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ORIGINAL ARTICLE

Evaluation of the clinical value of the newly identified urine biomarker HIST1H4K for diagnosis and prognosis of prostate cancer in Bulgarian patients

D. Kachakova[1], A. Mitkova[1], E. Popov[2], O. Beltcheva[1], A. Vlahova[3], T. Dikov[3], S. Hristova[3], V. Mitev[1], C. Slavov[2], R. Kaneva[1]

[1]*Department of Medical Chemistry and Biochemistry and Molecular Medicine Center, Medical University- Sofia, Sofia;* [2]*Department of Urology, Medical University –Sofia, Clinic of Urology, Alexandrovska University Hospital, Sofia;* [3]*Department of General and Clinical Pathology, Alexandrovska University Hospital, Sofia, Bulgaria*

Summary

Purpose: *Searching for diagnostic and prognostic biomarkers for prostate cancer (PC) is main public health priority. DNA methylation in body fluids is a stable, easily detectable and promising PC biomarker. The major advantages of urine-based assays are their noninvasive nature and the ability to monitor PC with heterogeneous foci. The aim of this study was to determine the diagnostic value of the recently identified candidate PC biomarker HIST1H4K.*

Methods: *We investigated DNA methylation of HIST1H4K in urine samples from 57 PC patients, 29 controls with benign prostatic hyperplasia (BPH) and 50 young asymptomatic men (YAM) by MethyLight real-time PCR.*

Results: *The frequency of HIST1H4K promoter hypermethylation significantly discriminated PC patients from YAM (AUC =0.763; 95% CI 0.672-0.839; p<0.0001), but did not show any statistical difference between PC patients and BPH controls (AUC=0.513, 95% CI 0.402-0.622; p=0.8255). HIST1H4K could not outperform the prostatic specific antigen (PSA) in our sample (AUC=0.785; 95% CI 0.679-0.870; p<0.0001). Methylation of HIST1H4K showed significant correlation with aging (r=0.5418; p<0.0001), but with no other clinicopathological characteristics.*

Conclusion: *The results suggest that the promoter hypermethylation of HIST1H4K is rather due to aging than related to prostate carcinogenesis. To elucidate this observation analysis of larger samples is needed.*

Key words: *aging, HIST1H4K, methylation, prostate cancer, urine biomarker*

Introduction

PC is the most commonly diagnosed malignancy in men and represents the 2nd leading cause of cancer-related deaths in the Western countries [1-3]. In Europe there are 2.6 million new PC cases per year. This cancer constitutes 11% of all malignant diseases among males in Europe and 9% of cancer-related deaths in the European Union [4]. According to the Bulgarian National Cancer Registry report the estimated new PC cases for 2009 in Bulgaria were 1666 that is 9.2% of all malignant diseases. In terms of incidence and mortality PC is the 3rd and 4th leading cancer in Bulgaria, respectively [5]. It is estimated that the incidence of PC and breast cancer increases by 31% in each decade [6].

Early and specific diagnosis of PC is very difficult due to the absence of reliable tumor specific biomarkers. The current diagnosis of PC relies on transrectal prostatic biopsy made due to high PSA levels and/or abnormal prostate after digital rectal examination [7]. PSA estimation that has shown reasonable sensitivity for detection of incipient cancer and prediction of response to treatment increased the number of diagnosed PC cases in the past two decades [8]. Disadvantage of PSA as biomarker is its low specificity, which results in huge numbers of unnecessary biopsies, uncertainty and discomfort of patients, and overburden

Correspondence to: Darina L. Kachakova MSc, PhD. Molecular Medicine Center, Zdrave 2 street, 1431, Sofia, Bulgaria.
Tel: + 359 291722 14, E-mail: darina_kachakova@mmcbg.org or darina.kachakova@gmail.com
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of the health system. More than 10% of patients with negative first biopsy are diagnosed with PC on repeat biopsy [9].

Because of the poor specificity of current methods, new robust biomarkers are needed to improve PC detection and management. Many researchers have focused on the discovery of appropriate PC biomarkers in body fluids. Urine, with less complexity than serum and relatively high thermodynamic stability, is readily available and can be used to detect either exfoliated cancer cells or secreted products. The main advantages of urine-based biomarker tests are their non-invasive nature and ability to monitor PC with heterogeneous foci. New tests will help not only early diagnosis but also the identification of aggressive tumors for radical therapy [10].

