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Original Article

miR-30b and miR-30d are potential diagnostic indicators and risk factors for endometriosis

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Abstract: Objective: This study aimed to analyze the diagnostic value of miR-30b and miR-30d in endometriosis (EMS) and whether they are risk factors. Methods: Altogether 184 infertile women admitted to Tongren Hospital of Wuhan University from March 2018 to March 2019 were selected. Among them, 99 patients with EMS were selected as the EMS group, and 85 patients with infertility and normal pelvic cavities were selected as the normal group. Serum miR-30b and miR-30d of the two groups were detected, and the possibility of their participation in the diagnosis and risk factors of endometriosis was analyzed. Results: Serum miR-30b and miR-30d were significantly down-regulated in the EMS group. AUC of miR-30b and miR-30d for diagnosis of EMS were 0.855 and 0.862. AUC of combined diagnosis of EMS was 0.949. Serum miR-30b and miR-30d were significantly negatively correlated with AFS stage and dysmenorrhea degree. Age, duration of infertility, dysmenorrhea, EMS family history, serum miR-30b and miR-30d were all risk factors affecting EMS in infertile women. Conclusion: miR-30b and miR-30d can be used as diagnostic indicators of EMS, and they are also risk factors for EMS.

Keywords: MiR-30b, miR-30d, endometriosis, diagnosis, risk factors

Introduction

Endometriosis (EMS) is a gynecological disease that may cause severe dysmenorrhea. Its symptoms are hidden and have potential negative effects on women's fertility and quality of life [1, 2]. According to EMS epidemiological statistics, it has an impact on 5-15% of women of childbearing age; about 37% of women with EMS suffer from infertility, and the diagnosis rate of EMS patients can be as low as 1% [3, 4]. As a chronic disease, EMS is difficult to diagnose because there are no obvious symptoms during the incubation period, and pelvic pain and infertility will be caused once the symptoms occur. Laparoscopy, the gold standard for diagnosis, has the disadvantages of high cost and operation risks, which brings great challenges to the diagnosis of EMS patients [5, 6]. EMS is a risk factor for abortion, ovarian cancer, and endometrial cancer, which brings great hidden dangers to women's health [7, 8]. Therefore, it is of great significance for improving women's health and quality of life to investigate the diagnosis and risk factors of EMS in infertile women.

miRNA is a small non-coding RNA that participates in the mechanisms of drug resistance and carcinogenesis of gynecologic tumors. It is widely used in the diagnosis and pathogenesis of gynecologic diseases [9]. An abnormal imbalance of miRNA in gynecologic diseases endows miRNA with the potential for effective diagnosis, and differential expression of miRNA in serum of EMS patients shows the potential for identifying EMS patients [10, 11]. The miR-30 family is a miRNA subgroup that is often used in the study of female gynecologic diseases [12]. Studies have reported that lower levels of miR-30b are significantly related to the recurrence of breast cancer patients, and miR-30d can inhibit the progression of ovarian cancer by targeted inhibition of Snail to block the further development of EMT process. Both may be involved in the pathological regulation of gynecologic cancer [13, 14]. Previous studies have also shown that miR-30b and miR-30d mediate

the secretion of endometrial cells in the menstrual cycle, and they also have the effect of regulating female endometrial receptivity [15]. However, the potential clinical value and specific regulatory mechanisms of miR-30b and miR-30d in EMS are still unclear.

At present, there is little research on the diagnosis and risk factors of miR-30b and miR-30d in infertile EMS patients. We will carry out correlation analysis by detecting the serum expression of miR-30b and miR-30d in patients, hoping to provide clinical reference value for diagnosis and prevention of infertile EMS patients.

