Correlation of HOXD3 Promoter Hypermethylation with Clinical and Pathologic Features in Screening Prostate Biopsies

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BACKGROUND. Molecular markers that can discriminate indolent cancers from aggressive ones may improve the management of prostate cancer and minimize unnecessary treatment. Aberrant DNA methylation is a common epigenetic event in cancers and HOXD3 promoter hypermethylation (H3PH) has been found in prostate cancer. Our objective was to evaluate the relationship between H3PH and clinicopathologic features in screening prostate biopsies.

METHODS. Ninety-two patients who underwent a prostate biopsy at our institution between October 2011 and May 2012 were included in this study. The core with the greatest percentage of the highest grade disease was analyzed for H3PH by methylation-specific PCR. Correlational analysis was used to analyze the relationship between H3PH and various clinical parameters. Chi-square analysis was used to compare H3PH status between benign and malignant disease.

RESULTS. Of the 80 biopsies with HOXD3 methylation status assessable, 66 sets were confirmed to have cancer. In the 14 biopsies with benign disease there was minimal H3PH with the mean percentage of methylation reference (PMR) of 0.7%. In contrast, the HOXD3 promoter was hypermethylated in 16.7% of all cancers and in 50% of high risk tumors with an average PMR of 4.3% ($P = 0.008$). H3PH was significantly correlated with age ($P = 0.013$), Gleason score ($P = 0.031$) and the maximum involvement of the biopsy core ($P = 0.035$).

CONCLUSIONS. H3PH is associated with clinicopathologic features. The data indicate that H3PH is more common in older higher risk patients. More research is needed to determine the role of this marker in optimizing management strategies in men with newly diagnosed prostate cancer. *Prostate 74:714–721, 2014.*
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KEY WORDS: HOXD3; hypermethylation; molecular markers

INTRODUCTION

Prostate cancer is one of the most frequently diagnosed cancers in males worldwide. Incidence rates are particularly high in developed countries largely due to the widely applied prostate-specific antigen (PSA) screening [1,2]. However, the benefit of the PSA-led diagnosis is controversial as it fails to reliably distinguish indolent cancer from aggressive ones, often leading to over diagnosis and potentially over treatment [3–5]. Most commonly used risk stratification criteria takes three parameters (PSA, clinical tumor stage, and Gleason score) into account in assessing patients' risk of having aggressive disease [6]. More recent efforts have attempted to incorporate tumor volume assessments such as percentage of positive biopsy cores and cancer volume in biopsy cores [7]. However, even the best prognosis indicator, Gleason score, can be biased by the accuracy and sampling limitations [8]. Clearly, new markers are needed to aid in difficult clinical management decisions.

Aberrant DNA methylation is a common epigenetic event in prostate cancer [9]. DNA hypermethylation in various genes was found to be associated with clinicopathologic factors indicative of poor prognosis in prostate cancer patients, suggesting a possible role in cancer progression [10]. Although the mechanism underlying DNA hypermethylation is not well understood, it may present an ideal prognostic marker in distinguishing indolent from aggressive disease [11]. GSTP1 promoter hypermethylation has been shown to occur in >80% of prostate cancers making it useful in distinguishing benign from malignant disease [12].

The homeobox (HOX) family of transcription factors has been shown to play a key role in the regulation of embryonic development, cellular proliferation and differentiation, and angiogenesis [13]. Global genomic CpG island microarray profiling from prostatectomy tissues of various grades identified several genes being hypermethylated; among the 100 most significantly hypermethylated genes, 27 belong to HOX gene family [14]. Specifically, analysis of HOXD3 promoter showed that HOXD3 DNA hypermethylation was associated with increasing tumor grade and stage and may help predict the risk of biochemical recurrence [15].

To be clinically useful, prognostic biomarkers should be readily assessable by minimal invasive procedures. As hypermethylated HOXD3 appears to be a potential candidate for predicting prostate cancer's aggressiveness, we examine HOXD3 methylation profile in prostate biopsy samples from patients undergoing a screening prostate biopsy.

MATERIALS AND METHODS

Patient Cohort and Pathology

Ninety-two patients who underwent a screening prostate biopsy at Georgetown University Hospital between October 2011 and May 2012 were included in this study. Prostate biopsy specimens were examined by Bostwick Laboratories® pathologists to determine Gleason grade/score and tumor burden. The maximum involvement of biopsy core (MIBC) was defined as the highest percentage of cancer presence in one biopsy core from all sampled cores [16]. The specimen with the highest MIBC was also subject to HOXD3 hypermethylation analysis at Bostwick Laboratories®. HOXD3 methylation status could not be assessed in eight patients. The eighty patients with conclusive HOXD3 methylation results were included in the analyses. Institutional IRB approval was obtained for this review.

