Urinary hsv2-miR-H9 to hsa-miR-3659 ratio is an effective marker for discriminating prostate cancer from benign prostate hyperplasia in patients within the prostate-specific antigen grey zone

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Purpose: Tumor microRNAs (miRNAs) are released to biofluids directly or indirectly. Although urinary miRNAs are promising non-invasive biomarkers for the diagnosis of prostate cancer (PCa), their clinical application is challenging for technical reasons. We examined the efficacy of urinary hsv2-miR-H9 to hsa-miR-3659 ratio as a non-invasive diagnostic biomarker of PCa.

Materials and Methods: The expression of urinary miRNAs was quantified by real-time PCR in 116 samples from 53 patients with benign prostatic hyperplasia (BPH) and 63 patients with PCa. The miRNA expression ratio was calculated using an upregulated miRNA (hsv2-miR-H9) as the numerator and a downregulated miRNA (hsa-miR-3659) as the denominator.

Results: The urinary miR-H9 to miR-3659 ratio was significantly higher in PCa than in BPH controls (p<0.001). The diagnostic accuracy of the urinary miRNA expression ratio was comparable with that of prostate-specific antigen (PSA) (receiver operating characteristic [ROC] curve comparison, p=0.287). The area under the curve for urinary miRNA expression ratio was 0.862 and that for PSA was 0.642 in the “PSA gray zone” (3–10 ng/mL) (ROC curve comparison, p=0.034). The use of the urinary miRNA expression ratio would have prevented 70.6% of unnecessary prostate biopsies; however, 28.6% of PCa cases could be missed in patients within the PSA gray zone.

Conclusions: The expression ratio of urinary miR-H9 to miR-3659 could be a relevant non-invasive biomarker for PCa diagnosis, particularly for patients within the PSA gray zone.

Keywords: Biomarker; Diagnosis; Microarray analysis; MicroRNAs; Prostatic neoplasms
INTRODUCTION

Serum prostate-specific antigen (PSA) is the most commonly used biomarker for prostate cancer (PCa) screening. However, the lack of specificity of this serum marker results in a high rate of negative biopsies [1]. A large population of men present with elevated serum PSA levels but show negative findings on prostate biopsy, and the lack of an accurate diagnostic test for PCa needs to be addressed [2]. Although several biomarkers have been identified to overcome the limitations of PSA, most of these show poor reproducibility or their detection requires complex protocols that limit their use in clinical practice [3]. Thus, identifying a novel diagnostic biomarker of PCa with a reliable detection method and good reproducibility is important.

There is a growing trend toward exploring the use of non-invasive “liquid biopsies” to identify diagnostic biomarkers of PCa [4,5]. In particular, urinary biomarkers have attracted attention because urine can be obtained in a convenient and non-invasive manner. In addition, circulating cell-free DNAs and circulating RNAs (microRNAs [miRNAs], long non-coding RNAs, and messenger RNAs) originating from tumor-derived necrosis or apoptosis are released into the urethra through the prostatic ducts [6-8]. Urine-based liquid biopsies are also promising because they can capture a snapshot of tumor heterogeneity [9]. miRNAs are small, single-stranded, non-coding RNA molecules of approximately 18–22 nucleotides that regulate gene expression [10]. Despite the potential of liquid biopsy, its clinical implementation is hindered by poor specificity and sensitivity, lack of standardization, and poor reproducibility [11]. Relative quantification is the most commonly used quantitative real-time PCR method for identifying urinary markers of PCa; however, there is a lack of reliable endogenous reference genes in urine [12]. To overcome these limitations, ratio analysis, which measures the expression patterns of multiple genes simultaneously, was developed. Because this strategy does not require an endogenous reference gene, ratio analysis is a reliable method to improve the accuracy of real-time PCR data.

