

Supplementary Information

Supplementary Materials and Methods

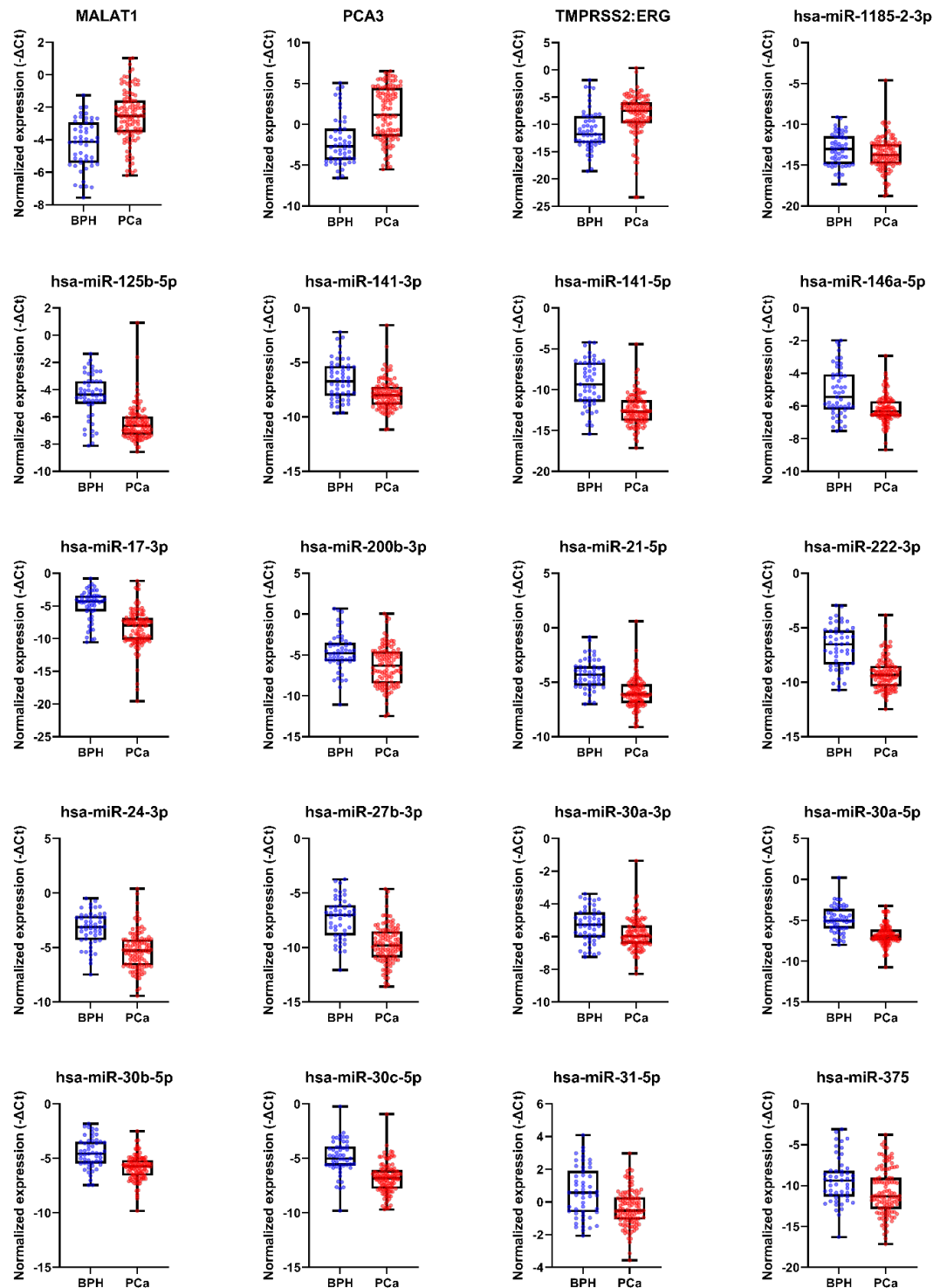
Urinary RNA extraction

Urinary circulating RNA was extracted and purified from urine using the QIAamp® Circulating Nucleic Acid Kit (QIAGEN, Hilden, Germany). For the isolation of urinary cell-free mRNA, 4 mL of urine sample was mixed with 500 µL of proteinase K, 4 mL of ACL lysis buffer, 5 µL of carrier RNA, and 1 mL of ATL buffer, and incubated at 60 °C for 30 min. Following the addition of 9 mL binding buffer, pulse-vortexing, and incubation on ice for 5 min, the mixture was added to a spin column in the circulating RNA binding step. Following this, 600 µL of washing buffer 1 (ACW1), 750 µL of washing buffer 2 (ACW2), and 750 µL of ethanol (96-100%) were sequentially passed through the silica membrane in the washing step. The spin column was then centrifuged at 20,000 x g for 3 min to dry the membrane and incubated at 56 °C for 10 min. Finally, 30 µL elution buffer (AVE) was applied to the center of the column membrane and centrifuged at 20,000 x g for 1 min. The extracted RNA was immediately frozen at -80 °C. For the isolation of urinary cell-free microRNA, 3 mL of urine sample was mixed with 400 µL of proteinase K, 3.2 mL of ACL lysis buffer, and 1 