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ASSESSMENT OF MICRORNA EXPRESSION IN RETROCERVICAL ENDOMETRIOTIC LESIONS

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Aim *To profile microRNA in the tissues of the ectopic and eutopic endometrium and identify signaling pathways and processes regulated by differentially expressed microRNAs.*
Material and methods *Differentially expressed microRNAs were detected using the next-generation sequencing technique. The sequencing data were validated by real-time polymerase chain reaction. Signaling pathways and processes were estimated by enriching information databases with bioinformatic analysis.*
Results *RNA sequencing resulted in the identification of 429 miRNAs with different patterns in the tissues of the eutopic and ectopic endometrium. As a result of statistical analysis based on the reliability of differences and representation in all comparison groups, 85 miRNAs were selected to determine the possible involvement of their target genes in intracellular cascades and the processes regulated by them. Bioinformatic analysis showed the involvement of regulated target genes into the processes of proliferation, migration, invasion, and into key cascades of intracellular signaling and inflammatory processes.*
Conclusion *The findings suggest that the change in the microRNA expression in the ectopic endometrium and subsequent disruption of the regulation of the expression of their target genes is an important pathogenic link and may contribute to the development of endometriosis.*

Keywords: *microRNA, endometriosis, expression of target genes, sequencing, polymerase chain reaction*

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Endometriosis is defined as the heterotopic presence of endometrium including endometrial epithelium and endometrial stroma outside the uterus. Endometriotic lesions show the diverse location and are found in retrocervical fiber, rectovaginal septum, ovary, bowel, bladder, ureter, peritoneum, and appendix [1]. The formation of endometriotic lesions is accompanied by the development of intra-abdominal fibrosis and adhesions, and a cascade of inflammatory processes and pain syndrome. Due to multiple locations and infiltrative nature of endometriotic lesions, the pathological process involves several organs impairing their physiological functions and leading to severe complications.

Endometriosis is a prevalent gynecological disorder, affecting about 10% of the female population and 20–50% of women with infertility and/or pain syndrome [2]. It is important to note that no effective pharmacotherapy has yet been proposed for this disease. To date, there are several concepts of the etiology and pathogenesis of endometriosis [3]. Nevertheless, current literature is lacking studies investigating key pathogenic mechanisms responsible for the onset of this disease to gain insight into molecular therapeutic targets.

Recently, the role of microRNA (miRNA) in the regulation of gene expression has been intensively studied, both in norm and in pathology. MicroRNA was found to induce post-transcriptional repression due to interaction with mRNA targets, which leads either to the degradation of the corresponding mRNA or to stopping translation [4, 5]. Analysis of the miRNA-mRNA binding sites showed that several miRNAs could bind to one mRNA, and one miRNA can interact with approximately 200 mRNA [6]. Besides, miRNA was found to regulate the activity of transcription factors, which in turn can affect the expression of a huge number of genes. By some estimates, up to 30% of human genes may be regulated by miRNAs [7]. Accumulating evidence suggests that miRNA regulates embryonic development, cell differentiation, metabolism, inflammation, and apoptosis [8]. The change in miRNA expression is associated with malignancy development, as evidenced by several studies that reported significant differences in the expression of miRNA in neoplasms compared to normal tissues [9].

Therefore, the variation of miRNA expression profile in cells, both in norm and pathology can be considered as an informative parameter for assessing the changes in cell homeostasis. Therefore, studying regulatory mechanisms involving miRNA is relevant for gaining insight into the stages of the disease pathogenesis. To address these problems, this work aimed to profile miRNA using the next-generation sequencing technique. MicroRNA expression was examined in the tissues of the eutopic endometrium and endometriotic lesions located in the rectum. Also, a bioinformatic

analysis of signaling pathways and processes regulated by differentially expressed miRNAs was carried out.

Material and methods

The tissues of the eutopic and ectopic endometrium were collected during surgical intervention or diagnostic procedures. Normal endometrial tissue was obtained during diagnostic curettage. The study groups comprised patients with retrocervical and rectal endometriosis and patients without endometriosis. All patients signed informed consent for the use of biomaterial. The ethical committee of the V.I. Kulakov NMRC for Obstetrics, Gynecology and Perinatology approved the plan and methods of biomaterial sampling.

