Cancer Management and Research

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ORIGINAL RESEARCH

Methylation and Expression Analysis of POU4F2, HOXA9, RBM46, and TSGA10 Genes in Bladder Cancer Using Methyl-Sensitive Restriction Enzyme PCR (MSRE-PCR)

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Purpose: Bladder cancer is a prevalent malignancy, with high recurrence rates for non-muscle invasive cases and significant progression risks. Traditional diagnostic methods, such as cystoscopy and urine cytology, are limited by their invasiveness and low sensitivity for detecting low-grade tumors, respectively. Advances in non-invasive diagnostics focus on biomarkers such as cfDNA and DNA methylation, offering promising tools for early detection.

Patients and Methods: This study investigated methylation and expression changes in POU4F2, HOXA9, RBM46, and TSGA10 genes in bladder cancer. A total of 22 patients and 30 controls were enrolled, with urine and plasma samples collected for analysis. cfDNA and total RNA were extracted using commercial kits. Methylation was assessed via MSRE-PCR, and gene expression was evaluated with Real-Time PCR. Statistical analysis was performed using GraphPad Prism software, with an unpaired *t*-test and ANOVA to compare the differences between the cancer and control groups. A p-value of less than 0.05 was considered statistically significant.

Results: Urine cfDNA from bladder cancer patients showed significantly higher promoter methylation of POU4F2, HOXA9, RBM46, and TSGA10 compared with controls (approximately 2.2-, 9.0-, 3.6- and 6.1-fold increases; all $p < 0.0001$), with smaller but still significant differences in plasma ($p = 0.0005–0.0278$). ROC analysis of urine methylation yielded AUCs of 0.97 for POU4F2, 0.95 for HOXA9, 0.88 for RBM46 and 0.98 for TSGA10 (sensitivities 95–100%, specificities 80–100%; all $p < 0.0001$). A PCA-derived composite methylation score further improved discrimination (AUC = 0.993, 95% CI 0.974–1.000; sensitivity 100%, specificity 95%; $p < 0.001$). Consistently, gene expression analysis in urine showed significant downregulation of POU4F2 and HOXA9 (≈7- and 9-fold decreases; $p = 0.0099$ and $p = 0.025$) and upregulation of RBM46 and TSGA10 (≈2.5- and 2.3-fold increases; $p = 0.0037$ and $p = 0.0114$) in patients versus controls.

Conclusion: The results indicate that these methylation and expression profiles can be used as non-invasive biomarkers for the early diagnosis of bladder cancer. The use of these methods provides both greater sensitivity and accuracy than traditional methods and can pave the way for the development of more effective screening tests.

Keywords: bladder cancer, DNA methylation, non-invasive biomarkers, cell-free DNA, PCR-based methods

Introduction

Bladder cancer is one of the most common urological malignancies worldwide, with an estimated 573,000 new cases and 213,000 deaths reported globally in 2020.[1] Approximately 70–75% of newly diagnosed cases present as non-muscle invasive bladder cancer (NMIBC), which is associated with a high recurrence rate of up to 70% and a 10–20% risk of progression to muscle-invasive disease.[2] In contrast, muscle invasive bladder cancer (MIBC) is clinically aggressive and often requires radical cystectomy and systemic therapy. Despite available treatment options, the long-term burden of recurrence and progression highlights the need for improved approaches for early detection and disease monitoring.[3]

Traditional diagnostic methods for bladder cancer include cystoscopy and urine cytology. While cystoscopy remains the gold standard due to its high sensitivity, it is an invasive procedure associated with discomfort and potential

https://doi.org/10.2147/CMAR.S557943
Received: 19 August 2025
Accepted: 26 December 2025
Published: 6 January 2026

complications. Urine cytology, on the other hand, has high specificity but limited sensitivity, especially for low-grade tumors and also, bladder cancer is a molecularly heterogeneous disease, comprising distinct genomic and epigenomic subtypes that differ in biological behavior, progression risk, and therapeutic response.[4] These limitations have spurred efforts to develop alternative diagnostic tests that are non-invasive, sensitive, and cost-effective. Several urinary biomarkers, including protein-based assays (eg, NMP22, BTA), and genomic or epigenomic markers (eg, DNA methylation), have been explored for their potential utility in bladder cancer detection. Recent studies have shown that DNA methylation patterns correlate with these molecular subgroups and can stratify patients into low-risk and high-risk categories. As an epigenetic mechanism that influences gene regulation, methylation profiling has emerged as a promising tool for refining diagnostic accuracy, subclassifying tumors, and predicting disease aggressiveness.[5,6]

The use of noninvasive techniques for cancer diagnosis and progression tracking has increased recently. These techniques are based on the understanding that parts of cancer cells are released into the bloodstream making it possible to investigate tumor-derived alterations through readily accessible liquid samples, although their diagnostic sensitivity varies across cancer types. Cell-free DNA (cfDNA), which is extracellular DNA found in plasma and urine, is one such instance. Numerous benign physiological conditions, such as pregnancy,[7] urinary tract infection,[8] and systemic inflammatory response syndrome,[9] as well as several malignancies,[10] have been shown to cause an increase in the amount of cfDNA. The percentage of mutated cfDNA that comes from cancer cells is known as circulating tumor DNA, or ctDNA.[11,12] These rare ctDNA fragments can be detected with advanced sequencing technologies, although detection sensitivity remains limited in low-tumor-burden cancers such as early-stage bladder cancer.[13]

As a result, a lot of work has gone into creating tests based on non-invasive urine biomarkers.[14] The basis for these tests is the fact that BCs are molecularly diverse and can be categorized into various molecular groups that help predict prognosis and treatment response.[15] One promising cancer biomarker is the measurement of epigenetic changes, such as DNA methylation. Without changing the DNA sequence, DNA methylation is an epigenetic alteration that influences gene expression. Methylated loci have been identified in several studies related to bladder cancer, suggesting that they may be used as a biomarker for diagnosis and prognosis.[16]

Also, based on previous evidence, it is believed that a combination of urine biomarkers from different methods can be a desirable strategy to improve bladder cancer screening. The present study also tries to continue this way by selecting suitable candidates and testing in the urine of patients to help develop a non-invasive and accessible screening test. A number of studies have investigated panels of DNA methylation in bladder cancer using array-based technologies.[17–20] Based on findings from previous genome-wide methylation studies, a group of genes showing significant methylation changes in bladder cancer tissues has been identified. Among these, the genes that have the most methylation changes were selected. Therefore, we selected two genes, HOXA9 and POU4F2, which have not been evaluated in urine cfDNA samples. Also, based on the strategy of expression changes, by reviewing the previous articles, two genes, TSGA10 and RBM46, which have been reported in some studies to show altered expression patterns in bladder cancer[20,21] but have not been studied in terms of methylation patterns, have been chosen. Despite the growing number of studies on methylation-based biomarkers, many existing assays lack sufficient sensitivity for early-stage or low-grade bladder cancer, and few have focused specifically on cfDNA in urine, which directly reflects the tumor environment in the bladder. Moreover, the combined evaluation of both methylation and gene expression patterns in POU4F2, HOXA9, RBM46, and TSGA10 has not been previously investigated in the context of non-invasive bladder cancer detection. By focusing on urinary cfDNA and integrating both methylation and expression analysis, this study aims to address an unmet need for a reliable and accessible screening tool. Demonstrating the diagnostic performance of these markers may contribute to developing a clinically applicable biomarker panel for early detection and patient monitoring. For this purpose, we investigated the methylation of HOXA9, POU4F2, TSGA10 and RBM46 genes in the urine samples of people with bladder cancer and bladder cancer cell lines (Cell lines are considered as hypermethylated controls), and their expression is also evaluated to explore whether methylation patterns correspond to changes in gene expression or whether additional regulatory factors may be involved. We hypothesized that methylation alterations in POU4F2, HOXA9, RBM46, and TSGA10, as detected through urine-derived cfDNA, are significantly associated with bladder cancer and can distinguish patients from healthy individuals.

Materials and Method

Sample Size

In this case-control study, 22 patients with bladder cancer referred to Velayat Hospital and 30 healthy volunteers were studied between September 2023 and May 2024.

Clinical Diagnosis, Inclusion Criteria, and Pathology Confirmation

A total of 27 individuals initially suspected of having bladder cancer based on clinical presentation and cystoscopic evaluation were assessed for eligibility. All cases underwent histopathological examination of tumor biopsy specimens to confirm diagnosis. Five individuals were excluded due to non-malignant pathology findings (eg, cystitis, adenoma), inadequate biopsy sampling, or insufficient RNA yield, leaving 22 confirmed bladder cancer cases for inclusion.

All included patients had histopathologically confirmed transitional cell carcinoma (urothelial carcinoma). According to the WHO/ISUP grading criteria, 15 cases were classified as low-grade, and 7 cases as high-grade tumors. First-morning urine samples were collected prior to any treatment (including TURBT, intravesical therapy, chemotherapy, or radiotherapy). The control group consisted of 30 age-matched healthy individuals without any known urological disease or hematuria. First-morning urine samples were also collected from this group. Demographic and pathological characteristics of the study groups are presented in Table 1.