HOXD3 Methylation

Genomic DNA was extracted from formalin-fixed, paraffin-embedded biopsy tissue samples and amplified by quantitative methylation-specific polymerase chain reaction (MSP) using probes and primers specific for methylated HOXD3 (AI Biotech, Richmond, VA) as described previously by Kron et al. [15]. Specifically, Bostwick Laboratories® identified abnormal tissue from patient's tissue blocks. The microdissected tissue was subjected to DNA extraction and MSP was performed to determine the methylation status. On average, less than 20% of each dissected sample contained normal tissue. MSP was performed with the 7500 Fast Real-time PCR System (Applied Biosystems) by way of fluorescent detection using primer/probe previously published [15]. The real-time PCR readout was normalized to a house-keeping gene (β-actin) in each sample to obtain the relative quantity of the target (HOXD3 methylation). The calculated percentage of methylated reference (PMR) was used to determine the degree of methylation with PMR ≤9% deemed negative and PMR >9% positive based on results of previous studies [15,17].

Statistical Analysis

Multivariate analysis of the pathological diagnosis with other clinical factors such as T-stage, Gleason scores, prostate volume and HOXD3 hypermethylation was performed using logistic regression. Chi-square (χ^2) analysis was used in analyzing categorical variables such as Gleason scores and race, as well as in analyzing the percent positive rate of HOXD3 methylation among D'Amico risk stratification groups. Kruskal–Wallis rank

test was used to analyze the percentage of positive cores (PPC) and MIBC rate between HOXD3 positive and negative groups; ANOVA and Student's *t*-test were used to analyze PMR between different stratified groups. Mann–Whitney *U*-test was used to analyze the correlation of age, Gleason scores, PSA, prostate volumes, race, clinical T-stage, PPC with HOXD3 methylation. Statistical analyses were performed with IBM SPSS Statistics 21 (Armonk, NY).

RESULTS

Table I showed the characteristics of ninety-two patients who underwent a screening prostate biopsy at Georgetown University Hospital between October 2011 and May 2012. The median age of the cohort was 65-year-old (range, 43–83). Among the 66 patients who had pathologically confirmed prostate carcinoma, 11 of them were positive for HOXD3 hypermethylation. In the benign group, no HOXD3 hypermethylation was detected in 14 non-cancer patients (17.5%) which

TABLE I. Patient Characteristics

	% Patients (N = 92)
Median Age (*range*)	65 y/o (*43–83*)
Race	
White	53.75%
Black	33.75%
Other	12.50%
Median PSA (*range*)	6.05 ng/ml (*0.9–114.4*)
T-Stage	
≤T1c	72.50%
T2a	11.25%
T2b	8.75%
T2c	5.0%
>T2c	2.5%
Gleason	
ASAP + HGPIN	17.5%
6	45.0%
7	27.5%
8	5.0%
9	5.0%
Medium Percentage of Positive Cores (*range*)	25.0% (*7.1–100*)
Medium Maximal Core Involvement (*range*)	37.5% (*2–100*)
D'Amico Risk Stratification	
Benign	17.50%
Low	38.75%
Intermediate	31.25%
High	12.50%

PSA, Prostate Specific Antigen; ASAP, atypical small acinar proliferation; HGPIN, high grade prostatic intraepithelial neoplasia.

TABLE II. HOXD3 Methylation in Prostate Cancer Patients

	P-values (univariate analysis)	P-values (multivariate analysis)
Age	0.013[a]	0.016[b]
Race	0.422	0.880
PSA	0.087	0.057
T-stage	0.098	0.052
Gleason	0.031[a]	0.052
Percentage of positive cores (PPC)	0.058	0.040[b]
Maximal involvement of biopsy core (MIBC)	0.035[a]	0.026[b]

[a]Significant in univariate analysis.
[b]Significant in multivariate analysis.

consisted of three with atypical small acinar proliferation (ASAP) and 11 with high grade prostatic intraepithelial neoplasia (HGPIN). Multivariate analysis showed HOXD3 hypermethylation correlated with the tissue diagnosis ($P < 0.0005$). Reflecting the demographics of the metropolitan Washington area, Caucasians comprised of about half of the cohort (53.75%; Table I). The correlation between race and HOXD3 methylation was not significant (Table II).

