miR-424-5p combined with miR-17-5p has high diagnostic efficacy for endometriosis

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Abstract

**Purpose** Endometriosis (EMT) is a chronic benign disease with high prevalence. This study investigated the diagnostic value of serum miR-17-5p, miR-424-5p, and their combined expressions for EMT.

**Methods** Total 80 EMT patients of reproductive age who underwent laparoscopy or laparotomy and were confirmed by pathological examination were included as the study subjects, and another 80 healthy women of reproductive age receiving gynecological examination and ultrasonography with no pelvic abnormalities were selected as the control group. The whole blood samples of enrolled subjects were collected and clinical characteristics were recorded. The miR-17-5p, miR-424-5p, VEGFA, IL-4, and IL-6 levels in the serum were measured. ROC curve was used to evaluate the diagnostic efficacy of miR-17-5p and miR-424-5p expressions for EMT. Pearson correlation was performed to analyze the correlation of miR-17-5p and miR-424-5p with clinical indexes in EMT patients.

**Results** miR-17-5p and miR-424-5p were downregulated in EMT patients. For diagnosing EMT, the AUC of miR-17-5p was 0.865 and cutoff value was 0.890 (91.3% sensitivity and 85% specificity), the AUC of miR-424-5p was 0.737, and cutoff value was 0.915 (98.8% sensitivity and 61.2% specificity), and the AUC of miR-424-5p combined with miR-17-5p was 0.938 and cutoff value was 2.205 (93.8% sensitivity and 88.7% specificity), with the diagnostic efficacy higher than miR-424-5p or miR-17-5p alone. miR-17-5p and miR-424-5p expressions were negatively correlated with dysmenorrhea, infertility, pelvic pain, and rASRM stage, but not with age, BMI, menstrual disorder, and nulliparity. VEGFA, IL-4, IL-6, and CA-125 were increased in EMT patients and were inversely associated with miR-17-5p and miR-424-5p.

**Conclusion** miR-424-5p combined with miR-17-5p has high diagnostic efficacy for EMT.

**Keywords** Endometriosis · miR-424-5p · miR-17-5p · Combined diagnosis · Receiver operating characteristic curve · VEGFA · Inflammatory cytokines

**Introduction**

Endometriosis (EMT) is a commonly diagnosed, chronic, and hormone-dependent gynecological disease featured by the endometrial stroma and glands in the outside of the uterine cavity [1, 2]. Since various inflammatory biomarkers such as interleukin (IL)-4, IL-6, IL-8, C-reactive protein, and tumor necrosis factor-α are increased in the serum of women with EMT, EMT is also regarded as an inflammatory disorder [3]. EMT affects 6–10% of reproductive women and 1–4% of postmenopausal women [4]. About 3.8–37% among the EMT patients will suffer from bowel involvement, mainly the rectosigmoid colon [5]. Chronic pelvic pain, dysmenorrhea, dyspareunia, infertility, deep dyspareunia, cyclical intestinal complaints, and fatigue/weariness are common symptoms [6, 7]. Advanced EMT may cause gynecological
malignancies, including ovarian cancer [8]. Diagnosis is a great challenge in EMT, whose diagnostic time is prolonged by an average of 6–12 years due to the lack of specific symptoms and noninvasive diagnostic tests [9]. EMT not only affects the physical and mental health of patients and their spouses but also brings great economic and medical burdens to society [10]. Therefore, it is extremely important to find effective diagnostic markers with strong specificity and high sensitivity for EMT.

MicroRNAs (miRNAs) are small, single-stranded, and non-coding RNAs, with a length of 20–24 nucleotides, which mediate the level of messenger RNA in numerous eukaryotic lineages [11, 12]. Abnormal miRNA expression is linked with various human disorders, including cardiovascular diseases, cancer, inflammatory diseases, and gynecologic pathology [13]. The differentially expressed miRNAs are key players in the occurrence of EMT and the associated infertility, with miR-17-5p being downregulated in the plasma from women with EMT [14]. The expression of miR-424-5p is reduced in the endometriotic mesenchymal cells, indicating its potential regulatory effect on EMT [15]. Since the sensitivity and specificity of a single miRNA as a biomarker to distinguish EMT patients from healthy women are relatively low [16], a combined diagnosis of miR-17-5p and miR-424-5p for EMT was investigated in this study.