In previous work from our group, we performed a urine miRNA array and identified differentially expressed urinary miRNAs between PCa and benign prostatic hyperplasia (BPH) controls [13-15]. Two candidate urinary miRNAs (hsv2-miR-H9 and hsa-miR-3659) were sequentially validated in urine [13,14]. Initially, urinary miRNA expression was normalized to the total RNA concentration using the Quant-iT Ribo-Green RNA Reagent and Kit (Invitrogen, Grand Island, NY, USA) [13]. However, normalization using total RNA concentration limits the clinical application of novel urinary miRNA markers because of high costs and time-consuming procedures. In this study, we calculated the expression ratio of urinary miRNAs that are differentially expressed between PCa and BPH controls. The aim of the present study was to determine the diagnostic value of urinary hsv2-miR-H9 to hsa-miR-3659 ratio as a non-invasive diagnostic biomarker of PCa.

MATERIALS AND METHODS

1. Patients and samples

The study complied with the applicable laws and regulations, good clinical practice, and ethical principles described in the Declaration of Helsinki. The study protocol was approved by the Ethics Committee of Chungbuk National University (IRB approval number: GR2010-12-010). All patients provided written informed consent prior to enrollment in the study. Sample collection and analysis procedures were approved by the Institutional Review Board of Chungbuk National University.

A total of 116 urine samples from 53 patients with BPH and 63 patients with PCa were obtained from the National Biobank of Korea between January 2016 and July 2018. First morning voided urine was collected prior to surgery. For patients undergoing biopsy, spot urine samples were obtained immediately before the procedure. Urine samples were centrifuged at 2,500 rpm for 15 minutes, and the aliquoted supernatant was stored at -20°C until use. PCa urine samples were obtained from patients who underwent radical prostatectomy (RP) or palliative transurethral resection of the prostate (TURP) and who had histologically confirmed primary adenocarcinoma. Patients with BPH who underwent TURP were selected as controls. BPH controls with serum PSA levels ≥3 ng/mL underwent transrectal 12-core prostate biopsy prior to TURP to rule out the presence of cancer. All tissue specimens were examined by an experienced senior pathologist, and Gleason grades and TNM 2002 staging were assessed. Serum samples were obtained on the morning of the operation and stored at -80°C until sample preparation. Serum PSA concentrations of 3–10 ng/mL are defined as the “PSA gray zone”.

2. miRNA purification from urine

Urine samples were purified using a Genolution urine miRNA purification kit (Genolution Pharmaceuticals Inc., Seoul, Korea). A volume of 500 µL of supernatant from each urine sample was added to a tube containing the Genolution
proprietary miRNA separation solution and vortexed for 20 seconds. Next, 200 µL chloroform was added and vortexed for 10 seconds, followed by centrifugation at 13,000 rpm for 10 minutes at 4°C. A sample of 650 µL of the top aqueous phase was removed without disturbing the white precipitate and transferred to a new 1.5 mL tube, and 0.8 mL isopropanol was added, followed by centrifugation for 20 minutes at 15,000 rpm at 4°C. The solution was decanted by tilting the tube in the opposite direction of the expected RNA pellet, and 500 µL of 70% EtOH was added, followed by centrifugation for 20 minutes at 15,000 rpm at 4°C. After removing the leftover ethanol, the pellet was dissolved in 40 µL RNase-free water and stored at -80°C until use.

3. cDNA synthesis of urinary miRNAs

The concentration of isolated RNA was measured using the Quant-IT RiboGreen RNA Reagent and Kit (Invitrogen). The cDNA was synthesized using the Mir-X™ miRNA First Strand cDNA Synthesis Kit (Clontech, TAKARA Bio Inc., Otsu, Japan) according to the manufacturer’s protocol.

4. Real-time PCR

To quantify the expression of urinary hsv2-miR-H9 and hsa-miR-3659, RT-PCR was performed using a Rotor-Gene Q instrument (Qiagen, Valencia, CA, USA) and SYBR Premix EX Taq (TAKARA Bio Inc.) in micro-reaction tubes (Corbett Research, Mortlake, NSW, Australia) in a final volume of 10 µL. Chemically synthesized RNA oligonucleotides (Integrated DNA Technologies [IDT], Seoul, Korea) corresponding to the target miRNAs were used to generate standard curves. The standard curves ranged from $2.25 \times 10^5$ to $2.25 \times 10^8$. The following forward primers were used for amplifying miRNAs hsv2-miR-H9, 5′-CTCGGAGGTGGAGTCGCGGT-3′; hsa-miR-3659, 5′-TGAGTGTTGTCTACGAGGGCAG-3′. All samples were run in triplicate, and RT-PCR conditions were as described in the manufacturer’s protocol. Rotor-Gene Q software 2.3.1.49 (Qiagen) was used for capturing and analyzing spectral data.

