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Elevated MMP-9, Survivin, TGB1 and Downregulated Tissue Inhibitor of TIMP-1, Caspase-3 Activities are Independent of the Low Levels miR-183 in Endometriosis

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Purpose: This study aimed to measure the correlation between miR-183 and gene expression that regulates apoptosis and adhesion mechanism that may be linked to the pathogenesis of endometriosis.

Patients and Methods: Forty-four subjects, including 22 control subjects, participated in this study. We collected ectopic endometriosis and endometrial samples. For the control, the sample was taken from endometrial tissue through pipelle biopsy. RNA was extracted from all tissues using RNA mini kit, and the expression was assessed using quantitative-real time PCR. Relative mRNA and miRNA expression were presented using the formula of the Livak method. The data were statistically analyzed using GraphPad Prism 8.

Results: The expression of Caspase-3, Survivin, Integrin β1 (ITGB1), matrix metalloproteinase-9 (MMP-9), and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) (adhesion- and apoptosis-related gene) were calculated using the relative expression method. We found significant differences in Caspase-3, Survivin, ITGB1, MMP-9, and TIMP-1 expression between ectopic endometriosis tissues of women with endometriosis compared to healthy endometrium. MMP-9, Survivin, and ITGB1 was significantly increased in the endometriosis group, while Caspase-3, TIMP-1, and miR-183 were significantly reduced in the endometriosis group. No correlation was found between the expression level of miR-183 and Caspase3, Survivin, ITGB1, and Cadherin in both tissue types.

Conclusion: Despite the difference in expression levels of miR-183 and associated adhesion- and apoptosis-related genes, there was no significant association between miR-183 with specific adhesion and apoptosis genes in endometriosis tissue.

Keywords: endometriosis, miR-183, MMP-9, TIMP1, apoptosis, antiapoptosis

Introduction

Endometriosis is a disease that affects 10–15% of women of reproductive age, with 40–50% of women with infertility experiencing the condition.[1] This disease is characterized by major complaints of pelvic pain, dysmenorrhea, and infertility. It is caused by the presence of ectopic endometrial stromal tissue and glandular tissue outside the uterus. In contrast, some patients are asymptomatic or have only mild symptoms.[2] The diagnosis of endometriosis often involves a thorough medical history, a physical examination, and imaging tests. Additionally, there is a familial tendency, with women having a sevenfold greater risk of developing endometriosis if a first-degree relative is affected.[1]

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The exact pathogenesis mechanism of endometriosis remains unclear, but several theories have been proposed. These include theories related to retrograde menstruation, altered immunity, coelomic metaplasia, metastasis, and possible stem cell and genetic origin.[3] These changes may contribute to the development and progression of endometriosis. Research on factors such as RNAs, proteins, or microRNAs (miRNAs) could provide new insights.[4] miRNAs, which play a role in normal biological processes by targeting specific genes, are also involved in the development and progression of endometriosis when dysregulated.[5] Several miRNAs are also involved in angiogenesis, cell adhesion, cell proliferation, and invasion.[6] Elevated miRNAs also play a pathological role in infertility-associated endometriosis. Elevated miRNA expression can induce inflammation, suppress apoptosis and cell proliferation, cause progesterone resistance and estrogen dominance, impair endometrial receptivity, and inhibit decidualization.[7,8]

Previous studies revealed that miR-183 was one of the most downregulated miRNAs in endometriotic tissue compared to normal tissue. miR-183 contributes to causing apoptosis in endometrial stromal cells and negatively regulates cell invasiveness by targeting specific genes.[9,10] Matrix metalloproteinases (MMPs) play a crucial role in the progression and invasion of endometriosis. In endometriosis, the expression of MMPs, especially MMP-2 and MMP-9, is increased.[11,12] MMPs have specific endogenous inhibitors known as tissue inhibitors of metalloproteinases (TIMPs). Strategies targeting either MMPs or TIMPs might suppress the progression of endometriosis.[13] Concurrently, Integrin B1 (ITGB1) plays a critical role by mediating the adhesion of endometrial cells to extracellular matrices and promoting their invasion into ectopic sites, thereby facilitating the establishment and expansion of endometriosis lesion.[14]

Additionally, endometriosis is characterized by a disruption in the balance between cell proliferation and apoptosis. Specifically, reduced expression of caspase-3, a key executor of apoptosis, has been observed in endometriosis tissues.[15] In contrast, Survivin assists endometriosis cells in evading apoptosis, allowing them to survive and proliferate outside the uterus.[15] Together, the combined effects of increased MMP activity, enhanced ITGB1-mediated cell adhesion and invasion, and altered apoptotic regulation contribute to the progression and persistence of endometriosis lesions.

