



MicroRNA-21 as a biomarker for ovarian cancer detection

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ABSTRACT Ovarian cancer is a lethal disease. One of the problems faced by patients with ovarian cancer is the lack of symptoms in its early stages, which results in it only being detected when it is at an advanced stage. Therefore, there is an urgent need for biomarkers that can predict ovarian cancer precisely. The purpose of this study was to determine the expression of microRNA-21 as a predictive biomarker candidate in both early- and advanced-stage ovarian cancer. This was a cross-sectional study using the blood plasma of 21 healthy control subjects and 37 blood plasma samples from patients with ovarian cancer. Blood plasmas were collected, from which the RNA was isolated. Based on the RNA, the cDNA was synthesized and run through qPCR, the results of which were analyzed using the Livak method. The results showed an upregulation of microRNA-21 in the advanced stage by 2.14 fold compared with the early stage, and 6.13 fold compared with the healthy controls ($p < 0.05$). The upregulation of microRNA-21 in early-stage ovarian cancer was 2.86 fold compared with the healthy control subjects ($p < 0.05$). In addition, there was an increase in the expression of microRNA-21 in ovarian cancer by 4.14 fold compared with the healthy controls ($p < 0.05$). Based on these results, it can be concluded that the expression of microRNA 21 upregulated with the severity of the disease.

KEYWORDS microRNA-21; minimally invasive; ovarian cancer; predictive biomarker

1. Introduction

Ovarian cancer is one of the leading causes of death with most first detections occurring in its late stages (Cannistra 2004). Only 20% of ovarian cancer patients are diagnosed when the cancer has not yet spread into the ovaries. Conversely, in 80% of cases, the cancer has metastasized, metastasis that often occurs in the peritoneum (Bast et al. 2009; Cho and Shih 2009). Poor prognosis is associated with multiple factors in ovarian cancer: weak invasive minimum detection, early detection tests, the development of invisible symptoms, and tumor chemoresistance (Qu et al. 2011). The International Agency for Research on Cancer (IARC) in GLOBOCAN 2012 stated that the total cases of ovarian cancer in Indonesia amounted to 10,238 cases, with the age of most patients being around 60–69 years, and 7,075 of the patients dying. Ovarian cancer

was ranked the third incidence in Indonesia, along with the fifth leading cause of cancer-associated mortality in Indonesia. This proves that the incidence of ovarian cancer is associated with a delay in the early detection process, and ovarian cancer causes a decrease in the life expectancy of the community. New biomarkers with high sensitivity and specificity are needed for the diagnosis and therapeutic targets for ovarian cancer. One of these biomarkers that can be used as a candidate for early detection of ovarian cancer is miRNA (Taylor and Gercel-Taylor 2008; Resnick et al. 2009).

MicroRNAs are noncoding RNA molecules that have a nucleotide length of 21–25, and bind to 3' UTR in mRNA where microRNAs inhibit protein translation or trigger mRNA degradation (Mezzanzanica et al. 2010). microRNAs can function as both oncomers and tumor suppressors. microRNAs also play an important role in cel-

lular differentiation, cell growth, stress response, and cell death, via modulation of the target mRNA transcription expression (Koutsaki et al. 2014). Specific types of microRNAs target specific mRNAs, microRNAs are stable and are excreted out of cells by vesicles and exosomes and circulate into the bloodstream (Yang et al. 2008; Chao et al. 2014). There are different patterns of miRNA expression in both the serum and plasma of cancer patients compared with healthy controls, which means miRNA can be used as a biomarker candidate to diagnose malignancy (Zhou et al. 2012; Chen et al. 2013).

