

6-gene promoter methylation assay is potentially applicable for prostate cancer clinical staging based on urine collection following prostatic massage

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Abstract. The detection of prostate cancer (PCa) biomarkers in bodily fluids, a process known as liquid biopsy, is a promising approach and particularly beneficial when performed in urine samples due to their maximal non-invasiveness requirement of collection. A number of gene panels proposed for this purpose have allowed discrimination between disease-free prostate and PCa; however, they bear no significant prognostic value. With the purpose to develop a gene panel for PCa diagnosis and prognosis, the methylation status of 17 cancer-associated genes were analyzed in urine cell-free DNA obtained from 31 patients with PCa and 33 control individuals using methylation-specific polymerase chain reaction (MSP). Among these, 13 genes indicated the increase in methylation frequency in patients with PCa compared with controls. No prior association has been reported between adenomatous polyposis coli 2 (*APC2*), homeobox A9, Wnt family member 7A (*WNT7A*) and N-Myc downstream-regulated gene 4 protein genes with PCa. The 6-gene panel consisting of *APC2*, cadherin 1, forkhead box P1, leucine rich repeat containing 3B, *WNT7A* and zinc family protein of the cerebellum 4 was subsequently developed providing PCa detection with 78% sensitivity and 100% specificity. The number of genes methylated (NGM) value introduced for this panel was indicated to rise monotonically from 0.27 in control

individuals to 4.6 and 4.25 in patients with highly developed and metastatic T₂/T₃ stage cancer, respectively. Therefore, the approach of defining the NGM value may not only allow for the detection of PCa, but also provide a rough evaluation of tumor malignancy and metastatic potential by non-invasive MSP analysis of urine samples.

Introduction

According to the most recent global estimates in 2012, ~307,000 and ~3,000 prostate cancer (PCa)-associated mortalities were reported globally and in Ukraine, respectively, and 1,111,700 and ~7,000 novel cases were diagnosed, respectively (1,2). Although the advent of relatively non-invasive prostate-specific antigen (PSA) testing has notably improved PCa diagnosis, its routine usage remains controversial, since a false positive diagnosis rate of PCa, over-treatment, and excessive medical spending has been reported (3-5). It has been reported that up to 75% of patients with elevated PSA level (>4 ng/μl) do not have any prostatic malignancy, although at least 1/3 of aforementioned patients undergo subsequent invasive follow-up evaluation, including prostate biopsy (6). Additionally, ~25% of individuals with normal PSA level exhibit biopsy evidence for PCa (7,8). Another important limitation of a canonical PSA assay is that it is neither a reliable discriminator between prostatic cancer and benign hyperplasia nor a precise staging indicator of PCa (4,5). Therefore, the development of an accurate, discriminative, and cost-efficient non-invasive PCa diagnostic tool is required.

Previous studies involving high-throughput techniques, including genome-wide sequencing, failed to identify a common genetic driver event (e.g. specific point mutation) in PCa tumorigenesis (9,10). None of the recurrent mutations have been reported to appear in 40-50% of PCa cases, including transmembrane serine protease 2-*ERG* fusion and phosphatase and tensin homolog (*PTEN*) deletion (10). Additionally, when examined for alterations in DNA sequences, PCa

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demonstrates comparatively high clonal heterogeneity or even distinct genomic origin, complicating the use of mutation hotspots as tumor biomarkers (11). Instead it has been indicated that epigenetic alterations, including cytosine base followed immediately by a guanine base island methylation, occur in a number of loci in 80-90% of PCa cases, including methylation of glutathione S-transferase π 1 (*GSTP1*) gene promoter, may drive the neoplastic transformation and would be a preferable target for prostate cancer diagnosis and its biological potential assessment (11-13).

It has been previously proposed that the free-floating DNA fragments originating from the apoptotic/necrotic malignant cells in bodily fluids may be used for the detection of cancer biomarkers (14). This approach, also referred to as liquid biopsy, would be particularly beneficial when performed on urine samples, due to the maximal non-invasive requirements for its collection, which notably improves patient's compliance and safety (15). Urine contains notable amounts of cell-free DNA (UcfDNA) with a concentration of up to 250 ng/ml and consisting of the following two size category fragments: Long (>1 kb), which are primarily cell-associated, including from the exfoliated epithelium; and short (150-250 bp), which are predominantly non-cell associated and originate from urogenital tract *per se* or circulation (16,17). This observation potentially makes UcfDNA the optimal source of data for diagnosing urogenital system cancer types. The prostate, whose lumen is continuously connected to the urogenital tract via prostatic sinuses, may be the optimal organ for investigation by means of UcfDNA analysis, which was successfully demonstrated by a number of studies (18-22).

