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DNA Ploidy as a Surrogate to Biopsy Gleason Score for Preoperative Organ vs. Non-Organ Confined Prostate Cancer Prediction

Sumit Isharwal, M.D.¹, M. Craig Miller, B.S.², Jonathan I. Epstein, M.D.¹, Leslie A. Mangold, M.S.¹, Elizabeth Humphreys, B.S.¹, Alan W. Partin, M.D., Ph.D.¹, and Robert W. Veltri, Ph.D.¹

¹ The James Buchanan Brady Urological Institute, The Johns Hopkins University School of Medicine, Baltimore, MD

² Quakertown, PA

Abstract

Background—Transformation of normal epithelium into cancer cells involves epigenetic and genetic changes and modifications in nuclear structure and tissue architecture. Nuclear morphometric alterations and clinicopathologic features were evaluated for organ vs. non-organ confined PCa prediction.

Methods—Of the 557 prospectively enrolled patients, 370 had complete information and sufficient tumor area for all evaluated parameters (281 organ-confined and 89 non-organ confined). Digital images of Feulgen-DNA stained nuclei were captured from biopsies using the AutoCyte™ imaging system and nuclear morphometric alterations were calculated. Logistic regression analysis with bootstrap resampling was used to determine factors important for differentiation of the two groups and to generate models for organ vs. non-organ confined PCa prediction.

Results—Several nuclear morphometric features were significantly altered and could differentiate organ and non-organ confined disease. DNA ploidy was the most important factor among the significant nuclear morphometric features and was the second most important factor for organ vs. non-organ confined PCa prediction when considered with tPSA, cPSA, f/tPSA, biopsy Gleason score and clinical stage. The combination of DNA ploidy with clinical stage, tPSA and biopsy Gleason score showed an improvement of 1.5% in the AUC-ROC compared to the combination of clinical stage, tPSA and biopsy Gleason (73.97% vs. 72.43%). The use of DNA ploidy in lieu of the biopsy Gleason score in each preoperative model evaluated resulted in equivalent or improved organ vs. non-organ confined PCa prediction.

Conclusions—DNA ploidy can serve as a surrogate biomarker that has the potential to replace biopsy Gleason scores for organ vs. non-organ confined PCa prediction.

Keywords

Prostate cancer; nuclear morphometry; biopsy; pathology; prediction

Author for correspondence: Dr. Robert W. Veltri, Associate Professor, James Buchanan Brady Urological Institute, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21287, Tel: 410 614 6380, Fax: 410 614 3695, E-mail: E-mail: rveltri1@jhmi.edu.

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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 186,320 newly diagnosed cases and 28,660 deaths in 2008¹. The majority of men with clinically localized PCa are treated with radical prostatectomy (RP), which provides excellent cancer control². However, there is no consensus regarding the optimal management of locally advanced PCa³. The preoperative ability to assess pathologic stage permits better counseling of patients as well as more appropriate selection of therapy and consideration of novel clinical trials for those with more advanced disease.

Diamond and associates⁴ were the first to employ nuclear morphometric alterations (nuclear roundness factor) for prediction of outcomes in PCa patients with stage B1 and B2 disease (Whitmore-Jewett staging). Subsequently, other investigators^{5–8} have used nuclear morphometric alterations to predict pathologic stage and prognosis for PCa patients. Nuclear morphometric alterations measured by computer assisted image analysis detect abnormal DNA content representing large scale chromosomal alterations and reflect genetic instability in tumor cells^{9,10}. The current study, utilizing a single 5µm section of biopsy tissue from a prospectively accrued patient cohort obtained at a single institution, assesses the ability of nuclear morphometric alterations and preoperative clinicopathologic parameters to predict organ vs. non-organ confined PCa after RP in 370 men.