Angiogenesis is an important step in the development of endometriotic lesions, hence EMT is also regarded as an angiogenic disease [17]. Vascular endothelial growth factor A (VEGFA) is described as an essential mediator of angiogenesis, which refers to the occurrence of new vessels from pre-existent ones [18]. VEGFA plays a pivotal role in the pathogenesis of EMT [19]. miR-17-5p can promote angiogenesis and inversely modulates the expression of VEGFA in EMT [20, 21]. miR-424-5p negatively targets VEGFA and lowers the angiogenic activity of VEGFA protein [22]. However, there is no report about the diagnostic value of miR-17-5p combined with miR-424-5p for EMT. This study therein explored their combined diagnosis of EMT to improve diagnostic accuracy and efficacy.

**Methods**

**Ethics statement**

This study was approved by the academic ethics committee of Hunan Province Maternal and Child Health care Hospital. All participants were fully informed and voluntarily signed the informed consent before sampling.

**Study subjects**

This study included 80 patients with EMT of reproductive age who underwent laparoscopy or laparotomy in the Department of Gynecology in Hunan Province Maternal and Child Health care Hospital from January 2019 to December 2020 and were confirmed by pathological examination as the experimental group (EMT group). Another 80 women of reproductive age with the healthy physical examination at the same period who underwent gynecological examination and ultrasonography with no pelvic abnormalities were selected as the control group. The fasting venous blood was collected from EMT patients before surgery and health controls, and the clinical characteristics were recorded, including age, body mass index (BMI), dysmenorrhea, menstrual disorder, infertility, parity, and pelvic pain. The diagnostic criteria referred to the Specifications for the Diagnosis and Treatment of Endometriosis [Chinese Journal of Obstetrics and Gynecology, 2015(3)] issued by the Obstetrics and Gynecology Branch of Chinese Medical Association. Endometriosis was classified as mild (stages I and II), moderate to severe (stages III and IV) according to the American Society for Reproductive Medicine (ASRM) staging criteria.

Inclusion criteria included: EMT patients confirmed by surgery and pathology; women of reproductive age, aged 24–46 years; first-time admissions without a history of surgery; not using hormonal drugs within 3 months and without other inflammatory diseases.

Exclusion criteria referred to the women: with adenomyosis, endometrial carcinoma, endometrial hyperplasia or polyps, chronic or acute inflammation, infectious diseases, malignancy, autoimmune diseases, and cardiovascular diseases.

**Reverse transcription quantitative polymerase chain reaction (RT-qPCR)**

RT-qPCR was used to determine the expression of miR-17-5p and miR-424-5p in the serum of the study population. The whole blood sample was placed in the 1.5 mL microcentrifuge tube without RNase, centrifuged at 3000 rpm for 20 min, and then the supernatant was stored in a 1.5 mL microcentrifuge tube without RNase and placed in a freezer at – 80 °C. Total RNA was extracted from samples according to the instructions of the TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was synthesized using the PrimeScript RT reagent kit (Takara, Tokyo, Japan). RT-qPCR was performed using the SYBR PCR Green Master Mix kit (Qiagen, Hilden, Germany) under the following reaction conditions: at
95 °C for 10 min, and then 40 cycles of 95 °C for 15 s, 55 °C for 30 s, and 70 °C for 30 s. U6 was used as an internal reference. The relative levels of miR-17-5p and miR-424-5p after normalization to U6 were calculated using the 2−ΔΔCt method. The primer sequences are shown in Table 1.

**Enzyme-linked immunosorbent assay (ELISA)**

The corresponding Quantikine ELISA kits of VEGFA (ab119566, Abcam, Cambridge, UK), IL-4 (ab215089, Abcam), IL-6 (EK0410, Boster, Pleasanton, CA, USA), and cancer antigen 125 (CA-125, XY-EH0361, X–Y Biotechnology, Shanghai, China) were employed to quantify their concentrations in serum samples.

