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Microarray analysis of microRNA deregulation and angiogenesis-related proteins in endometriosis

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ABSTRACT. We examined the aberrant microRNA (miRNA) expression profile responsible for the changes in angiogenesis observed in endometriotic lesions. This study revealed characteristic miRNA expression profiles associated with endometriosis in endometrial tissue and endometriotic lesions from the same patient, and their correlation with the most important angiogenic and fibrinolytic factors. miRNA expression was quantified using a microRNA array and reverse-transcription microRNA polymerase chain reaction. Levels of vascular endothelial growth factor A (VEGFA), epidermal growth factor receptor 2 (EGFR2), phosphatase and tensin homolog (PTEN), and C-X-C chemokine receptor type 4 (CXCR4) were quantified using enzyme-linked immunosorbent assay. The endometrial tissue showed significantly lower levels of miR-200b, miR-15a-5p, miR-19b-1-5p, miR-146a-5p, and miR-200c, and higher levels of miR-16-5p, miR-106b-5p, and miR-145-5p. VEGFA was significantly upregulated,

whereas EGFR2, PTEN, and CXCR4 were markedly downregulated, in the endometriotic tissues compared to that in the normal endometrial tissues. In conclusion, differences in the miRNA levels could modulate the expression of VEGFA, EGFR2, PTEN, and CXCR4, and may play an important role in the pathogenesis of endometriosis. The higher angiogenic and proteolytic activities observed in the eutopic endometrium might facilitate the implantation of endometrial cells at ectopic sites.

Key words: Endometriosis; MicroRNA; VEGFA; EGFR2; PTEN; CXCR4

INTRODUCTION

Endometriosis is a frequent, chronic, benign disease that is characterized by the growth of proliferative endometrial-like tissue outside the uterus. It commonly affects the ovary, uterosacral ligament, recto-uterine pouch, and the uterine serosa, causing pelvic pain and even infertility. Endometriosis affects 5-10% of all women of reproductive age, especially from age 25 to 45, and the prevalence rises to 20-50% in infertile women (Taylor et al., 2009). Although endometriosis is considered benign, it is associated with an increased risk of malignant transformation in approximately 1.0% of affected women, with the involvement of multiple pathways of development (Forte et al., 2014). However, the etiology and pathogenesis of endometriosis are still unclear.

microRNAs (miRNAs) are short, noncoding single-stranded RNA molecules that can regulate gene expression. Since miRNAs were first discovered, they have been identified as significant mediators in cellular physiology and pathology. Recently, studies have demonstrated that aberrant miRNA expression is strongly linked with tumor-related processes in humans (Fassan et al., 2011; Li et al., 2011; Manavalan et al., 2011; Matsuyama et al., 2011). Moreover, miRNAs appear to be potent regulators of gene expression in endometriosis and its associated reproductive disorders, raising the prospect of using miRNAs as biomarkers and therapeutic tools in endometriosis (Teague et al., 2010). miR-191 modulates malignant transformation in endometriosis by regulating TIMP3 (Dong et al., 2015), and inhibits the TNF-α-induced apoptosis of ovarian endometriosis and endometrioid carcinoma cells by targeting DAPK1 (Tian et al., 2015). Aberrantly expressed miR-20a contributes to endometriosis by decreasing NTN4 expression (Zhao et al., 2014). In addition, as with tumor metastasis, endometriosis is an angiogenesis-dependent disease, and endometriotic implants require neovascularization to proliferate and invade ectopic sites within the host (Taylor et al., 2009). Research has shown that endometrial miR-200c targets the expression of ZEBs, VEGFA, FLT1, IKKβ, KLF9, and FBLN5, and then influences cellular angiogenesis, transformation, and inflammation during normal and endometriotic progression (Panda et al., 2012).

The present study was designed to examine the miRNA expression profile in endometriotic tissues compared with normal human endometrium tissues. Reverse-transcription (RT) miRNA polymerase chain reaction (PCR) and miRNA microarray analysis revealed aberrant miRNA expression in the endometriotic tissues. We also determined the characteristic miRNA expression profiles associated with endometriosis in endometrial tissue and endometriotic lesions from the same patient, and their correlation with the most important angiogenic and fibrinolytic factors.

