

# miRNA-21 Serum Evaluation in BPH, Hormone Sensitive Prostate Cancer, and Castrate Resistant Prostate Cancer: Attempt for Diagnostic Biomarker Evaluation

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## ABSTRACT

**Background:** Some of prostate cancer cases could progress to be Castrate Resistant Prostate Cancer (CRPC). However it is still a challenge to early diagnose it since no reliable examination could be done except PSA, which has high variability. It is now known that miRNAs are involved in nearly all inflammatory responses. Several malignancies in humans that specifically express miRNA have been detected and identified. The expression values of miRNA-21 also correlates with the occurrence of resistant castration of prostate cancer and metastases, therefore miRNA-21 is expected to be a biomarker to estimate the progression of cancer. **Objective:** The purpose of this study was to analyze the expression values and cut-off markers of miRNA-21 as markers of CRPC progression. **Methods:** This study used a retrospective cohort design with observational analysis. The forty-eight total sample was obtained from serum, then the RT-PCR was performed to obtain expression values of miRNA-21. Data were analyzed using One Way ANOVA to see the difference in the expression values of miRNA-21. Furthermore, to determine the cut-off analysis was carried out using the ROC curve. **Results:** In the BPH group, an average expression value of miRNA-21 was  $33,785 \pm 1.80$  ng/dL, in the Prostate cancer group the average miRNA-21 was  $34.51 \pm 1.32$  ng/dL, while in the CRPC group, an average miRNA-21 was obtained, reaching to  $34.51 \pm 1.32$  ng/dL. The cut-off value of miRNA-21 from the BPH category was  $<33,595$ , PPV = 50%, NPV = 80% with a value of  $p = 0.081$ , the prostate Ca category was  $33.595-35.21$ , PPV = 87.5%, NPV = 66.7% with  $p$  value = 0.003, while the value of miRNA-21 in the CRPC category was  $> 35.21$ , PPV = 80%, NPV = 58.3 with a value of  $p = 0.04$ . **Conclusion:** There is a significant difference in the expression values of miRNA-21 between BPH with CRPC and Prostate cancer and CRPC, therefore, miRNA-21 cut-off point is potential to differentiate the diagnosis.

**Keywords:** miRNA-21, biomarker, prostate, cancer, progressivity.

## 1. BACKGROUND

Some of prostate cancer cases could progress to be Castrate Resistant Prostate Cancer (CRPC). However it is still a challenge to early diagnose it since no reliable examination could be done except PSA, which has high variability. The definition of Castrate Resistant Prostate Cancer (CRPC) based on the EAU Guideline in 2019 is concentrated serum testosterone level  $<50$  ng/dl or  $1.7$  nmol/L with one of increasing PSA values progression 3 times in a row at an interval of one week and two of them experienced a 50% increase in PSA nadir and  $PSA > 2$  ng/mL or presence of radiological progression is char-

acterized by the appearance of new lesions: namely the presence of two or more lesions on the bone or lesions in soft tissue assessed by RECIST (Response Evaluation Criteria in Solid Tumors) (1). Currently, PSA is the only modality used to detect CRPC however, use of PSA has drawbacks because the increase in PSA has long onset that the diagnosis of CRPC is always late, not to mention the variability of PSA is also high.

Therefore, it is necessary to have a new modality to detect CRPC. It was known that miRNAs are involved in almost all inflammatory responses and they have a significant impact on the magnitude of the inflammatory

response (2). Several malignancies in humans that specifically express miRNA have been detected and identified. One of the miRNAs that provide a large number of cancers is miRNA-21. MiRNA-21 controls the expression values of mRNA associated with microvascular proliferation and tumor invasion. Positive expression values of miRNA-21 are associated with weak biochemical recurrence, free survival, and predictive value for biochemical recurrence in prostate cancer patients after radical prostatectomy. The expression values of miRNA-21 also correlates with the occurrence of castrate-resistant of prostate cancer (CRPC) and metastases, therefore miRNA-21 is expected to be a biomarker to estimate the progression of cancer (2). Previously, research had been done by Seputra et al, (in 2021), that miRNA-21 are potential to be biomarker in meta-analysis study.