MATERIALS AND METHODS

Patients Cohort

Of the 557 patients enrolled in a prospective PCa study between October 1998 and January 2000 scheduled for RP at the Johns Hopkins Hospital (JHH), 370 patients had complete information for all evaluated parameters, including serum samples for evaluation of total PSA (tPSA), complex PSA (cPSA), free PSA (fPSA) and free/total PSA (f/tPSA), sufficient area of cancer on the biopsy section to determine the Gleason score on H&E section and a sequential biopsy section stained with Feulgen from which a minimum of ~125 intact prostate cancer nuclei had been captured using a computer assisted image analysis system. Patients were excluded from the cohort on the basis of microwave tissue over processing (which can degrade the ability to measure nuclear morphometric alterations; n = 39), preoperative hormone therapy (n = 6), no biopsy material available (n = 45), no cancer observed on re-cut biopsy material provided for the study (n = 56), no RP done (n = 9), PSA molecular forms missing from the evaluation (n = 22), or when patients declined to participate in study (n = 10). All of the men had a minimum of a sextant biopsy that sampled tissue cores from the apex, mid and base of the right and left lobes of the prostate gland and none of them underwent neoadjuvant hormonal therapy. All underwent RP at JHH with routine PSA follow-ups that consisted of assessment at 3 months postoperatively and annually thereafter. Pathological interpretation of the Gleason score on the biopsy tissue was performed by pathologists in the department of pathology at JHH. Table 1 summarizes the pre-treatment information available in this patient cohort.

Digital Measurement of Nuclear Morphometric Alterations

As noted above, using ~5µm tissue sections from the prostate biopsies, Feulgen DNA-staining was performed per the manufacturer's instructions (TriPath Imaging Inc, Burlington, NC). Next, a minimum of ~125 intact, Feulgen-stained nuclei were captured from the cancer areas of each biopsy using an AutoCyteTM Pathology Workstation [TriPath Imaging, Inc., Burlington, NC] and the QUIC-DNA software⁵. The QUIC-DNA software calculated 40 different nuclear morphometric alterations for each nucleus captured [listed in Ref 5]; including nuclear size, shape, DNA content and chromatin texture features (at a step size of one pixel). We utilized the variance of the nuclear morphometric features, determined using the nuclei captured for

each case, as our input variables, thereby reducing the complexity of the database to a single set of 40 variables for each case⁵.

To determine DNA ploidy, the image system was first calibrated using a control slide which contained Feulgen stained rat hepatocytes. Using densitometry, rat hepatocytes representing diploid (2C) and tetraploid (4C) states are measured, and the QUIC-DNA software generates two reference points to define a calibration curve which is then used for the calculation of DNA ploidy. Once again, we used the variance of the DNA ploidy measurements in the nuclei captured from each case to generate a continuous DNA ploidy variance variable for each case that was used in our statistical analyses.

Statistical Analysis

All data was analyzed using StataTM v10.0 statistical analysis software (Stata Corporation, College Station, TX). Univariate logistic regression analysis was performed first to determine the independent variables significant in the differentiation of organ and non-organ confined PCa. Next, using the univariately significant variables, 500 bootstrap samples were used to create multivariate logistic regression models using backward stepwise selection at a stringency of $P_z \leq 0.05$ “bootstrap resampling”). The number of times each variable was selected using the 500 bootstrap samples was determined and only variables that were selected in $>50\%$ of the bootstrap models were selected for the final multivariate modeling. Areas under the receiver operator characteristic (AUC-ROC) curves for the ability of the logistic regression models to differentiate between organ and non-organ confined PCa were calculated. Correlation of nuclear morphometric alterations with Gleason score, clinical stage and all PSA derivatives were evaluated using Spearman’s rank correlation coefficients.

RESULTS

The demographic, clinical and pathologic information for organ and non-organ confined PCa are shown in Table 1. There were significant differences between the two groups for all clinical and pathologic variables assessed with the exception of race.

