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Expression and Significance of miRNA-29 in Endometriosis

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Abstract

To explore the expression of miRNA-29b and miRNA-29c in the endometrial tissue of patients with endometriosis.Dataset GSE26346 was downloaded from GEO and the differentially expressed genes (DEGs) were analyzed by the Affy package in R.2 DEGs of the top 3 up-regulated genes miRNA-29b and miRNA-29c used for further experimental validation.120 patients with EMS in our hospital from June 2018 to August 2020 were selected as the study group, another 70 patients who underwent myoma of uterus were selected as the control group during the same period. The expression levels of miRNA-29b and miRNA-29c in the study groups and control groups were detected respectively. The receiver operating characteristic (ROC) curve was used to analyze the diagnostic value of the two separately and jointly. The study group was divided according to the r−AFS stage. The levels of miRNA-29b, miRNA-29c and Anti-mullerian hormone (AMH) in patients with different stages of endometriosis were measured and compared. The correlation between miRNA-29b, miRNA-29c and disease stages and serum AMH was analyzed. ① The expression levels of miRNA-29b and miRNA-29c in the study group were higher than those in the control group(P<0.05). ② The AUC of miRNA-29b that diagnosed endometriosis (EMS) was 0.729, and the AUC of miRNA-29c that diagnosed endometriosis (EMS) was 0.745. ③There is a positive correlation between the disease stage of EMS patients and the expression of miRNA-29b and miRNA-29c（P<0.05）, The expression of miRNA-29b and miRNA-29c in EMS patients was negatively correlated with serum AMH（P<0.05).

Keywords

Endometriosis; miRNA-29b; miRNA-29c; Anti-mullerian Hormone.

1. Introduction

Endometriosis (EMS), a condition in which endometrial-like tissue aberrantly grows outside the uterus, is a common disease in women of childbearing age, with the increase of cesarean rate in recent years, the incidence of the endometriosis is gradually increasing. In addition to causing dysmenorrhea, infertility is also one of important hazards. The main cause of infertility caused by endometriosis are the infiltration, erosion and destruction of the ovarian tissue, which has a certain impact on the reserve function of the ovary. However, its early clinical manifestations are not consistent with the severity of the disease, it brings many difficulties to the early diagnosis and treatment. There are many theories about the pathogenesis of the disease, including common theories of counter blood implantation, body cavity metaplasia,

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mulellar tube remnant, distant metastasis, genetic hormone immune inflammatory factors, etc., but it is still widely recognized as having endometrial implants [1-2].

Micro RNA (miRNA) is a single-stranded small molecule RNA of 21 to 25 bases. It has identified hundreds of miRNA in various species, including humans, Drosophila and plants, and plays an important role in cell differentiation and tissue development. Due to the universality and diversity of its existence, it suggests that miRNA has a very wide range of biological functions and may represent a higher level of gene expression regulation mode. As one of the regulatory ways of epigenetic modification, miRNA affects gene expression directly or indirectly in the cell, and plays an important role in human life activities by regulating cell proliferation, differentiation and apoptosis. Previous studies have proved that a variety of miRNA are closely related to the occurrence and development of endometriosis, among them, miRNA plays an important role in the regulation of angiogenesis, participating in cell invasion, metastasis, proliferation, apoptosis and immune modulation of participating cells [3]. the miRNA-29 (miR-29) family is one of the most abundant miRNA expressed in the pancreas and liver in mice and humans [4]. Ababnormal expression were detected in ectopic endometrium in both The Human microRNA Disease Database (version 3.2) and miRNet database. The microRNA-29 (miR-29) family consists of three miRNAs: miR-29a, miR-29b, and miR-29c[5] ,Among them, miRNA29b is also divided into miRNA-29b-1 and miRNA-29b-2.In humans, miRNA-29a is co-located with miRNA-29b-1 in chromosome 7, whereas miRNA-29b-2 is co-located with miRNA29c on chromosome 1.Several isoforms of the miRNA-29 family have almost identical coding sequences, all containing the sequence-AGCACCA at the 5′ cap end [6], Thus, we appears that the nucleotide sequence encoded by the miRNA-29 family has genetic conservation[7]. The miRNA-29 family is involved in the development of multiple diseases, such as tumors, cardiovascular disease, the development of diabetes, etc. Shaker's study on breast cancer indicated that miRNA29 expression is significantly elevated in breast cancer and is considered as a key gene involved in the development of breast cancer [8]. However, the expression is also different in different tumors, such as miRNA-29 is low expressed in gastric cancer, considering that it can inhibit the invasion and metastasis of gastric cancer cells[9]. The results of Xiaoxi Wang et al have confirmed that inhibiting miR-29 can promote angiogenesis and reduce fibrosis in patients with myocardial infarction[10]. Marsh EE's [11] study reported that all miR-29 family members (29a, 29b, 29c) were downregulated in leiomyomas when compared to the myometrium in vivo. However, the specific expression of miRNA29 in endometrial tissue in endometriosis patients is not clear. In this study, we examined the expression level of miRNA-29b and miRNA-29c in the endometrial tissue of the patients with endometriosis, to provide a reference for the early clinical diagnosis and treatment.

