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Evaluation of Urinary hsa-miR-21-5p Expression as a Non-Invasive Biomarker for Prostate Cancer

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Abstract

Background: Prostate cancer is one of the most common malignancies among men worldwide. Prostate-specific antigen (PSA) testing and prostate biopsy are commonly used for diagnosis. However, these methods may be invasive and can lead to delayed detection in some cases. This study aimed to evaluate the expression of hsa-miR-21-5p in urine samples from prostate cancer patients compared with healthy individuals to assess its potential as a non-invasive biomarker.

Methods: This observational study used a cross-sectional design and was conducted in Yogyakarta, Indonesia, between October and December 2018. Expression profiling of hsa-miR-21-5p was performed using quantitative real-time polymerase chain reaction (qRT-PCR) in urine samples obtained from 12 prostate cancer patients and 8 healthy controls. Relative expression levels were analyzed using GenEx software and calculated using the Livak (2^−ΔΔCt) method.

Results: The age of prostate cancer patients ranged from 60 to 83 years. In contrast, the healthy control group had a mean age of 53.88 ±2.59 years (range 50–58 years). A slight decrease in hsa-miR-21-5p expression was observed in urine samples from prostate cancer patients compared with healthy controls (fold change=0.7; p>0.05).

Conclusions: Expression of hsa-miR-21-5p shows a tendency toward downregulation in urine samples of prostate cancer patients, however, no statistically significant difference is identified. Further studies with larger sample sizes are required to clarify its potential role as a non-invasive biomarker for prostate cancer detection.

Keywords: Biomarker, hsa-miR-21-5p, prostate cancer, non-invasive screening, urine.

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Introduction

Prostate cancer is one of the most common malignancies affecting male worldwide and represents a significant global health burden due to its often late diagnosis and associated poor prognosis.[1–3] Current diagnostic methods, such as prostate-specific antigen (PSA) testing and tissue biopsy, have important limitations. PSA lacks adequate specificity and sensitivity, leading to false-positive results and potential overtreatment, whereas biopsy is invasive and not suitable for routine screening.[4] These limitations underscore the need for alternative, accurate, and non-invasive diagnostic strategies to improve early detection and clinical outcomes.

MicroRNAs (miRNAs), small non-coding RNAs that regulate gene expression at the post-transcriptional level, have emerged as promising biomarkers in cancer. Their high stability in body fluids and involvement in key cellular processes, including proliferation, apoptosis, and carcinogenesis, make them attractive candidates for non-invasive detection.[5–7] Among these, hsa-miR-21-5p has been widely recognized as an androgen-regulated 'oncomiR' with a pivotal role in prostate cancer pathogenesis. It promotes tumor progression primarily by targeting tumor suppressor genes such as phosphatase and tensin homolog (PTEN), thereby activating the PI3K/Akt signaling pathway, which drives cell proliferation and inhibits apoptosis.

Recent evidence, including meta-analyses and clinical studies published between 2020 and 2024, has consistently highlighted the diagnostic and prognostic value of miR-21-5p. Elevated expression levels have been associated with higher Gleason scores, advanced disease stages, and the development of castration-resistant prostate cancer (CRPC). Furthermore, its presence in urinary exosomes highlights its potential as a non-invasive biomarker, offering advantages over conventional serum-based testing.[8–12]

Urine represents a particularly suitable biological matrix for liquid biopsy in prostate cance. Due to the anatomical proximity of the prostate to the urethra, urine contains prostate-derived secretions and exfoliated cells, potentially providing a more specific reflection of the tumor microenvironment compared to blood. In addition, urine collection is non-invasive, cost-effective, and easily repeatable, thereby improving patient compliance and feasibility for large-scale screening.[13]

Despite growing evidence supporting miR-21-5p as a biomarker, data on its expression in urine samples remain limited, particularly using quantitative real-time PCR (RT-qPCR) approaches. Therefore, this study aimed to evaluate the expression of urinary hsa-miR-21-5p in prostate cancer patients and healthy individuals to assess its potential as a non-invasive screening biomarker. By focusing on a minimally invasive method with high translational potential, this study seeks to contribute to the development of improved early detection strategies for prostate cancer.