In an effort to extend the list of biomarkers applicable for non-invasive PCa detection, the methylation profile of 17 cancer-associated genes was examined using the approach of UcfDNA analysis in the urine from patients with prostate cancer. From a functional perspective, the genes investigated in the present study are considered well-established tumor suppressors from earlier reports and participate in PCa pathogenesis [forkhead box P1 (*FOXPI*), *FOXP3*, *FOXP4*, hypermethylated in cancer 1 (*HICI*), zinc finger protein of the cerebellum 4 (*ZIC4*), *PTEN*, cadherin 1 (*CDHI*), O-6-methylguanine-DNA methyltransferase (*MGMT*) and leucine rich repeat containing 3B (*LRRC3B*)] or are known to be associated with other malignancies [adenomatous polyposis coli 2 (*APC2*), homeobox A9 (*HOXA9*), Wnt family member 7A (*WNT7A*) and N-Myc downstream-regulated gene 4 protein (*NDRG4*)] (23,24). The protein products of *FOXPI*, *FOXP3*, *FOXP4*, *ZIC4* and *HOXA9* are members of three families of transcription factors, including Forkhead, Zic, and HOX, which are broadly involved in the processes of tissue morphogenesis and cell differentiation (13,22,25). A number of genes encode the extracellular and intracellular signaling proteins, including ligands (*WNT7A*), binding factors (*APC2*) and enzymes (*NDRG4* and *PTEN*), are known to take part in embryonic development and cell cycle regulation as well (26-28). E-cadherin encoded by the *CDHI* gene is a key adhesion molecule in the epithelial tissues crucial for the formation of adherent junctions, whose disruption results in tumor metastasis (29). The gene *MGMT*, which encodes the pivotal repair enzyme MGMT, has been extensively implicated in the neoplastic transformation, due to the increased mutation rate following its silencing (29). The

product of the *LRRC3B* gene is a 29-kDa membrane-bound protein, whose function, to the best of our knowledge, has yet to be defined; however, it has been reported to participate in the tumorigenesis of a number of human cancer types, including clear cell renal cell carcinoma (30).

In the present study this initial gene set was analyzed and 13 genes demonstrating a statistically significant increase in methylation frequency in the PCa group, compared with controls, were selected. A final panel of 6 genes, including *APC2*, *CDHI*, *FOXPI*, *LRRC3B*, *WNT7A*, and *ZIC4*, was formed based on zero/low methylation level in controls, with significant moderate-to-strong correlation with tumor stage, and no significant correlation with patient's age. Within the panel, the number of genes methylated (NGM) was observed to increase monotonically from control samples to highly developed and metastatic types of cancer, providing a simple and cost-efficient method to identify tumor stage using the NGM value in the urine sample.

Materials and methods

Patients sample collection. The present study was approved by the local Ethics Committee of the Institute of Molecular Biology and Genetics of the National Academy of Sciences of Ukraine (approval no. 18/4). Urine samples were collected between May and October 2017 from 64 individuals, including 31 patients diagnosed with PCa and receiving treatment at the Institute of Urology NAMSU, and 33 patients who were diagnosed as disease-free controls. Patient's detailed information is presented in Table I. None of the patients with PCa or control individuals underwent radical prostatectomy or any type of pharmacological treatment prior to sampling. All patients provided written informed consent to participate in the present study. In each case the diagnosis was further confirmed and the tumor was graded according to the Gleason scoring system on prostate biopsy followed by histological examination (31). The Tumour-node-metastasis (TNM) staging system was used to classify PCa cases according to their development and malignancy (32). Within the cancer group, individuals were categorized with localized, including T₁ and T₂N₀M₀/N_xM₀ (n=5 and n=9, respectively) PCa, locally-advanced, including T₃N₀M₀ (n=10) PCa, and metastatic, including T₂N_xM₁/N₁M₁ and T₃N₁M₁ (n=3 and n=4, respectively) PCa.

UcfDNA isolation. Voided urine (50 ml) was harvested from patients following prostate massage on the previous day of definitive surgery. Each sample was spun at 3,000 x g for 10 min at room temperature, the supernatant was removed, and the pellet was washed twice with 1X PBS. The resultant pellet was cryopreserved at -80°C. Genomic DNA was extracted using a Quick-gDNA MiniPrep kit (Zymo Research Corp., Irvine, CA, USA), according to the manufacturer's protocol. The quality of isolated DNA was checked by 3% agarose gel electrophoresis. For the DNA concentration and purity measurements, a spectrophotometer ND-2000 (NanoDrop Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was utilized.

