COMPARATIVE STUDY OF miRNA-205 EXPRESSION AND CERVICAL CANCER SEVERITY IN BALI

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ABSTRACT

Cervical cancer is one of the leading causes of death for women worldwide. It occurs due to infection with the human papilloma virus (HPV), which attacks the cervix in the female reproductive organs. The detection of cervical cancer method is by using pap smears and liquid-based cytology (LBC), and vaccinations. However, both methods are limited in detecting cervical cancer quickly and accurately. This study aims to find potential biomarkers that will be used to quickly detect the development of cervical cancer. Large-scale studies have shown that microRNA-205 (miRNA) regulation is very important in various types of cancer, including cervical cancer. The isolation and synthesis of cDNA from 36 preparations of cervical cancer and chronic cervicitis paraffin blocks were measured by quantitative real-time PCR to evaluate the expression of miRNA-205 as a biomarker of cervical cancer in cervical cancer samples. Statistical results showed that there was a significant difference in the expression of miRNA-205, which was relatively higher in cervical cancer patients ($p<0.05$) miRNA-205. In addition, the increased expression of miRNA-205 is associated with an increase in the severity of cervical cancer (stage 3). We conclude that miRNA-205 is a potential candidate for cervical cancer molecular biomarkers.

Keywords: HPV; Cervical cancer; miRNA-205; Biomarker

INTRODUCTION

Cervical cancer became the second cancer with the most sufferers in Indonesia, with a prevalence of 10.69%, according to Riskesdas data at the Ministry of Health's Data and Information Centre in 2019. Cervical cancer has the highest contribution to the prevalence of cancer in women in Indonesia. This type of cancer has a high mortality rate due to a delay in early detection.[1] Patients with this cancer are generally detected at the last stage of cancer. Based on the health profile released by the Bali Provincial Health Office in 2020, it is known that the percentage of cervical cancer detection examinations in Bali is still low. This becomes a reference source for increasing the promotion and development of detection methods so that the basis for women to detect cervical cancer can increase.[2]

Pap smear testing and vaccination as early detection and prevention of cervical cancer can reduce the risk of being infected

with the human papilloma virus (HPV). It has been shown to be able to reduce the mortality rate caused by cervical cancer. However, nowadays, detection methods cannot be used to detect the development of cervical cancer directly and easily.[3] Therefore, the discovery of new specific biomarkers is needed to improve detection capability and increase the accuracy of prognostic diagnoses in patients suspected of cervical cancer.

Preliminary research shows that the regulation of microRNA (miRNA) has a very important role in various types of cancer, including cervical cancer. miRNA is a non-coding RNA consisting of a short chain of nucleotides (18-25 nucleotides) and is involved in cancer pathological and physiological activities.[4] miRNA is not only expressed in cancer tissue but also in blood.[5] miRNA-205 is one of the types that act as an oncogene. It was reported in many previous studies that miRNA-205 could become a signal gene to ensure abnormal cell growth in cancer patients. It happened because the circulation of miRNA-205 was significantly increased in patients with cervical cancer. The type of miRNA increases the proliferative and migratory activity of cervical cancer.[6] In previous studies, it has been proven that miRNA-205 can be used as an effective biomarker for the early detection of lung cancer..[7] However, the significant difference in miRNA-205 regulation in cervical cancer patients with three different stages is still unclear.

MATERIAL AND METHODS

Subject

This study is quantitative research conducted in September-December 2019. The study used 36 samples of paraffin blocks of cervical cancer patients and chronic cervicitis from 2013-2019, which were grouped according to 3 different stages (Grades 1, 2, 3 with the number of sample sizes 18) and chronic cervicitis as control (n=18). The calculation of sample size by using single proportion formula is 18 samples.[8]

$$N = \frac{(Z\alpha)^2 \, p.q}{d2}$$

$$= \frac{(1,96)^2 \; (0,25)\,(0,75)}{(0,2)^2}$$

$$= 1\,8$$

Sampling was carried out after approval of the ethical committee of Warmadewa University (No:11/UN14.2.2.VII.14/LP/2019 date January 7th, 2019). The sample came from the Anatomical Pathology Laboratory, Balimed Hospital, Bali.