Methods

This analytical observational study employed a cross-sectional design and was conducted from October to December 2018 involving all positive prostate cancer patients in the Special Region of Yogyakarta. The study observed the expression of hsa-miR-21-5p in urine samples from prostate cancer patients and healthy control. Diagnosis of prostate cancer was confirmed through histopathological examination following transrectal ultrasound (TRUS)-guided biopsy. Furthermore, eligible patients had clinically elevated prostate-specific antigen (PSA) levels (>4 ng/mL) and/or abnormal digital rectal examination (DRE) findings, with biopsy-confirmed adenocarcinoma and documented Gleason score.

The control group consisted of healthy individuals with no history of malignancies or current urinary tract infections. Written informed consent was collected from all participants prior to sample collection. Ethical approval was obtained from the Faculty of Medicine, Public Health, and Nursing, Gadjah Mada University, Indonesia (No. KE/FK/0956/EC/2018).

Morning urine samples were collected from all participants. Midstream urine was obtained from non-catheterized patients to minimize contamination, while catheterized samples were collected using sterile syringe aspiration after temporarily stopping irrigation. Urine samples (15 mL) were transported to the laboratory within 4 hours and centrifuged at 10,000 × g for 10 minutes to remove debris.

Exosomes were isolated using the miRCURY™ Exosome Isolation Kit-Cells, urine, and CSF (EXIQON) according to the manufacturer's protocol. Urine sample (15 mL) was collected in a tube and centrifuged at 10,000 × g for 5 minutes. The supernatant (1 mL) was then transferred to a new tube. Precipitation Buffer B (400 µL) was added, and the mixture was gently inverted and vortexed for 5 seconds. The sample was subsequentlyincubated at 4°C for 60 minutes. After incubation, the sample was centrifuged again at 10,000 × g for 30 minutes at 20°C. The supernatant was carefully discarded, and the sample was briefly centrifuged again for 5 seconds to remove any residual liquid, after which the remaining supernatant was discarded.

Total RNA was extracted from isolated exosomes using the provided lysis and purification system. First, 350 µL of Lysis Solution was added to the sample and vortexed for 15 seconds until the solution became transparent. Next, 200 µL of 96% ethanol was added, and the mixture was vortexed for 10 seconds. The sample (550 µL) was then transferred into a Mini Spin Column placed in a collection tube, and additional 96% ethanol was added to reach a final volume of 600 µL. The column was centrifuged at 3,500 × g for 1 minute, and the flow-through was discarded. The column was washed three times by adding 400 µL of Wash Solution followed by centrifugation at 14,000 × g for 1 minute, with the flow-through discarded after each step. A final dry centrifugation step was performed at 14,000 × g for 2 minutes to remove residual buffer. The spin column was then transferred to a new 1.5 mL tube. RNA was eluted by adding 50 µL of Elution Buffer directly onto the membrane, followed by centrifugation

at 200 × g for 2 minutes and a subsequent centrifugation at 14,000 × g for 1 minute. The isolated RNA was immediately stored at −80°C.

For cDNA synthesis, frozen RNA samples were thawed on ice, vortexed, and briefly centrifuged. cDNA was synthesized using the Universal cDNA Synthesis kit II, 8-64 rxns (EXIQON). All reagents, including 5× reaction buffer, nuclease-free water, and Sp6 spike-in, were thawed on ice, vortexed, and briefly centrifuged. The enzyme mix was gently mixed and kept on ice. A master mix was prepared containing 2 µL of 5× reaction buffer, 4.5 µL of nuclease-free water, 1 µL of enzyme mix, and 0.5 µL of Sp6 spike-in per reaction. For each reaction, 2 µL of RNA template was combined with 8 µL of the master mix and resuspended using a micropipette. Reverse transcription was carried out using a C1000 Thermal Cycler (Bio-Rad) with the following conditions: incubation at 42°C for 60 minutes, enzyme inactivation at 95°C for 5 minutes, and cooling at 4°C. The synthesized cDNA was stored at −20°C.

