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NON-INVASIVE BIOMARKERS FOR BLADDER CANCER: A STUDY ON LNCRNAS AND DNA METHYLATION

Modern diagnostic methods for bladder cancer (BC) have some limitations, including invasiveness and low sensitivity, making the search for new molecular markers important. ***Aim.*** *To identify potential epigenetic markers for BC diagnosis and progression, including the methylation of gene promoters and the lncRNAs expression levels in tumor tissues*

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and liquid biopsies. ***Methods.*** *RT-qPCR, MSP.* ***Results.*** *Significant diagnostic value of the VIM, TMEFF2, NKX3.1, MYO3A, GDF15, and RASSF1A methylation in BC patients, increased the RASSF1A expression in tumor tissues and decreased the RASSF1A and ANRASSF1 in liquid biopsies. Correlations were identified between the studied markers and the clinical characteristics of patients.* ***Conclusions.*** *Methylation of the genes VIM, TMEFF2, NKX3.1, MYO3A, GDF15, and RASSF1A, and changes in the expression of the lncRNAs ANRASSF1, PANDAR, and GAS5 may play an important role in BC pathogenesis. The data obtained underscore the relevance of further studies on these markers for BC diagnosis and progression assessment.*

Keywords: *epigenetic biomarkers, DNA methylation, bladder cancer, lncRNAs, liquid biopsy, tumor tissues.*

Introduction

Bladder cancer (BC) is one of the most prevalent cancer types in the world, with over three-quarters of cases occurring in men [1]. Most newly diagnosed BC cases are non-muscle-invasive; nevertheless, recurrence or progression to the muscle-invasive type is observed in over half of cases despite available treatment [2, 3]. Common BC diagnostic methods have some limitations. This problem calls for research on the effective early detection and prognosis tools. Biomarker analysis in biological fluids (mainly urine for BC) is gaining popularity as a non-invasive, repeatable method. However, reliable biomarkers are needed to enhance specificity and reduce false positives [4].

In recent years, numerous studies have investigated epigenetic mechanisms in cancer, particularly DNA methylation. The *RASSF1* gene has eight splice variants, one of which, the *RASSF1A*, acts as a tumor suppressor in normal tissues, participating in the cell cycle regulation and apoptosis. Epigenetic silencing of the *RASSF1* gene through promoter hypermethylation is regulated via a sophisticated cascade involving the polycomb repressor complex 2 (PRC2). This process has been observed in several types of cancer, including BC, making the *RASSF1A* gene a potential biomarker [5].

Aberrant gene promoter methylation is a promising diagnostic and prognostic tool for BC. Methylation of genes *TMEFF2* (encoding transmembrane protein), *VIM* (encoding structural protein), *GDF15* (encoding growth differentiation factor), *MYO3A* (encoding transferase) and *NKX3.1* (encoding transcription factor) is being explored as a potential biomarker [6—8]. A 3-gene panel consisting of *VIM*, *TMEFF2*, and *GDF15* was studied in Ukrainian population. All three genes were found to be methylated in BC patients, proving their value as potentially useful markers [9].

Another key epigenetic mechanism in oncogenesis involves long non-coding RNAs (lncRNAs). Aberrant lncRNA expression significantly contributes to tumor development and progression, making them valuable targets for early cancer detection [10—12]. ANRASSF1, an antisense lncRNA of the *RASSF1*, has been reported to participate in its epigenetic regulation by binding to PRC2 and recruiting it to the *RASSF1* promoter [13]. Upregulation of ANRASSF1 has been observed in several cancers, including breast and gastric cancer [14, 15], but its role in BC pathogenesis remains unclear and requires further study.

Some lncRNAs are considered oncogenic and, therefore, can be useful in early diagnosis and prognosis. PANDAR is transcribed as an antisense RNA from the *CDKN1A* promoter and reduces pro-apoptotic gene expression by interacting with the transcription factor NF-YA and polycomb repressive complexes 1 and 2. Significant upregulation of PANDAR has been observed in BC tissues as well as in other cancer types, leading to inhibited apoptosis and increased aberrant cell proliferation [16, 17].

