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Methylation pattern of tumor-suppressor gene promoters as putative noninvasive diagnostic markers for prostate cancer

O. S. Mankovska[1], A. S. Korsakova[2], K. R. Cherniavskyi[2], O. A. Kononenko[3], E. O. Stakhovskyy[3], Yu. M. Bondarenko[4], V. I. Kashuba[1,5], G. V. Gerashchenko[1]

[1] Institute of Molecular Biology and Genetics, NAS of Ukraine
150, Akademika Zabolotnoho Str., Kyiv, Ukraine, 03143

[2] Educational and Scientific Center "Institute of Biology and Medicine",
Taras Shevchenko National University of Kyiv
64/13, Volodymyrska Str., Kyiv, Ukraine, 01601

[3] National Cancer Institute
33/43, Lomonosova Str., Kyiv, Ukraine, 03022

[4] State Institution «Institute of Urology of NAMS of Ukraine»
9-a, Yu. Kotsubyns'koho Str., Kyiv, Ukraine, 04053

[5] Karolinska Institutet
Stockholm SE-171 77, Sweden
mankovsska@gmail.com

Aim. To assess the rate of promoter methylation of putative TSGs for PCa in tumor tissue and in urine of PCa patients for better understanding of regulation of gene expression upon the PCa development and to evaluate the possibility to use the data on TSGs' methylation for the development of noninvasive PCa markers. **Methods.** A quantitative methyl-specific PCR (qMSP) was used for the analysis of a methylation rate in prostate tissues and cell lines, and an ordinary MSP was performed for the study of urine samples. **Results.** We found that the *RASSF1A* promoter demonstrated a higher methylation rate in the TMPRSS2:ERG fusion positive PCa. The methylation of *NKX3.1, PTEN* and *RASSF1A* in DNA from urine was more common for cancer patients than for healthy donors. The promoters of *CDH1* and *GDF15* were methylated more frequently in PCa patients, than in patients with inflammatory disease. **Conclusions.** The abovementioned five genes can form a panel for early non-invasive detection of PCa. This set can be combined with the detection of the TMPRSS2:ERG fusion transcript. More work should be done to understand the molecular mechanisms explaining the functional role of promoter methylation of the selected genes.

K e y w o r d s: gene promoter methylation, tumor suppressor genes, noninvasive diagnostics, prostate cancer

Introduction

Prostate cancer (PCa) is one of the most abundant malignancies among men worldwide [1]. In Ukraine, the number of PCa new cases in 2018 was estimated as 7 936 (*Cancer today https://gco.iarc.fr/*). For now, the blood-based PSA (Prostate Specific Antigen) test is the most widely used approach for prostate cancer screening but it shows rather low sensitivity and specificity (75 %–85 % and 25–35 %, respectively) [2]. Of note, tumors of a prostate gland are highly heterogeneous, demonstrating histological, cellular and molecular differences even within one tumor from the same patient [3, 4]. Therefore, it is important to seek additional PCa diagnostic markers of high sensitivity and specificity as well as the markers for estimation of disease severity and prediction of its progression.

The aberrant methylation of promoters of tumor-suppressor genes (TSGs) is one of the earliest events in cancer development and, therefore, the its detection can be used for early cancer diagnosis. However, many various cell populations with different methylome can be found in PCa lesions. Due to this fact, the quantitative analysis of gene methylation patterns and comparison with clinical tumor characteristics are needed to shed the light on a role of methylation events in PCa development [5, 6].

The methylation patterns can be easily assessed in liquid biopsies [7, 8]. Combining the investigations on PCa patients, patients with inflammation in the prostate gland and the healthy individuals, it is possible to study the impact of promoter methylation of defined TSGs on PCa development, and the optimal set of potential markers can be created.