DNA methylation is the best studied epigenetic mechanism and occurs in mammals mostly at cytosines within CpG dinucleotides. It is thought to promote epigenetic gene silencing. CpG dinucleotides are commonly found in clusters called CpG islands, which are preferentially seen at the 5' end (promoter, untranslated regions and exon 1) of human genes [11].

The link between methylation at the N5-position of cytosine in CpG sequences and cancer development is well established. Cancer formation is accompanied by dramatic changes in the cellular methylation profile such that global demethylation of the genome occurs in parallel with CpG hypermethylation at specific genes strongly linked to their transcriptional inactivation. Genes that are hypermethylated in PC have different functions - there are hormone receptors (*RAR-B, ESR1 and ESR2*); genes involved in cell-cycle control (*CCND2, CDKN2A*) or in repair and avoidance of DNA damage (*GSTP1, MDR1, MGMT*); signal transduction genes (*DAB2IP, DAPK, EDNRB, RASSF1*); cell adhesion genes (*CD44, CDH2, LAMA3, LABM3*); inflammation response genes (*PTGS2*) and many other tumor suppressor genes. Methylation may also be associated with tumor progression [12]. The most frequent epigenetic mark in PC is *GSTP1* promoter hypermethylation, which has been found in cancerous and precancerous prostate tissues [13], as well as in body fluids like urine, plasma/serum and ejaculate of PC patients [14-21].

Since epigenetic alterations are highly prevalent and occur early in prostate carcinogenesis they have been currently studied as stable, easily detectable and promising PC biomarkers [11].

Searching for genes with promoter hypermethylation in PC has advantages over the other markers as DNA methylation is relatively stable in body fluids in comparison to changes in RNA and proteins and is located in certain regions in comparison to DNA mutations. In addition, DNA methylation is a positive signal that may be identified among normal DNA, even when present in small amounts [22]. Therefore, promoter methylation of genes in DNA from body fluids can be used as reliable marker for improving diagnosis and monitoring of patients with PC.

Earlier studies have shown that PC-specific DNA methylation can be detected in body fluids such as urine and plasma and can be used as noninvasive biomarker in PC diagnosis [23-25]. The loss of glutathione-S-transferase P (*GSTP1*) expression as a result of promoter hypermethylation is the most common (>90%) molecular alteration found in PC [26,27]. Many studies reported promising results with high specificity and low or moderate sensitivity in detecting *GSTP1* methylation in urine samples collected after prostatic massage [18,28], but there are conflicting results in terms of predictive accuracy [23,24,29]. Hoque et al. found a combination of 4 genes (*p16, ARF (p14), MGMT* and *GSTP1*) that detected 87% of PC with specificity of 100% [24]. Roupert et al. did not observe correlation between methylation of p14 or p16 and PC but they suggested another gene combination panel (*GSTP1, RASSF1A, RARB* and *APC*) with 86% sensitivity and 89% specificity [16]. The 4 gene panel of *RASSF2, HIST1H4K, TFAP2E* and *GSTP1* suggested by Payne et al. did not significantly improve the test performance over that of the single biomarkers. The area under the curve of the 4 tested genes varied from 0.64 to 0.69 for discriminating patients with positive from those with negative biopsies [15].

In the current study we analysed the promoter hypermethylation of *HIST1H4K* in urine from PC patients and controls. The gene was selected as a promising candidate on the basis of previous data suggesting differences in urine DNA methylation between PC cases and controls [15]. In order to test the usefulness of the epigenetic changes of this gene as biomarker in prostate cancer while controling for the effect of age two different control groups were used - patients with BPH and YAM.

Methods

Sample collection

Urine samples of patients with PC and controls were collected as part of a project MU28/2010, funded

Table 1. Participants' demographic and clinical characteristics

Characteristics	*PC patients N=57 N (%)*	*Controls with BPH N=29 N (%)*	*YAM N=50 N (%)*
Age (years)			
Median	66.7	65.8	24.4
Range	51-85	49-83	17-34
T stage			
T1	19 (33.93)		
T2	23 (41.07)		
T3	13 (23.21)		
T4	0 (0)		
Unknown	2 (3.51)		
Gleason score			
Median	9		
Range	5-10		
5-6	11 (19.3)		
7	25 (43.86)		
8-10	19 (33.33)		
Unknown	2 (3.51)		
PSA serum levels (ng/ml)			
Median	43.38	9.75	
Range	2.3-136	1.79-31.30	
>2	0 (0)	1 (3.45)	
2-4	1 (1.75)	2 (6.9)	
4-10	9 (15.79)	14 (48.27)	
>10	41(71.93)	11 (37.93)	
Unknown	6 (10.53)	1 (3.45)	