Bisulfite treatment and methylation-specific real-time polymerase chain reaction (MSP). Extracted UcfDNA

Table I. Clinical characteristics of patients.

Variables	PCa, n=31 (%)	Control, n=33
Age, years		
Median	66	62
Range	29-82	37-88
Tumor stage (TNM classification)		
T ₁	5 (16)	-
T ₂ N ₀ M ₀ /N _x M ₀	9 (29)	-
T ₃ N ₀ M ₀	10 (32)	-
T ₂ N _x M ₁ /N ₁ M ₁	3 (10)	-
T ₃ N ₁ M ₁	4 (13)	-
PSA		
Median	37.7	2.0
Range	5.9-223.0	0.2-4.1
Gleason score		
6	10 (32)	-
7	11 (36)	-
8	3 (10)	-
9	2 (6)	-
9	5 (16)	-
Unknown	5 (16)	-

-, not applicable; PCa, prostate cancer; PSA, prostate-specific antigen; T, tumor; N, node; M, metastasis.

was subjected to bisulfite conversion using an EZ DNA Methylation kit (Zymo Research Corp.), according to manufacturer's protocol. MSP was conducted using 34 pairs of forward and reverse primers of methylated or unmethylated type. Nucleotide sequences are presented in Table II. All primers were designed with MethPrimer 2.0 online software (The Li Lab, Beijing, China; <http://www.urogene.org/methprimer>) and their performance was evaluated using 6% polyacrylamide gel electrophoresis. The size of the polymerase chain reaction products was within the range of 87-263 bp. Each reaction mix contained 2.5 μ l 10X DreamTaq buffer (Thermo Fisher Scientific, Inc.), 0.3 mM primers, 100 ng UcfDNA previously subjected to bisulfite conversion and nuclease-free water to a final volume of 25 μ l. MSP was performed using thermocycler CFX96 Real-Time system (Bio-Rad Laboratories, Inc., Hercules, CA, USA), according to the following protocol: Initial 12-min incubation at 95°C, followed by 40 cycles of denaturation for 15 sec at 95°C, annealing for 30 sec at 60°C and extension for 30 sec at 72°C.

Statistical analysis. Statistical analysis was performed using STATISTICA 7.0 software (StatSoft, Inc., Tulsa, OK, USA). Since the data obtained were not normally distributed, it was analyzed with a non-parametric approach. $P < 0.05$ was considered to indicate a statistically significant difference. The comparison of PCa and control groups for the methylation frequency in the 17 genes was conducted with Mann-Whitney U-test. The Spearman's rank correlation coefficient with

Bonferroni correction for multiple hypothesis testing was used to calculate correlation between methylation rate and patient's age or tumor stage. For this evaluation, the variables were measured on the ordinal scale. In the correlation analysis with regards to age, patients with PCa and controls were involved, while during the correlation analysis with regards to stage, only patients with cancer were taken into account. A resulting R-value > 0.3 along with $P < 0.05$ was considered to indicate a positive correlation between the values examined. The comparison of the control and five cancer groups after the NGM value was calculated was performed using the Kruskal-Wallis test with Conover's post-hoc analysis adjusted by the Benjamini-Hochberg false discovery rate method. The results are presented as the means \pm standard error of the mean.

Results

Examination of the methylation status of 17 genes in PCa and control groups. The methylation frequency of 17 gene promoters was evaluated in urine samples from patients with PCa and the control group (Table III). The increase in methylation frequency in tumor samples was the lowest for *FOXP2* (3.03-6.45%) and the highest for *ZIC4* (6.06-58.06%) genes, compared with controls. Among the panel analyzed, 8 genes, including *WNT7A*, *LRRC3B*, *FOXP3*, *FOXP4*, *CDH1*, *HOXA9*, *NDRG4* and *PTEN*, were not methylated in all of the control samples. However, aforementioned genes were indicated to be methylated to different extents in prostate tissues from patients with PCa. On the contrary, 9 genes, including Von Hippel-Lindau tumor suppressor (*VHL*), *FOXP1*, *FOXP2*, *APC2*, *ZIC4*, phospholipase C like 2 (*PLCL2*), *HIC1*, ubiquitin conjugating enzyme E2 (*UBE2E2*) and *MGMT* were identified to be methylated in control and cancer groups. A statistically significant difference was indicated in the methylation status of the following 13 genes: *WNT7A*, *LRRC3B*, *FOXP1*, *FOXP3*, *FOXP4*, *APC2*, *ZIC4*, *CDH1*, *HOXA9*, *NDRG4*, *PTEN*, *MGMT* and *HIC1*. All these genes, except *HIC1*, had a methylation frequency between 0-12% in the control samples, while in the PCa samples methylation frequency was between 13-58%. The *HIC1* gene demonstrated a notable methylation frequency even in control samples (~39%) and for this reason *HIC1* was excluded in subsequent analyses. The difference in methylation status of *VHL*, *FOXP2*, *PLCL2*, and *UBE2E2* genes was indicated to be statistically insignificant between cancer and control individuals (Table III).