LncRNA GAS5 (growth arrest specific 5) is a well-known tumor suppressor frequently silenced in cancers. GAS5 has been shown to inhibit oncogenic processes by regulating Wnt/β-catenin pathway activity through the miR-18a-5p/AXIN2/GSK3β axis. Studies have demonstrated that GAS5 downregulation correlates strongly with BC stage, grade, and metastasis [18].

In this study, we comprehensively investigate several known BC biomarkers related to DNA methylation (*VIM*, *TMEFF2*, *NKX3.1*, *MYO3A*, *GDF15*, and *RASSF1A*) and lncRNA expression (GAS5, PANDAR, ANRASSF1) in the Ukrainian population, aiming to clarify their diagnostic and prognostic characteristics. Additionally, to our knowledge, this is the first study to investigate the expression of PANDAR and ANRASSF1 in urine samples from patients, with the aim of characterizing them as markers for the non-invasive diagnosis of BC.

Materials and methods

Sample collection. Tissue samples were collected at the National Cancer Institute (Kyiv, Ukraine) from BC patients, including tumor and paired normal tissue (hereafter normal tissue), immediately frozen in liquid nitrogen, and stored at –80 °C until DNA extraction. Urine samples were collected from BC patients at the National Cancer Institute (Kyiv, Ukraine) and the State Institution 'Institute of Urology of NAMS of Ukraine before surgery. A cohort of conditionally healthy donors (hereafter healthy donors) was included as a control group for biomarker analysis in urine. Urine samples were transported in sterile containers to the Institute of Molecular Biology and Genetics at 2—8 °C and processed immediately.

All samples were collected following the Declaration of Helsinki and guidelines issued by the National Cancer Institute of Ukraine and the Ethics Committee of Institute of Molecular Biology and Genetics of the National Academy of Sciences of Ukraine (protocol number: 16, December 3, 2018). Urine samples were collected between 2015 and 2021; those obtained before 2018 lacked complete patient data and were excluded from correspondent statistical analyses to ensure reliable interpretation. The study was not conducted in parallel, resulting in partial overlap between methylation and lncRNA expression cohorts. Total sample count and clinical characteristics of patients are presented in Table 1.

Primary processing and nucleic acids extraction. Frozen tissue samples were ground into a fine powder using a mortar and pestle until completely homogenized. Urine samples were centrifuged at

Table 1. **Sample sizes and patient data on the samples used in the study**

Sample type		Sample number	
Tumor tissues		24	
Normal tissue		24	
Urine of patients		28	
Urine of patients for MSP		26	
Urine of healthy donors		15	
Clinical characteristics	Sample number of tumor tissues	Number of patient urine samples	Number of patient urine samples for MSP
Stage			
I-II	6	8	18
III-IV	15	15	6
TNM classification (tumor, nodulus and metastasis)			
T1-2	8	10	2
T3-4	11	13	5
N0	6	8	2
N1-2	5	4	2
M0	16	23	23
M1	3	0	1
Grade			
G1	2	2	0
G2	9	10	6
G3	8	8	2
Therapy with gemcitabine and cisplatin			
With	10	13	3
Without	10	7	4
Recurrency			
Yes	2	2	5
No	20	22	9
Resistance			
Yes	3	3	2
No	19	17	5
Age			
<61	10	12	6
>61	10	9	7

1500 rpm and 4 °C for 15 min to separate cells. The supernatant was collected to obtain cell-free fraction of urine (cell-free urine). DNA and RNA were extracted using TRIzol Reagent (Thermo Fisher Scientific, USA) following the manufacturer's protocol. Nucleic acid concentration and purity were assessed with NanoDrop 2000 (Thermo Fisher Scientific, USA).