Univariate logistic regression analyses of nuclear features showed that area, perimeter, circular form factor, maximum feret, minimum feret, feret X, feret Y, DNA ploidy, transmission and intensity were significantly altered and differentiated between the organ confined and non-organ confined PCa groups (Table 2). Next, a bootstrap resampling procedure employing 500 replications was used to perform backward stepwise logistic regression analyses. The goal was to identify the most important nuclear morphometric alteration(s) for differentiating organ and non-organ confined tumors. Each variable in the replicated models was counted with significance level of $P_e \leq 0.01$ for a variable that entered the model and $P_r \leq 0.05$ (variable selection cut-off) to remain in the model. Inclusion frequencies for area, perimeter, circular form factor, maximum feret, minimum feret, feret X, feret Y, DNA ploidy, transmission and intensity were 21.2%, 19.6%, 37.6%, 37.4%, 10.6%, 17.8%, 7.8%, 59.0%, 16.8% and 22.6% respectively. DNA ploidy had the highest inclusion frequency among all univariately significant nuclear morphometric features for differentiating organ and non-organ confined PCa.

All PSA molecular forms, i.e. tPSA, cPSA, fPSA and f/t PSA, were univariately significant for the differentiation of organ and non-organ confined tumors (Table 2). The AUC-ROC of cPSA was not significantly higher than tPSA (68.12% vs. 67.81% respectively, $p = 0.48$) for differentiating both groups. Biopsy Gleason score (stratified as <7 , 7 and >7) and clinical stage were also significant for differentiating organ and non-organ confined tumors in the cohort (Table 2). There was no advantage to further stratifying the biopsy Gleason score 7 group into 3+4 ($n=38$), and 4+3 ($n=22$) categories because there was an equal proportion of non-organ

confined tumors in these two categories (50% in both) (AUC-ROC: 61.81% vs. 61.81%; $p = 1.00$).

Next, we combined the most important nuclear morphometric measurement, DNA ploidy, with the univariately significant clinicopathologic parameters (i.e. tPSA, fPSA, cPSA, f/tPSA, biopsy Gleason score and clinical stage) in backward stepwise logistic regression analyses using 500 iterations of bootstrap resampling. Each variable in the bootstrapped model was counted with significance level of $P_e \leq 0.01$ for a variable to enter the model and $P_r \leq 0.05$ to remain in the model. fPSA was dropped from the model because of collinearity with other PSA molecular forms and their derivatives. Inclusion frequencies of clinical stage, biopsy Gleason score, DNA ploidy, tPSA, cPSA and f/tPSA were 92.2%, 62.0%, 82.2%, 80.4%, 24.8% and 16.0% respectively. Clinical stage was the best and DNA ploidy was second best predictor of organ and non-organ confined PCa. Among the PSA forms assessed, tPSA had the highest inclusion frequency for differentiation of organ and non-organ confined PCa.

Of note, the odds ratio (OR) with 95% confidence interval (95CI) for clinical stage, biopsy Gleason score, DNA ploidy, tPSA, cPSA, fPSA and f/tPSA were 1.90 (1.36 – 2.67), 3.09 (1.86 – 5.11), 12.95 (3.02 – 55.53), 1.17 (1.10 – 1.24), 1.19 (1.11 – 1.28), 1.80 (1.26 – 2.56) and 0.96 (0.92 – 0.99) respectively. Using a cut-off criterion of at least 50% inclusion frequency; clinical stage, DNA ploidy, tPSA and biopsy Gleason score were selected for a final multivariate logistic regression model which resulted in an AUC-ROC of 74.0% and the OR (95CI) of 1.83 (1.27 – 2.34), 9.91 (2.0 – 48.95), 1.15 (1.08 – 1.22) and 1.87 (1.08 – 3.24) respectively. Thus, combination of DNA ploidy with clinical stage, tPSA and biopsy Gleason score showed a modest improvement of 1.5% in the AUC-ROC compared to the multivariate model of clinical stage, tPSA and biopsy Gleason [AUC-ROC : 72.4%, OR (95CI): 1.83 (1.28 – 2.63), 1.15 (1.08 – 1.22), 2.05 (1.19 – 3.54) respectively].