MATERIAL AND METHODS

Endometriotic tissues and normal controls

Endometriotic tissues were obtained from 32 female patients with endometriosis (stages III to IV) diagnosed by laparoscopic findings according to the revised American Fertility Society classification of endometriosis guidelines (American Society for Reproductive Medicine, 1997). Control endometrial tissues were obtained from 19 premenopausal patients who had undergone hysterectomies for subserosal leiomyoma without evidence of endometriosis, as described in a previous study (Adachi et al., 2011). All the women had abstained from medication for at least 3 months prior to donating the tissues. The use of each specimen was formally allowed with written consent from each subject before the study, which was approved by the Ethics Committee of the Second Hospital of Jilin University. All the tissues were homogenized and incubated in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) before storage at -80°C.

miRNA microarray analysis

Total RNAs were extracted from the TRIzol reagent-dissolved tissues using a miRVana miRNA Isolation Kit (Ambion, Austin, TX, USA) according to the manufacturer instructions, and were quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). miRNA samples then were hybridized to Affymetrix GeneChip miRNA arrays using a FlashTag Biotin RNA labeling kit (Genisphere, Hatfield, PA, USA) according to the manufacturer instructions. The microarray message was analyzed using Feature Extraction v.9.5.3.1 (Agilent, Santa Clara, CA, USA).

Real-time PCR analysis for miRNA

Each miRNA sample was first reverse-transcribed with a Multiplex RT pool set, and was then quantified with a mirVana™ reverse transcription-quantitative real-time PCR (RT-qPCR) miRNA detection kit (Thermo Scientific, Rockford, IL, USA) according to the manufacturer instructions. Specific primer pairs for miR-106b-5p, miR-126, miR-142, miR-145-5p, miR-146a-5p, miR-148a, miR-150, miR-15a-5p, miR-16-5p, miR-181a, miR-19b-1-5p, miR-200b, miR-200c, miR-205, miR-423, miR-663, miR-675, and miR-21-5p were utilized for each target miRNA. Real-time PCR was performed on a StepOnePlus™ system (Applied Biosystems, Foster City, CA, USA) using the following conditions: heat activation of reverse DNA polymerase at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. Small nuclear U6 RNA was taken as a control, and the ΔΔCt method was used for relative quantification (Livak and Schmittgen, 2001).

Enzyme-linked immunosorbent assay (ELISA)

The levels of vascular endothelial growth factor A (VEGFA), epidermal growth factor receptor 2 (EGFR2), phosphatase and tensin homolog (PTEN), and C-X-C chemokine receptor type 4 (CXCR4) in the samples from each endometriotic tissue or the control were determined using an ELISA kit (Westang Bio, Shanghai, China), according to the manufacturer instructions. Each homogenized tissue sample was centrifuged for 30 min at 9400 *g* and 4°C, and the supernatant was collected. Serially diluted standard samples or endometriotic tissue

samples (100 μL) were added to the ELISA microplate and incubated at 37°C for 2 h, and then 100 μL diluted antibody against VEGFA, EGFR2, PTEN, or CXCR4 was added to the plate for further incubation at 37°C for 1 h. Finally, the plate was incubated with 100 μL horseradish peroxidase-conjugated secondary antibody for 30 min at 37°C, and with 100 μL substrate in the dark for 15 min. Each well was washed three times with 100 μL phosphate-buffered saline with Tween before each inoculation. The specific binding optical density of each well was determined immediately at 450 nm.

Statistical analysis

All quantitative data are reported as means ± standard error of the mean. Statistical analyses were performed using the Student *t*-test for comparisons of two groups, and P values <0.05 were considered statistically significant.