mL of ATL buffer, followed by incubation at 60 °C for 30 min. In the binding step, 9 mL of ACB buffer and 7 mL of isopropyl alcohol were added to the lysate and mixed thoroughly by pulse-vortexing for 30 sec. The remaining steps were conducted as described for the urinary cell-free mRNA isolation. The concentration and purity of RNA were assessed using a NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, MA, USA).

Reverse transcription

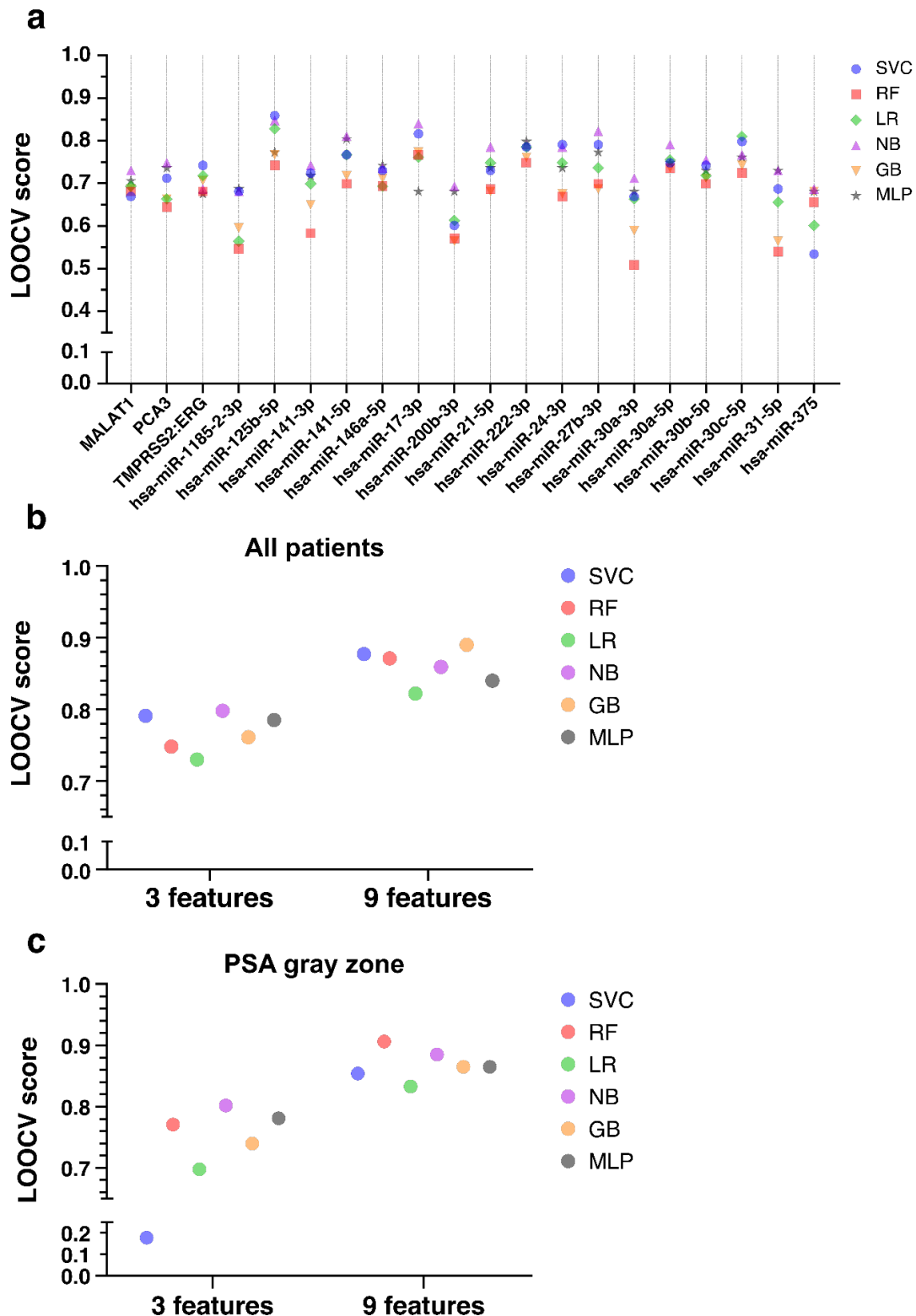
Complementary DNA (cDNA) was synthesized from mRNA and long non-coding RNA using the QuantiTect® Reverse Transcription Kit (QIAGEN). The mixture of gDNA Wipeout buffer in a sample tube was incubated at 42 °C for 2 min. Then, Quantiscript reverse transcriptase, RT buffer, and RT primer mix were added to the sample tube and incubated for 15 min at 42 °C and finally 3 min at 95 °C to inactivate reverse transcriptase. cDNA was synthesized from miRNA using the miRCURY Locked Nucleic Acid (LNA) Universal RT Kit (QIAGEN). For RT reactions, miRCURY RT Reaction Buffer, RNase-free water, miRCURY RT Enzyme Mix, UniSp6 spike-in, and template RNA were used. RT reaction mixture was incubated at 42 °C for 60 min, at 95 °C for 5 min, and then held at 4 °C. All cDNA samples were stored at -80 °C.

