RESEARCH ARTICLE

Editorial Process: Submission:04/30/2021 Acceptance:03/23/2023

miRNA-21 as High Potential Prostate Cancer Biomarker in Prostate Cancer Patients in Indonesia

Regina R Gunawan[1]*, Indwiani Astuti[2], H Raden Danarto[3]

Abstract

Objective: This study aimed to explore the diagnostic performance of miRNA-21 to differentiate between Prostate Cancer (PCa) and Benign Prostatic Hyperplasia (BPH) patients in Indonesia. **Methods:** Urine samples were collected from each PCa and BPH patient. miRNA-21 relative expression against the reference gene was analyzed and compared between the two. miRNA expression was then analyzed using the comparative quantification method to find the fold change. miR-21 validity in identifying PCa patients was performed by quantifying the sensitivity and specificity using samples in this study. **Result:** The results of this study indicated that miRNA-21 relative expression against miRNA-16 in PCa and BPH showed 12.95 differences in fold change. Moreover, using prostate biopsy as the gold standard to differentiate PCa and BPH, miRNA-21 Cq expression has 100% sensitivity and 75% specificity in differentiating the two. **Conclusion:** miRNA-21 relative expression can be used to discriminate PCa from BPH by using a urine sample. Furthermore, the expression of miR-21 has higher sensitivity than PSA; therefore, miR-21 has a high potential to be analyzed and developed further for clinical diagnosis of prostate cancer.

Keywords: Benign prostate hyperplasia- biomarker- miRNA-21- prostate cancer

Asian Pac J Cancer Prev, **24 (3)**, 1095-1099

Introduction

MicroRNA (miRNA) is a small non-coding RNA that regulates the gene expression through complementary bond towards mRNA targets and cause mRNA degradation. miRNA works as endogenous epigenetic regulators of gene expression, and it can promote or suppress cell proliferation; thus, studies focus more on finding the role of miRNA in carcinogenesis. One of the earliest known miRNA with an oncogenic role termed oncomiR is miR-21. miR-21 is overexpressed in cancers, including lung, breast, stomach, prostate, colon and pancreatic cancer (Sánchez et al., 2020). miR-21 is believed to promote proliferation, anti-apoptosis, cell cycle progression and invasion of tumor cells by down-regulating mammary serine protease inhibitor (Maspin), Fas ligand (Fas-L), a reversion-induced cysteine-rich protein with Kazal motifs (RECK), programmed cell death protein 4 (PDCD4), Sprouty homolog 1/2 (Spry 1/2), phosphatase and tensin homologue deleted on chromosome 10 (PTEN), tissue inhibitor of metalloproteinases 3 (TIMP3), acidic nuclear phosphoprotein 32 family member (ANP32A), tropomyosin 1 (TPM1), forkhead box 01 (FOXO1), RhoB, Cdc25a and also sec23a (Feng and Tsau, 2016; Li et al., 2016; Wu et al., 2017).

miRNA expression levels in cancer may offer another value of miRNA as a biomarker in cancer diagnosis because it is differentially expressed in cancer and normal tissues (Ahmad et al., 2013). One of the most common malignancies in men with dire need of novel biomarkers is prostate cancer (PCa). PCa diagnosis is confirmed by biopsy, a procedure to take prostate gland tissue as a sample to see its histopathological appearance. Prostate biopsy is offered when medical professionals find abnormalities during digital rectal examination or increased Prostate Specific Antigen (PSA) serum concentration (> 4 ng/mL). PSA is an organ-specific indicator but not cancer-specific. The PSA threshold used in suspecting PCa diagnosis is 4.0 ng/mL, having 20.5% sensitivity and 93.8% specificity (Nogueira et al., 2009). Because of this low sensitivity, PCa screening using PSA has been causing overdiagnosis and an increase in unnecessary biopsy indications to identify PCa, so the need for a new biomarker has been proposed to overcome this issue (Dall'Era et al., 2012; Nogueira et al., 2009). Hsa-miR-21-5p marker has been identified in the urine of Prostate Cancer (Pca) and Benign Prostatic Hyperplasia (BPH) patients (Kristanto, 2017). This research planned to explore the diagnostic performance of miR-21 to differentiate PCa and BPH patients.