Data and methods

General information

Altogether 184 infertile women admitted to Tongren Hospital of Wuhan University from March 2018 to March 2019 were selected. Among them, 99 patients with EMS were taken as the EMS group, aged 25-48 years, with an average age of (35.68±6.23). Another 85 patients with infertility and normal pelvic cavities were taken as the normal group, aged 24-49 years, with an average age of (35.02± 6.11). This study was approved by the Ethics Committee of Tongren Hospital of Wuhan University. The subjects and their families have been fully informed and signed an informed consent form. Inclusion criteria: the diagnosis was conformed EMS with diagnostic criteria [16]; patients did not receive any hormones or other drug treatment or surgical treatment for half a year; the study met the American Fertility Society (AFS) staging standard [17]; patients with clinical symptoms such as infertility and abnormal menstruation. Exclusion criteria: patients combined with malignant tumors or serious organ and system dysfunction; patients combined with other gynecological diseases; patients combined with infectious diseases; patients with mental illness or communication disorders and loss of cognitive ability. The inclusion criteria were applicable to patients in EMS group, and the subjects in control group were infertile with normal pelvic health.

Assessment of dysmenorrhea

Visual analogue scale (VAS) [18] was used to evaluate the degree of dysmenorrhea in patients in the EMS group. Patients with no pain and no need to take medicine to relieve their pain were given a score of 0 points; patients who needed to take medicine, with no or little influence on daily life, and with moderate pain were given 1-3 points; patients with mild pain, and the pain was relieved after taking medicine, but still had a great influence on daily life were given 4-6 points; patients taking medicine that did not relieve their pain, and patients with severe pain were given 7-10 points.

Detection method

Five mL of fasting venous blood from the elbow was collected from the subjects at 7-8 am, placed in a vacuum blood collection tube containing EDTA-K2, and centrifuged at 1500× g at 4°C for 10 min. Two mL of upper serum was collected and transferred to an EP tube, centrifuged at 16,000 g for 10 min to precipitate cell debris, and then the supernatant was stored in a new EP tube at -75°C for later use. The total RNA was extracted according to the instructions of mirVanaTM miRNA Isolation Kit (Mito Biotechnology Co., Ltd., Shanghai, China, AM1561). The concentration of RNA was measured by SpectroArt 200S ultraviolet-visible spectrophotometer (ANATECH Co., Ltd., Beijing, China). Referring to TaqMan MicroRNA Reverse Transcription Kit (Biolab Technology Co., Ltd., Beijing, China, MT0006-POH), RNA was reverse transcribed into cDNA, and PCR amplification experiments were conducted with cDNA as a template, U6 was used as the internal reference gene, and primer sequence was designed by Shanghai Xinfan Biotechnology Co., Ltd., China. miR-30b and miR-30d were quantitatively detected on ABI7500 real-time fluorescence quantitative PCR system (Biomerry Biotechnology Co., Ltd., Beijing, China) with reference to miRNA RT-qPCR research kit (Genetimes Technology, Inc., Shanghai, China, 110-001S). PCR amplification conditions: 90°C for 5 min, 90°C for 5 s, 60°C for 30 s, 72°C for 5 s, for a total of 40 cycles. All samples were repeatedly detected 3 times, and the results were expressed by $2^{-\Delta CT}$.

Statistical analysis

Graph pad prism 6 (GraphPad Software, San Diego, USA) was used to visualize the data. The counting data was expressed by the number/percentage (n/%), and the comparison of count-

ing data between groups was performed by chi-square test. When the theoretical frequency in chi-square test was less than 5, the continuity correction chi-square test was used, and the measuring data was expressed by mean ± SEM. Independent sample t test was used to compare the measurement data between groups. Receiver operating characteristic curve (ROC) was used to evaluate the diagnostic value of serum miR-30b/d for ESM patients. Spearman correlation coefficient was used to analyze the correlation of serum miR-30b/d with AFS staging and dysmenorrhea degree. SPSS 22.0 (EasyBio Inc., Beijing, China) Logistics Multivariate Regression Analysis was used to analyze the risk factors of EMS in infertile women. When P<0.05, the difference was statistically significant.