RESULTS

miRNA profiling by microarray analysis in endometriotic tissues

To screen the specific expression of miRNAs in endometrium and/or endometriotic tissues, miRNA profiling by microarray was performed on five endometriotic tissue samples and five control endometrium specimens. As shown in Table 1, there were 17 miRNAs with microarray signaling. Moreover, the microarray analysis indicated that there were three miRNAs with an upregulation of more than 1.5-fold: 3.154-fold for miR-145-5p, 2.735-fold for miR-106b-5p, and 2.471-fold for miR-16-5p in the endometriotic tissues compared with the normal endometrium tissues. However, miR-200b, miR-15a-5p, miR-19b-1-5p, and miR-146a-5p were downregulated by 1.931-fold, 1.704-fold, 1.536-fold, and 1.522-fold, respectively.

Table 1. miRNA microarray expression in the endometrium of women without endometriosis and in the endometriotic tissues of women with endometriosis.

Gene symbol	Microarray value		Fold-change
	Control (N = 5)	Endometriotic tissues (N = 5)	
miR-106b-5p	0.1116	0.3052	2.735
miR-126	0.3150	0.2668	0.847
miR-142	0.2085	0.2504	1.201
miR-145-5p	0.1203	0.3793	3.154
miR-146a-5p	0.1098	0.0721	0.657
miR-148a	0.0353	0.0312	0.885
miR-150	0.1683	0.1508	0.896
miR-15a-5p	0.1422	0.0835	0.587
miR-16-5p	0.1059	0.2617	2.471
miR-181a	0.1932	0.2376	1.230
miR-19b-1-5p	0.2280	0.1484	0.651
miR-200b	0.1125	0.0583	0.518
miR-200c	0.1510	0.1145	0.763
miR-205	0.2067	0.2286	1.106
miR-423	0.2229	0.2189	0.982
miR-663	0.1374	0.1841	1.340
miR-675	0.1995	0.1939	0.972

miRNA levels in endometrium and endometriotic tissues compared with the normal controls

To confirm the up- or downregulation of the miRNAs mentioned above, real-time

PCR analysis was performed with specific pared primers for each target miRNA in 19 control endometrium specimens and 32 endometriotic specimens. The relative quantitative analysis indicated that four miRNAs in the 32 endometriotic tissues were significantly upregulated (miR-106b-5p, miR-145-5p, miR-16-5p, and miR-21-5p); the relative levels of the four upregulated miRNAs were 2.10 ± 0.24, 3.62 ± 0.43, 1.87 ± 0.21, and 2.84 ± 0.31, respectively ($P < 0.001$ or $P < 0.0001$; Table 2). In contrast, six miRNAs were markedly downregulated in the endometriotic tissues (miR-126, miR-146a-5p, miR-15a-5p, miR-19b-1-5p, miR-200b, and miR-200c) ($P < 0.05$, $P < 0.01$, or $P < 0.001$; Table 2). These results demonstrate the aberrant miRNA expression profile in the endometriotic tissues.

Table 2. miRNA levels in the endometriotic tissues of women with endometriosis relative to those in the endometria of women without endometriosis.

Gene symbol	Endometrium		Significance
	Control (N = 19)	Endometriotic tissues (N = 32)	
miR-106b-5p	1.00 ± 0.11	2.10 ± 0.24	***
miR-126	1.00 ± 0.09	0.76 ± 0.08	*
miR-145-5p	1.00 ± 0.13	3.62 ± 0.43	****
miR-146a-5p	1.00 ± 0.13	0.50 ± 0.06	***
miR-15a-5p	1.00 ± 0.12	0.67 ± 0.06	**
miR-16-5p	1.00 ± 0.08	1.87 ± 0.21	***
miR-19b-1-5p	1.00 ± 0.09	0.73 ± 0.06	**
miR-200b	1.00 ± 0.11	0.62 ± 0.07	**
miR-200c	1.00 ± 0.09	0.81 ± 0.09	*
miR-21-5p	1.00 ± 0.10	2.84 ± 0.31	****

*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Expression of angiogenesis-related proteins

In addition, to explore the possible target markers for miRNAs in the endometriotic tissues, we then screened the homologous sequences in the untranslated region of possible protein markers with the miRNAs mentioned above. The possible target proteins for the 10 markedly regulated miRNAs are listed in Table 3. In particular, angiogenesis-related proteins such as VEGFA, EGFR2, PTEN, and CXCR4 were most frequently recognized as possible targets for these miRNAs in the endometriotic tissues. ELISA was conducted to determine the levels of these protein markers in endometriotic tissues. As shown in Figure 1, the results indicated that VEGFA was significantly upregulated and EGFR2, PTEN, and CXCR4 were markedly downregulated in the endometriotic tissues compared with the normal endometrial tissues.

