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Evaluation of microRNAs expression (miR-16 and miR-199a) in serum of endometriotic patients

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Abstract

Endometriosis is a complex, inflammatory disease, aberrant expression of miRNAs is commonly observed in pathological processes, including gynecological disorders. The aim of this study was to evaluate the serum miRNAs (199a and 16) expression level as a biomarker for endometriosis. 30 newly diagnosed endometriotic patients (admitted to the Gynecology Department of Al-Emamain Al-Kademian Medical City, Al-zahraa teaching hospital and Al-kut hospital of pediatric and obstetric) and 30 apparently healthy women as control group were enrolled in this case control study during the period from November 2016 to November 2017. Serum miRNAs were profiled by using TaqMan RT-PCR assay. The relative levels of miR-16 and miR-199a were found to be up regulated in endometriosis women compared with control group. The mean of the Log fold change values of gene expression of miR-199a showed statistically significant difference between early stages (stage I and stage II) and late stages (stage III and stage IV) of disease (P-value = **0.041**) so, miR-199a may be useful to discriminate between severe and mild endometriosis. In conclusion, this study demonstrated that the levels of circulating miRNAs (miR-199a and miR-16) could serve as noninvasive tools for diagnosis of endometriosis.

Key words:- miR-16, miR-199a, Endometriosis, microRNAs

INTRODUCTION

Endometriosis is a complex, inflammatory disease, almost half of the women with endometriosis experience infertility [1]. Although Sampson's theory of retrograde menstruation explains the migration of endometrial fragments to ectopic locations, additional steps are required for the development of endometriotic implants [2].

In recent years, there has been a significant focus on the etiology of endometriosis and the role of miRNAs in the pathogenesis of the disease. MicroRNAs (miRNAs) are small non coding RNAs (18-22 nucleotides) that function as modulators of gene expression. It is well-known that miRNAs are present in bio fluids (plasma, serum, urine, semen, peritoneal fluid and menstrual blood) and have been proposed as ideal candidates as disease biomarkers [3]. Lack of non-invasive diagnostic test contributes to the long delay between onset of symptoms and diagnosis of endometriosis [4], so the discovery of sufficiently sensitive and specific biomarkers for non-surgical detection of endometriosis promises earlier diagnosis and prevention of deleterious sequelae [5]. MiRNAs may thus be attractive candidates for novel diagnostic markers and therapeutic interventions in endometriosis, as demonstrated in other miRNA regulated diseases [6].

MiR-199a is well correlated with pelvic adhesion and lesion distribution implying that it may play an important role in the progression of the disease [7]. MicroRNA -16 targets B-cell lymphoma 2 (BCL2) [8], which considers anti-apoptotic protein and the reduced expression of this microRNA may contribute to increased activity of this anti-apoptotic protein in endometriosis. High miRNA-16 expression is consistent with repressed cell proliferation and enhanced cell survival in endometriosis [9].

MATERIALS AND METHODS

Thirty newly diagnosed endometriotic patients, admitted to the Gynecology Department of Al-Emamain Al-Kademian Medical City, Al-zahraa teaching hospital and Al-kut hospital of pediatric and obstetric their ages range between (18-45) years matched with 30 apparently healthy women as control group were enrolled in this study during the period from November 2016 to November 2017. Ethical approval and informed consent were obtained from each participant in this work, it was obtained from the Institutional Review Board of College of Medicine /AL- Nahrain University according to the declaration of Helsinki -ethical agreement.

The controls were fertile, not pregnant and non-smoker with no current infection (genital or systemic). The blood samples were collected before the operation. A total of 5 ml of venous blood was collected in gel tubes. For complete clotting, the tubes had been left at room temperature (15 - 25°C) for 20 min. and centrifuged for 10 min. at 1900 × g and 4°C, then the serum was transferred to a new tube and centrifuged for 10 min at 16000 × g and 4°C. Finally, the supernatant carefully transferred to a new tube and kept frozen in aliquots at -70°C until use.

Data collection

Data were collected by direct interview including demographic data, social status, period of symptoms, phase of menstrual cycle, familial history with endometriosis, previous operations, chronic diseases, and if patients with contraceptive drug.

