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miR-21-5p, miR-141-3p, and miR-205-5p levels in urine—promising biomarkers for the identification of prostate and bladder cancer

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Background: Early detection of cancers improves patients' survival and decreases the treatment cost. Unfortunately, the current methods for diagnosis of bladder and prostate cancers, two most common urothelial malignancies, suffer from a low sensitivity and specificity. MicroRNAs, as a group of endogenously produced non-coding RNAs, regulate gene expression and their expression is observed to be altered in many cancers and cancer progression phenomena. The remarkable stability of microRNAs in biofluids and their unique expression pattern in different pathological conditions make them an appealing, noninvasive diagnostic method in cancer diagnosis. Our objective is to identify microRNAs as biomarkers in urine samples of bladder and prostate cancers to improve the existing diagnostic methods in this field.

Materials and Methods: In this study, urine samples from 110 men with either bladder (n = 45) or prostate (n = 23) cancer, benign prostatic hyperplasia (n = 22) and healthy controls (n = 20) were collected. qPCR was used to evaluate the expression level of miR-21-5p, miR-141-3p, and miR-205-5p in these samples. The sensitivity and specificity of these microRNAs were determined using ROC curve analysis.

Results: The analysis of the data revealed that miR-21-5p, miR-141-3p, and miR-205-5p are differentially expressed in urine of bladder and prostate cancer patients. All these three microRNAs were upregulated in these samples and they were also able to differentiate benign prostatic hyperplasia from malignant cases. The statistical analyses revealed a good specificity for each individual microRNA.

Conclusion: The results show that these three urine-based microRNAs might be a good choice to implement a specific and non-invasive diagnostic tool for bladder and prostate cancer. The expression pattern of all three microRNAs was particularly useful to distinguish benign and invasive tumors in prostate cases. From the patients' perspective the improvement of the diagnostic situation is awaited eagerly.

KEYWORDS
miR-21-5p, miR-141-3p, miR-205-5p, prostate cancer, urothelial cancer

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1 | INTRODUCTION

Prostate (PCa) and bladder cancers (BCa) are the two most common and recurrent urothelial cancers with high mortality rate.[1]

In prostate cancer the amount of prostate-specific antigen (PSA) in serum is widely used as a routine and conventional method in tumor detection. However, due to lack of satisfying specificity in many cases, PSA amount cannot be considered as the only parameter in PCa diagnosis.[2]

The diagnosis of bladder cancer, as the second most frequent malignancy of the urinary tract after prostate cancer,[3–5] is mainly based on cystoscopy.[6,7] This invasive procedure is painful and not free from risks. The presently used tests with their apparent weak specificity demands to develop alternatives.[6] Although other biomarkers such as bladder tumor antigen (BTA stat, BTA TRAK), nuclear matrix protein-22 (NMP-22), and fibrin/fibrinogen degradation products (FDP) are used in BCa diagnosis, it is obvious that they harbor some disadvantages like the high amount of false-positive and false-negative results.[6]

microRNAs are now an established class of regulatory small non-coding RNAs involved in post-transcriptional and transcriptional gene regulation[8] which also show their usability as diagnostic markers. microRNAs are involved in many signaling pathways including cell survival and apoptosis, cancer migration, and progression.[8–11] Recent studies showed that microRNAs have abnormal expression profiles in a wide range of malignancies.[9,12,13] Some studies showed that they can be applied as suitable markers for tumor classification.[12,14]

Alterations in microRNA expression levels have been shown in different cancers including bladder and prostate tumors.[12] For example, the level of miR-21-5p has been shown to be increased in PCa.[15,16] miR-21-5p, targeting PTEN, promotes proliferation and migration of cancer cells.[16] The over expression of miR-21-5p abnormally activates TGFβ and Hedgehog signaling pathways which promotes invasion through the induction of EMT.[17]

