miR-424-5p Combined with miR-17-5p Has High Diagnostic Efficacy for Endometriosis

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Research Article

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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: A total of 80 EMT patients of reproductive age were included as the study subjects, and another 80 healthy women of reproductive age 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 significantly downregulated in EMT patients. For the diagnosis of 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), 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. The expressions of miR-17-5p and miR-424-5p were negatively correlated with dysmenorrhea, infertility, pelvic pain, and rASRM stage, but not with age, BMI, menstrual disorder, and nulliparity. VEGFA, IL-4, and IL-6 were remarkably increased in EMT patients, and both 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.

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, and infertility are common symptoms [6]. Advanced EMT may cause gynecological malignancies, including ovarian cancer [7]. Diagnosis is a great challenge in EMT, whose diagnostic time is prolonged by an average of 6 to 12 years due to the lack of specific symptoms and noninvasive diagnostic tests [8]. EMT not only affects the physical and mental health of patients and their spouses but also brings great economic and medical burdens to society [9]. 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 to 24 nucleotides, which mediate the level of messenger RNA in numerous eukaryotic lineages [10, 11].

Abnormal miRNA expression is linked with various human disorders, including cardiovascular diseases, cancer, inflammatory diseases, and gynecologic pathology [12]. 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 [13]. The expression of miR-424-5p is reduced in the endometriotic mesenchymal cells, indicating its potential regulatory effect on EMT [14]. Since the sensitivity and specificity of a single miRNA as a biomarker to distinguish EMT patients from healthy women are relatively low [15], a combined diagnosis of miR-17-5p and miR-424-5p for EMT were investigated in this study.

Angiogenesis is an important step in the development of endometriotic lesions, hence EMT is also regarded as an angiogenic disease [16]. 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 [17]. VEGFA plays a pivotal effect on the pathogenesis of EMT [18]. miR-17-5p can promote angiogenesis and inversely modulates the expression of VEGFA in EMT [19, 20]. miR-424-5p negatively targets VEGFA and lowers the angiogenic activity of VEGFA protein [21]. 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 confirmed by pathology in the Department of Gynecology in Hunan Province Maternal and Child Health care Hospital from January 2019 to December 2020 as the experimental group (EMT group). Another 80 women of reproductive age with the healthy physical examination at the same period were selected as the control group. The whole blood samples of all enrolled subjects were collected, and the clinical characteristics were recorded, including age, body mass index (BMI), dysmenorrhea, menstrual disorder, infertility, parity, pelvic pain, and revised American Society for Reproductive Medicine (rASRM) stage (I/II or III/IV). The diagnostic criteria referred to the *Specifications for the Diagnosis and Treatment of Endometriosis* [*Chinese Journal of Obstetrics and Gynecology*, 2015(3)] issued by 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 ASRM staging criteria.

Inclusion criteria included: women of reproductive age, aged 24-46 years, 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 expressions 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 minutes, 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 minutes, and then 40 cycles of 95°C for 15 seconds, 55°C for 30 seconds, and 70°C for 30 seconds. 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 $2^{-\Delta\Delta Ct}$ method. The primer sequences were shown in Table 1.

Table 1
Primer sequences

Gene	Forward 5'-3'	Reverse 5'-3'
miR-17-5p	GCGGCCAAAGTGCTTACAGTG	CAGCCACAAAAGAGCACAAT
miR-424-5p	GGCTAGTCAGCAGCAATTCATGT	GTGCAGGGTCCGAGGT
U6	GCTTCGGCAGCACATATACTAAAAT	CGCTTCACGAATTTGCGTGTCAT
Note: *miR-17-5p*, microRNA-17-5p; *miR-424-5p*, microRNA-424-5p.		

Enzyme-linked immunosorbent assay (ELISA)

The corresponding Quantikine ELISA kits of VEGFA (ab119566, Abcam, Cambridge, UK), IL-4 (ab215089, Abcam), and IL-6 (EK0410, Boster, Pleasanton, CA, USA) 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 hours 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 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, nulliparity, and rASRM stage 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).

Table 2
Comparisons of general data and characteristics

	Control (N = 80)	EMT (N = 80)	*P*
Age (years)	32.40 ± 2.67	31.50 ± 4.73	0.140
BMI (kg/m^2)	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%)	/

Note: EMT, endometriosis; BMI, body mass index; rASRM, revised American Society for Reproductive Medicine.

miR-17-5p and miR-424-5p were downregulated in the serum of EMT patients

In this study, RT-qPCR was used to determine the expressions of miR-17-5p and miR-424-5p in the serum of healthy controls and EMT patients, and revealed that the miR-17-5p and miR-424-5p levels in EMT patients were markedly lower than those of healthy controls (Figure 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 suggested 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) (Figure 2A); the AUC of miR-424-5p was 0.737 and the cutoff value was 0.915 (98.8% sensitivity and 61.2% specificity) (Figure 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 (Figure 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 (Figure 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 rASRM stage in EMT patients (all $P < 0.05$), but not associated with age, BMI, menstrual disorder, and nulliparity (Table 3).