*[1]Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia. [2]Department of Pharmacology and Therapy, Universitas Gadjah Mada, Yogyakarta, Indonesia. [3]Department of Surgery, Division of Urology, Sardjito General Hospital, Indonesia. *For Correspondence: reginagunawan02@gmail.com*

Materials and Methods

Sample Collection

Urine samples were collected from PCa and BPH patients in the morning. In patients without a urinary catheter, 15 mL mid-stream urine samples were collected independently, while in patients with a urinary catheter, the urine samples were collected using a syringe. Upon collection, the samples were directly sent to the laboratory to be analyzed in under 6 hours.

Laboratory Methods

Exosome isolation was performed with miRCURY™ Exosome Isolation Kit - Cells, urine and CSF (EXIQON) protocol. cDNA synthesis was done using the Universal cDNA Synthesis Kit II, 8-64 rxns (EXIQON) protocol. Synthesized cDNA was inserted into qPCR along with the ExiLENT SYBR Green Master Mix 2.5 mL (EXIQON) Kit, target primer microRNA Hsa-miR-16-5p, and Hsa-miR-21-5p. Data obtained from qPCR was then grouped into PCa and BPH groups.

Statistical Analysis

The miRNA data were analyzed with Biorad CFX Manager Software to find out the Quantification cycle (Cq), amplification curve, and melting curve from the qPCR result. The differences in Hsa-miR-21-5p expression in PCa and BPH urine were compared to the reference gene expression level with Livak equation:

$$\Delta CT(test)=CT(target,test)-CT(ref,test)\ \Delta CT$$

$$(calibrator)=CT(target,calibrator)-CT(ref,$$

$$calibrator)$$

$$\Delta\Delta CT=CT\ test-CT\ calibrator$$

$$Fold\ change=2-\Delta\Delta CT$$

Notes

: Cycles needed to reach the threshold

Δ, Relative quantification

target test, Hsa-miR-21-5p

reference gene, Hsa-miR-16-5p

Normality testing was performed on the acquired data to analyze both groups of patients. In addition, the data were also analyzed using an Independent T-test with Genex software. Statistical test results were regarded as significant if the p-value was < 0.05.

Table 1. Relative Quantification Test with Livak Method between PCa and BPH Group

Variable	Group	Obs	Mean Cq*
Hsa-miR-21-5p	PCa	20	28.204
Hsa-miR-21-5p	BPH	20	32.193
Hsa-miR-16-5p	PCa	20	30.603
Hsa-miR-16-5p	BPH	20	30.898

*Cq, quantification cycle, mean Cq units are cycles

Results

Characteristics of Sample Population

This study enrolled 40 patients

20 Prostate Cancer patients and 20 Benign Prostatic Hyperplasia patients. The mean age of PCa patients was 65.9 + 8.162 (range, 50 – 78) years, and the mean age of BPH patients was 62.95 + 10.61 (range, 44 – 88) years. Of the 20 prostate cancer patients, 7 patients (35%) had a total Gleason Score of 10, 3 patients (15%) had a total Gleason Score of 9, 7 patients (35%) had a total Gleason Score of 8, and the rest 3 patients (15%) each had a total Gleason Score of 7, 5, and 4. At the time of enrollment, 8 PCa patients (40%) had a clinical manifestation of metastasis in their bones and/or organ, proven by bone surveys and CT scans.

Normality Testing of Data Group

Normality testing (Shapiro-Wilk) performed over Cq of Hsa-miR-16-5p in PCa and BPH patients showed that the data were normally distributed in both groups. The independent T-test was then used to find the difference in Hsa-miR-16-5p expression in both groups. The data had a significance level of 0.411 (> 0.05), meaning that the null hypothesis was accepted, and there was no significant difference between the average Cq Hsa-miR-16-5p expression between PCa patients and BPH patients.