The formation of the 6-gene diagnostic/prognostic panel based on correlation analysis. The correlation between the methylation of the considered genes and tumor stage was evaluated using the Spearman's rank test. A positive correlation was identified in the following 14 genes: *VHL*, *WNT7A*, *LRRC3B*, *FOXP1*, *FOXP3*, *FOXP4*, *APC2*, *ZIC4*, *PLCL2*, *CDH1*, *HIC1*, *NDRG4*, *PTEN* and *MGMT*. The genes *FOXP3*, *FOXP4*, *NDRG4*, *PTEN* and *MGMT* were excluded from subsequent analyses, due to a weak correlation with the disease stage. Furthermore, *VHL* was also excluded due to low statistical significance ($P > 0.05$), following Bonferroni correction, in addition to a number of genes, including *FOXP3*, *FOXP4* and *PTEN*, indicating a weak correlation. In contrast, 3 genes, *FOXP2*, *HOXA9* and *UBE2E2*, indicated negative values of correlation coefficient. However, they were also rejected due to the low correlation with tumor stage.

Table II. Primers used for methylation-specific polymerase chain reaction.

Genes	Forward 5'-3'	Reverse 5'-3'	Product size (bp)
<i>APC2</i>			
M	ATTTCCGGGTCGGGATTTTC	GCTTACGTACAATAACTAACTAACG	135
U	GTTGTTTGTATTTGTTTGTTTTGA	AAACATAACCTTAAACTCCCCACT	136
<i>CDH1</i>			
M	GGTTTTGACGTCGAGAGTTATAC	TACGTAAATTCCAAAAATATCGTT	211
U	TTTGGTTTTGATGTTGAGAGTTATATG	TACATAAATTCCAAAAATATCATT	214
<i>FOXP1</i>			
M	CGGAGTTCGGAAAATTTAAATACGT	GTCTCGAAAAACGAAAACCGA	87
U	TGGAGTTTGGAAAATTTAAATATGT	TCATCTCAAAAAACAAAAACCAA	89
<i>FOXP2</i>			
M	CGTTTTTTCGAGGAGAGGTAGTTTC	GCGCGCGTATTATTAACAATACG	101
U	TGTTTTTTTGAGGAGAGGTAGTTTT	ACACACATATTATTAACAATACAAA	103
<i>FOXP3</i>			
M	GGATAGGGTAGTTAGTTTTTCGGAAC	GAATACGCCGAACCTTCATCGA	93
U	ATAGGGTAGTTAGTTTTTGAATGA	ACCAAATACACCAAACCTTCATCAAC	94
<i>FOXP4</i>			
M	TTCGTAGTTATTCGTAGTTTAGGTTAGTC	TCGCGAACTAAAACTCCGT	120
U	TTGTAGTTATTTGTAGTTTAGGTTAGTTG	TCCTCACAACCTAAAACTCCATCC	122
<i>HIC1</i>			
M	TTTTATTAGTAATTTAATTCGAATAGCGTC	AACCGCAATCCTAAAAATCG	138
U	TATTAGTAATTTAATTTGAATAGTGTGG	TACAAAACCACAATCCTAAAAATCAC	140
<i>HOXA9</i>			
M	ATCACCTAATAAATTAACCGACG	TCGGATTATTAATAGCGTGC	101
U	TGTAGTTTTTAGTTTAAGGTGATGG	AATAATAATAATACACCACAACAAA	100
<i>LRRC3B</i>			
M	GGTGCAGGAAGGTAGGC	ACCAATACCTCGCCGACG	222
U	TGGTGTAAGGTAAGGTGATGTTGT	AAACAAAAACAAAAAATCAAC	217
<i>MGMT</i>			
M	CGTTTGTAGTTGAGTAAGTATGAGTTTAG	AAACGACCCTAAATTCATCGAAAA	263
U	GTTTTGGATATGTTGGGATAGTTTG	ACACCTAAAAACACTTAAACACA	261
<i>NDRG4</i>			
M	GGTATTTTAGTCGCGTAGAAGGC	GTACCCGCGTAAATTTAACGAA	119
U	GTTAGATAGGTGGGTTTTGTAGATG	CAATCAAACTAAAAACAAAAACAC	120
<i>PLCL2</i>			
M	GTATTTTTTTTCGGGAGAGTAAGTC	CCAAAAACGACTAAAAATAAACGAT	105
U	TTTTTTTGGGAGAGTAAGTTGG	CCAAAAACAACCTAAAAATAACAAT	100
<i>PTEN</i>			
M	TTTTTTTATTTTCGTTGTCGTCGT	TTAACGATAACTAATACCCCTCGC	155
U	TTTTTTTATTTTGTGTTGTTGT	TTACAATAACTAATACCCCTCACT	156
<i>UBE2E2</i>			
M	ATTAGACGGTTCGTAGGGGATATTTTC	ATATCCGTACAAATCGCAAACCTCGA	180
U	GAGATTGAGATTATGGTGAAATTTT	ACCCAACTAAAATACAATAACACA	181
<i>VHL</i>			
M	TTATTCGGGAGGTTGAGGCGAGAC	CGCAAAAAATCCTCCAACACCGTAA	103
U	AGGTAGGATATATTTAGGGTGATGT	ACTCCAACCTAAACAACAAAACAA	105
<i>WNT7A</i>			
M	CGAAACCGTCTATCGATACG	GTAGTTCGGCGTCGTTTTAC	179
U	TTTTTTGATGTATATTAGGTTTGT	CTAAACCACACTACCACAATTTCAA	178
<i>ZIC4</i>			
M	GTTGTAGCGATAAGGTAGGAGTTTC	CCACTTTAACGAAATAAAAAATCGAT	202
U	TGTAGTGATAAGGTAGGAGTTTTGG	CCACTTTAACAAAAATAAAAAATCAAT	200