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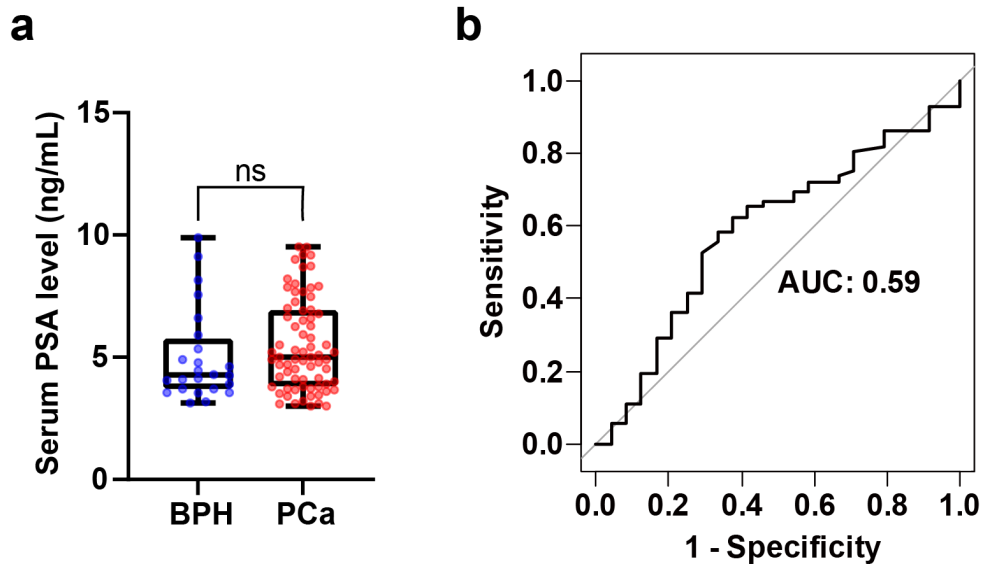
Supplementary Figure 1 Urinary RNA biomarker expression in patients with BPH (n = 51) or PCa (n = 112). Urinary RNA marker expression levels were normalized with mean β -actin and 18S rRNA values: Normalized expression $(-\Delta Ct) = (Ct_{\beta\text{-actin}} + Ct_{18S\text{ rRNA}})/2 - Ct_{\text{target gene}}$. Boxes represent the first and third quartiles, and median values are depicted as horizontal lines. Colored dots indicate normalized RNA expression levels of individual samples for each patient group. All of the above urinary RNA biomarkers except for has-miR-1185-2-3p were differentially expressed between patients with BPH and patients with PCa. P values from Wilcoxon test and area under the curve (AUC) of receiver operating characteristic (ROC) curve analysis for RNA biomarkers are in **Supplementary Table 3**. BPH = benign prostatic hyperplasia; PCa = prostate cancer.