Hsa-miR-21-5p Cq in both PCA and BPH patients were also tested for their normality using Shapiro-Wilk Test. The Hsa-miR-21-5p Cq of PCa and BPH patients had a significance of 0.095 and 0.713, respectively. These results confirmed that both samples were distributed normally. Furthermore, independent T-test results showed that the data had a significance of 0.00 (<0.05), meaning that the null hypothesis was rejected, and there was a significant difference between the average Cq Hsa-miR-21-5p expressions between PCa patients and BPH patients.

Hsa-miR-21-5p relative expression in PCa and BPH group

Hsa-miR-21-5p relative expression against Hsa-miR-16-5p quantified using the Livak equation

Table 2. Relative Quantification Test with Livak Method between PCa with and without Metastasis

Variable	Group	Obs	Mean Cq*
Hsa-miR-21-5p	PCa without metastasis	12	28.15
Hsa-miR-21-5p	PCa with metastasis	8	28.283
Hsa-miR-16-5p	PCa without metastasis	12	30.54
Hsa-miR-16-5p	PCa with metastasis	8	30.698

*Cq, quantification cycle, mean Cq units are cycles

Table 3. Relative Quantification Test with Livak Method between PCa with and without Metastasis

Variable	Prostate Cancer	Benign Prostate Hyperplasia
Cq miR-21 < 30 cycles	20	5
Cq miR-21 > 30 cycles	0	15

*Cq, quantification cycle, mean Cq units are cycles

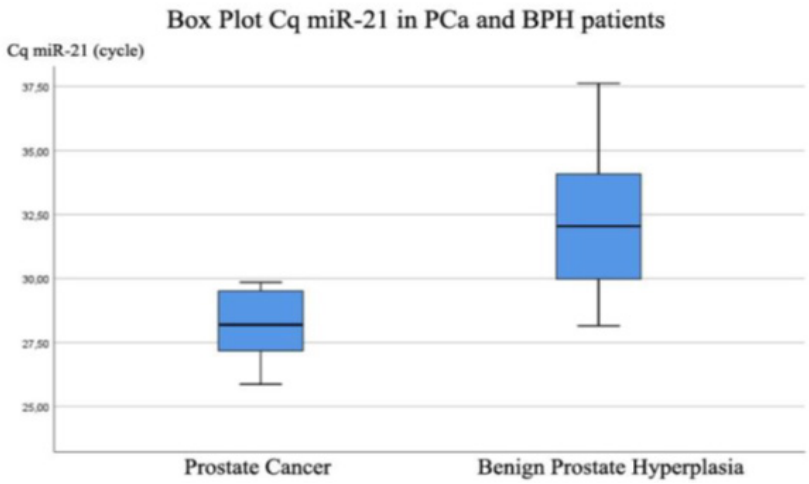

Figure 1. Box Plot of Hsa-miR-21-5p expression in Prostate Cancer and Benign Prostate Hyperplasia Patients

showed 12.9488-fold change differences between PCa and BPH group. The difference became more evident by arranging the miR-21 expression in a box plot and scatter plot (Figure 1 and 2). There was an observable difference between the two, with the Cq miR-21 values of BPH patients being generally higher than the Cq miR-21 values of PCa patients.

Hsa-miR-21-5p relative expression in Prostate Cancer group with and without Metastasis

Hsa-miR-21-5p expression in the PCa group was further analyzed by separating patients with bone and/or organ metastasis from those without metastasis (Table 2). The relative expression between the two groups was 1.017, meaning there was nearly no difference in Hsa-miR-21-5p