Results

Baseline data

There were no significant differences in the average age, menstrual cycle, infertility course, body mass index (BMI), hypertension history, diabetes history, drinking history, smoking history and residence between the two groups (P>0.05), but there were significant differences in age, infertility course, dysmenorrhea and EMS family history (P<0.05). See **Table 1**.

Expression of serum miR-30b and miR-30d in the two groups of patients

The relative expression of serum miR-30b and miR-30d in the EMS group and normal group was (1.01±0.30) and (1.54±0.40) respectively, while the relative expression of serum miR-30d was (0.87±0.26) and (1.34±0.35) respectively. The relative expression of serum miR-30b and miR-30d in the EMS group were significantly lower than those in normal group (P<0.05). See **Figure 1**.

Diagnostic value of serum miR-30b and miR-30d in EMS

The ROC curves of patients with EMS diagnosed by serum miR-30b and miR-30d were visualized. The AUC of patients with EMS diagnosed by serum miR-30b was 0.855 (95% CI: 0.799-0.910), the cut-off value was 1.35, the diagnostic sensitivity was 92.30%, and the specificity was 68.24%. AUC of patients with EMS diagnosed by serum miR-30d was 0.862 (95% CI: 0.807-0.916), cut-off value was 1.12, the diagnostic sensitivity was 87.88%, and the specificity was 72.94%. Furthermore, the ROC curve for diagnosing EMS patients was visualized by combining serum miR-30b and miR-30d. A logistic regression model was established by taking miR-30b and miR-30d as independent variables, Logit (P combined detection) =13.687+2.086 miR-30b+43.037 miR-30d, and the area under the ROC curve of combined detection was fitted by the probability value in the model. AUC of patients with combined diagnosis of serum miR-30b and miR-30d was 0.949 (95% CI: 0.921-0.977), cut-off value was 0.49, the diagnostic sensitivity was 89.90%, and the specificity was 84.71%. See **Figures 2**, **3** and **Tables 2**, **3**.

Correlation of serum miR-30b, miR-30d with AFS stage and dysmenorrhea degree in the EMS group

We set AFS stage I and dysmenorrhea degree with no pain as 1, AFS stage II and dysmenorrhea degree with mild pain as 2, AFS stage III and dysmenorrhea degree with moderate pain as 3, AFS stage IV and dysmenorrhea degree with severe pain as 4. Serum miR-30b in the EMS group was significantly negatively correlated with AFS stage and dysmenorrhea degree (r=-0.645, P<0.001; r=-0.652, P<0.001); serum miR-30d was significantly negatively correlated with AFS stage and dysmenorrhea degree (r=-0.635, P<0.001; r=-0.659, P<0.001).

Risk factors affecting EMS in infertile women

We included miR-30b and miR-30d into the analysis, listed them as dependent variables for assignment, and took the occurrence of EMS as a dependent variable. A logistic regression model was also used for multivariate analysis of factors with differences such as age, infertility course, dysmenorrhea and EMS family history. The results showed that age (P=0.001), duration of infertility (P=0.008), dysmenorrhea (P=0.022), EMS family history (P=0.031), miR-30b (P=0.001), miR-30d (P=0.005) were independent risk factors for EMS in infertile women. See **Tables 4**, **5**.

Discussion

EMS is a benign disease related to functional endometrial and stromal hyperplasia, and it is also one of the common causes of infertility,

Table 1. Baseline data of two groups of patients [n (%), Mean ± SD]

