Original Research Article

# Detection of miRNAs in urine of prostate cancer patients

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## ARTICLE INFO

## ABSTRACT

*Background and aim:* Prostate cancer (PCa) is the second most prevalent oncologic disease among men worldwide. Expression of various transcripts, including miRNAs, is markedly deregulated in cancerous prostate tissue. This study aimed at identifying a PCa-specific expression profile of miRNAs for subsequent use in noninvasive diagnostics.

*Materials and methods:* MiRNA expression was profiled in 13 PCa tissues using human miRNA microarrays. Highly expressed miRNAs were selected for the analysis in urine of patients with PCa ($N = 143$) and benign prostate hyperplasia (BPH; $N = 23$) by means of real time PCR, while miRNAs showing the expression differences in relation to clinical variables were further analyzed in 52 PCa and 12 noncancerous prostate tissues (NPT) on TaqMan Low Density Arrays (TLDA).

*Results:* Analysis of miRNA expression in prostate tissue linked miR-95 to aggressive form of PCa. This miRNA was up-regulated in high grade ($P = 0.041$), the *TMPRSS2-ERG* fusion-positive tumors ($P = 0.026$), and in patients with subsequently developed biochemical recurrence (BCR; $P = 0.054$) after radical prostatectomy. MiRNAs highly expressed in PCa tissues were also detectable in urine from PCa patients. Moreover, the urinary levels of miR-21 had significant discriminatory power ($P = 0.010$) to separate PCa patients from BPH, while the combined analysis of urinary miR-19a and miR-19b was prognostic for BCR. In PCa, the diagnostic potential of urinary miRNA panel (miR-21, miR-19a, and miR-19b) was higher than that of the PSA test (AUC = 0.738 vs. AUC = 0.514).

*Conclusions:* Measurement of urinary levels of PCa-specific miRNAs could assist in more specific detection of PCa and prediction of BCR.

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# 1. Introduction

Prostate cancer (PCa) is the second most prevalent male cancer worldwide and the predominant male cancer in developed countries [1]. While many prostate tumors grow slowly, are confined in the prostate and do not reduce the life quality of patients, others are aggressive, spread quickly and, therefore, should be detected at earlier stages. Despite its high false positive and false negative rates, the prostate-specific antigen (PSA) test remains the only widely available test for PCa screening and diagnostics [2]. Nowadays, studies of PCa are directed toward development of novel non-invasive tests that can improve the specificity of PSA testing and provide at least modest prognostic information about the disease aggressiveness. However, none of the novel PCa-specific molecular biomarkers is currently approved for routine clinical usage.

MicroRNAs (miRNAs) are single-stranded small (18–24 nt) non-coding RNAs that originate endogenously and function in the post-transcriptional regulation of gene expression. Target mRNA undergoes transcriptional repression or cleavage according to complementarity level of its 3′ UTR and the seed region (2–8 nucleotides) of miRNA [3]. In that way one single miRNA is able to have multiple targets as well as a single mRNA can be regulated by multiple miRNAs. Regulatory capabilities of miRNAs affect nearly all physiological processes such as cell growth, differentiation, and apoptosis. In contrary, many human diseases, including cancer, are associated with deregulation of miRNAs production [4]. In cancer research, in analogy with cancer-related genes, up-regulated and down-regulated miRNAs are called oncogenic miRNAs (onco-miRs) and tumor suppressor miRNAs, respectively [5]. In many forms of cancer, miRNA expression profile significantly differs between cancerous vs. non-cancerous tissues, between aggressive vs. indolent cases, and may change simultaneously with the disease progression [6]. MiRNAs are also detectable in cell-free form circulating in various body fluids [7]. During oncogenesis, the content of freely circulating miRNAs changes and can reflect the situation in tumor [8]. Circulating miRNAs are firmly packed and remain quite stable in body fluid even after collection [9], providing possibilities to serve as non-invasive tools for detection of disease-specific changes. Recent studies of circulating miRNAs in PCa [10,11] revealed their good diagnostic potential when used as a single biomarker or in combination with the PSA test [12]. The prevalent source to analyze circulating miRNAs in PCa is blood [12,13], whereas a quite limited number of studies has been done on more specific body fluids such as urine [11], seminal fluid [14] or ejaculate [15].

In the present study, miRNA profiling was performed in PCa tissues by miRNA microarrays, while expression levels of selected miRNAs were analyzed in PCa and noncancerous prostate tissues on custom designed TaqMan Low Density Arrays (TLDA). A subset of miRNAs was selected for the investigation in urine of PCa and benign prostatic hyperplasia (BPH) patients. Changes of miRNA levels in urine were disease-specific and correlated with the biochemical disease progression after surgery.