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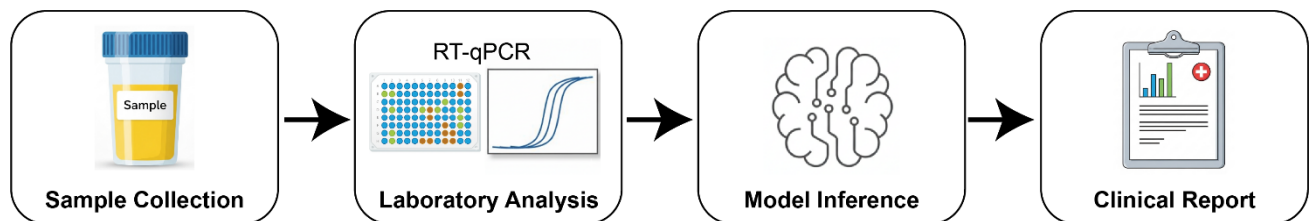


Supplementary Figure 2 Leave-One-Out cross-validation (LOOCV) scores for univariate or multivariate machine learning (ML) classifiers. **(a)** LOOCV scores for univariate ML classifiers for all patients. **(b, c)** LOOCV scores for multivariate ML classifiers using conventional 3 features and 9 features from RFECV analysis for all patients **(b)**, or PSA gray zone patients **(c)** (serum PSA level 3~10 ng/mL). In LOOCV, only one data point is used for testing, while other data points are used for training the ML classifiers, providing robust estimate of model performance.

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Supplementary Figure 3 Serum PSA level analysis for patients with PSA levels in the gray zone (serum PSA level 3~10 ng/mL). **(a)** The difference of serum PSA levels in patients with BPH (n = 27) and PCa (n = 73) were not significant among patients with PSA in the gray zone. Boxes represent the first and third quartiles, and median values are depicted as horizontal lines. Colored dots indicate serum PSA levels of individual samples for each patient group. **(b)** Receiver operating characteristic (ROC) curve for serum PSA levels for the diagnosis of PCa in the gray zone (AUC = 0.59). ns = not significant (p > 0.05); BPH = benign prostatic hyperplasia; PCa = prostate cancer; AUC = area under the curve.



Supplementary Figure 4 Schematic of the prospective clinical workflow for the PCASSO test. The process begins with a non-invasive urine sample collection and proceeds through a standardized RT-qPCR assay. The resulting data are processed and entered into a pre-trained model such as Gradient Boosting (GB) model to generate a final clinical report indicating the patient's probability of having prostate cancer.

Supplementary Information

Target	Forward primer (5'→3')	Reverse primer (5'→3')
PCA3	GAGAACAGGGGAGGGAGAG	ACGTTCTGGGATACATGTGC
TMPRSS2:ERG	CCTGGAGCGCGGCAGGAAGCCTTAT CAGTTG	TCCTGCTGAGGGACGCGTGGGCTCA TCTTG
MALAT1	CTTCCCTAGGGGATTTTCAGG	GCCACAGGAACAAGTCCTA
β-actin	ATGTACCCTGGCATTGCCGAC	GACTCGTCATACTCCTGCTTG
18s-rRNA	CCTGGATACCGCAGCTAGGA	GCGGCGCAATACGAATGCCCC

Supplementary Table 1 Primer sequences used for SYBR green qPCR detection of urinary RNA markers (mRNA and lncRNA) and reference RNAs.

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Biomarker	Biological pathway / key targets (PCa-relevant)	Reported diagnostic performance (AUC; specimen/model)	Performance in this study (AUC)	Representative references
PCA3	Prostate-specific lncRNA; overexpressed in PCa epithelial cells	0.66 (urine, multicenter validation); meta-analytic accuracy acceptable	0.78	1
TMPRSS2:ERG	AR-driven gene fusion; promotes invasion/ETS transcription	0.68 (urine T2:ERG); combined with PCA3/PSA improves AUC	0.74	2
MALAT1	Oncogenic lncRNA; splicing/transcriptional programs, metastasis	0.670 (all PSA) and 0.742 (PSA 4–10) in urine	0.78	3
hsa-miR-1185-2-3p	Putative tumor-suppressive roles; limited PCa literature	NR for single-marker diagnostic AUC; differential expression reported (tissue/liquid)	0.57	4
hsa-miR-125b-5p	p53 network (PUMA, p21), BAK1; AR-linked pro-survival signaling	NR (single urine); differential expression widely reported; panel data exist	0.84	5
hsa-miR-141-3p	AR axis; epithelial phenotype; associates with grade	Panel with miR-21/375 in urine reached 0.872	0.72	6
hsa-miR-141-5p	As above (arm variant); epithelial/AR programs	Panel performance as above (see Foj 2017)	0.82	6
hsa-miR-146a-5p	NF-κB/innate signaling; inflammation–tumor crosstalk	NR (single urine); differential expression reported	0.71	7
hsa-miR-17-3p	miR-17-92 cluster; c-MYC/Cell cycle	NR (single urine); tissue/plasma data; panels reported	0.83	8
hsa-miR-200b-3p	EMT suppression via ZEB1/2; epithelial maintenance	Panel (miR-100/200b) improved AUC vs PSA/base model in urine, incl. PSA gray	0.68	9