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

	EMT	miR-17-5p		miR-424-5p	
	(N = 80)	r	*P*	r	*P*
Age (years)	31.5 ± 4.73	0.086	0.447	0.124	0.274
BMI (kg/m^2)	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

Note: 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.

miR-17-5p and miR-424-5p were negatively correlated with VEGFA, IL-4, and IL-6 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 [22]. Therefore, we subsequently validated the correlation of miR-17-5p and miR-424-5p with VEGFA, IL-4, and IL-6 in the serum of EMT patients. Firstly, the expressions of VEGFA, IL-4, and IL-6 in the serum were measured using ELISA, and the results unveiled remarkably

increased levels of VEGFA, IL-4, and IL-6 in EMT patients compared with healthy controls (all $P < 0.05$) (Figure 3A). Next, the correlations of miR-17-5p and miR-424-5p expressions with VEGFA, IL-4, and IL-6 concentrations were assessed. Pearson correlation scatter plot showed a negative correlation of miR-17-5p expression with VEGFA ($P < 0.0001$; r = -0.8853), IL-4 ($P < 0.0001$; r = -0.6552), and IL-6 (P < 0.0001; r = -0.7438) concentrations (Figure 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), and IL-6 ($P < 0.0001$; r = -0.6870) concentrations (Figure 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 vector (WT or MUT) was 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 (Figure 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.

Discussion

EMT emerges as a common gynecologic disease with complicated pathogenesis, which mainly impacts women of reproductive age [23]. miRNAs are implicated in the nosogenesis of EMT and can be proposed as potential markers in EMT [9]. This study evaluated the diagnostic efficacy of miR-17-5p and miR-424-5p for EMT.

Aberrant levels of miRNAs are implicated in the occurrence and progression of EMT [24] and proposed as biomarkers for early diagnosis and prediction of EMT [25]. 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 clinical diagnosis of EMT [26, 27]. miR-424-5p is involved in the development of EMT and is lowered in EMT lesions [14, 28]. Thus, weak expressions 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. 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). The AUC of miR-424-5p was 0.737 and the cutoff value was 0.915 (98.8% sensitivity and 61.2% specificity). AUC was 0.939 and the cutoff value was 2.205 (93.8% sensitivity and 88.7% specificity) for combined diagnosis of miR-17-5p and miR-424-5p. Previous studies also elicit that the combination of several different miRNAs, with improved sensitivity and specificity, has elevated diagnostic accuracy relative to individual miRNA [15, 29]. Altogether, miR-17-5p and miR-424-5p both could be considered as biomarkers for EMT, and their combination had amplified this diagnostic efficacy.

Infertility, chronic pelvic pain, and dysmenorrhea are primary symptoms of EMT [30]. 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 [31], and 30–50% of women with EMT also suffer from pelvic pain and infertility [32]. 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 expressions with dysmenorrhea, infertility, pelvic pain, and rASRM stage in women with EMT. miR-17-5p is related to tubal factor infertility [33]. miR-424 is involved in the human estrogen receptor and progesterone receptor pathways [34]. 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 also defined as a chronic inflammatory disorder, and EMT-related pain often results from inflammation [35]. The abnormal expression of inflammatory factor IL-4 occurs in EMT patients [36]. IL-6 is a potential indicator for EMT and is positively related to the disease stage [37]. Angiogenesis is of great importance for the engraftment and development of endometriotic lesions [38]. VEGFA is an effective angiogenic factor and is regarded as a leading element in uterine angiogenesis [39]. Hence, we investigated the relationship of miR-17-5 and miR-424-5p with VEGFA, IL-4, and IL-6. Firstly, the ELISA unveiled the increased expressions of VEGFA, IL-4, and IL-6 in the serum of EMT patients. Similarly, VEGFA is also enhanced in the peritoneal fluid of EMT patients [40]. The previous studies have illustrated the increased concentration of IL-4 and IL-6, and the levels will increase as the disease progression [41–43]. Later, Pearson analysis revealed that miR-17-5p and miR-424-5p were negatively related with VEGFA, IL-4, and IL-6 respectively in EMT. Furthermore, the binding relationship of miR-17-5p and miR-424-5p with VEGFA was identified. miR-17-5p is an inflammation-associated miRNA and its overexpression can suppress the lipopolysaccharide-induced inflammatory response, including IL-6 level [44]. Consistently, there is a negative correlation between miR-17 level with IL-4 and IL-6 in EMT [22]. 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 [20]. miR-424-5p upregulation leads to the reduced IL-6 level [45]. miR-424 is inversely linked with the levels of serum Il-4 and IL-6 [46]. 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 [47]; PMID: 24608518). 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 expressions 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.