# 2. Materials and methods

## 2.1. Sample collection

Prostate specimens were obtained prospectively from PSA-screened and biopsy-proven intermediate-risk PCa patients treated with radical prostatectomy (RP). Surgery was performed at the Urology Department of Vilnius University Hospital Santariškių Klinikos from January 2008 to May 2011. The local Bioethics Committee approved the study before its initiation, and all patients gave informed consent for participation in the study.

The investigation was a part of the large-scale PCa biomarker study performed according to standardized protocols of sample collection and processing as reported previously [16,17]. Briefly, after RP, prostate tissue cores of 0.8 cm diameter were prepared from unfixed prostate and immediately frozen in liquid nitrogen. Before freezing, sections of tissue were placed on microscopic slides for quantification of tumor cells using hematoxylin and eosin staining. Cancerous (≥70% of cancer cells) and non-cancerous (0%) prostate tissues were sampled by expert histopathologist and transferred for molecular analysis. For the RNA extraction frozen tissue sections from 65 PCa patients were available, including 53 prostate adenocarcinomas and 12 noncancerous prostate tissues (NPT).

In addition, 166 samples of urine sediments were collected from 23 BPH and 143 PCa patients, 40 of which had a corresponding tissue sample. Urine was catheterized during RP, and 30 mL of collected urine were centrifuged within a half an hour at 1000 rpm for 15 min at 4 °C and PBS-washed three times, and stored at −70 °C until use.

Due to overlapping cases in tissue and urine analyses, the total number of PCa cases of our study was 156, with follow-up data available for 144 (92.3%) of them. Biochemical recurrence (BCR) was defined as an increase in PSA level to ≥0.2 ng/mL after RP. 34 (23.6%) of PCa patients experienced BCR in 22 months after surgery on average (range 0–64 months). The mean follow-up time of cases without progression was 36 months (range 4–66 months). Clinical–pathological characteristics are summarized in Table 1. The status of the fusion transcript TMPRSS2-ERG was identified in our previous study [16,18].

## 2.2. Total RNA purification from tissue

Total RNA from prostate tissue was isolated with mirVana™ miRNA Isolation Kit (Ambion, USA) according to manufacturer's protocol. About 30 mg of mechanically homogenized tissue were lysed with 600 μL Lysis/Binding Buffer and extracted total RNA was eluted in 100 μL of 95 °C temperature Elution Solution.

## 2.3. Total RNA purification from urine sediments

miRNeasy Mini Kit (Qiagen, USA) was used for RNA isolation from urine according to manufacturer's protocol, which adjusts kit for the purification of total RNA from liquid samples. Total RNA was extracted from 200 μL of urine

| Table 1 – Clinical and pathological characteristics of controls and patients with PCa. | | | | | |
|---|---|---|---|---|---|
| Variable | | Tissue | | Urine | |
| | | PCa (N = 53) | NPT (N = 12) | PCa (N = 143) | BPH (N = 23) |
| Age, years | Mean ± SEM | 61.8 ± 0.95 | 61.5 ± 1.2 | 61.4 ± 0.6 | 74.2 ± 1.4 |
| | Range | 48–73 | 53–67 | 42–74 | 59–83 |
| Pathological stage, N (%) | ≤pT2 | 35 (66.0) | – | 112 (78.3) | – |
| | pT3 | 18 (34.0) | – | 31 (21.7) | – |
| Gleason score, N (%) | G6 | 19 (35.8) | – | 74 (51.7) | – |
| | G ≥ 7 | 32 (64.2) | – | 68 (47.6) | – |
| | Unknown | – | – | 1 (0.7) | – |
| BCR, N (%) | Yes | 18 (34.0) | – | 27 (18.9) | – |
| | No | 34 (64.0) | – | 104 (72.7) | – |
| | Unknown | 1 (1.9) | – | 12 (8.4) | – |
| TMPRSS2-ERG, N (%) | Yes | 35 (66.0) | – | 57 (39.9) | – |
| | No | 18 (34.0) | – | 40 (28.0) | – |
| | Unknown | – | – | 46 (32.2) | – |
| Serum PSA, ng/mL | Mean ± SEM | 11.1 ± 1.9 | 7.2 ± 1.4 | 9.3 ± 1.0 | 7.8 ± 1.3 |
| | Range | 2.8–84.2 | 2.6–17.0 | 0.1–84.2 | 0.8–28.1 |
| | Unknown (%) | 2 (3.8) | – | 3 (2.1) | 2 (8.7) |

PCa, prostate cancer; NPT, noncancerous prostate tissue; BPH, benign prostatic hyperplasia; BCR, biochemical recurrence; PSA, prostate-specific antigen; pT, pathological tumor stage; G, Gleason score; SEM, standard error of mean.

sediments. Each sample was spiked with 25 fmol synthetic cel-miR-39 (Qiagen, USA), and purified RNA was eluted twice in 30 μL RNase-free water.