Several studies related to miR-21 expression in ovarian cancer, miR-21 were exposed to serum from Epithelial Ovarian Cancer (EOC) patients compared with healthy controls (Resnick et al. 2009), overexpression of miR-21 with exosome isolation in plasma of ovarian cancer patients compared to controls, i.e. Benign Ovarian Adenoma (Taylor and Gercel-Taylor 2008), upregulation of miR-21 in the serum of EOC tumor-type ovarian cancer patients was compared to healthy controls (Suryawanshi et al. 2013). The studies that have been conducted in association with the expression of miR-21 in ovarian cancer proved to be a change in expression to overexpression compared with healthy controls. However, the expression of miR-21 in plasma of ovarian cancer patients with the population of Indonesia especially Yogyakarta has not been previously identified. This study aimed to measure changes in miR-21 expression in the plasma of ovarian cancer patients compared with healthy controls using a minimally invasive method.

2. Materials and methods

2.1. Patient samples

A total of 19 early-stage ovarian cancer patients, 18 advanced-stage patients, and 21 healthy control patients being treated in Sardjito General Hospital, Yogyakarta, Indonesia, were included in the study during the period of September 2015 to May 2016. The subjects' ages ranged from 21 to 70 years old. Stage breakdown was $n = 17$ for stage I, $n = 2$ for stage II, $n = 14$ for stage III, and $n = 4$ for stage IV. Informed consent was obtained from each patient prior to sampling. Blood samples up to 5 mL in volume were drawn from a peripheral venous puncture prior to surgery. The blood was collected using an EDTA tube measuring 5 mL. Immediately after sample collection, the blood samples were centrifuged at 1,500 rpm for 10 min at 4°C. Plasma was transferred into 1.5 mL RNase-free tubes using a tip filter, and then the plasma was stored at -80°C until further use. Ethical approval was obtained from the Medical and Health Research Ethics Committee (MHREC), Faculty of Medicine, Universitas Gadjah Mada-Dr. Sardjito General Hospital (Ref: KE/FK/085/EC/2015) and all plasma samples were acquired with the informed consent of the respective individuals.

2.2. MicroRNA isolation and real-time quantitative PCR

The plasma sample of -80°C was melted and then centrifuged at 3000 g, for 5 min at 4°C and taken as much as 200 µL. Total RNA was extracted using miRCURY RNA Isolation Kit-Biofluid kit according to manufacturer's instruction (Exiqon, Denmark). RNA is stored in a refrigerator -80°C for reuse.

Immediately done DNA synthesis of RNA was obtained. Making cDNA is done using a Universal cDNA Synthesis kit according to manufacturer's instruction (Exiqon, Denmark). The preparation and distribution of the master mix was done by mixing the 4 µL 5x reaction buffer, 9 µL Nuclease free water, 2 µL Enzyme mix, and 1 µL Spike in (sp6), up to a total volume of 16 µL reagents and sprayed with 4 µL RNA samples up to total volume 20 µL per reaction. The reaction mixture was incubated at 42°C for 60 min, inactivated reverse transcriptase at 95°C for 5 min, and cooling down at 4°C. The cDNA synthesis results were stored in a refrigerator at -20°C.

Furthermore, cDNA was diluted with RNase-free water at a ratio of 1:80, ie 1 µL cDNA with 80 µL RNase-free water. Real Time Quantitative PCR using ExiLent SYBR Green master mix kit according to manufacturer's instruction (Exiqon, Denmark). primary set (forward and reverse) microRNA, cDNA that has been made before. The RNA is then prepared mixed master mix for the target miR-16 as the reference gene, miR-21, and 1 H₂O. As many as 5 µl SYBR Green master mix, and 1 µl mixed primary PCR mix (for each differentiated mixture). 4 µL cDNA coupled with 6 µL master mix. The reaction mixture was incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 60°C for 1 min ramp-rate 1.6°C/s optical read and analyzed melting curve. The result of qRT-PCR duplicate, and miR-16 was used as the internal control. The relative miR-21 expression was calculated using the equation $2 - \Delta\Delta Ct$, the difference between the original copy number of miR-21 in the ovarian cancer group and that in the healthy control, $-\Delta\Delta Ct = (Ct \text{ miR-21} - Ct \text{ miR-16})$ of the ovarian cancer group, $-(Ct \text{ miR-21} - Ct \text{ miR-16})$ of the healthy control.