The PMR for prostate cancer was significantly higher than that for benign disease (4.3% vs. 0.7%, respectively, $P < 0.009$) in this cohort. Among the HOXD3 (+) patients, the average PMR for patients over 70-year-old was higher compared to patients who were younger (Fig. 1a, Table II). The median age of patients with HOXD3 hypermethylation and those without was significantly different, with the former being 72-year-old and the latter 64-year-old ($P = 0.013$; Fig. 1b, Table II). Additionally, among the patients with HOXD3 methylation, age stratification showed a trend of rising prevalence of this marker with age (Fig. 2b).

The median PSA of this cohort was 6.05 ng/ml (range, 0.9–114.4; Table I). Among this cohort, the PSA medians for the benign, non-HOXD3 hypermethylated cancer and HOXD3 hypermethylated cancer subgroups were 5.1 ng/ml (range, 1.1–8.4), 6.3 ng/ml (range, 0.9–50.5), and 7.2 ng/ml (range, 3.7–114.4). While the average PMR was highest among patients with PSA > 10 ng/ml (Fig. 3a), there was no statistical significance between the HOXD3 (+) and HOXD3 (−) subgroups regarding their PSA levels (Fig. 3b, Table II).

The majority of patients included in this study had T1c tumor (72.5%; Table I). Both patients with tumors greater than T2c were positive for HOXD3 methylation. No statistical significance was found between
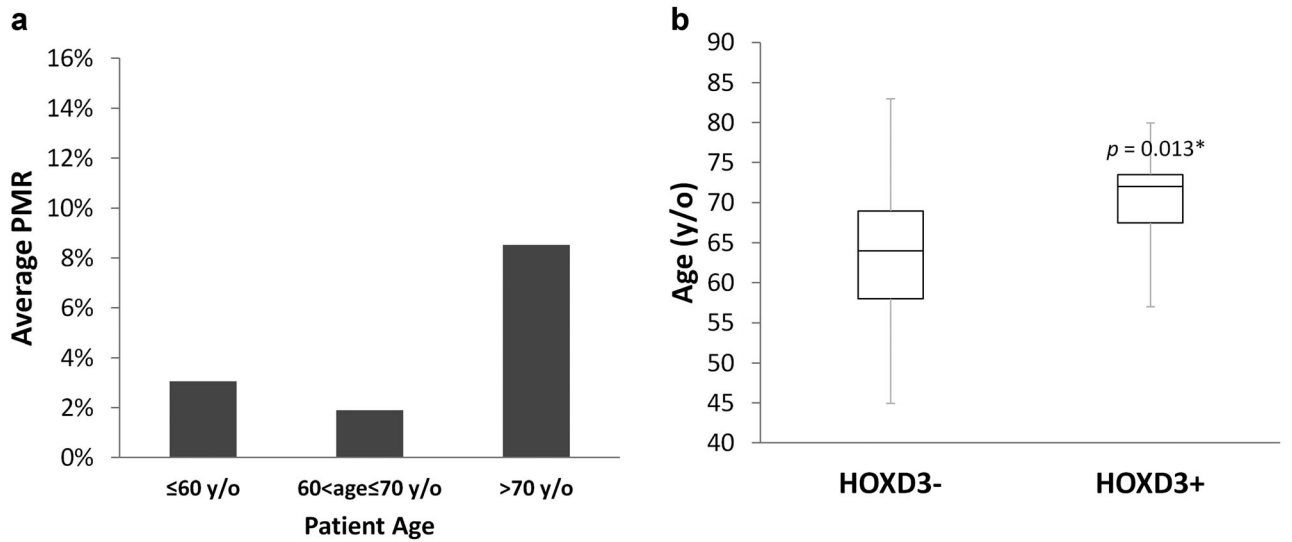
Fig. I. **a**: Average percentage of methylated reference (PMR) in three age groups; **(b)** Age distribution of patients stratified by the status of HOXD3 hypermethylation.