Thus, this study assessed the correlation between miR-183 and ITGB1 as a potential target gene. We also evaluated the correlation of miR-183 expression with apoptosis and the expression of genes related to apoptosis inhibition (Caspase-3 and Survivin) and between miR-183 expression and the expression of genes related to proliferation and invasion (MMP-9 and TIMP-1), as genes related to apoptosis inhibition, proliferation, and invasion could contribute to endometriosis development.

Materials and Methods

Sample Collection

The study, conducted between January to October 2021, involved the collection of samples from Dr. Cipto Mangunkusumo General Hospital. All experimental procedures, including sample processing and analysis, were performed at the Human Reproduction, Infertility, and Family Planning Research Center. All subjects included in this study provided informed consent for participation. All procedures were conducted in accordance with the ethical standards of the Ethics Committee of the Faculty of Medicine, University of Indonesia - Cipto Mangunkusumo General Hospital (KET-972/UN2.F1/ETIK/PPM.00.02.2020) and in line with the Declaration of Helsinki (1964) and its subsequent amendments. Twenty-two women diagnosed with endometriosis were recruited as the case group of this study. The endometriosis was diagnosed by ultrasound and laparoscopy, and no adenomyosis was found. Endometriosis tissues were collected during surgery and classified based on the American Society for Reproductive Medicine (ASRM) scoring system (stages I, II, III, and IV). The control group (n=22) was selected from women who underwent in vitro fertilization procedures and had no history of gynecological disorders. Endometriosis tissues were collected laparoscopically, while normal endometrial tissues were collected using a sterile Pipelle cannula (Pipelle de Cornier, Laboratoire CCD, Paris, France). None of the groups received hormonal drugs in the last three months.

Total RNA Extraction and Complementary DNA (cDNA) Synthesis

As per the manufacturer's protocol, RNA was extracted from each tissue sample using the Tissue Total RNA Mini Kit (Geneaid, Taipei, Taiwan). Subsequently, complementary DNA (cDNA) was synthesized using ReverTra Ace™ qPCR

RT Master Mix (Toyobo Co., Ltd., Osaka, Japan). The PCR procedure was carried out according to the manufacturer's instructions with the following conditions: 37°C for 15 minutes, 50°C for 5 minutes, and 98°C for 5 minutes.

Quantitative Real-Time PCR for mRNA Expression

The level of mRNA expression of the target genes (caspase-3, survivin, ITGB1, MMP-9, and TIMP-1) was determined using the software platform Primer3Plus (https://www.bioinformatics.nl/cgibin/primer3plus/primer3plus.cgi) (Table 1). Beta-actin (ACTB) was used as a reference gene for normalization. To conduct quantitative real-time PCR (qRT-PCR), the SensiFAST™ SYBR® No-ROX Kit (Meridian Bioscience, Tennessee, United States) was used. The qRT-PCR involved the initial denaturation step was run at 95°C for 2 min and was continued by 40 cycles of amplification at 95°C for 5 seconds, annealing at 55°C for 10 seconds, and extension at 72°C for 15 seconds.

Real-Time Quantitative Reverse Transcription for miR-183

The total RNA from each tissue was reverse transcribed using the TaqMan® MicroRNA Reverse Transcription Kit and the TaqMan™ MicroRNA Assay HSA-mir-183 (Applied Biosystems, Massachusetts, USA). The specific sequence of miR-183 was obtained from the miRbase database (https://www.mirbase.org). miR-183 was quantified using TaqMan® Fast Advanced Master Mix (Applied Biosystems, Massachusetts, United States). The cycling procedure was as follows: polymerase activation at 95°C for 20 seconds, 40 cycles at 95°C for 3 seconds, and annealing at 60°C for 30 seconds. The expression of the target gene miR-183 was normalized to that of U6 snRNA as a reference gene.