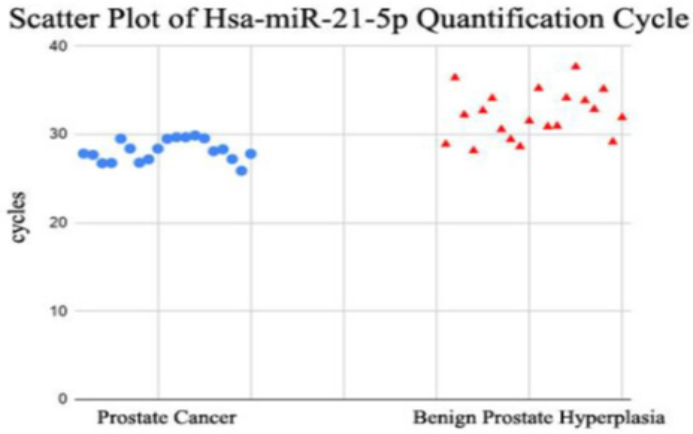

Figure 2. Scatter Plot of Hsa-miR-21-5p expression in Prostate Cancer and Benign Prostate Hyperplasia Patients

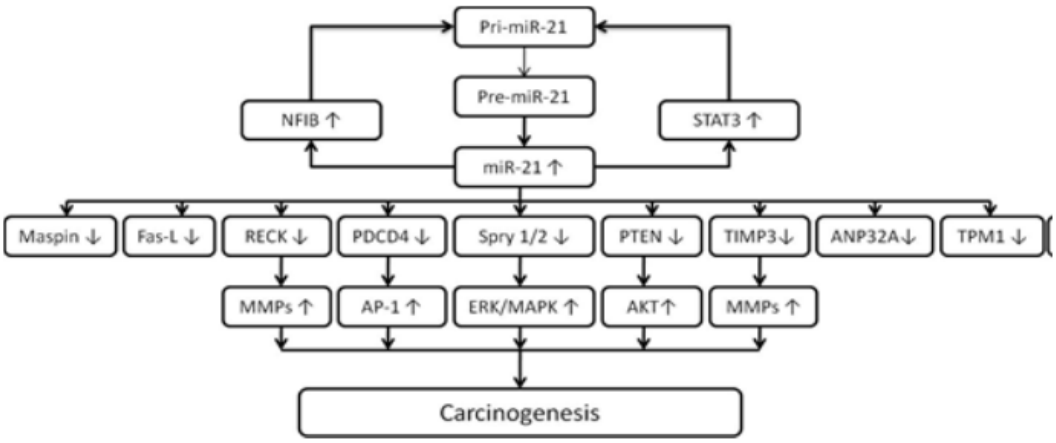

Figure 3. Path of miR-21 Involvement in Carcinogenesis (Feng et Tsao, 2016)

expression in PCa patients with and without metastasis.

Sensitivity and Specificity of Hsa-miR-21-5p expression

The utility of a diagnostic test is usually explained with several terms, such as sensitivity, specificity, positive predictive value, and negative predictive value. Utilizing the biopsy as the gold standard, miR-21 expression in differentiating PCa and BPH patients had 100% sensitivity and 75% specificity (Table 3).

Discussion

This study involved data from 40 samples, consisting of 20 PCa patients and 20 BPH patients, where miRNA expressions in each sample were then measured. miRNA expressions act as a potential biomarker to differentiate prostate cancer from BPH. Hsa-miR-16-5p was chosen as the reference gene in this study because Hsa-miR-16-5p is a miRNA expression that acts as a housekeeping gene, a gene needed to maintain the basic cell functions and can be expressed in all human cells, whether in normal or pathophysiological conditions (Lange et al., 2017). To prove that Hsa-miR-16-5p can be used as a reference gene, Hsa-miR-16-5p expressions levels were tested using an Independent T-test to prove that there was no significant difference between Hsa-miR-16-5p expressions in both groups of patients.

The difference between Hsa-miR-21-5p expressions and Hsa-miR-16-5p expressions (delta Cq) in PCa patients and BPH patients were processed using a quantifying comparative method or delta delta Cq proposed by Livak and Schmittgen, 2001. The results of the quantifying test using the aforementioned method can be seen in table 1, where the fold change value in PCa patients is 12.9488 times higher compared to BPH patients. Therefore, we can conclude that the copy numbers of Hsa-miR-21-5p in PCa patients are higher compared to BPH patients.