## 2. OBJECTIVE

The purpose of this study was to analyze the expression values and cut-off markers of miRNA-21 as markers CRPC.

## 3. MATERIAL AND METHODS

This study used a retrospective cohort design with observational analysis. Samples were taken by consecutive technique with a total sample of 48 patients divided into 3 groups of BPH, Prostate cancer and CRPC which each group consists of 16 samples. We got the sample from Saiful Anwar general hospital, Malang. The Criteria CRPC concentrated serum testosterone level <50 ng/dl or 1.7 nmol/L with one of increasing PSA values progression 3 times in a row at an interval of one week and two of them experienced a 50% increase in PSA nadir and PSA > 2 ng/mL or presence of radiological progression is characterized by the appearance of new lesions: namely the presence of two or more lesions on the bone or lesions in soft tissue assessed by RECIST (Response Evaluation Criteria in Solid Tumors).

Examination for miRNA-21 started from RNA extraction and then cDNA was synthesized and amplified in real time PCR. RNA extraction from serum by miRNeasy Serum/Plasma Advanced Kit catalog no#217204 by QIAGEN. Serum was added Buffer RPL and RPP to gain protein precipitation and added isopropanol. Bind total RNA including small RNA was obtained and was purified and eluted to get targeted nucleic acid. Synthesis of cDNA was performed by adding buffer optimized for Poly(A) polymerization and reverse transcription contains a universal reverse transcription primer for the Probe-based workflow, Mg<sup>2+</sup>, and dNTPs. Synthesis of cDNA was using miRCURY RT SYBR Green and Probe Reaction Buffer catalog no#339340 by QIAGEN. Reagens was mixed and added adjusted RNA template 5 ng/μL and then incubate at 42°C for 60 minutes and continued with 95°C for 5 minutes, then qPCR was performed.

Real time PCR was performed by diluted cDNA with RNase-free water with 1:60 ratio (10 μL cDNA with 590 μL RNase-free water). Prepare 7 μL of mixture into PCR tube and put 3 μL diluted cDNA into PCR tube continued with spindown and perform into qPCR machine. Results was analyzed by Ct value of each sample and was calculated for normalized expression ratio.

The inclusion criteria were patients who had undergone TURP surgery and/or prostate biopsy who diagnosed histopathologically with either BPH, prostate cancer, or CRPC, and are currently undergoing ADT treatment for a prostate cancer patient. We exclude other cancers besides prostate cancer, suffering from diabetes mellitus, hypertension, and/or other cardiovascular diseases. Criteria of CRPC was Samples were taken in serum and performed RT-PCR to obtain the expression value of miRNA-21. The BPH, prostate cancer, and CRPC categories were obtained from the results of the patient's histopathology. Before analyzing data, normality test was performed. The normality test used is the Kolmogorov-Smirnov test. If the data were normally distributed, the analysis T-test was carried out, if the data were not normally distributed, the Mann-Whitney analysis test was performed. Analysis of the ROC curve was used to determine the cut-off between nonCRPC and CRPC toward miRNA-21.

## 4. RESULTS

In this study, 16 samples were diagnosed with BPH, 16 patients with a diagnosis of prostate Ca and 16 patients with a diagnosis of CRPC. In the BPH group, an average expression value of miRNA-21 was 33,785±1.80 ng/dL, in the Prostate cancer group, the average miRNA-21 serum was 34.51±1.32 ng/dL, while in the CRPC group, an average miRNA-21 was obtained, up to 34.51±1.32 ng/dL. The results of the expression values of miRNA-21 were then analyzed using the ROC curve to determine the cut-off of the expression value of miRNA-21 in each group. Characteristic of study data showed in Table 1.