Statistical Analysis

All the statistical analyses were conducted using GraphPad Prism 8 (San Diego, CA, USA). The cycle threshold (Ct) values of each mRNA and miR-183 were normalized to a selected reference gene. Relative mRNA and miRNA expression levels were determined using the Livak method with the formula $2^{-\Delta\Delta Ct}$.[14] For normally distributed data, values were presented as mean ± standard error (SE) while non normally distributed data were reported as median and range. The T-independent test was used to determine the differences in caspase-3, Survivin, ITGB1, MMP-9, and TIMP-1 mRNA expression as well as miRNA-183 expression between ectopic endometriotic tissues and normal endometrial tissues. Additionally, the correlation between miRNA-183 and mRNA (caspase-3, Survivin, ITGB1, MMP-9, and TIMP-1) expression in endometriotic tissues was assessed using Spearman correlation. We also used a one-way ANOVA test to compare the phases of endometriosis and gene expression levels. Statistical significance was considered at p values < 0.05.

Results

We obtained 44 control samples from endometriotic and ectopic endometrial tissues. Our analysis revealed a significant age difference between control (36.13 ± 1.39) and endometriosis (33.73 ± 0.771). However, there were no significant

Table 1 Primers Sequence Used for mRNA Expression

Gene	Primer Sequences (5'-3')	Tm (°C)
Caspase3	Forward: CTG ACT GGA AAG CCG AAA CT	55
	Reverse: GTT CCA CTG TCT GTC TCA ATA CC	
Survivin	Forward: GGA TCA CGA GAG AGG AAC ATA AA	54
	Reverse: GGC TCT TTC TCT GTC CAG TTT	
ITG1B	Forward: TAA AAG CAA ATG CCA AAT CA	55
	Reverse: GGC ATT CCT TCC TGT AAA AA	
ACTB	Forward: CAC TCT TCC AGC CTT CCT TC	55
	Reverse: GTA CAG GTC TTT GCG GAT GT	
MMP-9	Forward: GTC TTC CAG TAC CGA GAG AAA G	55
	Reverse: CAG GAT GTC ATA GGT CAC GTA G	
TIMP-1	Forward: ACC ACC TTA TAC CAG CGT TAT G	55
	Reverse: TAT CCG CAG ACA CTC TCC A	

Table 2 Demography of Samples

Variable	Control (n=24)	Endometriosis (n=22)	p value
Age (years)	36.13 ± 1.39	33.73 ± 0.771	0.046*
Weight (kg)	63.93 ± 2.23	61.49 ± 1.85	0.247
Height (m)	1.58 ± 0.12	1.58 ± 0.01	0.989
Body mass index (kg/m^2)	25.63 ± 0.87	25.86 ± 0.73	0.237

Notes: The values were expressed as mean ± SE. T-independent test; *p<0.05.

differences between the two groups based on the baseline data. The demographic data of the patients is presented in Table 2.

The relative expression level of miR-183 was calculated using the relative expression method in both the endometriosis and the control groups. We observed a significant decrease in the expression of miR-183 in the endometriosis group (Figure 1A). Additionally, we conducted qPCR to determine the mRNA expression levels of caspase-3, survivin, ITGB1, MMP-9, and TIMP-1 in the two groups of samples (Figure 1B). Our results showed significant differences in the expression of survivin, ITGB1, and MMP-9 between endometriotic and control tissues ($p<0.05$). Furthermore, the expression levels of caspase-3 and TIMP-1 were lower in the endometriotic tissues compared to the control tissues ($p<0.05$).

We assessed the relative expression of miR-183 and its associated mRNAs in the normal endometrium, eutopic endometrium, and ectopic lesions (Figure 2). The relative expression levels of miR-183, ITGB1, and MMP-9 were not significantly different among the tissue types. Caspase-3 and TIMP-1 expression were significantly greater in normal tissue than in other tissue types ($p<0.05$). Moreover, Survivin expression was higher in the eutopic endometrium.

