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CLINICAL ARTICLE

Diagnostic accuracy of serum miR-122 and miR-199a in women with endometriosis

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Synopsis: Serum miR-122 and miR-199a are significantly increased in women with endometriosis and these microRNAs have a strong potential to act as biomarkers to diagnose endometriosis.

Abstract

Objective: To evaluate the value of serum microRNA-122 (miR-122) and miR-199a as reliable noninvasive biomarkers in the diagnosis of endometriosis.

Methods: During 2015–2016, at a teaching hospital in Egypt, a prospective cohort study was conducted on 45 women with pelvic endometriosis and 35 women who underwent laparoscopy for pelvic pain but were not diagnosed with endometriosis. Blood and peritoneal fluid (PF) samples were collected; interleukin-6 (IL-6) was detected by enzyme-linked immunosorbent assay and miR-122 and miR-199a expression was measured by quantitative real-time polymerase chain reaction.

Results: The serum and PF levels of IL-6, miR-122, and miR-199a were significantly higher in women with endometriosis than in controls ($P<0.001$ for all comparisons). Serum miR-122 expression was positively correlated with serum IL-6 ($r=0.597$), PF IL-6 ($r=0.603$), PF miR-122 ($r=0.934$), serum miR-199a ($r=0.727$), and PF miR-199a ($r=0.653$). Serum miR-199a expression was positively correlated with serum IL-6 ($r=0.677$), PF IL-6 ($r=0.678$), PF miR-122 ($r=0.744$), and PF miR-199a ($r=0.932$). Serum miR-122 and miR-199a had a sensitivity of 95.6% and 100.0% and a specificity of 91.4% and 100%, respectively, for the detection of endometriosis.

Conclusion: Serum miR-122 and miR-199a were significantly increased in endometriosis, indicating that these microRNAs might serve as biomarkers for the diagnosis of endometriosis.

1 INTRODUCTION

Endometriosis is a chronic gynecologic disorder. It is defined as the existence of glands and stroma of the endometrial type outside the uterine cavity especially in the pelvic cavity, with particular persistence in the ovaries and the peritoneum [1]. Endometriosis is present in 5%–10% of women of reproductive age, and 50% of the women with endometriosis experience fertility problems [2,3].

Endometriosis is currently diagnosed by laparoscopy, which is a highly invasive procedure with many potential risks. At the present time, there are other reliable diagnostic tests that can be both sensitive and specific for endometriosis [4].

There is a great need to find a reliable noninvasive diagnostic test for the disease to minimize the number of laparoscopy procedures without affecting the patients' clinical outcomes [5].

MicroRNAs are small (21–22 nucleotides) noncoding RNA molecules that regulate many biological processes through gene silencing at the post-transcriptional level or by catalyzing transcript degradation. The expression of microRNAs is precisely controlled to achieve optimum cell function and differentiation [6]. MicroRNAs have important target genes whose expression in turn affects many physiological and pathological conditions such as endometriosis [7].

The expression of several microRNAs and their target genes differs between normal endometrial tissues and tissues from women with endometriosis [8]. Microarray assays can detect the differential expression of these microRNAs in endometrium

obtained from women with and without endometriosis, and in normal versus ectopic endometrium obtained from women with endometriosis [9].Therefore, the differential expression of microRNAs in women with endometriosis and the detection of circulatory microRNAs in their serum may prove to be highly diagnostic [10].

Among the microRNAs that are dysregulated in endometriosis are miR-122 and miR-199a.Their serum levels are increased in women with endometriosis, and these markers may also be used to differentiate between mild and severe disease. The concentrations of these two microRNAs are correlated, and both target transcription factor *SOX4*, an apoptosis-related gene that also has a role in the differentiation of endometrial carcinomas. Given the high expression of miR-122 and miR-199a in the serum of patients with endometriosis, these microRNAs might exert their role in the pathogenesis of the disease through dysregulation of the expression of SOX4 [11].

Measurement of inflammatory markers in women with endometriosis has shown no consensus. Some studies have confirmed no differences between serum interleukin 6 (IL-6) between diseased and healthy women, whereas others reported high levels of IL-6 in women with endometriosis [12].

Biomarkers in endometriosis can be evaluated in blood or other body fluids such as peritoneal, menstrual, and endometrial fluids in addition to tissue samples [13].

The aim of the present study was to evaluate the value of serum and peritoneal fluid miR-122, miR-199a, and IL-6 as reliable noninvasive biomarkers in the diagnosis of endometriosis.