5. Statistical analysis

The expression ratio of urinary miRNAs was calculated using an upregulated miRNA (hsv2-miR-H9) as the numerator and a downregulated miRNA (hsa-miR-3659) as the denominator (Fig. 1). The Mann–Whitney U-test was used to compare the expression ratio of urinary hsv2-miR-H9 to hsa-miR-3659 between the BPH and PCa groups. The diagnostic performance of PSA and urinary miRNA expression ratio was evaluated by plotting receiver operating characteristic (ROC) curves and by calculating the area under the curve (AUC). A comparison of ROC curves was performed to test the difference between the AUC values for expression ratio of urinary miRNAs and PSA. Multivariate logistic regression analysis was performed to adjust for confounding variables such as age at diagnosis and prostate size. Statistical analysis was performed using IBM SPSS 24.0 (IBM, Armonk, NY, USA) and MedCalc software ver. 15.8 (MedCalc Software, Mariakerke, Belgium). Results with p-values <0.05 were considered to be significant and all reported p-values are two-sided.

RESULTS

1. Baseline characteristics

The clinical and pathological characteristics of 53 patients with BPH and 63 patients with PCa are shown in Table 1. The mean age of the PCa group was 67.27±6.76 (standard deviation) years, and the median baseline PSA was 11.62 (interquartile range [IQR], 5.15–17.90) ng/mL. The BPH control group was significantly older (70.85±8.16 years) compared to the PCa group (p-value = 0.011). The median baseline PSA was 1.87 (0.96–4.97) ng/mL for the BPH group and 11.62 (5.15–17.90) ng/mL for the PCa group (p-value < 0.001). The median Gleason score was 7 (3+4) for the BPH group and 7 (4+3) for the PCa group (p-value = 0.011). The median stage was T2 for the BPH group and T3 for the PCa group (p-value = 0.001). The median operation was TURP for the BPH group and Radical prostatectomy for the PCa group (p-value = 0.001).

Table 1. Clinical characteristics of the study subjects

| Variable | BPH | PCa | p-value |
|----------|-----|-----|---------|
| Patient number | 53 | 63 |         |
| Age (y) | 70.85±8.16 | 67.27±6.76 | 0.011* |
| PSA (ng/mL) | 1.87 (0.96–4.97) | 11.62 (5.15–17.90) | <0.001† |
| Operation | TURP (100.0) | Radical prostatectomy (93.7) |         |
| Gleason score | 7 (3+4) | 41 (65.1) |         |
| Stage | T2 (46.0) | T3 (44.4) |         |
| T4 or metastasis | 6 (9.5) |          |         |

Values are presented as mean±standard deviation, median (interquartile range), or number (%). BPH, benign prostate hyperplasia; PCa, prostate cancer; PSA, prostate-specific antigen; TURP, transurethral resection of the prostate. p-values were obtained using the *Student’s t-test and the †Mann–Whitney U-test.

Fig. 1. Schematic of the expression ratio of urinary miRNAs. Currently, there are no reliable housekeeping genes in urine samples. Expression ratio analysis does not require normalization based on housekeeping genes in urine. miRNA, microRNA.
Non-invasive urinary diagnostic marker for prostate cancer

and had a lower baseline PSA (1.87 ng/mL; IQR, 0.96–4.97 ng/mL) than the PCa group (both p<0.05). Among the 63 PCa patients, 59 (93.7%) underwent RP and the remaining patients underwent palliative TURP. At the time of diagnosis, 29 (46.0%) patients had T2 stage, 28 (44.4%) had T3 stage, and 6 (9.5%) had T4 stage or metastasis. In the PCa group, 57 (90.5%) patients had a Gleason score of 7 (3+4) or 7 (4+3).

