Investigating differential miRNA expression profiling using serum and urine specimens for detecting potential biomarkers for early prostate cancer diagnosis

Sevde HASANOĞLU1*, Beyza GÖNCÜ1, Emrah YÜCESAN2, Sezen ATASOY3, Yunus KAYALI4, Nur ÖZTEN KANDAŞ5
1Experimental Research Center, Bezmialem Vakıf University, İstanbul, Turkey
2Department of Medical Biology, Faculty of Medicine, Bezmialem Vakıf University, İstanbul, Turkey
3Department of Biochemistry, Faculty of Pharmacy, Bezmialem Vakıf University, İstanbul, Turkey
4Department of Urology, Faculty of Medicine, Bezmialem Vakıf University, İstanbul, Turkey
5Department of Pharmaceutical Toxicology, Faculty of Pharmacy, Bezmialem Vakıf University, İstanbul, Turkey

Background/aim: MicroRNAs (miRNAs) are known up-to-date candidate biomarkers for several diseases. In addition, obtaining miRNA from different body fluids such as serum, plasma, saliva, and urine is relatively easy to handle. Herein we aimed to detect miRNAs as biomarkers for early stage prostate cancer (PC). For this purpose, we used urine and serum samples to detect any significant differences in miRNA profiles between patients and healthy controls.

Materials and methods: Total ribonucleic acid (RNA) in urine and serum samples were isolated from eight untreated PC patients, thirty healthy individuals were screened for miRNA profile, and candidate miRNAs were validated. Whole urinary and serum miRNA profile was analyzed using Affymetrix GeneChip miRNA 4.0 Arrays. Candidate miRNAs were investigated by stem-loop reverse transcription-polymerase chain reaction.

Results: When we analyzed the urinary samples of PC patients, 49 miRNAs were detected to be upregulated and 14 miRNAs were found to be downregulated when compared with healthy controls. According to the serum samples, 19 miRNAs were found to be upregulated, and 21 miRNAs were found to be downregulated when compared with healthy individuals as well. Interestingly, we detected only four overlapping miRNAs (MIR320A, MIR4535, MIR4706, MIR6750) that commonly increase or decrease in both serum and urine samples. Among them, MIR320A was found to be downregulated, and MIR4535, MIR4706, and MIR6750 were found to be upregulated for urine samples. However, only MIR6750 was upregulated and the other three miRNAs were downregulated for serum samples.

Conclusion: Notably, the expression profile of MIR320A was significantly altered in urine specimens of prostate cancer patients. We considered that MIR320A has been evaluated as a valuable biomarker that can be used in the early diagnosis of PC.

Key words: Prostate cancer, biomarker, microarray, miRNA profiling

1. Introduction
Prostate cancer (PC) is the second common cancer in developed countries, and also the most frequent cause of cancer-related mortality in men after lung cancer [1]. The incidence and prevalence of PC varies between different geographical regions, e.g., the most common in North America and less common in South Asia [2].

The main diagnostic criteria in PC are digital rectal examination (DRE), serum prostate-specific antigen (PSA) level, and transrectal ultrasound-guided biopsy, respectively. The PSA is exclusively expressed in prostate tissue, yet it is not specific for diagnosis [3]. Increased levels may be associated with numerous clinical conditions such as benign prostate hyperplasia (BPH), prostatitis, other infections, trauma, and urinary retention. [4,5]. Currently, the measurement of PSA is the most common PC indicator [6,7]. However, PC was detected in only 25% of individuals who underwent prostate biopsy based on elevated PSA level (>4.0 ng/mL) [4,5]. In addition, the PSA test has some limitations [4] causing a low positive predictive value (approximately 25%–40%) [4,8]. The role of PSA in prostate cancer has become increasingly controversial [9]. False-negative results and low specificity have limited the application of the clinical use as a diagnostic parameter.

miRNAs are uncoded RNAs that transcribe gene expression with the average length from 19 to 25 nucleotides [10,11]. Each miRNA binds to the 3’ untranslated region (UTR) of its target mRNA [12], and inhibits the gene