2 MATERIALS AND METHODS

The present study was a prospective cohort study conducted at the Department of Obstetrics and Gynecology, Faculty of Medicine, Fayoum University, Fayoum, Egypt, from March 1, 2015, to April 30, 2016. It included all women with pelvic endometriosis, diagnosed laparoscopically and confirmed by histopathological examination, who were seen during the study period. Endometriosis was classified as minimal/mild (Stage I–II) or moderate/severe (Stage III–IV) according to the revised American Society for Reproductive Medicine classification [14]. The control group included all women who underwent laparoscopic surgery for pelvic pain, infertility, or benign neoplasms but who were not diagnosed with endometriosis. The exclusion criteria were receipt of medication during the 3 months prior to the laparoscopy, adenomyosis, endometrial cancer, hyperplasia, endometrial polyps, chronic or acute inflammatory disease, infectious disease, malignancy, autoimmune disease, and cardiovascular disease.

The study design, objectives, and methods were compatible with the 2013 version of the World Medical Association Declaration of Helsinki. Ethics approval for the study was obtained from the research ethics committee at Fayoum University. The protocol was explained to all study participants, and full written informed consent was obtained from each participant.

All participants underwent full history-taking including evaluation of the menstrual pattern, general and abdominal examination, local pelvic examination, and laparoscopic detection of the presence or absence of endometriosis.

Prior to surgery, blood samples were collected during the follicular phase (days 5–10). The samples were incubated at 37 °C for 15 minutes, centrifuged at 3000 g for 15 minutes to separate the serum, and stored in aliquots at –80 °C for the determination of interleukin-6 (IL-6), miR-122, and miR-199a levels. During laparoscopy, samples of peritoneal fluid were aspirated from the peritoneal cavity and the pouch of Douglas. The cellular components of the peritoneal fluid were removed by centrifugation at 3000 g for 20 minutes, and the supernatant was stored in aliquots at –80 °C for the determination of IL-6, miR-122, and miR-199a levels.

The concentration of interleukin-6 was determined by using a commercially available enzyme-linked immunosorbent assay (DRG International, Springfield, NJ, USA) according to the manufacturer's instructions.

Extraction of RNA was performed with the mirVana PARIS Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. The eluate containing the RNA was stored at –20 °C.

The expression of miR-122 and miR-199a was evaluated by quantitative real-time polymerase chain reaction (PCR) analysis. U6 RNA was used as endogenous control. In preparation, the eluted RNA was reverse-transcribed by incubation of 20 µL of each RNA sample for 1 hour at 42 °C and for 3 minutes at 93 °C using the miRNeasy Serum Reverse Transcription Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The reverse transcription products (cDNA) were then maintained at 4 °C.

For the real-time PCR reactions, 10 μL of the diluted cDNA, 12.5 μL SYBR Green Master Mix (Qiagen, Valencia, CA, USA), 0.5 μM of each specific primer, and RNase-free water were mixed to a total volume of 25 μL. The real-time PCR reactions were performed using the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the following thermal cycling parameters: 95 °C for 5 minutes followed by 35 cycles at 95 °C for 10 seconds, 57 °C for 30 seconds, and 75 °C for 30 seconds.

The expression level of the microRNAs was quantified using the cycle threshold (CT) method, with CT defined as the number of cycles required for the fluorescent signal to cross the detection threshold. The difference in CT (ΔCT) between the reference and target samples was calculated by subtracting the CT value for U6RNA from the CT values of the studied microRNAs. The resulting normalized ΔCT values were then used to calculate relative expression values using the equation $2^{-\Delta CT}$.

The statistical analysis was conducted using SPSS version 16.0 (SPSS; Chicago, IL, USA). Comparisons between groups were performed using the χ^2 test for qualitative data, and the *t* test and analysis of variance for quantitative data. Pearson correlation analysis was performed to test for correlation between quantitative variables; the value of the Pearson correlation coefficient (*r*) describes the strength and direction of a correlation ranging from +1 to –1. Receiver operating curve analysis was conducted to detect the best cutoff levels of serum and peritoneal fluid miR-122 and miR-199a that differentiate between cases and controls. The area under the curve (AUC) was calculated to evaluate the accuracy of the diagnostic tests, with an AUC

of 0.90–1.00 defined as excellent, 0.80–0.90 defined as good, 0.70–0.80 defined as fair, and 0.50–0.60 defined as fail.$P<0.05$ was considered statistically significant.

The minimum sample size required was calculated to be 38 based on an expected sensitivity of 80.0 and a specificity of 76.0% for serum miR-122to detect endometriosis [15], a prevalence of endometriosis of 33% in women with pelvic pain [16], and a marginal error of 5%.