Bisulfite conversion and methylation analysis. Bisulfite conversion was performed using EZ DNA Methylation-Lightning Kit (Zymo Research, USA) according to manufacturer's instructions. Converted DNA was analyzed by methylation-specific PCR (MSP) using 5X FIREPol® Master Mix Ready to Load (Solis BioDyne, Estonia). Primer sequences used for the reaction were:

F., m. *RASSF1A* 5'GGTTTTGCGAGAGCGCGTTTAGTTTC3'
R., m. *RASSF1A* 5'CTAAAAAAAACCGCGCAATAAAAAC3'
F., u. *RASSF1A* 5'GTGAGAGTGTGTTTAGTTTT3'
R., u. *RASSF1A* 5'ACCACACAATAAAAACCTAAATA3'
F., m. *VIM* 5'CGGTGAGTTATCGTCGGTGATTAAGC3'
R., m. *VIM* 5'CCAACTTCGAACGACGAAAATTACG3'
F., u. *VIM* 5'GTTATTGTTGGTGATTAAGT3'
R., u. *VIM* 5'CAACAAAAATTACAAAACAACAA3'
F., m. *TMEFF2* 5'ACCACACAATAAAAACCTAAATA3'
R., m. *TMEFF2* 5'GTGAGAGTGTGTTTAGTTTT3'
F., u. *TMEFF2* 5'GTGAGAGTGTGTTTAGTTTT3'
R., u. *TMEFF2* 5'ACCACACAATAAAAACCTAAATA3'
F., m. *MYO3A* 5'CGTCGGGTTTCGGGTTAAGGAAGCGC3'
R., m. *MYO3A* 5'ACCCGCAAATAAAAACCGCGCCATC3'
F., u. *MYO3A* 5'GTTTTGGGTTAAGGAAGTGT3'
R., u. *MYO3A* 5'AAAAACCACACCATCCACA3'
F., m. *GDF15* 5'TGTTGTTGGTGTTTTCGTGGTTGTC3'
R., m. *GDF15* 5'AAATCCCGAAAAACTTACGCGACTC3'
F., u. *GDF15* 5'GTTTTTGTGGTTGTTGTATGGGGGT3'
R., u. *GDF15* 5'AAAAACTTACACAACTCACCTCA3'
F., m. *NKX3.1* 5'CGGAGCGGGGTAGTGTTTAGGGTTC3'
R., m. *NKX3.1* 5'TCCCATTAACCGCGAAACGAACC3'
F., u. *NKX3.1* 5'GTAGTGTTTAGGGTTTGTAGATT3'
R., u. *NKX3.1* 5'CCACAAAACAAACCACACC3'

Reaction conditions were as follows: initial denaturation at 95 °C for 12 min, 40 cycles of denaturation at 95 °C for 20 sec, annealing at 60 °C for 20 sec and extension at 72 °C for 20 sec, with final step of inactivation at 72 °C for 7 min. After MSP all products were separated by gel electrophoresis using 2.5% agarose gel prepared with 0,5x TBE buffer (10 μl/100 ml of ethidium bromide was added to gel for further visualization of products under UV light).

cDNA Synthesis and RT-qPCR. 1 μg of total RNA was used for cDNA synthesis with the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific, USA) according to the instruction. Relative expression levels were analyzed by real-time qPCR using HOT FIREPol EvaGreen qPCR Mix (Solis BioDyne, Estonia) on the CFX96 Real-Time PCR Detection System (Bio-Rad, USA). Before qPCR, cDNA the samples were diluted with nuclease-free water in 1:10 ratio; 20 ng of DNA was used for the reaction with final concentration of primers 50 nM. Primers sequences used for PCR were as follows:

F. *ACTB* 5'TGACGTGGACATCCGCAAAG3'
R. *ACTB* 5'CTGGAAGGTGGACAGCGAGG3'
F. *RASSF1A* 5'CTCGTCTGCCTGGACTGTTG3'
R. *RASSF1A* 5'CAGGTGTCTCCCACTCCACAG3'
F. *ANRASSF1* 5'CGCGCAGAATTAGCCTCTCT3'
R. *ANRASSF1* 5'ACCCACTGAGATAGGTCGGG3'
F. *PANDAR* 5'CTGTTAAGGTGGTGG3'
R. *PANDAR* 5'GGAGGCTCATACTGGCTGAT3'
F. *GAS5* 5'TGGTTCTGCTCCTGGTAACG3'
R. *GAS5* 5'AGGATAACAGGTCTGCCTGC3'

The conditions for the reaction were: initial denaturation at 95 °C for 12 min, followed by 41 cycles of denaturation at 95 °C for 15 sec, annealing at 60 °C for 20 sec and elongation 70 °C for 15 sec with the plate read. Melt-curve analysis was also performed. *ACTB* was used as a reference gene to calculate the relative amount of gene products. Quantifications were performed using $2^{\Delta Ct}$ method ($\Delta Ct = Ct^{ACTB} - Ct^{gene\ of\ interest}$).