The study included a correlation analysis to investigate the impact of miR-183 on the mRNA expression of Caspase-3, Survivin, ITG1B, MMP-9, and TIMP-1 in endometriosis tissue (Table 3). The results indicated a significant positive correlation between miR-183 and Caspase-3 ($r=0.478$, $p<0.05$). Additionally, it was found that the expression of Survivin and ITGB1 showed a negative correlation in women with endometriosis. At the same time, MMP-9 and TIMP-1 exhibited a positive correlation with the upregulation of miR-183 in endometriosis tissue.

We analyzed miR-183 expression and its mRNAs based on the menstrual phase. Our statistical analysis revealed that only miR-183 and survivin showed significant differential expression between the secretory and proliferative phases ($p<0.005$). In the secretory phase, miR-183 expression was higher compared to the proliferative phase. On the other hand, survivin expression was higher in the proliferative cells than in the control cells (Table 4).

We also analyzed miR-183 and mRNA expression based on the endometriosis stage. The endometriosis stage was classified according to the ARM scoring system, and the distribution was as follows: stage II (n=5), stage III (n=8), and stage IV (n=9). However, we found no difference in the expression of miR-183, caspase-3, survivin, ITGB1, MMP-9, or TIMP-1 in eutopic or ectopic endometrial tissue based on the stage of endometriosis (Table 5).

Discussion

Endometriosis is a benign condition marked by the presence ectopic endometrial tissue, which might be due to increased angiogenesis or a lack of cells undergoing apoptosis. Some research emphasizes the pivotal role of inflammation in endometriosis, as it influences cell proliferation, apoptosis, and angiogenesis. Understanding these mechanisms could have significant implications for the diagnosis and treatment of endometriosis.[16] Cell death primarily regulates tissue homeostasis, and certain studies indicate that apoptosis increases during the menstrual cycle to maintain cell balance by removing older cells from the endometrium's functional layer.[17] In women with endometriosis, the apoptosis rate of endometrial cells is reduced, suggesting the potential role of apoptosis in disease onset.[18]

Apoptosis, a programmed cell death process, involves Survivin as a key component. In endometriotic tissue, we have observed a significant increase in Survivin expression. Survivin is crucial in inhibiting apoptosis and influencing intrinsic and extrinsic apoptotic pathways.[19] From an immune response perspective, Survivin adjusts the apoptotic threshold in