Concentration and quality of prostate tissue- and urine-derived RNA were measured using NanoDrop 2000 spectrophotometer (ThermoScientific, USA) and 2100 Bioanalyzer (Agilent Technologies, USA). RNA of proven quality was immediately used for cDNA synthesis or stored at −70 °C until use.

### 2.4. Microarrays

For the genome-wide miRNA expression profiling, total RNA from 13 PCa tissues was available for microarray hybridization following manufacturer's protocol for miRNA labeling and hybridization v2.4 (Agilent Technologies). Briefly, 100 ng of total RNA was used for labeling with cyanine3-pCp using miRNA Complete Labeling and Hyb Kit together with MicroRNA Spike-In Kit (both from Agilent Technologies). Cy3-labeled samples were dried for up to 3 h in the vacuum concentrator and then resuspended in nuclease-free water. Samples were hybridized onto Human miRNA 8 × 60 K format microarrays (Agilent Technologies) based on miRBase Release 16.0 at 55 °C for 20 h. After hybridization microarrays were washed with Gene Expression Wash Buffer Kit and scanned using SureScan microarray scanner (Agilent Technologies). Quantitative data values were obtained from the images using Feature Extraction software v10.7 with default parameters (Agilent Technologies).

For data analysis, GeneSpring software v13.0 was used (Agilent Technologies). All datasets were normalized using the same procedure. Saturated, non-uniform and outlier probe signals were removed and the rest of the data were $\log_2$ transformed and percentile normalized. Probe annotations were automatically uploaded from the eArray platform (Agilent Technologies). For the comparison of two groups, a fold change (FC) value was calculated and the unpaired t-test was applied. A P value of <0.050 was considered significant.

### 2.5. TaqMan Low Density Arrays

Expression levels of selected miRNAs were evaluated by means of custom-designed TaqMan Low Density Arrays (TLDA; Life Technologies, USA). TaqMan MicroRNA Reverse Transcription Kit (Life Technologies) was used for reverse transcription of purified RNA into cDNA as per manufacturer's protocol for cDNA synthesis without preamplification step. Final volume of reverse transcription (RT) reaction for custom designed TLDAs was performed in 15 μL volume and contained 350 ng of input RNA. Real-time PCR (qPCR) was carried out on ViiA™ 7 Real Time PCR System (Life Technologies). Cycle threshold values (Cq) were determined by using ViiA™ 7 Software v1.2.1.

Selected miRNAs were run in triplicates and required at least one valid well (amplification curve reaching the automatically set threshold) for data inclusion in further analyses. For relative quantification, global normalization was applied (the arithmetic Cq average of all the miRNAs subtracted from the certain miRNA); values were converted to relative quantities and $\log_2$-normalized.

### 2.6. Quantitative reverse transcription PCR (RT-qPCR)

MiRNA investigation in urine specimens was achieved using TaqMan Human MiRNA Assays (Life Technologies) by means of quantitative reverse transcription PCR (RT-qPCR). Urine-derived RNA was reverse transcribed for the evaluation of expression levels of mature miR-19a, -19b, and -21 (assay numbers 000395, 000396, and 000397, respectively), using TaqMan MicroRNA Reverse Transcription Kit (all from Life Technologies). Selected miRNAs for targeted RT-qPCR were run in triplicates. According to the manufacturer's protocol, RT reaction contained specific stem-loop primers and 5 μL of total urine-derived RNA, reaching the final reaction volume of 15 μL. TaqMan Universal PCR Master Mix, TaqMan probes, sequence specific primers and 1.33 μL of RT product composed qPCR reactions, which were performed on ViiA™ 7 Real Time

PCR System. ViiA™ 7 Software v1.2.1 was used to determine Cq values. For data normalization, spike-in control (cel-miR-39, assay number 000200, Life Technologies) was used as RNA purification and PCR efficiency control, and all the values were scaled to this miRNA standard before global normalization was performed.

## 2.7. Statistical analysis

A relative quantification method was used to evaluate expression changes of miRNAs (Genex v.6.0.1, MultiD Analyses AB, Sweden). The expression of miRNAs analyzed in urine was dichotomized into relatively high and low expression after the median value. According to the expression level of the *TMPRSS2-ERG* fusion gene, tumors were divided into the fusion gene-positive and -negative. BCR-free survival and receiver operating characteristic (ROC) analyses were done, and Kaplan–Meier survival and ROC curves, respectively, were generated using GraphPad Prism (demo v.). STATISTICA v.8.0 (StatSoft, USA) was used to perform logistic regression analysis for biomarker combinations.

