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Combination of Urinary MiR-501 and MiR-335 With Current Clinical Diagnostic Parameters as Potential Predictive Factors of Prostate Biopsy Outcome

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Abstract. *Background: The detection of prostate cancer (PCa) is currently based on prostate-specific antigen (PSA) quantification as an initial screening followed by ultrasound-guided transrectal biopsy. However, the high rate of false-negative biopsies often leads to inappropriate treatment. Therefore, new molecular biomarkers, such as urine microRNAs (miRNAs), are a possible way to redefine PCa diagnostics. Patients and Methods: Urine samples of 356 patients undergoing prostate biopsy (256 cases with confirmed prostate cancer, 100 cases with negative prostate biopsy) at the Masaryk Memorial Cancer Institute (Czech Republic) and additional 36 control subjects (healthy controls, benign prostatic hyperplasia – BPH) were divided into the discovery and validation cohorts and analyzed. In the discovery phase, small RNA sequencing was performed using the QIAseq miRNA Library Kit and the NextSeq 500 platform. Identified miRNA candidates were validated by the RT-qPCR method in the independent validation phase. Results: Using the small RNA sequencing method, we identified 12 urine miRNAs significantly dysregulated between PCa patients and controls. Furthermore, independent validation showed the ability of miR-501-3p and the quantitative miR-335:miR-501 ratio to distinguish between PCa patients and patients with negative prostate biopsy. The subsequent combination of the miR-335:miR-501 ratio with PSA and total prostate volume (TPV) using logistic regression exceeded the analytical accuracy of standalone parameters [area under curve (AUC)=0.75, positive predictive value (PPV)=0.85, negative predictive value (NPV)=0.51)] and discriminated patients according to biopsy outcome. Conclusion: Combination of miR-335:miR-501 ratio with PSA and total prostate volume was able to identify patients with negative prostate biopsy and could potentially streamline decision making for biopsy indication.*

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Key Words: Prostate cancer, diagnosis, prostate biopsy, microRNA, urine, non-invasive, NGS, miRNA ratio, regression model.

Prostate cancer (PCa) is the second most frequently diagnosed cancer and the fifth leading cause of cancer-related death among men (1). A crucial event for timely and effective therapy is the early detection of the disease which is maintained primarily using the measurement of prostate-specific antigen (PSA) levels in the blood serum. Based on increased PSA levels patients are further indicated for transrectal ultrasound-guided biopsies (TRUS) of the prostate (2). Unfortunately, the PSA test shows low specificity, as elevated levels may also reflect inflammation or trauma. This leads to a significant number of PSA-positive men who are unnecessarily subjected to biopsy. Furthermore, even if patients are correctly referred for biopsy due to abnormal screening results, there is a high rate of false-negative TRUS results (3). For this reason, there is an effort to refine or replace PSA testing with novel PCa-specific biomarkers (2, 3).

Modern tumor biomarkers are described as non-invasive, easily measurable, highly specific, sensitive, inexpensive, and producing rapid results. Within prostate cancer diagnostics, urine represents an ideal source of biomarkers as prostate cancer cells release proteins, DNA, RNAs, other small molecules, and exosomes in a detectable amount. In the context of urogenital malignancies, one of the most promising classes of potential biomarkers meeting the described criteria are microRNAs (miRNAs) (4), and more importantly urinary miRNAs. These cell-free molecules possess remarkable stability and good analytical performance and can reflect the clinical status of the malignancy or characteristics of the tumor (5, 6). Despite a considerable number of studies focused on the importance of urinary miRNAs within prostate cancer detection, there is, however, a low overlap in potential biomarker miRNAs.

In our current study, we performed high-capacity analysis of urine miRNAs to identify potential non-invasive biomarkers for the early detection of prostate cancer using a small RNA sequencing approach. Within the independent validation, we employed a more clinically relevant design and evaluated the diagnostic power of selected miRNAs in a group of prostate cancer patients and patients with negative prostate biopsy, where we compared the analytical performance of miRNAs with current clinical diagnostic parameters.

Patients and Methods

Patient characteristics. Between May 2016 and June 2020, 356 men undergoing prostate biopsy due to either abnormal PSA values or digital rectal examination were recruited to participate in this prospective study at the Department of Urologic Oncology, Masaryk Memorial Cancer Institute (MMCI). The study has been approved by the Ethics Committee of MMCI (the Ethics approval number MOU171816) and all participants signed an informed consent. Patients with urinary tract infection or a history of cancer were excluded from the control group. In total, 392 samples were used for analysis in this study.

Urine samples were obtained after digital rectal examination (DRE) before performing a biopsy. The prostate core biopsies were examined by an expert uropathologist; in patients where prostate cancer was detected, the grading was assigned according to the International Society of Urological Pathology 2014 consensus guidelines. Three subgroups were histologically defined based on the pathological evaluation of biopsy samples. The subgroups of indolent, intermediate, and aggressive PCa corresponding to ISUP grade 1, grade 2, and grade 3-5, respectively.

In the discovery phase, the control group included 18 healthy men who were seen in the urology outpatient clinic for benign urological conditions or as part of a cancer prevention program with long-term follow-up and low PSA values below 1.5 ng/ml. In addition, 18 patients with histologically confirmed benign prostatic hyperplasia (BPH) were included. In the validation phase, urine samples from 100 patients with negative prostate biopsy were used as a control group. The clinicopathological characteristics of the cohorts are summarized in Table I. A schematic overview of the workflow is depicted in Figure 1.

Table I. *Clinicopathological characteristics of prostate cancer patients and control groups*.