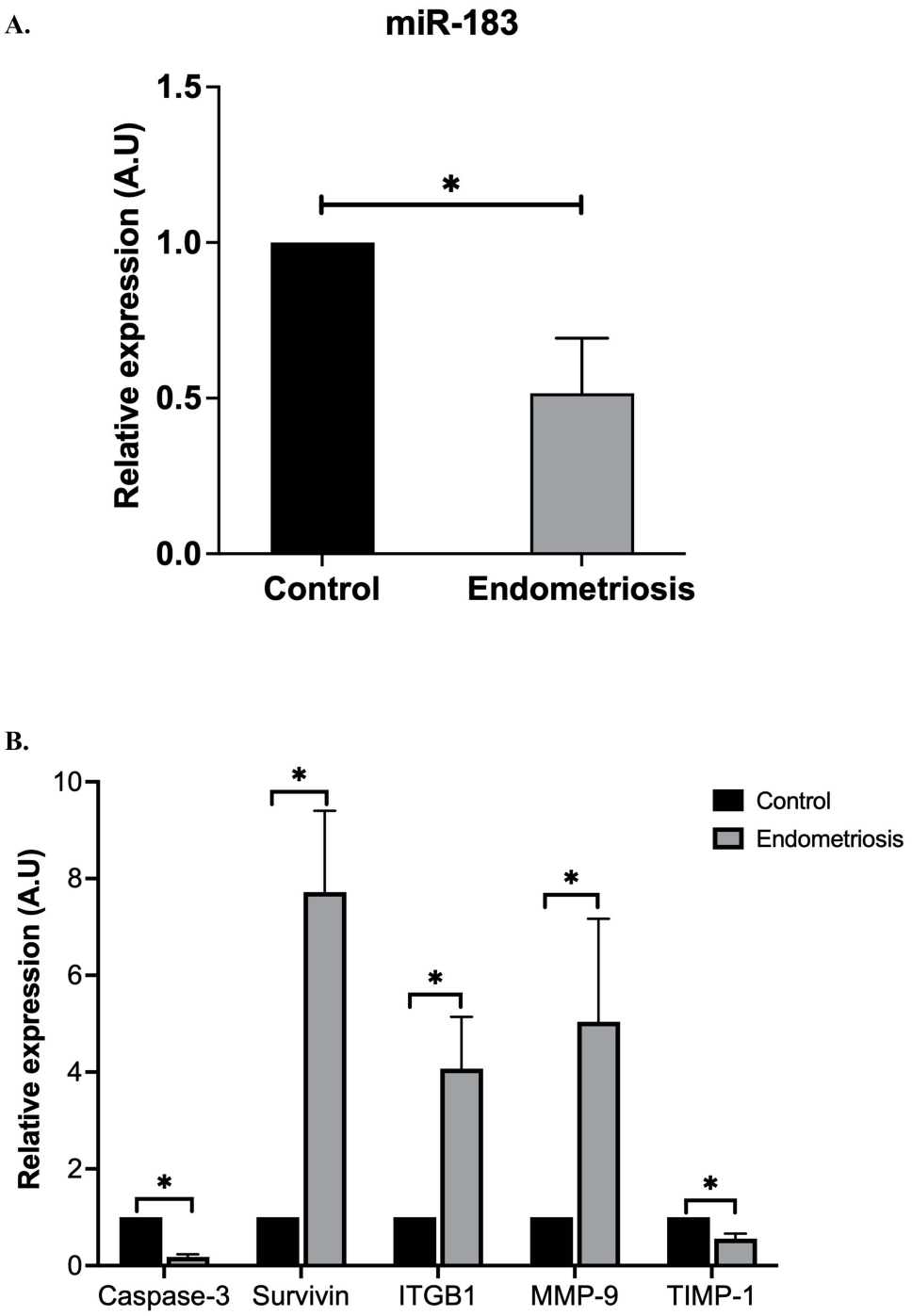

Figure 1 The relative expression (arbitrary unit/A. (U) miRNA and mRNA expression in control and endometriotic tissue samples. The relative expression of miR-183 was greater in the control group (**A**). The relative mRNA expression levels of Survivin, ITGB1, and MMP-9 were greater in endometriotic tissues, while the expression levels of caspase-3 and TIMP-1 were greater in control tissue (**B**). *p<0.05; T-independent test.

neutrophils and is upregulated during inflammatory responses. Furthermore, Survivin contributes to T-cell growth, maturation, activation, and homeostasis. Upon activation of naive T cells in lymphoid organs, their expression of Survivin intensifies, emphasizing the critical role of survivin in initiating immune reactions.[20] The expression of Survivin is essential for normal growth and organ formation. Its role in regulating endothelial cell survival and maintaining vascular integrity is crucial for neurogenesis, angiogenesis, and cardiogenesis. The survival of

