The role of circulating MicroRNA-21 (miR-21) in epithelial ovarian cancer

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ABSTRACT

Background: The development of a novel biological marker for diagnosis and prognosis prediction of epithelial ovarian cancer (EOC) remains urgent. Current literature shows that microRNA-21 (miRNA-21/miR-21) are involved in the growth, metastasis, and apoptosis of cancer cells. This study aims to determine the difference in miR-21 expression between stages, histopathology type, and residual tumor after surgery in EOC patients.

Methods: A cross sectional study was conducted among 40 EOC patients. Blood samples were taken prior to surgery, and when the pathological anatomy result confirmed the presence of EOC, the RNA was then isolated. Based on the RNA, the cDNA was synthesized and run through qPCR. All data were analyzed using the GenEx analyzing software.

Results: The expression of miR-21 in the advanced stage was 1.36 up-fold compared to the early stage (p=0.52). The expression of miR-21 in type II EOC was 1.33 up-fold compared to type I EOC (p=0.56). The expression of miR-21 in residual tumor >1cm after surgery was 1.30 up-fold compare to residual tumor after surgery < 1cm (p=0.59)

Conclusion: There was no significant increase in the expression of circulating miR-21 between stages, histopathology type, and residual tumor after surgery in EOC patients in this study. Circulating miR-21 may be a promising biomarker for EOC. Nevertheless, further studies with larger sample sizes are still needed to address the discrepancy and clarify the prognostic value of circulating miR-21.

Keywords: Circulating miR-21, epithelial ovarian cancer, non-invasive biomarker

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INTRODUCTION

Of all gynecological cancers, ovarian cancer is associated with the worst prognosis and the highest mortality rate. Ovarian cancer is the seventh most frequent cancer worldwide, with 238,700 new cases in 2012, and is the eighth most common cause of cancer mortality, with 151,900 deaths.1 In developed countries, ovarian cancer has the highest mortality rate compared to other types of cancers, while it is the second most common cause of cancer-related death in developing countries, following cervix cancer. In 2018, there will be approximately 22,240 new cases of ovarian cancer diagnosed and 12,070 ovarian cancer deaths in the United States. Ovarian cancer accounts for 2.5% of all malignancies among females but 5% female deaths because of low survival rates, largely driven by late-stage diagnoses.2

The most common ovarian cancer is the epithelial subtype.3 A study of epithelial cancer survival rate in Dr. Cipto Mangunkusumo Hospital showed that the survival rate in the first year was 95.8, second year 86.8%, while the percentage decreased in the third and fourth year becoming 77%. The survival rate based on the stage of the ovarian cancer was 32.7% (44.9%) for the early stage and 67.3% (55.1%) for the advanced stage. The highest incidence of ovarian cancer in Dr. Cipto Mangunkusumo Hospital belongs to the age of reproductive women (≤ 55 years old), particularly nulliparous women. Most ovarian cancer cases are diagnosed in the advanced stage.4 The primary reasons for this poor prognosis include the late stage of presentation due to the absence of symptoms, the lack of effective screening tools, and the development of recurrent disease that is resistant to chemotherapy.

Currently, there are no effective biomarkers for ovarian cancer prognosis that can provide information regarding the clinical treatment for ovarian cancer. The circulating biomarkers used in current clinical practice lack sufficient accuracy. Therefore, there is still a great need for simple and reliable biomarkers to predict the outcome of ovarian cancer patients.

MicroRNAs (miRNAs) are endogenous, small non-coding 18–25 nucleotide RNAs. These miRNAs could be post-transcriptional gene regulators that paired to complementary sequences in the 3’ untranslated region (3’ UTR) of target mRNAs, leading to mRNA degradation or translational repression. Not only do these miRNAs regulate gene expression, but they are also involved in various biogenesis that is associated with carcinogeneses, such as cell proliferation, cell cycle, apoptosis, angiogenesis, invasion, and metastasis. In
particular, circulating miRNAs in the serum and plasma, for example, could be used as non-invasive biomarkers for cancer. The plasma and serum were found to be rich in miRNAs that appeared to be protected from RNase degradation and remained highly stable at room temperature as well as under adverse conditions such as multiple freeze-thaw cycles. miRNAs have been detected in various fractions of circulating blood such as erythrocytes, anucleate platelets, apoptotic bodies and all other cells in the body, making these molecules suitable as biomarkers for cancer prognosis.

Circulating miRNAs as biomarkers have several advantages compared with tissue-based markers. Among them, miR-21 was the representative one since it has been extensively studied in various cancers. Many studies showed that the expression level of circulating miR-21 could distinguish cancer patients from healthy people and predict disease outcomes. However, there are inconsistent results for the diagnostic accuracy and the overall risk for prognosis associated with miR-21. miR-21 is overexpressed in multiple types of cancer and promotes the initiation of cancer, progression, and drug-resistance. It also impacts tumorigenesis by negatively regulating several targets.