In the present paper, we analyze the patterns of promoter methylation of a set of TSGs that was investigated previously in our laboratory [9]. Among them there are the genes, which are involved in cytoskeleton integrity (*KRT18, VIM, CDH1, RASSF1A*), cell division and signaling (*RASSF1A, PTEN, NKX3.1*), and other cancer-related cellular processes [9–14]. *KRT18* encodes keratin 18 [10], participating in maintenance of epithelial cell integrity, together with E-cadherin, encoded by *CDH1* [11]. Vimentin, encoded by *VIM* gene, oppositely, represents the type III intermediate filament, which is more common for the mesenchymal cells type [12]. The *PTEN* gene encodes the protein PTEN, the well-known inhibitor of phosphatidylinositol 3-kinase pathway, involved in carcinogenesis [13]. The proteins, encoded by *RASSF1A* and *NKX3.1* have multiple functions and were reported to participate in malignant transformation [14, 15]. In our previous work we described the differential expression of those genes in PCa compared to the paired conventionally normal tissues (CNT), which pointed to their potential role in development of PCa, and, especially, in epithelial-mesenchymal transition [9]. *GDF15* was included in our analysis as the cancer-related gene, according to the previously reported data [16].

Hence, the aim of the present study was to assess the rate of promoter methylation of the set of genes in tumor tissue and in urine of PCa patients for better understanding of regulation of gene expression upon the PCa development and to evaluate the possibility to use the data

on TSGs' methylation for the development of noninvasive PCa markers.

Materials and Methods

Cell culture. Three human PCa cell lines — PC3, DU145 and LNCaP were grown in a DMEM medium, supplemented with 10 % FBS at 37 °C in a humidified atmosphere of 5 % CO2.

Sample collection and primary processing. All samples were collected according to the Declaration of Helsinki and approved by the guidelines issued by the Ethic Committee of the Institute of Urology and National Cancer Institute, Kyiv, Ukraine.

Tissues. The PCa samples and the paired CNTs were collected at the Institute of Urology of National Academy of Medical Sciences of Ukraine and National Cancer Institute (NCI) (Kyiv, Ukraine) as described earlier [9]. The most important clinical and pathological characteristics (CPC), such as Gleason score (GS), PSA levels in blood, tumor stage, patient age and the presence of the TMPRSS2:ERG fusion transcripts in cancer samples are presented in S1.

Urine. Urine samples of PCa patients were collected at National Cancer Institute (NCI) (Kyiv, Ukraine). The urine samples of patients with inflammatory disease of genitourinary system as well as the samples of healthy donors were collected at the Institute of Urology of National Academy of Medical Sciences of Ukraine. The urine from cancer patients was taken before surgical treatment into sterile containers and immediately transported to Institute of Molecular Biology and Genetics.

Primary processing of urine. All samples were centrifuged at 1500 rpm for 20 minutes (at +4 °C) to collect all cells. The supernatant was replaced to other tubes and the CTAB reagent was added for precipitation of DNA. After incubation at +4 °C (until visually detected cloudy precipitate was formed in urine) the samples were centrifuged at 2000 rpm for 15 minutes. The supernatant was discarded and a pellet of CTAB and DNA was stored at –20 °C until the DNA isolation [17].

DNA isolation

DNA from cells, tissues and urine were extracted according to the protocol [18].

The bisulfite conversion and PCR

Bisulfite conversion of DNA was performed, using the EZ DNA Methylation lightning kit (Zymo Research, USA), according to the manufacturer's instructions.

Quantitative methylation-specific PCR (qMSP). The primers for methylated and unmethylated promoter sequences were designed, using MethylPrimer software, the sequences and product sizes are listed in the S2. qMSP was performed, using the HOT FIREPol® EvaGreen® qPCR Mix (Solis BioDyne), according to the manufacturer's instructions using the CFX96 Touch Real-time PCR Detection System (Bio-Rad, USA). Reaction conditions were 95 °C for 12 min, 40 cycles of dissociation at 95 °C for 20 sec, annealing at 60 °C for 20 sec and elongation at 72 °C for 20 sec, followed by melting of PCR products from 65 °C to 95 °C increment 0.5 °C, 5 msec, 60 repeats. The *Col2A1* gene was used as a reference gene. The quantification of relative amount of methylated and unmethylated forms of gene promoters was performed, using $2^{\Delta Ct}$ method, where ΔCt=Ct(Col2A1) – Ct(gene of interest), meth-

ylated or unmethylated. Then the methylation rate for each gene was calculated by the equation [relative amount of methylated gene]/[relative amount of methylated+relative amount of unmethylated gene].