Isolation RNA

A total of 36 paraffin blocks were isolated using the MiRNeasy PPFE Kit (Qiagen, Valencia, CA, USA) following the instructions for use contained in the Kit. The concentration and level of purity of RNA were measured with a spectrophotometer NanoDrop 2000 (260/280 nm) with a volume of 1 l/sample.

Synthesis of cDNA

Reverse Transcriptase (RT) PCR techniques using Transcript First Strand cDNA synthesis kit were performed to the cDNA synthesis and following its protocol (Qiagen). Then, cDNA samples as stored at -20^0C for further Analysis.

Amplification of cDNA fragment Target

cDNA were continued to the PCR quantification stage using the miRNA-specific SYBR Green I No Rox dari Qiagen. Quantitative PCR (qPCR) using the Applied Biosystems 7000 sequence detection system. First step for PCR quantification was created a mixed solution of PCR by plotting the reaction component into plate/well with specific composition following the kit instructions. The compositions were 10 µl master mix SYBR Green I, 0.8 µl primer forward, 0.8 µl primer reverse, 5.4 µL nuclease-free water, and 3 µL cDNA template. The total volume of each reaction is 20µL.

The plate with its composition is covered and vortexed, then spin down to reduce air bubbles. RT-PCR conditions for profiling reaction were incubation stage at 50^0C for 2 minutes, activation of the polymerase at 95^0C for 20 seconds, denaturation process at 95^0C for one second, and annealing stage at 60^0C for 20 seconds. The reaction was run for 40 cycles. The primers used in this expression study were GAPDH and miRNA205.

The mean expression levels from the sample miRNA-205 were normalized against the difference of gene expression between cervical cancer and the control group in determining the miRNA-205 expression value was determined by Livak (2008) method established by the formula $2^{-\Delta\Delta Ct}$.

Statistical Analysis

The number of expressions between miRNA-205 is the result of Ct (cycle threshold), which indicates the difference in Ct from the number of miRNA-205.[9] Analysis of changes in miRNA-205 expression based on severity by Kruskal Wallis analysis ($P<0.05$). Correlation level between miRNA-205 and severity using Spearman correlation ($P>0.5$).

RESULT

This study used cervical cancer tissue, and 18 samples of chronic cervicitis tissue as control. Each sample consisted of 6 samples of stage 1 cervical cancer, 7 samples of stage 2, 5 samples of stage 3, and 18 samples of chronic cervicitis tissue. The paraffin block sample has been stored for six years. The paraffin blocks used were tissue samples bound in paraffin and stored at room temperature.

Paraffin blocks are grouped according to the degree of severity from stages 1 to 3. The determination of this paraffin block is based on tracing stored data with preparations that have been made and determined. The lowest age for stage 1 cervical cancer was 35 years old, at grade 2 cervical cancer, the lowest was 25 years old, in grade 3 cervical cancer, the lowest recorded at age 26 years. This study used SYBR Green detection, so it requires a very specific primer to capture the fluorescent signal when the target miRNA-205 in the sample binds to the primer that has been designed. The primer optimization results for miRNA-205 and GAPDH have been previously designed to show a disassociation curve with a single peak. This single peak result explains that GAPDH and miRNA-205 target primers are specific, which is shown in Figure 1.

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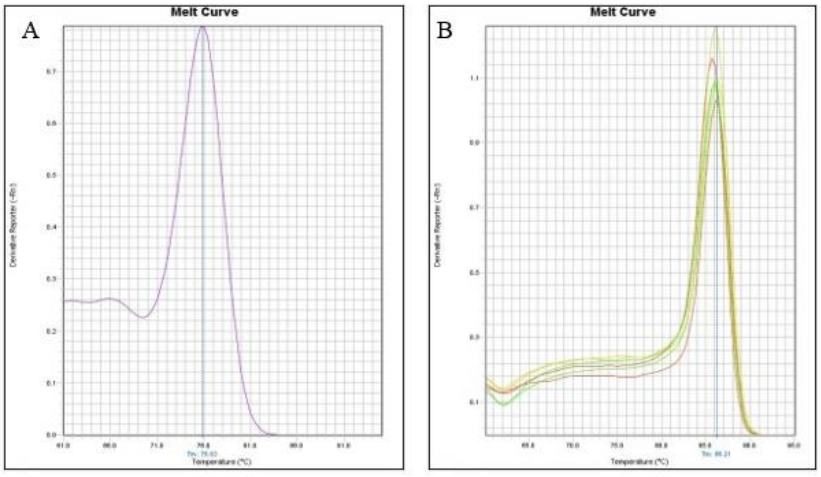

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Figure 1. The disassociation curve with a single peak, indicates a primer that has been designed to attach to the target gene, a specific primer for the GAPDH gene (A), a specific primer for a miRNA gene at three sample stages with triple repeats (B).

