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# MicroRNA 1825 up-regulation for discrimination Prostate Cancer versus Benign Prostatic Hyperplasia Patients

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**Abstract**

**Background:** Malignant tumor of Prostate consider the greatest form of malignant tumor, and it has the second rank of cancer leading death in Western nations. MicroRNAs represent a promising new class of noninvasive diagnostic markers for early detection such tumor.
**Aim:** Estimation of urine microRNA-1825 gene expression levels in patients of benign prostatic hyperplasia, prostate cancer, and apparently healthy control by using Real-Time PCR technique for discrimination the benign prostatic hyperplasia versus prostate cancer and correlate with age, grade and stage of tumors.
**Methods:** Stem-loop follows by Taq-Man Real-Time PCR was performed to identify the level of microRNA-1825 gene expression in the urine sample of patients of benign prostatic hyperplasia, prostate cancer, and apparently healthy controls. The expression levels of microRNA-1825 relative to messenger RNA of Glyceraldehyde 3-phosphate dehydrogenase were determined using the link method
**Results:** The gene expression of micro R-1825 in urine sample was significantly increased in prostate cancer cases relative to benign prostatic hyperplasia and apparently healthy controls. Receiver operating characteristic curve analyses indicated that use of urine microRNA-1825 gene expression has a high diagnostic sensitivity and specificity for early detection of prostate cancer. Urine microRNA-1825 levels were found to be highly significant with advanced stage and higher grade.
**Conclusion**: RT-PCR method for urine- microRNA-1825 gene fold change can act as a non-invasive way for early diagnosis of prostate tumor and have good screening properties for prostate cancer, especially in men with PSA range of 4-10ng ml $^{-1}$.
**Keyword:** MicroRNA-1825; RT-PCR; Urine sample; Prostate cancer; Benign Prostatic Hyperplasia.

## INTRODUCTION

Malignant tumor of Prostate considers the greatest form of malignant tumor, and it has the second rank of cancer leading death in Western nations [1]. "Prostate cancer (PCa)" has a multifactorial etiological and risk factors. Although with progress in the molecular pathogenesis of such disease, a sensitive method for diagnosis and precise treatment still in pursuit challenges. "Digital rectal examination (DRE) signified the crucial diagnostic test for PCa. In the 1980s, prostate-specific antigen (PSA)" screening was rapidly and broadly approved for PCa detection [2]. An Invasive (needle) prostate biopsies are essential to catching out the pathological presence of PCa. Furthermore, an increased serum level of PSA above 4.0 ng/ml reflect an increased risk of PCa considerably [3]. To our knowledge, blood PSA concentration may reflect many statuses, such as "benign prostatic hyperplasia (BPH)", inflammation condition, or pharmacological intervention, thus it seems a marker with low specificity for expecting tumor aggressiveness or therapy responsiveness [4,5]. Serum PSA testing for the early detection of PCa has a sensitivity of around 86% and a specificity of approximately 33%, depending on the patient's age and local prevalence of disease [5]. Due to the multifocal invasion of PCa, the chance for detecting PCa is low even with several prostate- biopsies are taken and inspected by a well-skilled pathologist. Furthermore, about 20% of PCa cases are not discovered with the first set of biopsies, which give an indication for frequent-biopsies in patients that may clinically do not have such disease [6]. and consequently, patients may develop superinfection as a complication of the invasive unsterile procedure [7]. Still, with regard of the important progress in early diagnosis and relapse assessment after radical prostatectomy, there is no proof that the PSA-test decreases the risk of death for such disease and it is not associated with both of tumor aggressiveness estimate, nor with therapy responsiveness. Accordingly, PSA level evaluation inevitably affects the false-positive rate of the malignant prostate tumor [8,9]. According to its low prognostic importance, PSA showing miss diagnosis and treatment in patients who are exposed to invasive or radical operation. However, low specificity (PSA testing) and low sensitivity (DRE) of these tests restrict their diagnostic significance. To overcome these drawbacks, there are many lines have been anticipated to develop cancer diagnostic accuracy of the PSA, which include detection of "PSA velocity" (change over time), "PSA density" (ratio between protein blood-level and prostate volume), in addition to PSA-free and protein-bound PSA levels. still, the clinical usefulness of these plans quiet solely experimental [9].