3 RESULTS

The present study included 45 women with pelvic endometriosis (nine with deep infiltrating disease) and 35 women without endometriosis. There was no significant difference between the two study groups in terms of age, body mass index, presence of pelvic pain, dysmenorrhea, or menstrual irregularities (Table 1). However, the groups differed significantly in terms of parity.

Women with endometriosis had significantly higher serum and peritoneal fluid levels of IL-6, miR-122, and miR-199a when compared with women in the control group (Table 2).

No significant difference was detected between the serum and peritoneal fluid levels ofmiR-122 and miR-199a at different stages of endometriosis (Table 3), indicating that miR-122 and miR-199a levels were not correlated with the stage of endometriosis.

There was a positive correlation between serum miR-122 and IL-6 (serum and peritoneal fluid), miR-122 (peritoneal fluid), and miR-199a (serum and peritoneal fluid); all correlations were significant (P<0.001) (Table 4).

There was also a positive correlation between serum miR-199a and IL-6 (serum and peritoneal fluid), miR-122 (peritoneal fluid), and miR-199a (peritoneal fluid); all correlations were significant (P<0.001) (Table 4).

Receiver operating curve analysis revealed a high diagnostic value for serum miR-122 and miR-199ain respect to the diagnosis of endometriosis (Table 5, Figure 1, Figure 2).

4 DISCUSSION

Endometriosis is mainly diagnosed and assessed by laparoscopy. Therefore, its detection and treatment are often delayed because there is a lack of symptoms and sensitive biomarkers in the early stages of the disease. In the present study, the serum and peritoneal levels of miR-122 and miR-199a and IL-6 were significantly increased in women with endometriosis compared with women in the control group, indicating that these molecules might serve as biomarkers for the diagnosis of endometriosis.

In our study serum miR-122 and miR-199a had a sensitivity of 95.6% and 100.0% and a specificity of 91.4% and 100%, respectively, for the detection of endometriosis.

According to our findings, serum miR-122 expression was positively correlated with IL-6 (serum and peritoneal fluid), miR-122 (peritoneal fluid), and miR-199a (serum and peritoneal fluid). In addition, serum miR-199a expression was positively correlated with IL-6 (serum and peritoneal fluid), miR-122 (peritoneal fluid), and miR-199a (peritoneal fluid).

The fact that 90% of women have retrograde menstruation but not all of these women have endometriosis [17] indicates that eutopic endometrium from women with and without endometriosis might differ at the molecular level. These molecular differences might result in the development of endometriosis in some but not all women. If these variations are indeed pathognomonic of endometriosis, the involved molecules might serve as biomarkers in tissue biopsies obtained by laparoscopy [18].

MicroRNAs play an essential role in several physiological and pathological conditions [19]. Altered plasma microRNA profiles have been detected in various types of cancer including solid tumors, leukemia, B-cell lymphoma, ovarian cancer, and in other disorders such as diabetes mellitus and liver cirrhosis [20]. Because serum markers are easy, uncomplicated, and direct to measure, the detection of changes in the serum level of specific circulating microRNAs may be of use in the diagnosis of endometriosis. Indeed, a number of microRNAs have been implicated to have a role in endometriosis [21]; however, previous studies have chiefly concentrated on the expression of microRNAs in endometrial tissue. For instance, the miR-9 and miR-34 microRNA families have been shown to be dysregulated in eutopic endometrial tissue from women with endometriosis compared with those

without the disease, and 50 microRNAs have been shown to be characteristically expressed in eutopic versus ectopic endometrium inpatients with ovarian endometriosis [22].

The consideration of circulating microRNAs as biomarkers for the detection of endometriosis is a relatively new field of research. Only a few studies investigating the predictive values of serum or plasma microRNAs have been published [23,24].

Wang et al. [15] were first to evaluate serum microRNA levels in women with endometriosis and healthy women. They found increased levels of miR-122 and miR-199a and decreased levels of miR-145, miR-141, miR-542-3p, and miR-9 in women with endometriosis and proposed that these compounds could be used as potential biomarkers for the disease.Their findings also indicatedthat evaluation of the expression of miR-122 and miR-199a might be of use in assessing the severity of the disease.

The present study found no association between the expression of miR-122 and miR-199a and the severity of the disease. This discrepancy with the findings by Wang et al. [15] could be explained by several factors. First, Wang et al. included women up to age 60 years, whereas the maximum age in the present study was 37 years. It is possible that the expression of the two markers varies by age; however, the involvement of older women is not relevant because most women with endometriosis are young. Second, microRNA expression might differ by race; the study population in the study by Wang et al. comprised Chinese women, whereas the present study was conducted in a population from Egypt. Third, Wang et al. used

a pool of 10 samples from women with the disease and another pool from controls. Using pooled blood may have different values compared with using individual samples as one sample in the pool may have extremely high levels, thus affecting the results. Of note, Wang et al. recommended validation of their finding in another study.