The collected endometrial tissue specimens were divided into two parts, one of which was instantaneously frozen in liquid nitrogen and stored further in a biobank at -80°C; the remainder of the specimen was sent to a pathomorphological laboratory for histological examination. Material for further work stages was selected based on the results of diagnostic investigations (ultrasound, magnetic resonance imaging), operative report and a histological report confirming the presence of epithelial and stromal components in foci of the ectopic endometrium.

Total RNA fractions containing miRNA were isolated from the tissue specimens using the miRNeasy Micro Kit and RNeasy MinElute Cleanup Kit (Qiagen, USA) according to the manufacturer's instructions. The resulting specimens were further used to make cDNA libraries and for subsequent sequencing, as well as to validate the differentially expressed (DE) miRNAs by real-time polymerase chain reaction (PCR). The quality of the isolated fractions was evaluated by on-chip microelectrophoresis with the Agilent 2100 Bioanalyzer instrument.

Assignment of specimens to different groups for microRNA sequencing is shown in Table. 1. The cDNA libraries from the isolated RNA specimens were prepared using the NEBNext Multiplex Small RNA Library Prep Set for Illumina (New England Biolab, Germany), according to the manufacturer's instructions Sequencing was performed using the Illumina NextSeq platform using Illumina reagents and consumables (NextSeq 500/550 High Output v2 kit). Qualitative and quantitative analysis of libraries was conducted by microelectrophoresis (Agilent) and fluorometry (Qubit, ThermoFisher Sci).

The sequencing quality was evaluated with the BaseSpace (Illumina) service using to the following parameters: cluster density, signal strength in the detection channels, and the percentage of clusters that passed the filter on the output of aligned reads. All parameters did not exceed the acceptable values. The sequence of nucleotides obtained as a result of sequencing passed the procedure of removing adapters, followed by alignment to miRBase version 21 miRBase database (http://mirbase.org).

The RNA isolated from the tissues, including miRNA, was converted to cDNA using the miScript II RT kit (Qiagen) according to the manufacturer's protocol. To prepare the reaction mixture and set up PCR, a miScript SYBR Green PCR Kit (Qiagen) was used. The reaction was carried out in a StepOnePlus thermal cycler (Applied Biosystems, USA) in the following sequence: first 15 min at 95°C, then 40 cycles of 15 sec at 94°C for 30 sec at optimized annealing temperature (52-60°C) 30 sec at 70°C. The SNORD 68 was used as an endogenous control for tissue specimens.

To assess the representation of each microRNA, the reads in the library were normalized per million. The differential expression analysis was performed using the DESeq2 package in R. For all comparison groups, the model included information about the group's membership, and for paired specimens information about the patient from whom the material was obtained.

Enrichment of intracellular signaling and biological processes using the KEGG database (http://www.genome.jp) was also carried out with the Cytoscape 3.4.09 program and the JEPETTO10 plug-in. The mRNA targets for miRNA were searched using the miRWalk 2.0 validated database (http://zmf.umm.uni-heidelberg.de).

Results and discussion

Sequencing identified 429 miRNAs with different patterns in the eutopic and ectopic endometrial tissues. MicroRNAs for further analysis were selected based on a multiplicity of changes in expression of more than two, and with an average number of reads of more than one hundred in the comparison groups.

Data processing resulted in sets of differentially expressed miRNA (DE miRNA) for different comparison groups (Table 1). Paired specimens (specimens from one patient) and unpaired specimens (specimens from different patients) were defined. Paired specimens included ectopic/eutopic endometrium in the first and second phases (Ec/Eu I, Ec/Eu II). Unpaired specimens included eutopic/normal (Eu/ N I, Eu/N II), ectopic/ normal (Ec/N I, Ec/N II).

Analysis of possible interactions of DE miRNA in all comparison groups is shown in Fig. 1. Below the axis of ordinates, the names of the comparison groups are given, the number of DE miRNA in each set is shown to the left of them, possible combinations of comparison groups are shown on the right side. Above the bars, the number of unique interactions for each combination of comparison groups is indicated. The figure also shows the combination of groups of paired and unpaired specimens, including the ectopic, eutopic and normal endometrium of the first and second phase specimens. In this combination, the highest number of interactions of differentially expressed miRNAs is found, equal to 85, of which 44 and 41 showed increased and decreased expression levels, respectively. It is important to note that the expression of miRNA in all combination groups demonstrates the independence of expression from the cycle phase and low sensitivity to the specimen source (paired, unpaired).

