Rwanda Medical Journal RMJ

ORIGINAL ARTICLE Open Access

Downregulation of Circulating MicroRNA-155 Expression in the Blood Plasma as a Promising Minimally Invasive Biomarker for Epithelial Ovarian Cancer

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ABSTRACT

INTRODUCTION: MicroRNA-155 (miR-155) is implicated in ovarian carcinogenicity by regulating gene expression post-transcriptionally. The objectives of the study include evaluating miR-155 expression in tissue and plasma from Epitherial Ovarian Cancer (EOC) patients, examining its association with histological sub-types and clinical stages, and assessing its diagnostic potential in plasma for EOC.

METHODS: Blood plasma was collected from healthy women (n=37) and EOC patients (n=42), while EOC tissues and paired adjacent normal ovarian tissues were collected from the 42 EOC patients. Tissue- and plasma-derived RNA was extracted and synthesized into cDNA. The relative quantification of miR-155 was achieved using qRT-PCR, followed by calculation using the $2^{-\Delta\Delta Ct}$ method.

Results: Significant downregulation of tissue- and plasma-derived miR-155 is observed in EOC groups compared to normal groups ($P<0.05$). Moreover, significant downregulation of tissue- and plasma-derived miR-155 is observed in advanced-stage compared to early-stage EOC ($P<0.05$). However, tissue- and plasma-derived miR-155 expression is not significantly different among histological subtypes ($P>0.05$). The AUC value of plasma-derived miR-155 in distinguishing EOC from healthy subjects was 0.962 (95% CI: 0.928-0.996), with 92.9% sensitivity and 86.5% specificity.

Conclusion: Consistent downregulation of miR-155 expression is observed in both tissue and blood plasma of EOC patients, and its downregulation is associated with advanced-stage EOC, suggesting its potential application as a minimally invasive biomarker for EOC.

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Received: 8th September 2026; **Initial decision given:** 18th December 2025; **Revised manuscript received:** 4th January 2026; **Accepted:** 16th March 2026.
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Citation for this article: Akbar Satria Fitriawan; Siti Nur Chasanah; Aprilia Indra Kartika et al. Downregulation of Circulating microRNA-155 Expression in the Blood Plasma as a Promising Minimally Invasive Biomarker for Epithelial Ovarian Cancer. Rwanda Medical Journal, Vol. 83, no.1, p. 59-71, 2026. https://dx.doi.org/10.4314/rmj.v83i1.7

INTRODUCTION

Epithelial ovarian cancer (EOC), ovarian malignancy that arises from malignant transformation of epithelial cells of the ovarian surface and fallopian tube, is a leading cause of death from gynecological malignancies [1–3]. Annually, it is estimated that ovarian cancer is responsible for the mortality of more than 150,000 women. Lack of specific symptoms and effective detection methods contributed to the late diagnosis and high mortality rate of EOC [4]. The advanced stage EOC, which accounted for 80% of all detected cases, had a significantly lower 5-year survival rate (<17%) compared to early stage EOC (>90%) [4,5], indicating that early detection of EOC is essential for improving patient prognosis. Currently available EOC biomarkers such as CA-125 and HE4 demonstrated a lack of sensitivity and specificity [6–8].

MicroRNAs (miRs/miRNAs) are a class of small non-coding RNA molecules with 18-25 nucleotides in length that negatively regulate protein-coding gene expression at the post-transcriptional level, either by inhibiting mRNA translation and/or stimulating mRNA degradation [9]. The ability of microRNA to regulate numerous protein-coding genes is crucial for modulating various key cellular phenotypes, and its aberrant expression contributes to the development of human malignancies by regulating almost all of cancer hallmarks, including cell cycle, apoptosis, invasion, metastasis, angiogenesis, chemoresistance, and immune escape phenotype [10,11].

MicroRNAs are secreted by cells into the extracellular environment and are expressed in various body fluids, including blood circulation, as cell-free microRNAs. Cell-free microRNAs are also secreted by cancer cells and facilitate intercellular communication within the tumor microenvironment, ultimately affecting tumorigenesis. The deregulation of cell-free microRNA correlates with tumor formation and progression. Moreover, the microRNA expression profile in blood plasma has been shown to correlate with its deregulation in cancer cells and tissues [12,13], suggesting its potential as both therapeutic targets and minimally invasive biomarkers for cancer diagnosis and prognosis.

Homo sapiens-microRNA-155-5p (miRNA-155/miR-155) is one of 3000 known human microRNAs with the potential ability to negatively regulate at least 147 protein-coding genes that play important roles in various cellular phenotypes, such as mitotic division, anti-apoptosis, differentiation, epithelial-to-mesenchymal transition (EMT), migration, and angiogenesis [14]. Extensive studies revealed the dual role of miR-155 in carcinogenicity; it could act as an oncogene or tumor suppressor microRNA depending on the cancer type [14–17].