2. Diagnostic value of urinary hsv2-miR-H9 to hsa-miR-3659 expression ratio

The expression ratio of urinary miR-H9 to miR-3659 was significantly higher in the PCa group (median, 17.27; IQR, 14.52–19.97) than in BPH controls (median, 13.45; IQR, 11.44–14.93) (p<0.001) (Fig. 2A). After classifying patients by baseline PSA value, the expression ratio of urinary miR-H9 to miR-3659 differed significantly between the PCa and BPH groups in patients within the PSA gray zone (3–10 ng/mL) (Fig. 2C) and in those with PSA >10 ng/mL (Fig. 2D) (both p<0.05); there was no significant difference in patients with PSA <3 ng/mL (Fig. 2B). Multivariate logistic regression analysis revealed that higher expression ratio of urinary miRNA was significantly associated with the diagnosis of PCa (odds ratios 1.479, 95% confidence interval [CI] 1.256–1.740, p<0.001; Table 2).

The AUC of the expression ratio of urinary miR-H9 to miR-3659 was 0.803 (95% CI, 0.725–0.881) (p<0.001), which was comparable with that of PSA (AUC, 0.857; 95% CI, 0.790–0.925) (p<0.001) (Fig. 3A). Comparison of ROC curves did not show significant differences between the expression ratio of urinary miR-H9 to miR-3659 and PSA value (p=0.287). Subgroup analysis showed that the AUC of urinary miR-H9 to miR-3659 ratio was significantly higher than that of the PSA test in patients within the PSA gray zone (ROC curve

| Variable                      | Odds ratio (95% confidence interval) | p-value |
|-------------------------------|-------------------------------------|---------|
| Age at diagnosis (continuous) | 0.952 (0.894–1.013)                 | 0.121   |
| Prostate size (continuous)    | 1.001 (0.969–1.034)                 | 0.941   |
| Urinary miRNAs expression ratio (continuous) | 1.479 (1.256–1.740) | <0.001 |

Fig. 2. The expression ratio of urinary miR-H9 to miR-3659 (A) in the total cohort, (B) in patients with PSA <3 ng/mL, (C) in patients within the PSA gray zone (3–10 ng/mL), and (D) in patients with PSA >10 ng/mL. miRNA, microRNA; BPH, benign prostate hyperplasia; PCa, prostate cancer; PSA, prostate-specific antigen. p-value was determined by Mann–Whitney U-test.
comparison, p=0.034) (Fig. 3B).

Regarding pathologic characteristics, there was no significant association between the expression ratio of urinary miRNAs and pathological characteristics such as stage or Gleason score in patients who underwent RP (data not shown).

The optimal cut-off value for the expression ratio of urinary miR-H9 to miR-3659, which provided optimal sensitivity and specificity for PCa detection, was 14.7 in those within the PSA gray zone (sensitivity and specificity, 71.4% and 70.0%, respectively). Application of this cut-off value would reduce 70.6% of unnecessary prostate biopsies, but 28.6% of PCa could be missed in patients within the PSA gray zone.

**DISCUSSION**

Here, we determined the diagnostic value of urinary hsv2-miR-H9 to hsa-miR-3659 expression ratio as a non-invasive diagnostic marker of PCa. The urinary hsv2-miR-H9 to hsa-miR-3659 ratio has comparable diagnostic power to the PSA test for discriminating PCa from BPH and showed superior diagnostic accuracy in the “PSA gray zone”.

Liquid biopsy is a topic of emerging interest because of its potential for cancer diagnosis and as a prognostic biomarker [16]. It has opened a plethora of clinical opportunities because tumor-derived material (circulating tumor cells, cell-free DNA, proteins, metabolites, extracellular vesicles, and cell-free RNA) is released into different biofluids such as blood, urine, saliva, cerebrospinal fluid, and pleural effusion [17]. Among various biofluids, urine has emerged as a promising material for minimally-invasive diagnostic detection of urologic cancer. Analysis of urine is a non-invasive method that provides abundant and readily accessible sample material [8]. In addition, urine represents an abundant source of tumor-derived material without background noise [18]. The aim of the present study was to identify a non-invasive urine biomarker for PCa diagnosis with particular focus on urinary miRNAs. Although RNAs are highly unstable molecules, miRNAs are stable in plasma and serum and resistant to RNase activity, as well as extreme pH and multiple freeze–thaw cycles [19]. Extracellular miRNAs are loaded into microvesicles and are protected from RNases [20,21]. Circulating blood miRNAs have been investigated as novel biomarkers, and urinary miRNAs have shown potential as markers of genitourologic malignancies. Recently, Mall et al. [22] investigated the stability of urinary miRNAs under various storage conditions. Despite modest degradation, sufficient miRNA for quantitative analysis was detected after a 5-day period of storage at room temperature and after ten freeze–thaw cycles. Besides instability and low abundance of urinary miRNAs, another technical hurdle is that appropriate reference genes are needed for comparison with target genes [12]. Serum miRNA expression is usually normalized using the expression of housekeeping genes such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-globin, U6, and β-actin [23]. Appropriate genes for normalization of miRNA expression in urine remain to be identified [24]. Concentration-based quantification methods have also