M, methylated; U, unmethylated.

Table III. Methylation status of 17 genes determined in urine.

Genes	Methylation frequency, %			Age correlation (R-value)		Stage correlation for PCa group (R-value)
	Control	PCa	U-test (P-value)	Control	PCa	
<i>APC2</i> ^a	12.12	35.48	0.03 ^a	0.21	0.23	0.32 ^b
<i>CDH1</i> ^a	0.00	48.39	<0.01 ^a	0.00	0.24	0.63 ^b
<i>FOXP1</i> ^a	9.09	58.06	<0.01 ^a	0.22	-0.20	0.41 ^b
<i>FOXP2</i>	3.03	6.45	0.81	0.04	0.07	-0.05
<i>FOXP3</i>	0.00	32.26	0.03 ^a	0.00	-0.13	0.03
<i>FOXP4</i>	0.00	12.90	0.03 ^a	0.00	-0.24	0.16
<i>HIC1</i>	39.39	90.32	<0.01 ^a	0.06	-0.10	0.47 ^b
<i>HOXA9</i>	0.00	35.48	<0.01 ^a	0.00	-0.09	-0.01
<i>LRRC3B</i> ^a	0.00	19.35	0.01 ^a	0.00	-0.06	0.31 ^b
<i>MGMT</i>	3.03	38.71	<0.01 ^a	-0.20	-0.18	0.01
<i>NDRG4</i>	0.00	32.26	<0.01 ^a	0.00	0.12	0.20
<i>PLCL2</i>	24.24	38.71	0.22	0.36 ^b	-0.18	0.22
<i>PTEN</i>	0.00	22.58	<0.01 ^a	0.00	0.06	0.05
<i>UBE2E2</i>	3.03	9.68	0.28	0.18	0.11	-0.20
<i>VHL</i>	6.06	22.58	0.06	-0.30 ^b	0.23	0.37 ^b
<i>WNT7A</i> ^a	0.00	41.94	<0.01 ^a	0.00	0.14	0.50 ^b
<i>ZIC4</i> ^a	6.06	58.06	<0.01 ^a	-0.08	0.22	0.41 ^b

^aSelected genes for panel. PCa, prostate cancer. ^aStatistical significance. ^bMild or strong correlation; APC2, adenomatosis polyposis coli 2; CDH1, cadherin 1; FOXP1, forkhead box P1; HIC1, hypermethylated in cancer 1; HOXA9, homeobox A9; LRRC3B, leucine rich repeat containing 3B; MGMT, O-6-methylguanine-DNA methyltransferase; NDRG4, N-Myc downstream-regulated gene 4 protein; PLCL2, phospholipase C like 2; PTEN, phosphatase and tensin homolog; UBE2E2, ubiquitin conjugating enzyme E2 E2; VHL, Von Hippel-Lindau tumor suppressor; WNT7A, Wnt family member 7A; ZIC4, zinc finger protein of the cerebellum 4.

