

CIRCULATING miRNAs – A POTENTIAL NON-INVASIVE BIOMARKER FOR DIAGNOSIS OF ENDOMETRIOSIS

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Abstract

Endometriosis is a debilitating disease affecting one in ten women worldwide. Due to several reasons the diagnosis is delayed for 7–11 years. Currently, the ultimate diagnostic procedure is laparoscopy followed by histological examination. The need for non-invasive biomarker is placed as a top priority according to the World Endometriosis Society.

The present study includes 45 females – 30 patients with endometriosis at different stages and 15 healthy controls. An expression analysis of 84 circulating miRNAs was performed comparing between different groups of participants. Ten miRNAs with the most significant expression difference between females at early stage of endometriosis, advanced stage of endometriosis and healthy controls were nominated as a potential diagnostic and prognostic biomarker using a panel of miRNAs.

Key words: endometriosis, diagnosis, biomarker, miRNA

Introduction. These days we know a lot about endometriosis but still we do not know enough. A citation from Emery Wilson claims that endometriosis

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“remains a riddle wrapped in a mystery inside an enigma” [1]. According to the World Endometriosis Society it is an inflammatory condition characterized by endometrium-like tissue at sites outside the uterus [2]. The endometrial heterotopic islets contain stroma and glands that may respond to exogenous, endogenous or local hormonal stimuli [1]. Endometriosis afflicts about 10% of women of reproductive age, which makes approximately 190 million women worldwide [3]. In the group of women with infertility and chronic pelvic pain endometriosis accounts for 35–50% of cases [4].

The clinical presentation of endometriosis consists of dysmenorrhea, dyspareunia, chronic pelvic pain and infertility. It is not uncommon that morphological changes negatively impact the quality of life, affecting many aspects of personal, social and sexual life [5]. Attention should be paid to the fact that symptoms do not always correlate to the stage of the disease, and no symptom is specific to endometriosis, as well [6].

Nowadays, endometriosis remains difficult to diagnose. The gold standard for a definitive diagnosis of endometriosis is the laparoscopy inspection of the abdominal cavity followed by histopathological exam of the found lesions [3]. Considering the fact that the symptoms of the disease are unspecific and a surgery is needed for confirmation of the diagnosis, 7–11 years of diagnostic delay is registered worldwide [7]. Therefore, one of the priorities in the field of endometriosis research, is finding a non-invasive biomarker to help with the diagnosis and prognosis of women suffering from the disease. A wide range of molecules are tested in different body fluids as potential biomarkers for endometriosis [8]. None of them had sufficient specificity and sensitivity to be used in the clinical practice [8]. MicroRNAs (MiRNAs) are a relatively new class of molecules that may be the key to endometriosis diagnosis.

MiRNAs are highly conserved noncoding single-stranded RNAs of about 22 nucleotides [9]. They were discovered in 1993 in the nematode *Caenorhabditis elegans* and are known to control more than half of the genes in human genome [10]. MiRNAs are posttranslational modulators of gene expression in many human pathologies, involving different gynecologic diseases and endometriosis [8]. They are detected in multiple body fluids – serum/plasma, peritoneal fluid, saliva, urine, etc. [11]. Such class of molecules is characterized by high stability in the biological fluids and tissue specificity. They are reproducible and consistent among individuals, as well [9]. Studies show that small changes in the miRNA expression may have an impact on biological processes [12]. Such changes indicate an alteration of the physiological processes and have been reported in different human diseases [9]. All this increases their potential for usage as a biomarker for endometriosis and other pathologies [10, 11].

Materials and methods. Peripheral blood (10 ml) was obtained from 45 females between the age of 24 and 51 after 10 h of fasting before surgery. Informed consent was signed by all of them. For the aim of the study, participants were

divided into three groups: group 1 ($n = 15$) – early stage of endometriosis (stage I-II); group 2 ($n = 15$) – advanced stage of endometriosis (stage III-IV); group 3 ($n = 15$) – controls. Endometriosis was confirmed in patients after laparoscopy was performed followed by a histological examination. Patients were classified according to the revised American Society for Reproductive Medicine score that is the most widely used system presently [4]. The control group involves females without endometriosis and needed hysteroscopy due to different pathologies – excessive menorrhagia or metrorrhagia, uterine polyps, chronic pelvic pain, etc.

Plasma RNA from all collected samples was isolated using the miRNeasy Serum/Plasma Kit with regard to Plasma Spike-in Control (Qiagen®) according to the manufacturer's specifications. Three pool samples, each containing 15 RNA samples, were constructed, using equimolar amounts of RNA extracted from the tissue samples: 1) early stage of endometriosis; 2) advanced stage of endometriosis; 3) controls. Reverse transcription of the three pool samples into cDNA was carried out via miScript II RT Kit (Qiagen®). MiScript miRNA PCR Array Human Serum & Plasma (Qiagen®) and miScript SYBR Green PCR Kit (Qiagen®) were used to determine miRNA expression profiles. All arrays were run on Applied Biosystems™ 7500 instrument. Screening for differences in the levels of MiRNA expression was performed by QIAGEN's GeneGlobe Data Analysis Center using a software-based tool. Bioinformatics tools were used for predicting miRNA targets – Diana tools, TargetScan, and PicTar. The GeneCodis web tool was used for prediction of the biological processes and pathways the target genes are involved in.