To validate the sequencing data by the PCR, some DE miRNA was selected based on the number of reads and topological parameters obtained in the analysis of miRNA interactions using the Cytoscape program (the number of interactions with targets, clustering, interaction with

clusters). The specimens used for sequencing and from other paired and unpaired combinations were analyzed (Table 2). The table shows the average number of readings for all specimens as a miRNA representation parameter from the sequencing data, the number of potential target genes (from the list obtained from the miRWalk database), and the nature of the changes observed when comparing the ectopic and eutopic endometrial specimens. For all the studied miRNAs, a similar pattern of expression changes was observed when comparing data obtained by sequencing (NGS) and real-time PCR.

Also, the validation of the results was carried out by evaluating the differential expression of miRNA in endometrial tissue according to the literature. We reviewed 9 studies which investigated miRNA expression using microchip technology [10-12], PCR [13-16] and high-throughput sequencing [17, 18]. Changes in the direction of miRNA expression reported in these studies were similar to our study findings: a decrease in miR-202-3p, miR-424-5p, miR-449-3p, miR-556-3p, miR-15, miR-17-5p, miR-20a, miR-200a, miR-200a, miR-200b, miR-200c, miR-182, miR-141, miR-34c, miR-451 and increase in miR-125a, miR-126 , miR-143, miR-145, miR-29c, miR-21, miR-22, miR-24, miR-99a.

Search for targets of DE miRNA was conducted using miRWalk 2.0 database generating potential miRNA interactions. The search resulted in detecting 5815 potential target genes. Further, the interactions of the selected miRNAs with one or more targets were evaluated. Of particular note is the presence of two miRNAs (hsa-miR-20a-5p and hsa-miR-93-5p), which had more than a thousand potential targets and six miRNAs (hsa-miR-574-5p, hsa-miR-24 -3p hsa-miR-30c-5p hsa-miR-320a hsa-miR-375 hsa-miR-15b-5p) with the number of targets ranged from 400 to 1000. Eight and 28 of the remaining miRNAs had from 200 to 400 and from 20 to 200 potential targets, respectively.

An analysis was also made of the number of miRNAs interacting with each target gene. As a result, target genes were distributed depending on the number of interactions

Figure 1. Analysis of possible interactions of differentially expressed microRNA in all comparison groups