Methods:

The expression of miRNAs (16 and 199a) genes in patients and controls were normalized to the one of their reference genes which was RNU6-2 gene that was also obtained from their serum. After finding the gene expression of each target and normalizing it with their own reference genes, they were all compared to the normalized target gene expression of a single calibrator (that was considered to be equal to 1) in order to find the final fold change in gene expression for each target.

Purification of total RNA, including microRNAs from serum

The purification of total RNA, including miRNAs was performed by the use of miRNeasy Mini Kit (Qiagen Cat. No. 217004). This kit combines phenol/guanidine based lysis of samples and silica membrane based purification of total RNA containing miRNAs.

Assessment of the extracted RNA

The concentration of the extracted total RNA was quantified by the use of Quantus fluorometer system following the manufacturer's instructions (Promega, USA).

Two – step RT- PCR reaction

The TaqMan MicroRNA Assays were used looped-primer RT-PCR, a new real-time quantification method, for detection mature miRNAs. Total RNA containing miRNA was the starting material in RT-PCR reaction which was performed in two-steps:

1. In the reverse transcription (RT) step, cDNA was reversed transcribed from total RNA samples using specific miRNA

primers from the TaqMan MicroRNA Assays and reagents from the TaqMan®MicroRNA Reverse Transcription Kit**.**

2. In the PCR step, PCR products were amplified from cDNA samples using the TaqMan MicroRNA Assay together with the TaqMan® Universal PCR Master Mix.

Statistical analysis

Data were organized in Microsoft office Excel software, 2010. The statistical package for social sciences (SPSS) software (version. 20) was used for all statistical analysis. Data were subjected to normality test (Shapiro-Wilk test) before statistically analyzed. Non-normally distributed data were further subjected to transformation into log value to get normal distribution. Continuous variables were expressed as median and analyzed either by independent t-test for comparison between two groups or analysis of variance (ANOVA) for comparison among more than two groups. Binomial variables were expressed as frequency and percentage and analyzed by Pearson chi-square. Receiver Operating Characteristic (ROC) test was used to find out the sensitivity, specificity, area under curve and the cut-off value of some variables which is supposed to have a diagnostic value. A P-value of ≤ 0.05 was considered a statistically significant.

RESULTS

Demographical picture and clinical presentation

In the present study, the mean age of patients was 31.86±8.52 years with a range of (18-45) years, while mean age of control group was 32.43±7.08 years with a range of (20-45) years.

Regarding the stage of disease, the highest incidence was the minimal stage I 17 (57.67%) followed by mild stage II 9 (30%) as in figure (1).

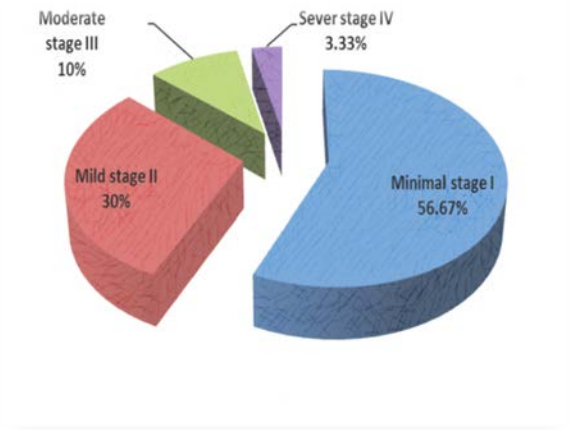

Figure (1): Stages of endometriosis in patients group.

About the menstrual cycle phases (secretory and proliferative), endometriosis patients in secretory phase were 21(70%) and in proliferative phase were 9 (30%), while in secretory phase the healthy controls were 13(43.33%) and 17(56.67) in proliferative phase. There was no statistically difference with a P-value more than 0.05 as shown in table (1).

Table 1: Distribution of patients and controls according to menstrual phases.

Phases of MC.	Patients group		Controls group		P-value
	No.	%	No.	%	
Secretory	21	70	13	43.33	0.067
Proliferative	9	30	17	56.67	
Total	30	100	30	100	

Results of the molecular investigations

In the current study, the mean Log fold change values of gene expression of miR-16 in serum of endometriosis patients was higher as in comparison to controls and showed statistically significant difference between them, the P-value was 0.041 as shown in figure (2).