Declarations

Ethics statement

This study was approved by the academic ethics committee of Hunan Province Maternal and Child Health care Hospital and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All participants were fully informed and voluntarily signed the informed consent before sampling.

Informed consent

Informed consent was obtained from all individual participants included in the study.

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Declaration of competing interest

The authors declare that they have no conflicts of interest.

Availability of data and materials

All the data generated or analyzed during this study are included in this published article.

Authors' contribution

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, d 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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Figures

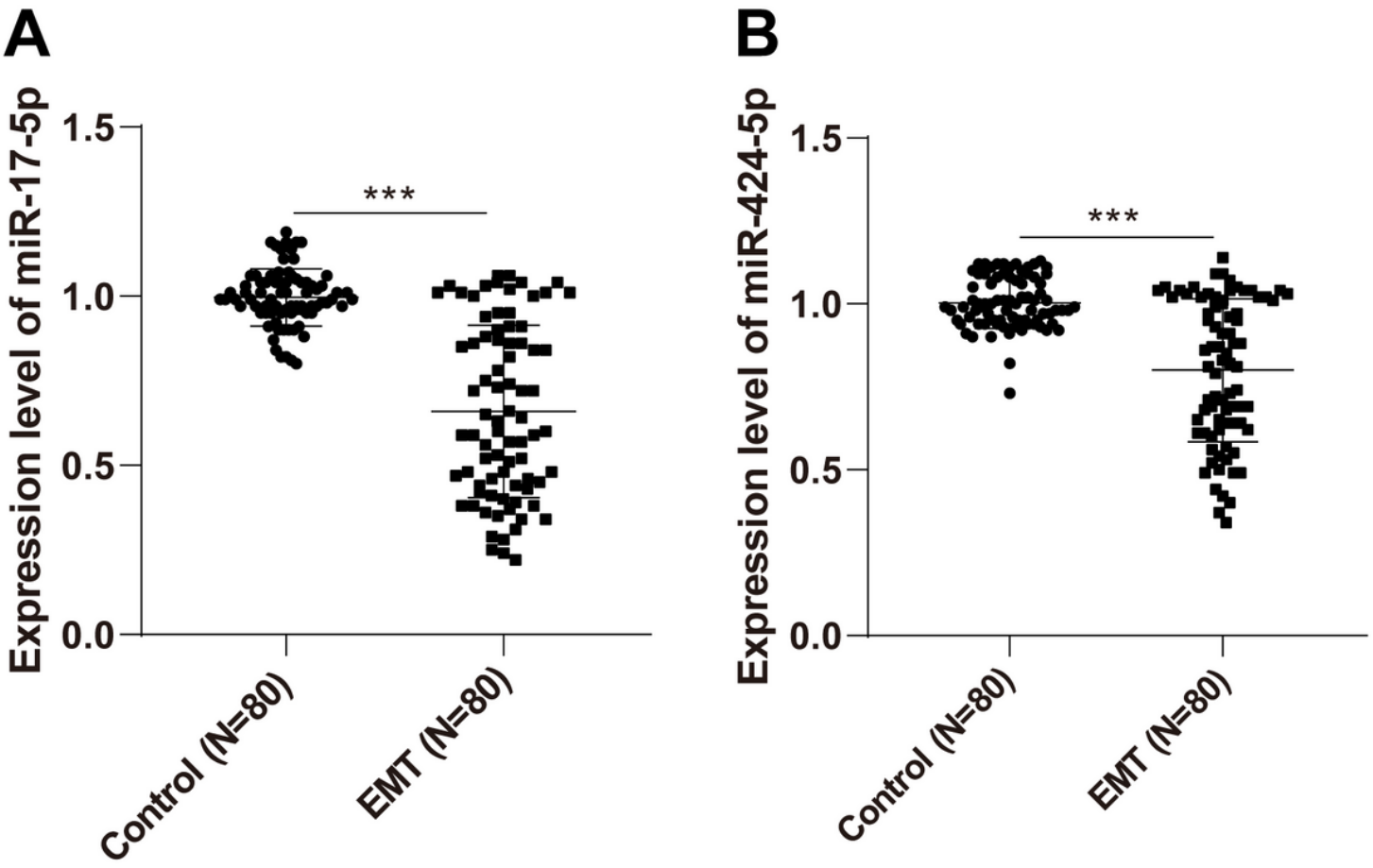

Figure 1

miR-17-5p and miR-424-5p were downregulated in the serum of EMT patients. (A) RT-qPCR was used to determine the expression of serum miR-17-5p; (B) RT-qPCR was adopted to measure the expression of serum miR-424-5p. Independent sample *t* test was employed to analyze panel A and B. ***$P < 0.001$.