2.3. Statistical analyses

All statistical analyses were carried out using SPSS. p values < 0.05 were considered statistically significant. Data were expressed as the mean \pm SD. Expression of miR-21 in both early-stage and advanced-stage ovarian cancer was analyzed using ANOVA and to compare two variables using the Mann-Whitney test.

3. Results

3.1. miR-21 undergoes upregulation in plasma samples of ovarian cancer

The non-parametric Mann-Whitney test was employed to compare miR-21 expression in patients with ovarian can-

TABLE 1 Expression of miR-21 in plasma samples of ovarian cancer and healthy control.

Plasma samples	miRNA targeted	Mean (ΔCq) \pm SD	$\Delta\Delta Cq$	Fold change ($2^{-\Delta\Delta Cq}$)
Ovarian cancer	miR-21 as target miR-16 as internal control	0.4078 \pm 1.3922	-2.0512	4.1445
Healthy control	miR-21 as target miR-16 as internal control	2.4590 \pm 1.6084		

cer and healthy controls. The results showed that miR-21 had a significant upregulation in ovarian cancer patients (Table 1). MiR-21 expression in the ovarian cancer samples was upgraded four times when compared with the control ($p < 0.05$). The mean \pm SD ΔCq value of miR-21 was 0.4078 \pm 1.3922 in the 37 ovarian cancer samples, and 2.4590 \pm 1.6084 in the healthy control subjects.

3.2. Plasma miR-21 is associated with cancerous malignancy rates in the FIGO stage

To determine the important role of plasma miR-21 in the development and progression of ovarian cancer, we examined the association of miR-21 expression at various stages of malignancy in ovarian cancer (Table 2). Classification of cancer based on FIGO stage, i.e. early-stage ovarian cancer patients (numbering 19 in total) had a mean \pm SD 0.9421 \pm 1.0198, and advanced-stage ovarian cancer patients (18 in total) had a mean \pm SD -0.1561 \pm 1.5321. Based on the results obtained, miR-21 in advanced-stage ovarian cancer patients experienced a two-fold upregulation compared with early-stage cancer patients ($p < 0.05$, ANOVA). Meanwhile, miR-21 in early-stage ovarian cancer of plasma undergoes upregulation three ($p < 0.05$). The highest increase of expression was found in miR-21 plasma of advanced ovarian cancer when compared with healthy control that was 6 times ($p < 0.05$). These results expand on the current knowledge in cancer research, showing that the expression of miR-21 in plasma will have upregulation in accordance with the stage of the malignancy. There is not only an increase at the advanced stage, but in the early stage, as well, with plasma miR-21 increasing

three-fold, which suggests that miR-21 can be a promising biomarker candidate for the early detection of ovarian cancer.

4. Discussion

Ovarian cancer is one of the most severe forms of cancer that can cause death and occur only in women. In its early stages, ovarian cancer does not show specific symptoms that might encourage patients to undergo an early examination. In addition, the location of the ovary organs in the abdominal cavity means that an examination cannot be done consciously like with breast cancer.

Weak examination using CA 125 biomarkers also causes a false positive ovarian cancer diagnosis. CA 125 is very weak for specificity (SP) and sensitivity (SN), so it is not a reliable biomarker in screening in early detection of ovarian cancer (U.S. Preventive Services Task Force 2004; Coticchia et al. 2008; Rauh-Hain et al. 2011). CA-125 values in normal serum range from 0–35 U/mL, with an excess of these values indicating a malignancy or non-cancerous disease (Bhatt et al. 2010). Based on observations on patient status in the Medical Record Room of Dr. Sardjito General Hospital from September 2015 to April 2016, the obtained data revealed that of 52 patients measured for the number of CA-125, eight patients that had CA-125 levels <35 U/mL were diagnosed with a tumor, 19 patients that had CA-125 levels >35 U/mL were diagnosed with a tumor, and 25 patients with CA-125 levels >35 U/mL were diagnosed with ovarian cancer (based on definitive PA results).