Since PCa is a relatively slow-growing cancer, the actual age-associated methylation status dynamics may be misinterpreted as the respective alterations caused by tumor progression (6). Therefore, the correlation between patient's age with methylation of the gene panel investigated was also assessed (Table III). In the control group, 8/17 genes indicated no correlation with age (*WNT7A*, *LRRC3B*, *FOXP3*, *FOXP4*, *CDH1*, *HOXA9*, *NDRG4* and *PTEN*), 8 demonstrated weak correlation (*VHL*, *FOXP1*, *FOXP2*, *APC2*, *ZIC4*, *HIC1*, *UBE2E2* and *MGMT*) and 1 exhibited moderate correlation (*PLCL2*). In the cancer group all genes were indicated to be weakly correlated with age. All 10 genes that indicated moderate correlation with tumor stage (*VHL*, *WNT7A*, *LRRC3B*, *FOXP1*, *FOXP3*, *FOXP4*, *APC2*, *ZIC4*, *CDH1* and *HIC1*) demonstrated a notably weaker correlation with age (Table III).

Subsequently, the genes *WNT7A*, *LRRC3B*, *FOXP1*, *APC2*, *ZIC4* and *CDH1* were selected to form a panel for PCa diagnosis in urine samples, according to the following four criteria: Low methylation frequency in control samples, statistically significant increase of methylation frequency in patients with PCa, notable correlation with PCa progression, and 0 or weak correlation with age.

PCa detection and determination of the tumor stage by 'number of genes methylated' (NGM) approach. For the interpretation of the results obtained with the 6-gene panel, the 'number of genes methylated' (NGM) value ranging from

Table IV. The methylation of genes from 6-gene panel in PCa and controls.

Number of genes methylated	Patients (n)	
	Control	PCa
0	24	3
1	9	6
2	-	6
3	-	4
4	-	9
5	-	3
6	-	-
Total	33	31

PCa, prostate cancer; -, not applicable.

0 to 6 was introduced. With the cut-off value established at 2, the panel provided PCa detection with 78% sensitivity and 100% specificity. In the control group NGM values were 0 for 24 patients and 1 for 9 patients, while none of them had ≥ 2 methylated genes (Table IV). The NGM values of patients with PCa were uniformly distributed within the range of 0-5 reflecting the tumor stage. The mean NGM value determined

Table V. The number of genes methylated values in patients with different stages of prostate cancer.

Panel genes methylated	T ₁		T ₂ N ₀ M ₀ /N _x M ₀		T ₃ N ₀ M ₀		T ₂ N _x M ₁ /N ₁ M ₁		T ₃ N ₁ M ₁	
	Samples. n	%	Samples. n	%	Samples. n	%	Samples. n	%	Samples. n	%
0	3	60	-	-	-	-	-	-	-	-
1	2	40	3	33	1	10	-	-	-	-
2	-	-	3	33	3	30	-	-	-	-
3	-	-	2	22	2	20	-	-	-	-
4	-	-	1	12	4	40	1	33	3	75
5	-	-	-	-	-	-	2	67	1	25
6	-	-	-	-	-	-	-	-	-	-
Total	5		9		10		3		4	

-, not applicable; T, tumor; N, node; M, metastasis according to TNM staging system.

for each PCa stage group (NGM_{aver}) was indicated to almost monotonously rise from 0.27-4.25 along with tumor progression and/or increase of its metastatic potential (Fig. 1). In particular, in the group with the least developed T₁ stage cancer the NGM values overlapped with those in controls (0-1); however, it included more NGM 1 values, reaching 40% of the total number (Table V). Among the patients with non-metastatic PCa of T₂/T₃ stages, 15/19 patients indicated NGM values between 2-4, while only 4 patients had NGM values between 0-1, as indicated in the control and T₁ groups. Furthermore, none of the patients with metastatic cancer of T₂/T₃ stages demonstrated an NGM value <4, and in a number of cases an NGM value of 5 was reached.

The NGM approach was further tested for its prognostic potential. Although the resolving power of this method was not adequate to discriminate between all 5 PCa and 1 control groups, it provided the discrimination between at least three categories of cases: controls and early cancer (T₁), developed cancer without metastases (T₂N₀M₀/N_xM₀ and T₃N₀M₀) and developed metastatic PCa (T₂N_xM₁/N₁M₁ and T₃N₁M₁) (Fig. 1). The NGM_{aver} of the control and T₁ groups was 0.27 ± 0.07 and 0.40 ± 0.24 , respectively, with no significant difference between the two groups, but with significant differences determined between neighboring groups without metastases, T₂N₀M₀/N_xM₀ and T₃N₀M₀, ($P=0.02$ and $P=0.0009$ between T₁/T₂ and T₁/T₃ groups, respectively). The latter groups also demonstrated an NGM_{aver} of 2.10 ± 0.35 and 2.90 ± 0.34 , respectively, and could have not been statistically separated from each other, but differed from metastatic groups T₂N_xM₁/N₁M₁ and T₃N₁M₁ ($P=0.0009$ and $P=0.001$ comparing non-metastatic T₂ and respective groups with metastases; $P=0.01$ and $P=0.02$ comparing non-metastatic T₃ and the respective groups with metastases). Finally, the patients with invasive cancer T₂N_xM₁/N₁M₁ and T₃N₁M₁ exhibited NGM_{aver} of 4.60 ± 0.33 and 4.25 ± 0.25 , respectively, and exhibited no statistically significant difference between each other. Collectively, these data indicated that NGM value could be used to discriminate between PCa of three types: Early T₁ cancer; T₂/T₃ cancer without metastases; and the highly invasive metastatic T₂/T₃ cancer stages.