Parameter	BPH (n :16) Mean ± SD	HSPC (n:16) Mean ± SD (n)	CRPC (n:16) Mean ± SD	p value
Umur (tahun)	64.875 ± 6.47	64.67 ± 5.28	66 ± 4.35	0.82
BMI (Kg/m <sup>2</sup> )	21.83 ± 1.59	21.66 ± 1.81	20.92 ± 1.70	0,28
VAS (nyeri)	0.65 ± 0.23	3.23 ± 0.56	5.23 ± 0.34	0.70
Hb (mg/dL)	9.77 ± 1.58	9.07 ± 0.90	8.83 ± 1	0.85
Ureum	33.37 ± 9.97	34.18 ± 7.44	38.37± 8.85	0.23
Creatinin	1.08 ± 0.36	1.19 ± 0.39	1.2 ± 0.41	0.644
Leukosit	8662 ± 1454.59	9068 ± 2155.21	10137.5 ± 2063.9	0.09
Volume Prostat (mm <sup>3</sup> )	69.36 ± 10.46	56.33 ± 18.22	60.45 ± 13.9	0.48
Metastasis				
iya	0	2	13	0.00
tidak	16	14	3	
PSA	1.26 ± 0.42	493.94 ±178.65	544.47 ± 370.325	0.04
Gambaran Histologi				
BPH	16	0	0	0.04
Adenocarcinoma	0	16	0	
Adenocarcinoma poorly differentiated	0	14	2	
Gleason Score		8.095 ± 1.72	8.4 ±1.14	0.53
Crnalic Prognostic skor		3.112 ± 0.8	0.43 ± 0.51	0.04

Table 1. Characteristic data on this study

Based on Table 2 above, the cut-off value was obtained. The ROC curve image shows the location for determining the cut-off, by looking at the point of the line closest to the top left corner, namely the sensitivity of 1,000 and 1-specificity of 0,000. Then, it is obtained in the variable miRNA-21 with a CRPC state with a sensitivity value of 62.5 and a specificity value of 68.75 cut-off value was 35.2150, above or equal to that value is included in CRPC category, and below that number is included in prostate cancer category. Based on these results, it can be concluded that the cut-off miRNA-21 value in the CRPC category is >35.215 with p value = 0.04.

As seen on Table 3, it is known that of the 32 samples, 16 samples were included in the CRPC category group, and 16 samples were included in the Prostate cancer category group based on pathology result. Analysis between the prostate cancer group and the CRPC group, 16 samples were included in the prostate cancer category group, and 16 samples were included in the CRPC cat-

Base	Comparison	Cut-off point	Sensitivity	Specificity	AUC	p value
Prostate Cancer	CRPC	35.2150*	62.5	68.75	70%	0.04

Table 2. miRNA-21 serum cut-off values and its specificity and sensitivity. \*p value was obtained <0.05 and statistically significant

miRNA-21 Ct value cut-off	Diagnosis (Gold Standard)				p-value	PPV	NPV
	CRPC		Prostate Cancer				
	n	%	n	%			
CRPC	10	62.5%	5	33.33%	0.05*	66.67%	64.71%
Prostate Cancer	6	35.29%	11	64.71%			
Sensitivity	62.5%						
Specificity	68.75%						
Normalized expression ratio	3.382**		1.654***				

Table 3. Description of Prostate Cancer and CRPC groups against the categories of miRNA-21 groups. \*p value was obtained <0.05 and statistically significant, \*\*Normalized expression ratio for Ct-value CRPC compared to Prostate cancer (calibrator), \*\*\* Normalized expression ratio for Ct-value Prostate cancer compared to BPH (calibrator)

miRNA21 category	Group Category		Frequency
	CRPC	NonCRPC (BPH + PCa)	
CRPC	9	6	15
Non CRPC	7	26	33