Statistical analysis. Analysis was performed using Prism GraphPad 8.4.3 software. Data were checked for normality, with most showing a non-Gaussian distribution. The Wilcoxon test (for dependent samples), Mann-Whitney U test (for independent samples), and Spearman correlation were used. For data with a Gaussian distribution, the corresponding t-test was applied. Additional-

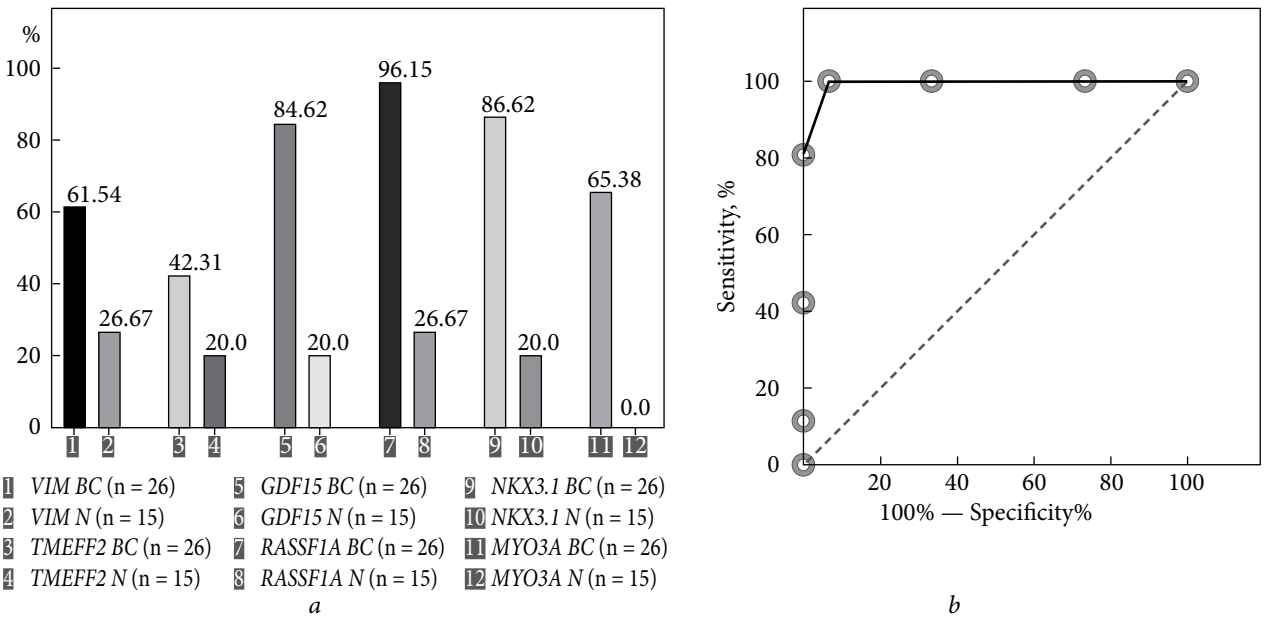

Fig 1. The methylation frequency of the studied genes relative to the total sample size: BC — patients with BC; N — healthy donors; n — total sample size (*a*). ROC curve for evaluating the diagnostic value of the methylation of the studied genes (*b*)

Table 2. **The statistical outcomes of Fisher's Exact Test**

Gene name	VIM	TMEFF2	GDF15	RASSF1A	NKX3,1	MYO3A
P value	0,052	0,186	<0,0001	<0,0001	0,0001	<0,0001
Relative Risk	1,680	1,414	6,160	10,340	3,520	2,667
Odds ratio	4,400	2,933	44,000	68,750	22,000	6,216 to Infinity
Sensitivity	0,615	0,423	0,917	0,962	0,846	0,654
Specificity	0,733	0,800	0,800	0,733	0,800	1,000

ly, Fisher's Exact Test and ROC curve analysis were conducted.