The possible role of microRNAs in bladder cancer development and progression was determined in a profiling study in 2007 detecting ten up regulated microRNAs including miR-205-5p.[18] In another profiling study, miR-21-5p and miR-145 were identified as the most up- and down regulated microRNAs.[18] Several large-scale profiling experiments have been done since then to elucidate the potential role of microRNAs in urothelial cancers.[19–21]

Detection of microRNAs, actively released from tumor cells into biofluids, like peripheral blood samples or urine, makes them a suitable choice to be used as biomarker.[22] The usage of biofluids such as urine has the advantage of the simple sampling and of moderate cost.[23,24] Therefore, the burden for the patient is leveraged. The direct contact of urine with bladder and prostate tissues makes it a promising source of released tumor microRNAs.[25] Although the exact cellular mechanism of microRNA secretion has not been clarified yet, it has been demonstrated that microRNAs are packaged into exosomes,[26] apoptotic bodies or microveiscles.[27] In this regards, there are some preliminary reports which show differential expression of microRNAs in urine samples of patients with BCa and PCa and make it as a good source to search for non-invasive tumor markers.[28–30]

The development of sensitive and specific urinary markers which provides a noninvasive tool in cancer diagnosis, is the goal of this study. We selected several microRNAs based on their involvement in either BCa or PCa, and their specific profile in urine samples. So, the expression level of miR-21-5p, miR-141-3p, and miR-205-5p were evaluated as oncogenic microRNAs in urine of patients with BCa and PCa using quantitative real time PCR (qPCR). The expression pattern of these three microRNAs are also compared between benign prostatic hyperplasia (BPH) and prostate cancer samples. To get insight into the quality of the biomarker candidates, the sensitivity and specificity was evaluated based on the receiving operating characteristic algorithm (ROC).

2 | MATERIALS AND METHODS

2.1 | Patient samples

Urine samples were collected from men with bladder ($n = 45$) and prostate cancer ($n = 23$) in Baqiatallah hospital (Tehran, Iran). The control group includes healthy normal ($n = 20$) and benign prostatic hyperplasia (BPH, $n = 22$) men with the same age distribution as the cancer patients. Men with bladder cancer had an age range of 45-82 with the average age of 65.7 ± 10.2. Prostate cancer patients had an age range of 59-81 with the average of 68.4 ± 6.0. The age range in the control group was 47-67 and the average was 56.9 ± 7.8 (Table 1). All demographic information concerning age and its distribution in different groups is included in Table 1. Because we did not own for every patient reliable information regarding the exact grading score, we could not statistically discriminate between high and low grade patients.

Specifically, for the prostate situation the inclusion criteria of healthy control group include normal prostate sonography and their PSA value below the threshold of 4 ng/mL. BPH and cancer patients showed a higher PSA value. Aside of estimating the PSA level additionally sonography and biopsy analysis by a pathologist was employed to discriminate BPH and prostate cancers cases. All collected cases had no other malignancies.

Urine samples were collected from the participants as first morning voided specimen and aliquots of 0.5 mL were kept in RNAase-free tubes and frozen at −20°C until RNA extraction. Informed consent was obtained from all participants for the use of their clinical samples. The study has been approved by the Clinical Research Ethics Committee of Baqiatallah Hospital, Tehran.

2.2 | Extraction of total RNA

Before RNA purification, 500 μL of urine was incubated at 56°C for 1 h with Proteinase K (Takara, Osaka, Japan). The proteinase K-treated urine was then used for total RNA extraction using Trizol Ls (Invitrogen Life Technologies, Carlsbad, CA), according to the manufacturer's protocol. RNA was quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc., Wilmington, DE).