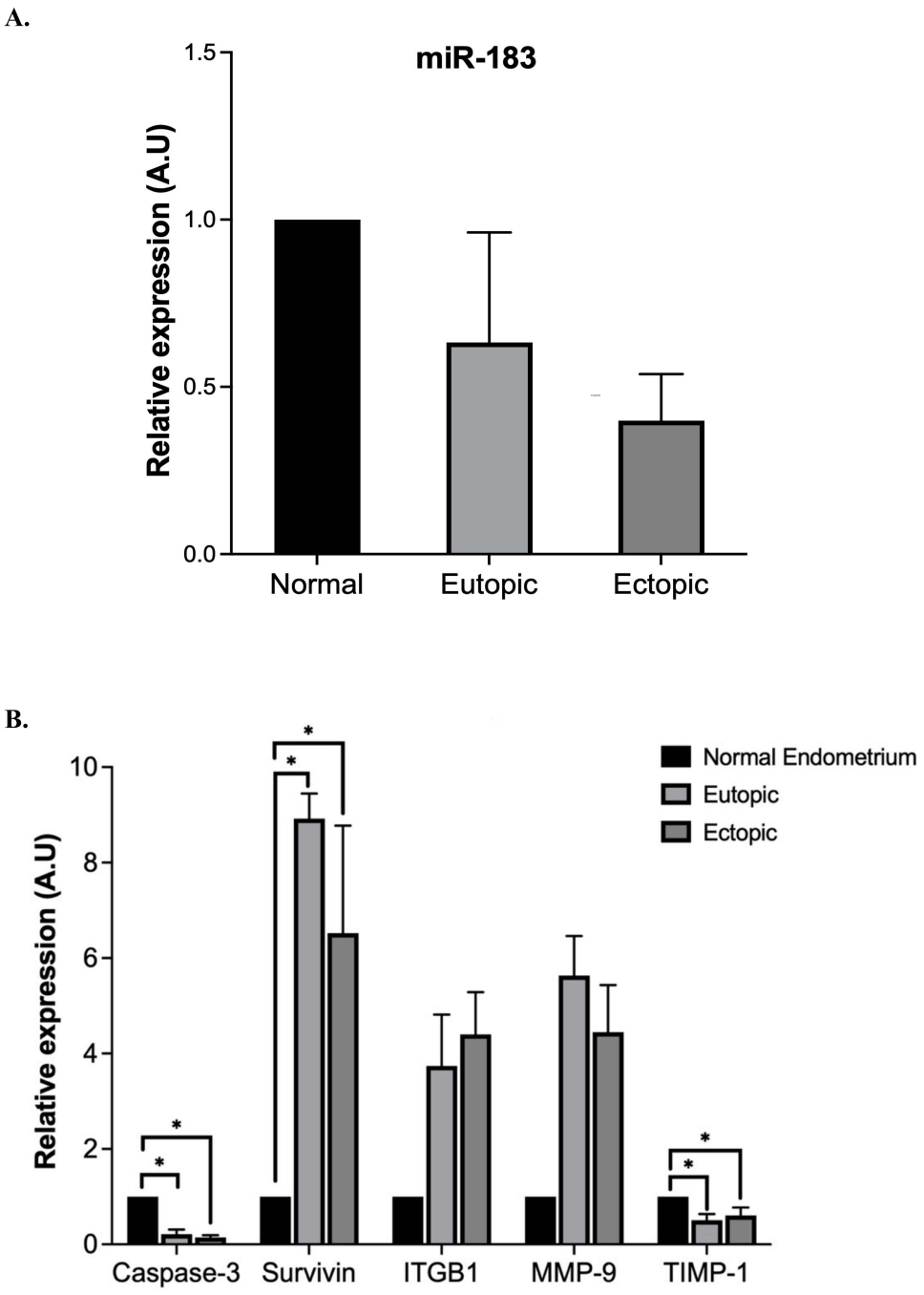

Figure 2 The relative expression of miR-183 and mRNA in the normal endometrium, eutopic endometrium, and ectopic lesion (**A**) No significant difference was found in miR-183, ITGB1, and MMP-9 expression between the type of tissue ($p>0.05$). Caspase-3, Survivin, and TIMP-1 expression significantly differed in normal endometrium, eutopic endometrium, and ectopic lesion (**B**) ($p<0.05$). *$p<0.05$; One-way ANOVA test.

undifferentiated pluripotent stem cells heavily relies on antiapoptotic factors, including Survivin.[21] Survivin is also overexpressed in embryonic stem cells, pluripotent cells, somatic stem cells pluripotent cells, and somatic stem cells.[22]

These observations support various hypotheses regarding the genesis of endometriosis. One such theory suggests the role of endometrial stem cells.[23] Another suggests an increase in transient progenitor cells, where stem cells from the bone marrow or the basal layer of the endometrium can transform into endometrial tissue at different anatomical sites. Additionally, another theory revolves around genetic and epigenetic alterations. According to this theory, regardless of the initial cell's origin, genetic variations or epigenetic modifications combined with changes in the peritoneal

Table 3 Correlation Between miR-183 and Various mRNA in Endometriosis Tissue

Variables	n	Coefficient Correlation (r)	p value
Caspase-3	22	0.478	0.000*
Survivin	22	−0.096	0.130
ITGB1	22	−0.211	0.437
MMP-9	22	0.075	0.546
TIMP-1	22	0.145	0.237

Note: *Spearman correlation test, p<0.05.