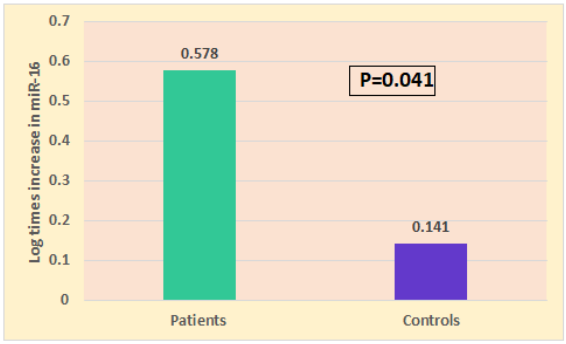

Figure (2): The mean Log fold change values of gene expression of miR-16 of endometriosis patients and controls.

The mean Log fold change values of gene expression of miR-199a in serum of endometriosis patients was higher as in comparison to controls but showed no statistically significant difference between them, the P-value was more than 0.05 as shown in figure (3).

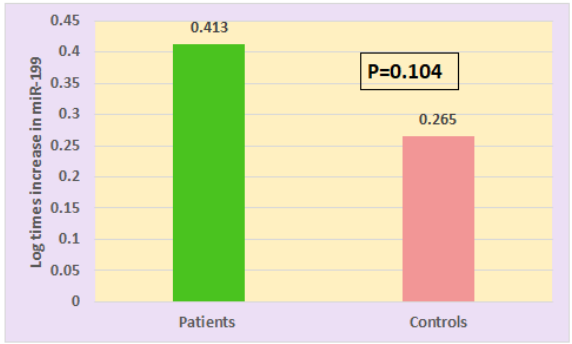

Figure (3): The mean Log fold change values of gene expression of miR-199a of endometriosis patients and controls.

The mean of the Log fold change values of gene expression of miR-199a showed statistically significant difference between early stages (stage I and stage II) and late stages (stage III and stage IV) of disease, the P-value was **0.041**. While miR-16 showed no statistically difference between early and late stages, the P-value was more than 0.05 as shown in table (2).

Table 2: MiRNAs log fold changes in early and late stages of endometriosis

MiRNAs	Stages	Mean	SE	No.	P-value (t-test)
MiRNA16 Log fold change	Early	0.3994	0.263	26	0.39
	Late	1.74	0.621	4	
MiRNA199a Log fold change	Early	0.778	0.161	26	0.041
	Late	1.578	0.218	4	

As shown in table (3), the mean of the Log fold change values of gene expression of miR-16 and miR-199a showed no statistically significant difference in secretory phase between patients and controls, the P-value was more than 0.05.

Table 3: MiRNAs log fold changes in secretory phase for endometriosis patients and controls.

MiRNAs	Groups	Mean	SE	No.	P-value (t-test)
MiRNA16 Log fold change	Patients	0.266	0. 142	21	0.234
	controls	0.163	0.103	13	
MiRNA199a Log fold change	Patients	0.194	0.094	21	0.657
	Controls	0.141	0.075	13	

On the other hand, the mean of the Log fold change values of gene expression of miR-16 and miR-199a showed statistically significant difference in proliferative phase between patients and controls, the P-value was **0.012** and **0.047** respectively as shown in table (4).

Table 4: MiRNAs log fold changes in proliferative phase for endometriosis patients and controls.

MiRNAs	Groups	Mean	SD	No.	P-value (t-test)
MiRNA16 Log fold change	Patients	1.306	0.106	9	**0.012**
	controls	0.125	0.092	17	
MiRNA199a Log fold change	Patients	0.925	0.088	9	**0.047**
	controls	0.36	0.142	17	

The mean of the Log fold change values of gene expression of miR-16 showed statistically significant difference between proliferative and secretory phase for endometriosis patients, the P-value was **0.03**. While the mean of the Log fold change values of gene expression of miR-199a revealed no statistically significant difference between two phases for endometriosis patients, the P-value was more than 0.05 as shown in table (5).

Table 5: MiRNAs log fold changes in endometriosis patients during secretory and proliferative phase.