Thus far, previous studies have consistently demonstrated that miR-155 is downregulated in ovarian cancer cells [18–20], ovarian cancer tissue [20], and urine of ovarian cancer patients [3], suggesting the potential tumor-suppressive nature of miR-155 in EOC. However, the published study evaluating the diagnostic power of miR-155 expression in EOC patients' blood plasma is still limited. This study aims to evaluate miR-155 expression in EOC tissue and EOC patients' blood plasma and the diagnostic value of plasma miR-155 to distinguish EOC patients from healthy subjects.

METHODS

Study Setting and Period

The sample collection process and miR-155 expression analysis (total RNA isolation, cDNA synthesis, and qRT-PCR) was performed sequentially between October 2015 and September 2016. The sample collection process was conducted at Dr. Sardjito General Hospital, whereas miR-155 expression analysis was performed at Laboratory of Molecular Biology, Faculty of Medicine, Universitas Gadjah Mada, Indonesia.

Sample size calculation

G*Power version 3.1.9.7 was used to determine minimum sample size in EOC groups and control groups. The calculation was carried out with the following parameters: statistical test=means: difference between two independent means (two groups); tailed=two; α=0.05; power level=0.95; and effect size (d) was determined based on the mean difference of miR-155 expression between ovarian cancer and control, which was 0.98 [14]. Therefore, the minimum sample size was 29 for each group.

Blood plasma collection

Between October 2015 and September 2016, 42 newly diagnosed patients with EOC who were diagnosed clinically and confirmed pathologically, irrespective of age, were consecutively recruited at Dr. Sardjito General Hospital (Yogyakarta, Indonesia) and assigned to the EOC group.

None of the EOC patients had a diagnosis of other cancers (simultaneous malignancies), had undergone chemotherapy, radiotherapy, hormone therapy, or cytoreductive surgery prior to blood sample collection. At the same period, 37 healthy women, irrespective of age, were also consecutively recruited as a control group. None of the healthy subjects had a history of cancers, benign gynaecological condition, chemotherapy, radiotherapy, hormone therapy, or cytoreductive surgery prior to blood sample collection. Moreover, for the control group, a thorough and rigorous gynaecological assessment to eliminate any indications of gynaecologic cancer and benign gynaecological condition was performed as a prerequisite prior to the collection of blood samples.

All the eligible participants signed the written informed consent as a prerequisite to participating in this study. After informed consent was obtained, 5 mL of venous blood samples were acquired by a registered nurse in Dr. Sardjito General Hospital using an EDTA vacutainer. For participants in the EOC group, the blood samples were collected before the patients received surgery treatment, chemotherapy, and radiotherapy. We maintained the cold chain during sample transfer. Immediately after the collection, blood samples were then centrifuged at 1500 rpm and a temperature of 4°C for 10 minutes. The plasma was extracted from the supernatant and placed into 1.5 mL RNase-free tubes utilizing a filter tip. The blood plasma samples were subsequently preserved at −80°C until they were used for RNA isolation. Detailed information about the sample collection process is shown in Figure 1.

EOC tissue collection

Besides collecting blood plasma samples, 42 fresh frozen (FF) EOC tissues and their matched adjacent normal ovarian tissues were also obtained from the surgically removed ovarian tissues from the same EOC patients. The tissue samples were collected by pathologists in the Department of Anatomical Pathology of Dr. Sardjito General Hospital. The tissue samples were then preserved at −80 °C until they were used for RNA isolation.

Total RNA extraction from tissue

Twenty milligrams of EOC and paired adjacent normal ovarian tissues were used for RNA extraction. The RNA extraction procedure was conducted according to the miRCURY RNA Isolation Kit-Tissue (Cat. No. 300111, Exiqon, Denmark). Next, the purity and concentration of RNA were measured using nanodrop (Maestrogen, MN-913A). Finally, total RNA was preserved at −80 °C until it was used for cDNA synthesis.

Total RNA extraction from blood plasma

All blood plasma samples were subjected to a single freeze–thaw cycle. Two hundred microliters of blood plasma were used for RNA extraction according to the miRCURY RNA Isolation Kit-Biofluid (Cat. No. 300112, Exiqon, Denmark). Next, the purity and concentration of RNA were analysed with nanodrop (Maestrogen, MN-913A). Finally, total RNA was preserved at −80°C until it was used for cDNA synthesis.

cDNA synthesis

Tissue- and plasma-derived RNA was synthesized into cDNA according to universal cDNA synthesis kit