Criteria for Inclusion in the Case Group

The inclusion criteria for the case group consisted of individuals over the age of 35 with documented exposure to known risk factors for bladder cancer. These risk factors included smoking, opium derivatives, occupational exposure to aromatic amines, or a history of recurrent painless visible hematuria with the passage of clots. Alternatively, individuals with two or more separate clinical laboratory diagnoses of microscopic hematuria, defined as the presence of five or more red blood cells per high-power field in the urine, were also included. Additionally, participants must not have undergone any bladder cancer-related treatments, such as Bacillus Calmette-Guérin (BCG) injections, chemotherapy, or radiotherapy, within the past two years. It is important to note that not all individuals presenting with hematuria are necessarily diagnosed with bladder cancer, as conditions such as infections or bladder stones can also cause hematuria. To confirm the definitive diagnosis of bladder cancer or rule out the disease, cystoscopy was performed on all participants to provide final confirmation of their cancer status.

Criteria for Inclusion in the Control Group

The control group consisted of individuals without a history of bladder cancer at the time of the study, as well as an absence of key symptoms such as recurrent painless visible hematuria with clot passage or two or more separate clinical laboratory findings of microscopic hematuria, with five or more red blood cells per high-power field in the urine. Furthermore, participants in the control group were matched to the case group based on age and gender.

Table 1 Clinical and Demographic Characteristics of the Study Participants

Study Groups	Subgroups Studied	Sex	Age	Clinical Diagnosis	Number
Patient group	Transitional carcinoma (TCC)	Male= 20	61.35±14.9	Low grade=15	22
		Female= 2		High grade= 7	
Healthy group	Normal in terms of urological diseases	Male= 26	63.23±8.9	Normal in terms of urological diseases	30
		Female= 4			

https://doi.org/10.2147/CMAR.S557943

Sample Collection

This study was conducted with the approval and ethical code (IR.MODARES.REC.1403.119) from the Tarbiat Modares University's ethics committee and in accordance with the "Declaration of Helsinki". All participants signed an informed consent form prior to sample collection and participation in the study. Individuals suspected of having bladder cancer were referred for further evaluations, including urine analysis and cystoscopy, to confirm the diagnosis and determine the tumor's grade and stage, if present. Sample collection from both cancer patients and healthy individuals was carried out by a trained professional. Urine samples (10 mL) were collected as first-void morning urine in sterile 15 mL Falcon tubes prior to any diagnostic or therapeutic intervention. Samples were immediately placed on ice and transferred to the laboratory within 1 hour. Urine was centrifuged at 2,500 g for 10 minutes at 4°C to remove cellular debris. The cell-free supernatant was aliquoted and stored at −80°C until cfDNA extraction to prevent DNA degradation.

For plasma collection, blood samples were initially collected from individuals with cancer and healthy controls in serum separator tubes containing a separator gel. The blood was centrifuged at 1500–2000 g for 10 minutes at room temperature to separate cells and other large particles from the serum. The collected serum was then subjected to a second centrifugation step at 10,000–12,000 g for 10 minutes to remove any remaining particles, including platelets.

To minimize measurement bias, all urine and plasma samples were anonymized and coded prior to laboratory analysis. The researcher performing DNA extraction, MSRE-qPCR, and gene expression analysis was blinded to the clinical grouping (cancer vs control). Sample processing order was randomized to prevent batch effects, and data decoding was performed only after completion of statistical analysis.

Extract Cell Free DNA and Total RNA from Urine

To separate cells and other large particles, the urine sample was centrifuged at 2000–3000 g for 10–20 minutes. For cfDNA extraction, urine was collected above the sediment (supernatant). Commercial Dena Zist Asia kits (Iran) were used to extract cfDNA from urine. This kit is based on the use of silica columns. The process involved adding a lysis buffer to the sample, separating the proteins and debris, and then binding the cfDNA to the silica column. The DNA was then released by washing off excess material and finally using a washing buffer. To ensure reliability, an internal comparison was carried out between this kit and the Magen cfDNA extraction kit (China), widely used in international studies. Both kits yielded comparable cfDNA concentration, purity ratios, and PCR amplification efficiency, confirming the suitability of the Dena Zist kit for use in this study.

The total RNA was used for sedimentation. RNA extraction was performed using the TriPure isolation reagent (Roche, Germany) according to the manufacturer's protocol. Initially, the cells were lysed using the provided extraction buffer. Chloroform was then added to separate the aqueous and organic phases. The RNA was precipitated from the aqueous phase using isopropanol. The RNA pellet was washed with 70% ethanol, air-dried, dissolved in DEPC-treated water, and stored at −80°C.

To assess the quantity and quality of the extracted cfDNA and Total RNA, a spectrophotometer and gel electrophoresis were utilized. The spectrophotometer provided precise measurements of DNA and RNA concentration and purity, while gel electrophoresis allowed for the evaluation of the DNA and RNA fragment sizes and overall integrity, ensuring the cfDNA and RNA were suitable for downstream applications.

Assessment of Methylation Status of POU4F2, HOXA9, RBM46, and TSGA10 Genes Using the MSRE PCR Method

The methylation status of POU4F2, HOXA9, RBM46, and TSGA10 genes was evaluated using the Methylation-Sensitive Restriction Enzyme followed by Polymerase Chain Reaction (MSRE-PCR) technique. To analyze the methylation status of the selected genes, CpG islands for all four genes were initially identified using the UCSC Genome Browser. The regions that were hypermethylated in this cancer were identified, and the selected restriction enzyme sites are precisely located within these regions. The POU4F2, RBM4G, and TSG10 genes were tested using the methylation-sensitive restriction enzyme HhaI, while the HOXA9 gene was analyzed with the enzyme HpaII. These enzymes specifically target unmethylated recognition sites, allowing for the detection of methylation patterns across the CpG

islands in the respective genes. To assess the efficacy of the restriction enzyme, the DNA samples were processed under two conditions. In the first condition, the DNA was treated with the enzyme, referred to as the Digested sample. In the second condition, water was added to the DNA instead of the enzyme, and this was designated as the Undigested control. The final reaction volume for both conditions was set at 10 µL, consisting of 8.8 µL DNA, 1 µL buffer, and 0.2 µL enzyme for the Digested sample. Both the Digested and Undigested samples were incubated at 37°C for 15 minutes according to the enzyme manufacturer's protocol (BioLabs). The primer and TaqMan probe sequences for the POU4F2 and HOXA9 genes and primer alone for the RBM46 and TSGA10 (as detailed in Table 2) were used at a concentration of 10 pmol/µL. These were combined with the Master Mix, water, and DNA samples. The reactions were then subjected to amplification using the ABI Step One Plus Real-Time PCR system, with annealing temperatures set at 62°C.

Cell Culture

The human bladder cancer cell lines 5637 and EJ138 were used in this study. Both cell lines were obtained from the Pasteur Institute of Iran, and their identity was verified using short tandem repeat (STR) profiling according to supplier documentation. In addition, both cell lines were routinely tested and confirmed to be free of Mycoplasma contamination. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Pen-Strep). Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Cells were passaged at 70–80% confluency using standard trypsinization procedures and used for experiments between passages 4–10 to minimize phenotypic drift.

The selection of 5637 and EJ138 as hypermethylated reference models was based on extensive literature demonstrating widespread promoter hypermethylation in bladder cancer cell lines. Prior studies have shown that these lines exhibit hypermethylation across several tumor suppressor genes,[22–25] and EJ138 has specifically been used as a hypermethylated control in MSRE-based methylation assays.[26] CCLE-based RRBS methylation profiling further confirms promoter hypermethylation of POU4F2 in both 5637 and T24/EJ138, HOXA9 in EJ138, and TSGA10 and RBM46 in 5637.

At the time of DNA extraction, cells were collected at approximately 80% confluency. Genomic DNA was isolated using the Dena Zist Asia commercial DNA extraction kit (Iran), following the manufacturer's protocol. For methylation analysis, extracted DNA was subjected to digestion with methylation-sensitive restriction enzymes. HhaI was used for the POU4F2, RBM46, and TSGA10 genes, and HpaII was used for the HOXA9 gene. Digested and undigested DNA samples were then analyzed using Methylation-Sensitive Restriction Enzyme PCR (MSRE-PCR) to determine the methylation status of each gene.

Table 2 Sequence of Primers and Probes for Genes Studied

Gene Name	Sequence 5'-3'	Product size (bp)
POU4F2	F: GTCAGGCATCCGTTCAGAC R: CGCCTCCGGACTCTGTA P: AAGGAGCGCGTAGCCGAGAT	79
HOXA9	F: GAGGCTGGCCCAGGGT R: GCAACTACTACGTGGACTCGT P: CTGAGCGTTGGCCGCTATGCGCC	93
RBM46	F: GATGAGGCACCACTCCTCGT R: ACCGTTTAGGGGACCAGTCG	158
TSGA10	F: GTGGGGCTCGTCTTCCAC R: CTTGTAGACCCCGCGAGAAG	114

https://doi.org/10.2147/CMAR.S557943

Gene Expression Analysis of POU4F2, HOXA9, RBM46, and TSGA10 Using Real-Time PCR

A total of 200 nanograms of RNA from each sample was used for cDNA synthesis. The cDNA synthesis was performed using the Easy™cDNA Synthesis kit (Parstous, Iran, cat#A101161) according to the manufacturer's protocol. Primers were designed using the Primer3 Plus software, and their specificity was verified with Primer-BLAST (as detailed in Table 3). Gene expression levels were analyzed using the SYBR Green Master Mix (Ampliqon, Denmark) on a Step One Plus Real-Time PCR system.