TABLE 1 Demographic characteristics of participants recruited to the study

Sample	Number	Age (minimum-maximum, mean) [years]
Control	23	(47-63, 56.9)
Benign Prostatic Hyperplasia	22	(50-75, 61.7)
Prostate cancer	23	(59-81, 68.4)
Bladder cancer	45	(45-82, 65.7)

2.3 | DNase treatment and cDNA synthesis

Extracted RNA was treated with DNase I (Fermentase, London, UK) at 37°C for 30 min. cDNA synthesis was done as the manufacturer's protocol with the universal cDNA synthesis kit (Exiqon, Vedbaek, Denmark) using 4 µL of DNase-treated RNA in a total volume of 20 µL. cDNA was diluted 1:40 and applied for quantitative real time PCR.

2.4 | Quantitative real-time PCR

qPCR was performed using SYBR green (Exiqon), universal cDNA synthesis kit (Exiqon) and microRNA LNA™ PCR primer set (Exiqon). The accession number of each mature microRNAs is as follows: miR-21-5p: MIMAT0000076, miR-141-3p: MIMAT0000432, and miR-205-5p: MIMAT0000266.

miR-21-5p, miR-141-3p, and miR-205-5p expression level, was evaluated in each sample after applying 5S rRNA as a reference internal control. All reactions were run in duplicate. An ABI 7500 system (Applied Biosystems, Foster City, CA) was employed for quantitative real-time assays. The real time thermal condition was as follows: an initial denaturation at 95°C for 5 min, 40 cycles of denaturation at 95°C for 10 s and annealing at 60°C for 30 s followed by a melt curve stage.

Comparative threshold cycle (Ct) was determined for each microRNA and relative amount of each microRNA in each individual sample was described as ΔCt ($Ct_{microRNA}$- $Ct_{5S\ rRNA}$). ΔCt values were used in the analysis for comparison of expression level of microRNAs in control and cancerous samples. 5S rRNA level was estimated using $qPCR_{in}$ all samples under the study. The fold change of each microRNA was calculated using the REST 2009 or R software based on the delta delta Ct value to estimate the fold change for each individual gene. Both approaches use the benefit of integrated randomization and bootstrapping method to test statistical significance of expression data.

2.5 | Statistical analysis

Distribution of raw data of each microRNAs in either prostate or bladder cancer groups was compared with control group using Mann-Whitney test as the raw data does not follow a normal distribution. Separately, the delta Ct expression of measured microRNAs (normalized with 5S rRNA as reference gene) in patients and normal samples were compared with the student's *t*-test. The data in the graphs is presented as mean of two replicates for each microRNA. To assess the specificity and sensitivity of these microRNAs in discriminating between normal and tumor samples, the ROC curve analysis[31] of GraphPad Prism 7 as well as the pROC package of R was used. In all analyses, statistical significance was assumed at a *P* value smaller than 0.05.

3 | RESULTS

The expression pattern differences of three microRNAs—miR-21-5p, miR-141-3p, and miR-205-5p—in urine sample of patients with BCa and PCa in comparison to normal and benign prostate hyperplasia (BPH) were analyzed. qPCR was used to evaluate the expression level of the microRNAs. To have more reliable results the expression pattern of two most common reference genes including 5S rRNA and U6 snRNA investigated for delta Ct calculation in 17 urine samples. Our results showed that 5S rRNA has more reliable expression pattern compared to U6 (Figure S1), so it was used for final analyses. Student's *t*-test was applied to evaluate the Ct distribution of 5S rRNA in all participants including patients and control groups. The results showed that there is no correlation between the expression level of 5S rRNA in the cancer and normal group, which is necessary constraint to utilize a control gene.[32]

3.1 | Distribution and expression pattern of miR-21-5p, miR-141-3p, and miR-205-5p in urine of BCa and PCa patients

The expression distribution of miR-21-5p, miR-141-3p, and miR-205-5p in urine of 45 men with bladder cancer, 23 PCa patients, 20 healthy normal group, and 22 BPH cases showed clear differences between the normal and cancer groups and the Mann-Whitney test (Table 2) assured this observation (Figure 1A). As shown in Figure 1, there were no significance differences between the BPH and the normal group. Comparison of the expression level of these microRNAs for prostate cancer reveals a significant up-regulation of all these three microRNAs in prostate cancer cases, which is as follows: miR-21-5p (15.11 fold, *P* value = 0.001), miR-141-3p (8.91 fold, *P* value = 0.005), and miR-205-5p (6.57 fold, *P* value = 0.020, Figure 1B). In bladder cancer as shown in Figure 1B, the results showed a significant up regulation of miR-21-5p (fold change = 5.54, *P* value = 0.002), miR-141-3p (fold change = 6.46, *P* value = 0.016), and miR-205-5p (fold change = 3.75 fold, *P* value = 0.022).