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		zone (numeric shown in paper figs)		
hsa-miR-21-5p	PTEN/PI3K, apoptosis regulators; oncomiR	0.872 when combined with miR-375 (urine)	0.83	6
hsa-miR-222-3p	Cell cycle (CDKN1B/p27), AR signaling crosstalk	Panel evidence (urinary three-miR ratio including miR-222-3p) with AUC 0.95 (training)/ 0.89 (validation)	0.85	5
hsa-miR-24-3p	Cell cycle (CDKN1B/p27, p16); apoptosis	Panel with miR-222-3p & miR-30c-5p AUC 0.95/0.89 (urine)	0.81	10
hsa-miR-27b-3p	MAPK/EMT; angiogenesis regulators	NR (single urine); differential expression reported	0.82	11
hsa-miR-30a-3p	KRAS/EGFR pathway members; EMT nodes	NR (single urine)	0.66	12
hsa-miR-30a-5p	As above (arm variant)	NR (single urine)	0.82	12
hsa-miR-30b-5p	MTA1/HOXA1; EMT signaling	Urinary EV studies report prognostic/diagnostic utility; numeric AUC variably reported across cohorts	0.77	13
hsa-miR-30c-5p	EMT (ZEB1/2 axis), cell motility	Panel with miR-222-3p & miR-24-3p AUC 0.95/0.89 (urine)	0.83	10
hsa-miR-31-5p	AR (direct coding-region targeting), E2F1/2; integrins ($\alpha 2/\alpha 5/\alpha V$, $\beta 3$)	NR (single urine); strong mechanistic tumor-suppressor data	0.68	14
hsa-miR-375	Endocrine/secretory phenotype; AR and epithelial programs	0.79 and 0.84 (urine; two cohorts) in combination with miR-148a; 0.85–0.90 with PSA in grey zone	0.65	6

Supplementary Table 2 List of 20 RNA markers employed in this study with their molecular and diagnostic background. Candidate markers were initially retrieved from the published literature, and those demonstrating significant individual diagnostic performance (AUC) within the internal cohort were selected as final targets.

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RNA biomarker	References	This study	RNA biomarker	References	This study
MALAT1	Up ¹⁵	Up	hsa-miR-200b-3p	Down ¹⁰	Down
PCA3	Up ^{16,17}	Up	hsa-miR-21-5p	Down (hsa-mir-21) ¹⁰	Down
TMPRSS2:ERG	Up ^{18,19}	Up	hsa-miR-222-3p	Down ^{10,20}	Down
hsa-miR-1185-2-3p	Up ⁴	Down	hsa-miR-24-3p	Down ¹⁰	Down
hsa-miR-125b-5p	Up or Down ^{10,20}	Down	hsa-mir-375	Up or Down ¹⁰	Down
hsa-miR-141-3p	Down (hsa-mir-141) ^{10,20}	Down	hsa-miR-27b-3p	Down ^{10,20}	Down
hsa-miR-141-5p	Down (hsa-mir-141) ^{10,20}	Down	hsa-miR-30a-3p	Up ¹⁰	Down
hsa-miR-146a-5p	Down ¹⁰	Down	hsa-miR-30a-5p	Up or Down ^{10,20}	Down
hsa-miR-31-5p	Down ¹⁰	Down	hsa-miR-30b-5p	Up or Down ^{10,20}	Down
hsa-miR-17-3p	Up (hsa-mir-17) or Down ^{4,10,21}	Down	hsa-miR-30c-5p	Up or Down ^{10,20}	Down

Supplementary Table 3 Overview of results from studies on urinary RNA markers of prostate cancer patients. The reported up and/or down regulations of urinary RNA markers in other studies are compared with this study. Up = up-regulated in cancer; Down = down-regulated in cancer.