Quantitative PCR (qPCR) was conducted using the ExiLENT SYBR Green Master Mix (EXIQON) and target primers for hsa-miR-21-5p. The synthesized cDNA was thawed on ice, vortexed, spun down, and diluted 1:40 with nuclease-free water (1 µL cDNA in 40 µL nuclease-free water). The PCR Master mix and Primer mix were thawed at room temperature, vortexed, and spun down. A reaction mix was prepared by combining 5 µL of PCR Master Mix and 1 µL of Primer Mix per reaction Subsequently, 4 µL of diluted cDNA was added and mixed thoroughly. Amplification was performed using a CFX 96 Real-Time System (Bio-Rad) under the following conditions: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds and annealing/extension at 58°C for 1 minute (ramp rate 1.6°C/s, with optical reading). Melting curve analysis was conducted from 60°C to 95°C to confirm amplification specificity. miRNA expression levels were quantified using the quantification cycle (Cq) method, with miR-16 serving as the endogenous reference gene for normalization.

Data were analyzed using Bio-Rad CFX Manager Software. Data normality was assessed prior to statistical analysis. Relative expression levels of hsa-miR-21-5p in urine samples from prostate cancer patients were calculated using the Livak (2^−ΔΔCt) method, with miR-16 as the reference gene, to determine fold-change values.[14,15] Differences between the two groups were evaluated using an independent t-test. A p-value of <0.05 was considered statistically significant.

Results

A total of 20 participants were included, comprising 12 prostate cancer patients and 8 healthy controls. The age of prostate cancer patients ranged from 60 to 83 years. The healthy control group had a mean age of 53.88±2.59 years (range 50–58 years).

The expression levels of hsa-miR-21-5p and hsa-miR-16-5p were assessed in prostate cancer patients (n=12) and healthy individuals (n=8) using quantitative PCR (Table 1 and 2). The Cq values of both miRNAs showed variability across samples in both groups.

Normality testing using the Shapiro–Wilk test showed that the data were normally

Table 1 Cq Values of hsa-miR-21-5p and hsa-miR-16-5p in Prostate Cancer Patients (n=12)

Sample	Cq Value	
	hsa-miR-21-5p	hsa-miR-16-5p
P1	26.01	29.76
P2	28.42	30.29
P3	25.86	27.42
P4	27.27	26.66
P5	23.47	24.13
P6	23.32	28.17
P7	32.38	34.41
P8	30.31	34.22
P9	23.61	22.65
P10	21.67	22.43
P11	22.74	24.72
P12	25.16	27.95

Note: Cq= cycle of quantification

Table 2 Cq Values of hsa-miR-21-5p and Hsa-miR-16-5p in Healthy Individuals (n=8)

Sample	Cq Value	
	hsa-miR-21-5p	hsa-miR-16-5p
N1	24.11	27.31
N2	23.52	26.78
N3	25.48	27.22
N4	26.79	30.19
N5	28.69	28.77
N6	25.25	27.20
N7	27.42	29.30
N8	29.65	32.29

Note: Cq= cycle of quantification

Table 3 Relative Quantification Test of hsa-miR-21-5p Expression Using the Livak ($2^{-}\Delta\Delta Cq$) Method

Group	Mean Cq		ΔCq (Target - Reference)	ΔΔCq (ΔCqtest– ΔCqcontrol)	Fold Change (2–ΔΔCq)
	Target : miR-21-5p	Reference: miR-16-5p			
Healthy Individuals (control)	26.36	28.63	−2.27	0.00*	1.00
Prostate cancer patients	25.85	27.73	−1.88	0.39	0.765

Notes: *Calibrator, Cq= cycle of quantification

distributed (p>0.05). Relative expression levels were calculated using the Livak (2^−ΔΔCt) method, with the healthy group used as the calibrator.