T-stages and HOXD3 methylation (Table II). Among those with prostate cancer, 45% patients were with Gleason 6 tumors (Table I). The median Gleason scores for HOXD3 (−) and HOXD3 (+) patients were 6 and 7, respectively ($P = 0.031$; Table II). Tumors with Gleason score ≥ 8 had the highest average PMR (Fig. 4a); the percent positive rate of HOXD3 methylation correlated positively with the Gleason scores, with 8.3%, 22.73%, and 37.5% positive for Gleason 6, 7, and ≥ 8 patients, respectively ($P = 0.014$; Fig. 4b). When the Gleason 7

tumors were further divided into $3 + 4$ and $4 + 3$ subgroups, the HOXD3 percent positive rate remained higher in the more aggressive $4 + 3$ tumors than in $3 + 4$ ones (data not shown).

Among the 66 cancer patients, the median percentage of positive cores (PPC) was 25% (range, 7.1–100%) with the median MIBC as 37.5% (range, 2,100%; Table I). Grouping by the status of HOXD3 methylation, the PPC was higher in the HOXD3 (+) population, but it was not statistically significant ($P = 0.058$;
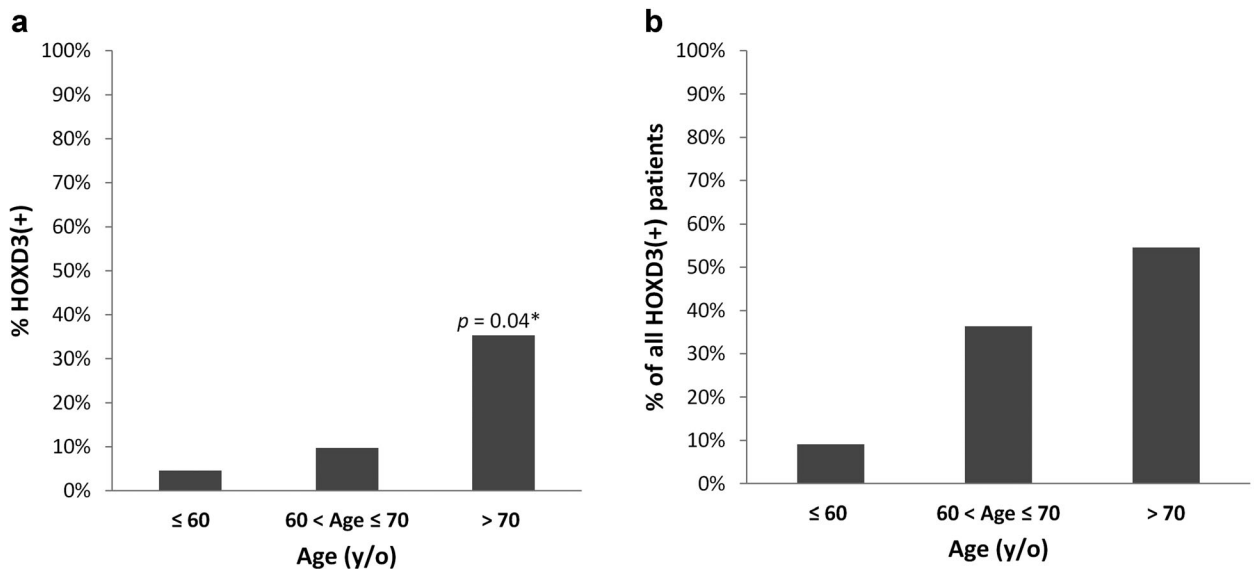