PC: prostate cancer, BPH: benign prostatic hyperplasia, YAM: young asymptomatic men

by the Medical Science Council, Medical University - Sofia. Urine samples from patients with PC and controls with BPH were collected at the Clinic of Urology, Alexandrovska University Hospital, Medical University–Sofia, after digital rectal examination. Urine from YAM was collected from volunteers, mainly students at the Medical University - Sofia.

All subjects were ethnic Bulgarians. Each participant provided written informed consent according to protocols approved by the Ethics Committee of the Medical University - Sofia. Patients from the Clinic of Urology underwent annual screening and biopsies were recommended to participants with abnormal digital rectal examination and/or an increase in PSA. The Gleason scoring system was used to classify tumors as low-grade (Gleason score ≤7) or high-grade (Gleason score >7). Controls with BPH were matched to the cases by ethnicity and age within 5-year categories. Our final study sample comprised 57 PC patients, 29 BPH patients and 50 YAM. The demographic and clinical characteristics of the included participants are shown in Table 1.

Isolation of DNA from urine and bisulfite conversion

Isolation of DNA from urine was performed with ZR Urine DNA Isolation Kit™ (Zymo Research) according to the manufacturer's instructions. Approximately 200 ng of extracted DNA were bisulfite converted using EZ DNA Methylation-Gold™ Kit (Zymo Research) following the conditions suggested by the manufacturer.

MethyLight analysis

Methylation status of promoter region of *HIST1H4K* was determined by previously described technique MethyLight [30-35].

Briefly, MethyLight is a sodium-bisulfite-dependent, quantitative, fluorescence-based, real-time PCR method to sensitively detect and quantify DNA methylation in genomic DNA. MethyLight relies on methylation-specific priming combined with methylation-specific fluorescent probing. This combination of methylation-specific detection principles results in a highly methylation-specific detection technology, with an accompanying ability to sensitively detect very low frequencies of hypermethylated alleles.

In MethyLight analysis two types of reactions are used: 1) MethyLight reactions: bisulfate-converted DNA is a substrate. The forward/reverse primers and probe are specific for methylated DNA and are also specific for bisulfate-converted DNA; 2) The bisulfite specific control reaction (ALU-C4): measures the loading of bisulfate-converted DNA. These reactions are not methylation-specific but are specific for bisulfate-converted DNA [34]. Consequently, bisulfate-converted DNA from each sample and from M.SssI-treated DNA was used for the two kinds of PCR reactions in duplicate. Four-point standard curve was built for every 384 well plate using M.SssI-treated DNA and the ALU-C4 control reaction over 6 wells in duplicate (a total of 12 wells).

Primers and probe for *HIST1H4K* were designed using Beacon Designer 7 software. Their sequences are shown in Tables 2 and 3.

The amount of methylated DNA (PMR, percentage of methylated reference at HIST1H4K promoter) was calculated by dividing the GENE: Alu-C4 ratio of a sample by the GENE: Alu-C4 ratio of M.SssI- treated human genomic DNA (presumably fully methylated) and multiplying by 100. Reactions using M SssI-treated DNA were used to normalize for any difference in amplification efficiencies between GENE and Alu-C4 control reaction.

Table 2. Primers sequences of MethyLight assays

Primer	*Sequence (5'→3')*
ALU-forward	GGTTAGGTATAGTGGTTTATATTTG-TAATTTTAGTA
ALU-reverse	ATTAACTAAACTAATCTTAAACTCCTA-ACCTCA
RASSF2-forward	GAGAGGATAGCGGACGAGTAGATT
RASSF2- reverse	CAACCAAACTAAACAAACGATAACCG
HIST1H4K-forward	GATCACCGCCATAAATATAATCTACG
HIST1H4K-reverse	TTGATAGAAAGGGACGTTTAATTATCG

Table 3. Probes sequences of MethyLight assays

Probe	*Sequence (5'→3')*
ALU-Probe	6FAM-CCTACCTTAACCTCCC-MGB-NFQ
RASSF2- Probe	6FAM-AAAACCGAACGCCCCGC-CCTCCTC-TAMRA
HIST1H4K- Probe	6FAM-AAACGCCAAAACCGCACCCTC-TACG-TAMRA