* Correspondence: shasanoglu@bezmialem.edu.tr
expression of multiple targets by binding to more than one region of mRNAs [13]. Recent studies have shown that miRNAs play important roles in many critical biological processes, including development, proliferation, differentiation, apoptosis, tumor formation, signal transduction, organ development, and hematopoietic lineage differentiation [14]. In the literature, there are several studies evaluating different miRNAs as biomarker for early diagnosis [15–18]. Although PSA levels are principally used for diagnosis in PC, despite some controversial cases, additional biomarkers are required to make a definite diagnosis. For this purpose, the present study demonstrated PC-related miRNAs such as MIR141, MIR21, MIR200B, MIR221, and MIR375. Particularly, MIR375, identified as a urine-associated miRNA, is directly related to stage two PC [19]. Importantly, a recent metaanalysis demonstrated 10 upregulated and 14 downregulated miRNAs that may discriminate PC from BPH/normal control tissues [20].

miRNAs have been suggested as a novel biomarker in cancer due to their presence in different body fluids such as serum, plasma, saliva, and urine [21]. They may also be used in the early diagnosis of PC [22]. As urine is one of the most easily accessible biofluids in primary care clinics, a urine-based new biomarker test may help to clarify PC diagnosis. Studies show that miRNAs are a more sensitive marker than PSA level for PC [19]. Furthermore, serum and urinary miRNA expressions, together, may define the diagnosis criteria for PC patients.

Understanding which miRNAs are present in urine and serum samples when comparing controls and patients may offer a novel insight into the early diagnosis of PC. The aim of this study is to determine a possible biomarker of miRNAs in serum and urine samples of PC patients using microarray expression analysis. Selected candidate miRNAs were investigated and validated in detail for each sample by stem-loop qRT-PCR.

2. Materials and methods

2.1. Ethical approval

This study was conducted after receiving approval from the Local Human Ethics Committee (approval number: 16926-22/30) from Bezmialem Vakif University. All protocols conformed to the ethical guidelines of the Helsinki Declaration and written informed consent was obtained from all subjects.

2.2. Donor recruitment

Eight patients with newly diagnosed untreated prostate cancer (mean age, 62 years; range, 49–72 years) who were referred from the Department of Urology were enrolled in this study. The clinical findings of the patients are listed in Table 1. Thirty healthy individuals (mean age, 35 years; range, 20–73 years) who did not have either suspected cancer or any metabolic diseases were used as a control group. Urine samples were collected and used immediately to maintain freshness for further experiments. Blood samples were collected using EDTA-coated tubes (Vacuette, Grenier Bio-One, Austria). Serum samples were separated by centrifugation at 1100 g for 10 min and stored at −80 °C until use.

For total RNA isolation from urine samples, freshly collected 50 mL of urine samples were centrifuged at 1210 × g for 20 min. The pellet was dissolved in 5 mL of 1X PBS (Phosphate Buffer Solution-Thermo Fisher Scientific, MA, USA) (pH 7.4) and continued through lysis phase. Total RNA isolation was performed according to the manufacturer’s protocol using the commercial kit (Urine Total RNA Purification Maxi Kit, Norgen, Ontario, Canada).

2.3. Total RNA isolation from serum samples

Serum samples were incubated on ice for a complete thaw process. Immediately, triazole reagent (1:1 ratio) (Thermo Fisher Scientific, MA, USA) and nuclease-free glycogen (1 μg/mL) (Thermo Fisher Scientific, MA, USA) were added, respectively. They were then incubated at room temperature for 10 min. Afterward, 200 μL of chloroform (Merck Millipore, Darmstadt, Germany) was added and the samples were incubated for 15 min. Next, they were centrifuged at 12,000 × g for 15 min at 4°C. Upper aqueous phase was transferred, and 1.2 mL of isopropanol (Merck Millipore, Darmstadt, Germany) was added to the samples. After that, the samples were vortexed and then incubated for 10 min at room temperature. They were centrifuged at 12,000 × g for 8 min. The supernatants were aspirated carefully. One milliliter of 75% ethanol was added and the samples werecentrifuged at 7500 × g for 5 min at room temperature. The supernatant was carefully removed, and the tubes were dried out at room temperature. Each

| Patient | Age | Serum PSA level (ng/mL) | Gleason score |
|---------|-----|------------------------|---------------|
| #1      | 52  | 40                     | 6 (3+3)       |
| #2      | 49  | 6.39                   | 6 (3+3)       |
| #3      | 60  | 36                     | 9 (5+4)       |
| #4      | 67  | 7.6                    | 7 (4+3)       |
| #5      | 72  | 3.3                    | 6 (3+3)       |
| #6      | 65  | 22.3                   | 7 (3+4)       |
| #7      | 70  | 15.11                  | 9 (4+5)       |
| #8      | 61  | 30                     | 7 (4+3)       |