Dai et al. [9] suggested an important role of miR-199a in the progression of endometriosis. They found that miR-199a inhibits endometrial stromal cell adhesions and its invasive power through direct action on IkappaB kinase beta, and suppresses the nuclear factor-kappa B pathway and interleukin-8 production inside endometrial stromal cells.

Many microRNAs have been investigated in the study of endometriosis. Wang et al. [25] evaluated microRNA expression in serum samples from 30 women with minimal to mild endometriosis and 20 control women. Using deep sequencing, they found that 98 microRNAs were downregulated and 10 microRNAs were upregulated. This dysregulation was significant in only 21 of the 98 downregulated and none of the 10 upregulated microRNAs. The difference in significance is likely attributable to differences in sampling technique and analytical methods. The authors concluded that circulating microRNAs may serve as potential markers for the diagnosis of early-stage endometriosis.

To the best of our knowledge, the present study is the first study with adequate sample sizes in both study groups (cases and controls) to evaluate the reliability of miR-122 and miR-199a in the diagnosis of endometriosis. The main limitation of the

present study was the inability to follow the patients after laparoscopy to assess the reliability of these markers with changing symptoms over time.

In conclusion, the present study showed that the serum levels of miR-122 and miR-199a are significantly increased in women with endometriosis compared with women in the control group. Accordingly, these microRNAs have a strong potential to serve as biomarkers in the diagnosis of endometriosis.

Use of these markers will allow early, noninvasive diagnosis of the disease, at a much lower cost than methods currently available. The proven correlation between serum level and peritoneal level of these markers may obviate the need for peritoneal fluid and tissue sampling through laparoscopic procedures. Further research is recommended on other serum microRNAs to reach an ideal gold standard test for the diagnosis of endometriosis.

Author contributions

WSD and AE carried out the recruitment of patients and participated in the randomization process, the data analysis,and the writing of the manuscript. AMM, SSZ,HS, MA, AAM, and MK participated in the design of the study, performed the statistical analysis, and participated in the writing of the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

No authors have no conflicts of interest.

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Figure legend

Figure 1 Receiver operating characteristic curve of serum microRNA-122 as a marker of endometriosis.

Figure 2 Receiver operating characteristic curve of serum microRNA-199a as a marker of endometriosis.

Table 1 Clinical characteristics of the study groups.[a]

Characteristic	Endometriosis (n=45)	Controls (n=35)	*P* value
Age, y	29.64 ± 3.44	29.46 ± 4.48	0.831
BMI[b]	21.71 ± 2.94	21.32 ± 2.70	0.542
Dysmenorrhea	24 (53.3)	16 (45.7)	0.499
Nulliparous	25 (55.6)	1 (2.9)	<0.001
Pelvic pain	15 (33.3)	11 (31.4)	0.802
Irregular menstrual cycle	20 (44.4)	12 (34.3)	0.354

Abbreviation: BMI, body mass index.
[a] Values are given as mean ± SD or number (percentage).
[b] Calculated as weight in kilograms divided by the square of height in meters.

Table 2 Serum and peritoneal fluid levels of IL-6, miR-122, andmiR-199a in the study groups.[a]

Marker	Endometriosis (n=45)	Controls (n=35)	*P* value
IL-6, pg/mL			
Serum	52.64 ± 17.39	18.70 ± 9.19	<0.001
Peritoneal fluid	53.52 ± 16.12	21.92 ± 11.05	<0.001
miR-122, pg/mL			
Serum	4.30 ± 0.68	1.78 ± 1.07	<0.001
Peritoneal fluid	4.02 ± 0.54	1.65 ± 0.93	<0.001
miR-199a, pg/mL			
Serum	4.74 ± 1.35	1.20 ± 0.33	<0.001
Peritoneal fluid	3.84 ± 1.38	1.09 ± 0.32	<0.001

Abbreviations: IL, interleukin; miR, microRNA.
[a] Values are given as mean ± SD.

Table 3 Relationship between markers and stage of endometriosis.