Methylation-specific PCR (MSP). PCR of bisulfite treated DNA from urine was performed, using the 5xFIREPol® Master Mix Ready to Load (Solis BioDyne). Reaction conditions were: 95 °C for 12 min, 40 cycles of dissociation at 95 °C for 20 sec, annealing at 60 °C for 20 sec and elongation at 72 °C for 20 sec, elongation 72 °C for 7 minutes.

Agarose gel electrophoresis. All samples after MSP were analyzed by the electrophoresis, using 2.5 % agarose gel in 0.5xTBE buffer, 100 mA, for the presence or absence of the methylated and unmethylated fragments.

Statistical Analysis

According to the fact, that obtained data cannot demonstrate the normal Gaussian distribution, a nonparametric statistical approach was used. The Mann-Whitney test was performed for comparison of independent samples. For comparison of the tumor-normal (TN) pairs we used the Wilcoxon matched pair test. The Kruskal-Wallis test with the Dunn-Bonferroni post-hoc test for multiple comparisons was performed to find differences between multiple experimental groups. The non-parametric Spearman correlation analysis was used to find correlations. Finally, the MDR, simple logistic regression and the receiver operator characteristic (ROC) analysis were used for identification of the combinations of the methylated studied genes with the best accuracy, sensitivity and specificity for PCa. The software STATISTICA10, Microsoft Excel, Sigma Plot, Displayr (https://app.displayr.com/), OpenEpi and GraphPadPrism were used to perform all listed tests and data visualization.

Results and Discussion

Methylation of genes' promoters in cell lines. Among all studied genes, *NKX3.1* was the most highly and constitutively methylated gene (Fig. 1*A*). In contrast, the *CDH1* gene demonstrated a low methylation rate in all cell lines, with the highest methylation in LNCaP and the lowest — in DU145. Promoter methylation of another epithelial cell marker, *KRT18,* was the highest in DU145 and lowest in PC3 cells. All data are shown in Fig. 1*A*.

Gene promoter methylation patterns in prostate tissues. Promoters of the genes described above (except *VIM*), showed differential methylation patterns in adenocarcinomas and paired CNTs (Fig. 1*B*). The *VIM* promoter was totally methylated in all samples. *NKX3.1* and *KRT18* were highly methylated whereas the methylation of *PTEN* and *CDH1* was low. *RASSF1A* demonstrated the most differential methylation, ranging from 0.03 to 1 in various samples (Fig. 1*B*).

There was no difference either in the methylation rate of PCa adenocarcinomas compared to CNTs, or in the groups, sorted by the early (1–2) and late (3–4) stages of the cancer disease according to results of the Wilcoxon matched pairs test.

A qualitative analysis of promoter methylation patterns in cell-free DNA samples from urine

Three groups of patients were included in this study: one group with confirmed PCa (n=41), one group with the inflammatory disease of a