	Discovery phase	Validation phase
Prostate cancer samples		
Number	36	220
Age (mean±SD), years	65.8±7.9	69.3±7.9
ISUP grade group, number (%)		
ISUP 1	18 (50)	67 (30.5)
ISUP 2	-	84 (38.2)
ISUP 3	8 (22.2)	37 (16.8)
ISUP 4	5 (13.9)	24 (10.9)
ISUP 5	5 (13.9)	8 (3.6)
Healthy control samples		
Number	18	-
Age (mean±SD), years	66.7±9.4	-
BPH samples		
Number	18	-
Age (mean±SD), years	63.5±8.1	-
Negative prostate biopsy samples		
Number	-	100
Age (mean±SD), years	-	68.7±7.8

BPH: Benign prostatic hyperplasia; SD: standard deviation.

Sample collection and RNA isolation and purification. For all patients, the morning voided urine after DRE was collected into 15 ml tubes with EDTA as a nucleic acid preservative agent. The whole urine was then centrifuged at 4˚C at 2,000 × g for 15 min and the supernatant was collected and stored at –80˚C until further analysis. Before RNA isolation, the urine supernatant was centrifuged again at 4˚C at 12,000 g for 15 min to separate cell fragments and debris. Finally, total RNA including small RNA and circulating and exosomal RNA was isolated from 1 ml of cell-free urine supernatant using the Urine microRNA Purification Kit (Norgen Biotek Corp., Thorold, ON, Canada) according to the manufacturer's instructions. The quality and quantity of RNA were determined using the NanoDrop™ 2000 spectrophotometer and/or the Qubit Fluorometer (both Thermo Fisher Scientific, Waltham, MA, USA).

Small RNA sequencing and data processing. Sequencing libraries were prepared using a QIAseq™ miRNA Library Kit and QIAseq™ miRNA NGS 96 Index IL (both Qiagen, Hilden, Germany) strictly according to the manufacturer's instructions. Adapter dilution and cycle numbers were set according to the user manual. In more detail, for 3' ligation adapter was diluted 10-fold and the starting volume of undiluted template RNA was 5 μl. For 5' ligation adapter was diluted 5-fold. In the library amplification step, the reaction was set according to the manual with the number of cycles programmed to 22. For the listed reactions, a ProFlex PCR System (Thermo Fisher Scientific) was used.

The quality and size profiles of the prepared libraries were analyzed using High Sensitivity D1000 ScreenTape and 2200 TapeStation (both Agilent, Santa Clara, CA, USA). The library concentration was measured by Qubit 2.0 Fluorometer using reagents provided in Qubit™ dsDNA HS Assay Kit (both Thermo Fisher Scientific). Each miRNA sequencing library was diluted with nuclease-free water to 4 nM, mixed at an equimolar ratio to create

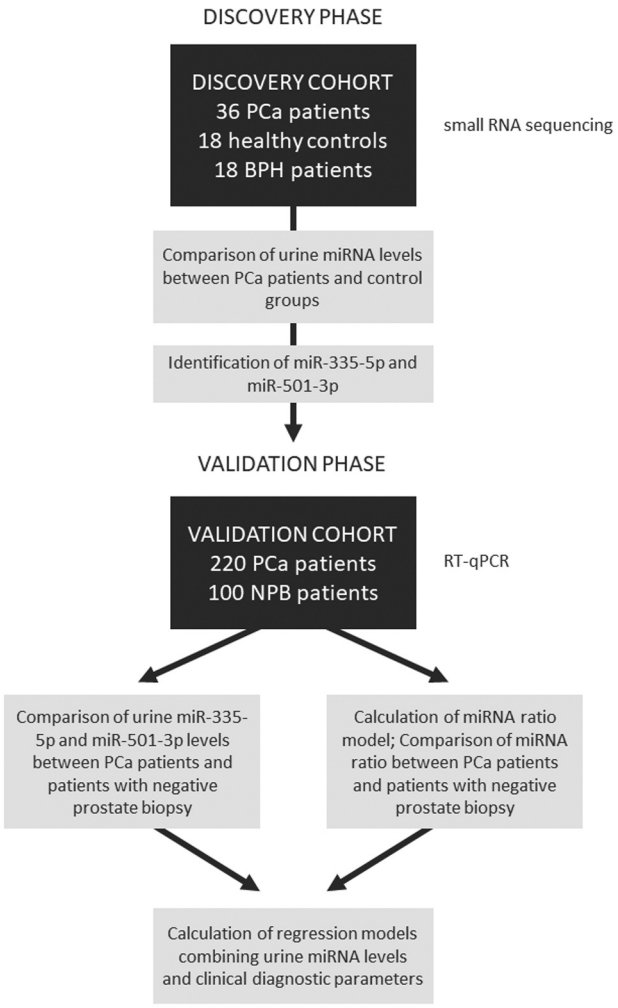

Figure 1. *Study flowchart. PCa: Prostate cancer; BPH: benign prostatic hyperplasia; NPB: negative prostate biopsy.*

a library pool, loaded onto the NextSeq 500/550 High Output v2 Kit (75 cycles) reagent cartridge, and sequenced on NextSeq 500 System (Illumina, San Diego, CA, USA).

The raw sequencing images from Illumina NextSeq 550 were converted to the fastq format using bcl2fastq (version 2.20.0). Raw reads were quality-checked with the FASTQC package (version 0.11.8). Adapter sequences were identified by Kraken package (15-065) and trimmed using Cutadapt (version 1.18). Subsequently, low-quality bases (Phred <10) were trimmed with Cutadapt and reads shorter than 15 nt were removed. Collapsing was performed utilizing unique molecular identifiers (UMIs) with FASTX-Toolkit (version 0.0.14). Reads originating from snoRNAs, snRNAs, rRNAs, tRNAs, piRNAs, and YRNAs (downloaded from Ensembl and RefSeq databases) were identified using Bowtie (version 1.2.2) and removed from the data. The remaining reads were mapped against the miRBase (version 21) and quantified using miraligner tool (version 1.2.4). Statistical analysis was carried out in R (version 3.4.3) with DESeq2 package (version 1.18.1).