Factor	n	EMS group (n=99)	Normal group (n=85)	χ^2/t	P
Age (years)				6.913	0.009
<35	89	39 (39.39)	50 (58.82)		
≥35	95	60 (60.61)	35 (41.18)		
Average age (years)	184	35.68±6.23	35.02±6.11	0.723	0.471
Menstrual cycle (d)	184	30.07±1.56	29.83±1.47	1.068	0.287
Course of infertility (years)	184	7.85±2.10	4.26±1.94	11.973	<0.001
BMI (kg/m^2)	184	22.96±2.62	22.54±2.55	1.098	0.274
History of hypertension				2.673	0.102
No	132	76 (76.77)	56 (65.88)		
Yes	52	23 (23.23)	29 (34.12)		
History of diabetes				0.798	0.372
No	146	81 (81.82)	65 (76.47)		
Yes	38	18 (18.18)	20 (23.53)		
Drinking history				0.287	0.592
No	87	45 (45.45)	42 (49.41)		
Yes	97	54 (54.55)	43 (50.59)		
Smoking history				0.654	0.419
No	98	50 (50.51)	48 (56.47)		
Yes	86	49 (49.49)	37 (43.53)		
Residence				0.300	0.584
Rural	74	38 (38.38)	36 (42.35)		
Urban	110	61 (61.62)	49 (57.65)		
Disease type					
Peritoneal type	33	33 (33.33)	-		
Ovarian type	30	30 (30.30)	-		
Recto-vaginal septum type	36	36 (36.37)	-		
AFS staging					
I	22	22 (22.22)	-		
II	30	30 (30.30)	-		
III	27	27 (27.27)	-		
IV	20	20 (20.21)	-		
Dysmenorrhea				6.288	0.012
No	103	47 (47.47)	56 (65.88)		
Yes	81	52 (52.53)	29 (34.12)		
Degree of dysmenorrhea					
No pain	16	16 (16.16)	-		
Mild	27	27 (27.27)	-		
Moderate	33	33 (33.33)	-		
Severe	23	23 (23.24)	-		
EMS family history				5.715	0.017
No	122	60 (60.61)	62 (72.94)		
Yes	62	39 (39.39)	23 (27.06)		

bringing physical and mental distress to infertile women [19, 20]. The lesions are widely distributed and can be involved in the peritoneum, ovary, and rectum, etc. The above lesions will cause inflammation, pelvic pain and other physiological distress in EMS women [21]. At present, the treatment methods of EMS include surgery, hormone therapy, and immunomo-

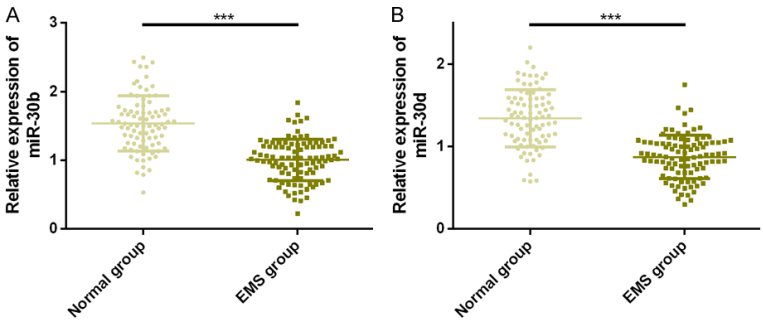

Figure 1. Expression of serum miR-30b and miR-30d in the two groups of patients. A: The relative expression of serum miR-30b in the EMS group was significantly lower than that in the normal group. B: The relative expression of serum miR-30d in the EMS group was significantly lower than that in the normal group. Note: *** indicates that P<0.001.

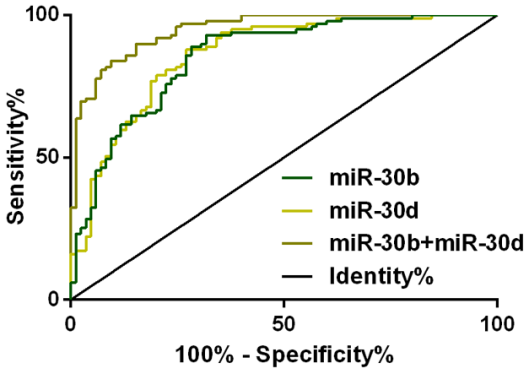

Figure 2. ROC curve of serum miR-30b and miR-30d for diagnosis of EMS.

dulators. The recurrence risk and potential toxic side effects may make the treatment very difficult for patients [22]. Therefore, it is of potential value to study the disease-related biomarkers of infertile women with EMS for the treatment of patients.