MiRNAs	Menstrual cycle Phases	Mean	SD	No.	P-value (t-test)
MiRNA16 Log fold change	Secretory	0.266	0.142	21	0.03
	Proliferative	1.306	0.106	9	
MiRNA199a Log fold change	Secretory	0.194	0.094	21	0.115
	Proliferative	0.925	0.188	9	

The area under the curve (AUC) for miR-16 was 0.657, and P equal to 0.037. The optimal cut-off value was 1.961-fold with sensitivity and specificity 61%, 63% respectively, 95% confidence interval equal to 0.517-0.796 as in figure (4).
The area under the curve (AUC) of miR-199a was 0.591, and P equal to 0.225. The optimal cut-off value was 0.808-fold with sensitivity and specificity 59%, 63% respectively, 95% confidence interval equal to 0.442-0.740 as in figure (5).

Discussion

The analysis of serum markers is direct and simple, changes in the levels of specific circulating miRNAs in the serum offer the potential for the detection of the disease. Two advantages are associated with using miRNA in clinical diagnosis. First, a serum-based biomarker would enable a relative comprehensive analysis of the disease without requiring surgery; this is especially important for endometriosis because the disease is diagnosed mainly by visualization at surgery. Second, a serum miRNA-based test is low cost and sample management is easy, including sample collection and processing [7].

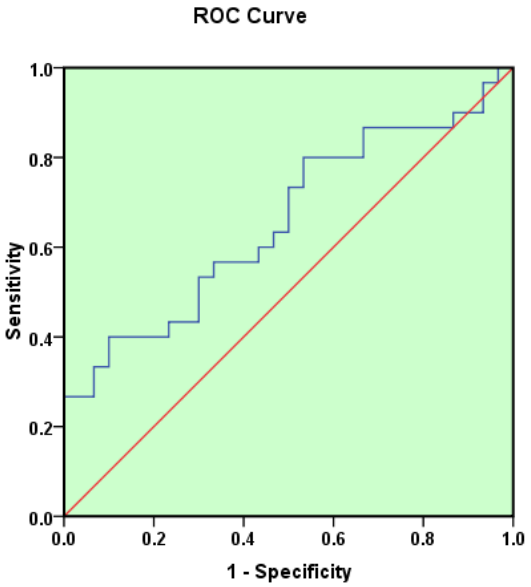

Figure 4: Receiver Operating Characteristic curve for miRNA-16 as a marker for endometriosis.

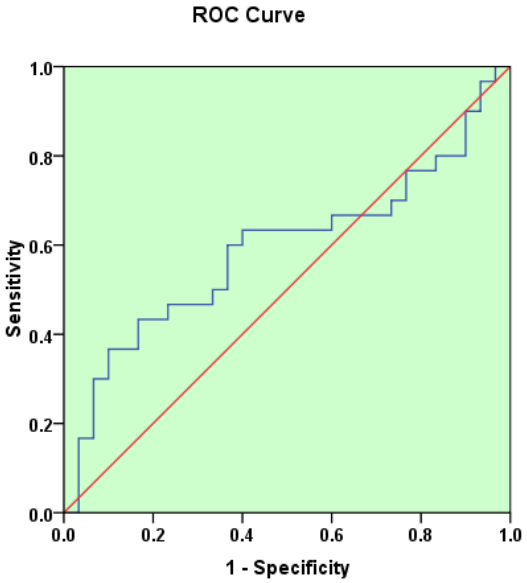

Figure 5: Receiver Operating Characteristic curve for miRNA-199a as a marker for endometriosis.

In the present study, there was an increased expression of both microRNA-16 and microRNA-199a in patients compared to healthy controls. This findings were agree with previous study by Wang WT and colleagues who found that the relative expression levels of miR-199a and miR-122 were higher in endometriosis patient samples than those in controls [7]. As well as studies by Ramon LA, *et al*. (2011) and by Braza-Boils A, *et al*. (2014) showed significant increase of miR-16 in endometriosis patients than control group [10, 11].
The current study was disagree with Pan Q and colleagues who found that both microRNAs (microRNA-16 and microRNA-199a) were down regulated in endometriosis when compared to women without endometriosis [12].MicroRNA-16 has been shown to regulate cell differentiation [13], and progression of the cell cycle, thereby inducing cell-cycle arrest and acting as potent tumor suppressors [14]. This effect is attributed to down-regulation of the expression of several cyclins (CCND1 and CCNE1), which are major inducers of cell-cycle progression.