Table II. *List of significantly dysregulated miRNAs in prostate cancer patients vs. control samples (BPH + healthy controls).*

miRNA	Base mean	Log2 FC	*p*-Value
hsa-miR-501-3p	6.68	2.74	0.00151
hsa-miR-191-5p	446.07	0.97	0.00176
hsa-miR-335-5p	16.17	2.07	0.00662
hsa-miR-342-3p	125.41	0.89	0.01583
hsa-miR-92b-3p	3.29	3.51	0.01783
hsa-miR-146a-5p	197.95	1.38	0.01786
hsa-miR-10b-3p	4.03	2.12	0.01881
hsa-miR-4286	2.92	–2.7	0.01993
hsa-miR-151b	4.85	2.03	0.02313
hsa-miR-196b-5p	100.94	–1.13	0.02340
hsa-miR-130a-3p	2.07	3.25	0.02363
hsa-miR-3168	97.92	2.5	0.02567

Base mean: The average of the normalized counts taken over all samples; FC: fold change.

RT-qPCR. The individual miRNAs were analyzed by standard TaqMan™ MicroRNA Assays (Thermo Fisher Scientific). First, the reverse transcription was performed using the TaqMan™ MicroRNA Reverse Transcription Kit, followed by real-time PCR with TaqMan probes and TaqMan® Universal Master Mix II, no UNG (all Thermo Fisher Scientific). All reactions and thermal cycling conditions were compliant with the standard protocol provided by the manufacturer. All reactions, including no-template controls and interplate controls, were run in duplicates. The RT-qPCR reaction was performed on the QuantStudio 12K Flex Real-Time PCR system (Thermo Fisher Scientific). The average Ct values of measured replicates were normalized using hsa-miR-101-3p as the most suitable reference gene identified with GenEx™ software 6.0 (MultiD Analyses AB, Göteborg, Sweden). Data normalization was performed using the $2^{-\Delta Ct}$ method where the ΔCt value corresponds to the difference between the Ct of the detected miRNA and the Ct of the reference gene. As an alternative normalization approach, the ratio of potential biomarker miRNAs was calculated by dividing the relative expression values of hsa-miR-501-3p and hsa-miR-335-5p (7).

Statistical analysis. Within the validation, the differential level of selected miRNAs between the compared cohorts was evaluated using the Mann-Whitney *U*-test and GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com). The discriminatory power of each biomarker and/or their combination, as well as the cut-off value for optimal analytical utilization, was established using ROC analysis (GraphPad Software).

For a combination of individual clinical biomarkers used for prostate cancer diagnosis, a stepwise logistic regression was performed using JMP® software (JMP®, Version 16, SAS Institute Inc., Cary, NC, 1989-2021). For all analyzes, a *p*-Value <0.05 was considered statistically significant.

Results

Small RNA sequencing. During the analysis of the small RNA sequencing data, samples with low miRNA coverage were