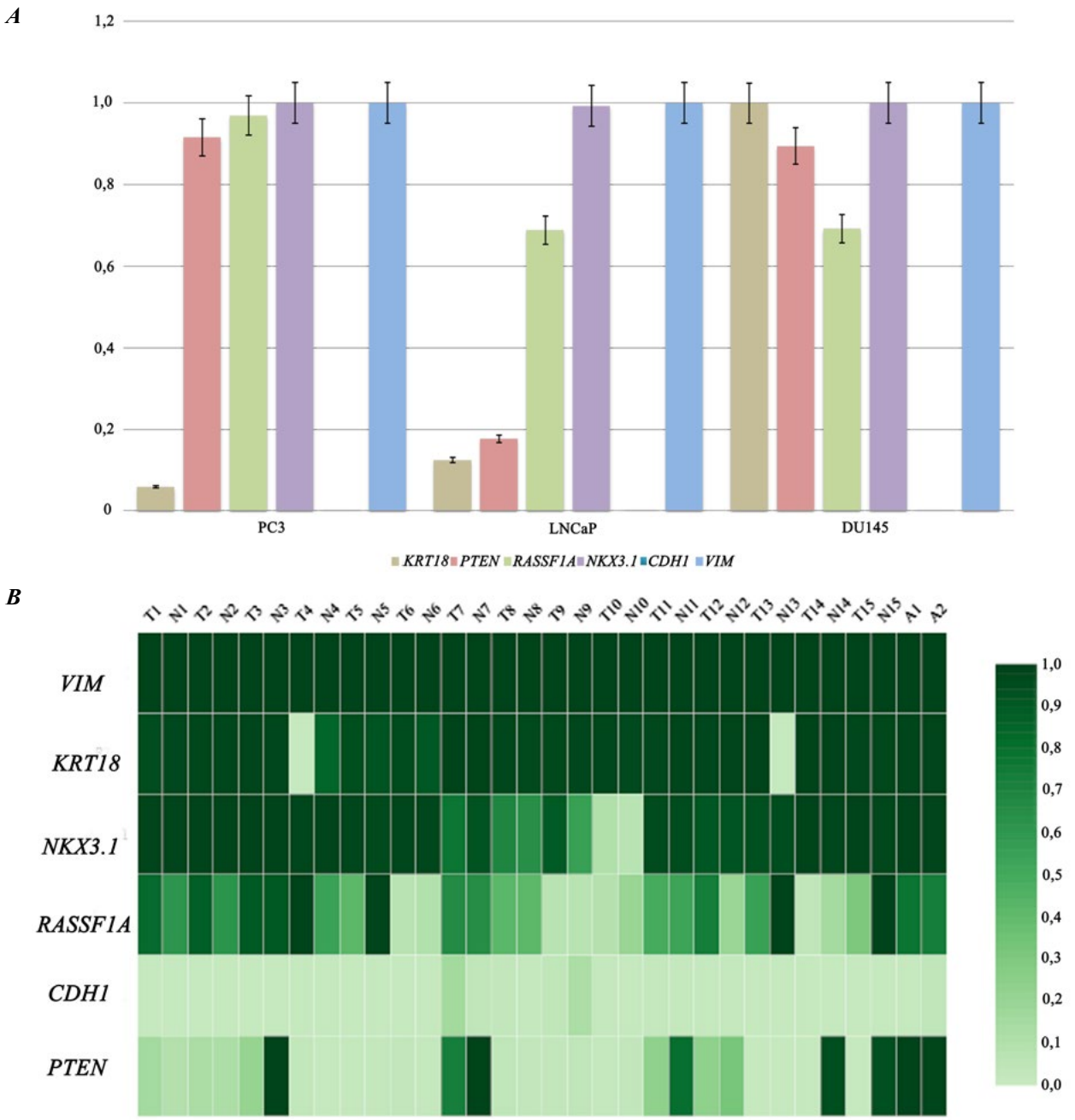

Fig. 1. Methylation rate of PTEN, CDH1, KRT18, RASSF1A and VIM in PCa cell lines and clinical samples. *A* — The fractions of methylated/unmethylated genes' promoters in PC3, LNCaP and DU145 cell lines. *B* — The fractions of methylated/unmethylated genes' promoters in adenocarcinomas (T), the paired CNT (N) and adenomas (A).

genitourinary system (n=12) and the control group of healthy donors (n=16).

The *NKX3.1* promoter was not methylated in any healthy donor but it was methylated more frequently in the samples of patients with inflammation than in PCa patients (75 % vs 63 %) (Fig. 2*A*). Oppositely, *CDH1* and *GDF15* were frequently methylated in the samples of PCa patients, but rarely in patients with inflammation. The *RASSF1A* promoter was methylated in the majority of PCa and inflammatory disease patients and only in 6 % of healthy donors. All data are presented in Fig. 2*A*). A representative image of MSP products is shown in Fig. 2B.