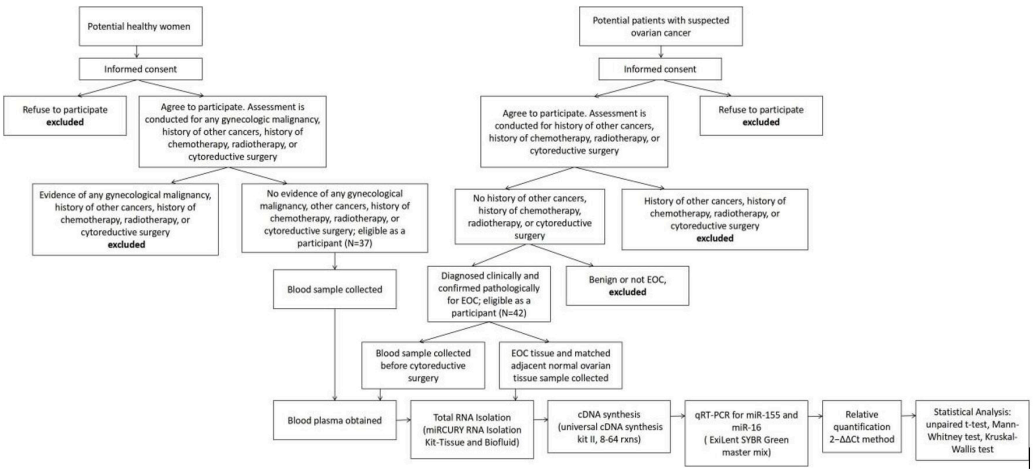

Figure 1: Sample collection process

II, 8-64 rxns (Cat. No. 203301, Exiqon, Denmark). All RNA samples were subjected to a single freeze–thaw cycle. A total of 16 µL of reagents were mixed, consisting of 4 µL of 5× reaction buffer, 9 µL of nuclease-free water, 2 µL of enzyme mix, and 1 µL of spike in (sp6). Afterwards, 4 µL of RNA was added to each reaction, resulting in 20 µL of final volume. Finally, samples were incubated at 42°C for 60 minutes for the reverse transcription step, followed by enzyme inactivation at 95°C for 5 minutes of incubation and a 4°C cooling-down step. The cDNA was preserved at -20°C until it was used for qRT-PCR.

Real-time quantitative polymerase chain reaction assay (qRT-PCR)

The PCR reaction was amplified using real-time quantitative polymerase chain reaction (qRT-PCR) according to the ExiLent SYBR Green master mix (Cat. No. 203 402, Exiqon) on the Bio-Rad CFX96 Real-time Thermal Cycler System (Bio-Rad, USA), and was performed in triplicate. The LNA microRNA PCR primer sets for miR-155 (Exiqon, Cat. YP00204308) and miR-16 (Exiqon, Cat. YP00205702), which have been optimized in earlier studies [21], were utilized to ensure a precise amplification reaction. All cDNA samples were subjected to a single freeze–thaw cycle.

Firstly, cDNA solution for qRT-PCR was made by reconstituting 1 µL of cDNA with 80 µL of RNase-free water. Afterwards, 4 µL of cDNA solution was combined with 5 µL of Exilent SYBR Green master mix and 1 µL of microRNA-specific primer, resulting in a total volume of 10 µL. Real-time PCR was amplified according to the following sequential procedure: initial denaturation at 95°C for 10 minutes, 40 cycles of 95°C for 10 seconds and 60°C for 1 minute with a ramp rate of 1.6°C/s for each cycle, optical reading, and melting curve analysis. Automatic threshold setting was used to determine the Ct cut-off threshold value. To confirm the specificity of the amplification during qRT-PCR, melting curve analysis was performed, and a single peak on the resulting melting curve indicates that only one, specific product was amplified.

According to previous studies, MiR-16 was used as the reference gene to normalize the cycle of threshold (Ct) values of plasma-derived miR-155 between EOC and healthy subjects [22–25]. The no template control (NTC) was incorporated to omit the contamination risk. As recommended by previous studies, relative quantification of miR-155 was achieved using the 2−ΔΔCt method [21,22,26]).

Classification of clinical stage and histological subtype of EOC

In this study, the clinical stage of EOC is classified according to the 2014 FIGO staging system [27]. According to this staging system, the clinical stage of EOC is classified into stage I (IA, IB, IC1, IC2, and IC3), stage II (IIA, IIB), stage III (IIIA1, IIIA2, IIIB, IIIC), and stage IV (IVA, IVB). Furthermore, stage I and stage II are regarded as early-stage EOC, and stage III and stage IV are regarded as advanced-stage EOC.

EOC is a heterogeneous disease and is divided into different histological subtypes as follows [28,29]: serous carcinoma which include high-grade serous carcinoma (HGSC) and low-grade serous carcinoma (LGSC), mucinous carcinoma (MC), endometrioid carcinoma (EC), clear cell carcinoma (CCC), carcinosarcoma, and undifferentiated carcinoma (UC). Furthermore, LGSC, MC, EC, and CCC are classified as Type I EOC, whereas HGSC, carcinosarcoma, and UC are classified as Type II EOC [30].

Statistical analysis

Mean and standard deviation (SD) were employed as univariate analyses for numerical variables exhibiting normal distribution, whereas median and interquartile range (IQR) were utilized for those having non-normal distribution. Univariate analysis for categorical data was characterized by frequency and percentage. All of the univariate analyses were performed using IBM SPSS Statistic for Windows version 26.