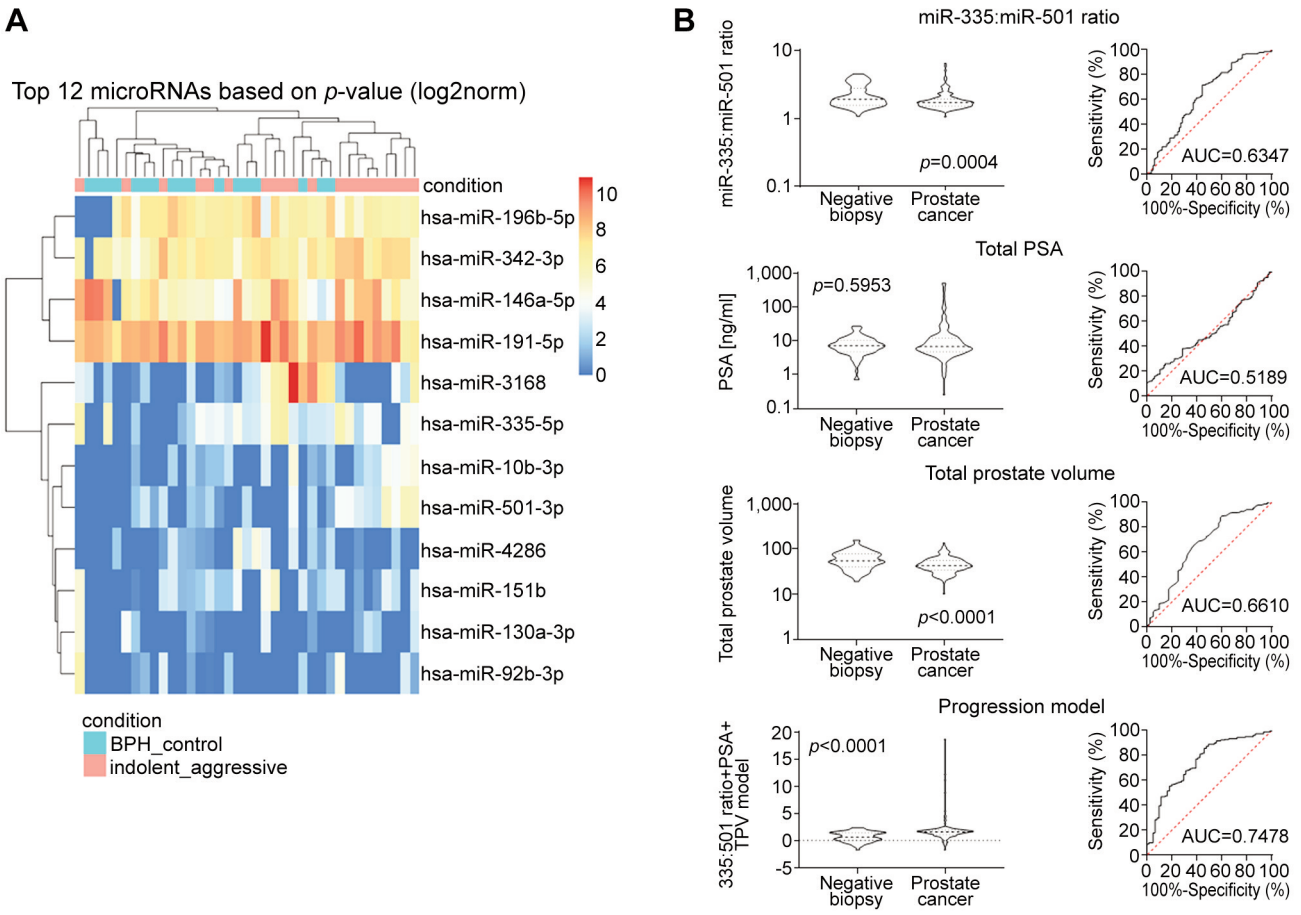

Figure 2. *Hierarchical clustergram discriminating prostate cancer patients (20 patients ISUP 1-5) and controls (17 healthy controls and BPH patients) according to differentially expressed urine miRNAs. The heatmap shows 12 significantly dysregulated miRNAs (p<0.05) (A). Analytical performance of standalone parameters (miR-335:miR-501 ratio, PSA, and TPV) and the regression model combining the parameters within the discrimination of PCa patients and patients with negative prostate biopsy. The regression model shows superior discriminatory power (AUC=0.7478) (B).*

discarded. Subsequently, the statistical analysis identified 12 miRNAs significantly dysregulated between a group of PCa patients (20 patients ISUP 1-5) vs a group of control samples (17 BPH patients and healthy individuals), thereof 10 miRNAs with higher levels and 2 miRNAs with lower levels in the urine of PCa patients (Table II and Figure 2A). For further validation in the independent cohort, we selected miR-335-5p and miR-501-3p based on their analytical parameters (fold change, *p*-value, and the average of normalized counts).

RT-qPCR analysis and clinical diagnostic parameters. In the validation cohort, we determined the urinary levels of miR-335-5p and miR-501-3p in cell-free urine samples of overall 220 prostate cancer patients and 100 patients with negative prostate biopsy as a control group. Of these two potential biomarkers, only the level of miR-501-3p was significantly dysregulated (p=0.02) in the urine of PCa patients compared to patients with negative prostate biopsy.

Table III. *Average values of clinical diagnostic parameters within control and testing groups.*

Parameter (mean±SD)	PSA	fPSA	PSAD	TPV
NPB	8.3±5.3	0.7±1.1	0.2±0.1	60.5±27.6
ISUP 1	7.8±12.9	0.7±0.7	0.2±0.4	44.0±17.6
ISUP 2	12.6±31.7	0.6±0.9	0.3±0.4	46.6±18.1
ISUP 3	26.7±82.1	0.4±0.5	0.7±2.5	47.8±18.4
ISUP 4	65.0±118.4	1.0±1.6	1.7±4.2	50.1±26.0
ISUP 5	94.4±156.7	6.9±5.4	2.4±3.9	45.6±13.2
Overall PCa	41.3±80.4	1.9±1.8	1.0±2.3	46.8±18.7

PSA: Prostate specific antigen; TPV: total prostate volume; fPSA: free prostate-specific antigen; PSAD: prostate specific antigen density; NPB: negative prostate biopsy patients; SD: standard deviation.

We performed the same analysis for available clinical diagnostic parameters: total PSA, free PSA (fPSA), prostate-specific antigen density (PSAD), and total prostate volume

Table IV. *Analytical performance of standalone urine miRNAs and clinical parameters within the comparison of patients with negative prostate biopsies with PCa patients (ISUP 1-5) (I), patients with indolent PCa (ISUP 1) (II), and patients with clinically insignificant PCa (ISUP 1-2) (III). Additionally, analytical parameters were obtained from comparison of group combining patients with negative prostate biopsy with indolent PCa (NPB + ISUP 1) from significant PCa (ISUP 2-5) (IV), comparison of patients with indolent (ISUP 1) and aggressive (ISUP 3-5) prostate cancer (V) and comparison among ISUP grade groups (VI).*

	PCa *vs*. NPB		PCa ISUP 1 *vs*. NPB		PCa ISUP 1-2 *vs*. NPB		PCa ISUP 1 *vs*. ISUP 3-5		NPB + ISUP 1 *vs*. ISUP 2-5		ISUP grade group
	p-Value	AUC	*p*-Value	AUC	*p*-Value	AUC	*p*-Value	AUC	*p*-Value	AUC	*p*-Value*
miR-335	0.1480	0.5551	0.6351	0.5238	0.2630	0.5455	0.2116	0.5678	0.0736	0.5627	0.3204
miR-501	0.0260	0.5822	0.0009	0.6593	0.0036	0.6139	0.0039	0.6512	0.6800	0.5140	0.0295
335:501 ratio	0.0004	0.6347	0.0002	0.6844	<0.0001	0.6626	0.0105	0.6410	0.3958	0.5302	0.0477
PSA	0.5953	0.5189	0.0039	0.6326	0.2394	0.5445	<0.0001	0.7522	0.0001	0.6259	<0.0001
fPSA	0.9485	0.5028	0.4511	0.5424	0.9929	0.5004	0.0991	0.6205	0.5783	0.5240	0.0670
PSAD	0.0019	0.6177	0.7909	0.5131	0.0895	0.5684	<0.0001	0.7141	<0.0001	0.6550	<0.0001
TPV	<0.0001	0.6610	<0.0001	0.6926	<0.0001	0.6729	0.2258	0.5615	0.0848	0.5579	0.7704

PSA: Prostate-specific antigen; TPV: total prostate volume; fPSA: free prostate-specific antigen; PSAD: prostate-specific antigen density; NPB: negative prostate biopsy patients. *Association with ISUP grade group was evaluated using the Kruskal-Wallis test.