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RNA biomarker	Ct difference ($-\Delta\Delta\text{Ct}$)	Median in PCa ($-\Delta\text{Ct}$)	Median in BPH ($-\Delta\text{Ct}$)	Wilcoxon test (BH-adjusted)	AUC
MALAT1	1.59	-2.53	-4.12	1.66E-07	0.78
PCA3	3.88	1.18	-2.70	1.66E-07	0.78
TMPRSS2:ERG	4.3	-7.49	-11.79	6.63E-06	0.74
hsa-miR-1185-2-3p	-0.74	-13.75	-13.01	1.38E-01	0.57
hsa-miR-125b-5p	-2.27	-6.64	-4.37	8.15E-11	0.84
hsa-miR-141-3p	-1.28	-8.02	-6.74	6.85E-05	0.72
hsa-miR-141-5p	-3.33	-12.67	-9.34	9.07E-10	0.82
hsa-miR-146a-5p	-0.87	-6.32	-5.45	1.26E-04	0.71
hsa-miR-17-3p	-3.68	-7.99	-4.31	1.72E-10	0.83
hsa-miR-200b-3p	-1.5	-6.30	-4.80	7.10E-04	0.68
hsa-miR-21-5p	-1.83	-6.12	-4.29	4.80E-10	0.83
hsa-miR-222-3p	-2.82	-9.35	-6.53	3.38E-11	0.85
hsa-miR-24-3p	-2.18	-5.29	-3.11	2.08E-09	0.81
hsa-miR-27b-3p	-2.79	-9.81	-7.02	5.74E-10	0.82
hsa-miR-30a-3p	-0.68	-5.96	-5.28	3.29E-03	0.66
hsa-miR-30a-5p	-1.89	-6.95	-5.06	7.69E-10	0.82
hsa-miR-30b-5p	-1.16	-5.72	-4.56	1.88E-07	0.77
hsa-miR-30c-5p	-1.8	-6.80	-5.00	1.73E-10	0.83
hsa-miR-31-5p	-1.09	-0.52	0.57	7.10E-04	0.68
hsa-miR-375	-1.91	-11.30	-9.39	5.98E-03	0.65

Supplementary Table 4 Comparison of urinary RNA biomarker expression levels in PCa and BPH.

Quantitative relative expression of RNA biomarkers was calculated using comparative Ct method, where Ct difference between PCa and BPH samples was calculated as follows: Ct difference ($-\Delta\Delta\text{Ct}$) = Median normalized expression ($-\Delta\text{Ct}$) in PCa samples – Median normalized expression ($-\Delta\text{Ct}$) in BPH samples. Statistically significant p-values ($p < 0.05$) are marked in bold. BPH = benign prostatic hyperplasia; PCa = prostate cancer; BH = Benjamini-Hochberg; AUC = area under the curve.

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Study	Biomarker(s)	Analysis method	Performance		Patient sample	Quantification method	Informative Cohort (Negative, Positive)	Note
This study	PCA3, TMPRSS2:ERG, hsa-miR-125b-5p, hsa-miR-141-5p, hsa-miR-17-3p, hsa-miR-24-3p, hsa-miR-30b-5p, hsa-miR-30c-5p, hsa-miR-31-5p	GB (And 5 more machine learning algorithms)	AUC	0.99	Urine	RT-qPCR	51, 112	Train-test set split (train:75%, test:25%), Cross-validation (LOOCV, Stratified-K-fold)
			Stratified-K-fold	0.912				
			LOOCV	0.89				
			AUC (PSA 3~10)	1.00				
			Stratified-K-fold (PSA 3~10)	0.885				
			LOOCV (PSA 3~10)	0.865				
1 ²²	SPINK1, PCA3, GOLPH2, TMPRSS2:ERG	Logistic regression	AUC for all data	0.758	Urine	RT-qPCR	105, 152	All samples were used for training
			AUC for LOOCV optimized model	0.736				
2 ¹	PCA3	ROC	AUC	0.66	Urine	RT-qPCR	360, 174	Patients with serum PSA levels 3~15 ng/mL
			sensitivity	65%				
			specificity	66%				
	Serum PSA	ROC	AUC	0.57	Serum	Clinical assay		
			sensitivity	65%				
			specificity	47%				
3 ²	TMPRSS2:ERG, PCA3, serum PSA	Logistic regression	AUC	0.88	Post-DRE urine, serum	RT-qPCR	30, 15	.
			sensitivity	80%				
			specificity	90%				
	TMPRSS2:ERG		AUC	0.77				
	PCA3		AUC	0.65				
	serum PSA		AUC	0.72				
4 ²³	PCA3, PSA transcripts	Manually designed expression (PCA3/PSA mRNA)	AUC	0.786	Post-DRE urine	RT-qPCR	64, 35	.
5 ²⁴	TMPRSS2:ERG, PCA3, serum PSA	Logistic regression	AUC	0.772	Post-DRE urine and serum	Transcription-mediated amplification assays	707, 518	Validation of pretrained models