2. Methods

2.1. Source of Gene Expression Profiling Data

In this study, the endometriosis-related expression profile dataset GSE26346 was downloaded from the GEO database (https://www.ncbi.nlm.nih.gov/geo/),which contains 3 ovarian endometrium samples and 3 paired in-place endometrium samples obtained from 3 patients with ovarian endometriosis. All samples were taken on the GPL7372 chip platform (LCSciences _miRNA_ Human_v9.1) to detect the differentially expressed genes.

2.2. Analysis of the Differentially Expressed Genes

First, we use the Affy [12] package in R language software to read the chip raw data, Data were then standardized and preprocessed (including background correction, normalization, and expression value calculation) using the RMA (robust multi-array average) method [13]. Probes were subsequently annotated using the GPL7372 platform annotation files to remove probes

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not matching to the gene names. When the different probes were mapped to the same gene, the average of the different probes was taken as the final expression value for this gene. Finally, the affy package was used to screen for differentially expressed genes, and | log2 FC | 2 was used as the threshold for screening for differentially expressed genes. two miRNAs (miRNA-29b and miRNA-29c) from top 3 were selected for further analysis.

2.3. General Information

Ectopic endometrium tissues were collected from 120 patients with endometriosis from June 2018 to August 2020 at Department of Gynecology of The Affiliated Hospital of Chengde Medical College as study group, age 22-47 years, mean (36.85 ± 6.25) years; body weight 47-67.5kg, mean (57.54 ± 5.44) kg. Eutopic endometrium tissues were collected from 70 patients with uterine fibroids at the same time as control group, Age 22-46 years, mean (37.26 ± 6.11) years, body weight 47.5-68kg, mean (56.21 ± 4.56) kg. Inclusion criteria for endometriosis patients:①Preoperative pelvic ultrasound suggested that ovarian cyst, cyst diameter more than 4cm, mainly cystic, its visible dense dotted weak echo, postoperative pathology confirmed as endometriosis,② with regular menstruation, the operation time choose 3-7 days after menstruation, ③No history of taking hormone drugs in the past half a year, ④Estrogen levels were greater than 200 pg/ml, ⑤There were no other serious endosurgical comorbidities that affected surgery or hormone determination. Inclusion criteria for uterine fibroids: ①pelvic ultrasound suggested uterine fibroids preoperative, pathology confirmed as uterine fibroids, without degeneration, ②with regular menstruation, the operation time choose 3-7 days after menstruation, ③No history of taking hormone drugs in the past half a year, ④There were no other serious endosurgical comorbidities that affected surgery or hormone determination. The studies involving human participants were reviewed and approved by the Research Ethics Committee of The Affiliated Hospital of Chengde Medical College. The patients/participants provided their written informed consent to participate in this study.

2.4. Instruments and Reagents

The miRNA extraction kit was purchased from Beijing Total Gold Biotechnology Co., Ltd; The miRNA reverse transcription kit and miRNA-NA real-time fluorescence (qRT-PCR) kit were purchased from Harbin Haiji Biotechnology Co, LTD; The miRNA-29b, miRNA-29c were purchased from Shanghai Biotechnology Co.

2.5. Method and Collection

AMH collection of specimens: Serum AMH of all study subjects was extracted on the morning venous blood before surgery, centrifuged at 3500 rpm. AMH was detected by Roche E411 automatic chemiluminescence analyzer in the hospital.

miRNA-29b, miRNA-29c detection: The subject endometrial tissues were collected by professional physicians, washed the samples with phosphate buffer salt solution and measured miRNA-29b (qRT-2 9 c expression levels of miRNA-29b and miRNA-29c expression levels by formula 2-ct. The sequences of the qRT-PCR primers are shown in Table 1.