Fig. 2. **a**: HOXD3 hypermethylation rate in each age group; **(b)** age distribution of HOXD3 positive patients.
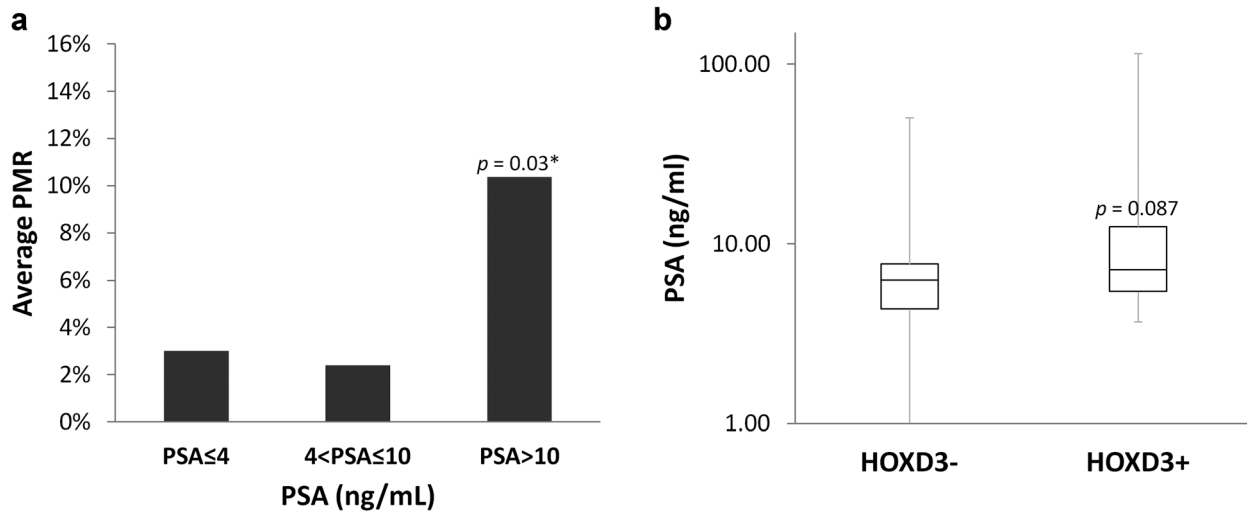
Fig. 3. HOXD3 hypermethylation and PSA. **a**: Stratified by PSA levels; (**b**) Stratified by HOXD3 methylation.

Fig. 5a). However, the MIBC is significantly higher among HOXD3 (+) population with the median MIBC 55% compared with 15% of the HOXD3 (−) population ($P = 0.035$; Fig. 5b).

In this cohort, 38.75% were with low risk and 31.25% with intermediate risk tumor according to D'Amico risk criteria (Table I). The average PMR was highest in high risk patients and also HOXD3 hypermethylation was found to be most prevalent among high risk patients (50%; $P = 0.003$; Fig. 6a and b).

DISCUSSION

HOX genes are a group of transcription factors that regulate morphogenesis and cell differentiation [18–

20]. Dysregulation of HOX genes was described in a wide variety of cancers such as leukemia, melanoma, cancers of lung, kidney, ovary, prostate, and breast [21–24]. Recent analysis of HOXD3 promoter hypermethylation in prostatectomy specimens revealed that it was associated with clinical and pathologic factors and could potentially serve as a tumor progression marker [15]. Studies of HOXD3 in cancer cell lines suggested possible mechanisms for cellular motility and invasiveness [25–27], but how HOXD3 expression contributes to prostate tumorigenesis or tumor progression is not clear. In this study, we examined the correlation of HOXD3 methylation with different clinical and pathologic parameters in our cohort.

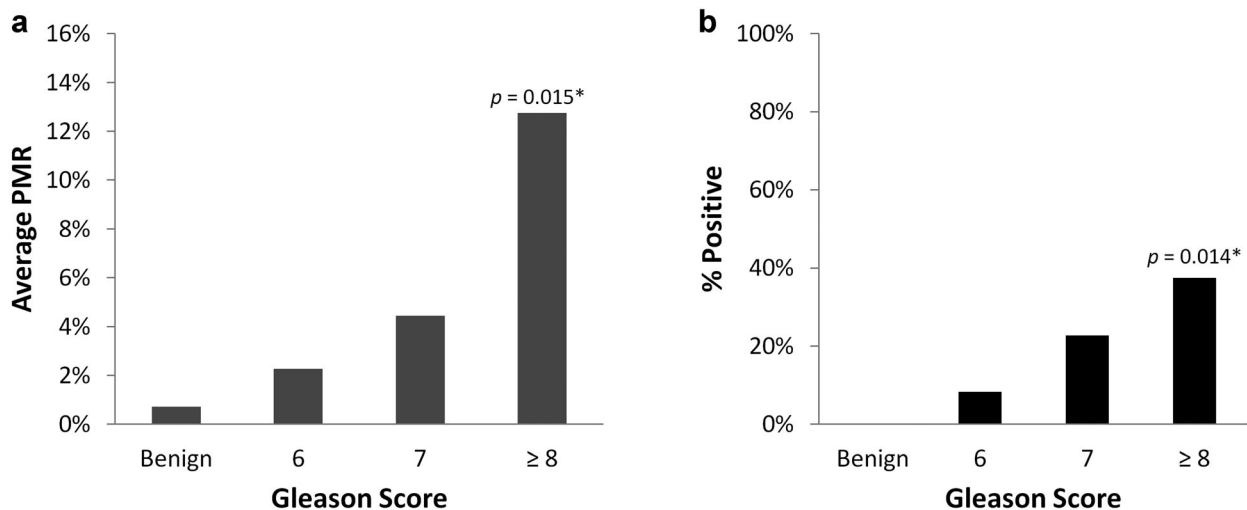In this cohort, HOXD3 methylation was positively correlated with increasing patient age. Age-related