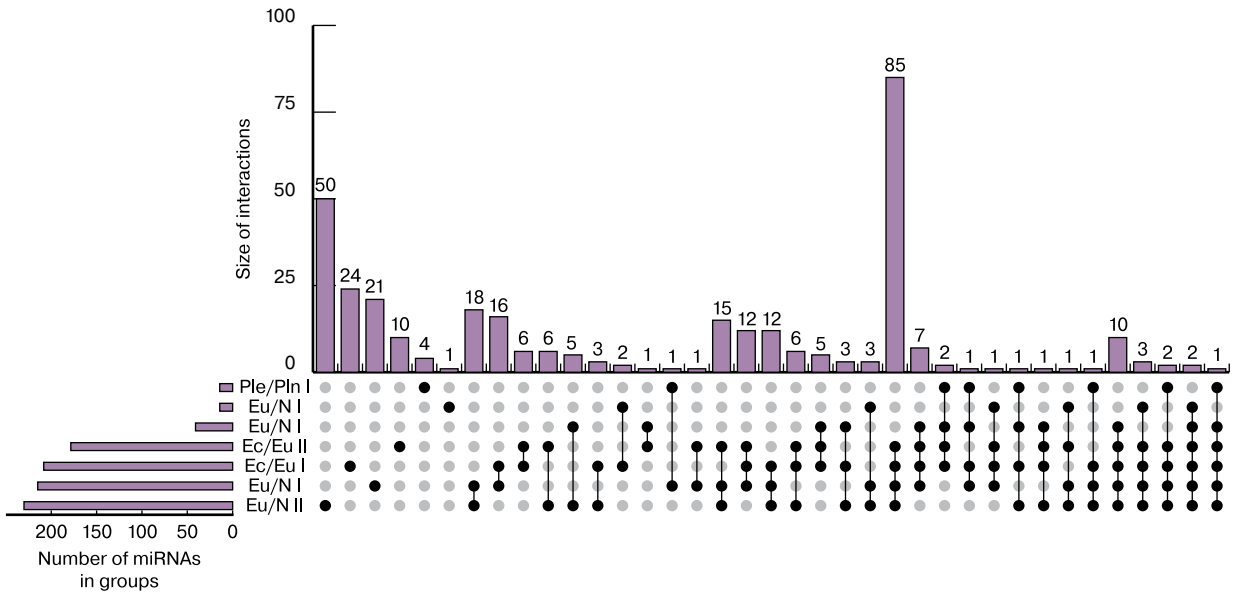


Table 1. Assignment of specimens to groups

Endometriosis		Without pathology	
Proliferative phase	Secretory phase	Proliferative phase	Secretory phase
Eutopic endometrium (Eu I) Ectopic endometrium (Ec I)	Eutopic endometrium (Eu II) Ectopic endometrium (Ec II)	Eutopic endometrium (N I)	Eutopic endometrium (N II)

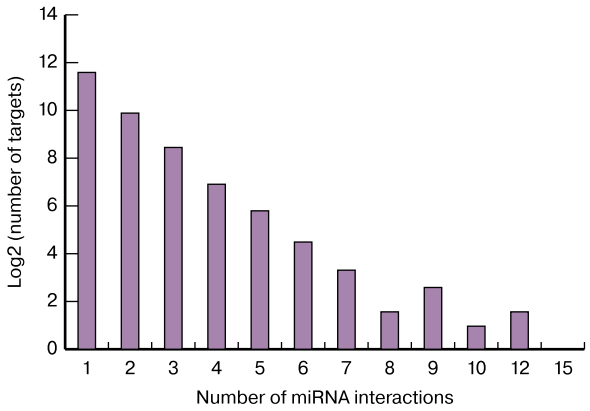
Table 2. Validation of sequencing results by real-time PCR

microRNA	Average number of reads	Number of target genes	Nature of changes	
			NGS	PCR
hsa-miR-let7e-5p	6235	297		
hsa-miR-143-3p	193400	17		
hsa-miR-148a-3p	25830	81	-	-
hsa-miR-20a-5p	213	187	-	-

(Fig. 2). From the list of identified targets, the IGF1R and NUFIP genes had the maximum number of possible interactions with the detected DE miRNAs (12). Target genes, which could have 8 or more interactions, are shown in the table in Fig. 2. Thus, it can be assumed that target genes and miRNAs capable of a maximum number of interactions are potential master regulators of cellular processes associated with the development of endometriosis.

It is important to note that proteins encoded by these target genes are key regulators of the cell's vital activities. Among these target genes, representatives of the families of receptors and growth factors (IGF1R, VEGFA, FRS2), transcriptional regulators (GATA6, MYC, E2F3, NRBP1, ZNF264, ZNF460), regulators of apoptosis (BCL2, XIAP) and cell division (CCND1, CCND2, CDKN1A, CDK6), and miRNA co-effector proteins (AGO1, TNRC6A). It is still an open issue

Figure 2. Distribution of target genes as a function of the number of interactions between them and differentially expressed microRNAs



Number of microRNA	Target genes
12	IGF1R, NUFIP2
11	CCND1, GATA6, MYC
10	BCL2, CCND2, TNRC6A
9	AGO1, CDKN1A, E2F3, KLHL15, VEGFA, XIAP
8	CDK6, CRK, FRS2, MAPK1, NRBP1, SCD, SEC24A, YWHAZ, ZNF264, ZNF460

Table 3. Bioinformatic analysis of the participation of sets of target genes of the detected differentially expressed miRNA in intracellular cascades and the processes regulated by them