The results of this study are consistent with the results of research conducted by Kumar et al., 2018, proving that Hsa-miR-21-5p levels increase in prostate cancer relative to the expression of the reference gene, which in this case is Hsa-miR-16-5p (Kumar et al., 2018). This study proves that Hsa-miR-21-5p is associated with biochemical recurrences as a continuous variable and with Gleason scores and the staging of prostate cancer. This result is consistent with Feng and Tsao's conclusion regarding the role of miR-21 in carcinogenesis as oncomiR (Feng and Tsao, 2016).

This study did not analyze the association of clinical staging based on histopathological changes and miR-21 expressions. However, in 2009, Guan et al., 2019 found that miR-21 expression increased along with Gleason scores, and miR-21 expression was higher in androgen-independent prostate cancer cells than in androgen-dependent prostate cancer cells. Guan also concluded that an increase in miR-21 expression would result in a decreased rate of apoptosis (decrease of Bax protein and increase of Bcl2); mir-21 is closely associated with migration and invasion of prostate cancer by targeting KLF5, GSK3B, and upregulating Akt pathway (Guan et al., 2009). The role of miR-21 in carcinogenesis is not solely to increase the rate of proliferation from the cell. In the review by Feng and Tsao, 2016, miR-21 is also associated with increased cancer resistance to drug treatment. The inhibition of miR-21 can also effectively reverse drug resistance (Figure 3). In prostate cancer, miR-21 is also believed to increase resistance toward Docetaxel via PDCD4 (Feng and Tso, 2016).

We obtained a sensitivity value of 100% and a specificity value of 75% of Cq miR-21 expression in prostate cancer patients. If these sensitivity and specificity values are compared to the usage of PSA with a threshold of 4 ng/mL (Sn 20.5% and Sp 93.8%) (Nogueira et al., 2009), it can be seen that Cq miR-21 expressions have the potential to replace PSA serum levels since Cq miR-21 has a higher sensitivity value.

The overexpression of miR-21 in body fluids has been comprehensively studied to develop miR-21 as a diagnostic marker for prostate cancer and other forms of cancer. Porzycki et al., 2016 has found positive results in his evaluation of miR-21 as a diagnostic marker to detect prostate cancer, showing that ROC (Receiver Operating Characteristic) curve analysis for the miR-21 marker has a large AUC (Area Under the Curve) between groups of prostate cancer patients and control groups (Porzycki et al., 2016). A meta-analysis by Zhou and Zhu, 2019 confirms that miR-21 has a good prognosis as a diagnostic biomarker for prostate cancer, with 91% sensitivity, 88% specificity and 0.95 Area Under the Curve (Zhou et Zhu., 2019).

Hsa-miR-21-5p expression did not differ between PCa with metastases and without metastases. This result shows that despite a relatively high increase between PCa and BPH patients, the miR-21 levels do not experience a significant change when bone metastasis is present. This may happen because our samples in this present study were not large enough, as studies by Kumar in 2018, Luu in 2017, Bonci in 2016, and Ribas in 2013 show that miR-21 promotes prostate cancer invasion and metastasis.

This research compares the level of Hsa-miR-21-5p expressions and the amount of Prostate Specific Antigen serum in PCa and BPH patients and found a 12.498-fold change difference. Thus, this study proves that miR-21 expression can be used to differentiate PCa from BPH patients. However, more validation samples and CoV of fold changes are needed for miR-21 to be used as aggressive biomarkers to differentiate Pca patients from BPH patients.

Author Contribution Statement

RRG carried out the molecular genetic studies, performed the statistical analysis and drafted the manuscript. HRD carried out the sample collection, designing the study and help to draft the manuscript. IA participated in the design of the study, help coordination between all the authors, and drafted the manuscript.

Acknowledgements

This study was supported by the Ministry of Research Technology and Higher Education Republic of Indonesia

(SK Dirjen RistekDikti No. 2909/UNI.DITLIT/DIT-LIT/LT/2019). Parts of this study were used in student thesis.