Results and Discussion

Our goal was to identify potential diagnostic markers for BC that could assist clinicians in early detection or assessing the disease progression, as well as evaluating them as potential therapeutic targets. We focused on studying the relationship between methylation of the cancer-related genes and clinical characteristics of BC patients. Based on the literature data, several promising gene candidates were selected. The control group consisted of healthy urine donors. To assess the diagnostic potential of the *VIM*, *TMEFF2*, *NKX3.1*, *MYO3A*, *GDF15* and *RASSF1A* genes methylation, we calculated the methylation frequency of the studied genes in both groups. These results are presented in Fig. 1*a*. Also, we determined whether the methylation status of each gene could significantly differentiate BC patients from healthy donors. The statistical outcomes are summarized in Table 2. Since we hypothesized

that these genes could form a potential biomarker panel, we generated a methylation score for each individual by summing the number of methylation events across the selected genes. Using these cumulative scores, we constructed a ROC curve, revealing an AUC of 0.9936, $p < 0.0001$ (Fig. 1*b*). This strongly suggests that the selected methylation markers are highly prevalent in BC patients and might hold a significant diagnostic value.

Further research is needed to determine whether these methylation changes occur early in tumor development and can be used as early diagnostic biomarkers. However, if these markers reflect persistent tumor-related alterations, they may also be useful for post-surgical monitoring of patients for disease recurrence.

The significantly high methylation level of the *RASSF1A* gene in BC patients prompted us to expand the range of considered markers and turn our attention to ncRNAs. Since lncRNAs are known to be secreted into biological fluids, particularly within exosomes, we hypothesized that they also could serve as non-invasive biomarkers for the BC detection. ANRASSF1 has been reported to promote the *RASSF1A* methylation and potentially silence its expression. Therefore, we assessed its relative quantification (RQ), along with several other lncRNAs known to be involved in cancer-related epigenetic regulation, to obtain a more comprehensive picture. To explore the utility of ANRASSF1, PANDAR and GAS5 in non-invasive diagnostics using liquid biopsies, we measured their levels in cell-free urine samples from BC patients and healthy donors. To complement our findings, we also examined the *RASSF1A* mRNA levels in urine. The results are shown in Fig. 2.

RASSF1A showed significantly decreased abundance in BC urine samples compared to healthy donors ($p = 0.0004$). ANRASSF1 also showed a significantly decreased abundance in BC urine samples compared to those from healthy donors ($p = 0.0017$), supporting its potential as non-invasive BC biomarker. No significant differences were found for PANDAR ($p = 0.4998$) and GAS5 ($p = 0.1847$), but an overall trend of their lower abundance in BC patients suggests further investigation in larger cohorts.

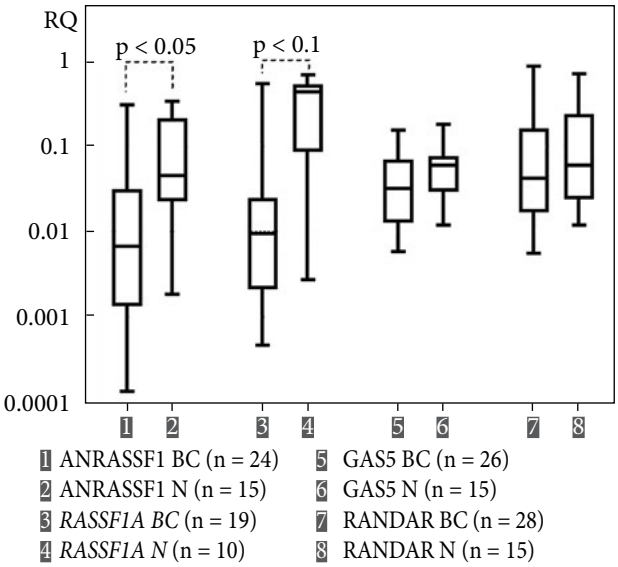

Fig 2. Histogram showing relative levels of ANRASSF1, *RASSF1A*, PANDAR, and GAS5 in urine samples: BC — BC patients; N — healthy donors; n — total sample size

Table 3. **The statistical outcomes of ROC curves analysis**

lncRNA/Gene name	Area	P value
ANRASSF1	0,7944	0,0022
RASSF1A	0,8923	0,0004
GAS5	0,5620	0,4898
PANDAR	0,5643	0,4914

We also performed ROC curve analysis for studied transcripts, the results of which are presented in Fig. 3 and Table 3. Obtained AUC values also indicate the high significance of *RASSF1A* and ANRASSF1 expression dysregulation in BC, emphasizing the importance of further research on them as potential therapeutic targets and diagnostic markers.