The expression of miR-21 has been found to be markedly increased in ovarian cancer. Few have investigated the role of miR-21 in regulating ovarian cancer chemoresistance which will affect the prognosis of ovarian cancer patients. The expression of miR-21 in the plasma of ovarian cancer patients in Indonesia, particularly in Dr. Sardjito Hospital Yogyakarta, has not been previously identified. This study aimed to measure the difference in miR-21 expression in the plasma between stages, histopathology type, and residual tumor after surgery in epithelial ovarian cancer (EOC) patients.

MATERIAL AND METHODS

Subject Criteria
This study was a cross-sectional study conducted in Dr. Sardjito General Hospital Yogyakarta from September 2015 to May 2016. The inclusion criteria were adult patients diagnosed with EOC at any stage who underwent primary surgery. We excluded patients who had a history of malignancy other than EOC, had previous ovarian cancer surgery and chemotherapy. The diagnosis of EOC was confirmed with histopathology results. Consecutive sampling was performed, with 33 subjects as the minimum sample size required based on the formula. Ethical approval was obtained from the Medical and Health Research Ethics Committee (MHREC), Faculty of Medicine, Universitas Gadjah Mada-Dr. Sardjito General Hospital. Informed consent was obtained from each patient prior to sampling.

Sampling Technique for DNA Preparation
Blood samples up to 5 mL in volume were drawn from a peripheral venous puncture before surgery. The blood was collected using EDTA tubes measuring 5 mL. Immediately after sample collection, the blood samples were centrifuged at 1,500 rpm for 10 minutes at 4°C. The 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. After histopathology result confirmed EOC, samples were proceeded to qRT-PCR microRNA in the Biomolecular Laboratory, Faculty of Medicine, Universitas Gadjah Mada.

The plasma sample that was stored at −80°C was melted and then centrifuged at 3000 g for 5 minutes at 4°C, and as much as 200 μl were taken. Total RNA was extracted using miRCURY RNA Isolation Kit-Biofluid kit according to the manufacturer’s instruction (Exiqon, Denmark). RNA was stored in a refrigerator at −80°C for reuse. Immediately DNA synthesis of RNA was obtained. The making of cDNA was done by using a Universal cDNA Synthesis kit according to the 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, i.e., 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 the 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 an- alyzed melting curve. The result of qR-PCR duplicate and miR-16 was used as the internal control. The relative miR-21 expression was calculated using the equation 2 − ΔΔ Ct.
Histopathology classification
The stage, histopathology type, and residual tumor were determined following the surgery. The stage of EOC was determined using the International Federation of Gynecology and Obstetrics (FIGO) classification. Early stage was defined as FIGO I and II, while the advanced stage was defined as FIGO III and IV. The histopathology type was divided into type 1 and type 2. Type 1 was low-grade serous, low-grade endometrioid, clear cell, mucinous carcinomas, and Brenner tumors. Type 2 was high-grade serous, high-grade endometrioid, malignant mixed mesodermal tumors carcinomas, and undifferentiated carcinomas. Residual tumor was divided into <1 cm and > 1 cm.

Data Analysis
The data were analyzed using GenEx 5 qPCR data analysis software. Data were expressed as mean ± SD. P values < 0.05 were considered statistically significant.

RESULTS
Baseline characteristics of respondents
A total of 40 patients with EOC were included in this study. Patient characteristics can be seen in Table 1. In this study, 52.5% of patients were >50 years old. Most patients were in the early stages of EOC (57.5%). Type I EOC (60%) was more common than type II EOC (40%). Residual tumor less than 1 cm after surgery was achieved in the majority of the patients (65%). Ca-125 level less than 500 U/ml were found in 23 patients (57.5%), while 17 (42.5%) patients had Ca-125 level more than 500 U/ml (Table 1).

The expression of miR-21 in Epithelial Ovarian Cancer
All samples were measured three times in the qRT/PCR measurement. The expression of miR-21 was detected in plasma serum samples. As shown in Table 2, the expression of miR-21 in patients in the early stage and advanced stage of EOC was -1.15 ± 2.38 and 0.45 ± 1.91 respectively. However, the difference was not statistically significant (p = 0.5253). Based on the fold change calculation using the Livak method from Figure 1, the expression of miR-21 in patients in the advanced stage of EOC was 1.3 fold up-regulated compared with the early stage (Table 2).

According to the histopathology, the expression of miR-21 in EOC was 2.22 ± 2.54 for type I and 0.41 ± 1.51 for type II. The difference of miR-21 expression between type I and type II was not statistically significant (p = 0.5635). The expression of miR-21 in type II was 1.3 fold up-regulated compared to type II (Figure 1 and 2).

The expression of miR-21 was -6.34 ± 2.35 in patients with residual tumor more than 1 cm and -0.38 ± 2 in those with residual tumor less than 1 cm. There was no significant difference in the expression of miR-21 between these group of patients (p = 0.59716). However, the fold change of miR-21 expression in residual tumor more than 1 cm was 1.3 fold up-regulated compared to residual tumor less than 1 cm (Figure 1 and 2).