DNA ploidy showed a significant correlation with biopsy Gleason score ($\rho = 0.211$, $p < 0.0001$) and no correlation with clinical stage ($\rho = 0.066$, $p = 0.206$), tPSA ($\rho = 0.057$, $p = 0.276$), cPSA ($\rho = 0.061$, $p = 0.242$), fPSA ($\rho = 0.028$, $p = 0.588$) and f/tPSA ($\rho = -0.037$, $p = 0.478$). To further explore the importance of DNA ploidy, we replaced biopsy Gleason score with DNA ploidy for making preoperative organ vs. non-organ confined PCa predictions and AUC-ROC results showed equivalent or slightly improved prediction in each model (Figure 1).

DISCUSSION

Identification of parameters predictive of pathologic stage of PCa has become a major focus in the area of prostate cancer biology. The commonly used Partin Tables¹¹ rely exclusively on clinicopathologic parameters i.e. tPSA, clinical stage and biopsy Gleason score to predict pathologic stage. Numerous simple and complex parameters have been evaluated for prediction of pathologic stage; location of tumor, volume of cancer in needle biopsy, percentage of positive cores, percentage of biopsy tissue with cancer, Gleason pattern 4/5, perineural invasion, vascular/lymphatic invasion, angiogenesis, DNA ploidy, reverse transcriptase polymerase chain reaction detection of circulating PSA-positive cells, fluorescence *in situ* hybridization for chromosomal anomalies, and radiographic imaging methods^{12–15}.

One of the limitations of biopsy Gleason score can be inter-observer and intra-observer reproducibility; exact agreement is reported in 43–78% of cases, and agreement within ± 1 score is reported in 72–87% of cases^{16–18}. Dr. Donald Gleason noted that on reexamination exact duplication of Gleason scores occurred in only approximately 50% cases, and were within ± 1 point in approximately 85% cases¹⁹. Under-grading of prostate adenocarcinoma is the most common problem, occurring in up to 45% of cases, with the over-grading of carcinoma

occurring in up to 32% of cases^{16–18}. However, Fine and Epstein²⁰ recently reported improvement in Gleason score reporting during last decade, which can most likely be attributed to increasing the number of cores that are being sampled from the prostate, comprehensive educational efforts via courses at meetings, online websites and the medical literature.

Nuclear morphometric alterations can be accurately measured after capturing only ~125 Feulgen stained nuclei from “an expert pathologist selected” tumor area. Such approach is objective and reproducible; hence reduces human error in making precise outcome predictions. The approximate cost of measuring nuclear morphometric alterations using the AutoCyte imaging system was about \$150.00 and a case took about 15 minutes to complete. In this study, we showed that nuclear morphometric measurements of area, perimeter, circular form factor, maximum feret, minimum feret, feret X, feret Y, DNA ploidy, transmission and intensity are significantly altered and capable of differentiating between organ and non-organ confined PCa (Table 2). DNA ploidy had the highest inclusion frequency among all the univariately significant nuclear morphometric alterations for differentiating organ and non-organ confined PCa.

DNA ploidy was the second best predictor for differentiating organ and non-organ confined tumors when combined with tPSA, cPSA, f/tPSA, biopsy Gleason score and clinical stage in a multivariate logistic regression model. Integration of DNA ploidy with clinical stage, tPSA and biopsy Gleason score model showed a modest improvement of 1.5% in AUC-ROC. Replacing biopsy Gleason score with DNA ploidy for preoperative models showed equivalent or slightly improved organ vs. non-organ confined PCa prediction (Figure 1). Use of tPSA was a better predictor for differentiating organ and non-organ confined tumors than cPSA and f/tPSA in our cohort, and hence it should not be replaced by cPSA, as suggested by some investigators, for stage prediction^{21,22}. Clearly, a limitation of our study is the lack of quantitative biopsy pathology information, which was not routinely determined, that could have been used to compare their predictive ability with nuclear morphometric alterations for differentiating organ and non-organ confined tumors.