## 3. Results

### 3.1. MiRNA expression profiling in PCa tissue

The presence of 547 of 1347 (40.6%) miRNAs was identified in at least one of the analyzed PCa samples ($N = 13$), while expression level of 800 miRNAs (59.4%) was below the detection limit in all PCa samples. Of these 547 expressed miRNAs, 450 (82.3%) were identified at detectable levels in ≥30% of the samples (Fig. 1), and these miRNAs were included in further analysis.

A quarter of miRNAs (26.2%; 118 of 450) was detectable in all PCa specimens and showed relatively high hybridization signal values. The list of highly expressed miRNAs included miR-19a, miR-19b, miR-21, and other well-known cancer-related miRNAs, like miR-15a, miR-15b, miR-30a, miR-30b, miR-34a, miR-17, miR-20a, miR-22. A complete list of highly expressed miRNAs is provided in Table S1. The large group of

miRNAs (53.1%; 239 of 450), including miR-95, miR-206, miR-623, miR-885-3p, miR-1180, miR-3692*, and others, showed relatively weak expression levels in PCa (Table S1). However, the most significant associations with clinical variables of PCa were identified in this subgroup of weakly expressed miRNAs.

Deregulated expression (FC > 1.5, $P < 0.050$) of 26 miRNAs, including miR-1180, miR-3692*, miR-623, miR-206, and miR-885-3p, was observed in BCR-positive PCa samples as compared to BCR-free cases (Tables 2 and S2), while miR-95 showed a borderline significance ($P = 0.054$). Expression of 46 miRNAs differed between pT3 and pT2 tumors, including up-regulation of above-mentioned miR-206, miR-3692*, miR-885-3p, and miR-95 (FC ≥ 13.5, $P < 0.050$) in tumors of higher stage, while expression change of miR-451 (FC = 1.8, $P = 0.016$) was the most significant among the down-regulated miRNAs. Comparison of Gleason score 7 and 6 tumors revealed significant differences in expression of 21 miRNAs, of which 12 were up- and 9 down-regulated in Gleason score 7 tumors (Table S2).

Based on significant differences and correlation to clinical–pathological characteristics, 6 miRNAs (Table 2) were chosen for further analysis in cancerous and noncancerous prostate tissues on custom-designed qPCR-based array (TLDA), while highly expressed oncogenic miRNAs, miR-19a, miR-19b, and miR-21, were examined as potential diagnostic biomarkers in urine samples from PCa and BPH patients.

### 3.2. MiRNA expression analysis in prostate tissue

Expression profile of 6 selected miRNAs (miR-95, -206, -623, -885-3p, -1180, and -3692*) was further analyzed in cancerous ($N = 52$; including 13 PCa specimens from microarray analysis) and noncancerous ($N = 12$) tissues from PCa patients. Real-time PCR-based study revealed weak but detectable expression levels of miR-206, -885-3p, and -3692*, while miR-95, -623, and -1180 showed more robust expression in prostate tissues.

Expression levels of selected miRNAs were compared between PCa and NPT, and between PCa tumor subgroups divided according to clinical–pathological variables (pT, Gleason score, BCR status, and *TMPRSS2-ERG* positivity). Higher level of miR-95 was detected in PCa than in NPT, but
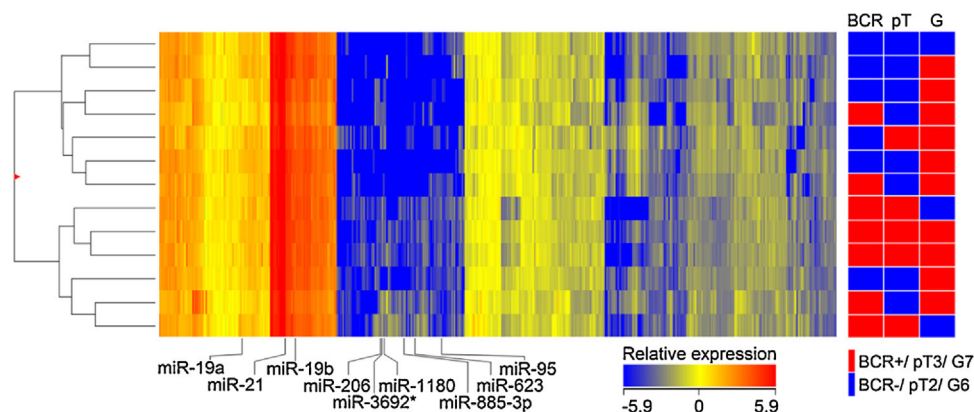


Fig. 1 – MiRNA expression profile in PCa tissue samples. Only miRNAs, expressed at detectable levels in ≥30% of samples, were included. Those miRNAs that were selected for further analyses are depicted below the heat map. Clinical–pathological patients' characteristics are given on the right. BCR+/−, biochemical disease recurrence status (positive/negative); pT, pathological tumor stage; G, Gleason score.