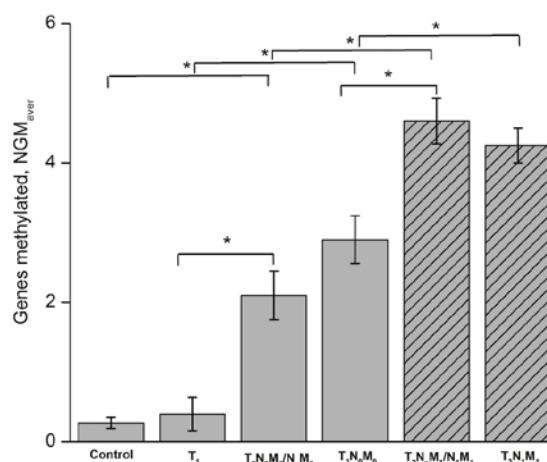


Figure 1. The dependence of NGM value on PCa tumor progression and metastasis development. The metastatic groups are marked with grid filling. NGM, number of genes methylated; PCa, prostate cancer; T, tumor; N, node; M, metastasis; NGM_{aver} , average NGM. * $P < 0.05$.

Discussion

The present study identified 13 tumor suppressor genes undergoing aberrant methylation in prostate cancer, where 4 of these genes have no prior reported association with PCa pathogenesis (24-26). Furthermore, the present study proposes the diagnostic and prognostic panel of 6 genes, which may be used for non-invasive PCa detection and prognosis by non-invasive UcfDNA analysis.

Additionally, the correspondence of polymerase chain reaction data obtained from tissue samples and urine was examined. Despite the fact that the present study did not question the reliability of MSP analysis conducted on urine samples without examining the tumor *per se*, previous aforementioned advanced techniques made it possible to detect methylation events in urine, regardless of apparent UcfDNA fragmentation and its comparatively low amount (20). In a similar investigation, including the parallel examination of PCa tumor samples, it was demonstrated that a positive signal for a specific biomarker could be registered from urine in

85% of cases compared with the solid tissue samples (21). Furthermore, the urine was indicated to be the most amenable source of UcfDNA for cancer detection if compared with other types of bodily fluids, including blood and semen (21).

Novel genes associated with PCa were examined and the data of the present study indicated that the genes *APC2*, *HOXA9*, *WNT7A* and *NDRG4* are associated with PCa pathogenesis. To the best of our knowledge, this has never been demonstrated on a DNA sequence alteration level or in epigenetic events. No methylation was indicated in *HOXA9*, *WNT7A* and *NDRG4* genes in any of the control samples, while *APC2* was methylated in 12% of the control samples. In the PCa samples methylation frequencies of aforementioned genes reached 35, 42, 32 and 35%, respectively. It should be noted that the actual methylation rate of these genes in PCa may be even higher when determined in the tumor samples themselves, due to seemingly increased DNA integrity and amount in solid tissue, compared with the UcfDNA pool (17). Further detailed investigation is required to clarify the significance of these genes for PCa pathogenesis.