Statistics

Fisher's exact test was used to check the correlation between methylation and development of PC. To evaluate the diagnostic power, the sensitivity and specificity of the investigated biomarker ROC curve analysis was performed and AUC was also calculated after dichotomization of the results from the MethyLight analysis. Samples with PMR value above 10 were considered methylated. Pearson's correlation coefficients were calculated in order to evaluate the associations between promoter methylation and clinicopathological characteristics. These correlations were evaluated in addition to Fisher's exact test. The odds ratios were also calculated. The statistical package SPSS, version 20.0.0 and MedCalc software for Windows, version 9.5.0.0 (MedCalc Software, Mariakerke, Belgium) were used for statistical analyses.

Results

The levels of promoter hypermethylation of *HIST1H4K* did not demonstrate any differences between PC patients and BPH controls, (p=1, two tailed, Fisher's exact test). Methylation was observed in 52.63% of PC patients and in 55.17% of BPH controls. In contrast, urine samples from YAM did not show any methylation at this locus and when they were compared to PC samples statistically significant results were achieved ($p=5.63x\ 10^{-11}$ two tailed, Fisher's exact test).

Since the ratio of free to total PSA has been implicated as a useful tool for predicting prostate biopsy outcome [36] we have further examined its utility in our study population. It was found that PSA (with a threshold of 4 ng/ml) had a sensitivity of 98.04% and low specificity of 14.29% for PC (AUC=0.785, 95% CI: 0.679-0.870, p<0.0001) in our patients (Figure 1). The statistical analysis has shown that the criterion corresponding to the highest Youden index (maximum potential effectiveness of a biomarker) was PSA value of 11.38 (sensitivity of 74.51% and specificity of 78.57%) (Figure 2). This may be explained by the recruitment strategy, which was to collect urine from pa-

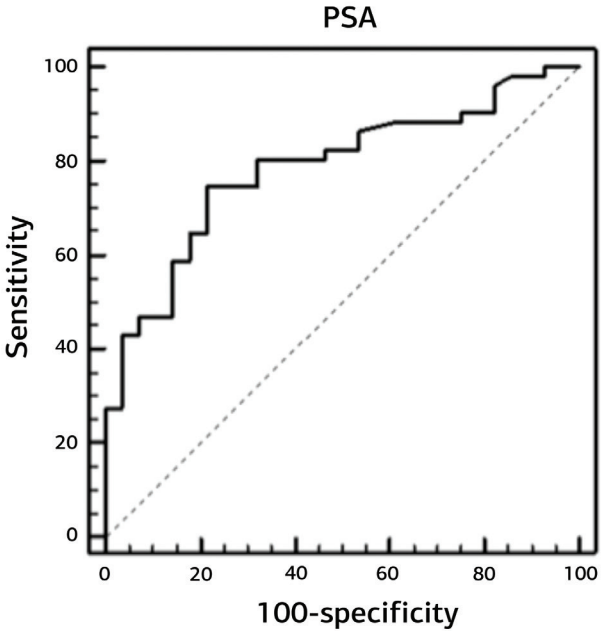

Figure 1. Receiver operative curve (ROC) analysis determination of sensitivity and specificity of serum PSA. Continuous line: PSA, dotted line: diagonal reference.

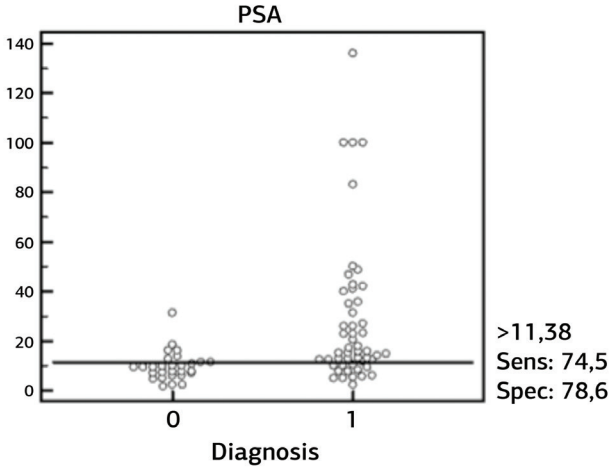

Figure 2. Interactive dot diagram of PSA level showing the cut off value to be 11.38.
O:controls with benign prostate hyperplasia,
1: patients with prostate cancer.