Results. MiRNA expression analysis was performed on each of the three pool samples. The results confirm there is a difference between the patients and the control group. For result normalization all miRNAs with $Ct > 35$ were excluded. According to the manufacturer's instructions a double deviation in expression level was assumed as a significant difference.

First, the group of patients with early stage of endometriosis was compared to the control one. The analysis of the 84 miRNAs included in the array showed a difference in the expression levels of 29 of them. Increase in the expression of the patient group was recorded in all samples compared to the control group.

In order to specify the miRNAs with the most pronounced expression change, a conditional difference of twice the mean value from the fold change in the expression of all miRNAs was assumed. Such a division gives possibility to define the miRNAs with the most significant expression change in plasma when comparing patients with early endometriosis and healthy controls. The three miRNAs fulfilling the abovementioned conditions are listed in Table 1.

The follow-up analysis included the group of patients with advanced stage of endometriosis compared to the control one. The results showed that 41 of the 84 miRNAs had increased expression in patients compared to controls. Using the same criteria as in the first comparison five miRNAs fulfilling the conditions were detected. They are listed in Table 2.

T a b l e 1

Circulating miRNAs with an expression change in case of comparison between patients with early endometriosis and healthy controls

MiRNA	$2^{-\Delta Ct}$		Fold change
	Early stage of endometriosis	Control group	
hsa-miR-21-5p	231.8556918	2.284428571	101.49
hsa-miR-26b-5p	451.0311714	5.051925392	89.27
hsa-miR-18a-5p	51.82525243	0.610825981	84.84

T a b l e 2

Circulating miRNAs with an expression change in case of comparison between patients with advanced endometriosis and healthy controls

MiRNA	$2^{-\Delta Ct}$		Fold change
	Advanced stage of endometriosis	Control group	
hsa-let-7a-5p	3641.540899	8.466896968	430.09
hsa-miR-195-5p	7456.760725	18.55625065	401.84
hsa-miR-26b-5p	1650.094957	5.051925392	326.62
hsa-miR-106b-5p	662.7556865	2.090478685	317.03
hsa-miR-21-5p	714.7690389	2.284428571	312.88

T a b l e 3

Change in the expression of circulating miRNAs between both stages of endometriosis

MiRNA	Expression change in early stage of endometriosis group	Expression change in advanced stage of endometriosis group	Fold change
hsa-miR-93-5p	9.38	168.37	↑ 17.99
hsa-miR-20a-5p	11.41	200.92	↑ 17.6
hsa-miR-106a-5p	16.13	226.2	↑ 14.02
hsa-miR-17-5p			

We would like to emphasize the reported expression change in miRNAs disguised in both groups of patients with endometriosis. Table 3 shows the fold change increase in the expression of these miRNAs; information about patient groups separately is available, as well as a comparison between the two groups. Three miRNAs with the most significant expression change were detected.

Discussion. MiRNAs are novel and promising molecules in the field of biomarkers research. Most attempts to establish biomarkers for endometriosis are based on nonspecific hormonal and inflammatory markers [13]. The increas-

ing number of miRNA studies regarding the diagnosis of endometriosis still remain controversial. They lack enough specificity and sensitivity to be used in clinical practice [8]. Moreover, miRNAs expression is dependent on many factors, including the health status and different lifestyle factors. This is why studies on miRNAs potential as biomarkers for endometriosis should be unified and performed under similar conditions. The current study was performed according to the guidelines of World Endometriosis Research Foundation, so that the results correspond to the international standards.

We chose to use pool samples to detect the most significant changes in miRNA expression status that occur in a large percentage of patients and to minimize the effect of any individual differences, as well. Comparison of the miRNA expression profile between females with and without endometriosis, as well as between those with endometriosis at different stages, allows for detection of disease-specific differences that may be the basis for identification of non-invasive diagnostic and prognostic biomarkers. Based on the comparison between different groups in the current study ten miRNAs (miR-21, miR-18a, let-7a, miR-195, miR-20a, miR-17, miR-26b, miR-106b, miR-93, and miR-106a miR-17) with the highest potential to be used as non-invasive biomarkers were nominated.

Currently, it is impossible to use miRNAs independently as a biomarker because no single miRNA is disease specific. Also, each miRNA controls the expression of many genes in various molecular pathways and vice versa – the expression of a gene may be modulated by many miRNAs. Consequently, we suggest usage of a panel of miRNAs as a more reliable approach to synthesize various pathological changes typical of the disease. In order for the non-invasive biomarker to serve both for diagnosis and prognosis of endometriosis, the panel includes the ten miRNAs with the most significant expression alteration in plasma of patients at different stages compared to the control group, as well as with each other. The differences in the expression of miRNAs with the most significant potential for diagnostic and prognostic biomarkers are shown in Fig. 1.