Table 4 Gene Expression Based on Menstrual Cycle

Genes Expression	Secretory (n=10)	Proliferative (n=12)	p value
Eutopic endometrium			
miR-183	1.175 ± 0.702	0.181 ± 0.052	0.007*
Caspase-3	0.112 ± 0.031	0.298 ± 0.177	0.142
Survivin	3.945 ± 2.183	13.072 ± 3.951	0.002*
ITGB1	4.645 ± 2.150	2.987 ± 0.883	0.260
MMP-9	8.411 ± 8.012	3.322 ± 2.548	0.143
TIMP-1	0.587 ± 0.197	0.436 ± 0.190	0.674
Ectopic endometrium			
miR-183	0.562 ± 0.269	0.262 ± 0.120	0.106
Caspase-3	0.122 ± 0.063	0.164 ± 0.073	0.781
Survivin	4.302 ± 1.551	8.373 ± 3.924	0.099
ITGB1	5.285 ± 4.078	3.668 ± 0.990	0.122
MMP-9	5.961 ± 3.775	3.184 ± 1.942	0.140
TIMP-1	0.435 ± 0.124	0.752 ± 0.283	0.100

Note: T-independent test *p<0.05.

Table 5 Gene Expression Based on Endometriosis Phases

Genes Expression	Stage II (n=5)	Stage III (n=8)	Stage IV (n=9)	p value
Eutopic endometrium				
miR-183	0.189 ± 0.127	0.463 ± 0.347	1.031 ± 0.748	0.597
Caspase-3	0.512 ± 0.428	0.167 ± 0.039	0.088 ± 0.030	0.248
Survivin	7.002 ± 5.580	8.203 ± 3.211	10.632 ± 4.851	0.853
ITGB1	3.514 ± 1.050	5.271 ± 2.676	2.506 ± 1.042	0.549
MMP-9	0.324 ± 0.253	1.059 ± 0.571	12.653 ± 9.131	0.327
TIMP-1	0.589 ± 0.339	0.629 ± 0.269	0.347 ± 0.150	0.641
Ectopic endometrium				
miR-183	0.477 ± 0.265	0.645 ± 0335	0.136 ± 0.042	0.661
Caspase-3	0.114 ± 0.052	0.101 ± 0.066	0.202 ± 0.099	0.558
Survivin	6.347 ± 5.588	8.800 ± 5.043	4.596 ± 1.733	0.306
ITGB1	4.038 ± 2.207	7.774 ± 4.938	1.609 ± 0.464	0.770
MMP-9	1.556 ± 0.994	3.836 ± 2.904	6.593 ± 4.160	0.661
TIMP-1	0.429 ± 0.270	0.466 ± 0.089	0.833 ± 0.370	0.380

Note: One-way ANOVA test.

environment, such as inflammation, immunological and oxidative stress, could trigger the disease through diverse manifestations, for example, ovarian, peritoneal, deep, or extra pelvic lesions. These theories could provide insight into the complexities of this disease.[24]

Our study found that caspase-3, a pro-apoptotic factor, showed lower gene expression levels in endometriotic tissue compared to control tissue. The development of endometriosis may involve both extrinsic and intrinsic apoptotic pathways. The FasL/Fas interaction in the extrinsic pathway triggers a sequence of activations involving initiator caspases (caspase-8 and −9) and effector caspases (caspase-3, −6, and −7), leading to apoptosis.[25] Caspase-3, a member of the cysteine protease family, plays a central role in driving apoptosis. Caspase-3, a cysteine protease family member, plays a central role in driving apoptosis.[26] Due to its crucial role in apoptosis, we examined the gene expression caspase-3 in endometriotic tissue and observed a significant reduction in its expression. Similarly, a study by Wei et al[27] also reported markedly decreased expression of the caspase-3 protein in both ectopic and eutopic endometrial tissues of endometriosis patients compared to endometrial tissues from the control group.