Statical Analysis

Undetectable Ct values were normalized to 47 to allow inclusion in comparative analyses. For each gene, methylation-sensitive restriction enzyme (MSRE) digestion was performed, and quantitative PCR (qPCR) was conducted on both enzyme-digested (methylation-sensitive) and undigested DNA samples. The ΔCt value was calculated as:

$$\Delta\ \mathrm{Ct} = Ct_{undigest} - Ct_{digest}$$

Relative methylation levels were then determined using the comparative ΔΔCt method (Livak and Schmittgen, 2001), employing control samples as the calibrator:

$$Fc : 2^{-\Delta\Delta Ct((\Delta Ct\,digest - \Delta Ct\,undigest)cancer - (\Delta Ct\,digest - \Delta Ct\,undigest)Control)}$$

These ΔΔCt-based fold-change values were used to calculate the methylation rate shown in Figure 1 and to perform statistical comparisons between bladder cancer and control groups.

In addition, for comparative visualization of methylation across different sample types (cell lines, urine, and plasma), the methylation percentage was calculated using the following equation:

$$\mathrm{Methylation\%} = \left(\frac{Intensity\ or\ Ct\ of\ Undigested\ Sample}{Intensity\ or\ Ct\ of\ Digested\ Sample}\right) * 100$$

This calculation represents the relative ratio between digested and undigested DNA amplification signals and was used for Figure 2 to illustrate proportional methylation changes. However, all statistical analyses were performed using the ΔΔCt-based fold change data.

In this study, statistical analysis was carried out using GraphPad Prism software, version 9. All experiments were conducted in duplicate to ensure the reliability and reproducibility of the results. Prior to statistical testing, data distribution was assessed using the Shapiro–Wilk normality test, and homogeneity of variances was evaluated using

Table 3 Sequence of Primers for Genes Studied

Gene Name	Sequence 5'-3'	Product Size (bp)
POU4F2	F: TTCCAACCCCACCGAGCAA R: GAGACGATGTCCACGGCTG	88
HOXA9	F: CCCCATCGATCCCAATAACCC R: GGTGAGGTTGAGCAGTCGAG	172
RBM46	F: CCCGAAAAGTTAATACAGAGCAGA R: CGATGGAAATTGTATCCAAATGA	103
TSGA10	F: ATGAGCGCCATTTGGCAGAA R: GGGTAATTTCTTCCTGTGCCTG	102
B2M*	F: AGATGAGTATGCCTGCCGTGT R: TGCGACATCTTCAAACCTCCAT	106

Note: * The B2M gene was used as a housekeeping gene.

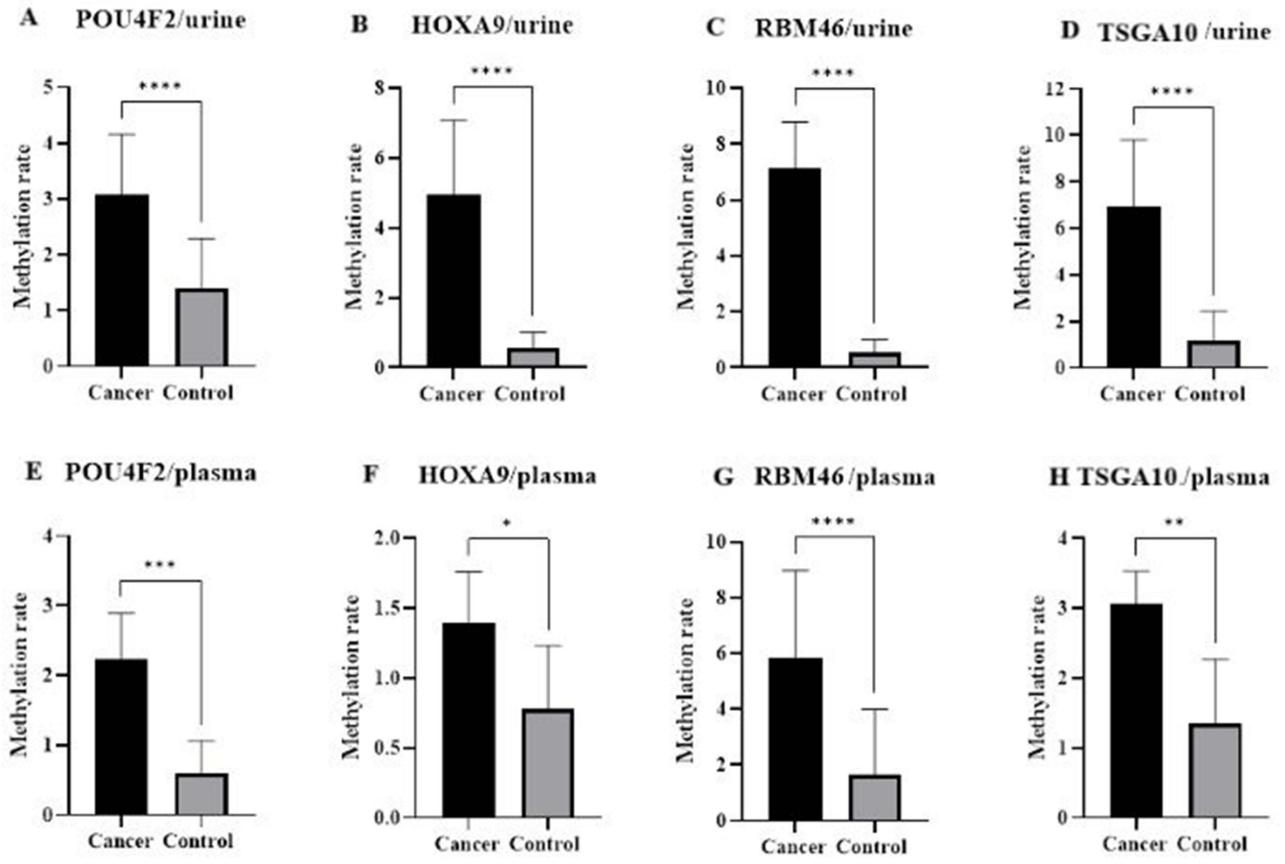

Figure 1 Methylation analysis of POU4F2 in urine (**A**), HOXA9 in urine (**B**), RBM46 in urine (**C**), TSGA10 in urine (**D**), POU4F2 in plasma(**E**), HOXA9 in plasma (**F**), RBM46 in plasma (**G**), TSGA10 in plasma (**H**), from n = 22 bladder cancer, n = 30 controls. The data are presented as mean ± standard deviation. Part A-D show significantly higher methylation rates in cancer patients compared to controls in urine samples ($p < 0.0001$). Parts E-H show similar trends in plasma samples, with highly significant differences observed ($p < 0.0005$ for most genes; $p = 0.0256$ for HOXA9). Stars indicate statistically significant differences between groups, indicating that these methylation changes may have potential diagnostic value. Statistical significance is indicated by stars (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

Levene's test. Differences between cancer and control groups were assessed using unpaired t-tests, and one-way ANOVA followed by Tukey's multiple comparison post-hoc test was applied for analyses involving multiple groups (cell lines, urine, and plasma). A p-value of less than 0.05 was considered statistically significant, indicating a less than 5% probability that the observed differences occurred by chance. This threshold was used to determine the significance of differences in gene expression or methylation levels across the various experimental conditions.

To evaluate the diagnostic performance of methylation status as a biomarker for bladder cancer, Receiver Operating Characteristic (ROC) curves were created for every gene. The accuracy of each biomarker was ascertained by calculating the area under the curve (AUC) and assessing the sensitivity and specificity at various cut-off points. Because the four genes (POU4F2, HOXA9, RBM46, TSGA10) showed strong inter-correlations and multicollinearity, multivariate logistic regression did not converge reliably. Therefore, Principal Component Analysis (PCA) was performed to derive a composite biomarker score representing the combined methylation pattern. Sampling adequacy was assessed using the Kaiser–Meyer–Olkin (KMO) test, and factorability was confirmed using Bartlett's test of sphericity. The first principal component (eigenvalue > 1) was extracted and used as the combined biomarker index.

Results

The clinicopathological characteristics of the patient and control groups are summarized in Table 1. The patient group consisted of 22 individuals diagnosed with transitional cell carcinoma (TCC), including 15 low-grade and 7 high-grade cases, while the control group consisted of 30 urologically healthy individuals.