**Dual-luciferase assay**

The binding sites of miR-17-5p or miR-424-5p with VEGFA were predicted by the online database (http://www.targetscan.org/vert_71/). The wild type (WT) or mutant (MUT) of VEGFA 3’-UTR were constructed and cloned into the pMIR vector (Ribobio, Guangzhou, China). HEK293T cells (CL-0005, Procell, Wuhan, China) were seeded into 48-well plates, and the constructed luciferase reporter vectors were co-transfected with miR-424-5p mimics, miR-17-5p mimics, or mimics NC using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 h of transfection, cells were collected and detected using the dual-luciferase assay kit (Promega, Madison, WI, USA).

**Statistical analysis**

SPSS 21.0 statistical software (IBM Corp. Armonk, NY, USA) and GraphPad Prism 8.0.1 software (GraphPad Software Inc., San Diego, CA, USA) were employed for the statistical analysis and mapping. Shapiro–Wilk test was used to verify the normal distribution. Measurement data of normal distribution were expressed as mean ± standard deviation (SD) and independent sample t test was adopted for comparisons between groups. Measurement data of non-normal distribution were presented as quartile and Mann–Whitney U test was used for comparisons between groups. The enumeration data were exhibited as cases and percentages, and Chi-square test was performed for comparisons between groups. Receiver operating characteristic (ROC) curve was used to evaluate the diagnostic efficacy of miRNAs and obtain the cutoff values. MedCalc was introduced to compare and analyze the differences of the area under the curve (AUC). The correlation between the expressions of miRNAs and indexes was analyzed using Pearson correlation.

**Results**

**Comparative analysis of general data and characteristics of participants**

A total of 160 subjects were included in this study, including 80 healthy controls and 80 EMT patients. We compared and analyzed the general data and clinicopathological characteristics of EMT patients and healthy controls. The results showed no statistical differences in age, BMI, menstrual disorder, and nulliparity between EMT patients and healthy controls (all \( P > 0.05 \)), while the proportions of dysmenorrhea, infertility, and pelvic pain were remarkably increased in EMT patients (\( P < 0.05 \)) (Table 2).

|                    | Control (\(N=80\)) | EMT (\(N=80\)) | \(P\)  |
|--------------------|---------------------|-----------------|-------|
| Age (years)        | 32.40 ± 2.67        | 31.50 ± 4.73    | 0.140 |
| BMI (kg/m²)        | 26.76 ± 6.25        | 27.39 ± 5.63    | 0.504 |
| Dysmenorrhea       | 8 (10.00%)          | 36 (45.00%)     | < 0.001 |
| Menstrual disorder | 16 (20.00%)         | 21 (26.25%)     | 0.454 |
| Infertility        | 12 (15.00%)         | 53 (66.25%)     | < 0.001 |
| Nulliparity        | 39 (48.75%)         | 42 (52.50%)     | 0.752 |
| Pelvic pain        | 25 (31.25%)         | 47 (58.75%)     | 0.001 |
| rASRM stage        |                     |                 |       |
| Stage I/II         | –                   | 32 (40.00%)     | –     |
| Stage III/IV       | –                   | 48 (60.00%)     | –     |

**EMT endometriosis, BMI body mass index, rASRM revised American Society for Reproductive Medicine**

**Table 1** Primer sequences

| Gene          | Forward 5’–3’ | Reverse 5’–3’ |
|---------------|---------------|---------------|
| miR-17-5p     | GCCGCCCAAAAGTGCTTACAGTG | CAGCCACAAAAGAGCACACAAT |
| miR-424-5p    | GGCTAGTCAGCAGCAATTCATGT | GTGCAAGGGTGTCGAGGT |
| U6            | GCTTCGCGACGACATAATCTAAAAT | CGCTTCAGAATTTGCGTGTCAT |

\(miR-17-5p\) microRNA-17-5p, \(miR-424-5p\) microRNA-424-5p
miR-17-5p and miR-424-5p were downregulated in the serum of EMT patients

RT-qPCR results showed that the miR-17-5p and miR-424-5p levels in EMT patients were markedly diminished (Fig. 1A, B, all \( P < 0.05 \)).

