HAT domain is essential for physiological processes of cell proliferation, differentiation and apoptosis (35-37). Mammals lacking *p300* gene exhibit defects in neurulation, cell proliferation and heart development (38). In addition to histone

modifications, p300/CBP can acetylate and modify activity of several non-histone proteins [reviewed in Ref.(39)] including p53 (40,41), HMG I(Y) (42), HMG14 (43), GATA-1 (44,45), c-Myb (46), E2F-1 (47), EKLF (48), ACTR, TIF2, SRC-1 (49), Tat (50,51), TCF (52), TFIIE and TFIIF (53). Further, p300/CBP depletion causes cyclin E down-regulation (17), which in association with CDK2, controls DNA replication, centrosome duplication and histone gene expression (54).

Additionally, *p300*/CBP is required for effective ligand-dependent gene activation by nuclear receptor (55). The *p300* protein acetylates the androgen receptor (AR) at three lysine residues in its DNA binding domain (21). Point mutations in these AR acetylation sites selectively prevent androgen-induction of androgen responsive genes, hampers coactivation of the AR by SRC-1, p300, Tip60 and Ubc9, and results in a 10-fold increase in the binding of the co-repressor NCoR (56). High levels of AR are associated with aggressive clinicopathologic parameters and decreased PCa recurrence free survival (57). Furthermore, IL-6 cytokine mediated transactivation of AR-dependent genes in the absence of androgens requires *p300* HAT activity, implicating *p300* in PCa progression (20).

The role of *p300* in PCa molecular pathogenesis is an important event that impacts transcription of several genes involved in key pathways critical to PCa recurrence and progression. Hence, our observation on the prognostic clinical value of *p300* protein expression and its potential role in transcription and effects on chromatin organization provide confirmation of results from other laboratories (16,20-22,37,38,43,55,58) and extend our understanding of its role in PCa progression.

In conclusion, *p300* expression in PCa tissue may be a useful biomarker for predicting progression and is one step in a series of finding additional tissue biomarkers that will improve early prognostic decisions on PCa patient management.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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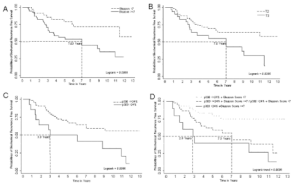

Figure 1.
Kaplan-Meier plots showing ability of Gleason score (A), pathological stage (B), p300 expression (C), and Gleason score & p300 combined (D) to predict biochemical recurrence free survival. Logrank test and Logrank trend test were used to test equality of survivor functions across two groups and three ordered groups respectively.

Table 1

Prostate Cancer Patients Demographics

Variable Description	No Biochemical Recurrence (N = 56)	Biochemical Recurrence (N = 36)	p value
Median Age in Years (range)	65.5 (47-76)	64 (42-77)	0.274[a]
Pathologic stage (%)			
T2a	9 (16.1)	2 (5.6)	
T2b	32 (57.1)	17 (47.2)	0.010[b]
T3a	13 (23.2)	9 (25.0)	0.028[c]
T3b	2 (3.6)	8 (22.2)	
Gleason Score (%)			
5	6 (10.7)	0 (0)	
6	21 (37.5)	10 (27.8)	0.024[b]
7	27 (48.2)	23 (63.9)	0.106[c]
8	1 (1.8)	2 (5.6)	
9	1 (1.8)	1 (2.8)	
Race (%)			
White	51 (91.1)	32 (88.9)	0.574[b]
Black	1 (1.8)	3 (8.3)	0.505[c]
Others	3 (5.3)	1 (2.8)	
Unknown	1 (1.8)	0 (0)	

[a] Median test

[b] Wilcoxon ranksum test

[c] Fisher's exact test

Table 2

Cox proportional hazards regression

Variable	N	Univariate		Multivariate		Stratification based upon Gleason Score & *p300* status	
		HR (95% CI)	P value	HR (95% CI)	P value	HR (95% CI)	P value
TNM Stage							
T2	60	1.00	0.034	1.00	0.170		
T3	32	2.03 (1.06 - 3.92)		1.60 (0.82 - 3.15)			
Gleason Score							
<7	37	1.00	0.013	1.00	0.060		
≥7	55	2.54 (1.22 - 5.30)		2.07 (0.97 - 4.43)			
p300							
<=24%	78	1.00	0.013	1.00	0.032		
>24%	14	2.47 (1.21 - 5.03)		2.19 (1.07 - 4.47)			
Gleason*p300*							
1¶	33					1.00	
2§	49					2.81 (1.19 - 6.66)	0.018
3*	10					5.18 (1.88 - 14.30)	0.002

¶Gleason Score <7 and p300 ≤24%

§Gleason Score <7 and p300 >24% / Gleason Score ≥7 and p300 ≤24%

*Gleason Score ≥7 and p300 >24%