Pathway or process type	Pathway or process name	XD	q-val	Ov/S
Cancer	Bladder cancer	1.71	>0.00001	19/38
	Pancreatic cancer	1.18	>0.00001	32/70
	Prostate cancer	1.34	>0.00001	38/84
	Myeloid leukemia	1.37	>0.00001	31/69
	Colorectal cancer	1.33	>0.00001	27/61
	Glioma	1.31	>0.00001	24/60
	Renal carcinoma	0.84	>0.00001	26/68
	Endometrial cancer	1.09	>0.0001	20/50
	Small cell lung cancer	0.89	>0.0001	27/82
	Melanoma	1.14	>0.001	22/62
Intracellular cascades	p53 signaling cascade	1.29	>0.00001	25/62
	Neurotrophic cascade	0.73	>0.00001	39/121
	Insulin cascade	0.60	>0.0001	36/123
	Adipocytokine cascade	0.77	>0.01	18/57
	ErbB signaling cascade	0.54	>0.01	23/84
	mTOR signaling cascade	0.52	0.011	15/49
	TGF-b signaling cascade	0.02	0.024	20/79
	Wnt signaling cascade	0.05	0.041	28/128
Intracellular processes	Ribosome	2.39	>0.00001	40/77
	Protein processing in the endoplasmic reticulum	0.33	>0.0001	36/139
	Reconstruction of the actin cytoskeleton	0.20	>0.01	42/187
Cell adhesion	Thick contacts	0.76	>0.00001	28/70
	Focal adhesion	0.30	>0.01	44/186
Gametogenesis, embryogenesis	Cell cycle	0.64	>0.00001	44/120
	Oocyte meiosis	0.14	>0.05	22/94
	Progeserone-dependent oocyte maturation	0.33	>0.05	19/79
	Formation of dorsoventral axis	0.79	0.07	7/20
Infectious process	Bacterial invasion of epithelial cells	0.56	0.011	18/64
	Epithelial reactions in *Helicobacter pylori* infection	0.15	0.060	15/59
	Pathogenic *E. coli* infection	0.20	0.048	13/47
	Shigellosis	0.47	>0.0001	21/56

whether the DE miRNAs regulate the expression of all possible targets or each miRNA is specific mainly for a single target. Nevertheless, the presence of several miRNAs exhibiting different levels of expression capable of interacting with a particular target may indicate a flexible mechanism for regulating the expression of the respective master regulators in the tissues and cells under investigation.

To determine the possible participation of sets of target genes in intracellular cascades and the processes regulated by them, we conducted a bioinformatic analysis using pathway and process enrichment with the KEGG database by selecting a list of target genes with which two or more miRNAs can interact. The pathways and processes were ranked by several parameters: XD-score (the parameter evaluates the degree of participation of the list of genes in one or another pathway in the network of molecular interactions). Positive and higher values indicate a more likely association of the detected pathways with the selected group of genes), q-value (the probability of an error in calculating XD), overlap/size (the ratio of the number of genes from the list to the number of annotated genes *en route*). A total of 177 pathways and processes were identified from which 34 were selected (Table 3) based on the q values (> 0.1).

Of particular note is the involvement of regulated genes in the processes of proliferation, migration, and invasion (characteristic of carcinogenesis), in key cascades of intracellular signaling, inflammatory processes (as evidenced by enrichment along the routes characteristic of infectious processes). At the same time, the proliferative process is maximally represented by the number of involved genes (25). Taking into account the complex and multilevel regulation of such a fundamental process as cell division, it seems appropriate to identify the groups of genes encoding cell adhesion proteins, receptors and their ligands, signal cascade and cytoskeleton proteins, enzymes and transcription factors. The endometrium periodically regenerates under strict control over its proliferative activity. The development of endometriosis is associated with disrupted regulation of the cell cycle and programmed cell death of the ectopic endometrium [3], which is also evidenced by the change in expression of miRNA, potentially controlling the synthesis of proteins regulating these processes, revealed in this study. It is also noteworthy that for most of the miRNA potential target genes, as determined by our analysis, there is no information on the expression of the proteins encoded by them in endometriotic lesions. The main body of data on the relationship between the protein products of the analyzed genes and the signaling pathways in which they participate has been obtained in cancer and embryonic cell studies.

Conclusion

Therefore, further studies are needed using omix and bioinformatics technologies aimed to identify gene protein products and their participation in signaling pathways and biological processes in cells and tissues of ectopic and eutopic endometrium. Such studies may help to create a relatively complete interactome of signaling pathways involved in the pathogenesis of endometriosis and identify possible therapeutic targets for the treatment of this disease.

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