Table 3. miRNAs expressed in the microarrays and targets of the miRNAs selected for the polymerase chain reaction (PCR) experiments.

miRNA	miRNA sequence (5'-3')	Targets
miR-19b-1-5p	aguuuugcagguuugcauccagc	FGFR2$^{\varphi}$, CCND1, KDR, CASP8, ITGB8
miR-200b-3p	uaauacugccugguaaugauga	ZEB2, EGFR2$^{\Psi}$, BAP1, E2F3, CREB1, VEGFA$^{\varphi}$
miR-16-5p	uagcagcacguaaauauuggcg	VEGFA, EGFR2$^{\Psi}$, BCL2, FGFR1, COX2
miR-126-3p	ucguaccgugaguaauaaugcg	VEGFA, CRK, SPRED1, PIK3R2, IRS1
miR-15a-5p	uagcagcacauaaugguuugug	BCL2$^{\varphi}$, VEGFA, CCND1, CCNE1, MYB, CCND2
miR-106b-5p	uaaagugcugacagugcagau	CDKN1A, VEGFA$^{\varphi}$, E2F1, RB1, PTEN, STAT3
miR-200c-3p	uaauacugccggguaaugaugga	ZEB1, BMI1, VEGFA, FN1, FBLN5
miR-21-5p	uagcuuaucagacugauguuga	PDCD4, PTEN$^{\Psi}$, RPS7, RECK, EFNA3, BMPR2
miR-146a-5p	ugagaacugaauuccauggguu	CXCR4$^{\Psi}$, IRAK1, TRAF6$^{\Psi}$, EGFR2$^{\Psi}$, BRCA1
miR-145-5p	guccaguuuucccaggaaucccu	IRS1, CBFB, FSCN1, KLF4, MUC1, EGFR2$^{\Psi}$

$^{\varphi}$Possible targets for the downregulated miRNAs; $^{\Psi}$Possible targets for the upregulated miRNAs.