TABLE 2 The *P*-value of Mann Whitney test for each microRNA

	BPH	PCa	BCa
miR-21-5p	0.91	0.000	0.001
miR-141-3p	0.68	0.0069	0.01
miR-205-5p	0.55	0.01	0.04

Raw data of each microRNA was compared using a non-parametric test between normal and BPH, PCa, BCa, respectively.

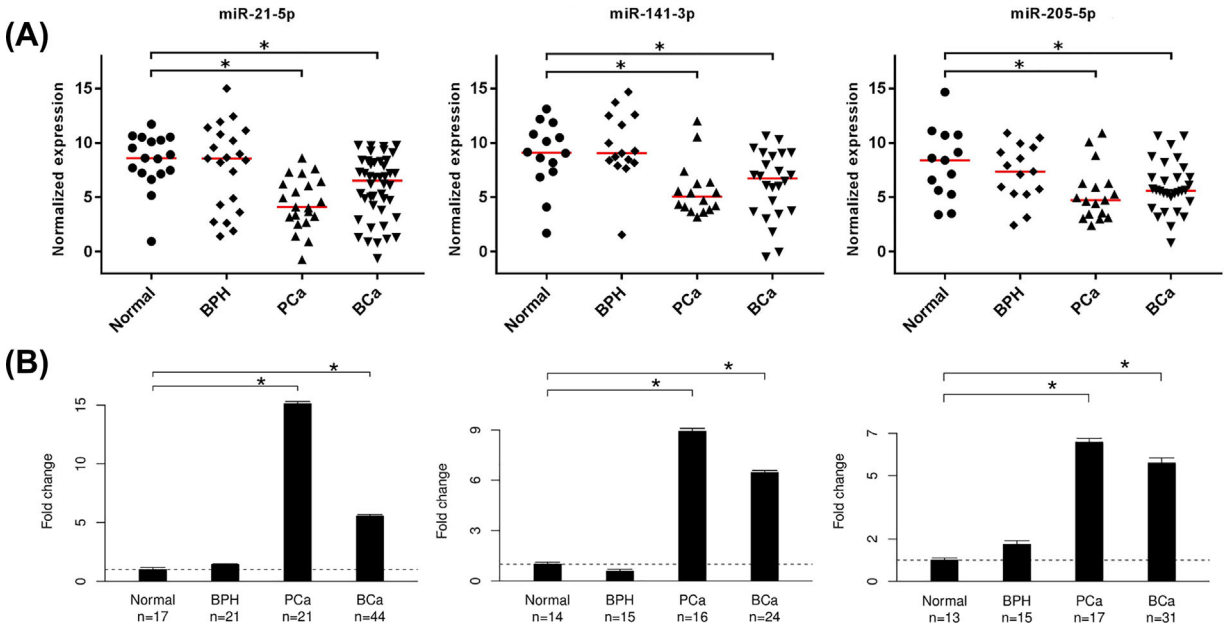

FIGURE 1 A, Scatter plot of raw data of miR-21-5p, miR-141-3p, and miR-205-5p. Normalized raw data of BPH, PCa, and BCa were compared to control group. In both bladder and prostate cases significant differences were observed but the difference was not significant regarding BPH group. B, miR-21-5p, miR-141-3p, and miR-205-5p fold changes in BPH, PCa, and BCa compared to control group. The results showed a significant up regulation of miR-21-5p (15.11 folds, *P* value = 0.001), miR-141-3p (8.91-folds *P* value = 0.005), and miR-205-5p (6.57 folds, *P* value = 0.020) regarding PCa and also again significant up regulation of miR-21-5p (5.54 folds, *P* value = 0.002), miR-141-3p (6.46-folds *P* value = 0.016), and miR-205-5p (3.75 folds, *P* value = 0.022) regarding BCa. Comparison of expression level of miR-21-5p, miR-141-3p, and miR-205-5p between BPH and normal urine samples showed no significant difference ($P > 0.05$)