Sensitivity: 56.2%, Specificity: 81.2% PPV: 60%, NPV: 78,7% Odds ratio: 9.0

Table 4. Description of Prostate Cancer and CRPC groups against the categories of miRNA-21 groups

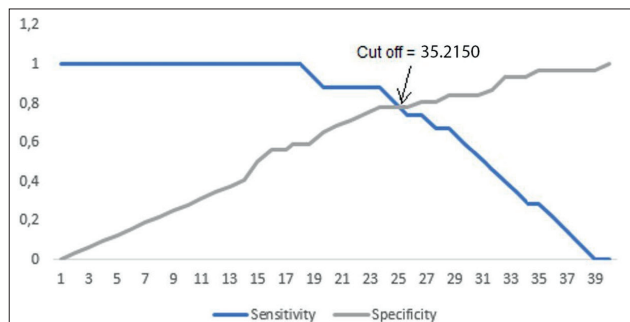


Figure 1. ROC curve, Total of miRNA-21 in the Prostate Cancer and CRPC groups

$$\Delta C_{T(\text{test})} = C_{T(\text{target, test})} - C_{T(\text{ref, test})}$$

$$\Delta C_{T(\text{calibrator})} = C_{T(\text{target, calibrator})} - C_{T(\text{ref, calibrator})}$$

Second, normalize the  $\Delta C_T$  of the test sample to the  $\Delta C_T$  of the calibrator:

$$\Delta \Delta C_T = \Delta C_{T(\text{test})} - \Delta C_{T(\text{calibrator})}$$

Finally, calculate the expression ratio:

$$2^{-\Delta \Delta C_T} = \text{Normalized expression ratio}$$

Figure 2. Normalized Expression Ratio Formula (Schmittgen et al, 2008).

egory group. For CRPC group, there were 10 samples detected as CRPC category, and 5 samples included the prostate cancer group by miRNA-21 method. Then, there were 6 out of 16 CRPC pathology samples detected as prostate cancer by miRNA-21 method, and the remaining 11 samples included to prostate cancer. Positive and negative predictive value of the proposed method being calculated. PPV was found 66.67% follower by NPV was around 64.71%. Sensitivity and specificity were performed, and the results were 62.5% and 68.75% respectively. After

the PCR expression result released, calculation for normalized expression ratio was performed using the standardized formula as it shown in Figure (Schmittgen et al, 2008).

Normalized expression ratio obtained by comparing each CRPC and prostate cancer group with its control by pathogenesis and natural history of its disease. The normalized expression ratio for CRPC group was found around 3.382 compared to prostate cancer group as control. The normalized expression ratio for prostate cancer group was 1.654 compared to the benign type as control by pathogenesis and for differentiate the CRPC and non-CRPC patients using miRNA-21 serum expression portrayed in Table 4.

### 5. DISCUSSION

MicroRNA is a small non-coding RNA that can inhibit the number of genes at the post-transcription level and has a target on mRNA so that it can reduce the amount of protein. The formation of miRNA through a series of processes begins with transcription of the miRNA gene by RNA polymerase II which forms the long primary precursor (pre-miRNA). These precursors are thousands of nucleotides long and have a hairpin structure. The hairpin structure is cut by the Drosha enzyme and the DGCR8 gene (DiGeorge syndrome chromosomal region 8). After this process, a hairpin structure with a length of approximately 80 basepairs is formed called pre-miRNA (3, 4). The miRNA profile in prostate cancer suggests that the expression value of miRNAs summed differently between prostate cancer and adjacent normal tissue, thereby contributing to the development of prostate cancer.