Matrix metalloproteinases (MMPs) and their inhibitors, termed tissue inhibitors of matrix metalloproteinases (TIMPs), serve as natural regulators of the extracellular matrix remodeling observed throughout the endometrial cycle. The eutopic endometrium produces TIMP-1. However, patients with endometriosis may show low expression of TIMP-1 in their peritoneal fluid (PF) and serum. An imbalance between MMPs and TIMPs or the presence of excess ectopic endometrial tissue can contribute to increased aggressiveness.[28] Our research revealed a notable decrease in TIMP-1 expression in patients with endometriosis, which was contrasted by a marked increase in MMP-9 expression.

Multiple factors contribute to the development and progression of endometriosis, including immune and hormonal elements. Research suggests that miRNAs may also play a role in the pathophysiology of endometriosis due to their involvement in processes such as apoptosis and cell proliferation.[29] For instance, miR-183, a member of the miR/182/183/96 family located at the 7q31-34 locus, has been found to be overexpressed in numerous tumors and associated cell lines, hinting at its potential role in tumor formation.[30] Additionally, MMP-9, which is involved in breaking down the extracellular matrix, has been linked to the invasiveness of various diseases.[31]

Various transcription factors significantly influence miR-183 expression across different conditions The data indicate that miR-183 levels are increased in 18 types of cancer, including prostate, ovarian, and breast cancer. In contrast, its levels are decreased in 6 types of tumors, such as osteosarcoma and melanoma. When miR-183 is upregulated, it often assumes an oncogenic role, whereas when it is downregulated, it tends to exert tumor-suppressive effects. However, its role is controversial in 7 specific cancers, namely, hepatocellular cancer (HCC) and endometrial cancer. Several studies have reported elevated miR-183 levels in tissues or cell lines, while others suggest the opposite. This highlights the dual roles of miR-183 in tumor progression.[32] In the context of HCC, a study by Li et al[10] revealed that out of 25 samples, 17 exhibited a significant increase (ranging from twofold to 367-fold) in miR-183 expression compared to that in paired noncancerous liver tissues.

Various studies have shown a strong association between miR-183 and tumor invasion and metastasis.[33] miR-183 influences approximately 45 tumor-associated genes that are integral to the adhesion, migration, and invasion of diverse cancer cells. Among these genes, programmed cell death factor 4 (PDCD4), early growth response 1 (EGR1), and integrin beta 1 (ITGB1) are the most researched. ITGB1, in particular, has a pivotal role in tumor migration and invasion.[34] miR-183 can function as an antioncogene by targeting ITGB1. Increased expression of ITGB1 and activation of ITGB1-related signaling mechanisms are observed in many human tumors. miR-183 markedly decreased ITGB1 expression, reducing cell migration and /adhesion However, as revealed by statistical analysis, the lack of a direct correlation between miR-183 and ITGB1 highlights the need for further research in this area. In addition, annexins, which are involved in critical cellular processes such as membrane trafficking, inflammatory modulation, and cell signaling, may also contribute to the mechanisms influenced by miR-183. By investigating how annexins interact with miR-183-regulated pathways, particularly those governing tumor-like behaviors such as migration and adhesion, we can achieve a more comprehensive understanding of endometriosis pathophysiology.[35]

Conclusion

The expression levels of miR-183 and its associated adhesion- and apoptosis-related genes varied. However, we found no significant association between miR-183 and specific adhesion- and apoptosis-related genes in endometriotic tissue. The lack of correlation between miR-183 and various mRNAs in this study may be due to the diverse roles of miR-183 in cell migration and invasion across different tissues. This diversity in miR-183's roles in cell migration and invasion not only highlights the complexity and depth of our research, but also underscores the urgent need for further investigation. We hypothesize that this outcome occurs because a single miRNA might target hundreds of genes. Using a miRNA target gene prediction network, we determined that miR-183 potentially targets oncogenes, tumor suppressor genes, cell signaling molecules, cell cycle regulators, and genes and molecules linked to invasion and metastasis. As for its function, miR-183 can act as a tumor suppressor by downregulating oncogenes and inhibiting cell migration and invasion. Conversely, it can promote oncogenic properties by downregulating tumor suppressor genes and encouraging cell movement and invasion. These findings indicate that miR-183 functions differently under various conditions, and its underlying molecular mechanisms are complex and multifaceted, warranting further investigation.

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Disclosure

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