The Shapiro-Wilk test was conducted using IBM SPSS Statistic for Windows version 26 as a prerequisite for selecting the appropriate statistical test for our study. A Shapiro-Wilk test result of $p > 0.05$ indicates a normal data distribution. Shapiro-Wilk test result confirmed that the data have non-normal distribution (Table 1), therefore Mann-Whitney test and Kruskal-Wallis test were employed as bivariate analysis. If the Kruskal-Wallis test yielded a p-value less than 0.05, Dunn's test was subsequently conducted as a post hoc analysis. All of the bivariate analysis were performed using GraphPad Prism Version 9.5.0 for macOS.

Receiver operating characteristic (ROC) curve analysis and the area under the curve (AUC) were used to evaluate diagnostic value of circulating cell-free miR-155 in the blood plasma for EOC.

Table 1: Data distribution according to the Shapiro-Wilk test

Variables	Shapiro-Wilk test					
	Tissue derived miR-155			Plasma derived miR-155		
	Statistic	df	Sig.	Statistic	df	Sig.
Malignancy status						
Normal	0.803	42	0.000	0.925	37	0.016
EOC	0.753	42	0.000	0.917	42	0.005
Clinical stage						
Early stage	0.894	12	0.133	0.948	12	0.603
Advance stage	0.834	30	0.000	0.859	30	0.001
Histological subtype						
Normal	0.803	42	0.000	0.925	37	0.016
Serous	0.688	20	0.000	0.932	20	0.165
Mucinous	0.797	15	0.003	0.925	15	0.226
Endometrioid	0.771	3	0.048	0.987	3	0.782
Clear cell	0.927	4	0.579	0.967	4	0.823
Type of EOC						
Normal	0.803	42	0.000	0.925	37	0.016
Type I EOC	0.802	23	0.000	0.909	23	0.038
Type II EOC	0.703	19	0.000	0.915	19	0.091

Youden index (J = sensitivity+specificity −1) was maximized to select the optimal cut-off for plasma miR-155 [31,32]. The ROC analysis was performed using IBM SPSS Statistic for Windows version 26.

The Ethical Review Board, Faculty of Medicine, Universitas Gadjah Mada, Indonesia, granted ethical clearance for this study (number: KE/FK/1085/EC) on 28 August 2015. Prior to data collection, comprehensive study information was explained to the subjects, and their informed consent was acquired before initiating the study. Over the course of the research period, the study data were maintained in confidentiality, and their access was exclusive for researchers.

RESULTS

Clinicopathological characteristic of the patients

The clinicopathological characteristics of the newly diagnosed EOC patients and healthy subjects were provided in Table 2. The mean age between the EOC (mean = 47.69 years; SD = 10.91 years) and healthy control groups (mean = 45.11 years; SD = 11.54 years) is not significantly different (independent sample t-test; P=0.312). The clinical stage of EOC was classified using the FIGO staging system 2014, and in this study, 22 EOC patients (52.4%) were classified as FIGO stage III, whereas 8 EOC patients (19.0%) were classified as FIGO stage IV. Since FIGO stage III and IV were regarded as advanced-stage EOC, this finding indicates that advanced-stage EOC was diagnosed in the majority of patients (n=30; 71.4%). Based on histological sub-types, most of the EOC patients (n=20; 47.6%) were diagnosed with serous EOC, followed by mucinous EOC (n=15; 35.7%), suggesting these sub-types are the most prevalent among EOC patients. Based on the type of EOC, most of the patients (n=23; 54.8%) were categorized as Type I EOC. The elevation of the CA-125 protein expression level (> 35 U/mL) was observed in all of the EOC patients. None of the EOC patients and healthy subjects had a history of hormonal therapy.

Significant downregulation of tissue-derived miR-155 from EOC patients, especially with advanced disease

To determine the expression pattern and the role of miR-155 in EOC, we analyse the expression of miR-155 in EOC tissue and its paired adjacent normal ovarian tissue (Figure 2). Our study

Table 2: Cinicopathological characteristic of the EOC patients for miR-155 expression analysis

Characteristic	EOC Groups (n=42)	Healthy Control Groups (n=37)	p
	F(%)	F(%)	
Age (years)	47.69 (10.91)	45.11 (11.54)	0.312*
< 45	18 (42.8%)	15 (40.5%)	
≥ 45	24 (57.2%)	22 (59.5%)	
FIGO Stage			
I	2 (4.8%)		
II	10 (23.8%)	N/A	
III	22 (52.4%)		
IV	8 (19.0%)		
Histological Sub-type			
Serous	20 (47.6%)		
Mucinous	15 (35.7%)	N/A	
Endometrioid	3 (7.1%)		
Clear Cell	4 (9.5%)		
Type of EOC			
Type I	23 (54.8%)		
Type II	19 (45.2%)		
CA-125 (U/mL)			
0-35	0 (0%)	N/A	
>35	42 (100%)		
History of Hormonal Therapy			
Yes	0 (0%)	0 (0%)	
No	42 (100%)	37 (100%)	

Mean (SD)
**Independent sample t-test*

demonstrated significant downregulation of miR-155 expression in EOC tissue (median = 0.06, IQR = 0.17) compared to normal tissue (median = 1.03, IQR = 0.96) (Mann-Whitney test; P<0.0001). Moreover, significant downregulation of tissue miR-155 expression is also observed in advanced stage (median = 0.05, IQR = 0.03) compared to early stage (median = 0.33, IQR = 0.26) (Mann-Whitney test; P<0.0001). There is no significant difference in tissue miR-155 expression among histological subtypes (Kruskal-Wallis test; P>0.05). The Kruskal-Wallis test also indicated that, based on the type of EOC, significant downregulation of tissue miR-155 is observed in both Type I (median = 0.09, IQR = 0.20) and Type II EOC (median = 0.05, IQR = 0.10) compared to healthy subjects (P<0.0001). However, its expression between Type I and Type II EOC is not significantly different (P>0.05).

