

MiRNA EXPRESSION PROFILES IN AN ECTOPIC
ENDOMETRIUM OF PATIENTS AT DIFFERENT STAGE
OF ENDOMETRIOSIS

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Received on October 13, 2020

Accepted on October 29, 2020

Abstract

Endometriosis is a debilitating disease that affects up to 15% of women worldwide. Delayed diagnosis, lack of noninvasive biomarker and unexplained pathogenesis are key problems for specialists and patients alike. MiRNAs are a class of noncoding RNAs that regulate myriad of cellular functions. These gene expression regulators may prove to be attractive biomarkers with diagnostic and prognostic value for endometriosis.

Tissue samples were collected from the participants enrolled in the study during laparoscopy (patients) and hysteroscopy (control group). After RNA isolation (miRNeasy MiniKit, Qiagen) based on the disease stage, three pools – each containing 15 RNA samples, were constructed: 1) early stage endometriosis, 2) late stage endometriosis, and 3) healthy controls. Each pool sample was subjected to reverse transcription via miScript II RT Kit, Qiagen to obtain cDNA. SYBR Green based Real-time PCR assay was used to determine the expression profile of 84 miRNAs (Human miFinder miRNA PCR Array, Qiagen).

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This research was supported by contract No. 212/12.12.2018, grant No. 4731/03.07.2018 of the Council of Medical Science at the Medical University of Sofia, Bulgaria.

DOI:10.7546/CRABS.2023.10.14

We detected 32 differently expressed miRNAs between early stage endometriosis and control group, and 51 differently expressed miRNAs between advanced stage endometriosis and control group. Three miRNAs were differentially expressed with more than 10-fold change in the early stage group and thn miRNAs showed more than 10-fold change in the advanced stages group. The three miRNAs with the highest fold change in the expression levels in both case groups were nominated as potential biomarkers for endometriosis. The results of the analysis of their target genes supports the role of deregulation of apoptosis, angiogenesis, epithelial-mesenchymal transition, and various other cellular signalling pathways in endometriosis development.

Key words: endometriosis, miRNA, expression analysis, biomarker

Introduction. Endometriosis is a chronic estrogen-dependent disorder, which is characterized by the presence of endometrial tissue outside the uterine cavity [1]. With prevalence of 10–15% among women of reproductive age and of about 70% among women with chronic pelvic pain, endometriosis is one of the most common benign conditions in gynecological practice [2,3]. Symptoms of endometriosis negatively impact the quality of life of affected women as they include dysmenorrhea, dyspareunia, chronic pelvic pain and infertility [4]. These lead to increased morbidity in women and significant costs for healthcare systems [5]. The average delay of diagnosis is 8–10 years due to the nonspecific endometriosis symptoms, the attitude of healthcare professionals and the lack of noninvasive diagnostic tests [6,7]. At present the “gold standard” for diagnosis of endometriosis is direct visualization of the lesions during laparoscopy, followed by a histological confirmation [4]. Therefore, World Endometriosis Society prioritizes the development of a non-invasive biomarker for the disease [8]. Although the significance of a variety of possible markers in plasma, urine, endometriosis tissue, etc. has been investigated, none of them proved to be specific and sensitive enough for clinical use [9,10].

Despite the high prevalence in the population and the large number of scientific studies dedicated to endometriosis, the complete etiopathogenesis of the disease still remains elusive [11]. The classical theories of retrograde menstruation and celomic metaplasia cannot singlehandedly explain the full spectrum of the disease’s pathology [4]. Studies so far have reported several dysregulated pathways such as inflammation, angiogenesis, cell adhesion, cell proliferation, apoptosis, etc. [12]. Elucidating the pathological processes which are responsible for the development of endometriosis would help the screening, diagnosis and treatment of the disease.

MicroRNAs (miRNAs) – a class of molecules involved in gene expression regulation of multiple cellular processes – may reveal new aspects in the pathogenesis of endometriosis, and may prove to be promising biomarkers for diagnosis and prognosis of the disease. MiRNAs are noncoding, single-stranded, evolutionary conserved RNAs, approximately 21–25 nucleotides in length [13]. Their

biological role is translational repression and/or mRNA degradation as they bind complementary to a target mRNA [14]. MiRNAs have been associated with cell division, proliferation, immune response, and apoptosis [15]. Changes in miRNAs expression profiles are implicated in the pathogenesis of multiple human disorders, including gynecological diseases [14]. Multiple studies report altered miRNA expression profile in women with endometriosis and healthy controls [12]. Several of them that evaluate the expression pattern differences in ectopic and eutopic endometrium shed more light on the molecular pathways involved in endometriosis [11].