Moreover, Sensitive diagnostic markers are necessary in order to reduce overdiagnosis, overtreatment, biopsy side effects, and psychological stress [10]. Therefore, noninvasive methods for diagnosis and prognosis of PCa are essential. Recent data mention that extracellular vesicles, which are small membrane vesicles, are released by PCa cells into the extracellular space. And the anatomical site of the prostate, the extracellular vesicles could be detected in urine [11,12], and their concertation can be increased after DRE massage [13]. Since urine can effortlessly be collected after DRE massage. Accordingly, testing for urine content- extracellular vesicles seems a hopeful goal as a diagnostic test for PCa. And due to them with microRNAs. Thus, can serve as PCa markers.

MicroRNAs (miRs) are a group of small noncoding RNA which have the capability to control its goal gene expression. PCa as many malignant tumor types with regards to expression of a miRs is categorically deregulated. The significance of miRs in PCa progress is highlighted by many papers that establish the abnormal miR expression in PCa tissues compared to normal tissues [14,15]. Since miRs have such unique roles in the carcinogenesis process [17,18]. Thus, the aim of the present study is to investigate whether mature miR-1825 have a unique expression pattern in the urine of PCa patients compared to apparently healthy control.

## PATIENTS AND METHODS

### Patients, urine samples, and histological examinations

The study was conducted during the period from March 2014 to January 2018. This is a prospective study, whereby 30 patients with newly diagnosed BPH ,20 patients with PCa, and 20 apparently healthy controls were enrolled, were recruited at the Surgical Department, AL-Diawaniah Teaching Hospital in Diawaniah City. Ultrasound-guided for DRE biopsy was done as a measure of the standard diagnostic procedure for patients with BPH and PCa in the Pathological Department, AL-Diawaniah Teaching Hospital in Diawaniah City. DRE needle biopsy for histopathological evaluation was performed in all patients with BPH and PCa. Great care was used to collect material only from urine with the help of DRE massage. A total of 70 cases in which, 20 with PCa, 30 patients with BPH and 20 cases of apparently

healthy controls. A urine sample was collected from the patients (n=50) before the operation and apparently healthy controls(n=20) were diagnosed without any tumor or physical illness and preserved in Diethylpyrocarbonate (DEPC) water for total RNA extraction and for RT-PCR. The histopathological examination for all cases with BPH and some cases with PCa which under wine for surgical resection (open supra-pubic prostatectomy). The histopathological classification was performed according to the WHO classification, tumor staging was carried out according to AJCC for patients which suitable for operation.

**MiR isolation from urine:** urine samples were collected between 8:00 and 9:00 a.m. following centrifugation for 30 min at 2,650 g, then urine samples were stored at $70^{o}$ c. Isolation of total RNA (RNA was extracted from urine using the Trizol reagent (Bioneer, Korea) according to the manufactures instructions. RNA quality was assessed with a NanoDrop 1000 spectrophotometer.

**Real-time RT-PCR for miR-1825 quantification:** miR-1825 was evaluated according to "TaqMan miR RT-kit procedure (Applied Biosystems, Foster City, CA, USA)" which involving use miR-specific primer (according to miR- database to design the primers) [19] in addition to essential TaqMan probes. Reverse transcriptase reactions were done to make cDNAs in a volume of 15 ml using, "10 ng total RNA for each sample, 50 nM stem-loop RT primer, 1 RT buffer, 1 mM each of dNTPs, 3.33 U/ml and 0.25 U/ml RNase inhibitor". Real-time PCR was performed in triplicate in a 96-well optical plate using "Applied Biosystems 7700 Sequence Detection System". The volume of 20 ml of each sample included TaqMan Universal PCR Master Mix, 1 ml specific miR Assay Mix, and 1.34 ml RT product. The reactions were incubated at 50 8C for 2 min and 95 8C for 10 min, followed by 40 cycles of 95 8C for 15 s and 60 8C for 1 min. All miR-1825 quantification data were normalized to a housekeeping gene. The messenger RNA(mRNA) of GAPDH gene primers and probe were designed using NCBI- GeneBank database and Primer 3 plus design online. The cDNAs primer of GAPDH as design as Random Hexamer primer and the primer used in qPCR was: forward, CAGCCGCATCT-TCTTTTGC and reverse, TTAAAAGCAGCCCTGGTGAC. Taq-Man probe for mGAPDH was: FAM-CCAGCCGAGCCACATCGCTC-TAMRA. The primer used in qPCR for miR-1825 was: Forward, 5'TCCAGTGCCCTCCTCT3, and reverse, 5'GTCGTATCCAGTGCAGGGTCCGAGG TATTCGCACTGGATACGACATCGG3'. The data results of RT-qPCR for miR-1825 and GAPDH were analyzed by the relative quantification gene expression levels (fold change) were based on the Ct values by using the Livak method (Fold change = $2^{-\Delta\Delta CT}$) that described by (Livak and Schmittgen, 2001) [20].