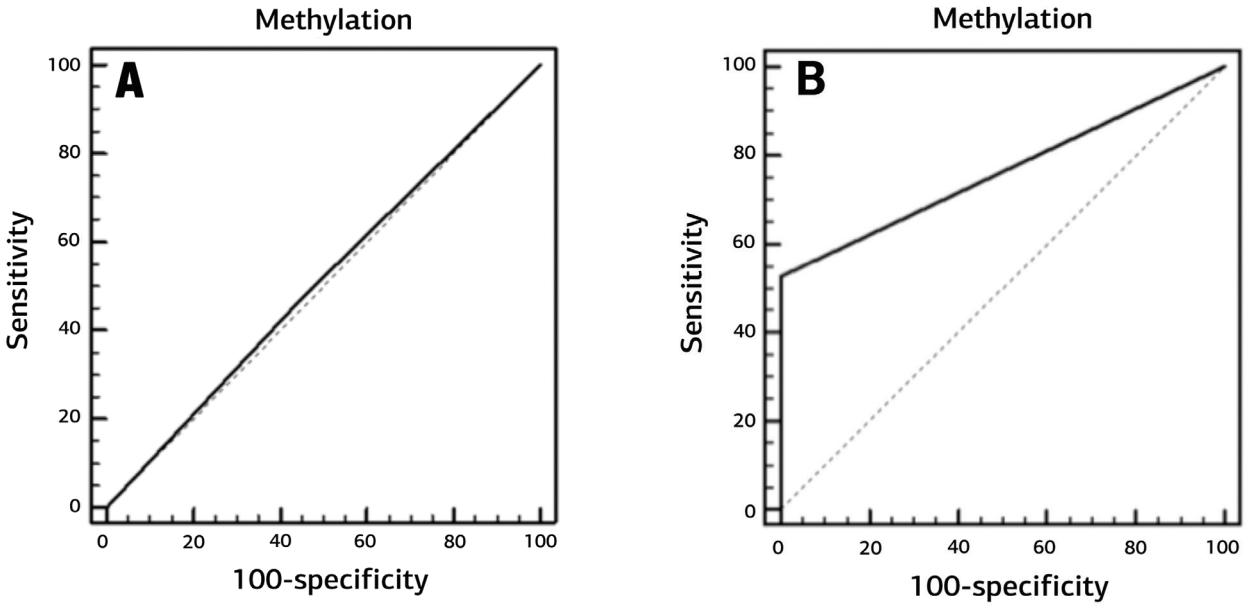

Figure 3. Receiver operative curve (ROC) analysis determination of sensitivity and specificity of DNA methylation of HIST1H4K biomarker in urine. **A**) when compared PC patients vs BPH controls; **B**) when compared PC patients vs YAM controls. Continuous line: HIST1H4K, dotted line: diagonal reference. PC: prostate cancer, BPH: begin prostate hyperplasia.

tients that show higher PSA values and/or abnormal result after digital rectal examination. This was also the reason for the high median serum PSA value (9.75 ng/ml) among the BPH controls.

ROC curve analysis was performed in order to evaluate the sensitivity and specificity of DNA methylation of *HIST1H4K* biomarker in urine by comparing PC patients vs BPH controls (Figure 3A) and YAM (Figure 3B), respectively. Methylation analysis of urine DNA successfully diagnosed 30 out of 57 studied PC cases and its sensitivity was 0.526 (Figure 3A). Of the 29 patients with BPH and no evidence of disease, 13 showed no aberrant methylation at *HIST1H4K* promoter site (specificity 0.45). The area under the ROC curve (AUC) was 0.513 (95% CI: 0.402-0.622), but the results were not statistically significant (p= 0.8255). On the contrary, when in the ROC curve analysis the methylation in urine from PC patients was compared to the methylation in urine from YAM statistically significant results were obtained (AUC=0.763, 95% CI: 0.672-0.839, p<0.0001). A specificity of 100% and sensitivity of 52.63% of the *HIST1H4K* methylation biomarker were achieved.

Correlation analysis did not show any statistically significant association of the methylation status with Gleason score, PSA and tumor stage. We were not able to check if there was a correlation between metastasis and promoter methylation because only one patient with metastasis participated in the present study.