| Index                  | AUC   | 95% CI          | p-value |
|------------------------|-------|-----------------|---------|
| PSA                    | 0.857 | 0.790–0.925     | <0.001  |
| Urinary miRNA ratio    | 0.803 | 0.725–0.881     | <0.001  |

**Fig. 3.** Receiver operating characteristic curve for discriminating PCa from BPH controls (A) for the total cohort (n=116) and (B) for patients within the PSA gray zone (3–10 ng/mL) (n=40). miRNA, microRNA; BPH, benign prostate hyperplasia; PCa, prostate cancer; AUC, area under the curve; CI, confidence interval.
been used for normalization of miRNA expression in urine; however, normalization using total RNA concentration is a costly and time-consuming procedure. In the present study, we calculated the ratio of the expression of two genes, which does not require an internal control. A similar approach was previously used by our group to analyze urinary cell-free DNA and urinary miRNAs [14,25,26]. Analysis of the ratio of urinary miRNAs is a relatively simple method that shows good reproducibility and could thus be applied in clinical practice. In this study, we showed that the diagnostic accuracy of the expression ratio of our novel urinary miRNAs was comparable to that of PSA. Furthermore, the diagnostic performance of the urinary miRNA expression ratio was superior to that of PSA in patients within the PSA gray zone. The expression ratio of urinary miR-H9 to miR-3659, alone or together with serum PSA, is a relevant non-invasive biomarker for PCa diagnosis.

The present study had both limitations and strengths. Limitations include the single center retrospective design, which could lead to selection bias. The small sample numbers may also reduce statistical power. There was no significant association between the urinary miRNA ratio and the pathologic aggressiveness of PCa. The negative findings can be attributed to the small cohort of patients who underwent RP (n=59). Our study should be viewed as exploratory and preliminary investigation. Further collaborative studies are required to validate the diagnostic accuracy and clinical application of the expression ratio of urinary miR-H9 to miR-3659 as a non-invasive diagnostic and prognostic biomarker of PCa. Despite the above limitations, the present study represents an important step toward the practical use of urinary miRNAs for PCa diagnosis.

CONCLUSIONS

The expression ratio of urinary miR-H9 to miR-3659 could be a relevant non-invasive biomarker for PCa diagnosis, particularly in patients within the PSA gray zone. Ratio analysis of urinary miRNA is a relatively simple method that shows good reproducibility and does not require correction with an internal control. Implementation of the urinary miRNA ratio represents a promising alternative or adjunct to serum PSA tests for PCa diagnosis.

CONFLICTS OF INTEREST

The authors have nothing to disclose.

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AUTHORS’ CONTRIBUTIONS

Research conception and design: Ho Won Kang, Young Joon Byun, Seok Joong Yun, and Wun-Jae Kim. Data acquisition: Sung Min Moon, Kyeong Kim, Xuan-Mei Piao, and Chuang-Ming Zheng. Statistical analysis: Ho Won Kang and Young Joon Byun. Data analysis and interpretation: Won Tae Kim and Yong-June Kim. Drafting of the manuscript: Ho Won Kang and Young Joon Byun. Critical revision of manuscript: Sung-Kwon Moon, Yong Hyun Choi, Sang-Chool Lee, Seok Joong Yun, and Wun-Jae Kim. Administrative, technical, or material support: Seok Joong Yun and Wun-Jae Kim. Supervision: Seok Joong Yun and Wun-Jae Kim. Approval of the final manuscript: All authors.

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