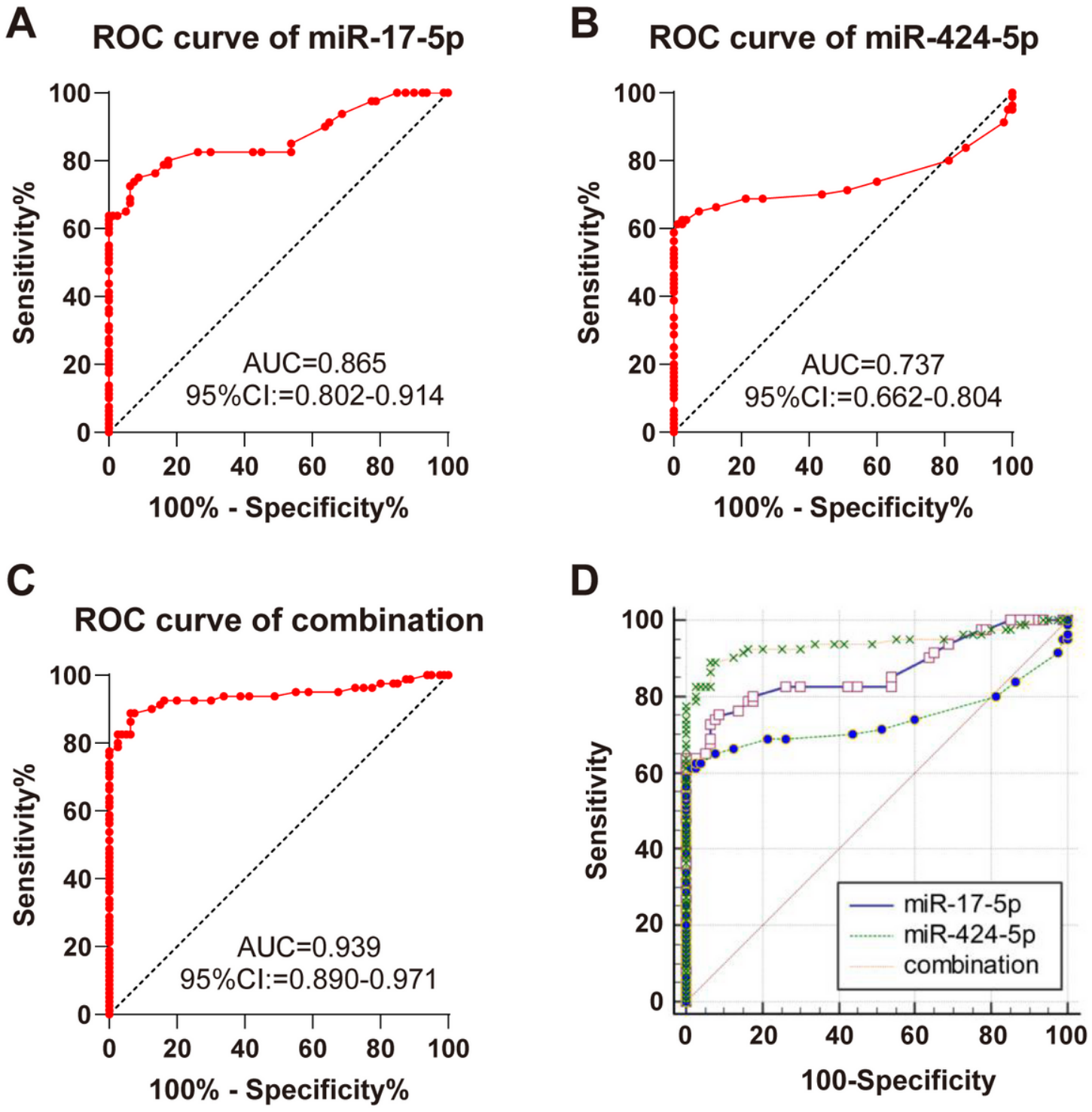

Figure 2

ROC curve of miR-17-5p and miR-424-5p. (A) ROC curve of miR-17-5p; (B) ROC curve of miR-424-5p; (C) ROC curve of miR-17-5p combined with miR-424-5p; (D) The differences of AUC were compared and analyzed using MedCalc.