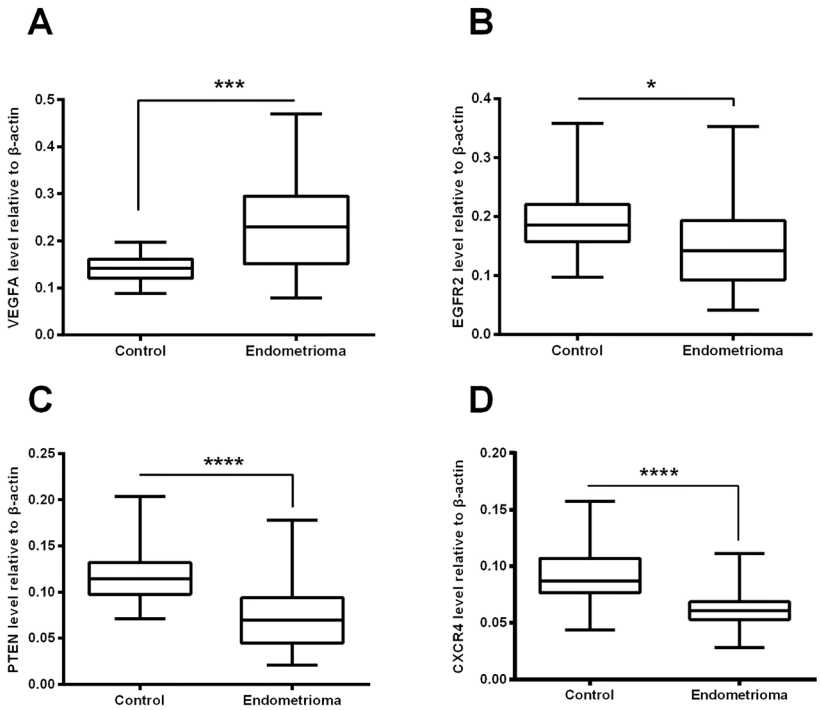

Figure 1. miRNA expression of angiogenesis-related proteins. The levels of vascular endothelial growth factor A (VEGFA), epidermal growth factor receptor 2 (EGFR2), phosphatase and tensin homolog (PTEN), and C-X-C chemokine receptor type 4 (CXCR4) were quantified by enzyme-linked immunosorbent assay (ELISA). **A.-D.** Expression of angiogenesis-related proteins (VEGF-A, EGFR2, PTEN, and CXCR4) compared with the control group and relative to β-actin. Statistical significance shown as *P < 0.05, ***P < 0.001, and ****P < 0.0001.

DISCUSSION

miRNAs are small (approximately 22 nucleotides in length), noncoding single-stranded RNA molecules that can regulate numerous biological processes, including cellular differentiation (Nolte-'t Hoen et al., 2015), proliferation (Zhao et al., 2012), apoptosis (Yeh et al., 2012), angiogenesis (Caporali and Emanueli, 2011), the development of cancer (Liu et al., 2015), and the inhibition of tumor angiogenesis and growth (Luo et al., 2013; Tu et al., 2015). Endometriosis is a common, although mysterious and fascinating, gynecological condition with diverse clinical manifestations and high variability. It has an unpredictable clinical course and results in diminished quality of life (Okeke et al., 2011). Despite extensive research, the pathogenesis of endometriosis remains unclear because it is complex. Recently, several studies have revealed that aberrant miRNA profiles are correlated with endometriosis (Ohlsson Teague et al., 2009; Braza-Boïls et al., 2014; Rekker et al., 2015). In particular, our own and previous studies indicated that members of the miR-200 family (miR-200a, miR-200b, and miR-200c) were downregulated in endometriosis-associated tissues (Ohlsson Teague et al., 2009; Braza-Boïls et al., 2014; Rekker et al., 2015). However, Saare et al. (2014) found that miR-200b was upregulated in such tissues. In addition, miR-20a plays an important role in the pathogenesis of ovarian endometriosis by suppressing NTN4 (Zhao et al., 2014), miR23a

and miR23b are potential biomarkers of ovarian endometriosis (Shen et al., 2013), and miR-196b targets c-myc and Bcl-2 expression, inhibiting proliferation and inducing apoptosis in endometriotic stromal cells (Abe et al., 2013).

The current study was conducted to detect aberrant miRNA expression in endometriotic tissues compared with normal endometrium tissues. Using a microRNA array and RT microRNA PCR, we found four upregulated miRNAs and six downregulated miRNAs in the endometriotic tissues (Tables 1 and 2). Furthermore, we explored the correlation between angiogenesis-related proteins (VEGFA, EGFR2, PTEN, and CXCR4) and endometriotic tissues using ELISA. Compared with the normal controls, the level of VEGFA was significantly increased, and the levels of EGFR2, PTEN, and CXCR4 were reduced in the endometriotic tissues (Figure 1). As listed in Table 3, the (5'-3') sequences of these miRNAs, and their targets in the endometriotic tissue samples were examined by PCR experiments. In particular, VEGF gene polymorphisms are thought to contribute to the risk of endometriosis (Li et al., 2013). We speculated that the deregulated miRNAs might regulate VEGF, thereby contributing to the pathogenesis of endometriosis.

In summary, we found aberrant expression of certain miRNAs in endometriotic tissues compared with those in normal endometrium tissues. We provided evidence that angiogenic and proteolytic activities, and VEGFA, EGFR2, PTEN, and CXCR4 expression levels were modulated by differentially expressed miRNAs in the endometriotic tissue samples. These findings may promote the use of miRNAs as biomarkers and therapeutic tools for endometriosis, and may provide important insight into the pathogenesis of endometriosis.

Conflicts of interest

The authors declare no conflict of interest.

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