Fig. 4. HOXD3 hypermethylation and Gleason Scores. **a**: Average PMR; (**b**) Percent positive rate for HOXD3 methylation.
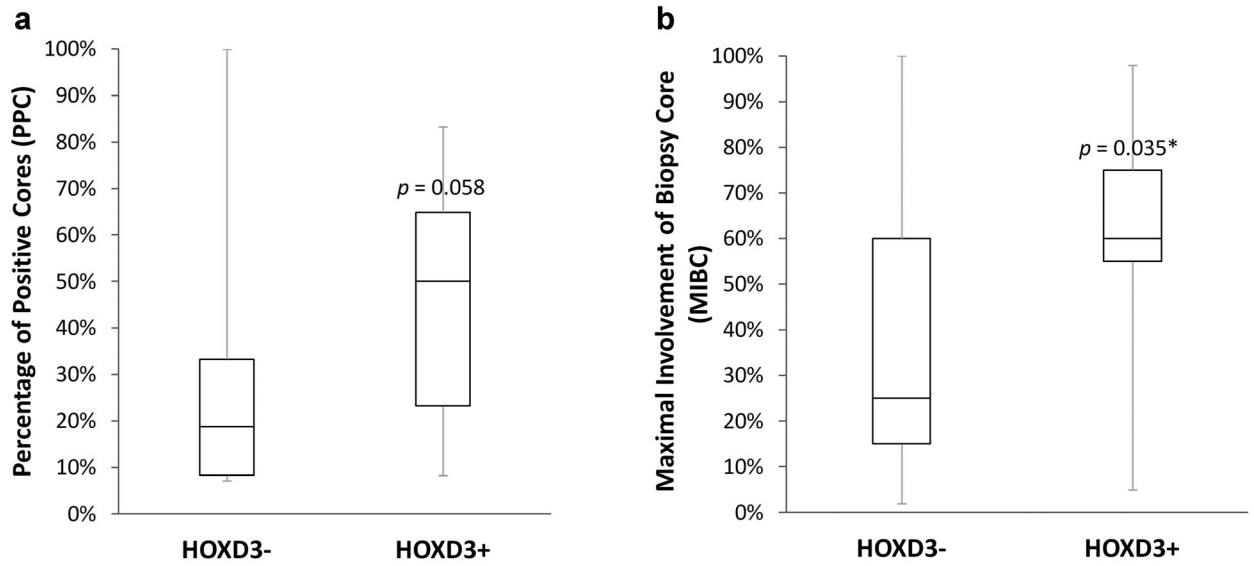
Fig. 5. HOXD3 hypermethylation and biopsy cores. **a**: Percentage of positive cores (PPC); (**b**) Maximal involvement of biopsy core (MIBC).

changes in methylation have been reported for a variety of genes [28–32]. For example, CpG island methylation rate increases for estrogen receptor α (ESR1) as the patient ages, and the severity of the promoter methylation is positively correlated with Gleason scores [32]. Older age is a well known risk factor for prostate cancer. There are two possible explanations for this finding. One is that HOXD3 methylation increases in normal prostate with age regardless of the presence of cancer. Alternatively, high grade prostate cancer, which represents