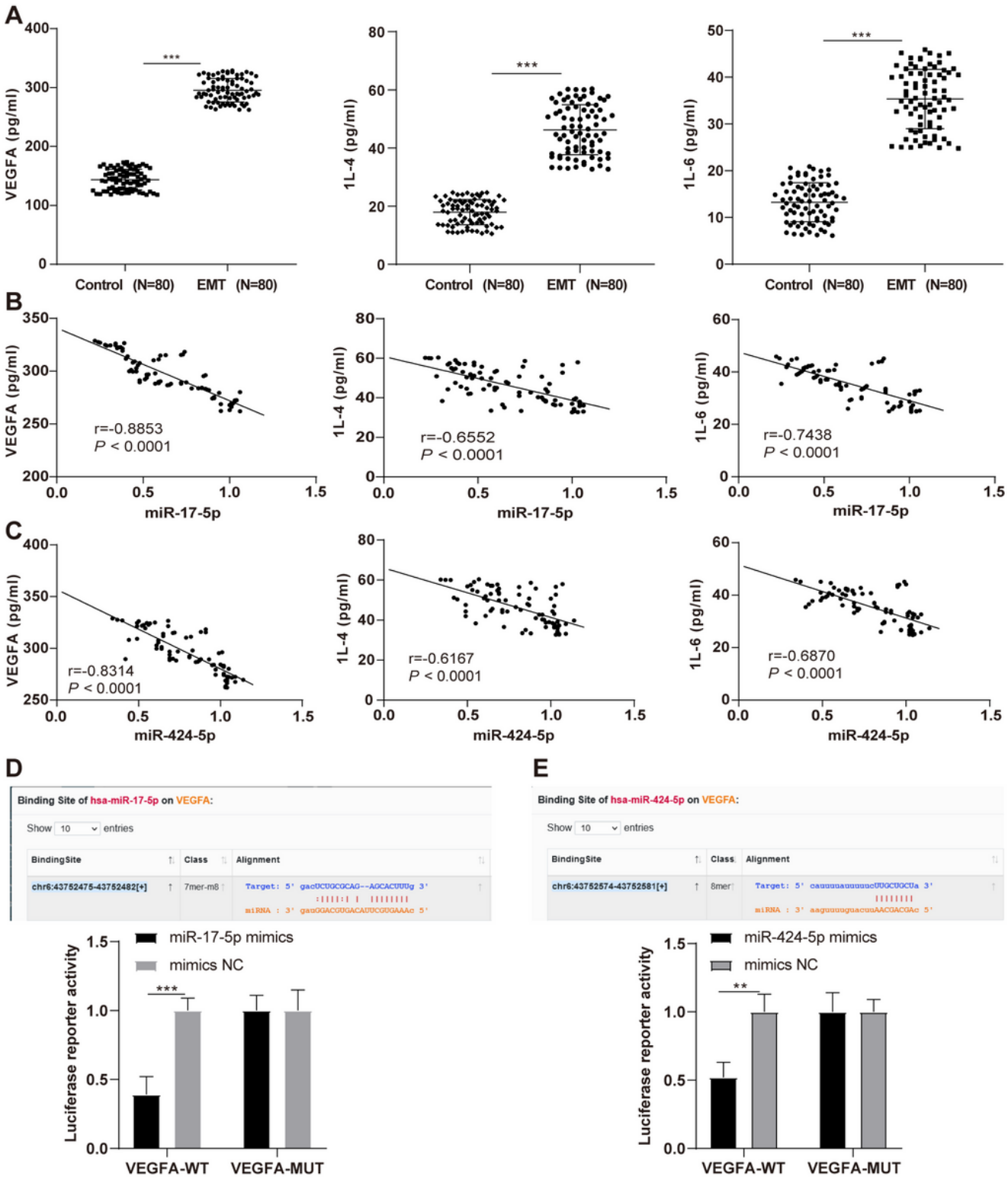

Figure 3

miR-17-5p and miR-424-5p were negatively correlated with VEGFA, IL-4, and IL-6 in the serum of EMT patients. (A) The expressions of serum VEGFA, IL-4, and IL-6 were measured using ELISA; (B) The correlation of miR-17-5p with serum VEGFA, IL-4, and IL-6 in EMT patients was assessed by Person analysis; (C) The association of miR-424-5p with serum VEGFA, IL-4, and IL-6 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 and E. $^{**}P < 0.01$, $^{***}P < 0.001$.