| Systematic name | BCR+ vs. BCR− | | pT3 vs. pT2 | | G7 vs. G6 | |
|---|---|---|---|---|---|---|
| | P value | FC | P value | FC | P value | FC |
| *Tissue analysis* | | | | | | |
| hsa-miR-1180 | **0.009** | 15.1 | 0.102 | 6.8 | 0.851 | 1.3 |
| hsa-miR-206 | **0.021** | 15.7 | **0.012** | 20.4 | 0.693 | −1.9 |
| hsa-miR-3692* | **0.004** | 40.8 | **0.006** | 39.8 | 0.469 | −3.7 |
| hsa-miR-623 | **0.009** | 13.2 | 0.174 | 4.6 | 0.535 | −2.3 |
| hsa-miR-885-3p | **0.006** | 17.6 | **0.020** | 13.4 | 0.217 | −5.7 |
| hsa-miR-95 | 0.054 | 10.6 | **0.033** | 14.0 | 0.385 | −3.9 |
| *Urine analysis* | | | | | | |
| hsa-miR-19a | 0.473 | −1.15 | 0.352 | 1.21 | 0.119 | 1.4 |
| hsa-miR-19b | 0.495 | −1.14 | 0.846 | 1.03 | 0.178 | 1.3 |
| hsa-miR-21 | 0.418 | 1.15 | **0.004** | 1.55 | 0.926 | 1.0 |

**Table 2 – Deregulated miRNAs selected for analyses in prostate tissue or urine.**

BCR+/−, biochemical disease recurrence status (positive/negative); pT, pathological tumor stage; G, Gleason score; FC, fold change. Statistically significant P values are highlighted in bold.

the difference was not statistically significant (Fig. 2A). Meanwhile, in PCa, the over-expression of miR-95 was observed in more aggressive forms of cancer: in PCa of higher Gleason score ($P = 0.041$) and positive for the oncogenic *TMPRSS2-ERG* transcript ($P = 0.026$). Moreover, a borderline difference ($P = 0.054$) in this miRNA levels was identified between the cases with and without BCR. No significant associations were revealed for other miRNAs, except of 2.5-fold higher expression of miR-206 in *TMPRSS2-ERG*-positive cases ($P = 0.049$).

### 3.3. MiRNA analysis in urine samples

Attempts to assess miR-95 in urine of PCa and BPH patients demonstrated low to undetectable levels of this urinary miRNA. Thus, for the investigation in urine highly expressed cancer-related miRNAs were selected, namely miR-19a, miR-19b, and miR-21.

Comparison of miRNA levels in urine from PCa ($N = 143$) and BPH ($N = 23$) cases, revealed 1.4-fold lower ($P = 0.010$) levels of miR-21 in specimens from PCa patients (Fig. 2B), while the levels of miR-19a and miR-19b differed insignificantly. No significant associations were identified between the levels of urinary miRNAs and clinical variables, like pathological stage and Gleason score, or the fusion gene status.

The prognostic value of urine circulating miRNA as biomarkers for BCR-free survival prediction was evaluated by generating Kaplan–Meier survival curves. BCR-free survival was best predicted by the level of miR-19b ($P = 0.014$, Fig. 3A). Moreover, combined detection of both miR-19a and miR-19b in urine of PCa patients was similarly prognostic for BCR ($P = 0.016$, Fig. 3B).

The sensitivity and specificity of urine-circulating miRNAs as possible biomarkers of PCa were tested by ROC analysis (Fig. 3C–F). Good diagnostic value was reached by measurement of urine amount of miR-21 (AUC = 0.633, $P = 0.041$; Fig. 3D). The specificity of this urinary miRNA was higher than that of the PSA test (76.22% vs. 63.57%), but sensitivity remained quite similar to that of PSA (47.83% vs. 52.38%). Combined analysis of all tested miRNAs was even more promising. The panel of the three miRNAs demonstrated high diagnostic power (AUC = 0.738, $P < 0.001$; Fig. 3E) that markedly outperformed the same value of the PSA test (AUC = 0.514; Fig. 3C). Moreover, the panel of urinary miRNAs showed high specificity to PCa (82.61%). Improved sensitivity (76.19%), but not specificity (65.71%) was achieved assessing the diagnostic
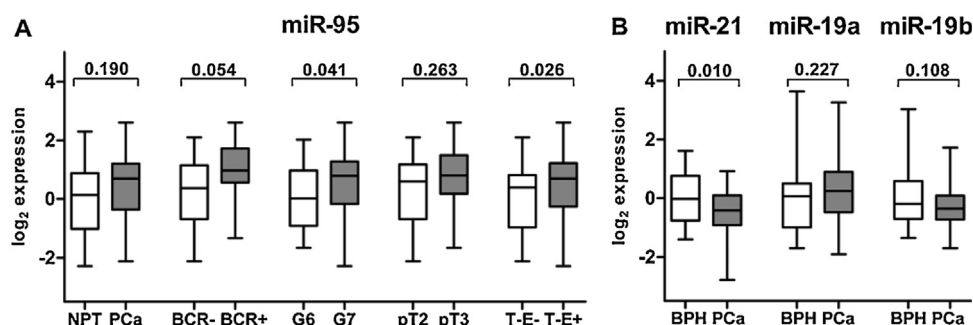