**Statistical analysis:** SPSS version 16 and Microsoft Office Excel 2007 was used as an analysis of these data, the Chi-square test, and Fisher exact test was used to study the association between any two nominal variables. P-value of less than or equal to 0.05 was considered significant.

## RESULTS

### 1- Clinicopathological characteristics of patients:

A total of 70 cases, whereby 30 patients with clinically diagnosed BPH, 20 patients with PCa, and 20 apparently healthy controls were enrolled in the study, as shown in table 1:

**Table (1): shown the characteristic categories of patients' study**

| No. | BPH age | BPH fold change in urine | PSA ng/ml serum BPH | PCa age | PCa stage | PCa Gleason score | PCa fold change in urine | PSA ng/ml in the serum of PCa |
|---|---|---|---|---|---|---|---|---|
| 1 | 70 | 0.3 | 7 | 75 | C | G6 | 7 | 10 |
| 2 | 73 | 0.4 | 6 | 65 | B | G3 | 5.5 | 6 |
| 3 | 76 | 0.2 | 6 | 85 | D | G10 | 10 | 8 |
| 4 | 79 | 0.4 | 7 | 90 | D | G10 | 10.5 | 8 |
| 5 | 75 | 0.5 | 5 | 67 | C | G7 | 7.5 | 8 |
| 6 | 70 | 0.3 | 6 | 69 | C | G7 | 8 | 7 |
| 7 | 80 | 0.4 | 7 | 70 | C | G6 | 7 | 8 |
| 8 | 71 | 0.2 | 6 | 75 | C | G7 | 8 | 10 |
| 9 | 65 | 0.4 | 4 | 88 | C | G7 | 7.5 | 7 |
| 10 | 69 | 0.3 | 7 | 70 | B | G3 | 6 | 6 |
| 11 | 60 | 0.5 | 4 | 85 | C | G6 | 7 | 9 |
| 12 | 77 | 0.4 | 7 | 80 | D | G10 | 10.5 | 8 |
| 13 | 75 | 0.3 | 6 | 85 | D | G10 | 9 | 10 |
| 14 | 73 | 0.5 | 7 | 80 | C | G7 | 8 | 7 |
| 15 | 72 | 0.2 | 6 | 75 | C | G7 | 7.5 | 6 |
| 16 | 71 | 0.4 | 4 | 77 | C | G7 | 8 | 10 |
| 17 | 75 | 2.5 | 7 | 80 | D | G10 | 10 | 6 |
| 18 | 77 | 1.5 | 7 | 75 | C | G6 | 7 | 7 |
| 19 | 80 | 3 | 6 | 75 | B | G2 | 0.4 | 5 |
| 20 | 85 | 2 | 6 | 75 | B | G2 | 0.35 | 5 |
| 21 | 79 | 0.4 | 5 | | | | | |
| 22 | 80 | 0.3 | 7 | | | | | |
| 23 | 81 | 0.4 | 7 | | | | | |
| 24 | 70 | 0.2 | 5 | | | | | |
| 25 | 77 | 0.1 | 6 | | | | | |
| 26 | 73 | 0.2 | 6 | | | | | |
| 27 | 72 | 0.5 | 7 | | | | | |
| 28 | 70 | 0.3 | 7 | | | | | |
| 29 | 75 | 0.4 | 6 | | | | | |
| 30 | 75 | 0.3 | 7 | | | | | |

A total of 30 cases of BPH where clinically diagnosed as BPH (from history, DRE, PR/US, DR/US needle biopsy and PSA serum level (where were in gray-zone (PSA level 4-10 ng $ml^{-1}$), but after open prostatectomy and histopathological examination revealed (26 cases of 30) with BPH truly and (4cases) diagnosed as BPH with microscopical foci of PCa with stage A and Gleason score 2 (as shown in cases number 17,18,19, and 20) as labeled in red color, as shown in table 1. According to the stage of patients with prostate carcinoma were classified into 4 (16.7 %), 4 (16.7 %), 11 (45.8 %) and 5 (20.8 %) having stage A, B, C, and D, respectively, as shown in table 2:

**Table 2: Frequency and percentage of patients with carcinoma of prostate according to stage**

| Stage | *n* | % |
|---|---|---|
| A | 4 | 16.7 |
| B | 4 | 16.7 |
| C | 11 | 45.8 |
| D | 5 | 20.8 |
| Total | 24 | 100 |

***n*: number of cases**

In addition, they were classified according to grade into 6 (25 %), 2 (8.3 %), 4 (16.7 %), 7 (29.2%) and 5 (20.8%) having Gleason grades of 2,3,6,7 and 10, respectively, as shown in table 4.

**Table 3: Frequency and percentage of patients with carcinoma of prostate according to Gleason grade score.**

| Gleason grade | *n* | % |
|---|---|---|
| 2 | 6 | 25.0 |
| 3 | 2 | 8.3 |
| 6 | 4 | 16.7 |
| 7 | 7 | 29.2 |
| 10 | 5 | 20.8 |
| Total | 24 | 100 |

***n*: number of cases**

Mean age of patients with PCa was significantly higher than that of both patients with BPH and healthy control subjects, 77.42 ±6.63 years, 73.38 ±4.83 years and 70.30 ±9.58 years, respectively; and *p*-values were (0.001 and 0.048), respectively. As shown in table 4.

**Table 4: Mean age in study groups**

| Group | *n* | Range (min.-max.) | Mean ± *SD* | *P** | *P** |
|---|---|---|---|---|---|
| Control | 20 | 35 (50 -85) | 70.30 ±9.58 | Reference | |
| BPH | 26 | 21 (60 -81) | 73.38 ±4.83 | 0.146 | Reference |
| Carcinoma | 24 | 32 (65 -90) | 77.42 ±6.63 | 0.001 | 0.048 |
| Total | 70 | 40 (50 -90) | 73.89 ±7.52 | | |

*n*: number of cases; min.: minimum; max.: maximum; *SD*: standard deviation; * post hoc LSD test

Mean serum PSA was highest in patients with carcinoma followed by patients with BPH and lastly by healthy control group, 7.38 ±1.56 ng/ml, 6.08 ±1.02 ng/ml, and 1.60 ±0.42 ng/ml, respectively, as shown in figure 1; the difference between any two groups was highly significant ($P < 0.001$).

**2-Comparison microRNA-1825 gene expression level between prostate carcinoma , benign prostatic hyperplasia patients, and apparently healthy control .**

The mir-1825fold change was up-regulated in the majority of cases (18 cases of PCa (and clinically diagnose PCa prove after histopathological diagnosis) of (91.6%) out of 20 cases of PCa and only 2cases of PCa remain unchanged for MiR -1825-fold change comparison to heath filthy the control. The mean the urine level of miR-1825 was significantly higher in patients with carcinoma of the prostate in comparison with control group, 7.5 (1.75) versus 1 ($P<0.001$) , as shown in figure 3. MiR-1825 fold change was un-change in 26 cases out of 30 cases of BPH (which clinically diagnose BPH and prove pure BPH after histo-pathological diagnosis) and only 4csaes of BPH (13.3%) out of 30 cases (which clinically diagnose BPH but after histopathological examination revealed BPH with microfoci of PCa with stage A, were for old Chaa one up-a regulated an in comparison to healthy control and these cases will be added to cases of PCa. The mean urine level of miR-1825 was significantly higher in patients with carcinoma of prostate in comparison with BPH patients ($P<0.001$), and it was not significantly changed in patients with BPH when compared to that of control group, 1.35 (0.12) versus 1 ($P > 0.05$), as shown in figure 3.