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	TMPRSS2:ERG, PCA3, Prostate Cancer Prevention Trial high-grade risk calculator (PCPThg)		AUC	0.779				
6 ¹⁰	miR-222-3p, miR-24-3p, miR-30c-5p	Manually designed expression (miR-222-3p*miR-24-3p/miR-30c-5p)	AUC for cohort 1	0.95	Urine	RT-qPCR	(Cohort 1) 20, 188	Cohort 1 (training) / Cohort 2 (validation)
			AUC for cohort 2	0.89			(Cohort 2) 20, 197	
7 ¹⁷	PCA3, hK2, total PSA	Logistic regression	AUC	0.841	Peripheral blood	RT-qPCR	57, 43	.
			sensitivity	69.8%				
			specificity	84.2%				
8 ²⁵	PCA3, PSA transcripts	ROC (PCA3/PSA)	AUC	0.717	Urine after prostatic massage	RT-qPCR	84, 24	.
9 ²⁶	Four TMPRSS2:ERG fusion markers	Existence of fusion markers	sensitivity	45.4%	Urine after DRE	RT-qPCR	55, 46	Included females (n=18) and post-radical prostatectomy patients (n=12).
			specificity	100%				
10 ²⁷	ANXA3, PSMA, ERG, ENG	Random forest / Neural network	AUC	0.99	Urine before/after DRE	Dual-gate field-effect transistor	26, 25	Urinary protein biomarkers, Random set split (train: 70%, test: 30%)

Supplementary Table 5 Overview of prostate cancer diagnostic studies using biomarkers. The results from each study are listed including the combination of biomarkers, performance of diagnostic model, quantification method, and the size of patient cohort. Patient sample describes the types of materials used (e.g., urine or blood) as well as methods (e.g., urine collection with no-DRE or post-DRE). DRE = digital rectal examination; AUC = area under the curve.

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Biomarker	AUC	p value (vs. GB with the 9 biomarkers)
MALAT1	0.78	1.66E-08
PCA3	0.78	2.82E-08
TMPRSS2:ERG	0.74	2.73E-08
hsa-miR-1185-2-3p	0.57	< 2.2E-16
hsa-miR-125b-5p	0.84	0.000105
hsa-miR-141-3p	0.72	1.44E-09
hsa-miR-141-5p	0.82	1.11E-05
hsa-miR-146a-5p	0.71	1.83E-09
hsa-miR-17-3p	0.83	9.76E-06
hsa-miR-200b-3p	0.68	7.13E-13
hsa-miR-21-5p	0.83	1.02E-06
hsa-miR-222-3p	0.85	9.57E-06
hsa-miR-24-3p	0.81	1.36E-06
hsa-miR-27b-3p	0.82	2.46E-06
hsa-miR-30a-3p	0.66	2.55E-11
hsa-miR-30a-5p	0.82	3.52E-06
hsa-miR-30b-5p	0.77	1.42E-07
hsa-miR-30c-5p	0.83	9.83E-06
hsa-miR-31-5p	0.68	7.65E-11
hsa-miR-375	0.65	4.98E-15

Supplementary Table 6 Comparison of single-marker non-ML diagnostic model and the trained GB model with nine biomarker combinations (PCA3, TMPRSS2:ERG, hsa-miR-125b-5p, hsa-miR-141-5p, hsa-miR-17-3p, hsa-miR-24-3p, hsa-miR-30b-5p, hsa-miR-30c-5p, hsa-miR-31-5p). The ROC curves from single marker models were compared with the ROC curve for GB model with nine biomarkers (AUC: 0.99). The AUC values for single-marker diagnostic models are listed in each row. To analyze the measurement uncertainties in the ROC curves, bootstrap method of 2000 replicates is applied^{28,29}. GB = Gradient Boosting classifier; AUC = area under the curve.