Therefore, the present study proposed a 6-gene panel consisting of *APC2*, *CDH1*, *FOXPI*, *LRRC3B*, *WNT7A* and *ZIC4* allowing PCa detection with 78% sensitivity and 100% specificity. In a number of previous studies, other individual genes or their respective panels were considered as candidate PCa biomarkers for urine-based polymerase chain reaction diagnostics (20-21,33-35). In the present study, promoter hypermethylation in the *GSTPI* gene, the most frequent molecular event in PCa pathogenesis occurring in 80-90% of its cases, was detected in only 27% of urine samples, while the respective biopsy samples indicated methylation frequency of 79%, which is consistent with previous reports (12,20). Although the sensitivity of this test appeared to be inadequate for clinical application, it demonstrated the practical feasibility of urine polymerase chain reaction/MSP examination for PCa diagnosis (20). In another study, it was reported that the detection rate in UcfDNA analysis may be increased notably by selecting more appropriate experimental conditions: When using a DNA isolation kit developed for blood/tissue samples, the study reported *GSTPI* promoter hypermethylation in only 36% of urine samples from patients with PCa, but switching to a viral kit allowed the detection of this molecular event in 76% of cases investigated (21). Furthermore, the use of differential display code 3 (*DD3^{PCA3}*), a gene expressing a non-coding RNA highly specific for prostate tissue, was proposed as a non-invasive PCa detection method. The diagnostic test based on the quantitative determination of its transcripts in urine was reported to exhibit 67% sensitivity and a 90% negative predictive value (33). In another study, the use of the *p16*, *p14^{ARF}*, *MGMT* and *GSTPI* gene combination, instead of focusing on a single gene, provided a diagnostic test with 87% sensitivity along with 100% specificity (34). Additionally, a gene panel consisting of *GSTPI*, Ras association domain family member 1A, retinoic acid receptor β 2 and *APCI* was proposed for detection of localized PCa, yielding a sensitivity and accuracy of 86 and 89%, respectively (35).

The main advantage of the 6-gene panel proposed in the present study over the aforementioned is the prognostic value

it may bear, as the tumor stage could be at least roughly determined based on a NGM value between 0-6. NGM_{aver} values calculated in the cohort rose monotonically from the control group, 0.27, to groups with T₂ and T₃ metastatic cancer types, 4.6 and 4.25, respectively. This indicated an increased probability of identifying malignant and metastatic tumor types in a patient with an increased individual NGM value. The number of genes included in this 6-gene panel is increased, compared with the 4-gene panels proposed by Hoque *et al* (34) and Roupret *et al* (35), which indicates a disadvantage in terms of cost-efficiency; however, it is necessary for distinguishing between PCa cases of different stages and metastatic potential. It is also important that a 6-gene set is amenable so that it can be applied in a single-tube multiplex polymerase chain reaction screen, providing maximal cost-efficiency and convenience. The cut-off NGM value of 2 was selected as a diagnostic criterion of PCa, since the NGM values of individuals without a diagnosed prostate malignancy and T₁ cancer extensively overlapped in the range of 0-1. The aforementioned disadvantage is mitigated by the fact that prostate cancer at T₁ stage is usually a small localized slow-growing tumor frequently left untreated, due to its asymptomatic nature, and the additional age-associated health problems that the patient with PCa may exhibit.

The main limitation of the present study is the relatively small patient cohort, including 31 individuals with PCa, while other studies of this type usually involve a broader cohort of patients, including 67 PCa cases in the study by Salvi *et al* (22), and 52 cases in the study by Hoque *et al* (34). Nevertheless, the number of patients involved in the present study allowed the identification of methylation for 13 genes in PCa, and the establishment of the correlation with tumor stage for 8 of them with adequate statistical significance. Therefore, although the 6-gene panel proposed in the present study may not be yet applicable in a clinical setting, it could still be potentially used for this purpose following additional verification. Furthermore, separate genes from the selected panel, whose methylation frequency, according to the present data, was increased in the PCa group, compared with controls, could be introduced in other detecting or prognostic panels. The same applies for the comparison of cancer subgroups with different PCa stages using the NGM value parameter. Although the NGM value tends to rise with tumor development, the application of this tool for clinical use requires more precise determination of the correspondence between its particular values and the PCa stage.

Furthermore, the prognostic potential of the NGM approach has its own limitations. Clinical conclusions drawn can only be of a probabilistic nature, according to the present data, as NGM values obtained from patients with close PCa stages partially overlapped. However, at more distant tumor stages, NGM values significantly differed, including 0-1 at T₁ and 4-5 at late metastatic stages. Therefore, it was demonstrated that the NGM approach serves as a valuable tool for further development of the panel proposed here or other gene panels designed for PCa prognosis.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request

Authors' contributions

MVV, IVI, VMG and ROD provided clinical material from patients with prostate cancer and control individuals. AGK and KAN designed the initial panel of cancer-associated genes and performed primer design. KAN and EER conducted the UcfDNA isolation and its MSP analysis. LAS, BRS and VIK conducted data analysis and wrote the article.

Ethics approval and consent to participate

The study was approved by local Ethics Committee of the Institute of Molecular Biology and Genetics of the National Academy of Sciences of Ukraine (approval no. 18/4 from 15th July 2016). All patients provided written informed consent to take part in the present study including further publication of the results obtained.

Patient consent for publication

All patients provided written informed consent for this study.

Competing interests

The authors declare no conflict of interests.

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