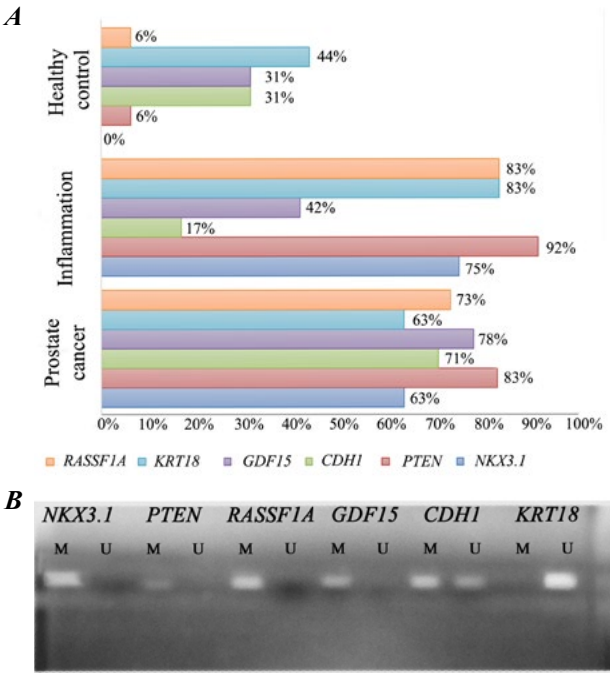

Fig. 2. *A* — A methylation pattern of promoters of the PTEN, CDH, KRT18, NKX3.1, RASSF1A and GDF15 genes in cell-free DNA, isolated from urine. *B* — A representative image of MSP products for PCa samples; M-methylated, U-unmethylated.

All five genes showed differences in methylation between healthy donors and PCa patients (Table 1). Three genes — *NKX3.1, PTEN* and *RASSF1A* differ between healthy donors and patients with inflammation. Only one gene, *CDH1*, demonstrated the differential methylation pattern in patients with inflammation and PCa.

Relation of the methylation pattern with a tissue type, gene expression levels and CPCs. We found that for the same patient a higher methylation rate of *CDH1*, *PTEN* and *NKX3.1,* observed in tumors, corresponds to a higher methylation rate of these genes in surrounding CNTs. The correlation coefficient for *CDH1* was $r_s = 0.771$, $p = 0.000231$, for *PTEN* — $r_s = 0.668$, $p = 0.00614$, and for *NKX3.1* — $r_s = 0.851$ with $p<0.0001$.

Using the presented and observed earlier data on the relative gene expression [11], the putative correlations between methylation and expression of studied genes were analyzed. We found that the methylation rates of *NKX3.1* and *CDH1* negatively correlated with each other ($s = –0.68633$, $p <0.01$) in tumor tissues,

Table 1. **The statistically significant differences between promoter methylation of DNA, isolated from urine of patients with PCa and inflammation, and from healthy donors.**

Gene/Groups	Healthy vs Infl	Healthy vs PCa	Infl vs PCa
NKX3.1	0.0022	0.0006	-
PTEN	0.0001	0.0000	-
CDH1	-	0.0138	0.0138
GDF15	-	0.0190	-
KRT18	-	-	-
RASSF1A	0.0016	0.0003	-

Note: $p<0.05$ in the Dunn-Bonferroni post hoc test for multiple comparisons

whereas their relative expression levels correlated positively (s = 0.696429, p <0.01). Methylation of *RASSF1A* negatively correlated with the expression of *PTEN* gene (s = –0.78929, p<0.01).

Of note, the level of *KRT18* methylation in PCa tissues negatively correlated with Gleason score (GS) (s = –0.593, p<0.05). There were no correlations between the methylation rate of the studied genes and other CPCs (S 3).

The differential methylation pattern in adenocarcinomas, bearing and negative for the TMPRSS2:ERG fusion transcript. Earlier, we detected TMPRSS2:ERG fusion transcript in PCa samples [9].

We analyzed the promoter methylation rates of all studied genes in PCa samples, grouped depending on the presence or absence of the TMPRSS2:ERG fusion (F+ and F-, respectively), using the Mann-Whitney rank sum test (Fig. 3*A–E*). The significantly higher levels of methylation of the *RASSF1A* promoter were shown for the F+, than for the F- groups (t=4.98, p<0.001) (Fig 3*A*). There were no other differences in methylation rates (Fig. 3*B–E*).