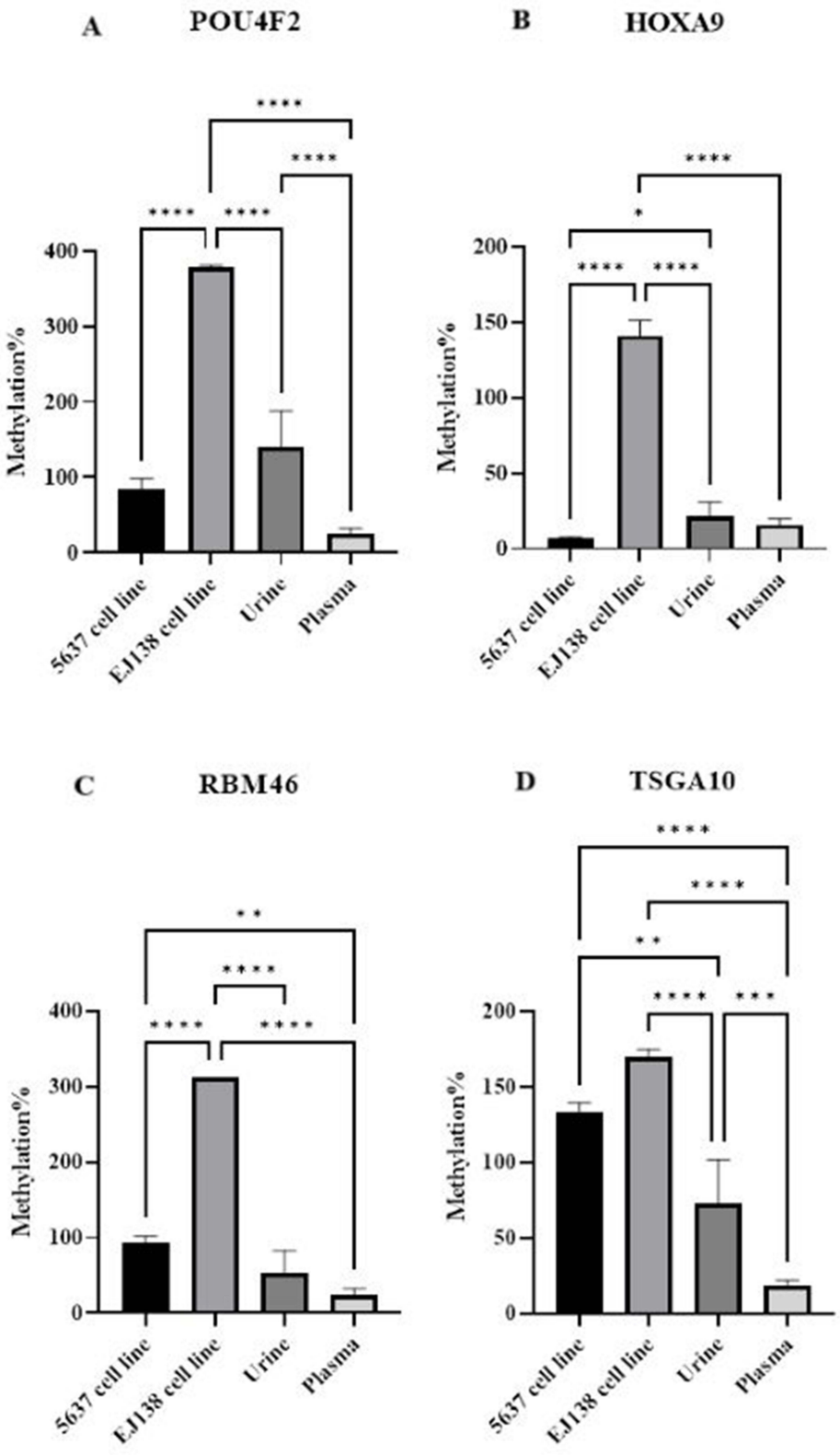

Figure 2 Methylation percentages of POU4F2 (**A**), HOXA9 (**B**), RBM46 (**C**), and TSGA10 (**D**) genes in different sample groups (Cell lines: 5637 and EJ138 (n = 3 technical replicates each); Urine and plasma samples: n = 22 bladder cancer patients). Healthy control samples were not shown in this figure because MSRE digestion resulted in undetectable Ct values, preventing methylation percentage calculation. 5637 and EJ138 cell lines were included as hypermethylation reference controls based on previous reports of promoter methylation in bladder cancer cell lines. Control (healthy) urine and plasma samples represent baseline non-tumor methylation levels. The data are presented as mean ± standard deviation. Tukey's multiple comparisons test was applied for statistical analysis. Stars indicate statistically significant differences between groups (* P<0.05, ** P<0.01, *** P<0.001 and **** P<0.0001).

Methylation Results in POU4F2, HOXA9, RBM46, and TSGA10 Genes and Their Comparison in Cancer and Healthy Individuals

To investigate methylation changes in the POU4F2, HOXA9, RBM46, and TSGA10 genes in bladder cancer, both urine and plasma samples were analyzed. Urine samples proved more effective for detecting gene methylation compared to plasma, offering greater sensitivity in identifying methylation changes. This may be related to the higher concentration of tumor-derived cfDNA typically observed in urine samples, which are in closer proximity to the bladder tumor environment.

In Figure 1A, the methylation status of the POU4F2 gene in urine samples from bladder cancer patients and the control group was significantly higher in the cancer group. The mean methylation rate in the cancer group was 3.076 ± 1.075, while in the control group it was 1.404 ± 0.8855. Statistical analysis revealed that this difference was highly significant ($p < 0.0001$). Figure 1B: The mean methylation rate of HOXA9 in the cancer group was 4.952 ± 2.133, while in the control group it was 0.5486 ± 0.4592. Statistical analysis revealed that this difference was highly significant ($p < 0.0001$). Figure 1C: The mean methylation rate of the RBM46 gene in the cancer group was 5.865 ± 3.105, while in the control group it was 1.643 ± 2.342. Statistical analysis revealed that this difference was highly significant ($p < 0.0001$). In Figure 1D: The mean methylation rate of the TSGA gene in the cancer group was 6.965 ± 2.834, while in the control group it was 1.147 ± 1.29. Statistical analysis revealed that this difference was highly significant ($p < 0.0001$).

In Figure 1E, the methylation status of the POU4F2 gene in plasma samples from bladder cancer patients was significantly higher compared to the control group. The mean methylation rate in the cancer group was 2.239 ± 0.6484, while in the control group it was 0.5944± 0.4679. Statistical analysis confirmed that this difference was highly significant (p: 0.0005). In Figure 1F; the mean methylation rate of HOXA9 in the cancer group was 1.396 ± 0.3618, whereas in the control group it was 0.7813 ± 0.4472. The difference between the groups was statistically significant (p: 0.0256). In Figure 1G; the mean methylation rate of RBM46 in plasma was 1.743 ± 0.5903 for the cancer group and 0.9342 ± 0.3854 for the control group. Statistical analysis indicated a highly significant difference (p: 0.0278). In Figure 1H; the mean methylation rate of TSGA in the cancer group was 3.061 ± 0.4664, while in the control group it was 1.345 ± 0.9242. This difference was also statistically significant (p: 0.0023).

In Figure 2, the Methylation percentage of the POU4F2, HOXA9, RBM46, and TSGA10 genes is compared across four different samples: the 5637-cell line, the EJ138 cell line, urine samples from bladder cancer patients, and plasma.

In Figure 2A, representing the POU4F2 gene, the methylation percentage in the EJ138 cell line is significantly higher than in the other groups (mean 378.8 ± 1.979), indicating pronounced hypermethylation in this cell line, consistent with previous studies reporting elevated methylation levels in bladder cancer cell lines. Tukey's multiple comparisons test revealed a significant difference between the EJ138 cell line and the 5637 cell line ($p < 0.0001$), as well as between the EJ138 cell line and urine ($p < 0.0001$) and plasma ($p < 0.0001$). Urine samples from cancer patients also exhibit a notable methylation percentage (mean 140 ± 48.56), which is significantly higher than plasma ($p < 0.0001$) but does not show a significant difference when compared to the 5637 cell line ($p = 0.1419$). Plasma displays the lowest methylation percentage (mean 24.85 ± 7.195), which may reflect the lower abundance of tumor-derived cfDNA typically found in plasma.

In Figure 2B, representing the HOXA9 gene, the methylation percentage in the EJ138 cell line is significantly higher than in the other groups (mean 141.1 ± 10.2), indicating strong hypermethylation in this cell line, which serves as a positive control. Tukey's multiple comparisons test revealed a significant difference between the EJ138 cell line and the 5637 cell line ($p < 0.0001$), as well as between the EJ138 cell line and urine ($p < 0.0001$) and plasma ($p < 0.0001$). Urine samples from cancer patients show a moderate methylation percentage (mean 21.51 ± 9.262), which is significantly higher than the 5637 cell line ($p = 0.031$) but does not show a significant difference when compared to plasma ($p = 0.449$). Plasma exhibits the lowest methylation percentage (mean 15.7 ± 4.07), likely due to the reduced concentration of tumor-derived cfDNA. The 5637 cell line has the lowest methylation percentage (mean 6.399 ± 1.573), reflecting minimal methylation activity for this gene.