miR-17-5p combined with miR-424-5p had a high diagnostic value for EMT

To further explore the clinical diagnostic efficacy of miR-17-5p and miR-424-5p in EMT, the ROC curve was plotted to distinguish EMT patients from healthy controls by the expression of miR-17-5p, miR-424-5p, or miR-17-5p combined with miR-424-5p. The results showed that for the diagnosis of EMT, the AUC of miR-17-5p was 0.865 and the cutoff value was 0.890 (91.3% sensitivity and 85% specificity) (Fig. 2A); the AUC of miR-424-5p was 0.737 and the cutoff value was 0.915 (98.8% sensitivity and 61.2% specificity) (Fig. 2B). The diagnostic efficacy of miR-17-5p combined with miR-424-5p for EMT was further evaluated, and the results indicated an AUC of 0.939 and a cutoff value of 2.205 (93.8% sensitivity and 88.7% specificity) for combined diagnosis (Fig. 2C). MedCalc was employed to compare and analyze the AUC, and the results illustrated that miR-17-5p combined with miR-424-5p had prominently higher diagnostic efficacy than miR-424-5p or miR-17-5p alone (Fig. 2D) (all \( P < 0.05 \)). Altogether, serum miR-17-5p and miR-424-5p could be used as biomarkers for EMT diagnosis, and their combination had a high diagnostic efficacy for EMT.

Correlation analysis of serum miR-17-5p and miR-424-5p expressions with clinical indexes in EMT patients

To investigate the correlation of serum miR-17-5p and miR-424-5p levels with clinical indicators of EMT patients, Pearson correlation analysis was performed and it demonstrated that the serum expressions of miR-17-5p and miR-424-5p were significantly inversely correlated with dysmenorrhea, infertility, pelvic pain, and revised ASRM (rASRM) stage in EMT patients (all \( P < 0.05 \)), but not associated with age, BMI, menstrual disorder, and nulliparity (Table 3).

miR-17-5p and miR-424-5p were negatively correlated with VEGFA, IL-4, IL-6, and CA-125 in the serum of EMT patients

EMT is an inflammatory disease and the levels of inflammatory cytokines IL-4 and IL-6 are increased in the serum and tissues of EMT patients [23]. CA-125 is identified as a biomarker for EMT detection, late recurrence, and treatment [24, 25]. Therefore, we subsequently validated the correlation of miR-17-5p and miR-424-5p with VEGFA, IL-4, IL-6, and CA-125 in the serum of EMT patients. First, the expressions of VEGFA, IL-4, IL-6, and CA-125 in the serum were measured using ELISA, and the results revealed remarkably increased levels of VEGFA, IL-4, IL-6, and CA-125 in the serum of EMT patients compared with healthy controls (all \( P < 0.05 \)) (Fig. 3A). Next, the correlations of miR-17-5p and miR-424-5p expressions with VEGFA, IL-4, IL-6, and CA-125 concentrations were assessed. Pearson correlation scatter plot showed a negative correlation of miR-17-5p expression...
VEGFA (P < 0.0001; r = −0.8853), IL-4 (P < 0.0001; r = −0.6552), IL-6 (P < 0.0001; r = −0.7438), and CA-125 (P < 0.0001; r = −0.8405) concentrations (Fig. 3B). Similarly, miR-424-5p expression was negatively related with VEGFA (P < 0.0001; r = −0.8314), IL-4 (P < 0.0001; r = −0.6167), IL-6 (P < 0.0001; r = −0.6870), and CA-125 (P < 0.0001; r = −0.8531) concentrations (Fig. 3C). The binding sites of miR-17-5p or miR-424-5p with VEGFA were predicted, respectively, through the online database (http://www.targetscan.org/vert_71/). The constructed VEGFA 3′-UTR vectors (WT or MUT) were co-transfected with miRNA or NC to verify the targeted inhibitory relationship of miR-17-5p and miR-424-5p with VEGFA. The dual-luciferase assay suggested that the cell luciferase activities of miR-17-5p mimics or miR-424-5p mimics co-transfection with VEGFA-WT were markedly lowered relative to the mimics NC group (all P < 0.05), while the cell luciferase activities of co-transfection with VEGFA-MUT expressed no apparent change (Fig. 3D–E), confirming the targeted relationship of miR-17-5p and miR-424-5p with VEGFA. Collectively, both miR-17-5p and miR-424-5p might play a regulatory role in EMT by targeting VEGFA.