To sum up, the same expression pattern regarding up regulation was observed in PCa like in BCa, although, the expression level of evaluated microRNAs is relatively higher in prostate compared to bladder cancer.

3.2 | All microRNAs were unchanged in urine of BPH patients

Comparison of the expression level of miR-21-5p, miR-141-3p, and miR-205-5p between BPH ($n = 22$) and normal ($n = 20$) urine samples showed no significant difference ($P > 0.05$, Figure 1B). A slightly up regulation in expression pattern of miR-205-5p in BPH compared to the normal group can be observed but reveals no significance.

These results point toward that the three microRNAs are involved in the cancer processes but not in the inflammatory processes which is responsible for the phenotype of benign prostate hyperplasia.

3.3 | Determination of the biomarker quality for PCa

The ROC curve analysis was used to evaluate the sensitivity and specificity of the expression level of miR-21-5p, miR-141-3p, and miR-205-5p to discriminate normal and malignant samples of prostate cancer (Figure 2A). The areas under the curve (AUC) is 0.88 for miR-21-5p ($P < 0.0001$), 0.79 for miR-141-3p ($P = 0.0022$), and 0.76 for miR-205-5p ($P = 0.0060$), respectively. The specificity is 0.88, 0.86, and 0.69 regarding miR-21-5p, miR-141-3p, and miR-205-5p, respectively (Figure 2A).

3.4 | Determination of the biomarker quality for BCa

In bladder cancer the ROC analyses to discriminate normal and bladder cancer showed a good sensitivity and specificity for miR-21-5p, miR-141-3p, and miR-205-5p (Figure 2B). The AUC is 0.76 ($P = 0.0002$), 0.74 ($P = 0.0049$), and 0.73 ($P = 0.0189$) regarding miR-21-5p, miR-141-3p, and miR-205-5p, respectively. The specificity is 0.59, 0.71, and 0.62 regarding miR-21-5p, miR-141-3p, and miR-205-5p, respectively. The reasonable specificity values observed in all the three microRNAs support their usage in the PCa diagnosis.

An AUC > 0.70 in the ROC curve analysis indicates that the tested marker is strong enough to discriminate the examined groups of samples.[33] As shown in Figure 2A, the AUC of all three microRNAs is above this guiding threshold which indicates that these microRNAs can discriminate patients and healthy people with satisfying specificity, and sensitivity. As shown in Figure 2A, specificity is nearly higher than 0.60 in all situation regarding BCa and PCa. This showed that microRNAs expression level can be used as a reliable alternative of PSA test which shows an extremely lower specificity value of 33% in prostate tissues.[32] This can significantly reduce the number of false positive cases regarding BPH which are diagnosed as cancer cases.

In total, we observed stable up regulation of all estimated microRNAs in PCa and BCa patients. ROC curve analysis showed that these microRNAs are promising markers regarding PCa and BCa. Moreover, these three microRNAs can efficiently discriminate BPH patients from the invasive forms of prostate cancer. This is remarkable