**Fig. 2 – MiRNA analysis in tissue (A) and urine (B) of PCa patients and controls. MiR-95 expression differences between cancerous (PCa) and noncancerous prostate tissue (NPT), and between tumors grouped according to clinical–pathological characteristics (A); relative miRNA levels in urine of prostate cancer (PCa) and benign prostatic hyperplasia (BPH) patients (B). Lines within boxes indicate median values; whiskers – min and max for miRNA values; miRNA expression is presented in $\log_2$-scale. BCR+/−, biochemical disease recurrence status (positive/negative); G, Gleason score; pT, pathological tumor stage; T-E+/−, TMPRSS2-ERG transcript status in PCas (positive/negative).**
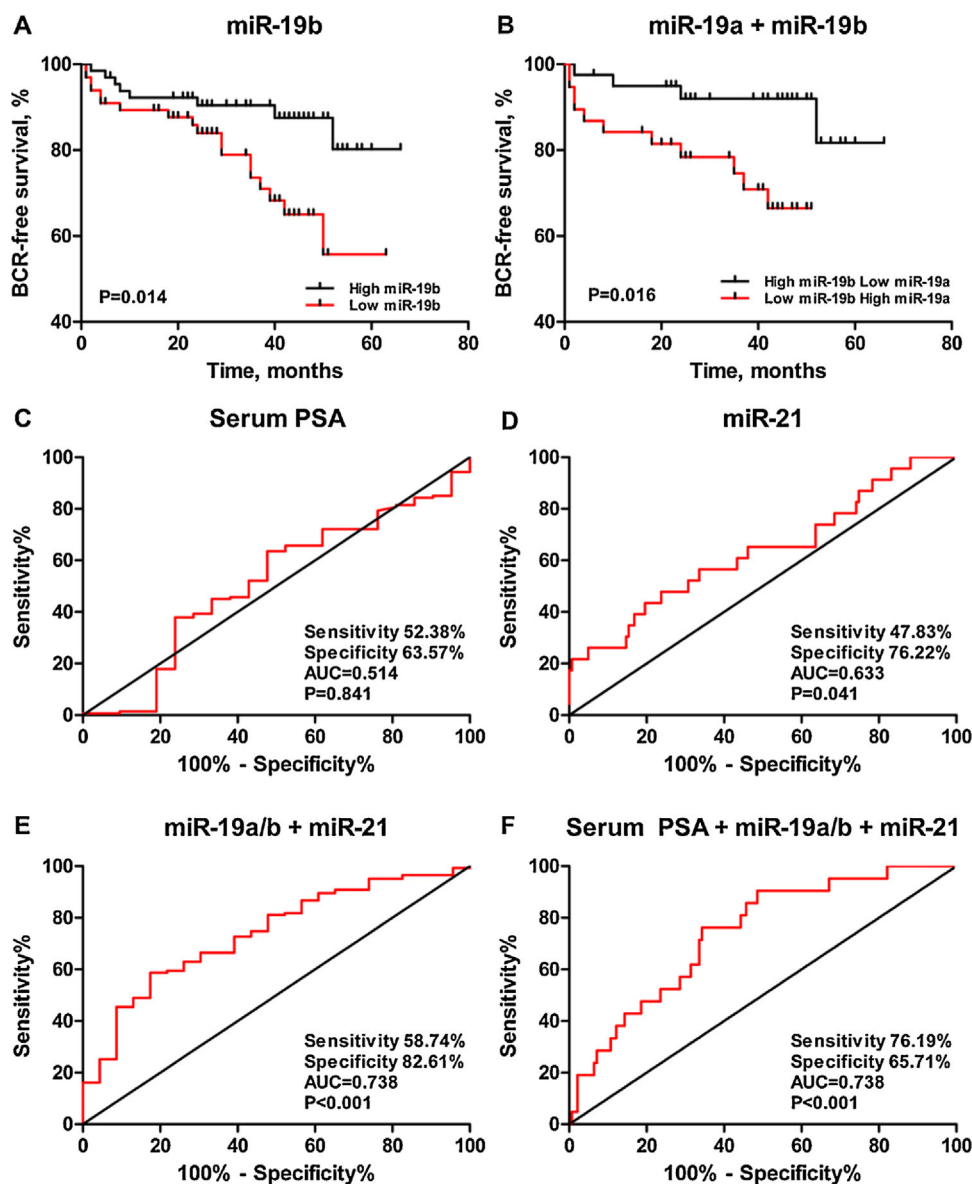
Fig. 3 – Prognostic and diagnostic value of urinary miRNAs. Kaplan–Meier curves show BCR-free survival predicted by miR-19b (A) and combined analysis of miR-19a/b (B). Higher level of miR-19b is shown in black (upper curve), lower – in red (lower curve) in both graphs. Receiver operating characteristic (ROC) curves show sensitivity and specificity of serum PSA (C), urine circulating miR-21 (D), combination of miR-19a/b and miR-21 (E), and all urinary miRNAs combined with PSA (F). AUC, area under the ROC curve.