approximately half of the newly diagnosed disease among patients older than 75-year-old [33], is characterized by dysregulation of genome-wide methylation [34]. Although in our cohort, the median age of the benign group (63.5-year-old) is similar to that of the HOXD3 (−) cancer patients (64-year-old), and both are significantly younger than the HOXD3 (+) group (72-year-old, range, 57–80-year-old), the complete lack of HOXD3 methylation detected in benign group (age range, 43–77-year-old) makes the first reason less likely, namely, this
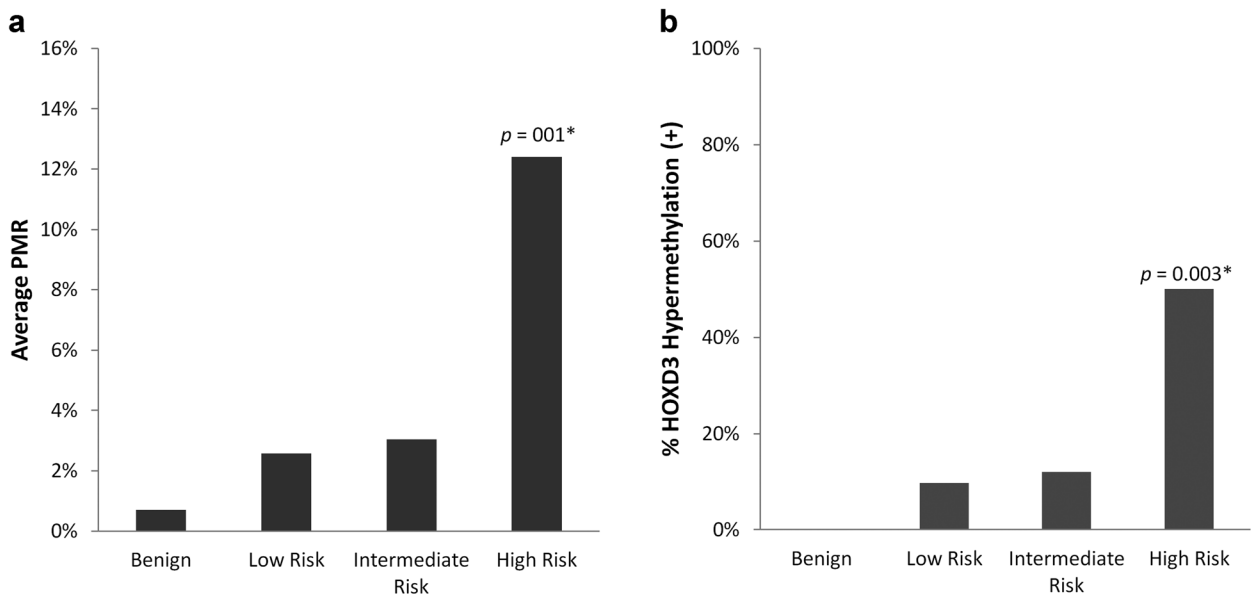

Fig. 6. D'Amico risk stratification: (**a**) average PMR in each risk group; (**b**) percent positive rate for HOXD3 methylation in each risk group.

epigenetic event is unique to carcinogenesis rather than simply due to aging. Whether HOXD3 methylation and down regulation drive the aggressiveness of the cancer or it is a consequence of other transformation events in prostate cancer cells is yet to be determined.

The prevalence of hypermethylated HOXD3 increased with increasing Gleason score in this cohort, and not present in benign biopsy samples. The strikingly positive correlation of Gleason scores and percent positive rate of HOXD3 methylation in this biopsy analysis echoed results of the studies done with prostatectomy tissue samples [15,17]. The increase in HOXD3 methylation with cancer progression indicates that HOXD3 could serve as a biomarker for risk assessment.

High PPC and MIBC are predictive for early biochemical failure for newly diagnosed patients [16,35]. We examined the PPC and MIBC in our cohort stratified by their HOXD3 methylation status, and found the HOXD3 (+) group with higher PPC and MIBC (albeit only with MIBC did it reach statistical significance; Fig. 5a and b), suggesting the potential need of adjuvant treatment for HOXD3 (+) patients, similar to those with high PPC and/or MIBC.

The limitations of this study include a relatively low incidence of HOXD3 positivity. There were also a modest but noteworthy number of non-assessable cases due to limited biopsy tissue. In addition, longer follow-up is necessary to determine the impact of HOXD3 expression on biochemical recurrence following treatment. Nevertheless, this study, which used readily accessible biopsy tissues, was able to yield similar findings from studies with radical prostatectomy specimens [15].

In conclusion, we showed that HOXD3 methylation in prostate biopsy samples may serve as a useful adjunct to the PSA to identify men at risk for developing aggressive prostate cancer. Further research is underway to determine whether this epigenetic event can be detected in easily accessible biological specimens such as serum and urine. Since one of the biggest challenges in managing prostate cancer is to distinguish the slow-growing tumors from the aggressive ones, the incorporation of bio-markers such as HOXD3 methylation offers a new parameter in risk evaluation that can potentially spare many patients of unnecessary treatment. The detection of HOXD3 methylation in prostate biopsy samples by PCR-based method also potentiates an easier and less-invasive clinical venue to detect this marker.

ACKNOWLEDGMENT

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