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MiR-141-3p Relative Expression Level from FFPE Samples as Biomarker of Prostate Adenocarcinoma Carcinogenesis in Yogyakarta, Indonesia

Sari Eka Pratiwi[1], Sri Nuryani Wahyuningrum[2], Rachma Greta Perdana Putri[3], Danarto[4], Didik Setyo Heriyanto[5], Nur Arfian[6], Sofia Mubarika Haryana[7], Indwiani Astuti[8]

[1]Department of Biology and Pathobiology, Faculty of Medicine, Universitas Tanjungpura, Indonesia

[2]Magelang Unit of Health Research and Development, Ministry of Health, Indonesia

[3]Department of Histology, Faculty of Medicine, Universitas Ahmad Dahlan, Yogyakarta, Indonesia

[4]Department of Urology, Sardjito Hospital, Yogyakarta, Indonesia

[5]Department of Anatomy Pathology, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia

[6]Department of Anatomy, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia

[7]Department of Histology and Cell Biology, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia

[8]Department of Pharmacology, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia

Correspondence:
Sari Eka Pratiwi,
Jl. Prof. Dr. Hadari Nawawi, Pontianak,
West Kalimantan, Indonesia
Zip Code: 78124

Email: sariekapratiwi@medical.untan.ac.id

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Abstract

Globally, prostate cancer (PCA) is the second leading cause of male cancer-associated mortality. Micro-RNAs (miRNAs) are small non-coding RNAs considered promising biomarkers for diagnosis, prognosis, and treatment options. A miR-141 expression is frequently dysregulated and influences the development and progression of PCA. This study aimed to identify miR-141 expression level as a marker to differentiate PCA from another prostate anomaly, especially in Yogyakarta-Indonesia. Formalin-fixed paraffin-embedded (FFPE) tissues for each three groups: benign prostatic hyperplasia/BPH, high-grade prostatic intraepithelial neoplasia/HGPIN, and PCA (n=7/group) were stored in a commercial clinical laboratory in Yogyakarta. The total RNA was extracted from FFPE sections using miRNeasy FFPE kit, followed by the quantification of miR-141-3p expression level by RT-PCR. The result showed that miR-141 relative expression level on PCA was higher than other groups and significantly different ($P<0.05$, Kruskal Wallis test). The mean of the miR-141 relative expression level of BPH, HGPIN, and PCA were 1.04±0.87, 6.44±7.8, and 7.06±8.83, respectively. The relative expression level of miR-141 can potentially be a prognostic biomarker in PCA and could differentiate aggressiveness in prostate anomaly, especially BPH, HGPIN, and PCA.

Keywords

BPH, HGPIN, miR-141, PCA, Prostate Anomaly Markers.

INTRODUCTION

Prostate cancer (PCA) is the most common malignancy in men and the second leading cause of male cancer-associated mortality around the world (1,2). The early PCA stage is localized and can be treated with a range of treatments, including chemotherapy, radiation therapy, radical prostatectomy, cryotherapy, and others. Unfortunately, following initial treatment, around 23-40% of these patients will acquire metastatic cancers. Prostate tumors frequently spread to the bone and other organs, resulting in death (2).

Although PCA is ordinary, there are no diagnostic or prognostic biomarkers that can be used to determine its aggressiveness. The prostate-specific antigen (PSA) test, utilized in the clinic as a standard assay, is not specific for PCA. Infections, inflammation, hyperplasia, and other things can affect PSA levels to vary. These limitations of PSA level usually lead to overdiagnosis and overtreatment (1,2). Recent research has demonstrated the capability of nucleic acids as a disease marker. MicroRNA (miRNA) is a type of RNA that has been demonstrated to be a disease marker (1–3).

Micro-RNAs (miRNAs) are small non-coding RNAs and one of the regulatory RNAs arranged by 18-25 nucleotides. miRNAs play a role in the post-transcriptional regulation of gene expression by targeting almost 30% of all messenger RNAs (mRNAs). It silences the mRNAs by degrading or inhibiting the mRNA translation and expression. The dysregulation of miRNAs can lead to some diseases such as cancer, indicating their essential role in carcinogenesis (4–7).

Today, miRNA is considered as promising biomarker for diagnosis, prognosis, and treatment options in several solid cancers, including PCA (8). miRNAs tend to be oncomir or tumor suppressor miRNAs. As a tumor suppressor, miRNA targets the oncogenic mRNA (6,7). miR-141 is one member of the miR-200 family (mir-200) that plays a role in proliferation, epithelial-mesenchymal transition (EMT), migration, invasion, and drug resistance (8,9).