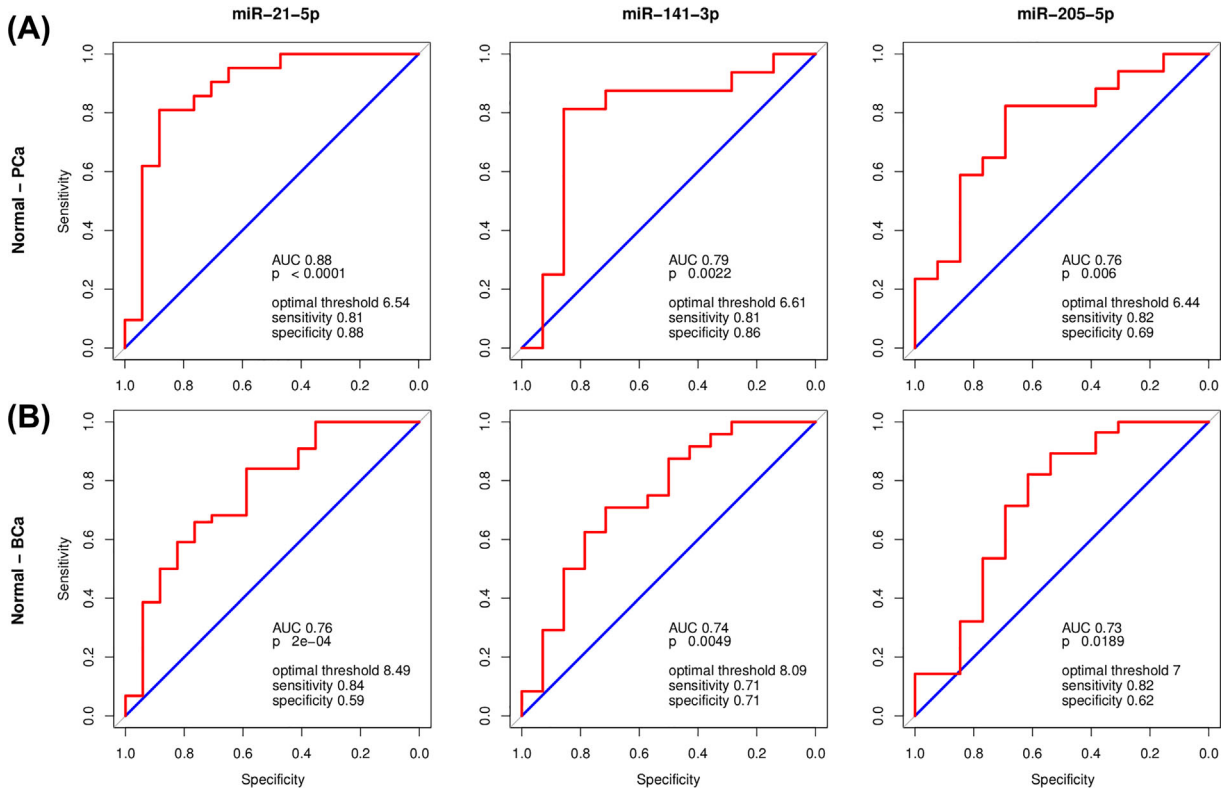

FIGURE 2 A, The ROC curve of miR-21-5p, miR-141-3p, and miR-205-5p expression level in urine sample of prostate cancer. miR-21-5p could significantly discriminate bladder cancer from control group by an AUC of 0.88 (*P* value < 0.0001). miR-141-3p and miR-205-5p could also significantly discriminate bladder cancer from healthy ones by an AUC of 0.79 (*P* value = 0.0022) and AUC of 0.76 (*P* value = 0.0060), respectively. B, The ROC curve of miR-21-5p, miR-141-3p, and miR-205-5p expression level in urine sample of bladder cancers. miR-21-5p could significantly discriminate bladder cancer from control group by AUC of 0.76 (*P* value = 0.0002). miR-141-3p and miR-205-5p could also significantly discriminate bladder cancer from healthy ones by an AUC of 0.74 (*P* value = 0.0049) and AUC of 0.73 (*P* value = 0.0189), respectively

because conventional PSA diagnostics is unable to discriminate these two forms.

3.5 | Does the microRNA discriminate BPH and PCa?