In Figure 2C, representing the RBM46 gene, the methylation percentage in the EJ138 and 5637 cell lines is significantly higher than in the other groups (mean 311.1 ± 0.01921, 93.1 ± 8.654, respectively), highlighting pronounced

hypermethylation in these cell lines, which serve as positive controls. Tukey's multiple comparisons test revealed a significant difference between the 5637 and EJ138 cell lines ($p < 0.0001$), as well as between the 5637 cell line and plasma ($p = 0.0019$), and between the EJ138 cell line and plasma ($p < 0.0001$). Urine samples from cancer patients show moderate methylation percentages (mean 53.98 ± 28.58), which are substantially higher than plasma, but the difference between urine and plasma is not statistically significant ($p = 0.0596$). Plasma displays the lowest methylation percentage (mean 24.48 ± 8.294).

In Figure 2D, representing the TSGA10 gene, the methylation percentage in the EJ138 and 5637 cell lines is significantly higher than in the other groups (mean 169.8 ± 5.16, 133.8 ± 5.645, respectively), indicating substantial hypermethylation in these cell lines, which serve as positive controls. Tukey's multiple comparisons test revealed significant differences between the 5637 cell line and urine ($p = 0.0019$), the 5637 cell line and plasma ($p < 0.0001$), as well as between the EJ138 cell line and urine ($p < 0.0001$), the EJ138 cell line and plasma ($p < 0.0001$), and urine and plasma ($p = 0.0003$). Urine samples from cancer patients exhibit a notable methylation percentage (mean 72.41 ± 29.46), which is clearly higher than that of plasma. Plasma has the lowest methylation percentage (mean 18.8 ± 2.865.

In healthy control urine and plasma samples, Ct values were undetermined after MSRE digestion, indicating complete digestion of unmethylated target regions. Therefore, methylation percentage could not be calculated for controls and these samples were excluded from Figure 2.

The methylation percentages in the cell lines, serving as positive controls, are significantly higher compared to urine and plasma samples. Urine samples from bladder cancer patients, as a rich source of tumor-derived cfDNA, exhibit higher methylation percentages than plasma.

Figure 3 presents the ROC curves for four genes—POU4F2, HOXA9, RBM46, and TSGA10—each demonstrating the ability to distinguish between cancer and control conditions.

Figure 3A shows the ROC curve for the POU4F2 gene, with an Area Under the Curve (AUC) of 0.9735 (95% CI: 0.9212 to 1.000, $P < 0.0001$). The sensitivity (95.45%) indicates the test's high ability to correctly identify true positive cases, while the specificity (100%) highlights its exceptional capacity to accurately identify true negatives. Overall, the POU4F2 gene-based test is highly effective in diagnosing bladder cancer, suggesting low rates of false positives and false negatives within the studied cohort.

Figure 3B presents the ROC curve for the HOXA9 gene, with an AUC of 0.95 (95% CI: 0.8545 to 1.000, $P < 0.0001$). The sensitivity (100%) indicates that, within this sample set, all cancer cases were detected, while the specificity (95%) shows its high ability to correctly identify true negatives. The HOXA9 gene-based test also demonstrates excellent performance in diagnosing bladder cancer, with a very low risk of both false positives and false negatives.

Figure 3C shows the ROC curve for the RBM46 gene, with an AUC of 0.8825 (95% CI: 0.7607 to 1.000, $P < 0.0001$). The sensitivity (100%) indicates the perfect detection of all true positive cases, while the specificity (80%) demonstrates a relatively lower ability to identify true negatives compared to the other genes. Despite this, the RBM46 gene-based test is still highly effective, with no false negatives and a manageable false positive rate.

Figure 3D illustrates the ROC curve for the TSGA10 gene, with an AUC of 0.9775 (95% CI: 0.9420 to 1.000, $P < 0.0001$). The sensitivity (100%) highlights its perfect identification of all true positive cases, while the specificity (80%) suggests a moderate false positive rate. Despite this, the TSGA10 gene-based test is highly effective in diagnosing bladder cancer, with no false negatives and an acceptable level of false positives.

Due to the high separation and potential multicollinearity among the four studied genes (POU4F2, HOXA9, RBM46, and TSGA10), the logistic regression model did not converge properly. Therefore, a principal component analysis (PCA) was performed to extract a composite score representing all four genes. The sampling adequacy was acceptable, with a Kaiser–Meyer–Olkin (KMO) value of 0.695, indicating that the data were suitable for dimensionality reduction. Bartlett's test of sphericity was significant, $\chi^2(6) = 40.72$, $p < 0.001$, confirming the presence of sufficient correlations among variables for PCA (Table 4).

The analysis identified one principal component with an eigenvalue greater than 1, which accounted for 58.06% of the total variance (Table 5). All genes loaded positively on this component, with loadings ranging from 0.625 (RBM46) to 0.872 (TSGA) (Table 6), indicating that the component captured a shared expression pattern across all

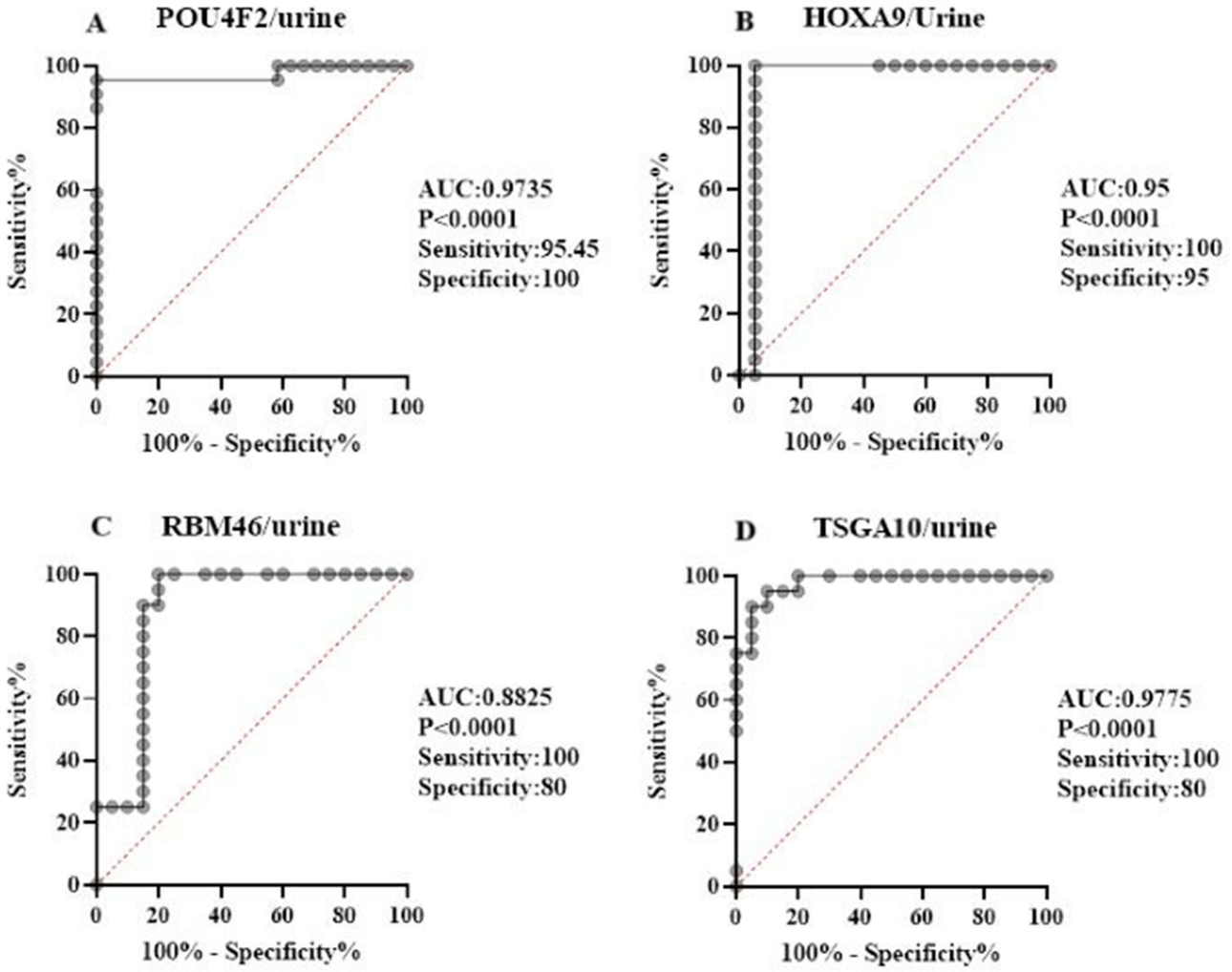

Figure 3 ROC curve analysis of four genes for bladder cancer diagnosis (n=22). (**A**) POU4F2 gene (AUC = 0.9735, 95% CI: 0.9212–1.000, P < 0.0001) shows excellent sensitivity (95.45%) and perfect specificity (100%). (**B**) HOXA9 gene (AUC = 0.95, 95% CI: 0.8545–1.000, P < 0.0001) demonstrates perfect sensitivity (100%) and high specificity (95%). (**C**) RBM46 gene (AUC = 0.8825, 95% CI: 0.7607–1.000, P < 0.0001) exhibits perfect sensitivity (100%) and moderate specificity (80%). (**D**) TSGA10 gene (AUC = 0.9775, 95% CI: 0.9420–1.000, P < 0.0001) shows perfect sensitivity (100%) and moderate specificity (80%).

four genes. The extracted component scores were subsequently used as a composite genetic index for outcome discrimination.