Table 3 Correlation analysis of serum miR-17-5p and miR-424-5p expression with clinical indexes in EMT patients

| EMT (N=80) | miR-17-5p | miR-424-5p |
|-----------|-----------|-----------|
| Age (years) | 31.5 ± 4.73 | 0.086 | 0.447 | 0.124 | 0.274 |
| BMI (kg/m²) | 27.39 ± 5.63 | 0.045 | 0.692 | 0.015 | 0.892 |
| Dysmenorrhea | 36 (45.00%) | −0.334 | 0.003 | 0.295 | 0.008 |
| Menstrual disorder | 21 (26.25%) | 0.068 | 0.552 | 0.080 | 0.479 |
| Infertility | 53 (66.25%) | −0.301 | 0.007 | −0.271 | 0.015 |
| Nulliparity | 42 (52.50%) | 0.108 | 0.340 | 0.119 | 0.292 |
| Pelvic pain | 47 (58.75%) | −0.541 | <0.001 | −0.534 | <0.001 |
| rASRM stage | | | | | |
| Stage I/II | 32 (40.00%) | | | | |
| Stage III/IV | 48 (60.00%) | −0.601 | <0.001 | −0.585 | <0.001 |

miR-17-5p microRNA-17-5p, miR-424-5p microRNA-424-5p, EMT endometriosis, BMI body mass index, rASRM revised American Society for Reproductive Medicine
**Discussion**

EMT emerges as a common gynecologic disease with complicated pathogenesis, which mainly impacts women of reproductive age [26], and influences female fertility, life quality and long-term health [27]. miRNAs, which are normally found in exosomes, are implicated in the nosogenesis of EMT and can be proposed as potential markers in EMT [10, 28]. This study evaluated the diagnostic efficacy of miR-17-5p and miR-424-5p for EMT.

**Fig. 3** miR-17-5p and miR-424-5p were negatively correlated with VEGFA, IL-4, IL-6, and CA-125 in the serum of EMT patients. A The expressions of serum VEGFA, IL-4, IL-6, and CA-125 were measured using ELISA; B The correlation of miR-17-5p with serum VEGFA, IL-4, IL-6, and CA-125 in EMT patients was assessed by Person analysis; C The association of miR-424-5p with serum VEGFA, IL-4, IL-6, and CA-125 in EMT patients was evaluated by Person analysis; D The targeted inhibitory relationship of miR-17-5p and VEGFA was verified using the dual-luciferase reporter assay; E The targeted relationship of miR-424-5p and VEGFA was elucidated by the dual-luciferase assay. Independent sample t test was performed for comparisons between panels (D, E). **P < 0.01, ***P < 0.001
Aberrant levels of miRNAs are implicated in the occurrence and progression of EMT [29] and proposed as biomarkers for early diagnosis and prediction of EMT [30]. First, the RT-qPCR revealed decreased expressions of miR-17-5p and miR-424-5p in women with EMT. Consistently, increasing reports unveil that miR-17-5p is weakly expressed in EMT patients, indicating its potential utility in the clinical diagnosis of EMT [31, 32]. miR-424-5p is involved in the development of EMT and is lowered in EMT lesions [15, 33]. Thus, weak expression of miR-17-5p and miR-424-5p in EMT patients may be potential biomarkers.

Next, we further investigated the diagnostic value of miR-17-5p and miR-424-5p for EMT. Our results revealed that miR-17-5p and miR-424-5p expression both had diagnostic values for EMT, and the diagnostic value of miR-17-5p combined with miR-424-5p for EMT was higher. Previous studies also elicit that the combination of several different miRNAs, with improved sensitivity and specificity, has elevated diagnostic accuracy relative to individual miRNA [16, 34]. Altogether, miR-17-5p and miR-424-5p both could be considered biomarkers for EMT, and their combination had amplified this diagnostic efficacy.