Table V. *Most significant regression models combining urine miRNAs and clinical parameters within the comparison of patients with negative prostate biopsy with PCa patients, patients with indolent PCa, clinically insignificant PCa and the comparison of indolent and aggressive PCa. For each model we state the AUC, PPV, NPV, and the cut-off value.*

Combination	AUC	PPV	NPV	Cut-off
PCa *vs*. NPB				
miR-335:miR-501 ratio + PSA + TPV	0.748	0.848	0.507	0.892
PCa ISUP 1 *vs*. NPB				
335:501 ratio + PSA + TPV + PSAD	0.772	0.656	0.769	–0.131
PCa ISUP 1-2 *vs*. NPB				
miR-335 + PSA + TPV + fPSA	0.752	0.767	0.647	0.492
PCa ISUP 1 *vs*. ISUP 3-5				
335:501 ratio + PSA	0.762	0.712	0.745	–0.281

PSA: Prostate-specific antigen; TPV: total prostate volume; fPSA: free prostate-specific antigen; PSAD: prostate-specific antigen density; NPB: negative prostate biopsy patients; AUC: area under curve; PPV: positive predictive value; NPV: negative predictive value.

measured by transrectal ultrasonography (TPV). For average values of clinical diagnostic parameters within control and testing groups, see Table III.

Next, to better discriminate the compared groups, we applied a quantitative ratiometric approach and calculated the ratio of miR-335-5p and miR-501-3p levels. This miRNA ratio outperformed individual miRNAs and distinguished PCa patients from patients with negative prostate biopsy with higher discriminatory power (p=0.0004, AUC=0.64). For comparison, only PSAD (p=0.019, AUC=0.62) and total prostate volume (p<0.0001, AUC=0.66) were able to distinguish prostate cancer patients from controls among the monitored clinical diagnostic parameters (Table IV). Since PCa patients and the control group were both subjected to the prostate biopsy based on increased PSA level, this parameter could not significantly discriminate the cohorts (p=0.595).

Additionally, both miR-501-3p and the miR-335:miR-501 ratio were able to distinguish indolent (p=0.0009 and p=0.0002, respectively) and clinically insignificant (p=0.0036, and p<0.0001, respectively) prostate cancer from controls. Indolent prostate cancer (ISUP 1) was distinguished also by the use of total PSA level (p=0.0039) and total prostate volume (p<0.0001) which significantly discriminated also clinically insignificant PCa (p<0.0001). Neither miR-501-3p, miR-335, nor the miRNA ratio could not discriminate clinically insignificant group combining patients with negative prostate biopsy with indolent PCa from significant PCa (ISUP 2-5).

Furthermore, miR-501-3p and the miR-335:miR-501 ratio (p=0.0039 and p=0.0105, respectively) as well as total PSA level and PSAD could discriminate between indolent (ISUP 1) and aggressive PCa (ISUP 3-5), which was later confirmed with the Kruskal-Wallis test showing association

of all these parameters/potential biomarkers with the ISUP grade. All statistical comparisons are shown in Table IV.

Combination of urine miRNAs and clinical diagnostic parameters. Since individual biomarkers did not show satisfactory discriminative power, we decided to use nominal logistic regression and apply a combination of all clinical diagnostic parameters with and without individual miRNAs and the miRNA ratio. ROC analysis and whole model test significance were performed for all conditions. The most significant combinations of miRNA biomarkers and clinical parameters for each condition are summarized in Table V. Standalone miRNAs or the miRNA ratio could not discriminate group combining patients with negative prostate biopsy with indolent PCa from significant PCa, therefore no regression models were calculated for this condition.

Prostate cancer patients vs. patients with negative prostate biopsy. For comparison of prostate cancer patients and patients with negative prostate biopsy, the highest increase in discriminatory power for miR-335 was achieved in combination with PSAD, PSA, and fPSA (AUC=0.7325). Only a minor improvement for miR-501 was reached in combination with PSAD, PSA, and fPSA (AUC for the model 0.7215). The highest discriminatory power was reached for the miR-335:miR-501 ratio when combined with PSA + TPV [AUC=0.7478, PPV=0.848, NPV=0.507; DxScore=3.55618451 + (–0.6526161 * miR-335:miR-501 ratio) + (0.03623058 * PSA) + (–0.028844 * TPV); Figure 2B; Table V]. For the PSA grey zone (patients with total PSA level between 4-10 ng/ml) this regression model reached the AUC of 0.7178. Among other models with the highest discriminatory power, we detected the miR-335:miR-501 ratio + PSA + TPV + PSAD or the miR-335:miR-501 ratio + TPV + PSAD (both conditions AUC=0.7476) and PSAD + PSA + fPSA (AUC=0.7475) (Table VI).

Indolent prostate cancer vs. patients with negative prostate biopsy. Next, we compared the ability of computed regression models to distinguish indolent ISUP1 PCa from patients with negative prostate biopsy. We observed a significant increase in discriminatory power for miR-335 when combined with PSA + TPV + fPSA (AUC=0.7548). Interestingly, every combination of miR-335 involving total prostate volume allowed to detect indolent PCa with AUC greater than 0.74. On the contrary, regression models based on the urinary level of miR-501 did not show a significant improvement from the standalone miRNA. The best analytical parameters were observed for the miR-335:miR-501 ratio combined with PSA + TPV + PSAD [AUC=0.7717; DxScore=2.75372875 + (–0.8142955 * miR-335:miR-501 ratio) + (–0.0915265 * PSA) + (–0.0252965 * TPV) + (4.04533464 * PSAD)], although more regression models reached the AUC of 0.75. Furthermore, when we evaluated significant regression models within the PSA grey zone, the additional refinement of the models was observed with AUC increasing to around 0.8. All comparisons and results are listed in Table VI.