Braza-Boıls A, *et al* (2013) found a significant correlation between the decrease in miR-16 and the increase in vascular endothelial growth factor-A (VEGF-A) in response to peritoneal fluid exposure in endometrial and endometriotic cell cultures [15], and this correlation could indicate regulation of VEGF-A translation by miR-16. So, it has been shown that VEGF-A is a target gene for miR-16 in several cell types, indicating that miR-16 could be an important regulator of angiogenesis [10]. In addition, miR-16 negatively regulates VEGF expression by directly targeting the 3 UTR of VEGF mRNA. VEGF, acting as a key angiogenesis promoter, plays a significant role in physiological and pathophysiological vascular development and maintenance [16]. A previous study indicated that miR-16 inhibits the proliferation, migration and angiogenesis-regulating potential of mesenchymal stem cells [17].

Concerning the proliferative phase, the mean of the Log fold change values of gene expression of miR-16 and miR-199a showed statistically significant difference between patients and controls, cyclic variations were only noted in sera of patients with endometriosis, but not in control group. These findings are in agreement with a study that showing the circulating miRNAs levels do not fluctuate in healthy women through the menstrual cycle [18]. Cyclic differences in circulating miRNAs were associated with endometriosis, this further evidence of the role of aberrant miRNA regulation as an essential component of endometriosis [19].Previous studies have shown that miR-199a is dysregulated in both eutopic and ectopic endometrial tissues compared with normal controls [20].

Dai and Di (2011) found that miR-199a can inhibit the adhesion, migration and invasion of the human eutopic endometrial stromal cells (ESC), Ikappa B kinase beta (IKKβ) is the target gene of miR-199a in ESC, which means one of the mechanisms of the inhibition effect is probably that miR-199a inhibits the activation of nuclear factor-kappa B (NF-κB) signaling pathway by targeting IKKβ gene. In addition, miR-199a is correlated with pelvic adhesion and lesion distribution as well as with hormone-mediated signaling pathways, demonstrating that it may play an important role in the progression of the disease [21].

Endometriosis is one of precancerous lesion of endometrial cancer that is a target for early diagnosis. A combination of miR-199a and miR-542-3p can be used to diagnose endometriosis with a sensitivity of 96.61 % and specificity of 79.66 %, and this approach may serve as a noninvasive marker in practice [22]. Further analysis also indicated that the relative concentration of miR-122 might be correlated with that of miR-199a. Interestingly, in silico functional analysis showed that both miRNAs could target SOX4, a well-known gene associated with apoptosis. It has also been reported that SOX4 plays a positive role in the differentiation of endometrial carcinomas [23]. The up-regulation of these two miRNAs in the serum of endometriosis patients implies that they might be involved in the disease pathogenesis via the regulation of SOX4 expression; this hypothesis deserves further investigation [7].

In the present study there were several limitations. The main one was that most of the patients included in the study were stage I and II (73.33%) because that most of patients discovered incidentally, also the lack of differentiation between patients with ovarian endometriomas (OE) and patients with deeply infiltrative endometriosis DIE. On the other hand, OE and DIE are considered two distinct entities of endometriotic disease, and accordingly it is accepted that endometriosis progresses to cystic ovarian disease and pelvic adhesions in some women, to deeply infiltrating disease in other women and sometimes both stages of severe disease in the same woman.

The current study revealed that the mean of the Log fold change value of gene expression of miR-199a showed statistically significant difference between early stages(stage I and stage II) and late stages(stage III and stage IV) of disease. This finding is agree with previous study by Wang *et al.*(2013) which found that, miR-199a and miR-122 were differentially expressed between severe (including stage III/IV) and mild endometriosis (stage I/II) samples, and their expression showed progressive changes along with the severity of the disease. The miR-199a concentration in the serum continues to increase as disease progresses. So, these miRNAs may be useful for diagnosing disease severity, although this will require validation in a large cohort of samples. Thus, the present study highlighted the possibility of using combined serum miRNA for management of endometriosis [7].

Therefore, studies on the expression, regulation and function of miRNAs in patients with endometriosis will provide the unique insights for the development of specific miRNAs as diagnostic markers and therapeutic targets for endometriosis in the future [24].

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