Materials and methods. Forty-five premenopausal women between 27 and 47 years of age with regular menstrual cycles were included in the study. All participants signed an informed consent and the study was approved by the Ethical Committee of Medical University of Sofia. Patients were divided into three groups based on the stage of the disease: group 1 ($n = 15$) – early stage endometriosis (stage I–II); group 2 ($n = 15$) – advanced stage endometriosis (stage III–IV); group 3 ($n = 15$) – controls. The staging of the disease was performed according to the reversed American Fertility Society scoring system. None of the participants reported suffering from a chronic disease such as diabetes, glomerulonephritis, autoimmune disorders, and chronic infections. Participants had not been subjected to hormonal treatment during the 3 months before the surgical procedure. In the control group were included women with no ultrasound signs of the disease who underwent hysteroscopy due to irregular bleeding, pain syndrome or infertility.

Tissue samples were collected under sterile conditions from each participant – for endometriosis patients ectopic endometrium was obtained during laparoscopy and for controls eutopic endometrium was obtained during hysteroscopy. Tissues were stabilized in RNAlater Tissue Reagent, Qiagen and total RNA was isolated from these stabilized samples with miRNeasy MiniKit, Qiagen according to the manufacturer's protocol. The quality and yield of RNA was evaluated on Thermo Scientific™ NanoDrop™.

Three pools, each one containing 15 RNA samples were constructed, using equimolar amounts of RNA extracted from the tissue samples: 1) early stage endometriosis; 2) advanced stage endometriosis; 3) controls. Reverse transcription of the pool samples into cDNA was carried out via miScript II RT Kit, Qiagen.

Expression profiles of 84 well-characterized miRNAs (Human miFinder miRNA PCR Array, Qiagen) were assessed by SYBR Green-based real-time PCR array on Applied Biosystems™ 7500 instrument. Data analysis was performed by QIAGEN's GeneGlobe Data Analysis Centre using a software-based tool. MiRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method. MiRNAs were considered present when cycle threshold (CT) values were lower than 35. Further miRNA characterisation and prediction of target genes was accomplished using miRBase.org, Targetscan.org and Diana tools.

Results. The expression analysis of 84 miRNA revealed:

- 32 dysregulated miRNAs in the early stage group compared to the control group of which 31 downregulated miRNAs and one upregulated miRNA;
- 51 dysregulated miRNAs in the advanced stage group compared to the control group of which 47 downregulated miRNAs and four upregulated miRNAs.
- The expression level of all other miRNAs was within normal range.

In order to detect the miRNAs with the most significant expression alterations between cases and controls we classified dysregulated miRNAs according to their expression level into two groups: 1) fold-change between 2 and 9.9; 2) fold-change ≥ 10 . The three miRNAs with ≥ 10 -fold change in the expression levels in the early endometriosis group are listed in Table 1. The ten miRNAs whose expression level change is ≥ 10 -fold in the advanced endometriosis group are presented in Table 2.

Table 1

MiRNAs with altered expression ≥ 10 -fold in patients in the early stage endometriosis versus healthy controls

MiRNA	Fold-change
miR-21-5p	-11.33
miR-200c-3p	-30.40
miR-196b-5p	-19.28

Table 2

MiRNAs with altered expression ≥ 10 -fold in patients in the advanced stage endometriosis versus healthy controls

MiRNA	Fold-change
miR-26b-5p	-12.59
miR-155-5p	-10.77
miR-21-5p	-27.52
miR-200c-3p	-151.22
miR-23a-3p	-14.32
miR-23b-3p	-16.15
miR-196b-5p	-139.54
let-7f-5p	-41.57
miR-20a-5p	-10.35
miR-100-5p	-13.55

Three miRNAs (miR-21-5p, miR-200c-3p, and miR-196-5p) were selected as potential diagnostic and prognostic biomarkers because changes in their expression profile were present in both early and late stage endometriosis groups. Moreover, we found a significantly higher fold-change in the advanced stages compared to the early stages (Fig. 1), which demonstrates their progressing dysregulation with the advancement of the disease.

MiRNA databases (miRBase.org, Targetscan.org and Diana) were used to identify the possible gene targets of the three most significantly dysregulated miRNAs. A large number of predicted target genes were registered for the three miRNAs – 260 top predicted targets and 1954 transcripts with conserved sites. We selectively searched for the most likely targets based on previously reported pathways that contribute to the pathophysiology of endometriosis and we identified

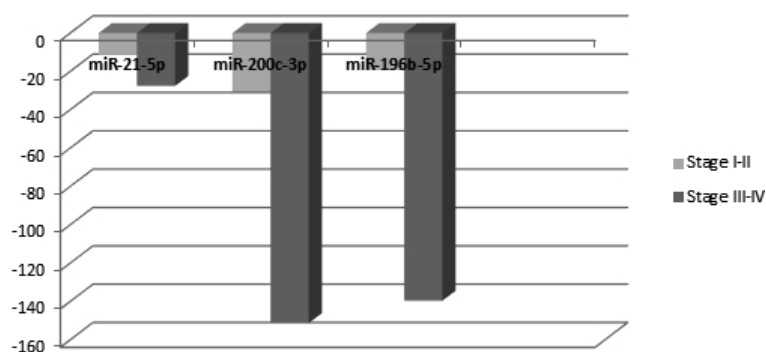


Fig. 1. Changes in miRNA expression profiles in the early stage endometriosis group compared with the late stage endometriosis

the most relevant gene targets with their predicted role for the disease pathophysiology (Table 3).