Clinically insignificant prostate cancer vs. patients with negative prostate biopsy. Interestingly, in the comparison of clinically insignificant prostate cancer and patients with negative prostate biopsy, we observed the most marked increase in discriminatory power in combinations with miR-335. Specifically, the highest analytical parameters were detected in the regression model combining the miR-335 level with PSA + TPV + fPSA [AUC=0.7516; DxScore=2.34331463 + (–0.0001012 * miR-335) + (0.0693638 * PSA) + (–0.0384259 * TPV) + (–0.4683634 * fPSA)]. Subsequent analysis of these conditions within the PSA grey zone did not improve discriminatory capabilities (Table VI).

Indolent vs. aggressive prostate cancer. Finally, we verified the ability of identified miRNAs and clinical diagnostic parameters to differentiate between indolent and aggressive prostate cancer. The highest predictive power was detected for miR-501 in combination with PSA (AUC=0.7565) and analogously for the miR-335:miR-501 ratio combined with PSA [AUC=0.7619; DxScore=–1.5082927 + (0.54160878 * miR-335:miR-501 ratio) + (0.03907273 * PSA)] (Table VI).

Discussion

Prostate cancer detection is dependent on PSA as an initial screening test and ultrasound-guided transrectal biopsy as the gold-standard diagnostic method. However, about 70% of PCa cases subjected to biopsy turn out negative (8) causing unnecessary physical and psychological harm to the patients. Moreover, a high rate of false-negative biopsies (15-30%) leads to commonly repeated biopsies (9, 10) and undertreatment. Thus, the legitimate indication for biopsy remains an important clinical issue. Despite several biomarkers available for PCa detection, they are not included in routine practice. Therefore, new molecular biomarkers are a possible way to improve and streamline PCa diagnostics.

In this study, we performed a high-capacity urinary miRNA analysis to identify a set of miRNAs significantly dysregulated between PCa patients and healthy controls and BPH patients. In subsequent validation, we employed a clinically more relevant design and evaluated the discrimination power of the selected miR-335-5p and miR-501-3p in a group of prostate cancer patients and patients with negative prostate biopsy. In this cohort, only miR-501-3p was able to significantly distinguish patients with negative prostate biopsy (p=0.02), however, with rather unsatisfactory analytical parameters. To further refine the analytical accuracy of identified miRNAs, we applied the ratiometric approach and calculated the miR-335:miR-501 ratio. This approach emerges to be widely

Table VI. *Discriminatory power of regression models combining clinical parameters and urinary miRNAs to compare patients with negative prostate biopsies with PCa patients (ISUP 1-5) (I), patients with indolent PCa (ISUP 1) (II), patients with clinically insignificant PCa (ISUP 1-2) (III), and to compare patients with indolent (ISUP 1) and aggressive (ISUP 3-5) prostate cancer.*