Significant downregulation of plasma-derived miR-155 from EOC patients, especially with advanced disease

To determine the possible application of circulating cell-free miR-155 as a biomarker for EOC, we examined the plasma miR-155 expression among EOC patients and healthy women (Figure 3). Our study elucidated significant downregulation of plasma miR-155 expression in EOC patients (median = 0.21, IQR = 0.26) compared to healthy subjects (median = 0.93, IQR = 0.66) (Mann-Whitney test; p<0.0001). We also compared plasma miR-155 expression based on clinical stage to determine its potential application as a biomarker of EOC progression. Significant downregulation of plasma miR-155 expression is observed in advanced stage (median = 0.18, IQR = 0.22) compared to early stage (median = 0.39, IQR = 0.23) (Mann-Whitney

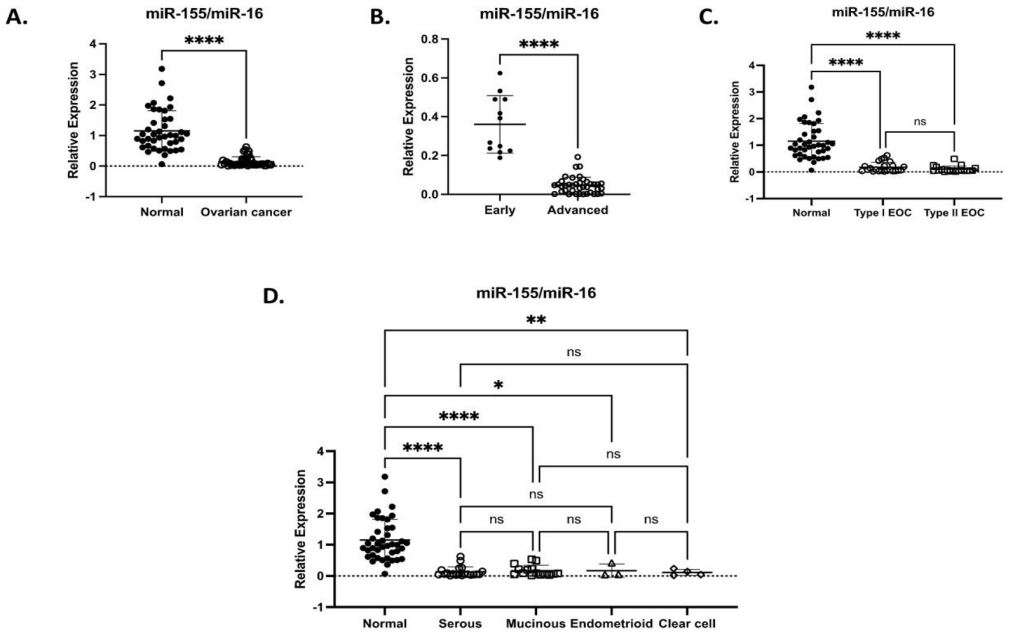

Figure 2: Downregulation of miR-155 is observed in EOC tissue.

A. *The relative expression of miR-155 is significantly downregulated in EOC tissue compared to normal ovarian tissue (Mann-Whitney test).* ***B.*** *The relative expression of miR-155 is significantly downregulated in EOC tissue of advanced stage compared to early stage (Mann-Whitney test).* ***C.*** *The relative expression of miR-155 is not significantly different between Type I and Type II EOC tissue (Kruskal-Wallis test).* ***D.*** *There is no significantly difference in relative expression of tissue miR-155 between histological sub-type (Kruskal-Wallis test). *=p<0.05; **=p<0.01; ***=p<0.001; ****=P<0.0001; ns = not significant (p>0.05).*