DISCUSSION
Ovarian cancer is one of the most lethal gynecological cancers. Routine diagnostic procedures such as pelvic examination, serum Ca-125, and transvaginal ultrasonography usually fail to detect ovarian cancer at an early stage. Consequently, within six months to two years after treatment,
60-80% of patients will have a recurrence, where recurrent cancer becomes more malignant, fast-spreading and resistant to previously used drugs, which will ultimately worsen the prognosis. This poor prognosis is due to the insidious asymptomatic nature of this cancer in the early stage, tumor resistance to chemotherapy and the lack of robust and minimally invasive methods for early detection.

miRNAs have been reported to be correlated with the progression of various cancers, and some of them have been identified as potential biomarkers. Circulating miRNAs were first detected in the plasma and serum in 2008 and subsequently found in various body fluids like urine, breast milk, saliva and many more. Circulating miRNAs were found to be stable even in harsh environments such as high temperatures, extreme pH values, and long term storage. The expression of miRNAs may act as a novel class of tumor suppressor genes or oncogenes. miRNAs have been found to be differentially expressed in solid tumors and hematopoietic tumors. miR-21 is aberrantly expressed and functions as an oncogenic miRNAs in many tumors including ovarian cancer. It has a role in resistance to hypoxic conditions which inhibit tumor growth.

This study was conducted to evaluate the expression levels of circulating miR-21 in EOC. The result showed there were increased miR-21 expression levels. We also evaluated the expression levels of miR-21 between patients in the early stage and late stage of EOC but found no significant difference. With regards to other patient characteristics that we evaluated, including type I and II histopathology as well as optimal and suboptimal surgery procedure, this study found no significant difference in the expression levels of miR-21 in these characteristics. Despite this, we found a trend that miR-21 was upregulated in patients who were in the advanced stage, had type II EOC and had residual tumor after surgery >1cm, which is related to negative prognostic factors for patients.

A study conducted by Kartika, which compared miR-21 expression between ovarian cancer patients and healthy control, found a significant up-regulation in ovarian cancer patients. The results showed that miR-21 expression in the ovarian cancer group was upgraded four times compared with the control group (p < 0.05). Hanzada et al. reported that miR-21 expression levels could distinguish patients in the early stage (I and II) and late stage (III and IV) of breast cancer. Jianhong Wu et al. also reported that circulating miR-21 could be used as an indicator in the diagnosis of gastric cancer in early and late stages. Another study conducted by Yun Zhao Xu et al. confirmed that the level of
serum miR-21 was elevated in EOC patients and was associated with FIGO stage and tumor grade.\textsuperscript{17}

Yun Zhao Xu et al. reported no significant correlation between plasma serum miR-21 levels and pathological type or patient’s age (≥59.2 versus <59.2) in their study.\textsuperscript{17} Lou et al. examined tissue samples of ovarian cancer patients and found no significant difference between miR-21 expression and histopathology type of ovarian cancer (serous, mucinous, and endometrioid).\textsuperscript{18} Enas et al. reported no correlation between miR-21 serum level and histopathology of ovarian cancer patients (p=0.643).\textsuperscript{19} Ovarian cancer is hereditary and is associated with germline mutations of breast cancer 1 (BRCA1) and breast cancer 2 (BRCA2), mismatch repair genes and, on rare occasions, p53. BRCA1 and BRCA2 affect gene expression profiles and abnormalities in high grade serous ovarian cancer. Almost 96% of p53 mutations are found in high grade serous ovarian cancers which can regulate miRNAs.\textsuperscript{21} Dong et al. reported that among miR-21 targets, phosphatases and tensin homolog (PTEN) and programmed cell death protein 4 (PDCD4) were up-regulated upon miR-21 deletion, regardless of p53 status. They found that there were fewer apoptotic cells when PTEN inhibited, which confirms the critical role of PTEN in linking miR-21 with p53 as independent apoptosis.\textsuperscript{26}

Debulking surgery followed by chemotherapy are widely used treatments for EOC at present. Although overall survival rates have increased slightly over the past 25 years, 5-years survival rate remains <50%.\textsuperscript{8} Ramos et al. identified miR-21-5p as a predictive marker response to neoadjuvant chemoradiation in rectal cancer patients. Patients with a complete clinical response based on very stringent criteria and high levels of miR-21-5p expression may be ideal candidates for alternative treatment strategies to radical surgery including a watch and wait approach.\textsuperscript{24} In present study from Ghisoni et al. made predictive score of cytoreductive (PSC) with age > 60, CA-125 ≥ 550, and peritoneal cancer index (PCI) >16 as predictor surgical outcome variables. Among these three variables, PCI was the best predictor of surgical outcome, with an accuracy of more than 80%. Prediction tools might help surgeons to give a surgical chance to all patients that could be completely debulked, therefore limiting the number of suboptimal surgeries at 16.5%.\textsuperscript{28}

**CONCLUSION**

In this study, no significant increase in the expression of circulating miR-21 was found in the advanced stages of EOC, type II EOC and residual tumor >1 cm after surgery. Despite this, circulating miR-21 can still be a promising biomarker for EOC. Further studies with larger sample sizes are needed to address the discrepancy and clarify the prognostic value of circulating miR-21.

**CONFLICT OF INTEREST**

Author declares there is no conflict of interest regarding all aspect of the study.

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