Zhang et al found a set of 46 miRNAs in serum Transgenic Adenocarcinoma of Mouse Prostate (TRAMP) and followed the study by measuring the homologous subset of miRNA in CRPC patients compared to healthy controls. In that study, it was found that the expression value of miRNA-21, miRNA-14 and miRNA-37 increased in the serum of patients with CRPC. This strengthens the potential of miRNA-21 in the diagnosis and prognosis of prostate cancer stage because miRNA-21 plays an important role in tumor cell proliferation, apoptosis, and invasion (5). Among its functions, miRNA plays a role in development, organogenesis, hematopoiesis, proliferation and apoptosis as well as tumorigenesis and its progression. miRNA has a role in the development of various tumors such as leukemia, neuroblastoma, pituitary adenoma, breast cancer, thyroid cancer, hepatocarcinoma, colorectal cancer, and lung cancer. Upregulation and downregulation of various miRNAs in these tumors are found mostly in target miRNAs located in the tumor region associated with the genome, fragile sites, loss of heterozygosity and region of amplification, thereby exerting the same effects as oncogenes or tumor suppression genes.

Several research studies have explained how miRNA-21 affects AR and CRPC relationships such as oncomirs and suppressors. Zhang et al in their study described the correlation between elevated serum miRNA in CRPC patients. Using qRT-PCR, serum miRNA-21 levels were calculated in CRPC, Androgen Dependent Prostate Cancer (ADPC), localized prostate cancer, and BPH. CRPC patients had significantly higher miRNA-21 levels compared to ADPC, localized prostate cancer and BPH. This was concluded by the researchers that miRNA-21 has the potential as a clinical biomarker in prostate cancer (6, 7).

As a biomarker in prostate cancer, microRNA can be measured and evaluated as an indicator of normal or pathogenic biological processes and pharmacological responses to therapy. For prostate cancer, miRNA can act as a biomarker for early detection or diagnosis of prostate cancer, enabling prediction of patient prognosis and therapeutic efficacy. RNase found in body fluids can degrade molecules, especially mRNA. MiRNA-21 can remain stable on heating, very low or high pH levels, long storage times, and repeated freeze-thawing (8). Apart from being stable, miRNA-21 testing is easy to detect and shows accurate results by using standard techniques such as RT-PCR, microarray and small RNA sequencing (9). On this basis, miRNA-21 was studied to be used as an alternative biomarker to predict the occurrence of CRPC.

miRNA-21 is majorly induced by androgen receptors. The role of miRNA-21 and signal androgen receptors play an important role in inhibiting the TGF $\beta$  II receptor which is added to the prostate ca. which in turn will result in positive feedback inhibition of growth response (4). An excessive expression of miRNA-21 is dependent on androgens, thus mediating castration resistance. The analysis related to miRNA-21 showed that the upregulation of miRNA-21 led to the downregulation of PTEN, which resulted in a significant apoptosis reduction in the prostate cancer state. miRNA-21 also regulates myristoylated alanine-rich protein kinase-C substrate (MARCKS), this causes resistance to apoptosis and leads to increased proliferation. This situation that occurs

continuously will cause a CRPC state, so it can be concluded that the level of CRPC is higher than prostate cancer and BPH (10, 11). This theory is following the results of research conducted where the expression value of miRNA-21 in the CRPC category is higher than with prostate cancer and BPH. These results are also supported by a study conducted by Yemen, the miRNA-21 studied saw a significant increase between patients with prostate cancer and healthy patients.

## 6. CONCLUSION

There was a significant difference in the expression levels of miRNA-21 in the BPH category group with CRPC and the BPH category group with CRPC. The cut-off value of miRNA-21 in the CRPC category was > 35.21 ng/dL with normalized expression ratio was 3.382 compared to prostate cancer group as control.

- **Ethics approval and consent to participate:** The study was held after approval from the Ethic Committee, Faculty of Medicine, Universitas Brawijaya Saiful Anwar General Hospital Malang (study number : 400/180/K.3/302/2020).
- **Author's contribution:** All authors were involved in all steps of preparation this article. Final proofreading was made by the first author.
- **Competing Interest:** The authors declare no competing interest in this study.
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