Funding Statement

We would like to express special thanks of gratitude to LPDP Scholarship for funding this study.

Statement conflict of Interest

There are no conflicts of interest regarding the publication of this mAanuscript.

References

Ahmad J, Hasnain SE, Siddiqui MA, Ahamed M, Musarrat J (2013). Microrna In Carcinogenesis and Cancer Diagnostics: A New Paradigm. *Indian J Med Res*, **137**, 680–94.

Bonci D, Coppola V, Patrizii M, et al (2016). A Microrna Code For Prostate Cancer Metastasis. *Oncogene*, **35**, 1180–92.

Dall'Era MA, Albertsen PC, Bangma C, et al (2012). Active Surveillance For Prostate Cancer: A Systematic Review Of The Literature. *Eur Urol*, **62**, 976–83.

Feng YH, Tsao CJ (2016). Emerging Role Of Microrna-21 In Cancer (Review). *Biomed Rep*, **5**, 395–402.

Guan C, Zhang L, Wang S, et al (2019). Upregulation Of Microrna-21 Promotes Tumorigenesis Of Prostate Cancer Cells By Targeting. *Cancer Biol Ther*, **2019**, 1–13.

Kristanto J (2017). Ekspresi Hsa-Mir-21-5p Pada Sampel Urin Kanker Prostat Dan Benign Prostatic Hiperplasia (BPH). Universitas Gadjah Mada, pp 1–7.

Kumar B, Rosenberg AZ, Choi SM, et al (2018). Cell-Type Specific Expression Of Oncogenic And Tumor Suppressive Micrornas In The Human Prostate And Prostate Cancer. *Sci Rep*, **8**, 7189.

Lange T, Stracke S, Rettig R, Lendeckel U, Kuhn J (2017). Identification Of Mir-16 As An Endogenous Reference Gene For The Normalization Of Urinary Exosomal Mirna Expression Data From Ckd Patients. *PLoS One*, **2017**, 1–13.

Li C, Zhao L, Chen Y, et al (2016). Microrna-21 Promotes Proliferation , Migration , And Invasion Of Colorectal Cancer , And Tumor Growth Associated With Down-Regulation Of Sec23a Expression. *BMC Cancer*, **16**, 1–11.

Livak KJ, Schmittgen TD (2001). Analysis Of Relative Gene Expression Data Using Real- Time Quantitative Pcr And The 2 -Δδcq. *Method*, **408**, 402–8.

Luu HN, Lin H, Sørensen KD, et al (2017). Mirnas Associated With Prostate Cancer Risk And Progression. *Bio Med Central Urol*, **17**, 1–18.

Nogueira L, Corradi R, Eastham JA (2009). Prostatic Specific Antigen For Prostate Cancer Detection. *Int Braz J Urol*, **35**, 521 – 31.

Porzycki A, Begic E, Hiros M (2016). Usefulness Of Total Psa Value In Prostate Diseases Diagnosis. *Acta Inform Med*, **24**, 156–61.

Ribas J, Lupold SE (2013). The Role Of Mir-21, An Androgen-Regulated Microrna, In Prostate Cancer. In: Androgen-Responsive Genes In Prostate Cancer: Regulation, Function And Clinical Applications. Springer Science+Business Media, pp 285–305.

Sánchez DB, Canon CA, Torres AP, et al (2020). The Promising Role Of Mir-21 As A Cancer Biomarker And Its Importance In Rna-Based Therapeutics. *Mol Ther Nucleic Acids*, **20**, 409-20.

Wu Y, Song Y, Xiong Y, et al (2017). Microrna-21 (Mir-21) Promotes Cell Growth And Invasion By Repressing Tumor Suppressor Pten In Colorectal Cancer. *Cell Physiol Biochem*, **43**, 945–58.

Zhou H, Zhu X (2019). Microrna-21 And Microrna-30c As Diagnostic Biomarkers For Prostate Cancer : A Meta-Analysis. *Cancer Manag Res*, **11**, 2039–50.