Finally, we analyzed correlations between all potential biomarkers (both methylation and ex-

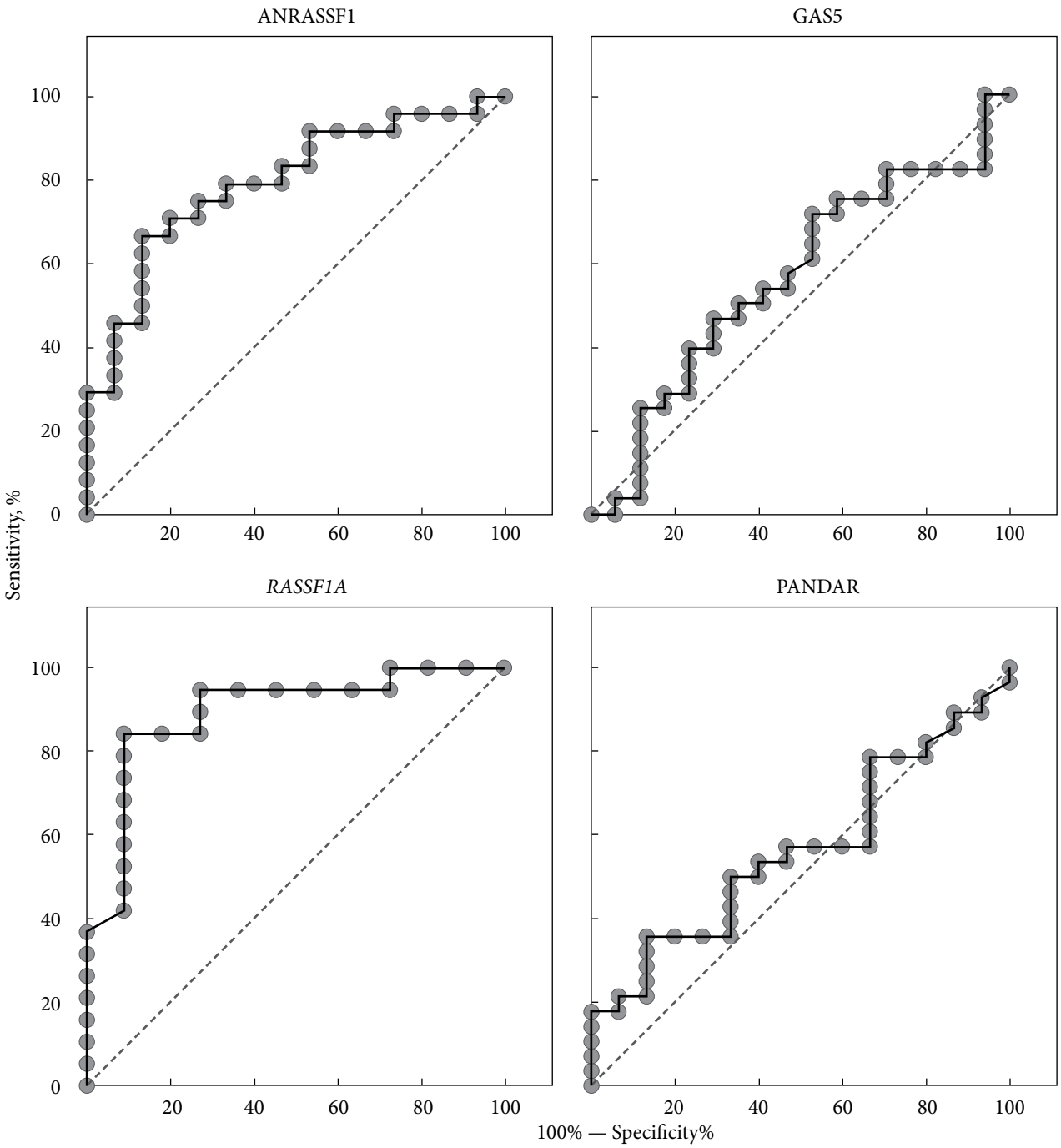

Fig 3. ROC curves for evaluating the significance of the RQ of the studied transcripts