	PCa *vs*. NPB		PCa ISUP 1 *vs*. NPB		PCa ISUP 1-2 *vs*. NPB		PCa ISUP 1 *vs*. ISUP 3-5	
	AUC	WMT (Prob>ChiSq)	AUC	WMT (Prob>ChiSq)	AUC	WMT (Prob>ChiSq)	AUC	WMT (Prob>ChiSq)
PSA + fPSA	0.5195	0.0900	0.3982	0.7656	0.5105	0.2765	0.5171	0.1788
PSA + PSAD	0.6714	<0.0001	0.6804	0.0006	0.6547	0.0041	0.7234	<0.0001
PSA + TPV	0.6979	<0.0001	0.6917	0.0001	0.6829	<0.0001	0.7262	<0.0001
PSAD + fPSA	0.6609	0.0002	0.5807	0.1668	0.6436	0.0014	0.5317	0.1679
PSAD + TPV	0.6964	<0.0001	0.6882	0.0002	0.6787	<0.0001	0.7226	0.0001
fPSA + TPV	0.6781	<0.0001	0.6953	0.0006	0.6781	0.0001	0.4951	0.1848
PSA + fPSA + PSAD	0.7208	<0.0001	0.6829	0.0196	0.7089	0.0002	0.5015	0.2554
PSA + fPSA + TPV	0.7116	<0.0001	0.7027	0.0011	0.7047	<0.0001	0.5200	0.3285
PSA + PSAD + TPV	0.6965	<0.0001	0.6900	0.0004	0.6781	<0.0001	0.7192	0.0002
PSAD + TPV + fPSA	0.7119	<0.0001	0.6965	0.0017	0.7047	<0.0001	0.5376	0.3107
PSA + fPSA + PSAD + TPV	0.7118	<0.0001	0.7000	0.0040	0.7042	<0.0001	0.5181	0.3418
miR-335 + PSA	0.5891	0.0004	0.5373	0.0173	0.5556	0.0004	0.7353	0.0004
miR-335 + PSA + TPV	0.7229	<0.0001	0.7481	<0.0001	0.7259	<0.0001	0.7282	0.0004
miR-335 + PSA + TPV + fPSA	0.7284	<0.0001	0.7548	0.0002	**0.7516**	**<0.0001**	0.6517	0.0531
miR-335 + PSA + TPV + fPSA + PSAD	0.7319	<0.0001	0.7498	0.0006	0.7504	<0.0001	0.6517	0.0705
miR-335 + PSA + TPV + PSAD	0.7182	<0.0001	0.7489	<0.0001	0.7222	<0.0001	0.7216	0.0006
miR-335 + TPV	0.6915	<0.0001	0.7487	<0.0001	0.7180	<0.0001	0.6332	0.0182
miR-335 + TPV + PSAD	0.7185	<0.0001	0.7434	<0.0001	0.7225	<0.0001	0.7242	0.0007
miR-335 + TPV + PSAD + fPSA	0.7297	<0.0001	0.7498	0.0002	0.7509	<0.0001	0.6544	0.0520
miR-335 + PSAD	0.6713	<0.0001	0.6314	0.0006	0.6625	<0.0001	0.6547	0.0040
miR-335 + PSAD + PSA	0.7054	<0.0001	0.6804	0.0006	0.7090	<0.0001	0.7212	0.0003
miR-335 + PSAD + PSA + fPSA	0.7325	<0.0001	0.7346	0.0008	0.7435	<0.0001	0.6122	0.0675
miR-335 + PSA + fPSA	0.5517	0.1095	0.5391	0.1262	0.5908	0.0284	0.6176	0.0331
miR-335 + PSAD + fPSA	0.6842	<0.0001	0.6868	0.0050	0.7156	<0.0001	0.6190	0.0331
miR-335 + TPV + fPSA	0.7029	<0.0001	0.7485	<0.0001	0.7245	<0.0001	0.6476	0.0252
miR-501 + PSA	0.5809	0.0051	0.6403	0.0401	0.5947	0.0457	0.7565	<0.0001
miR-501 + PSA + TPV	0.6864	<0.0001	0.7145	0.0006	0.6924	<0.0001	0.7464	<0.0001
miR-501 + PSA + TPV + fPSA	0.6965	0.0002	0.7056	0.0058	0.6927	0.0008	0.6666	0.0607
miR-501 + PSA + TPV + fPSA + PSAD	0.7119	0.0002	0.7056	0.0126	0.7037	0.0014	0.6711	0.0939
miR-501 + PSA + TPV + PSAD	0.6939	<0.0001	0.7084	0.0010	0.6917	0.0001	0.7461	<0.0001
miR-501 + TPV	0.6618	0.0001	0.7148	0.0002	0.6869	<0.0001	0.6176	0.2341
miR-501 + TPV + PSAD	0.6905	<0.0001	0.7132	0.0005	0.6924	<0.0001	0.7441	<0.0001
miR-501 + TPV + PSAD + fPSA	0.7083	<0.0001	0.7056	0.0060	0.7005	0.0006	0.6688	0.0584
miR-501 + PSAD	0.6127	0.0004	0.5895	0.0830	0.5839	0.0189	0.7377	<0.0001
miR-501 + PSAD + PSA	0.6630	<0.0001	0.7004	0.0009	0.6619	0.0012	0.7479	<0.0001
miR-501 + PSAD + PSA + fPSA	0.7215	0.0002	0.6976	0.0344	0.7125	0.0014	0.6633	0.0629
miR-501 + PSA + fPSA	0.5420	0.1367	0.5508	0.6428	0.5499	0.3865	0.6600	0.0310
miR-501 + PSAD + fPSA	0.6601	0.0014	0.5817	0.2111	0.6411	0.0096	0.6622	0.0305
miR-501 + TPV + fPSA	0.6595	0.0009	0.7008	0.0040	0.6696	0.0014	0.6600	0.0530
335:501 ratio + PSA	0.6466	0.0002	0.6779	0.0057	0.6501	0.0031	**0.7619**	**0.0005**
335:501 ratio + PSA + TPV	**0.7478**	**<0.0001**	0.7676	<0.0001	0.7480	<0.0001	0.7322	0.0011
335:501 ratio + PSA + TPV + fPSA	0.7359	<0.0001	0.7411	0.0022	0.7365	0.0001	0.6825	0.2786
335:501 ratio + PSA + TPV + fPSA + PSAD	0.7389	0.0001	0.7392	0.0052	0.7400	0.0003	0.6681	0.3587
335:501 ratio + PSA + TPV + PSAD	0.7476	<0.0001	**0.7717**	**<0.0001**	0.7482	<0.0001	0.7212	0.0017
335:501 ratio + TPV	0.7230	<0.0001	0.7653	<0.0001	0.7433	<0.0001	0.6402	0.0613
335:501 ratio + TPV + PSAD	0.7476	<0.0001	0.7663	<0.0001	0.7478	<0.0001	0.7336	0.0021
335:501 ratio + TPV + PSAD + fPSA	0.7384	<0.0001	0.7348	0.0023	0.7394	0.0001	0.6782	0.2767
335:501 ratio + PSAD	0.7057	<0.0001	0.7068	0.0012	0.6996	0.0001	0.7379	0.0044
335:501 ratio + PSAD + PSA	0.7263	<0.0001	0.7640	<0.0001	0.7291	<0.0001	0.7244	0.0007
335:501 ratio + PSAD + PSA + fPSA	0.7475	<0.0001	0.7260	0.0087	0.7394	0.0003	0.6479	0.2757
335:501 ratio + PSA + fPSA	0.6087	0.1380	0.6303	0.2858	0.6363	0.1136	0.6695	0.1701
335:501 ratio + PSAD + fPSA	0.7013	0.0005	0.6976	0.0337	0.7056	0.0011	0.6637	0.1697
335:501 ratio + TPV + fPSA	0.7051	0.0002	0.7373	0.0014	0.7235	0.0001	0.6796	0.1658

PSA: Prostate specific antigen; TPV: total prostate volume; fPSA: free prostate-specific antigen; PSAD: prostate specific antigen density; AUC: area under curve. The whole model test (WMT) (Prob>ChiSq) shows probability of obtaining the chi-square statistic given that the null hypothesis is true. Bold values represent the combinations with the highest AUC in particular comparison.

accepted, especially in body fluids, where stable reference genes might be challenging to find. The quantitative ratio does not require additional normalization and should be independent of the detection system. In fact, in our validation cohort the miR-335:miR-501 ratio outperformed standalone miRNAs and most clinical parameters in terms of distinguishing between PCa patients and patients with negative prostate biopsy (p=0.0004).