Table 1. Gleason grading system and PSA levels for patients who have untreated prostate cancer (Gleason score; >7: high grade, 7: intermediate grade, <7: low grade). PSA: prostate-specific antigen.
pellet was resuspended with 20 μL of nuclease-free water (AppliChem, Gatersleben, Germany) and stored at –80 °C for further evaluation.

2.4. microRNA expression array
Stored RNA samples were incubated on ice for a complete thaw process. The quantitative analysis of RNA was conducted by measuring optical density at 260 nm and 280 nm using a Multiskan GO (Thermo Fisher Scientific MA, USA) for microarray expression analysis. Between 100 and 300 ng of total RNA samples from the urine and serum control and patient groups were pooled. GeneChip® miRNA 4.0 Array-Affymetrix (Affymetrix, Santa Clara, California, US, catalog number 902411) microarray analysis was performed to identify miRNAs that were significantly differentially expressed. All microarray experiments were performed at Ayka Laboratories (Ayka Medikal, Ankara, Turkey). Identification of differentially expressed miRNAs at each stage was achieved by using linear regression and hierarchical clustering analysis with the Transcriptome Analysis Console software from Affymetrix. The filter settings were as follows: "Transcription Cluster ID": contains hsa-miR does not contain mmu-miR, "Fold change": +5 and –5, "False discovered rate (FDR)"; >2.0 or <0.5 "Adjusted P-value threshold": 0.05.

2.5. Stem-loop RT-PCR
A total of 76 samples’ (eight patients and 30 control samples of urine and serum) reverse transcription of 500 ng total RNA was performed using miScript II RT Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The obtained cDNAs were stored at –20 °C for further evaluation. Real-time PCR for miRNAs was performed on a CFX-connect Real-Time PCR Instrument (Biorad, CA, USA) using miScript SYBR Green PCR Kit and miScript Primer Assay (cat no: MS00014707 and MS00046543) both from Qiagen (Hilden, Germany) according to the manufacturer's instructions. As an endogenous reference control, RNU6-2 was used [23]. The primers were as follows: hsa-miR-6750-5p: 5’CAGGGAACACGUGGGUGACGCU, hsa-miR-320a: 5’AAAAGGUGGUUGAGAGGGGGA. Relative miRNA expression levels were calculated using the 2ΔΔCT method.

2.6. Statistical analysis
The false discovery rate (FDR) was set to <0.05 and the minimum fold change (FC) was set to >2.0 or <0.5 for microarray analysis. For the RT-PCR data, statistical analysis was performed using Graph Pad (Prism 7.0, GraphPad Software, Inc., CA, USA). The data were compared using the Mann–Whitney U test and Sidak's multiple comparisons test. A p-value of <0.05 was considered to be statistically significant.

3. Results
The yield of total RNA from serum and urine specimens was determined, and it showed significant differences among samples of the patients and the healthy control groups. The overall medians of urine and serum samples were 46.5 ng/μL and 483.9 ng/μL, respectively.

To identify miRNAs differentially upregulated and/or downregulated in PC patients, miRNA microarrays were used to determine the total expression profile. Patients' and healthy controls’ urine and serum samples were pooled into four independent groups (group 1: patients with serum sample, group 2: patients with urine sample, group 3: healthy controls with serum sample, group 4: healthy controls with urine sample) and were profiled on Affymetrix GeneChip miRNA 4.0 Arrays. Affymetrix GeneChip miRNA 4.0 Array covers of all the 2.578 mature human miRNAs available in miRBase version 20 (June 24, 2013) were used to profile miRNA expressions [24].