Receiver operating characteristic (ROC) analysis demonstrated that the derived factor score exhibited an excellent ability to differentiate between the outcome groups, with an area under the curve (AUC) = 0.993 (95% CI [0.974, 1.000]; $p < 0.001$) (Table 7 and Figure 4). Using the Youden index, the optimal cutoff point for the principal component score was determined to be −0.256, yielding 100% sensitivity and 95% specificity (J = 0.95).

Table 4 Kaiser–Meyer–Olkin (KMO) Measure and Bartlett's Test of Sphericity for Sampling Adequacy Among the Four Genes

Test	Statistic	Interpretation
KMO	0.695	Acceptable (values ≥ 0.6 indicate adequate sampling for factor extraction). Values between 0.6 and 0.7 are considered mediocre but usable.
Bartlett's Test	$\chi^2(6) = 40.72$, $p < 0.001$	Highly significant — the correlation matrix is not an identity matrix; correlations exist, so PCA is appropriate.

https://doi.org/10.2147/CMAR.S557943

Table 5 Total Variance Explained by Each Extracted Component in Principal Component Analysis (PCA)

Component	Eigenvalue	% Variance	Cumulative %
1	2.323	58.06	58.06
2	0.82	20.48	78.54
3	0.53	13.25	91.79
4	0.33	8.21	100.00

Table 6 Component Matrix Showing Loadings of the Four Genes on the Extracted Principal Component

Gene	Loading
TSGA	0.872
POU4F2	0.796
HOXA9	0.732
RBM46	0.625

Table 7 Discriminative Performance of the PCA-Derived Factor Scores Based on the Receiver Operating Characteristic (ROC) Analysis

AUC	95% CI for AUC		Best Cutoff	Sensitivity	Specificity
	Lower	Upper			
0.993	0.974	1.000	−0.256	1.000	0.950

At the optimal cutoff, classification accuracy was further evaluated (Table 8). Out of 40 total samples, 39 were correctly classified—yielding an overall accuracy of 97.5%. Specifically, all 20 cases with a positive outcome were correctly identified (true positives = 20, sensitivity = 100%), while 19 of 20 negative cases were correctly classified (true negatives = 19, specificity = 95%). Only one case (2.5%) was misclassified as a false positive.

Changes in the Expression of POU4F2, HOXA9, RBM46, and TSGA10 Genes and Their Comparison between Cancer Patients and Healthy Controls

In this study, the expression levels of the POU4F2, HOXA9, RBM46, and TSGA10 genes were evaluated in urine samples from patients with bladder cancer and the control group in Figure 5. Figure 5A shows that the expression level of POU4F2 in the cancer group was significantly lower, with a mean of 0.1376 ± 0.1691, compared to 0.9544 ± 0.971 in the control group (p = 0.0099). Figure 5B illustrates that HOXA9 expression was also significantly decreased in the cancer group, with a mean of 0.1228 ± 0.2244, whereas the control group had a mean of 1.097 ± 1.537 (p: 0.025). Figure 5C shows that RBM46 expression was elevated in the cancer group, with a mean of 3.189 ± 2.752, compared to the control group, which had a mean of 1.293 ± 1.363 (p: 0.0037). Figure 5D demonstrates that TSGA10 expression was

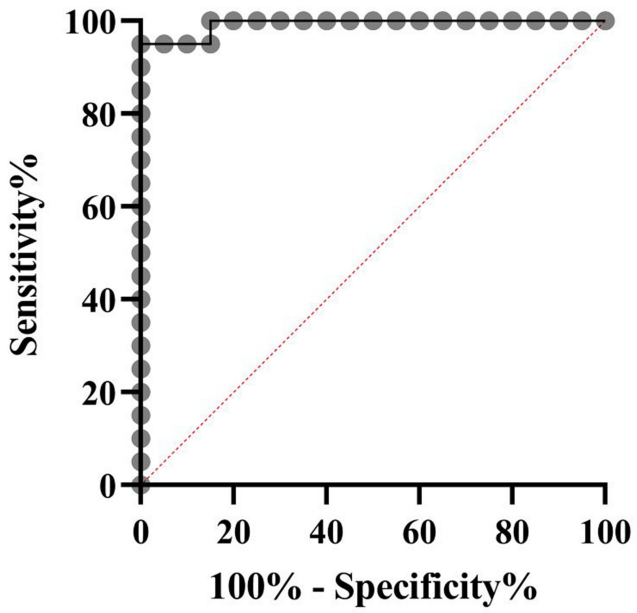

Figure 4 Receiver Operating Characteristic (ROC) Curve Depicting the Discriminative Ability of the PCA-Derived Gene Expression Factor Score.

significantly higher in the cancer group, with a mean of 2.731 ± 1.612, while the control group had a mean of 1.173± 1.891 (p: 0.0114).

Discussion

Urine cytology, as previously mentioned, despite its high specificity, has a poor performance in the diagnosis of low-grade tumors.[27,28] This limitation is due to factors such as the small sample volume that can be processed, the difficulty in distinguishing tumor cells from inflammatory cells such as leukocytes and red blood cells, and the lack of objective markers to distinguish reactive cells from low-grade tumors; factors that also cause heterogeneity in diagnosis among observers.[29] Although cystoscopy has a very high sensitivity and specificity, it is an invasive and uncomfortable procedure and carries risks such as pain, bleeding, infection, and patient anxiety; therefore, it is not suitable for widespread screening programs.[30–32] Even if the diagnosis is slightly delayed, it usually does not have a major clinical impact, although prompt treatment of recurrent cases can prevent disease progression.[33] In such a situation, the development of noninvasive, cost-effective, and reliable urinary biomarkers could play an important role in reducing morbidity and financial burden for patients.

Table 8 Observed and Predicted Classification Results at the Optimal Cutoff Determined by the Youden Index

Predicted	Observed		Total
	No	Yes	
No	19	0	19
Yes	1	20	21
Total	20	20	40

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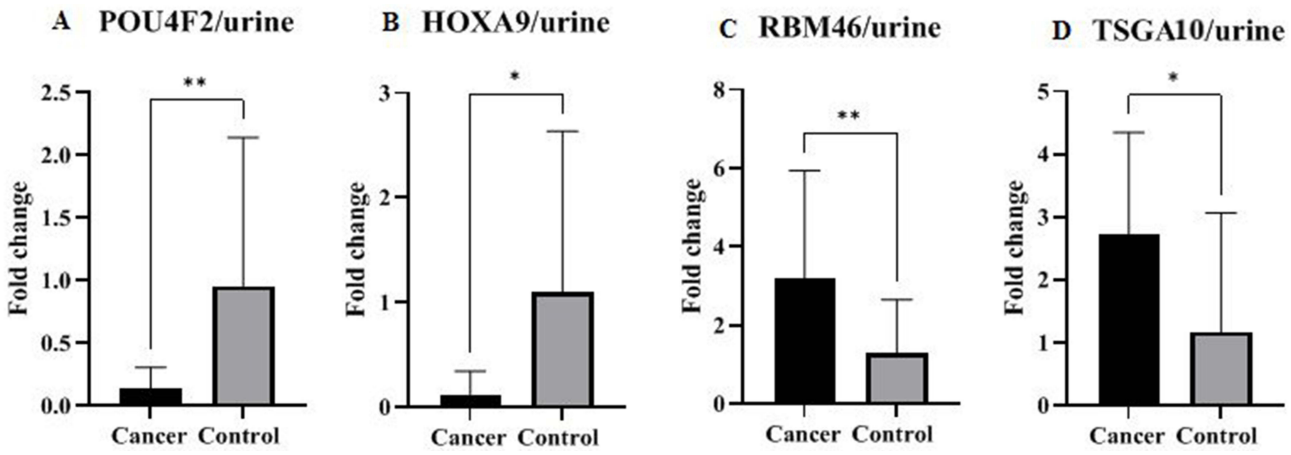

Figure 5 Gene Expression analysis of POU4F2 (**A**), HOXA9 (**B**), RBM46 (**C**), and TSGA10 (**D**) genes in urine samples from n = 22 bladder cancer, n = 30 controls. POU4F2 (**A**) and HOXA9 (**B**) show significantly reduced expression in the cancer group compared to controls (p = 0.0099, p = 0.025). RBM46 (**C**) and TSGA10 (**D**) demonstrate significantly increased expression in the cancer group (p = 0.0037, p = 0.0114). Statistical significance is indicated by stars (* p<0.05, ** p<0.01).