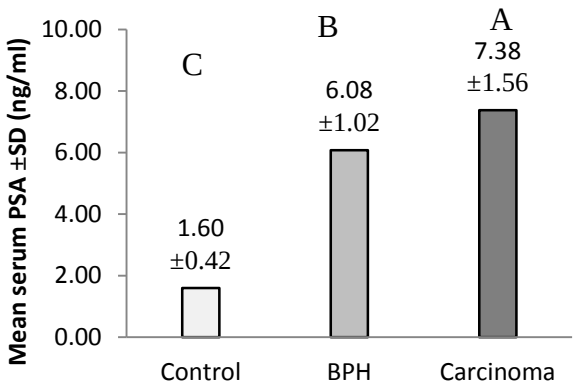


**Figure 1: Mean serum PSA in study groups.**

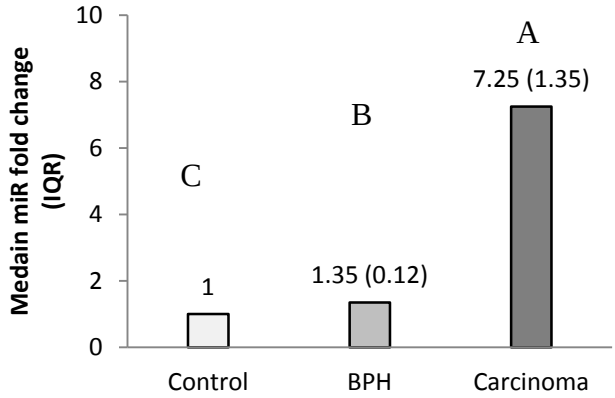


**Figure 3: Median fold change of the microRNA-1825 in study groups relative to average healthy control.**

**-Predictive value of microRNA-1825 in the urine sample of patients**

Receiver operator characteristic (ROC) curve analysis was conducted in order to evaluate the role of miR-1825 and serum PSA in the diagnosis of PCa and the results are shown in figure 4 and table 3. MiR- fold change of > 1.5 was the cutoff value that predicted PCa with a highly significant level of accuracy (97.9%) since the area under the curve was (0.979). This level was associated with a sensitivity of 87.5% the specificity of 100.0%. Serum PSA also was associated with a highly significant cutoff value of > 7 ng/ml, with a level of accuracy of 84.7%; however, it was far less specific than miR-1825- fold change 41.7% versus 90.0%, as shown in table 5.

**Table 5: ROC curve parameters**

| Variable | Cutoff | AUC | 95% CI | *P* | Sensitivity | Specificity |
|---|---|---|---|---|---|---|
| miR | > 1.5 fold change | 0.979 | 0.912 to 0.998 | <0.001 | 87.5 % | 100% |
| PSA | > 7 ng/ml | 0.847 | 0.741 to 0.922 | <0.001 | 41.7 % | 100 % |
| miR versus PSA | ------- | 0.132 | ----- | <0.001 | Better | Same |

CI: confidence in the interval; AUC: area under the curve; miR: micro RNA

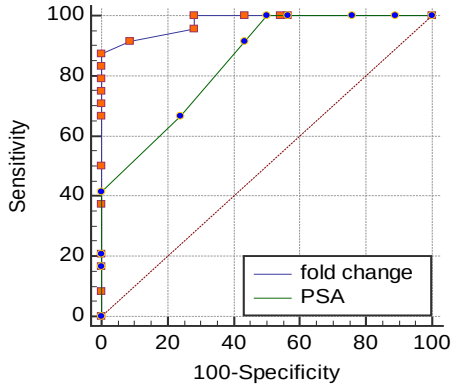


**Figure 4: Receiver operator characteristic (ROC) curve analysis.**

**The validity of microRNA-1825 gene expression folds change as a prognostic marker.**

Both stage and grade of carcinoma showed positive significant correlation with miR-1825-fold change. While Both stage and grade of carcinoma showed no significant correlation with serum level of PSA**,** as shown in table 6.

**Table 6: Correlation of stage and grade to age, microRNA-1825-fold change and serum level of PSA**

| Characteristic | Stage | | Gleason grade | |
|---|---|---|---|---|
| | *r* | *P* | *r* | *P* |
| Age | 0.282 | 0.183 | 0.378 | 0.069 |
| Fold change | 0.925 | <0.001 | 0.954 | <0.001 |
| PSA ng/ml in serum | 0.493 | 0.064 | 0.541 | 0.066 |

***r*: correlation coefficient.**

## DISCUSSION

The clinical application of miRs as a diagnostic tool is getting high attention from both clinicians and pharmacy tical manufacturing since they seek less invasive diagnostics strategy [21]. many biological fluids such as breast milk, sputum, CSF, blood, seminal fluid and also urine are known to contain secreted miRs. Urine is considering an attractive biological fluid due to its easy way of collection and have a rich source for free miRs produced by a selected group of organs (prostate, bladder, kidneys and urothelial cells). Due to such easy way of collection, and the fact that prostate cells are directly released into the urethra through prostatic ducts after DRE massage, thus, miRs in urine pave the way for noninvasive biomarker testing for PCa . In contrast with blood serum and plasma as bio-fluids that contain extracellular vesicles from all organs of the body and consider to be less specific [22].