The miRNA QC tool was used to assess the quality of the array data and the principal component analysis (PCA) mapping shown in Figure 1A. Afterwards, the data were normalized by global normalization with robust multichip average (RMA) and detection above background (DABG) using the Expression Console software (Affymetrix, Santa Clara, CA, USA) (Figure 1B). The differentially expressed miRNAs between patient and control groups of urine and serum samples are shown in the form of a scatter plot in Figures 2A and 2B. In order to better characterize the differential miRNAs to distinguish between patient and healthy control samples, the miRNAs were further used to heat map clustering analysis and urine and serum sample clusters shown in Figures 2C and 2D, respectively. The mature human miRNAs represented on the microarray showed that 103 (3.99%) miRNAs were expressed totally in all groups (patient and healthy control groups from urine and serum samples). There were 49 (1.9%) and 14 (0.54%) up- and downregulated urine miRNAs, respectively, when the patient and healthy control groups were compared (Table 2). There were 19 (0.73 21 (0.81%) up- and downregulated serum miRNAs (Table 2), when the patient and healthy control groups were compared.

Overlapping miRNAs in all groups of urine and serum samples are summarized in Table 2. Two of the four miRNAs demonstrated significant correlations between the urine and serum samples. In this study, the MIR4532 (Gene ID:100616353) and MIR4706 (Gene ID:100616490) were upregulated in urine and downregulated in serum samples. The MIR320A (Gene ID:407037) was downregulated in both urine and serum samples (fold change -4.2 and -2.05 respectively); in addition, MIR6750 (Gene ID:102466192) was upregulated in both urine and serum samples (fold change +2.23 and +2.37, respectively). Moreover, to check the accuracy of the microarray miRNA quantification, the
two miRNAs (MIR320A and MIR6750) were reexamined by using qRT-PCR in 76 samples (38 urine and 38 serum samples from eight patients and 30 healthy controls). The annealing temperatures were evaluated before qRT-PCR reactions and the results were 56 °C for the reference gene (RNU6-2), 58 °C for MIR320A, and 55 °C for MIR6750.

The selected candidate miRNAs in urine and serum samples of eight patients were compared. The MIR320A expression levels of the urine and serum samples of the healthy controls were higher in serum samples (p = 0.0023), and a similar result was observed in patient samples (p = 0.0026) (Figures 3A and 3B, respectively). In addition, the MIR320A expression levels between the patient and healthy control groups were compared, and higher levels were observed in patients (p = 0.0168) (Figure 3C). However, these levels were not found to be significant in serum samples (p > 0.05) (Figure 3D). The MIR6750 expression levels between urine and serum samples of
the healthy controls were not different (p > 0.05) (Figure 4A). The MIR6750 increased only in serum samples of the patients when compared to urine specimens (p = 0.0079) (Figure 4B). In addition, the comparisons of urine and serum samples between the control and patient groups were not found to be significant (p > 0.05) (Figures 4C and 4D, respectively).
Table 2. Transcript ID and fold change of miRNAs in urine and serum specimens of patients and healthy groups. Overlapping miRNAs both for serum and urine specimens are marked as bold. –: downregulated, +: upregulated.