A regression analysis of the qualitative methylation of genes' promoters in DNA isolated from urine and a putative combination of markers for non-invasive PCa diagnostics

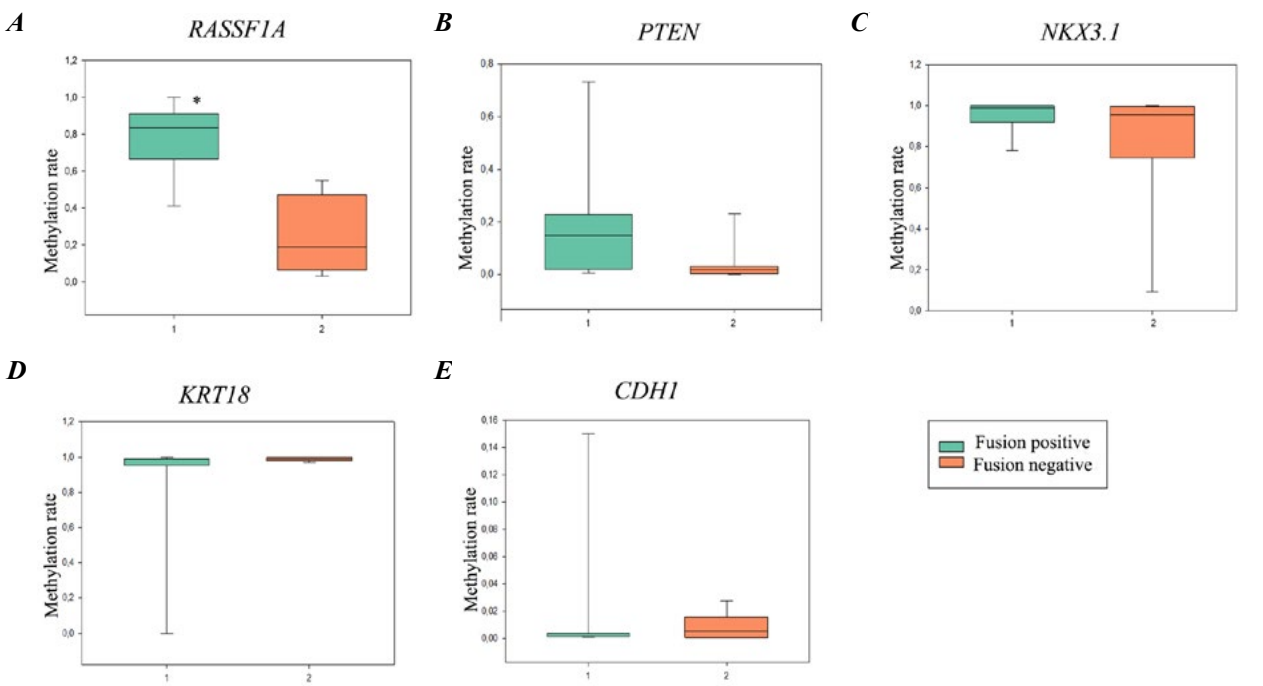

Fig. 3. The relative methylation rate of promoters of the PTEN, CDH, GDF15, KRT18 and RASSF1A genes between the TMPRSS2:ERG fusion positive and negative PCa samples. a) The methylation rate of the RASSF1A gene promoter; b) the methylation rate of the PTEN gene promoter; c) the methylation rate of the NKX3.1 promoter; d) the methylation rate of the KRT18 promoter; e) the methylation rate of the CDH1 promoter.