Discussion. MiRNAs are new molecules that hold great potential to overcome some of the deficits in our knowledge of the pathophysiology of endometriosis and to notably aid the diagnosis and prognosis of the disease. MiRNAs have been reported to be differently expressed in various disorders, including gynecological pathologies such as leiomyomas, pregnancy disorders, different malignant conditions of the endometrium, cervix, and ovaries [11,14]. Multiple studies have revealed altered miRNA expression profiles in various tissues and gynecological diseases [14].

The results of our study confirm the hypothesis that miRNAs expression profiles differ between women with endometriosis and controls. Considering the differences between the expressed miRNAs in endometriosis lesions in affected women and eutopic endometrium in unaffected women, and also the higher mag-

T a b l e 3

Most relevant target genes of miR-21-5p, miR-200c-3p, and miR-196b-5p and their possible contribution to endometriosis

MiRNA	Gene	Impact on endometriosis
miR-21-5p	<i>TGFBI</i>	Cell adhesion and differentiation
	<i>PDCD4</i>	Tumour suppressor
	<i>BCL7A</i>	Cell death regulation
	<i>FASLG</i>	Cell death regulation
miR-200c-3p	<i>RECK</i>	Tissue remodelling
	<i>ESRP1</i>	Epithelial-mesenchymal transition (EMT)
	<i>ZEB1</i>	EMT
	<i>VEGFA</i>	Angiogenesis
miR-196b-5p	<i>FLT1</i>	Angiogenesis
	<i>HOXA5, HOXB7,</i>	Transcription factors which are part of a developmental regulatory system
	<i>HOXB8, HOXC8,</i>	
	<i>HOXA10</i>	

nitude of the expression changes in the late stages of the disease, we defined the three miRNAs with the highest potential to be used as biomarkers. Literature review of previous studies in the field has elucidated the target molecular pathways of these miRNAs associated with endometriosis.

In the present study miR-21-5p was found to be downregulated with -11.33 -fold change in the early stages of endometriosis and with -27.52 -fold change in the advanced stages compared to controls. As abnormal apoptosis is supposed to be one of the possible causes for ectopic lesion survival, miRNAs that regulate this process may be of key importance for the pathophysiology of the disease [16]. MiR-21-5p targets Programmed Cell Death 4 (*PDCD*), B-cell lymphoma 2 (*BCL7A*), and Fas ligand (*FASLG*). These genes are cell death regulators as they transduce the apoptotic signal into the cells [12,17]. Downregulation of this anti-apoptotic miRNA may result in disease process and can be used for distinguishing ectopic and eutopic tissue. Also, miRNA-21 downregulation leads to overexpression of *RECK*, which targets MMPs-2 and -9 [12]. MMPs-2 and -9 are found to be increased in affected women compared to controls [12]. Targeting of *TGFB1* results in an increased cell differentiation and adhesion when miR-21 is downregulated [18].

One of the most studied miRNA families in regard to endometriosis is miR-200. We found miR-200c-3p to be downregulated in all stages of endometriosis. MiR-200c inversely correlates with the expression of *ZEB1* which is responsible for epithelial-to-mesenchymal transition (EMT) and progression through adhesion repression molecules such as E-cadherin [19]. MiR-200c also regulates *ESRP1*, *VEGFA*, *FLT1* – genes associated with EMT and angiogenesis [19]. The alteration of these processes promotes pathological tissue transformation that may facilitate the development of endometriosis.

The third miRNA found dysregulated in the early and late stages of endometriosis compared to controls is miR-196b-5p. It is known to target *HOXA5*, *HOXB7*, *HOXB8*, *HOXC8*, *HOXA10*, which reportedly regulate endometrial function [16]. Moreover, miR-196b plays a role in tissue vascularization, wound healing and common endometrial pathology [16,20].

Thus, this study nominates miR-21-5p, miR-196b, and miR-200c-3p as the top candidates for further validation as biomarkers in endometriosis.

Conclusion. Our research revealed: 1) significantly altered expression of miR-21-5p, miR-200c-3p, and miR-196b-5p in patients with endometriosis compared to healthy controls; 2) correlation between the level of dysregulation of miR-21-5p, miR-200c-3p, and miR-196b-5p and the stage of the disease; 3) dysregulated miRNAs are involved in key pathways for endometriosis development. Taken in their entirety, our results support the hypothesis that miRNA expression profiles differ between ectopic endometrium in affected women and eutopic endometrium in women without the disease. Further validation studies in larger patient cohorts are needed to confirm the role of miR-21-5p, miR-200c-3p, and

miR-196b-5p for the development of endometriosis and to assess their suitability as diagnostic and prognostic markers.

Acknowledgements. The authors gratefully acknowledge the support from the Bulgarian Ministry of Education and Science under the National Research Programme “Young Scientists and Postdoctoral Students.”

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