| miR ID     | Accession #   | Specimens     | Fold Change |
|------------|---------------|---------------|-------------|
| MIR4532    | MIMAT0019071  | Urine/Serum   | +3.99/−2.05 |
| MIR6750    | MIMAT0027400  | Urine/Serum   | +2.23/+2.37  |
| MIR4706    | MIMAT0019806  | Urine/Serum   | +2.66/−2.72  |
| MIR320A    | MIMAT000510   | Urine/Serum   | −4.2/−2.05  |
| MIR744     | MIMAT0004945  | Urine         | +6.21        |
| MIR6726    | MIMAT0027353  | Urine         | +5.49        |
| MIR6726    | MIMAT0027353  | Urine         | +5.34        |
| MIR6813    | MIMAT0027526  | Urine         | +4.33        |
| MIR3180−4  | MIMAT0018178  | Urine         | +4.24        |
| MIR4532    | MIMAT0019071  | Urine         | +4.21        |
| MIR6750    | MIMAT0027400  | Urine         | +2.23        |
| MIR4706    | MIMAT0019806  | Urine         | +2.66        |
| MIR3197    | MIMAT0015082  | Urine         | +2.66        |
| MIR4532    | MIMAT0019071  | Urine         | +4.39        |
| MIR4674    | M0017305      | Urine         | +3.57        |
| MIR4449    | MIMAT0018968  | Urine         | +3.52        |
| MIR4701    | MIMAT0019887  | Urine         | +2.66        |
| MIR6819    | MIMAT0027538  | Urine         | +2.84        |
| MIR6798    | MIMAT0027496  | Urine         | +2.74        |
| MIR4706    | MIMAT0019806  | Urine         | +2.66        |
| MIR3178    | MIMAT0015055  | Urine         | +2.6         |
| MIR6075    | MIMAT0023700  | Urine         | +2.57        |
| MIR3937    | MIMAT0018352  | Urine         | +2.47        |
| MIR1275    | MIMAT0005929  | Urine         | +2.42        |
| MIR423     | MIMAT0004748  | Urine         | +2.4         |
| MIR6787    | MIMAT0027474  | Urine         | +2.4         |
| MIR6845    | MIMAT0027590  | Urine         | +2.39        |
| MIR4649    | MIMAT0019711  | Urine         | +2.38        |
| MIR6782    | MIMAT0027464  | Urine         | +2.37        |
| MIR3648−1  | M0016048      | Urine         | +2.36        |
| MIR4674    | MIMAT0019756  | Urine         | +2.32        |
| MIR4758    | M0017399      | Urine         | +2.31        |
| MIR6808    | MIMAT0027516  | Urine         | +2.3         |
| MIR6090    | M0020367      | Urine         | +2.3         |
| MIR1909    | MIMAT0007883  | Urine         | +2.29        |
| MIR6848    | MIMAT0027596  | Urine         | +2.29        |
| MIR3781H   | MIMAT0018984  | Urine         | +2.27        |
| MIR6802    | MIMAT0027504  | Urine         | +2.24        |
| MIR6750    | MIMAT0027400  | Urine         | +2.23        |

miR ID | Accession # | Specimens | Fold Change |
MIR4466 | M0016817 | Urine | +2.23 |
MIR6510 | MIMAT0025476 | Urine | +2.21 |
MIR6778 | MIMAT0027456 | Urine | +2.2 |
MIR4750 | MIMAT0019887 | Urine | +2.16 |
MIR4673 | MIMAT0019755 | Urine | +2.15 |
MIR7150 | MIMAT0028211 | Urine | +2.14 |
MIR1224 | MIMAT0005458 | Urine | +2.13 |
MIR4467 | MIMAT0018994 | Urine | +2.09 |
MIR7110 | MIMAT0028117 | Urine | +2.09 |
MIR320E | M0014234 | Urine | +2.08 |
MIR4417 | MIMAT0018929 | Urine | +2.05 |
MIR4274 | MIMAT0016906 | Urine | +2.02 |
MIR4492 | MIMAT0019027 | Urine | +2.02 |
MIR4701 | MIMAT0019799 | Urine | −2.08 |
MIR6812 | MIMAT0027524 | Urine | −2.12 |
MIR6124 | MIMAT0024597 | Urine | −2.13 |
MIR3613 | MIMAT0017990 | Urine | −2.15 |
MIR4440 | MIMAT0018958 | Urine | −2.16 |
MIR575 | MIMAT0003240 | Urine | −2.28 |
MIR6840 | MIMAT0027583 | Urine | −2.31 |
MIR6716 | MIMAT0025845 | Urine | −2.52 |
MIR8071–1 | MIMAT0030998 | Urine | −2.76 |
MIR320C1 | MIMAT0005793 | Urine | −2.99 |
MIR320D1 | MIMAT0006764 | Urine | −3.19 |
MIR6790 | MIMAT0027480 | Urine | −3.58 |
MIR3188 | MIMAT0015070 | Urine | −3.63 |
MIR320A | MIMAT000510 | Urine | −4.2 |
MIR486−1 | MIMAT002177 | Serum | +11.02 |
MIR149 | MIMAT0004609 | Serum | +5.57 |
MIR1237 | MIMAT0022946 | Serum | +4.94 |
MIR4687 | MIMAT0019775 | Serum | +4.84 |
MIR4463 | MIMAT0018987 | Serum | +4.38 |
MIR4529 | MIMAT0019068 | Serum | +3.93 |
MIR4508 | MIMAT0019045 | Serum | +3.28 |
MIR4763 | MIMAT0019913 | Serum | +3.15 |
MIR7706 | M0025242 | Serum | +2.82 |
MIR6756 | MIMAT0027412 | Serum | +2.8 |
MIR6776 | M0022621 | Serum | +2.72 |
MIR6715A | MIMAT0025841 | Serum | +2.55 |
MIR6869 | MIMAT0027638 | Serum | +2.55 |
MIR3196 | MIMAT0015080 | Serum | +2.48 |
MIR409 | MIMAT0001639 | Serum | +2.43 |
MIR6750 | MIMAT0027400 | Serum | +2.37 |
miR ID | Accession # | Specimens | Fold Change
--- | --- | --- | ---
MIR4728 | MIMAT0019849 | Serum | +2.13
MIR4700 | MIMAT0019797 | Serum | +2.09
MIR6819 | MIMAT0027539 | Serum | +2.06
MIR6803 | MIMAT0027506 | Serum | –2.01
MIR4785 | MIMAT0019949 | Serum | –2.03
MIR320A | MIMAT000510 | Serum | –2.05
MIR4532 | MIMAT0019071 | Serum | –2.05
MIR4490 | MIM0016852 | Serum | –2.06
MIR1180 | MIMAT0026735 | Serum | –2.08
MIR3613 | MIMAT0017991 | Serum | –2.09
MIR6850 | MIMAT0027600 | Serum | –2.22
MIR6780B | MIMAT0027572 | Serum | –2.28
MIR4668 | MIMAT0019745 | Serum | –2.45
MIR1908 | MIMAT0007881 | Serum | –2.48
MIR1228 | MIMAT0005582 | Serum | –2.52
MIR940 | MIMAT0004983 | Serum | –2.65
MIR4706 | MIMAT0019806 | Serum | –2.72
MIR668 | MIMAT0026636 | Serum | –2.95
MIR602 | MIMAT0003270 | Serum | –3.00
MIR4497 | MIMAT0019032 | Serum | –3.19
MIR4707 | MIMAT0019807 | Serum | –3.96
MIR6511B1 | MIMAT0025847 | Serum | –5.07
MIR6126 | MIMAT0024599 | Serum | –18.39
MIR4484 | MIMAT0019018 | Serum | –23.97