Based on differences in severity, we were divided into 4 groups, namely chronic cervicitis, stage 1, 2 and 3. The statistical test used to determine the relationship between the expression of miRNA-205 and the degree of severity was the Kruskal Wallis non-parametric test. The use of a non-parametric test because the sample data was not normally distributed (<0.05).

Table 1. miRNA205 Gene Relative Expression on Cervical Cancer and Control

Gene	Sample	(Mean ± SE)		Mean(Min-Max)	P
		ΔCt	ΔΔCt	Relative Expressions using $2^{-\Delta\Delta Ct}$	
miRNA205	Stage 1	8.63 ± 5.65	18.70 ± 5.08	0.01 (0.00–0.09)	0,00
	Stage 2	3.29 ± 5.14	6.41 ± 5.17	6.26 (0.00-4381)	
	Stage 3	-7.95 ± 3.38	-3.23 ± 4.24	7.24 (0.00-362)	
	Control	-13.47 ± 1.72	0.00 ± 0.00	1 (1-1)	

Table 1 above shows the results of expression analysis after normalization with the internal GAPDH gene and control. Normalization used chronic cervicitis as control samples, which resulted in the expression of miRNA-205 in the sample being 0. This expression value was obtained from calculations using Livak Analysis.[9] The table shows that stage 3 has a very high expression value, an increase in the average expression of up to 362 compared to stages 1, 2, and chronic cervicitis. Although the increase in stage 3 was too excessive, the trend at each stage showed an increase in the expression of miRNA-205 from stage 1 and the highest at stage 3.

Statistical results using the Kruskal Wallis non-parametric test between groups of severity based on stages 1 to 3 on mRNA-250 expression were significantly different ($p < 0.05$) with various groups of severity stages. The results of the Spearman test showed a correlation between the expression of miRNA-205 at stages 1, 2, and 3 had a correlation with each stage. This correlation has a varying strength with a direction that is always in the same direction ($p > 0.5$). Specifically, stage 1 had a strong correlation with stage 2 and stage 3.

Table 2. Kruskal Wallis test results

Cervical cancer severity	n	p
Chronic cervicitis	18	0.000
Stage 1	6	
Stage 2	7	
Stage 3	5	
Total	36	

Significancy (P) <0.05

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Table 3. Spearman correlation between miRNA-205 and severity

		Stage 1	Stage 2	Stage 3
Spearman's Rho	Stadium 1			
	r		0,13	0,70
	p		0,80	0,18
	n		6	5
	Stadium 2			
	r			0,20
	p			0,74
	n			5

DISCUSSION

Cervical cancer is one of the four malignancies in women that poses a challenge to the health sector worldwide. The subtype of human papilloma virus (HPV) is the cause of almost all types of cervical cancer. Squamous cell carcinoma and adenocarcinoma are the most common cervical cancers recorded histologically. In its early stages, cervical cancer often has no symptoms and may be diagnosed if you are screened regularly. Cervical cancer can be prevented by not having multiple sexual partners, using contraception during sexual intercourse, and HPV vaccination. HPV vaccination is the most effective prevention. Cervical cancer is diagnosed through histopathological assessment of cervical biopsy. Women with symptoms of cervical cancer require pelvic examination and visualization of the cervix and vaginal mucosa.[10]

The samples used in this study were 36 samples, 18 from cervical cancer patients, 18 samples from patients suffering from chronic cervicitis. 18 samples of cervical cancer were recorded as cervical cancer, 6 samples were at stage 1 cervical cancer, 7 samples were at stage 2 cervical cancer, 5 samples were at stage 3 cervical cancer, and 18 samples were at chronic cervicitis tissue. Cervicitis is inflammation of the cervix, especially the columnar epithelial cells of the endocervical glands. Cervicitis is divided into two, namely acute and chronic. Acute cervicitis occurs due to infection from bacteria. Cervicitis is usually asymptomatic or followed by mild symptoms. Patients usually complain of vaginal discharge and pelvic pain.[11] Some types of HPV can also cause cervicitis. Cervicitis is mostly transmitted through sexual contact.[12]