Stage of endometriosis	**N**	**Concentration, pg/mL**	***P* value**
miR-122, serum			
I	9	4.25 ± 0.77	0.919
II	11	4.39 ± 0.60	
III	19	4.311 ± 0.82	
IV	6	4.15 ± 0.04	
miR-122, peritoneal fluid			
I	9	3.96 ± 0.59	0.515
II	11	4.20 ± 0.38	
III	19	3.91 ± 0.66	
IV	6	4.12 ± 0.06	
miR-199a, serum			
I	9	5.04 ± 1.24	0.171
II	11	4.51 ± 1.22	
III	19	4.42 ± 1.30	
IV	6	5.72 ± 1.66	
miR-199a, peritoneal fluid			
I	9	4.24 ± 1.30	0.101
II	11	3.46 ± 0.96	
III	19	3.53 ± 1.45	
IV	6	4.92 ± 1.57	

Abbreviation: miR, microRNA.
[a] Values are given as mean ± SD.

Table 4 Correlation of serum miR-122 and serum miR-199a with other markers.

Marker	Serum miR-122		Serum miR-199a	
	r	***P* value**	***r***	***P* value**
IL-6				
Serum	0.597	<0.001	0.677	<0.001
Peritoneal fluid	0.603	<0.001	0.678	<0.001
miR-122				
Serum	—	—	0.727	<0.001
Peritoneal fluid	0.934	<0.001	0.744	<0.001
miR-199a				
Serum	0.727	<0.001	—	—
Peritoneal fluid	0.653	<0.001	0.932	<0.001

Abbreviations: IL, interleukin; miR, microRNA.

Table 5 Receiver operating curve analysis of serum miR-122 and miR-199a for the diagnosis of endometriosis.

Marker	AUC	Cutoff point, pg/mL	Sensitivity	Specificity	PPV	NPV	Accuracy
SerummiR-122	0.963	3.24	95.6%	91.4%	93.5%	94.1	93.75
SerummiR-199a	1.000	2.30	100.0%	100.0%	100.0%	100.0%	100.0%

Abbreviations: AUC, area under the curve; miR, microRNA; NPV, negative predictive value; PPV, positive predictive value.

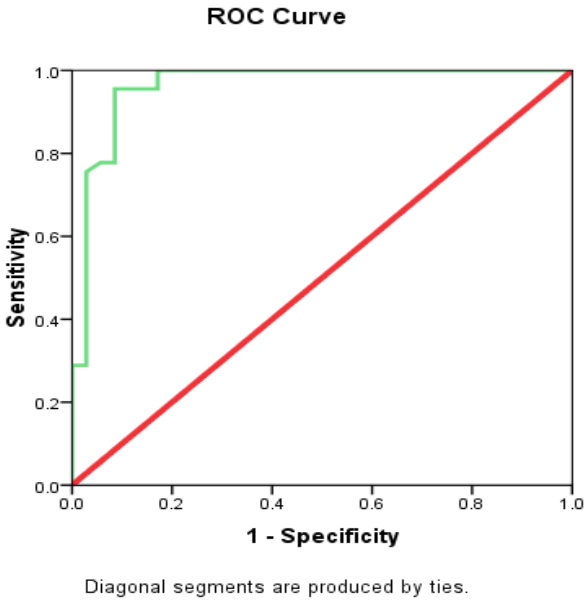

Figure 1 Receiver operating characteristic curve of serum microRNA-122 as a marker of endometriosis.

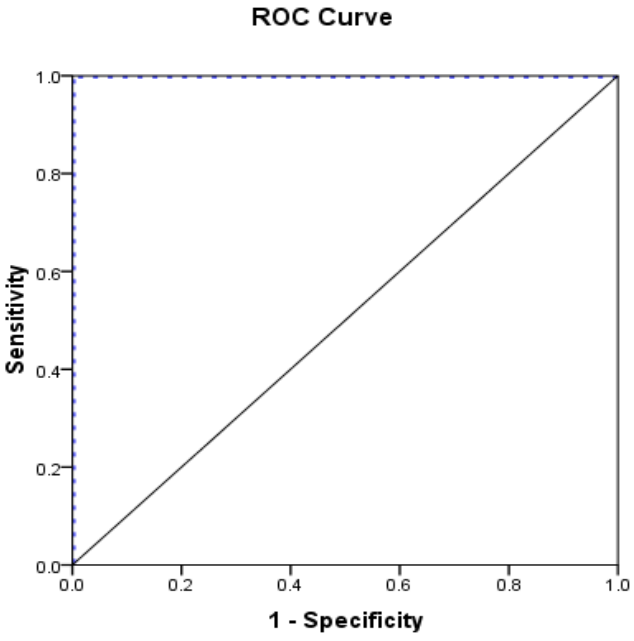

Figure 2 Receiver operating characteristic curve of serum microRNA-199a as a marker of endometriosis.