14 miRNAs were downregulated. In addition, up-regulated serum samples contain 19 and 21 miRNAs, respectively, when compared with healthy controls as well. The overlapping miRNAs are MIR4532, MIR4706, MIR320A, and MIR6750 between urine and serum samples which were detected for both patients and healthy controls. However, the microarray results were not found to be completely consistent. According to the overlapping miRNAs between patient and control groups, only two miRNAs (MIR4532 and MIR4706) were upregulated in urine although they were downregulated in serum samples, which may be due to the different samples being pooled for microarray analysis and/or the possible effects of different ages of the subjects and varying pathological cancer grades of patients. The MIR4532 is listed as having possible oncogene regulatory properties which have not been investigated specifically [28]. Only one report demonstrated that MIR4532 was found in malignant B cells [29]. However, this miRNA was withdrawn by miRBase, and NCBI reported that the record was discontinued on 14-Mar-2018. On the other hand, MIR4706 was identified in breast tissue [30], and there were no data for functional properties for this miRNA yet.

Interestingly, the MIR320A was downregulated for both urine and serum samples in microarray expression analysis. On the contrary, urine samples of the patient group showed a significant increase in the expression of MIR320A when compared to the control group by qRT-PCR. This result is also consistent with that of a previous study by Porkka et al., which showed upregulation of MIR320A in prostate carcinomas when compared to BPH specimens [31]. In addition, Ristou et al. found nine different miRNAs in plasma samples of colorectal cancer patients where MIR320A level was upregulated before surgery, and decreased expression was recorded after surgery [32]. Moreover, Okato et al. reported a significant decrease in the expression of MIR320A in PC tissue when compared to normal prostate tissue. Furthermore, a previous study proved that cell proliferation, cell migration, and cell invasion are significantly inhibited in the PC cell line by silencing lysosomal-associated protein 1 (LAMP1), which is the target of MIR320A; consequently, the data suggested that MIR320A may function as a tumor suppressor [33]. It has been recently observed that MIR320A suppresses tumor cell proliferation and invasion of renal cancer cells by targeting forkhead box protein M1 (FoxM1) [34].