Both miR-335-5p and miR-501-3p were already shown to be associated with prostate cancer. Previously, Haldrup *et al*. identified new potential serum miRNAs including miR-501-3p able to identify PCa patients (11). More importantly, miR-501-3p was among miRNAs significantly dysregulated in urine exosomes from prostate cancer patients detected by deep sequencing analysis (12). As we employed a similar study design, these results could serve as independent validation of our NGS analysis data. Whole-genome miRNA profiling studies also described upregulation of miR-335-5p in the tissue of high grade PCa tumors (13) and in exosomes secreted by human PCa cells under hypoxic conditions (14). The only exploratory study describing miR-335-5p as a potential biomarker of prostate cancer is the work by Koh *et al*., where this miRNA was differentially expressed in urine, plasma, and tissue samples of PCa patients (15).

As for diagnostic tests or biomarkers, there are currently several alternatives to total PSA levels, such as free PSA, PSA density and velocity, PHI, 4Kscore, or PCA3. However, they are often of limited value and are not routinely used for economical or technical reasons. One possibility is to combine available parameters into one model which could significantly improve diagnostic performance. For example, Liu *et al*. used the multivariate model combining PSAD and multiparametric magnetic imaging (mpMRI), which performed significantly better than standalone parameters for the detection of PCa even within the PSA grey zone (3). Even more beneficial should be models that merge genomic biomarkers with clinical parameters. Adopting this approach, we used nominal logistic regression and tested a combination of available clinical diagnostic parameters (total PSA, free PSA, TPV, PSAD) with miR-335-5p and miR-501-3p and the miRNA ratio. Among all combinations, the highest discriminatory power was reached for the model based on the miR-335:miR-501 ratio with PSA and TPV which discriminated PCa patients according to the biopsy outcome with AUC of 0.7478, PPV=0.848, and NPV=0.507. Based on the NPV, therefore, our model can potentially reduce the number of unnecessary biopsies by 50%.

Additionally, we identified regression models discriminating patients with negative prostate biopsy from indolent (335:501 ratio + PSA + TPV + PSAD) and clinically insignificant PCa (miR-335 + PSA + TPV + fPSA). When comparing the patients with indolent PCa, the highest discriminating power reached combinations based on the miR-335:miR-501 ratio involving total prostate volume since this parameter alone allowed to distinguish compared groups (p<0.0001, AUC=0.69). However, the combination of markers significantly refined the analytical parameters (AUC=0.77). Moreover, an additional enhancement of the models was observed when evaluated within the PSA grey zone (AUC=0.8). For clinically insignificant prostate cancer, we observed the most marked increase in discriminatory power in combinations involving miR-335 and free PSA, despite the fact that these factors alone could not distinguish compared cohorts.

Lastly, several regression models based on the level of miR-501-3p or the miR-335:miR-501 ratio could differentiate between indolent and aggressive prostate cancer. A similar result was observed for miR-501-3p alone, whose level also decreased with the higher ISUP grade group. This is in agreement with the literature describing miR-501-3p as a potential tumor suppressor (16) that restricts prostate cancer cell growth by targeting CREPT to inhibit the expression of cyclin D1.

Our study has several potential limitations. For the identification of miRNA biomarkers, we used samples from healthy controls and BPH patients as a control group. Since a different control group was used in validation, this could lead to overlooking of miRNAs enabling better discrimination of patients with negative prostate biopsy. Given the high rate of false-negative biopsies, our data may be affected by this inaccuracy, as we do not possess detailed follow-up data showing whether the patient was indicated for re-biopsy or was diagnosed with PCa in time. The analytical power of identified regression models combining genomic biomarkers with clinical parameters could be improved by using other promising tests (PHI, PCA3, mpMRI), however, these are not routinely evaluated within the standard protocol at MMCI and could not be used. The weakness is also the one-level validation, which we preferred due to the increase in the homogeneity of the patient group. For these reasons, further independent studies are needed to confirm our results and demonstrate the potential clinical utility of the model. Another limitation of our study is a relatively high rate of positive biopsies (65%) in our patient cohort, which is higher when compared to the commonly observed positivity rates. This may reflect the specific pattern of patient referral to our tertiary hospital. Noteworthy, this fact may disturb the analytical outcomes for PSA in the prediction of positive prostate biopsy.

Conclusion

Current diagnostic procedures result in a large number of patients being misclassified for prostate biopsy. Using NGS analysis, we identified urine miRNAs for noninvasive detection of PCa. In independent validation, we successfully tested the ability of miR-335-5p and miR-501-3p and the miRNA ratio to discriminate PCa patients and patients with negative prostate biopsy. Next, we showed that combination of genomic biomarkers with clinical parameters could significantly

improve diagnostic accuracy. Moreover, the combination of miR-335:miR-501 ratio + PSA + TPV was able to identify patients with negative prostate biopsy and could potentially improve decision making for biopsy indication.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

Study conceptualization, J.J., M.S. and O.S.; Patients enrollment, samples characterization, M.S.; Laboratory analysis, J.J., M.M., M.R., and J.B.; Data analysis and statistical analysis, K.T., M.M. and J.J.; Original draft preparation and visualization, J.J.; Review and editing, all authors; Supervision, O.S. All Authors have read and agreed to the published version of the manuscript.

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