Another limitation of our study is the lack of long term follow-up information available for a majority of patient cohort in order to assess potential prognostic value of nuclear morphometric alterations detected in the needle core biopsy specimens. However, our group^{5,6} has demonstrated the prognostic value of nuclear morphometric alterations detected in RP specimens for biochemical recurrence, distant metastasis, and death in men with PCa. Further, our group²³ showed that there are significant alterations in nuclear structure between and within Gleason grading patterns 3, 4, and 5. Recently, Makarov et al.²⁴ used quantitative nuclear structure alterations to predict conversion to unfavorable biopsy pathology during surveillance, based upon the Epstein criteria²⁵ use for selection of Expectant Management cases of PCa. Since alterations in nuclear structure are so important, identification of proteins that can modify nuclear chromatin organization, such as p300 expression, and others that have been shown to play an important role in PCa cell proliferation and PCa progression, is important^{26,27}. Recently, our group demonstrated that valproic acid (histone deacetylase inhibitor) causes dose- and time-dependent changes in nuclear structure in PCa cells both *in vitro* and *in vivo*²⁸. Clearly, the use of quantitative nuclear structure alterations and the molecular mechanisms which cause such changes provide a foundation for continued research in this area that can eventually change the management of PCa patients.

In conclusion, nuclear morphometric alterations provide an objective and reproducible quantitative measurement of nuclear structure and DNA ploidy feature can serve as a surrogate biomarker that has the potential to replace biopsy Gleason scores for organ vs. non-organ confined PCa prediction.

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Figure 1.

Figure 1(A–D): DNA ploidy as surrogate to biopsy Gleason scores for preoperative organ vs. non-organ confined prostate cancer prediction.

Table 1

Prostate Cancer Patients Demographics (N = 370)

Variable Description	Organ Confined Tumor(N = 281)	Non Organ Confined Tumor(N = 89)	p-value
Mean age in years [Median]	58.21 ± 6.03 [58.5]	59.75 ± 6.06 [60.3]	0.028 *
Mean tPSA (ng/ml) [Median]	5.95 ± 3.39 [5.32]	8.83 ± 5.47 [7.49]	<0.0001 *
Mean cPSA (ng/ml) [Median]	5.10 ± 3.03 [4.53]	7.70 ± 4.92 [6.49]	<0.0001 *
Mean fPSA (ng/ml) [Median]	0.85 ± 0.57 [0.72]	1.13 ± 0.82 [0.92]	0.0009 *
Mean f/t PSA ratio [Median]	15.5 ± 7.1% [14.7%]	13.6 ± 5.8% [13.1%]	0.0432 *
Clinical stage			
T1c	210 (74.7)	48 (53.9)	0.001 **
T2a	51 (18.2)	26 (29.2)	
T2b/c	20 (7.1)	15 (16.9)	
Biopsy Gleason Score (%)			
<7	247 (87.9)	57 (64.0)	<0.0001 **
7	30 (10.7)	30 (33.7)	
>7	4 (1.4)	2 (2.3)	
Race (%)			
White	268 (95.4)	86 (96.6)	1.000 **
Black	8 (2.9)	2 (2.3)	
Others	5 (1.7)	1 (1.1)	

* Wilcoxon's rank-sum test

** Fisher's exact test

Table 2
Univariate Logistic Regression Analysis – Significant Predictors of Organ vs. Non-Organ Confined Prostate Cancer

Nuclear Morphometric Alteration	%AUC-ROC	p-value	Clinicopathologic parameters	%AUC-ROC	p-value
DNA Ploidy	62.91	0.001	tPSA	67.81	<0.0001
Circular Form Factor	59.57	0.015	cPSA	68.12	<0.0001
Maximum Feret	62.65	0.001	fPSA	61.63	0.001
Intensity	58.47	0.05	f/tPSA	57.11	0.027
Area	60.04	0.014	Biopsy Gleason Score	61.81	<0.0001
Perimeter	61.71	0.005	Clinical stage	60.89	<0.0001
Feret X	59.25	0.01			
Transmission	56.61	0.035			
Minimum Feret	60.01	0.02			
Feret Y	60.48	0.004			