We also analyzed by microarray and verified by qRT-PCR the expression levels of MIR6750 in serum and urine samples which were found not to be significant between the patient and control group. In one report, MIR6750 was listed as human splicing-derived miRNAs (“mirtrons”) [35]. Currently, this is the only data about MIR6750 and
its relation with PC which was found in both urine and serum samples. However, apart from prostate cancer, only one study in nonsmall cell lung cancer found 59 downregulated miRNAs by silencing the insulin-like growth factor 1 receptor (IGF-1R) gene, and MIR6750 has been shown to be downregulated [36].

The limitations of our study are that we did not evaluate the cellular sources of miRNAs. Particularly urine specimens have podocytes, inflammatory cells, renal tubular, and urinary tract epithelial cells. In addition, urine samples possibly did not include exosomes which are the major sources of miRNAs in body fluids. Moreover, the low g value of centrifugation process also explains the low concentrations of total RNAs from urinary samples. Therefore, the underlying outcome of the changes and correlations observed in this study needs further investigation with more patient samples.

5. Conclusion
There are conflicting results in numerous studies of miRNA expression patterns in prostate cancer. In sum, several studies reported that miRNAs are downregulated in tumors, whereas others reported them to be upregulated [6]. The complexity of miRNAs is not fully clarified either in cancer development or PC in general.

In conclusion, the microarray expression data provided valuable results for possible noninvasive biomarkers for PC patients. Particularly the expression profile of MIR320A was significantly altered in urine specimens of the PC patients. Further studies with large cohorts that include both patients and healthy controls are needed to validate the expression profiles of overlapping miRNAs.

Abbreviations
BPH: Benign prostate hyperplasia; DRE: Digital rectal
examination; FC: Fold change; FDR: False discovery rate; FoxM1: Forkhead box protein M1; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; IGF-1R: Insulin-like growth factor 1 receptor; LAMP1: Lysosomal-associated protein 1; miRNA: Micro RNA; PC: Prostate cancer; PCA: Principal component analysis; PSA: Serum prostate-specific antigen; RMA: Robust multichip average; RT-PCR: Reverse transcription polymerase chain reaction; UTR: Untranslated region

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Figure 4. MIR6750 expression results. A) Urine and serum sample expression levels of the control group (p > 0.05). B) Urine and serum sample expression levels of the patient group (p = 0.0079). C) Urine sample expression levels of the control and patient group (p > 0.05). D) Serum sample expression levels of the control and patient group (p > 0.05).

Conflict of interest
The authors declare that there are no conflicts of interests.

Informed consent and ethical approval
Informed consent was obtained from all individual participants included in the study. This study was conducted after receiving approval from the Local Human Ethics Committee (approval number: 16926-22/30) from Bezmialem Vakif University.
References

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. CA: A Cancer Journal for Clinicians 2013; 63 (1): 11-30. doi: 10.3322/caac.21166

2. Bashir MN. Epidemiology of Prostate Cancer. The Asian Pacific Journal of Cancer Prevention 2015; 16 (13): 5137-5141. doi: 10.7314/APJCP.2015.16.13.5137

3. Akbayir S, Muslu N, Erden S, Bozlu M. Diagnostic value of microRNAs in prostate cancer patients with prostate specific antigen (PSA) levels between 2, and 10 ng/mL. Turkish Journal of Urology 2016; 42 (4) : 247-255. doi: 10.5152/tud.2016.52463

5. Lilja H, Ulmert D, Vickers AJ. Prostate-specific antigen and prostate cancer: prediction, detection and monitoring. Nature Reviews Cancer 2008; 8 (4): 268-278. doi: 10.1038/nrc2351

6. Vanacore D, Boccellino M, Rossetti S, Cavaliere C, D’Aniello et al. Biological functions of microRNAs: a review. Cell and Developmental Biology 2017; 5: 86. doi: 10.3389/fcell.2017.00086