Hoffman et al[34] shows that the PSA value is moderately discriminating between these two entities with a sensitivity of 86% but a low specificity of 33%.

The results in Figure 3 show that all three microRNAs are comparable with PSA on the sensitivity level but remarkably improve the specificity level. The specificity for miR-21-5p is 0.62 (AUC 0.76, *P* = 0.0013), for miR-141-3p it is 0.93 (AUC 0.85, *P* < 0.0001) and for miR-205-5p it is 0.87 (AUC 0.71, *P* = 0.0255).

4 | DISCUSSION

Recent findings have suggested the potential of urine/blood-based markers for urological malignancies to be a good replacement for the existing, invasive methods such as cystoscopy. Moreover, the conventional urine-based markers lack acceptable sensitivity and specificity which shows unmet need for an additional alternative for screening, initial diagnosis or follow up studies of bladder and prostate cancers.[28,35,36] As an alternative to conventional diagnostic methods, microRNAs serve as a promising biomarkers in human cancers. There are several studies which have shown the aberrant expression of microRNAs in BCa and PCa.[15,37–40] A recent microRNA profiling study showed a moderate correlation of microRNAs deregulation in tumor tissues and urine exosomes in BCa[15] but no correlation was found between tumor and plasma in this study. This emphasizes on the appropriateness of urinary microRNAs but not serum microRNAs regarding the detection of bladder cancer.

In the present study, we assessed the expression level of three oncogenic microRNAs including miR-21-5p, miR-141-3p, and miR-205-5p in urine samples of two most prevalent urological malignancies. A significant up-regulation of all three measured microRNAs was observed. miR-141-3p and miR-21-5p had the highest expression level in bladder and prostate cancers, respectively. miR-205-5p had the least expression level in both tested malignancies. We also evaluated the expression level of the aforementioned microRNAs in benign prostatic hyperplasia and compared it with control group to search for a non-invasive diagnostic method. Interestingly, all these three microRNAs had no significant alteration in BPH compared to normal. This is beneficial in stratifying benign patients from invasive ones while PSA, the commonly used biomarker, is unable to do such a stratification.

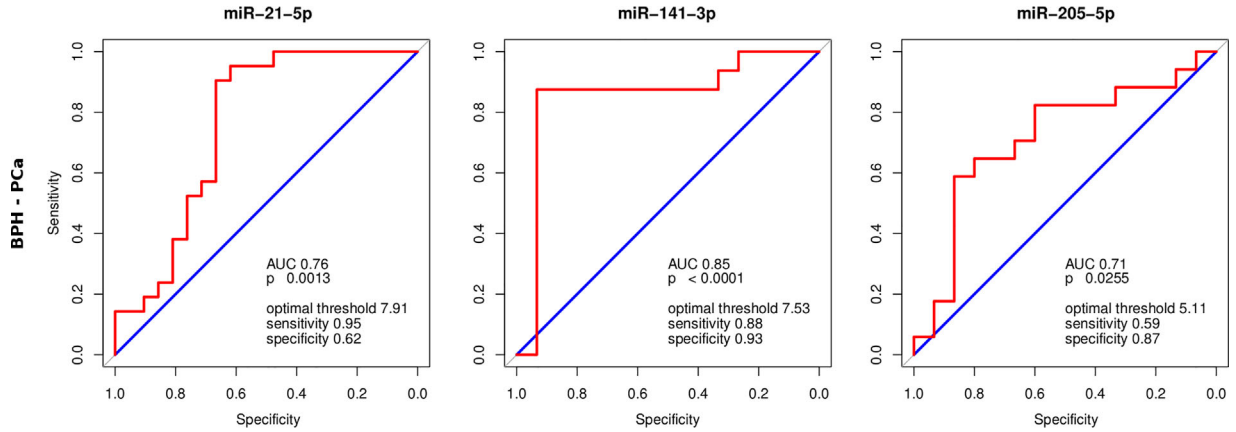

FIGURE 3 The ROC curve of miR-21-5p, miR-141-3p, and miR-205-5p expression level in urine sample of BPH compared to PCa. miR-21-5p showed an AUC of 0.76 (*P* value = 0.0013) in discriminating prostate cancer from BPH cases. miR-141-3p and miR-205-5p could also significantly prostate cancer from BPH by an AUC of 0.85 (*P* value < 0.0001) and AUC of 0.71 (*P* value = 0.025), respectively

The patient's advantage is that the presented approach avoids the painful cystoscopy to differentiate benign and invasive malignancies; an objective worth to be approached.