Currently, six FDA-approved urinary biomarker tests are available for the diagnosis or monitoring of bladder cancer, but they have significant limitations. These tests include FISH (UroVysion), fluorescence immunocytochemistry (ImmunoCyt), qualitative NMP22 (BladderChek), quantitative NMP22 (Alere NMP22), qualitative bladder tumor antigen (BTA stat), and its quantitative variant (BTA TRAK). Although some of them (such as qualitative BTA and NMP22) can be performed at the point of care, others require a laboratory.[31] However, 12–26% of individuals without bladder cancer may have false-positive results, and about 43% of truly cancerous cases are not detected due to insufficient sensitivity.[34,35] For this reason, despite the higher sensitivity of some of these tests compared to cytology, they have not yet found a place in clinical guidelines.[36]

In the meantime, urinary cfDNA offers important advantages as an emerging diagnostic platform. cfDNA is derived from apoptotic tumor cells and has greater specificity for tumor detection compared to cell pellets, which contain a mixture of normal and cancer cells. The use of urine also allows for noninvasive sampling.[37] Studies have shown that urinary cfDNA can have high sensitivity and specificity in the diagnosis of bladder cancer,[37,38] and specific methylation patterns, such as APC promoter methylation, further enhance the diagnostic accuracy.[39] Furthermore, cfDNA fragmentation features with a detection limit of less than 1% are able to distinguish cancer from normal samples very accurately.[38] However, relying solely on cfDNA may miss some aspects of tumor heterogeneity or false-negative results at very early stages, and its integration with other diagnostic methods can improve performance.[40,41]

This study aimed to investigate the methylation and expression patterns of four genes POU4F2, HOXA9, RBM46, and TSGA10 in bladder cancer. The findings showed that all four genes were significantly hypermethylated in urine and plasma samples from patients, indicating the presence of a distinct epigenetic signature in patients. The significant difference in methylation levels between the patient and control groups—especially in urine samples—indicates that these genes can be considered as reliable biomarkers for disease diagnosis. The superiority of urine over plasma can also be explained by the higher concentration of tumor cfDNA and the direct proximity to the anatomical site of involvement.

The results of gene expression analysis in urine samples also showed a dual pattern: a significant decrease in POU4F2 and HOXA9 expression in patients was consistent with the classic "hypermethylation-gene silencing" model. In contrast, the increased expression of RBM46 and TSGA10 despite their hypermethylation suggests a more complex regulatory pattern that could be due to the effect of methylation on non-promoter regions, the use of alternative promoters, or the silencing of upstream repressors. This functional distinction suggests that methylation in bladder cancer does not necessarily mean gene silencing and may also lead to increased expression in some regulatory pathways.

Several biological mechanisms may underlie the observed methylation and expression patterns in this study. Promoter hypermethylation is a well-established mechanism of transcriptional silencing in bladder cancer, particularly affecting developmental and differentiation-related genes such as HOXA9 and POU4F2. Previous studies have shown that

HOXA9 and POU4F2 promoter hypermethylation is frequent in urothelial tumors and that urinary methylation of these genes can be used for diagnosis and recurrence surveillance in bladder cancer patients. These findings support a tumor-suppressive role for HOXA9 and POU4F2, where promoter methylation is associated with reduced transcriptional activity and adverse clinical outcome in high-risk NMIBC and hematuria cohorts.[42,43] In contrast, TSGA10 has been characterized as a cancer-testis–related tumor suppressor that can inhibit angiogenesis, cell migration, and epithelial–mesenchymal transition and promote apoptosis in several malignancies, suggesting that its dysregulated expression may modulate key oncogenic pathways.[44] RBM46, on the other hand, is an RNA-binding protein involved in post-transcriptional regulation and β-catenin mRNA turnover, and members of the RBM protein family have been implicated in cancer-related processes such as proliferation, migration, and apoptosis.[45] Taken together, these data indicate that the methylation and expression changes we observed in HOXA9, POU4F2, RBM46, and TSGA10 are biologically plausible and may reflect gene-specific epigenetic dysregulation within pathways that are already linked to carcinogenesis.

Analysis of the diagnostic power of the genes showed that POU4F2, HOXA9, and TSGA10 were particularly sensitive and specific, and had a high ability to distinguish patients from healthy individuals. Although RBM46 showed lower specificity, its full sensitivity maintained its diagnostic value. Due to the strong correlation between the genes, multivariate modeling was not possible; However, principal component analysis (PCA) produced a composite score that performed almost perfectly (AUC = 0.993) and showed that combining epigenetic information from multiple genes significantly increased diagnostic accuracy.

Overall, the findings suggest that combining methylation and expression analysis of these four genes—particularly in urine samples—provides a suitable framework for the development of noninvasive tests for the early detection of bladder cancer. The superior performance of urine compared to plasma, its higher sensitivity, and its ease of sampling make this matrix a practical and effective option for disease screening and monitoring.

Bladder cancer is a heterogeneous disease, and biomarker performance may vary according to tumor grade and stage. In the present study, the patient group included 15 low-grade and 7 high-grade TCC cases; however, due to the limited sample size, subgroup statistical comparisons between these clinical categories were not performed. Therefore, the current results reflect overall biomarker behavior in bladder cancer rather than grade-specific diagnostic performance. Future studies with larger cohorts are necessary to evaluate whether methylation and expression patterns of POU4F2, HOXA9, RBM46, and TSGA10 differ between low-grade and high-grade tumors, and to determine the potential of these markers for disease stratification and recurrence monitoring.

Previous studies have confirmed the important role of cfDNA in the diagnosis of bladder cancer. Studies have shown that many genomic and epigenetic alterations identified in tumor tissues—including somatic mutations, CNVs, INDELs, and methylation patterns—can also be detected in cfDNA and can be used in liquid biopsy.[46] In addition, DNA methylation markers in urine samples, including APC methylation (68% in patients vs 16% in healthy subjects), and genes such as ZNF154, EOMES, and POU4F2, have shown remarkable sensitivity and specificity in previous studies.[39,47] Systematic reviews have also confirmed the high accuracy of these markers and have proposed ucfDNA as a promising approach for early diagnosis.[37,48] Despite these advances, methodological consensus on protocols for collection, processing, and analysis of cfDNA has not yet been fully established, and further studies are needed to standardize this process.

The study's findings by Rose et al indicated that ECRG4 and ITIH5 in particular might be good options for improving the existing bladder cancer biomarker panels and platforms. ECRG4 and ITIH5, two putative tumor suppressor genes, were described as novel urine DNA methylation biomarkers appropriate for noninvasive bladder cancer diagnosis. ECRG4-ITIH5 was able to differentiate bladder tumors from healthy, benign lesions and/or inflammatory diseases with an overall sensitivity of 64–70% and specificity of 80–92%. In comparison to other biomarkers, ECRG4 demonstrated a sensitivity of 73%, which rose to 76% with a specificity of 97% when paired with the well-known candidate biomarker NID2.[49]

Since the methylation process is crucial to the early phases of bladder cancer tumorigenesis, it is a promising early screening technique. Numerous studies have shown that methylation markers in tissue and urine samples can be used as non-invasive diagnostic tools to identify bladder cancer early on. Another study reported significantly higher global DNA methylation levels in bladder cancer patients, reaching a sensitivity of 92% with a methylation cutoff of 6.5%.[50] High

levels of hypermethylation in bladder tissue were found in studies on particular gene promoters, suggesting a possible epigenetic field effect in the development of cancer.[51] For the diagnosis of bladder cancer, the GHSR/MAL marker panel displayed an area under the curve (AUC) of 0.87 with a sensitivity of 79% and a specificity of 80%.[52] The potential of the CARE bladder assay for tracking treatment response was highlighted by the fact that it detected bladder cancer recurrence an average of 7–35 months earlier than traditional cystoscopy.[53] Although there is potential for early detection with methylation markers, issues with specificity and the requirement for larger validation studies to validate their clinical utility still exist.

However, bladder cancer is a multifaceted disease characterized by various genetic and molecular markers that influence its diagnosis, prognosis, and treatment. In the context of bladder cancer, key genes like POU4F2, HOXA9, and TSGA10 have been found to be significant, especially in their function in tumor recurrence and patient outcomes. POU4F2 and HOXA9 are both promising biomarkers for the recurrence of bladder cancer. Higher-grade tumors are linked to their hypermethylation in urine samples, indicating their possible use in surveillance and early diagnosis. These genes may be important predictors of patient prognosis because hypermethylation of these genes is linked to an increased hazard ratio for disease recurrence.[42] TSGA10 is known to be a tumor suppressor in bladder cancer, where its expression is associated with apoptosis and reduced cell migration. Higher levels of TSGA10 are associated with better overall survival rates in patients. Modulating TSGA10 expression could provide therapeutic avenues, as its overexpression increases apoptosis and reverses epithelial-mesenchymal transition (EMT) phenotypes in cancer cells.[54]

In one study, 192 urine samples from patients with microscopic or gross hematuria, 97 of whom had bladder cancer and 95 of whom had benign diseases, were used. The study was divided into two phases: validation and assay development. Eight genes' hypermethylation was examined in 81 urine samples, and a four-gene panel comprising HOXA9, PCDH17, POU4F2, and ONECUT2 was created. 111 more urine samples were examined for independent validation using the four-gene panel. Calculations were made for the combined methylation assay's positive predictive value (PPV) and negative predictive value (NPV). The relationship between each predictor variable and bladder cancer was determined using univariate and multivariate binary logistic regression analyses. Based on the findings of the multivariate logistic regression analysis in stage one, the combined assay of HOXA9, PCDH17, POU4F2, and 1-OCUT2 was chosen. In a population with general hematuria, the combined methylation biomarker produced an overall NPV of 98%, with an estimated prevalence of 10% for bladder cancer. In addition to potentially preventing 60% of needless cystoscopies, this combined methylation biomarker could correctly predict more than half of bladder cancer cases. Nevertheless, there were certain drawbacks to the study, including its small sample size and case-control population.[19]