value of combined testing of the urinary miRNA panel and the PSA test (Fig. 3F).

## 4. Discussion

In the present study, genome-wide and targeted approaches were used for the assessment of miRNA profiles in prostate tissues and urine from PCa patients. In tissue analysis, miR-95 was validated as onco-miR, as its expression was increased in more aggressive tumors. In urine analysis, lower levels of miR-19b alone or combined with higher levels of miR-19a were associated with worse BCR-free survival rates. Importantly,

the diagnostic potential of urinary miR-21 alone or in combination with miR-19a and miR-19b exceeded the diagnostic value of the PSA test.

In our study, miR-95 was selected from the microarray analysis as a miRNA showing associations with the disease aggressiveness. Validation study confirmed this notion: expression level of miR-95 was significantly increased in poorly differentiated cases according to Gleason score, tended to be up-regulated in BCR-positive cases, was more pronounced in pT3 than pT2 tumors, and also over-produced in *TMPRSS2-ERG*-positive cases. Similarly, up-regulated expression of miR-95 has been shown in PCa by other authors [19] and connected with the tumor aggressiveness and resistance to

ionizing radiation through sphingosine-1-phosphate phosphatase 1 (SGPP1), a novel target of miR-95. In addition, the same group [19] demonstrated that according to The Cancer Genome Atlas (TCGA), the expression of miR-95 was significantly elevated in primary PCa in comparison with healthy controls. It is worth to accent that our study for the first time revealed miR-95 up-regulation in the *TMPRSS2-ERG* fusion-positive prostate tumors. *TMPRSS2-ERG* fusion is the most common chromosome rearrangement in PCa, occurring approximately in 50% of cases. This aberration is PCa-specific and marks a distinct pathway of prostate oncogenesis [20] that also involves deregulated expression of multiple transcripts, including miRNAs [21].

Though showing significant associations with clinical variables of aggressive form of PCa, miR-95 was only moderately expressed in prostate tissues and almost undetectable in urine from PCa cases. Therefore, well-known oncogenic miRNAs showing high expression in PCa tissue were selected for identification of urinary biomarkers. In our study, urine-circulating miR-21 was found in significantly lower levels in PCa patients vs. those diagnosed with BPH. Moreover, this difference was proved to have a clinical value by ROC analysis. Similarly, Sapre and colleagues [22] found it at lower levels in urine of high-risk PCa compared to low-risk cases in the validation cohort. In PCa tissues, elevated levels of miR-21 have been detected [23], and showed significant associations with PCa progression [24]. Higher levels of serum miR-21 were found in both androgen-dependent PCa (ADPC) and hormone-refractory PCa (HRPC) patients with low PSA levels [25]. Moreover, some studies revealed associations between blood miR-21 levels and aggressive course of PCa [26,27]. The difference of miR-21 levels in urine and blood can be explained by specific miRNA regulation in separate body compartments and selective secretion of this miRNA into body fluids. MiR-21 plays a crucial role in prostate carcinogenesis by promoting cell proliferation, inhibiting apoptosis, enhancing tumor invasion and metastasis [28], therefore can be considered as a PCa-specific biomarker accessible by lowly invasive ways in urine or blood.