In malignant tumors, miR-141 expression is frequently dysregulated and influences the tumor's development and progression. Dysregulation of miR-141 (MicroRNA141) in many types of cancer also modulates cellular motility and regulates stemness. miR-141 is commonly known to have a dual role in tumor development, as a tumor suppressor gene or an oncomir depends on the type and severity of cancer. This phenomenon provides more benefit of miR-141 utilization for therapeutic targeting agents, diagnostic or prognostic biomarkers (10).

miR-141-3p (microRNA-141-3p) directly inhibits mRNA ZEB1 (zinc finger E-

box binding homeobox 1) and ZEB2 (zinc finger E-box binding homeobox 2), a gene that plays an essential role in the EMT process of invasive tumors (11). Conversely, one study in pancreatic, colorectal, and breast cancer cell lines showed that ZEB1 triggers a miRNA-mediated feed-forward loop by directly inhibiting miR-141 transcription, which stabilizes EMT and promotes invasion of cancer cells (12).

MiR-141 also repressed STAT4 (Signal Transducer and Activator of Transcription 4) and targeting transcriptional co-activator with PDZ-binding motif that regulates cancer invasion and proliferation in gastric cancer (13). Meanwhile, as an oncomir, miR-141 was found up-regulates in many prostate cancer studies compared to healthy controls (14). In patients with bone-metastatic prostate cancer, the miR-141 expression level was elevated and positively correlated with the bone lesion (15).

Dysregulation of miR-141 contributes to a deeper understanding of tumorigenesis and becomes a potential diagnostic and prognostic biomarker in the development of prostate cancer. Studies of miR-141 in prostate cancer and another prostate anomaly were rarely done in Indonesia, especially in Special Region of Yogyakarta, in the form of FFPE sample. Therefore, in this study, we aimed to identified miR-141 expression level as a marker to differentiate prostate cancer (PCA) to high-grade prostatic intraepithelial neoplasia (HGPIN) and benign prostatic hyperplasia (BPH), based on formalin-fixed paraffin-embedded (FFPE) tissue sample of patients from Yogyakarta, Indonesia.

MATERIALS AND METHODS

Sample collection

The study was approved by the Medical and Health Ethics Committee, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, with approval number of KE/FK/0888/EC/2018. Samples were collected from Formalin-Fixed Paraffin-Embedded (FFPE) blocks stored in a commercial clinical laboratory in Yogyakarta from 2017 until 2018. As many as seven FFPE blocks from each BPH, HGPIN, and PCA group were sliced into five sections with a 10-µm thickness each and prepared for RNA extraction.

Total RNA extraction and Reverse Transcriptase-PCR (RT-PCR)

Total RNA extracted from FFPE sections with miRNeasy FFPE Kit (Qiagen, Germany, Cat No. 217504). After FFPE blocks were sliced, the sections were placed in safe lock tubes (1.5 mL), and 160 µL Xylene was added to each tube for the deparaffinization process. The RNAs were eluted using 30 µL RNase-free water. cDNA synthesis was performed with miRCURY LNA RT Kit (Qiagen, Germany, Cat. No 339340). For the synthesis of cDNA from miRNA, the RNA template was diluted by adding nuclease-free

water until the concentration achieved 5 ng/ µL, then placed 2 µL into 0.2 mL tube.

Quantitative Real Time PCR For miR-141-3p

Quantification of miR-141-3p (3' ggUAGAAAUGGUCU--GUCACAAu 5') in FFPE section used miRCURY LNA miRNA PCR Assay Kit (Qiagen, Germany, Cat No. 339306) in BIORAD CFX96 real-time machine. The preparation step was started by diluting 1 µL cDNA template with 59 µL nuclease-free water (1:60), then placed 4 µL diluted cDNA template into a white qPCR strip tube.

The initial PCR condition was 95^0C for 10 minutes, followed by 40 cycles of denaturation step at 95^0C for 10 seconds, with annealing and extension steps at 58^0C for 1 minute. This study used U6 for internal control and normalized miRNA, H_2O for negative control, and all the samples were performed in duplicate.

miR-141-3p expression was analyzed based on the value of ΔCq of miR-141-3p, which was normalized with ΔCq of U6. Then, the expression was calculated using the Livak method.