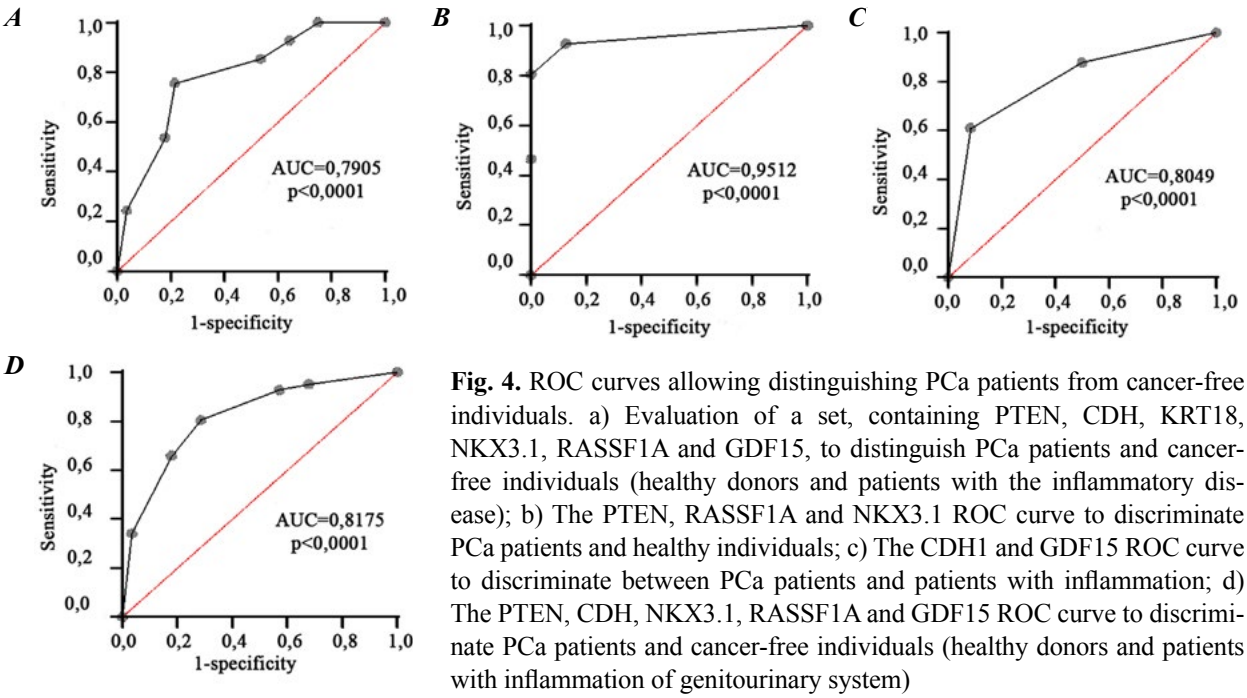

Fig. 4. ROC curves allowing distinguishing PCa patients from cancer-free individuals. a) Evaluation of a set, containing PTEN, CDH, KRT18, NKX3.1, RASSF1A and GDF15, to distinguish PCa patients and cancer-free individuals (healthy donors and patients with the inflammatory disease); b) The PTEN, RASSF1A and NKX3.1 ROC curve to discriminate PCa patients and healthy individuals; c) The CDH1 and GDF15 ROC curve to discriminate between PCa patients and patients with inflammation; d) The PTEN, CDH, NKX3.1, RASSF1A and GDF15 ROC curve to discriminate PCa patients and cancer-free individuals (healthy donors and patients with inflammation of genitourinary system)

Taking into consideration that our study included PCa patients and patients with inflammation of genitourinary system, the Odds ratio (OR) was calculated for each methylated gene, associated with PCa. The results of such analysis are presented in S4.

The calculations demonstrate, that the study of all genes in combination can help to distinguish PCa patients from non-PCa individuals. The area under ROC curve (AUC) is 0.7905, $p<0.0001$ (Fig. 4*A*).

Using the MDR analysis, we selected *PTEN, RASSF1A* and *NKX3.1* that are differentially methylated in PCa patients and healthy individuals. The AUC for those genes is estimated as 0.9512, $p<0.0001$ (Fig. 4*B*). The best combination for differentiation of PCa patients and patients with inflammation was *CDH1* with *GDF15*. The AUC for those genes was estimated as 0.8049 (Fig. 4*C*). We generated ROC curve, combining promoter methylation of *PTEN, RASSF1A* and *NKX3.1* with *CDH1* and *GDF15*, to evaluate their potential for diagnostics. The AUC was 0.8175, $p<0.0001$ (Fig. 4*D*).