Infertility, chronic pelvic pain, and dysmenorrhea are primary symptoms of EMT [35]. Initially, the analysis of general data and clinic characteristics of EMT patients and healthy women unraveled the elevated proportions of dysmenorrhea, infertility, and pelvic pain in EMT patients. EMT individuals usually have a prolonged history of dysmenorrhea [36], and 30–50% of women with EMT also suffer from pelvic pain and infertility [37]. These clinical parameters could assist the diagnosis of EMT. Moreover, Pearson correlation analysis demonstrated a remarkable inverse correlation between miR-17-5p and miR-424-5p expression with dysmenorrhea, infertility, pelvic pain, and rASRM stage in women with EMT. miR-17-5p is related to tubal factor infertility [38]. miR-424 is involved in the human estrogen receptor and progesterone receptor pathways [39]. There are limited studies about the relationship of miR-17-5p and miR-424-5p levels with clinical indicators in EMT patients. Our results initially identified the strong association of miR-17-5p and miR-424-5p with EMT.

EMT is an inflammatory disease with autoimmune and chronic features, and EMT-related pain is often caused by inflammation [40, 41]. The abnormal expression of inflammatory factor IL-4 occurs in EMT patients [42]. IL-6 is a potential indicator for EMT and is positively related to the disease stage [43]. Angiogenesis is of great importance for the engraftment and development of endometriotic lesions [44]. VEGFA is an effective angiogenic factor and is regarded as a leading element in uterine angiogenesis [45], which is highly expressed in plasma and peritoneal fluid of EMT individuals [44, 46]. Preoperative CA-125 is identified as an effective marker for diagnosing EMT [25], and CA-125 expression is upregulated in EMT patients [47]. The previous studies have illustrated the increased concentration of IL-4 and IL-6, and the levels will increase as the disease progression [48–50]. miR-17-5p is an inflammation-associated miRNA and its overexpression can suppress the lipopolysaccharide-induced inflammatory response, including IL-6 level [51]. Consistently, there is a negative correlation between miR-17 level with IL-4 and IL-6 in EMT [23]. Our results showed that VEGFA, IL-4, IL-6, and CA-125 were upregulated in the serum of EMT patients, and miR-17-5p and miR-424-5p were negatively related with VEGFA, IL-4, IL-6, and CA-125, respectively. VEGFA is a target of miR-17-5p and miR-17-5p can repress cell migration, proliferation, and invasion in EMT by directly inhibiting VEGFA expression [21]. miR-424-5p upregulation leads to the reduced IL-6 level [52]. miR-424 is inversely linked with the levels of serum IL-4 and IL-6 [53]. miR-424-5p can bind to the VEGFA mRNA and miR-424-5p overexpression significantly reduces the expression of VEGFA protein in primary cells cultured from EMT patients [22, 54]. Briefly, miR-17-5p and miR-424-5p could regulate EMT by targeting VEGFA.

In conclusion, this study first determined the expression of miR-424-5p in the serum of EMT patients and explored the diagnostic efficacy of miR-17-5p combined with miR-424-5p expression for EMT using the ROC curve. Moreover, we analyzed the correlation of miR-17-5p and miR-424-5p levels with clinical indicators in EMT patients by Pearson correlation to provide a new entry point for clinical judgment. However, this study only investigated these two miRNAs. In addition, our study included a small number of cases and events. Addressing these deficiencies requires us to carry out the study of multiple miRNAs with significant expression differences, and expand the sample size to enhance the reliability of results. Furthermore, prognostic studies can be continued to further clarify the diagnostic value of miR-17-5p and miR-424-5p. The regulatory mechanism of miR-17-5p and miR-424-5p to target VEGFA in EMT is also worth exploring.

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Author contributions CLL is the guarantor of integrity of the entire study; CLL contributed to the study concepts, study design, definition of intellectual content, literature research, clinical studies, experimental studies, data acquisition, data analysis, statistical analysis, manuscript preparation, manuscript editing and manuscript review; SLZ contributed to the study concepts, study design, definition of intellectual content, literature research, clinical studies, data acquisition, data analysis, manuscript preparation, manuscript editing and manuscript review; MJL contributed to the study design, clinical studies, experimental studies, data acquisition, data analysis, statistical analysis, manuscript preparation, manuscript editing and manuscript review; all authors read and approved the final manuscript.
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