To check whether promoter hypermethylation of *HIST1H4K* is age-dependent process we performed correlation analysis which included all 3 studied groups - PC patients, BPH and YAM controls (Figure 4). Pearson's correlation coefficient r was 0.542 (95% CI: 0.408-0.653), so there was a positive statistically significant correlation between age and methylation (p<0.0001). Apparently aging had effect on methylation of *HIST1H4K* promoter in the investigated sample but these results need to be proven in a larger sample.

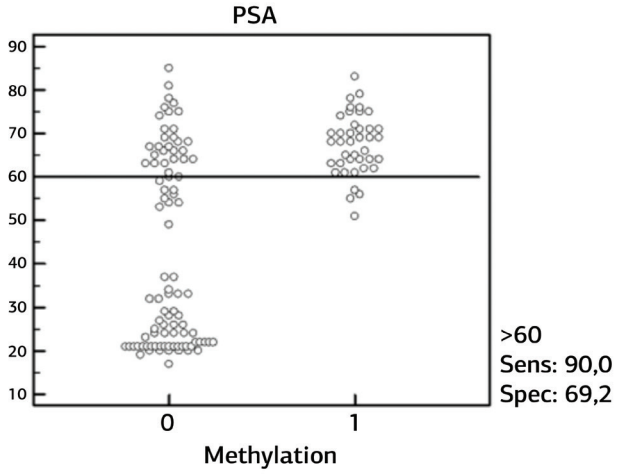

Figure 4. Interactive dot diagram representing the correlation between methylation and age and the sensitivity and specificity when cut off value is 60 years. O: unmethylated samples, 1: methylated samples.

Discussion

We have applied a MethyLight analysis to determine DNA promoter methylation of *HIST1H4K* in urine from PC cases (n=57), as well as from cancer-free controls (n=79). The selected gene was previously shown to discriminate the presence of PC when compared to controls using urine samples [15]. It encodes a member of the histone H4 family and resides within a large cluster of replication-dependent histone genes at chromosome 6p21-22 [37]. In the study of Payne et al. [15], promoter hypermethylation of *HIST1H4K* showed greater frequency in PC patients in comparison to genes *GSTP1* and *RASSF2* and lower frequency in YAM in comparison to *RASSF2* and *TFAP2E*.

Therefore we further explored the promoter hypermethylation of *HIST1H4K* as a promising biomarker for PC. Since it was demonstrated that the measurement of biomarkers in urine DNA was more sensitive than in plasma DNA [15] we concentrated our interest in studying urine samples only.

MethyLight was chosen as a highly sensitive assay, capable of detecting methylated alleles in the presence of a 10 000-fold excess of unmethylated alleles. The assay is also highly quantitative and can very accurately determine the relative prevalence of a particular pattern of DNA methylation [32].

It has been known that several genes such as *GSTP1*, *RARβ2* and *RASSF1A* that are hypermethylated in prostate cancer tissue may undergo methylation in healthy prostate epithelium in age-dependent manner [38]. In order to determine whether methylation of *HIST1H4K* is related to prostate carcinogenesis or due to changes related to aging we performed the analysis using two types of control samples - age adjusted controls with BPH (n=29) and YAM (n=50) (Table 1).

Comparable rates of methylation were found in both PC patients (52.63%) and BPH controls (55.17%), whereas no methylation was observed in YAM. Consequently in the ROC analysis when the PC patients were compared with BPH controls (Figure 3A) the predictive accuracy (AUC-0.513)) of the *HIST1H4K* biomarker was not statistically significant (95% CI: 0.402-0.622, p= 0.8255) in comparison to the study of Payne et al. [15] where AUC reached statistically significant value of 0.64 (95% CI: 0.55–0.72, p=0.01). The sensitivity (0.526) and specificity (0.45) of the marker in the current assay were similar to that reported by Payne et al. [15] (sensitivity 0.64 and specificity 0.34, respectively). The comparatively low specificity of *HIST1H4K* promoter methylation in both studies may be partially explained with the possibility some of the BPH controls to be diagnosed with PC on repeat biopsy.

The methylation of *HIST1H4K* successfully discriminated PC patients from YAM (AUC=0.763, 95% CI:0.672-0.839, p<0.0001) (Figure 3B) and demonstrated greater specificity (100%), but less sensitivity (52.63%) when compared to the previous data of Payne et al. [15] (AUC=0.64, 95% CI: 0.55–0.72, p<0.00001).