The microRNA amplification curve was read as Cycle Quantification (Cq). The Cq of miR-141-3p value was then normalized with the Cq of U6 value, and the value of ΔCq was conducted. Then the ΔΔCq quantification with the comparison was the average value of the BPH group's ΔCq (As a comparative group). After the ΔΔCq results were obtained, the next step was to analyze the target microRNA expression by the Livak method with the formula was $2^{-(\Delta\Delta Cq)}$. The type of quantification in this study used relative quantification by comparing the groups of HGPIN and PCA to BPH. The Mann-Whitney test and Kruskal-Wallis test were used to analyze the differences of miR-141 expression levels in the tumor tissue according to the clinicopathological characteristics (P value significant < 0.05).

RESULTS

Subject characteristics

This study involved 21 subjects that consisted of 7 BPH patients, 7 HGPIN patients, and 7 PCA patients. Patients were dominated by the elderly, with an average of age was 68 years old. Gleason score for PCA patients showed 2 people having medium-grade cancer (score 7) and 5 people having high-grade cancer (score 8 and 9). Detail for subject characteristics is shown in Table 1.

Mean difference of miR-141-3p relative expression

MiR-141 relative expression level of three groups of subjects was significantly different (P<0.05, Kruskal Wallis test). The difference showed that the miR-141 level in PCA samples was higher than HGPIN and BPH samples. The mean difference of miR-141 in the three groups is described in Figure 1.

Table 1. Subject characteristics of samples

Characteristics of Subject	
Age (years), mean ± SD (min – max)	67.71±11.48 (44 - 83)
BPH	67.71±11.64 (55 - 83)
HGPIN	63.29±12.34 (44 - 80)
PCA	72.14±10.27 (55 - 82)
Prostate anomaly status (n, %)	
BPH	7 (33.3)
HGPIN	7 (33.3)
PCA	7 (33.3)
Gleason score for PCA samples (n)	
Score 7	2
Score 8	2
Score 9	3
Grade of PCA samples (n)	
Grade 3	2
Grade 4	2
Grade 5	3
Relative expression mean of miR-141-3p	
BPH	1.04±0.87 (0.07-2.17)
HGPIN	6.44±7.8 (0.59-22.15)
PCA	7.06±8.83 (1.04-26.44)

BPH: Benign prostatic hyperplasia, HGPIN: High-grade prostatic intraepithelial neoplasia, PCA: Prostate cancer

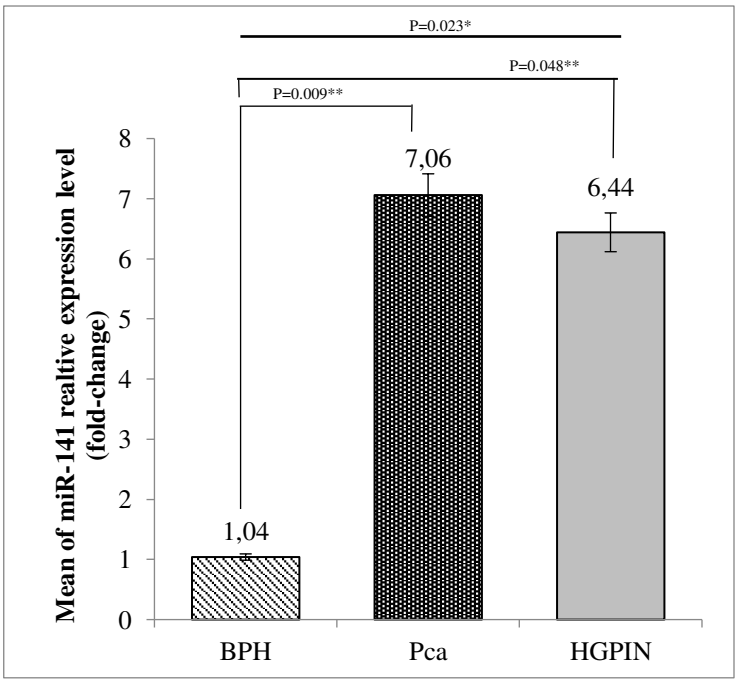

Figure 1. Clustered coloumn of miR-141 mean relative expression in benign prostatic hyperplasia (n=7, BPH), prostate cancer (n=7, PCA), and high-grade prostatic intraepithelial neoplasia (n=7, HGPIN). *P=0.023,three-way Kruskal-wallis test; **P=0.009 BPH versus Pca, **P=0.048 BPH versus HGPIN, P=565 Pca versus HGPIN, Mann-whitney test

DISCUSSION

Regulation of miR-141 in prostate cancer development involves complex mechanisms between the target gene and other interfering molecules. Considering that regulation of miR-141 was cancer type-dependent, the expression level of miR-141 could be different based on cancer severity. Our finding shows that the miR-141 expression levels of prostate cancer patients was significantly higher than HGPIN and BPH patients. Sample of prostate cancer tissue with upregulating miR-141 expression was known as a prostate cancer patient in a condition of grades 3, 4, and 5. In this term, we could report that miR-141 in the high stadium of prostate cancer, in this case, grades 3-5, played a role as oncomir. Oncomirs or oncogenic miRNAs are miRNA which are associated with cancer.