pression-based) and clinical characteristics. Statistical analysis revealed a negative correlation between the RQ of GAS5 and the methylation of *NKX3.1*, as well as between the RQ of ANRASSF1 and the methylation of *NKX3.1*, within a 90% confidence interval (CI) (r = –0.878, p = 0.1, n = 6 for both). Further studies in larger cohorts are required to validate and elucidate these associations. Also, correlations between transcript abundance and clinical data showed a positive association between *RASSF1A* mRNA levels and patients' age (r = 0.5771, p = 0.0332, n = 16), suggesting an increased possible role of *RASSF1A* in BC pathogenesis with age. Notably, PANDAR and GAS5 exhibited a strong positive correlation in BC urine samples (r = 0.812, p < 0.0001, n = 26), which was absent in healthy donors (r = 0.2286, p = 0.4114, n = 15). This may suggest their potential involvement in the BC-related regulatory networks and the key role of their dysregulations in carcinogenesis. A positive correlation was also found between *RASSF1A* and ANRASSF1 in both samples (r = 0.503, p = 0.049, n = 16 in BC patients and r = 0.903, p = 0.001, n = 10 in healthy donors), raising questions about whether ANRASSF1 truly acts as a negative regulator of *RASSF1A*, warranting further study. Correlations within 90% CI were also found between PANDAR and *RASSF1A* (r = 0.449, p = 0.054, n = 19) in the sample of cancer patients, and between ANRASSF1 and GAS5 (r = 0.454, p = 0.092, n = 15) in the sample of healthy donors. No other significant correlations were found.

We also analyzed *RASSF1A*, ANRASSF1, PANDAR, and GAS5 expression in paired tumor and conventionally normal tissues from BC patients. RQ are represented at Fig 4.

Paired Mann-Whitney U test confirmed significantly higher RQ of *RASSF1A* (p = 0.0268) in tumor tissues. Upregulation was also observed for PANDAR (p = 0.0973, within the 90% CI). Other lncRNAs showed increased RQ trends but without strong statistical confidence, which may be clarified in larger cohorts. These results suggest that dysregulation of *RASSF1A,* and potentially

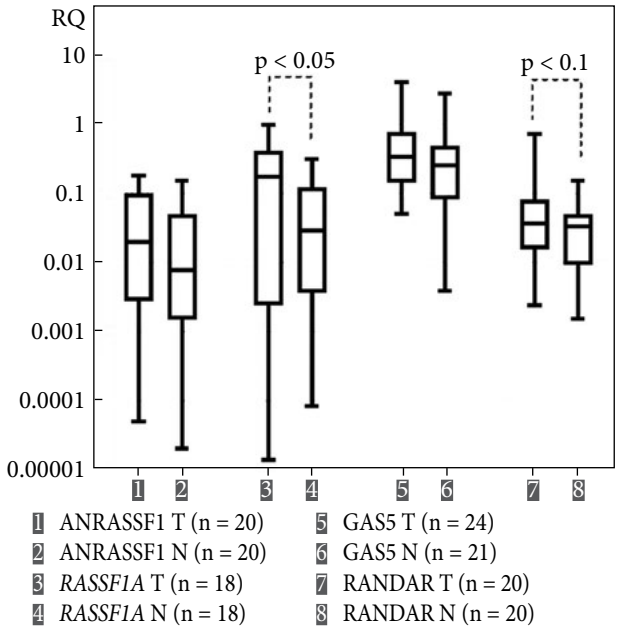

Fig 4. Expression levels of analyzed transcripts in tissues: T — tumor tissues; N — normal tissue; n — total sample size

PANDAR, may be associated with BC development and progression. Further analysis revealed a negative correlation between PANDAR RQ and patients' age (r = –0.509, p = 0.046, n = 16), which may suggest a decrease in its role in carcinogenesis with age. No other statistically significant correlations with clinical characteristics were found. We also compared lncRNAs expression in tumor tissues with their levels in the corresponding patients' cell-free urine but found no correlations. Notably, the decreased target levels in cell-free urine contrast with their upregulation in tumor tissues. This discrepancy may be explained by individual metabolic characteristics of tumors, or differences in transcript release mechanisms [19].