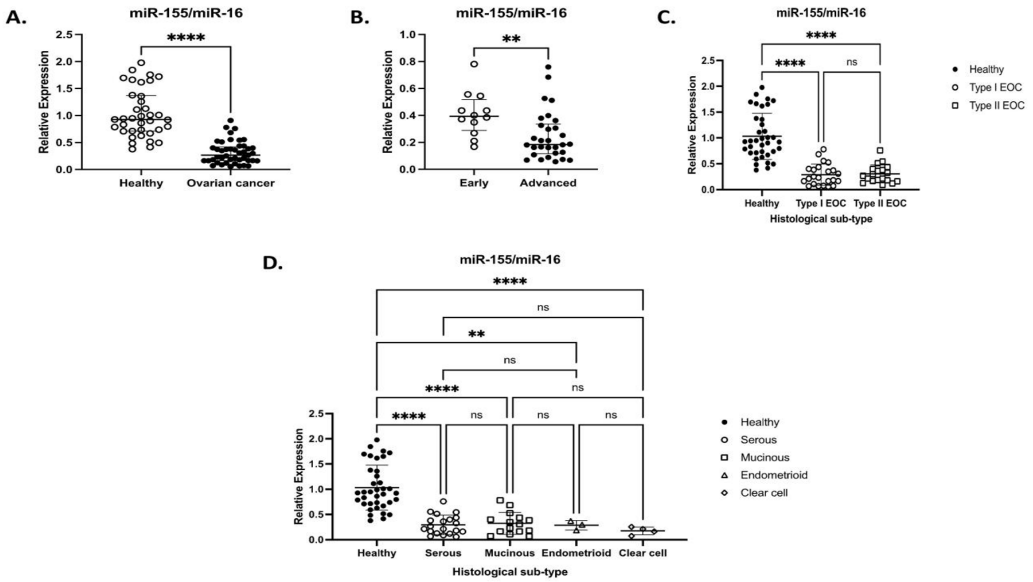

Figure 3: Downregulation of circulating cell-free miR-155 is observed in the blood plasma of EOC patients.

A. *The relative expression of plasma miR-155 is significantly downregulated in EOC groups compared to healthy groups (Mann-Whitney test).* ***B.*** *The relative expression of plasma miR-155 is significantly downregulated in advanced stage compared to early stage (Mann-Whitney test).* ***C.*** *The relative expression of plasma miR-155 is not significantly different between Type I and Type II EOC (Kruskal-Wallis test).* ***D.*** *There is no significantly difference in relative expression of plasma miR-155 between histological sub-type (Kruskal-Wallis test). **=p<0.01; ***=p<0.001; ****=P<0.0001; ns = not significant (p>0.05).*

test; p<0.01). There is no significant difference in plasma miR-155 expression among histological subtypes (Kruskall-Wallis test; P>0.05). The Kruskal-Wallis test also indicated that, based on the type of EOC, significant downregulation of plasma miR-155 is observed in both Type I (median = 0.18, IQR = 0.33) and Type II EOC (median = 0.27, IQR = 0.23) compared to healthy subjects (P<0.0001). However, its expression between Type I and Type II EOC is not significantly different (P>0.05).

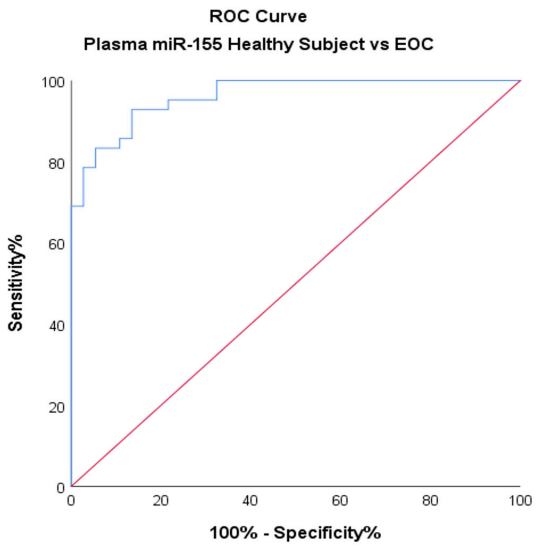

Figure 4: ROC curve analysis for plasma-derived circulating cell-free miR-155 for EOC patients relative to healthy subjects.

Diagnostic value of plasma derived circulating cell-free miR-155 for EOC

The diagnostic value of plasma miR-155 was evaluated using ROC curve analysis for distinguishing EOC from healthy subjects. Based on Figure 4 and Table 3, the AUC (95% CI) plasma miR-155 in distinguishing EOC from healthy subjects was 0.962 (95% CI: 0.928-0.996). Plasma miR-155 exhibited 92.9% sensitivity and 86.5% specificity with the cut-off value of ≤ 0.5741 for ΔCt miR-155 plasma.

DISCUSSION

It has been well established that microRNAs are secreted by cancer cells and facilitate intercellular communication within the tumour microenvironment, ultimately affecting tumorigenesis, and deregulation of cell-free microRNAs is correlated with tumour formation and progression. Moreover, the microRNA expression profile in blood plasma has been shown to correlate with its deregulation in cancer cells and tissues [12,13].