Chronic cervicitis in this study was used as a control sample. This refers to a study conducted by Bumrungthai et al. (2015) in Thailand using 20 samples of normal cytology diagnosed by Pap smear and 91 samples of fresh cervical tissue with colposcopy. The results of this study stated that the negative HPV with normal cytology had lower miRNA-21 expression than the HPV positive samples in abnormal tissue and squamous cell carcinoma (SCC). Cervicitis with detectable HPV negative had significantly high miRNA-21 expression compared to normal HPV negative cells.[13]

In this study, the samples used were also derived from formalin-fixed paraffin-embedded (FFPE) blocks obtained from biopsies or taken during surgery. FFPE is mainly used for pathological diagnosis as well as for studying the genomic characteristics of cancer. In genomic cancer studies, FFPE samples have several advantages over new frozen tissue samples, including that FFPE tissue can be used for long-term research. Ripoli et al. (2016) was looking for a comparison of gene expression patterns between FFPE and fresh frozen (FF) tissue with 109 samples of FFPE and 93 samples of FF. The results of this study showed that FFPE specimens showed lower gene expression than FF samples. This may be due to the effect of a long storage process.[14]

miRNAs are small, single-stranded RNA molecules that bind to complementary sequences in the mRNA molecule. miRNAs are formed from longer precursor RNAs that fold back and form one or more short double-stranded hairpin structures (hairpins). Each is linked by hydrogen bonds, and each hairpin is trimmed by enzymes broken down into short double-stranded fragments of 20 long nucleotide pairs. One strand is degraded, while the other strand is a miRNA that forms a complex with one or more proteins. miRNA allows the complex to bind to any mRNA molecule containing a complementary sequence. The miRNA-protein complex then degrades the target mRNA or blocks its translation.[15]

miRNA-205 is one of the miRNAs that have been discovered and investigated in the last few decades. Several studies have stated miRNA-250 has the potential as a biomarker of cervical cancer. Research conducted[16] on miRNA-205 extracted from cervical cancer serum samples and cervical cancer tissue showed a significant increase compared to samples from normal patients. The same results were also obtained in a study conducted by Farzanehpour et al. (2019). In this study, the expression of miRNA-205 will be analyzed with the internal control used in the form of the GAPDH gene.[17]

In this study, the sample experienced an increase in expression value. The most extreme increase in expression was in stage 3. The results of the Kruskal Wallis statistical test concluded that there was a relationship between the expression value and the degree of severity. Based on the test results, it also shows a trend of increasing expression from stage 1 to the highest stage 3. Likewise, the results of the correlation test for each stage with the degree of severity indicate a correlation. These results are in line with the research of Ma et al. (2014), which used 60 samples of cervical cancer patients and 60 healthy people as controls. The results showed an increase in miRNA-205 expression in serum and cervical cancer tissue samples from cervical cancer patients. According to Ma et al., miRNA-205 acts as an oncogene that regulates the expression of multiple cancer-related genes in target genes. In addition, miRNA-205 is also over-expressed in human cervical cancer tissue and cervical cancer cell migration by targeting CYR61 and CTGF.[16]

A recent study conducted by Xie et al. (2017) stated that miRNA-205 was constantly dysregulated in cervical cancer tissue compared to normal cervical tissue. The study also stated there was a significant increase in the expression of miRNA-205 in cervical cancer tissue compared to surrounding normal tissue.[16] In addition to cervical cancer, miRNA 205 was also detected in several other cancer tissues, such as breast cancer, lung cancer, and colorectal cancer.[18] According to Lan et al. (2014) it makes miRNA-205 has the potential as a biomarker because stage 3 cervical cancer patient tissue has an abnormal expression (overexpression) of it.

CONCLUSION

The value of miRNA-205 expression was significantly increased in cervical cancer samples, and this increase had a positive correlation. These results show that miRNA-205 is a potential candidate as an important biomarker for cervical cancer screening that is useful for diagnosing the development of cervical cancer. The higher the severity of cervical cancer, the more overexpression of the miRNA-2015. Further, we suggest a study with a larger population to compare the expression values of miRNA-205 between normal and cancer tissue samples.

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