In our study promoter hypermethylation of *HIST1H4K* was not able to outperform the reported specificity and sensitivity of PSA as diagnostic biomarker. On the contrary, the measured preoperative PSA levels showed higher sensitivity in the discrimination of biopsy-positive from biopsy-negative patients (AUC=0.785, p< 0.0001, sensitivity of 74.51% and specificity of 78.57%) when using 11.38 ng/ml as criterion value (Figure 2). In the Payne et al. study [15] PSA showed lower AUC and the analysis was not statistically significant.

The frequency of hypermethylation of *HIST1H4K* in PC samples and controls in the present study differed significantly from previously published data. We found similar rate of methylation in both PC patients (52.63%) and of BPH controls (55.17%), whereas 92% of the PC cases and 84% of the BPH controls were methylated in a previous study [15]. Interestingly we did not observe any methylation among YAM in comparison to 14% found by Payne et al. [15] These differences might be due to differences between the collected samples, the methods used or might be due to different CpG positions covered in the analysis. Payne et al. [15] used HeavyMethyl assay to determine the methylation of DNA while MethyLight assay was used in the current study [15]. Although it is shown that MethyLight and HeavyMethyl assays have similar sensitivity and specificity the results can be strongly influenced by the methylation biology because the two assays are not able to cover exactly the same CpG positions [39].

The correlation analysis showed statistically significant age-dependent hypermethylation of *HIST1H4K* promoter region in the studied cohort of PC patients and cancer-free controls (BPH and YAM). This correlation is not surprising because aging is a multifaceted process characterized by genetic and epigenetic changes in the genome. Epigenetic mechanisms have now emerged as key contributors to the alterations of genome structure and function accompanying aging [40]. The relationship between epigenetics and aging was proposed years ago [41] and many authors have

shown that genomic DNA methylation decreases with age in different organisms like salmon [42], rat, mouse and cow [43-45]. Similarly, other authors have found an age-dependent decrease in global methylation levels in human lymphocytes [46] and peripheral blood cells [47,48]. Together with global hypomethylation of the genome, a variety of specific loci become hypermethylated in normal tissues during aging [49]. The molecular mechanisms behind these changes in DNA methylation patterns during aging remain unknown. Future studies need to determine whether transcriptional changes are indeed responsible for the accumulation of DNA methylation alterations during aging, and whether these changes increase cancer susceptibility with age. [40]. The gene reported to be hypermethylated in non-tumorigenic tissues include estrogen receptor (*ER*), myogenic differentiation antigen 1 (*MYOD1*), tumor-suppressor candidates 33 (*N33*), insulin like growth factor II (*IGF2*), *MLH1*, *p14ARF*, lysisl oxidase (*LOX*), p16INK4a, runt-related transcription factor 3 (*RUNX3*), E-cadherin, c-fos and many others [41]. Other authors have shown significant increase in promoter methylation levels correlating with age for CpG islands at *RARB2*, *RASSF1A*, *NKX2-5*, *ESR1* and even in normal prostate tissue samples [38]. In another study *HIST1H4K* showed decreased expression in skeletal muscles from older (65-71 years old) people in comparison with skeletal muscles from young people (20-29 years old). High-density oligonucleotide arrays were used to probe the patterns of gene expression in skeletal muscle and *HIST1H4K* showed 11% decreased expression in older in comparison with young skeletal muscles which might be due to promoter hypermethylation [50]. The question is still open whether promoter hypermethylation of *HIST1H4K* in prostate gland predisposes aging cells to neoplastic transformation. To answer this question, a larger cohort has to be analyzed and BPH controls with hypermethylated *HIST1H4K* have to be followed in order to determine whether they will develop cancer after the first biopsy. It will be useful if patients with high grade prostatic interepithelial neoplasia could be included in the analysis.

Conclusions

In summary, urinary biomarkers for prostate cancer are subjects of ongoing research and represent a promising alternative or addition to serum-based biomarkers. We have investigated a new DNA methylation biomarker, *HIST1H4K*, in urine from PC cases and controls. Our study could not convincingly replicate the previous findings for this marker in urine samples from PC and BPH patients [15]. Methylation of *HIST1H4K* showed correlations with neither PC nor with clinicopathological characteristics in the studied sample, but demonstrated statistically significant correlation with age. To further validate HIST1H4 as a potential biomarker for PC diagnosis or prognosis analysis in larger samples is required.

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