Deregulation of miR-155 expression in various human cancers is observed in numerous studies. However, its utilization as a candidate biomarker and target therapy in cancer remains challenging because extensive evidence revealed the dual role of miR-155 in carcinogenicity, and it could act as an oncogene or tumour suppressor microRNA depending on the cancer type. miR-155 has been found to be upregulated in several cancers, such as breast cancer [14,16,33], nasopharyngeal carcinoma [17,34], and hepatocellular carcinoma [35], suggesting miR-155 acted as an oncogene. Contrarily, miR-155 was downregulated in gastric cancer [15,36], multiple myeloma [37], and prostate cancer [38] and regarded as a tumour-suppressor microRNA. Evidence regarding the nature of miR-155 in EOC is needed before it could be used as a candidate biomarker and target therapy for clinical management of EOC. Our present study aimed to evaluate the expression pattern of miR-155 in EOC tissue and blood plasma of EOC patients.

Our study revealed significant downregulation of miR-155 expression in EOC tissue compared to adjacent normal ovarian tissue. Our finding provided additional evidence that supports the tumour suppressive role of miR-155 in ovarian carcinogenicity. Furthermore, our study also revealed significant downregulation of plasma-derived miR-155 expression from EOC patients compared to healthy subjects. This finding provided additional evidence regarding similar aberration of the miR-155 expression pattern between the cancer tissue and body fluid, specifically blood plasma, suggesting that aberrant expression of plasma microRNA could represent its aberrant expression in cancer tissue. Consistent with our finding, previous studies demonstrated that miR-155 is downregulated in ovarian cancer cells [18–20], ovarian cancer tissue [20], serum of ovarian cancer patients [39], and urine of ovarian cancer patients [3].

Hsa-miR-155-5p was predicted to negatively regulate over 147 protein-coding genes [14], and several studies have verified that miR-155 negatively regulated the expression of numerous oncogenes that significantly contributed to EOC carcinogenicity. For example, miR-155 has been found to directly target Cyclin D1 mRNA,

and treatment with anti-miR-155 stimulated upregulation of Cyclin D1, increased inhibitory phosphorylation of Rb, and promoted a G1-S phase transition [40]. In a recent study, miR-155 negatively regulated IGF-2 expression via direct interaction with the 3'UTR of IGF-2 mRNA, and upregulation of miR-155 stimulated IGF-2 downregulation and perturbation of the downstream PI3K-AKT-mTOR signalling pathway, ultimately inhibiting mitotic division and stimulating apoptosis [41]. Another study verified that miR-155 targets the c-myc gene through direct binding to the 3'UTR of c-myc mRNA and stimulates downregulation of c-myc expression. The downregulation of miR-155 stimulated upregulation of c-myc, which in turn increased cancer cell proliferation and survival as well as disrupted cell adhesion, while restoration of miR-155 expression inhibited this process [36]. K-RAS is also identified as a direct target of miR-155 and contributes to mitotic division and the anti-apoptosis phenotype [42]. miR-155 also had the ability to inhibit mitotic division by targeting AGTR1 and inhibiting the AGTR1-NF□B-CXCR4 signalling pathway [43].

Our study unveiled the significant downregulation of tissue- and plasma-derived miR-155 in advanced-stage compared to early-stage EOC. Consistently, previous study found that the miR-155 was downregulated in advanced-stage EOC [39]. This finding suggested that downregulation of miR-155 potentially contributed to the molecular mechanism that promotes the migration, invasion, and metastasis phenotype in EOC and leads to the progression of EOC from the early stage into the advanced stage.

Several studies attempted to unveil the role of miR-155 in cancer cell metastasis. For instance, by negatively regulating IGF2 expression, miR-155 stimulates perturbation of the PI3K-AKT-mTOR signalling pathway, leading to suppression of migration and invasion [41]. miR-155 also had the capability to inhibit migration and invasion by targeting SPOCK1 [38]. By targeting SeL1-Like (SEL1L), miR-155 could also regulate expression of TIMP-1 and TIMP-2, which leads to inhibition of ECM remodelling [44]. In another study, downregulation of miR-155 promoted migration and invasion phenotype in OC stem-like cells (CD44+/CD117+) and was mediated by downregulation of Claudin-1 (CLD-1) and E-cadherin expression, and restoration of miR-155 expression reversed this mechanism (Qin et al., 2013), indicating that the tumour suppressive role of miR-155 in inhibiting metastasis is mediated by suppressing the EMT process. Through hybridization to the 3'UTR of AGTR1 and HIF-1α mRNA, miR-155 stimulates downregulation of AGTR1 and HIF-1α, leading to suppression of EMT, invasion, and angiogenesis [18,43].

Although the precise mechanism behind the miR-155 downregulation in EOC remains underexplored, several possible mechanisms are proposed and should be validated in future studies. An epigenetic mechanism, specifically DNA methylation, regulates the transcriptional silencing of both protein-coding genes and microRNA genes by either recruiting proteins that facilitate gene repression or inhibiting the binding of transcription factors to the gene's promoter; this process may lead to deregulation of microRNA expression in cancer [45]. A recent study demonstrated that DNA methylation of CpG islands present in the promoter and first exon of the BIC gene stimulated downregulation of miR-155 expression, and this reaction is catalysed by DNMT1 [46,47]. Since DNMT1 expression is upregulated in ovarian cancer [48], this phenomenon could stimulate DNA methylation of the miR-155 host gene and subsequent downregulation of miR-155 in ovarian cancer.