Various miRNAs act as EMT repressors, particularly inhibiting the *ZEB1* expression, which involves miR-200s families (miR-200a, miR-200b, miR-200c, miR-141, and miR-429). The function of the miR-200s families is to promote MET and inhibit EMT by targeting *ZEB1*. In other ways, *ZEB1* represses the gene transcription of miR-200s by directly binding on the gene's promoter region (16,17). MiR-141-3p is involved in targeting mRNA *ZEB1* and regulates the Androgen Receptor (AR) mRNA expression by targeting the AR's open reading frame (ORF), particularly at early PCA but not in the advanced stages (18).

The results of this study are in line with studies conducted by Cheng et al., (19) which showed an increment of miR-141-3p expression in the serum of prostate adenocarcinoma patients who have undergone metastasis compared to the serum of healthy patients.

Cancer metastases involve angiogenesis, a formation of new blood vessels, which is required for tumor expansion, and sufficient tumor nutrition. A previous study showed that overexpression of miR-141 correlated with higher blood vessel formation in mouse models and inducing secretion of VEGFA (vascular endothelial growth factor-A), one of the potent blood vessel growth factor (20,21).

Another mechanism that relates miR-141 in worsening tumor conditions is inducing oxidative stress response. A prior study reported that miR-141 could modulate higher reactive oxygen species (ROS) in oxidative stress conditions, including tumor microenvironment, by directly targeting the p38a gene that blocks proliferation and promotes apoptosis. This condition implies disruption of cellular components, induces cell proliferation, and stimulates tumor growth (22).

Fluctuation of miR-141 expression level in any cancer was capable of distinguishing malignant or non-malignant tissue and used

to differentiate cancer staging (10). In a prostate cancer, overexpression of miR-141 is the most potent biomarker of metastatic phase. It can differentiate prostate cancer patients with local advanced from those with metastasis (14). In line with our results, a study reported that a higher level of miR-141 was related to more aggressive and advanced disease of prostate cancer (high Gleason score, large tumor size, and lymph node metastases). Upregulation of miR-141 also associated with increased risk of biochemical recurrence (23). Other studies confirm that overexpression of serum miR-141 could be used to differentiate non-metastatic from metastatic group patients with 69% sensitivity and 94% specificity (24). Otherwise, a recent study revealed that higher expression of miR-141 enforces a strong epithelial phenotype and minimizes partial loss of mesenchymal phenotype by directly binding to the ZEB1 gene, an EMT transcription factor. This mechanism correlated with miR-141 down-regulation in metastatic prostate cancer patients (25).

Our study, to our knowledge, is the first study to look for the possible role of miR-141 on prostate cancer compared to high-grade prostatic intraepithelial neoplasia and benign prostatic hyperplasia in Indonesian people using FFPE samples. Our result suggests that elevating miR-141 expression shows a higher stadium of prostate cancer. This study had limitations in representing the small number of the patients. A large number of prostate cancer patient subjects is required to validate diagnostic and prognostic relevance of miR-141 to represent prostate cancer progression.

CONCLUSIONS

In conclusion, our research has shown that miR-141 level in a higher grade of PCA plays a role as oncomir and can be a prognostic biomarker. The miR-141 level also could differentiate aggressiveness in BPH, HGPIN, and PCA.

AUTHOR CONTRIBUTIONS

Sari Eka Pratiwi: conceptualization, methodology, investigation, writing-reviewing and editing. Sri Nuryani Wahyuningrum: software, data curation, and writing-editing. Rachma Greta Perdana Putri: investigation. Danarto: conseptualization: Didik Setyo Heriyanto: supervision. Nur Arfian: methodology, supervision, and validation. Sofia Mubarika Haryana: supervision and validation. Indwiani Astuti: methodology, supervision, writing - reviewing and editing.

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CONFLICT OF INTEREST

There are no conflicts of interest.

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