More and more researchers focus on the application of miRNA in EMS, and have conducted relevant research. For example, in the research of Nothnick [23] et al., miR-451a can be used as a serum diagnostic marker of EMS, which shows abnormally high expression in EMS patients and baboons with EMS, and has a close positive correlation with lesion content. Moreover, Hsu [24] et al. showed that miR-199a-5p is significantly down-regulated in EMS patients. Over-expression treatment of miR-199a-5p can inhibit cell proliferation, motility and other pathological processes of ectopic stem cells, and animal model tests have also confirmed that it is helpful for the reduction of endometriosis foci. In this study, serum miR-30b and miR-30d were significantly down-regulated in the EMS group, suggesting that they may participate in the pathological process of EMS and have the potential to diagnose EMS. In the research of Cai et al. [25], miR-30d can be regulated by polychlorinated biphenyls. After up-regulation treatment, miR-30d can target and inhibit epithelial-mesenchymal transition markers, thus reversing the damage of polychlorinated biphenyls to endometrial receptivity to a certain extent. It was also pointed out by Balaguer [26] et al. that the development of fetuses and the receptivity of the endometrium in women with low miR-30d levels before pregnancy will be negatively affected to a certain extent. According to Katzorke et al. [27], miR-30b and miR-30d were significantly increased in secretory endometrium of fertile women, suggesting that the two may have potential value in distinguishing fertile women from infertile women. All of the above research results showed that low levels of miR-30b and miR-30d may be potential risk factors affecting female endometrial receptivity and infertility. In addition, the development of miR-30b and miR-30d expression enhancers may become a new therapeutic direction for EMS patients or infertile patients. Further analysis showed that AUC of serum miR-30b and miR-30d for diagnosis of EMS were 0.855 and 0.862, while AUC of combined diagnosis of both was as high as 0.949, indicating that combined diagnosis of the two for EMS has higher value. In the research of Gaziel et al. [28], the mechanism of miR-30b and miR-30d in melanoma was also indicated. Both can promote malignant activities such as melanoma metastasis by directly targeting a GalNAc transferase and enhancing the immune suppression of the body.

We also analyzed the correlation of serum miR-30b and miR-30d with AFS stage and dysmenorrhea degree. The results showed that both were significantly negatively correlated with AFS stage and dysmenorrhea degree, which indicated that both have certain predictive value for the pathological development

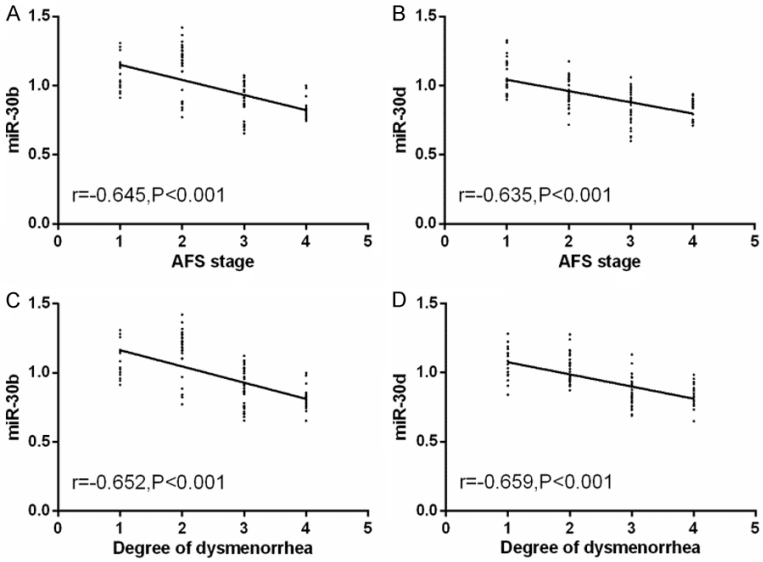

Figure 3. Correlation of serum miR-30b and miR-30d with AFS stage and dysmenorrhea degree in EMS group. A, B: Serum miR-30b, miR-30d in the EMS group were significantly negatively correlated with AFS staging (r=-0.645, P<0.001; r=-0.635, P<0.001). B-D: Serum miR-30b and miR-30d in the EMS group were significantly negatively correlated with dysmenorrhea (r=-0.652, P<0.001; r=-0.659, P<0.001).