Six of these miRNAs are part of two miRNA families – miR-17 and miR-18. They are included in the so-called group “oncoMiR” because of their essential role in tumourigenesis [14]. Several studies prove their role in the pathogenesis of cancer, but the way in which individual miRNAs modulate different strands in this process should be considered individually. In addition to the association to carcinogenesis, members of the miP-17 family – miP-20a, miP-93, miP-17, and miP-106a – are specifically expressed in undifferentiated and differentiated embryonic stem cells [15]. They are suspected to play a major regulatory role in cell differentiation during the early stages of fetal development [15]. This finding supports one of the endometriosis pathogenesis theories and gives even greater credibility to the results obtained.

Despite the general knowledge of the origin and function of large groups of miRNAs, it is important each one of them to be considered separately in order

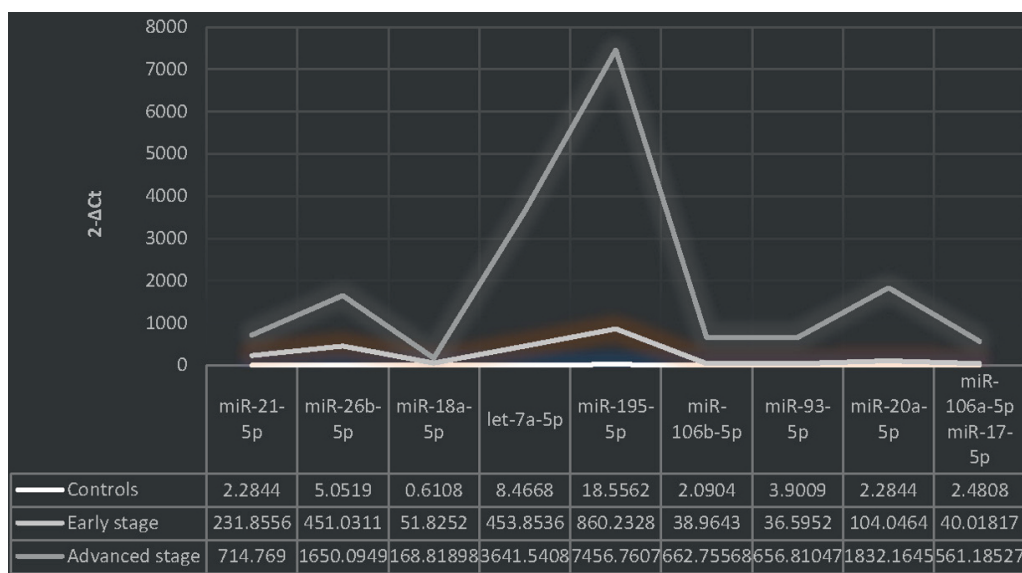


Fig. 1. Differences in the miRNA expression with highest potential for a non-invasive biomarker for endometriosis

to gain more information of its potential use as a biomarker. MiR-21 increased expression is associated with poor prognosis of endometriosis and ovarian cancer [16]. In addition, animal studies detect that miR-21 is a key factor for successful implantation and it is a potential biomarker for fertility potential [17]. MiR-18a and miR-195 are known as expression modulators of key oncogenes and tumour-suppressor genes [18, 19]. Different studies detect increased expression of the circulating miR-18a and miR-195 in females with endometriosis which correlates to our findings [20]. On the other hand, let-7 and miR-20a – regulators of key processes such as angiogenesis, DNA repair, and apoptosis, have been detected decreased in the plasma of endometriosis patients which is the opposite of our results. Interestingly, a change in the expression of miR-26b, miP-106b, miR-93, and miR-106a as, to our knowledge, have not been mentioned in other endometriosis studies before. All this confirms the inconclusiveness of miRNA studies in search of endometriosis noninvasive biomarker and the need of standardized study design.

Taken together, our findings of differences in miRNA expression profiles reveal specific disease changes. The level of these changes allows for a distinction between females with and without endometriosis, as well as in females with endometriosis at different stages. This data indicates that miRNAs may be used as reliable diagnostic and prognostic biomarkers for endometriosis. The miRNAs that are included in the panel demonstrate a significant expression change to become part of the diagnostic process in endometriosis patients.

There are some limitations in the current study that need to be mentioned – small sample size, lack of individually tested miRNAs expression profile, and lack

of a second method to confirm the results. Undoubtedly, this is the beginning of our studies in this field and we hope that in the near future we will be able to continue this research. However, we strongly believe that the results are novel and substantial knowledge among the large amount of information regarding the possibilities of non-invasive endometriosis diagnosis.

Conclusion. Endometriosis is still a disease with many unanswered questions. It is a great problem for patients, their families, physicians, and society as a whole. Therefore, the need for non-invasive biomarker increases. Studies in this field nowadays show that individual miRNAs are not suitable as biomarkers. Consequently, we suggest a miRNA panel consisting of ten miRNAs as a better solution.

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