TABLE 2 Expression of miR-21 in plasma samples of early and advanced ovarian cancer compared with the healthy controls.

Plasma samples	miRNA targeted	Mean (ΔCq) \pm SD	Remarks	$\Delta\Delta Cq$	Fold change ($2^{-\Delta\Delta Cq}$)
Early-stage ovarian cancer	miR-21 as target miR-16 as internal control	0.9421 \pm 1.0198	Advanced-stage ovarian cancer compared with early-stage	-1.0982	2.1410
Advanced-stage ovarian cancer	miR-21 as target miR-16 as internal control	-0.1561 \pm 1.5321	Advanced-stage ovarian cancer compared with healthy control	-2.6151	6.1269
Healthy control	miR-21 as target miR-16 as internal control	2.4590 \pm 1.6084	Early-stage ovarian cancer compared with healthy control	-1.5169	2.8618

Research related to miRNA has been widely performed for the purpose of diagnosis and prognosis physiopathologic malignancy. Multiple profiling has indicated that microRNA expression significantly changes in the development of ovarian cancer (Iorio et al. 2007; Nam et al. 2008). The microRNA marker in circulating tumor exosomes in ovarian cancer patients demonstrates a high level of correlation with the expression of microRNA in the primary tumor (Taylor and Gercel-Taylor 2008).

MicroRNA is contained in small particles and these particles can protect against microRNA from RNase activity (Mo et al. 2012). MicroRNA plays an important role in cellular differentiation, cancer development, angiogenesis, immune response, cell growth, stress response, and cell death, via modulation of the target mRNA transcription expression (Kuehbachner et al. 2008; Davidson-Moncada et al. 2010; Song et al. 2010; Liu et al. 2011; Koutsaki et al. 2014).

Research has been widely conducted on the expression of miR-21 in ovarian cancer, whether on ovarian tissue, serum, or plasma. One study using a malignant and normal ovarian tissue sample reported an overexpression of miR-21 in ovarian cancer compared with normal tissue (Iorio et al. 2007). Taylor and Gercel-Taylor (2008) studies using serum samples in serous adenocarcinoma patients, and controls using benign ovaries, the results showed several upregulations of miR-21, -141, -200a, -200c, -203, -20, and -214 in ovarian cancer. Xu et al. (2013) also showed that patients with EOC had significantly increased serum miR-21 compared with healthy controls, and serum expression of miR-21 had a correlation with FIGO stage.

The recent used both ovarian cancer plasma and healthy controls aimed at developing a minimally invasive biomarker. The results showed that plasma miR-21 in ovarian cancer had upregulated four times compared with the healthy control ($p < 0.05$, Mann-Whitney test). Based on the FIGO stage, the expression of miR-21 increased along with the increase in stages. The expression of miR-21 in early-stage ovarian cancer was upregulated when compared with the healthy controls ($p < 0.05$, ANOVA). An increased expression of miR-21 also occurred in advanced ovarian cancer compared with the early stage. Based on these results, miR-21 can be used as a diagnostic biomarker candidate of ovarian cancer as it was found to undergo significant expression changes in the early stages of ovarian cancer.

5. Conclusions

This study revealed that the level of miR-21 in the plasma of patients with ovarian cancer showed an increase, and can be associated with FIGO stage. Increased miR-21 expression in malignancy conditions can aid in cancer research, especially in the development of diagnostic and prognostic biomarkers, and may be targeted for a personalized medicine ovarian cancer treatment.

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Authors' contributions

The first author, AIK conducted a collection of presurgical patient plasma samples, carried out the laboratory work such as RNA isolation, cDNA synthesis, qRT-PCR, analyzed the data and wrote the manuscript, SNC and ASF assisted in collecting plasma samples of patients with ovarian cancer, informed consent patient, and looking for secondary data in medical record, DST wrote the manuscript. AT, HP, TA, SMH designed the study, providing guidance in ovarian cancer research and microRNA.

Competing interests

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