Table 2. ROC parameters of serum miR-30b/d for diagnosis of EMS patients

Indicators	AUC	95% CI	S.E	Cut-off	Sensitivity (%)	Specificity (%)
miR-30b	0.855	0.799-0.910	0.028	1.35	92.30	68.24
miR-30d	0.862	0.807-0.916	0.028	1.12	87.88	72.94
miR-30b/d	0.949	0.921-0.977	0.015	0.49	89.90	84.71

Table 3. Results of binary logistic regression analysis

Variable	B	Std. Error	Wals	*P* value	OR	95% CI
miR-30b	-5.671	1.028	30.455	<0.001	0.003	0.000-0.026
miR-30d	-5.981	1.040	33.049	<0.001	0.003	0.000-0.019
Constant	13.687	2.086	43.037	<0.001	8.795E5	-

Table 4. Logistic multivariate regression analysis assignment

Factor	Variable	Assignment
Age (years)	X1	<35=0, ≥35=1
Course of infertility	X2	Continuous variable
Dysmenorrhea	X3	No =0, Yes =1
EMS family history	X4	No =0, Yes =1
miR-30b	X5	Continuous variable
miR-30d	X6	Continuous variable

process of EMS and dysmenorrhea degree of patients. Finally, we analyzed the risk factors of EMS in infertile women. The results confirmed that age, duration of infertility, dysmenorrhea, EMS family history, serum miR-30b and miR-30d are all risk factors of EMS in infertile women; indicating that old age was related to a long duration of infertility, high risk of EMS in infertile women with dysmenorrhea, EMS family history, as well as low level of miR-30b and miR-30d. We further explored miR-30b and miR-30d and found that miR-30b can target oncogenic gene KRAS to regulate tumor growth, and KRAS is abnormally up-regulated in ectopic endometrium of EMS patients and may participate in the pathogenesis [29, 30]. It was also found that miR-30d can regulate 176 genes, some of which are involved in physiological and pathological processes such as reproduction, endocrine system and tissue development [31]. We suspected that miR-30b and miR-30d may mediate female reproductive ability and dysmenorrhea by regulating one or more EMS pathogenic target genes, but the exploration of relevant pathological mechanisms still needs further experiments to verify this, and this study has not yet investigated these ideas.

To sum up, serum miR-30b and miR-30d can not only be used as biological indicators for combined diagnosis of EMS, but may also be risk factors for infertile women suffering from EMS. However, there is still room for improvement in this study. First of all, we can supplement the cellular biology research on EMS on both of them and further explore their specific regulatory mechanisms on EMS. In addition, we can also increase the diagnostic value of the two for clinical patho-

Table 5. Multivariate analysis of influencing factors of EMS in infertile women

factor	β	S.E	Wald	P	OR	95% CI
Age	0.407	0.121	10.775	0.001	1.526	1.174-1.921
Course of infertility	0.523	0.195	7.741	0.008	1.642	1.140-1.927
Dysmenorrhea	1.109	0.486	5.336	0.022	2.984	1.187-7.654
EMS family history	1.348	0.653	5.820	0.031	3.903	1.276-12.968
miR-30b	1.416	0.594	5.944	0.001	6.725	2.018-30.875
miR-30d	1.024	0.462	6.987	0.005	4.780	1.135-19.752

logical indexes of EMS patients and expand the potential clinical value of the two in EMS.

Disclosure of conflict of interest

None.

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