Interestingly, the expression level of the *RASSF1A* tumor suppressor was higher in the tumor tissues compared to the normal tissue, which contradicts our expectations. It is hypothesized that the functional inactivation of the *RASSF1A* gene in BC may be unrelated to a direct reduction

in its expression [20]. Secondly, according to the literature data, the relationship between its hypermethylation and expression in BC remains unclear [21]. Thus, tissue-specific upregulation of the *RASSF1A* expression in BC may occur in response to increased genomic instability, while its tumor-suppressive functions remain impaired in this cancer type. It is important to consider that other explanations for this phenomenon may exist. Thus, further research is needed to clarify these findings.

Conclusions

This study explored DNA methylation and lncRNA expression as potential biomarkers for BC. Our findings highlight increased frequency of methylation of *VIM*, *TMEFF2, GDF15, NKX3.1*, *MYO3A* and *RASSF1A* genes in liquid biopsy as a potential promising diagnostic panel for personalized diagnosis of BC.

We also observed that *RASSF1A* and ANRASSF1 showed significantly decreased abundance in BC urine samples. This may indicate that ANRASSF1 has potential as a non-invasive diagnostic marker. However, contrary to the existing literature [13], the observed positive correlation between *RASSF1A* and ANRASSF1 raises doubts about the role of ANRASSF1 as a negative regulator of *RASSF1A*, suggesting a more complex relationship between them. Moreover, *RASSF1A* was upregulated in tumor tissues, raising questions about its tissue-specific tumor suppressor role in BC. Consistent with the literature data [16], PANDAR was also upregulated in tumor tissues, highlighting its potential as a therapeutic target. We also observed several other correlations and trends for investigated transcripts, which may indicate their significant role in BC development.

These findings raise several questions about the functional properties of the studied targets and highlight the potential of DNA methylation and lncRNA expression markers for the BC detection and monitoring, while also emphasizing the need for further research and validation in larger and more diverse patient cohorts.

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НЕІНВАЗИВНІ БІОМАРКЕРИ РАКУ СЕЧОВОГО МІХУРА: ДОСЛІДЖЕННЯ ДНРНК ТА МЕТИЛЮВАННЯ ДНК

Сучасні методи діагностики раку сечового міхура (РСМ) мають обмеження, включаючи інвазивність і низьку чутливість, що актуалізує пошук нових молекулярних маркерів. ***Мета.*** Виявити потенційні епігенетичні маркери діагностики та прогресії РСМ, зокрема метилювання промоторів генів і рівень експресії днРНК у пухлинних тканинах і рідких біопсіях. ***Методи.*** РТ-кПЛР, МСП. ***Результати.*** Показано значну діагностичну цінність метилювання *VIM, TMEFF2, NKX3.1, MYO3A, GDF15* і *RASSF1A*, підвищення експресії *RASSF1A* у пухлинних тканинах і зниження рівнів *RASSF1A* і ANRASSF1 у рідких біопсіях. Виявлено кореляції між маркерами та клінічними характеристиками пацієнтів. ***Висновки.*** Метилювання генів *VIM*, *TMEFF2*, *NKX3.1*, *MYO3A*, *GDF15* і *RASSF1A* та зміни експресії днРНК ANRASSF1, PANDAR, GAS5 можуть відігравати важливу роль у патогенезі РСМ. Отримані дані кажуть про актуальність їх подальших досліджень для діагностики та оцінки прогресії РСМ.

***Ключові слова*:** епігенетичні біомаркери, метилювання ДНК, рак сечового міхура, днРНК, рідинні біопсії, тканини пухлин.