Four gene classes (HOX A, HOX B, HOX C, and HOX D) that are found on various chromosomes are used to further categorize HOX homeobox genes. Outside of the HOX clusters, non-HOX homeobox genes are dispersed throughout the genome.[55] Numerous human cancers, including bladder, breast, colorectal, gastric, renal, liver, lung, ovarian, and prostate cancers, have been shown to exhibit dysregulation of homeobox genes.[56–61] Cancers at different stages of tumorigenesis, such as the epithelial-to-mesenchymal transition (EMT), cell proliferation, invasion, and metastasis, have been found to exhibit dysregulated expression of homeobox genes.[62–64] Numerous processes, such as DNA hypermethylation, loss of heterozygosity, gene amplification, and histone deacetylation, contribute to the dysregulation of homeobox genes.[65,66]

Urine samples from bladder cancer patients and healthy controls showed significantly different methylation statuses of a 4-gene panel (POU4F2, EOMES, ZNF154, and HOXA9) in one study. Urine cytology was 84% less sensitive than the 4-gene panel methylation assay. According to these results, methylation of the 4-gene panel may be employed as a urine biomarker for the early identification of bladder cancer.[67] Furthermore, compared to healthy controls, the urine of patients with bladder cancer had higher levels of methylation in a 6-gene panel (EOMES, HOXA9, TWIST1, POU4F2, ZNF154, and VIM). The sensitivity of the 6-gene panel hypermethylation was 88–94% for bladder cancer recurrence detection and 82–89% for bladder cancer detection. According to these results, methylation of the 6-gene panel may be employed as a biomarker for the identification and tracking of bladder cancer recurrence.[42]

For patients with hematuria, the methylation status of a panel of urinary biomarkers (HOXA9, PCDH17, TWIST1, PENK, VIM, ONECUT2, and ZNF154) in urine samples demonstrated a high predictive accuracy in identifying bladder cancer.[68] In patients with hematuria, a different panel of urinary biomarkers (HOXA9, PCDH17, ONECUT2, and POU4F2) demonstrated strong positive and negative predictive values.[19] These results may lessen the need for

cystoscopies in low-risk individuals by aiding in the diagnosis of bladder cancer in hematuria patients. Nine genes (HOXA9, DBNDD2, ADD1, GCNT4, RAPGEF5, TLR4, TSTD1, EPAS1, and ZNF582) were expressed differently in cisplatin-resistant bladder cancer cell lines than in cisplatin-sensitive ones regarding chemoresistance. Greater resistance to chemotherapy based on cisplatin was linked to. HOXA9 promoter methylation. According to these results, methylation of the HOXA9 promoter may be a useful biomarker for anticipating chemoresistance in patients undergoing chemotherapy based on cisplatin.[69]

A panel of hypermethylated genes (HOXA9, TWIST1, HOXA11, CSPG2, SOX1, and HS3ST2) was linked to muscle invasion in high-grade MIBC. Furthermore, cancer-specific survival was predicted by a different panel of hypermethylated genes (HOXA9, EYA4, CSPG2, APC, EPHA5, IPF1, ISL1, SOX1, PITX2, TWIST1, and JAK3). It is possible that these overmethylated genes could be used as biomarkers for bladder cancer prognosis.[70] Patients with NMIBC showed hypermethylation of a three-gene panel (ALDH1A3, HOXA9, and ISL1) when compared to healthy controls. The 3-gene panel is a promising biomarker for early detection, diagnosis, and monitoring of bladder cancer recurrence, as evidenced by the fact that hypermethylation of the panel predicted bladder cancer recurrence.[71] When comparing relapsed and advanced NMIBC to their non-relapsed counterparts, the mean methylation of HOXA9 and ISL1 was noticeably higher. Within a year, tumor progression and recurrence were predicted by concurrent HOXA9 and ISL1 methylation with a positive predictive value of 91.7% and a specificity of 90.9%. Methylation of ISL1 and HOXA9 had a specificity of 57.1% and a negative predictive value of 70.6% and 60%, respectively, concerning disease-specific mortality.[72] These results imply that ISL1 methylation and HOXA9 may be useful prognostic biomarkers for bladder cancer.

In bladder cancer, the interaction between RBM46 and TSGA10 demonstrates the intricate interaction of tumor suppressor genes (TSGs) in this cancer. Because of its correlation between tumor stage and patient survival, TSGA10 has been recognized as a potential prognostic biomarker. RBM46, on the other hand, has a less clear function in bladder cancer, although it is a component of a larger network of TSGs that affect carcinogenesis. In comparison to normal tissues, bladder cancer tissues exhibit an upregulation of TSGA10, which is linked to variables like tumor stage and gender.[54] According to in vitro research, downregulating TSGA10 results in less apoptosis and more cell migration, which may indicate a role for the protein in the development of tumors.[54] In bladder cancer patients, TSGA10 expression levels may be a predictor of overall survival.[54] Although there are few studies specifically examining RBM46 in bladder cancer, it is known to be a component of the TSG landscape, which also includes genes like TP53 and RB1 that are crucial for the development of bladder cancer.[73,74] Although more investigation is required to elucidate its precise role, RBM46 may interact with other TSGs and influence pathways that control cell growth and apoptosis.[74] To completely comprehend RBM46's role in tumor suppression and cancer progression, more research is necessary. Overall, the findings suggest that methylation and expression changes of HOXA9, POU4F2, RBM46, and TSGA10 genes can be used as reliable and noninvasive biomarkers for early detection and monitoring of bladder cancer. However, further research is necessary to confirm clinical efficacy and develop combination panels to increase diagnostic accuracy.

Although the biomarkers investigated in this study do not directly guide therapeutic decision-making, their diagnostic performance suggests potential utility in clinical management. Specifically, methylation and expression changes in POU4F2, HOXA9, RBM46, and TSGA10 may support early detection, assist in monitoring treatment response, and help identify recurrence, all of which indirectly contribute to optimizing therapeutic strategies.

This study has several important limitations that should be acknowledged. First, the sample size was modest (22 bladder cancer cases and 30 controls), which limits the statistical power and may reduce the generalizability of the findings. Although the observed methylation and expression differences were statistically significant, small or moderate diagnostic effects may have remained undetected due to limited power. Therefore, the results should be interpreted as preliminary, and larger multi-center validation studies are required. Second, although pathology-confirmed clinical data such as tumor grade (low-grade vs high-grade) were available, the number of cases in each subgroup was insufficient to allow meaningful stratified statistical analyses. As a result, the study could not determine whether biomarker performance differs across tumor grades, stages (NMIBC vs MIBC), or histological subtypes. Future studies with larger and more diverse cohorts are necessary to clarify whether these methylation markers are broadly applicable across bladder cancer subtypes or exhibit stage-specific diagnostic value. Third, although methylation changes were correlated with altered gene expression, the underlying biological mechanisms driving hypermethylation and dysregulated expression of

POU4F2, HOXA9, RBM46, and TSGA10 were not fully explored. Several of these genes have been implicated in oncogenic pathways and epigenetic regulation in bladder cancer and other malignancies, including promoter methylation, enhancer silencing, alternative promoter usage, chromatin remodeling, and context-dependent transcriptional activation. Further functional studies are required to elucidate the precise regulatory interactions responsible for the observed patterns. Finally, while urine cfDNA-based biomarkers show strong potential for use in non-invasive screening, their performance in post-treatment surveillance or recurrence monitoring was not evaluated here. Longitudinal sampling and follow-up studies will be required to determine whether these markers can support clinical decision-making in patient management after therapy.

Conclusion

The present study showed that methylation and expression changes of HOXA9, POU4F2, RBM46, and TSGA10 genes in patients with bladder cancer were significantly different from those in controls. Increased methylation in these genes and associated changes in their expression levels have great potential for use as non-invasive biomarkers in early diagnosis and monitoring of this disease. In addition, the use of cfDNA and urinary RNA as non-invasive samples represents a more accurate and less invasive method for the diagnosis of bladder cancer. However, further studies with larger sample sizes and longitudinal designs are recommended to evaluate the clinical value of these biomarkers and to investigate their incorporation into diagnostic panels. The observed interplay between methylation and gene expression in these markers may also reflect biologically meaningful dysregulation in pathways related to urothelial differentiation, apoptosis, and epithelial–mesenchymal transition, suggesting a potential contribution to bladder cancer pathogenesis.

In this study, the four-gene methylation panel demonstrated high diagnostic performance, with individual genes showing AUC values ranging from 0.88 to 0.98, and the combined PCA-derived score achieving an AUC of 0.993 with 100% sensitivity and 95% specificity. These validated performance metrics highlight the strong potential of HOXA9, POU4F2, RBM46, and TSGA10 as non-invasive biomarkers for bladder cancer detection. Overall, the findings lay essential groundwork for the future development of clinical urine-based diagnostic kits and may facilitate the practical application of cfDNA-based testing in bladder cancer screening and recurrence monitoring.

Disclosure

The author(s) report no conflicts of interest in this work.

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