MiR-19 family is a key oncogenic component of the miR-17∼92 cluster located on chromosome 13 [29]. Though both miR-19a and miR-19b were highly expressed in PCa tissues in our microarray-based screening, these miRNAs showed an opposite abundance in urine of PCa and BPH patients: miR-19a was predominant in urine from PCa patients, while miR-19b in urine of BPH cases. Mature sequences of miR-19a and miR-19b differ by a single nucleotide aside the seed region. Due to this minor difference both miRNAs can recognize different targets. It was shown [30] that miR-19b but not miR-19a directly targets *PTEN* and *TP53*, and consequently reduces levels of its downstream components like Bax and p21 [31]. Besides, miR-19a is regulated by AR [32], whereas miR-19b has two homologues, located on chromosome 13 (cluster miR-17∼92) and on chromosome X (cluster miR-106b∼363), and shows more complex regulation. In general, miR-19 family is reported to be over-expressed in PCa comparing to normal prostate tissue [33,34]. Moreover, the amounts of circulating miR-19a/b were found and validated to be markedly decreased in serum of PCa patients in compare to controls and were declared as an independent prognostic markers for high risk PCa [35]. Our

study for the first time reports the difference in amounts of miR-19a and miR-19b in urine of cases with PCa and BPH, and shows diagnostic and prognostic value of these urinary miRNAs. In our study, lower levels of urinary miR-19b showed increased risk of BCR, and the prognostic potential of this urinary miRNA was similar in complementary analysis with urinary miR-19a. Moreover, the combination of urinary miR-19a/b and miR-21 was highly specific for PCa and showed diagnostic power higher than the PSA test.

With a similar approach, after pre-screening in cancerous and non-cancerous prostate tissues, urinary miRNAs showing diagnostic potential for PCa identification have been identified in several previous studies [36,37]. Four out of eight miRNAs selected by PCR-based array were validated by RT-qPCR in prostate tissue in the study of Srivastava et al. [36], and two of these miRNAs (miR-205 and miR-214) possessed a potential to discriminate PCa cases from controls. In another study [37], after microarray-based screening, two miRNAs (miR-182 and miR-187) were selected for further validation in PCa tissue and urine, and urinary miR-187 together with PSA and PCA3 showed capability to predict PCa in diagnostic biopsies. In our study, microarray-based screening identified multiple miRNAs showing diverse expression in correlation with clinical–pathological features of PCa, but only few associations were validated by means of RT-qPCR. Moreover, a set of miRNAs with deregulated expression in microarrays was hardly detectable with RT-qPCR. Real time-based methods are the main approach to confirm gene expression changes identified on microarrays, however, multiple disagreements between these two methods exist, and the causes are widely discussed in other publications [38,39]. In our study, real time-based TLDA plates were used for miRNA expression analysis in tissues. This method is known as a highly sensitive and reliable tool for gene expression analysis [40], allowing analysis of multiple samples at once with the avoidance of pipetting errors and discrepancies between plates. With this approach we were able to confirm the findings of microarray-based study for miR-95, but not for other miRNAs.

Besides, in our study, miRNAs well expressed in PCa tissue (miR-21, miR-19b, miR-19b) were not uniformly well detectable in urine of PCa patients. Moreover, the levels of miR-21 and miR-19b were higher in urine from BPH patients. Similar discrepancies between the abundance of miRNAs in tissues and body fluids have been reported in other studies as well [36,41]. Moreover, differences in miRNA levels between various body fluids were also reported [42] and the variation was seen between samples of different cohorts [22]. Urine by itself is quite a variable material, reflecting possible urinary tract diseases and depending on hydration status. Spike-in control is usually used in urinary miRNA studies to normalize the amount of input miRNA and to control the variation in RT-qPCR efficacy. In addition to this, urine from BPH cases was used as the control in our study, which enabled us to control for the age-related deregulation in miRNA profile. To avoid the variation caused by urine collection, all biosamples from cancer and BPH patients were collected by catheterization during prostatectomy. However, due to the rare prostatectomies in BPH cases, our control group remained quite small.

Despite the limitations, our study enabled identification of several miRNAs suitable for noninvasive diagnostics of PCa

using urine as a source of cancer-derived transcripts. Moreover, the panel of three urinary miRNAs outperformed serum-based diagnostic PSA test and showed prognostic value by predicting the risk of BCR.

## 5. Conclusions

In summary, miRNA expression profile markedly differs between PCa groups stratified according to clinical variables. Some miRNAs highly expressed in PCa tissues can also be detected in urine. The panel of urinary miR-21, miR-19b, and miR-19a shows a potential to serve as a tool for non-invasive diagnostics of PCa and prediction of the disease relapse. Although this panel of urinary miRNA could improve the overall diagnostic value of the PSA test, the specificity of such combined test remains quite low.

## Authors' contributions

K.S. performed the TLDA and RT-qPCR experiments and statistical analysis, drafted the manuscript. K.D. performed the microarray experiments and conducted bioinformatics analysis. F.J. provided the samples and clinical data, critically revised the manuscript. S.J. conceived and designed the study and drafted the manuscript. All authors read and approved the final manuscript.

## Conflict of interest

The authors state no conflict of interest.

## Acknowledgements

## Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.medici.2016.02.007.

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