As shown in Table 3, the mean Cq value of hsa-miR-21-5p was slightly lower in prostate cancer patients compared with healthy individuals (25.85 vs 26.36), indicating relatively higher expression. Similarly, the mean Cq value of the reference gene hsa-miR-16-5p was lower in prostate cancer patients than in controls (27.73 vs 28.63). The calculated ΔCq values were −1.88 for prostate cancer patients and −2.27 for healthy individuals.

Relative quantification using the Livak ($2^{-}\Delta\Delta Cq$) method demonstrated a ΔΔCq value of 0.39, corresponding to a fold change of 0.765 in prostate cancer patients relative to healthy controls. This finding indicates that hsa-miR-21-5p expression was reduced in prostate cancer patients compared with healthy individuals. However, this difference was not statistically significant (p=0.342).

Discussion

This study demonstrated the feasibility of isolating and detecting microRNAs (miRNAs), specifically hsa-miR-21-5p, from urine samples using a non-invasive workflow. The detection of miRNAs has been widely recognized as an important tool in the prognosis of prostate cancer.

Previous studies have shown that prostate cancer prognosis can be assessed through the expression profiles of specific miRNAs.[9,16,17] A recent meta-analysis further reported that elevated miR-21 expression is significantly associated with advanced clinical stage, higher Gleason scores, and high-risk groups in prostate cancer.[9] These findings support the growing body of evidence suggesting that Hsahsa-miR-21-5p has potential as a biomarker for prostate cancer prognosis and disease stratification.

In contrast to previous findings reporting upregulation of miR-21 in prostate cancer tissue and plasma,[9] this study observed a slight, non-significant downregulation of hsa-miR-21-5p in prostate cancer patients compared with healthy controls. The lack of statistical significance is most likely attributable to the limited sample size and high inter-individual variability in urinary exosome concentration. Urinary miRNA profiling is particularly susceptible to variations in hydration status and renal function, which may dilute biomarker concentrations, leading to false-negative results or non-significant findings in small cohorts.[16]

Furthermore, the observed downregulation also contrasts with the expected oncogenic overexpression of miR-21. One plausible explanation lies in the normalization strategy. In this study, miR-16-5p was used as a single reference gene. However, reliance on a single endogenous control in urine-based assays may be insufficient. Previous studies have demonstrated that miR-16 expression can fluctuate in biofluids due to factors such as hemolysis or inflammation, and the use of multiple reference genes has been recommended to improve quantification accuracy.[17,18,19] If miR-16 expression was inadvertently elevated in the cancer group due to non-tumor-related factors, the relative quantification of miR-21 may have been artificially downregulated. Additionally, urinary miRNA profiles may not directly reflect tissue expression due to selective exosomal packaging mechanisms.[20]

Despite the lack of statistical significance, the detection of hsa-miR-21-5p in urine highlights its potential as a non-invasive biomarker. The measurable Cq values confirm its presence in urinary exosomes, supporting the technical validity of the detection method. Future studies should include larger, multi-center cohorts and apply validated panels of multiple reference genes to minimize technical variability and improve reproducibility.

In conclusion, urinary hsa-miR-21-5p can be successfully detected using a non-invasive approach however, its expression is not significantly different between prostate

cancer patients and healthy individuals in this study. With methodological optimization and validation in larger cohorts, urinary miR-21-5p may still serve as a complementary biomarker alongside prostate-specific antigen (PSA) to improve prostate cancer screening and reduce unnecessary invasive procedures.

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Author's Contributions
RPM contributed to conceptualization, methodology, validation, investigation, and resources. AFR contributed to software, formal analysis, data curation, and writing original draft.

Conflict of Interest
The authors declare no conflicts of interest.

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