14. Huang Y, Shen XJ, Zou Q, Wang SP, Tang SM et al. Biological functions of microRNAs: a review. Journal of Physiology and Biochemistry 2011; 67 (1): 129-139. doi: 10.1007/s13105-010-0050-6

15. Spakova I, Zelko A, Rabajdova M, Kolarick P, Rosenberger J et al. MicroRNA molecules as predictive biomarkers of adaptive responses to strength training and physical inactivity in haemodialysis patients. Scientific Reports 2020; 10 (1): 15597. doi: 10.1038/s41598-020-72542-1

19. Wu D, Ni J, Beretov J, Cozzi P, Wilcox M et al. Urinary biomarkers in prostate cancer detection and monitoring progression. Critical Reviews in Oncology/Hematology 2017; 118: 15-26. doi: 10.1016/j.critrevonc.2017.08.002

21. Wang K. The ubiquitous existence of MicroRNA in body fluids. Clinical Chemistry 2017; 63 (3): 784-785. doi: 10.1373/clinchem.2016.267625

22. Bryant RJ, Pawlowski T, Catto JW, Marsden G, Vessella RL et al. Changes in circulating microRNA levels associated with prostate cancer. British Journal of Cancer 2012; 106 (4): 768-774. doi: 10.1038/bjc.2011.595

27. Guzel E, Karatas OF, Semercioz A, Ekici S, Aykan S et al. Identification of microRNAs differentially expressed in prostatic secretions of patients with prostate cancer. International Journal of Cancer 2015; 136 (4): 875-879. doi: 10.1002/ijc.29054

1773
28. Reza AM, Choi YJ, Yasuda H, Kim JH. Human adipose mesenchymal stem cell-derived exosomal-miRNAs are critical factors for inducing anti-proliferation signalling to A2780 and SKOV-3 ovarian cancer cells. Scientific Reports 2016; 6: 38498. doi: 10.1038/srep38498

29. Jima D.D, Zhang J, Jacobs C, Richards KL, Dunphy CH et al. Deep sequencing of the small RNA transcriptome of normal and malignant human B cells identifies hundreds of novel microRNAs. Blood 2010; 116 (23): e118-27. doi: 10.1182/blood-2010-05-285403

30. Persson H, Kvist A, Rego N, Staaf J, Vallon-Christersson J et al. Identification of new microRNAs in paired normal and tumor breast tissue suggests a dual role for the ERBB2/Her2 gene. Cancer Research 2011; 71 (1): 78-86. doi: 10.1158/0008-5472.can-10-1869

31. Porkka KP, Pfeiffer MJ, Waltering KK, Vessella RL, Tammela TL et al. MicroRNA expression profiling in prostate cancer. Cancer Research 2007; 67 (13): 6130-6135. doi: 10.1158/0008-5472.can-07-0533

32. Ristau J, Staffa J, Schrotz-King P, Gigic B, Makar KW et al. Suitability of circulating miRNAs as potential prognostic markers in colorectal cancer. Cancer Epidemiology, Biomarkers & Prevention 2014; 23 (12): 2632-2637. doi: 10.1158/1055-9965.epi-14-0556

33. Okato A, Goto Y, Kurozumi A, Kato M, Kojima S et al. Direct regulation of LAMP1 by tumor-suppressive microRNA-320a in prostate cancer. International Journal of Oncology 2016; 49 (1): 111-122. doi: 10.3892/ijo.2016.3522

34. Zhao S, Wang Y, Lou Y, Wang Y, Sun J et al. MicroRNA320a suppresses tumour cell proliferation and invasion of renal cancer cells by targeting FoxM1. Oncology Reports 2018; 40 (4): 1917-1926. doi: 10.3892/or.2018.6597

35. Ladewig E, Okamura K, Flynt AS, Westholm JO, Lai EC. Discovery of hundreds of mirtrons in mouse and human small RNA data. Genome Research 2012; 22 (9): 1634-1645. doi: 10.1101/gr.133553.111

36. Ma W, Kang Y, Ning L, Tan J, Wang H et al. Identification of microRNAs involved in gefitinib resistance of non-small-cell lung cancer through the insulin-like growth factor receptor 1 signaling pathway. Experimental and Therapeutic Medicine 2017; 14 (4): 2853-2862. doi: 10.3892/etm.2017.4847