There is a recent study that reports the over expression of miR-21-5p, miR-141-3p, and miR-205-5p in cancerous tissue of upper urinary tract of urothelial cancer (UUTUC) patients. They could demonstrate a significant increase of miR-141 in serum of UUTUC patients.[41] In other studies, over expression of miR-21-5p[15] and miR-141[42] in tumor tissues and serum samples of patients with PCa was reported.

In our study we observed 4.7 and 9.77-fold up regulation of miR-21-5p in urine of BCa and PCa patients, respectively. miR-21-5p act as an oncogene which is up-regulated in nearly all epithelial cell-derived tumors including breast, pancreas, lung, gastric, esophageal, colon, and PCa.[43] The majority of its reported target genes are tumor suppressors.[44] In particular, miR-21-5p exerts its oncogenic function predominantly through the inhibition of cellular apoptosis by targeting some important tumor suppressor genes including *Fas ligand* (*FasL*), *pten*, *TAp63*, and *bax*.[44] Another study showed that miR-21-5p directly targets MARCKS which promotes apoptosis inhibition and cellular invasion in prostate cancer cells.[44,45] These findings underline the oncogenic action of miR-21-5p in urothelial tracts. Our observation regarding up regulation of miR-21-5p in BCa and PCa highlight these oncogenic activities regarding these malignancies.

We also observed over expression of miR-141-3p in urine of BCa (7.19 folds) and PCa (12.21 folds) patients, which is also in line with the result of a study by Wang el al,[46] which showed increased urinary miR-141-3p level in BCa patients.

There is an ongoing controversy about the expression level of mir-205-5p in BCa and PCa, another upregulated microRNAs in our tested malignancies. In contrast with some studies that reported down regulation of miR-205-5p in cancerous tissues and urine samples of PCa patients,[47] our results as well as some other reports showed a significant up regulation of this miRNA in urine of PCa and BCa patients.[18,48]

To conclude, significant up regulation of these three microRNAs was observed consistently in this study which advocate for these miRNAs as promising biomarkers for BCa and PCa.

Employing a sensitivity/specificity analysis (ROC), we underline that these microRNAs are valuable diagnostic tumor markers for BCa and PCa.

Currently, PSA evaluation and biopsy specimens are widely used in PCa and BCa diagnosis. Biopsy is an invasive procedure which might affect the patient's quality of life. PSA is a common biomarker in the clinical PCa diagnosis,[49] however, the specificity of PSA is limited to make a definite diagnosis on PCa.[50] As we could show with these three microRNAs that at least the specificity could be improved notably over Hoffman et al.[32] Obviously there need to be more research and validation on those still preliminary results, but the tendency of all three microRNAs is promising. So, in this case, urinary microRNAs might be an appropriate non-traumatic alternative.

A further advantage of the chosen microRNAs in contrast to PSA is, that these tumor-associated miRNAs, are able to noninvasively discriminate PCa and BPH individuals. This is a remarkable gain for diagnostics.

5 | CONCLUSION

The expression level of miR-21-5p, miR-141-3p, and miR-205-5p in urine samples are deregulated in bladder- and prostate cancer. The detection of the increased miR-141-3p, miR-21-5p, and miR-205-5p levels allows identification of patients with bladder and prostate cancers and also stratification of BPH patients from malignant tumors.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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SUPPORTING INFORMATION

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