In the present study, the mean age of patients with PCa was significantly higher than that of both patients with BPH and healthy control subjects, and mean serum PSA was highest in patients with carcinoma followed by patients with BPH and lastly by the healthy control group, the results of the present study were in agreement with other studies [23-25]. We identified the urine miR-1825 was up-regulated in the majority of cases with PCa with a significant difference in the expression compared to patients with BPH and apparently healthy controls. We speculated that urine miR-1825 may originate from tumor cells or a product of tumor cell death and lysis. The results of the present study were in agreement with other studies [23-25]. The levels of urine miR-1825 gene expression were a un-change comparison to normal healthy control in all cases of BPH, and only 4cases of BPH,13.3%, which none of whom had at that time yet been diagnosed with PCa. But these cases, which clinically diagnose as BPH later on after open prostatectomy, and histopathology examination, reveals as BPH with microscopical microfoci of PCa with stage A and Gleason score 2), these numbers coupled with the fact that DR/US needle biopsy of prostate can occasionally miss PCa. in addition, these cases with PSA serum level was in gray-zone, range from 4-10 ng ml $^{-1}$ (lo sensitivity and specificity) and DR U/S, PR/US needle biopsy and CT examination have limited value for the prediction of microscopical foci of PCa. Although, in order to distinguish between these possibilities, especially in cases with clinically diagnose of BPH patients and PSA in gray-zone, we should collect urine for miR -1825 gene expression level for performing the diagnoses. The results of the present study were in agreement with other studies [23-27]. MiR-fold change of > 1.5 was the cutoff value that predicted PCa with a highly significant level of accuracy (97.9%), area under the curve was (0.979). The sensitivity of 87.5% and a specificity of 100.0%. The results of the present study were in agreement with other studies [23, 24]. Serum PSA also was associated with a highly significant cutoff value of > 7 ng/ml, with a level of accuracy of (84.7%); however, it was far less specific than miR-1825- fold change (41.7%) versus (90.0%). The results of the present study were in agreement with other studies [23, 24]. In fact, PSA, DR U/S, CT, and MRI have limited value for the prediction of lymph node metastasis; hence it is crucial to find novel biomarkers that can be used for this purpose and also as a standard for optimizing therapy and long-term follow-up care. Consider to prognostic markers, the results of present study demonstrated that up-regulation of miR-1825 in PCa was statistically significant difference in PCa with higher stage (C and D) from that patient with early stage of cancer (stage A and B), suggesting its role in metastasis and functional analyses of miR-1825 revealed its involvement in migration, invasion, proliferation and cell cycle. The results of the present study were in agreement with other studies [16, 23, 24, 28]. The concept of using urine for the detection of miRs gene expression as a biomarker is relatively new and use of urine as a specimen for tumor marker remains challenging in view of the fact that urine contains a wide variety of biomolecules including a high amount of nucleases and RNases. However, due to the small size, miRs are more stable against RNase degradation which advocates their merit as biomarkers [29]. The best for your knowledge, this study could be the first study of its type to be conducted in Iraq, evaluating urine miR-1825 gene expression level by RT-qPCR, in a sample of Iraqi patients. There was no baseline study regarding urine miR-1825 gene expression level stratification in apparently healthy control in Iraqi individuals. Although, similar studies were conducted abroad to stratify urine miR-1825 in BPH and PCa patients in other countries [23-29].

## CONCLUSION

MiR-1825 can easily be purified from the urine sample and semi-quantitated by RT-PCR. since they are protected from enzymatic degradation. Thus, free urine miR-1825 can serve as a potential non-invasive biological diagnostic marker for highly sensitive and specific for early detection of PCa and can significantly improve the specificity and sensitivity of PSA test, especially in men who fall in the intermediate PSA range of 4-10ng ml $^{-1}$.

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