Conclusions

Summarizing the obtained data on methylation in prostate tissues and urine, we found that the *RASSF1A* promoter demonstrates a higher methylation rate in the TMPRSS2:ERG fusion positive PCa. The frequencies of methylated *NKX3.1, PTEN* and *RASSF1A* in DNA, isolated from urine, were higher in cancer patients compared to healthy donors. The promoters of the *CDH1* and *GDF15* genes were methylated more frequently in PCa patients, than in patients with inflammatory disease. *PTEN, RASSF1A,*

NKX3.1, *CDH1* and *GDF15* can form a panel for early non-invasive detection of PCa. This set can be combined with detection of the TMPRSS2:ERG fusion transcript. Further work should be done to understand the molecular mechanisms explaining the functional role of promoter methylation of the selected genes.

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Патерн метилювання промоторів генів-онкосупресорів як набір можливих неінвазивних діагностичних маркерів раку передміхурової залози

О. С. Маньковська, А. С. Корсакова,
К. Р. Чернявський, О. А. Кононенко,
Є. О. Стаховський, Ю. М. Бондаренко,
В. І. Кашуба, Г. В. Геращенко

Мета. Оцінити метилювання промоторів низки потенційних генів-супресорів росту РПЗ у пухлинній тканині та сечі хворих на РПЗ для кращого розуміння регуляції експресії генів при розвитку РПЗ та оцінити можливість використання метилювання генів-онкосупресорів як неінвазивних маркерів РПЗ. **Методи.** Для кількісного аналізу метилювання промоторів досліджуваних генів використовували кількісну метил-специфічну ПЛР (qMSP), для виявлення метилювання у зразках сечі проводили метилспецифічну ПЛР, результати якої перевіряли за допомогою електрофорезу. **Результати.** Рівень метилювання промотора *RASSF1A* є значно вищим у TMPRSS2: ERG позитивних аденокарциномах. Метилювання промоторів *NKX3.1*, *PTEN* та *RASSF1A* є частою подією для пацієнтів із РПЗ у порівнянні з умовно здоровими особами. Метилювання *CDH1* та *GDF15* часто зустрічається у пацієнтів з РПЗ, у порівнянні із пацієнтами із запаленням. **Висновки.** Вищезазначені п'ять генів можуть утворювати панель для раннього неінвазивного виявлення РПЗ. Цей набір можна поєднати з виявленням TMPRSS2:ERG транскрипту. Потрібно провести більше роботи, щоб зрозуміти молекулярні механізми, що пояснюють функціональну роль метилювання промотору вибраних генів.

Ключові слова: метилювання промотора гена, гени-онкосупресори, неінвазивна діагностика, рак передміхурової залози

Паттерн метилирования промоторов генов-онкосупрессоров как предполагаемый набор неинвазивных диагностических маркеров рака предстательной железы

О. С. Маньковская, А. С. Корсакова,
К. Р. Чернявский, О. А. Кононенко,
Е. О. Стаховский, Ю. М. Бондаренко,
В. И. Кашуба, Г. В. Геращенко

Цель. Оценить метилирование промоторов ряда потенциальных генов-супрессоров роста РПЖ в опухолевой ткани и в моче пациентов с РПЖ для лучшего понимания регуляции экспрессии генов при развитии РПЖ и оценить возможность использования метилирования генов онкосупрессоров в качестве неинвазивных маркеров РПЖ. **Методы.** Для количественного анализа метиллирования промоторов исследуемых генов использовали количественную метилспецифичную ПЦР (qMSP), для выявления метилирования в образцах мочи проводили метилспецифическую ПЦР, результаты которой проверяли с помощью электрофореза. **Результаты.** Уровень метилирования промотора *RASSF1A* значительно выше в TMPRSS2: ERG положительных аденокарциномах. Метилирования промоторов *NKX3.1, PTEN* и *RASSF1A* является частым событием для пациентов с РПЖ по сравнению с условно здоровыми лицами. Метилирования *CDH1* и *GDF15* часто встречается у пациентов с РПЖ, по сравнению с пациентами с воспалением. **Выводы.** Вышеупомянутые пять генов могут образовать панель для раннего неинвазивного выявления РПЖ. Этот набор можно совместить с выявлением TMPRSS2: ERG транскрипта. Нужно провести больше работы, чтобы понять молекулярные механизмы, объясняющие функциональную роль метилирования промотора выбранных генов.

Ключевые слова: метилирования промотора гена, гены-онкосупрессоры, неинвазивная диагностика, рак предстательной железы

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