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Machine learning model	Biomarker with the highest AUC	AUC	p value (vs. GB with nine biomarkers)
SVC	hsa-miR-17-3p	0.84	0.01305
RF	hsa-miR-17-3p	0.80	0.004762
LR	hsa-miR-141-5p	0.82	0.009229
NB	hsa-miR-141-5p	0.82	0.008512
GB	hsa-miR-30c-5p	0.81	0.007853
MLP	hsa-miR-141-5p	0.82	0.008794

Supplementary Table 7 Comparison of single-marker ML models and the trained GB model with the nine biomarker combinations. For each ML model, the biomarkers with highest AUC values were chosen for analysis. The ROC curves from single-marker ML models were compared with the ROC curve for GB model with nine biomarkers (AUC: 0.99). The AUC values for given ML model and single biomarker combinations are listed in each row. Bootstrap method of 2000 replicates is applied to compute the uncertainties of measures in ROC analysis. SVC = Support Vector classifier; RF = Random Forest classifier; LR = Logistic Regression; NB = Naïve Bayes; MLP = Multi-Layer Perceptron; GB = Gradient Boosting classifier; AUC = area under the curve.

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Biomarker	Targeted gene(s) / pathway(s)	References
hsa-miR-125b	Wnt, PI3K/Akt, STAT3, MAPK, NF- κ B, p53	30-36
hsa-miR-141	RUNX1, Shp, PEITC, STAT4, PTEN, AR	37-39
hsa-miR-17	c-MYC, RhoA GTPase, AIB1, PCAF	40-42
hsa-miR-24	CDKN1B, p27, p16, FSCN1	43,44
hsa-miR-30b	Snail, HOXA1, EGFR, NT5E	45-47
hsa-miR-30c	KRAS, UBC9, MTA1, HMBOX1	48,49
hsa-miR-31	AR, E2F1, E2F2, EZH2, integrin- α 2/5/V	14,50-52

Supplementary Table 8 List of miRNA biomarkers used in the 9 biomarker models. The most relevant and representative regulatory targets of each miRNA in relation to cancer progression are listed.

Test	Sample	Biomarker	DRE-dependent	AUC	Cost (\$)
4Kscore	Serum	tPSA, fPSA, iPSA, hK2	No	0.82	500 ⁵³
PHI	Serum	tPSA, fPSA, p2PSA	No	0.7	80 ⁵³
PCA3	Urine	PCA3	Yes	0.76	300 ⁵³
ExoDx	Urine	PCA3, TMPRSS2:ERG, SPDEF	No	0.73	760 ⁵³
SelectMDx	Urine	HOXC6, DLX1, PSA	Yes	0.86	500 ⁵³
MiPS	Urine	PCA3, TMPRSS2:ERG, PSA	Yes	0.77	700 ⁵³
This study	Urine	PCA3, TMPRSS2:ERG, 7 miRNAs.	No	0.99	91

Supplementary Table 9 Overview of biomarker-directed prostate cancer assays. tPSA = total PSA; fPSA = free PSA; p2PSA = [-2]proPSA; hK2 = human kallikrein 2; MiPS = Michigan-Prostate Score; AUC = area under the curve. Costs for commercially available assays are listed. The cost calculation for this study is described in **Supplementary Table 10**

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Procedure	Materials	Cost/sample (\$)
RNA extraction (mRNA, lncRNA)	the QIAamp® Circulating Nucleic Acid Kit	24.74
	Plastic wares/consumables	1.00
RNA extraction (miRNA)	the QIAamp® Circulating Nucleic Acid Kit	24.74
	Plastic wares/consumables	1.00
cDNA synthesis (mRNA, lncRNA)	QuantiTect® Reverse Transcription Kit	8.34
	Plastic wares/consumables	0.50
cDNA synthesis (miRNA)	miRCURY LNA Universal RT Kit	6.23
	Plastic wares/consumables	0.50
qPCR (mRNA, lncRNA)	ORA™ qPCR Green ROX L Mix, 2X	1.03
	Reference primer set1	0.01
	Reference primer set2	0.01
	mRNA primer set1	0.01
	mRNA primer set2	0.01
	Plastic wares/consumables	2.00
qPCR (miRNA)	ORA™ qPCR Green ROX L Mix, 2X	1.03
	Reference primer set1	0.01
	Reference primer set2	0.01
	miRNA primer set1	2.42
	miRNA primer set2	2.42
	miRNA primer set3	2.42
	miRNA primer set4	2.42
	miRNA primer set5	2.42
	miRNA primer set6	2.42
	miRNA primer set7	2.42
	Plastic wares/consumables	2.50
Total		90.61

Supplementary Table 10 Analysis of costs per sample to perform all the experimental steps in this study. The costs for commercially available kits at the time of purchase are listed. The costs for plastic wares and other consumables are approximated.

Supplementary Information

References

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