Previous studies found that transcriptional activation of the miR-155 host gene is induced by numerous transcription factors that bind in the specific binding sites upstream of the miR-155 host gene, including SMAD4, in which this interaction occurs in the DNA sequence located at-454 bp from the transcriptional start site; IRF family members in the ISRE sequence both in the promoter region (-3111 bp from transcriptional start site) and enhancer; and Ets family members, including PDEF, in the numerous Ets binding sites in the promoter region, especially adjacent to the TATA box [49,50]. Previous studies elucidated that SMAD4, IRF-1, and PDEF expression was downregulated both in EOC tissue and cell lines [51–53]. Loss of transcription factor SMAD4, IRF-1, and PDEF expression in EOC could lead to the decrease of transcriptional activation of the microRNA-155 gene and subsequent downregulation of miR-155 expression in EOC. Moreover, a previous study demonstrated downregulation of SMAD4 expression in ovarian carcinoma cells with a high metastasis phenotype compared to a low metastasis phenotype [51] and downregulation of PDEF expression is associated with advanced-stage EOC [52], which could explain

the downregulation of miR-155 in advanced stage observed in our study.

Finally, we evaluated the diagnostic power of plasma-derived miR-155 using ROC analysis. Plasma miR-155 exhibited an AUC of 0.962 for discriminating EOC from healthy subjects, with 92.9% sensitivity and 86.5% specificity. Due to the low prevalence of ovarian cancer, the sensitivity above 75% and a specificity of at least 99.6% are expected for any biomarker to meet an acceptable PPV and be regarded as an effective screening method for EOC [54–56]. Our finding suggests that plasma miR-155 exceed the minimum sensitivity threshold of 75% but failed to achieve ideal specificity threshold of 99.6% for EOC screening test. Since downregulation of circulating cell-free miR-155 is also observed not only in the blood plasma of EOC patients but also in the blood plasma of other cancers such as gastric cancer [15,34], multiple myeloma [37], and prostate cancer [38], we recommend assessing the utilization of plasma miR-155 in combination with another molecular minimally invasive biomarker, whether transcriptomic and/or proteomic, such as CA-125 and HE-4 protein, to further increase its sensitivity and specificity. Previous study in hepatocellular carcinoma demonstrated that a panel biomarker of circulating microRNA in combination with Alpha-Fetoprotein (AFP) protein expression level had higher sensitivity and specificity compared to microRNA alone or AFP alone [35]. A recent study demonstrated that the combination of plasma CA-125 and exosomal miR-223 increased its diagnostic value in distinguishing EOC patients from healthy subjects compared to CA-125 and miR-223 alone [31], suggesting that a panel biomarker should be assessed in the future as an effort to develop a highly sensitive and specific biomarker for EOC detection.

Our finding demonstrated consistent downregulation of miR-155 expression between EOC tissue and blood plasma, supporting the potential tumour suppressor role of this microRNA and potential application of circulating plasma miR-155 as a minimally invasive biomarker for EOC. However, several limitations of our study should be considered. First, we did not control for participants' menstrual cycle phase at the time of sample collection. Previous studies have consistently demonstrated that fluctuations in hormone levels throughout the menstrual cycle may alter circulating cell-free microRNA expression profiles in blood plasma [57,58]. Because microRNA-regulated genes are involved in ovarian steroidogenesis, and their expression is modulated by ovarian steroid hormones and hormone antagonists [58], the menstrual cycle should be considered a potential confounding factor in plasma microRNA analysis. Second, we did not perform a haemolysis assessment, which is critical to prevent plasma contamination from microRNAs highly expressed in erythrocytes. Specifically, the housekeeping gene used in our study, miR-16, is one of the most abundant microRNAs in erythrocytes, and many studies have shown that haemolysis may increase plasma miR-16 levels by 20-30-fold [59,60]. Third, we utilized miR-16 as a housekeeping gene, which is commonly used for the relative quantification of plasma microRNA expression in the previous EOC biomarker studies [22–25]. However, the evidence from the stability analysis that supports the utilization of miR-16 as a stable and ideal housekeeping gene for plasma microRNA analysis in EOC is still limited. Moreover, since miR-16 is highly expressed in erythrocytes, the utilization of miR-16 as a housekeeping gene in plasma-based microRNA biomarker studies has been questioned [61]. Stability analysis study to evaluate the most stable housekeeping gene for plasma microRNA expression analysis in EOC should be conducted in the future, and if miR-16 is intended for use in subsequent experiments, haemolysis analysis should be performed as a prerequisite.

CONCLUSION

Our study provided evidence that supports consistent downregulation of tissue- and plasma-derived miR-155 expression from EOC patients, especially among those with advanced stages of the disease. Our findings unveil the potential tumour suppressive role of miR-155 in EOC and its diagnostic value as a less invasive biomarker for EOC. Future studies to unveil the mechanism behind miR-155 downregulation in EOC, as well as validation of oncogenes potentially regulated by miR-155 and its impact on EOC carcinogenicity, are needed.

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