Table 1. qRT-PCR Primer sequences

Variable	forward primer (5′-3′)	reverse primer(5′-3′)
miRNA-29b	ACACTCCAGCTGGGTAGCAC	GCTGTCGTGGACTCGGCAAT
miRNA-29c	CTGACCTTAGCACCATTTGA	TATCGTTGTACTCCACTCCT
U6	ATTGGAACGATACAGAGAAG	GGAACGCTTCACGAATTTG

ISSN: 1813-4890

2.6. Statistical Analyses

All data were presented as examples or percentage, comparison between groups using theχ^2test, normally distributed measurement data were represented as mean ± SD. T-test were presented between two groups, correlations were analyzed by Pearson, the diagnostic value of miRNA-29b and miRNA-29c were evaluated by the subject working characteristic curve (ROC curve). Statistical significance was determined according to p-values <0.05. SPSS19.0 was used for statistical analysis.

3. Results

3.1. Screening for DEG miRNA

Endometriosis-related expression profiling dataset GSE26346, which contains 3 ovarian endometrial samples and 3 matched incumbent endometrial samples, GSM647040, GSM 647 041 and GSM647042, were retrieved and downloaded from the GEO database (https: //www. ncbi.nlm.nih.gov/geo/). DEGs were identified based on a 2 log fold change (logFC) cut-off. LogFC cut-off was defined as the mean of the sums of the absolute value of logFC and its two standard deviations in the whole expression. A total of 142 differentially co-expressed miRNA were selected, and downregulated top 3 was miRNA-200b, miRNA-200c, miRNA-542-5p, and upregulated top 3 was miRNA-1, miRNA-29b, and miRNA-29c,respectively.The miRNA-200 family has been reported in several publications of the endometriosis.

3.2. General Data

No significant difference of general data on age and weight between the two groups (P> 0.05) was comparable, as shown in Table 2.

Table 2. General clinical data [N (%), ($\overline{x}$±S)]

Variable	Study group (n=120)	Control group (n=70)	t/X²	P
Age(years)	36.85±6.25	37.26±6.11	0.725	0.471
Weight(kg)	57.54±5.44	56.21±4.56	1.721	0.094
Marital status				
Married	110(91.67)	64(91.43)	0.956	0.581
Unmarried	10(8.33)	6(8.57)		
Delivery situation				
Yes	99(82.50)	57(81.43)	0.035	0.871
No	21(17.50)	13(18.57)		

3.3. Expression of miRNA-29b and miRNA-29c in Endometrial Tissue in Both Groups

Expression of miRNA-29b in the study group of endometrial tissue (0.898 ± 0.159), miRNA-29c expression (2.028 ± 0.159) was higher than control miRNA-29b (0.365 ± 0.067) and miRNA-29c (1.017 ± 0.156) (t=26.531,38.927), The difference was statistically significant(P<0.05).

3.4. The Expression of miRNA-29b and miRNA-29c in Different Stages of Endometrisis

With miRNA-29b and miRNA-29c increased in ectopic tissues, the expression levels of miRNA-29b and miRNA-29c gradually increased(P<0.05), table 3.

ISSN: 1813-4890

Table 3. miRNA-29b, miRNA-29c with different stages of the endometriosis($\overline{x}$±S)

Stages	n	miRNA-29b	miRNA-29c
Ⅰ	15	0.734±0.021	1.742±0.158
Ⅱ	26	0.768±0.056	1.962±0.290
Ⅲ	35	0.890±0.025	2.007±0.386
Ⅳ	46	0.988±0.046	2.180±0.158
F		229.14	61.429
P		<0.01	<0.01

3.5. Correlation between miRNA-29b, miRNA-29c and AMH

Pearson correlation analysis showed that miRNA-29b, miRNA-29c were inversely associated with serum AMH (r1= 0.468, r2= 0.512), The difference was statistically significant(P<0.05).

3.6. The Diagnostic Value of miRNA-29b, miRNA-29c for Endometriosis

The ROC curve analysis showed that the diagnosis of miRNA-29b with AUC was 0.745, sensitivity of 0.625, specificity of 0.771, and AUC of miRNA-29c was 0.820, sensitivity of 0.658 and specificity of 0.800 (Figure.1).

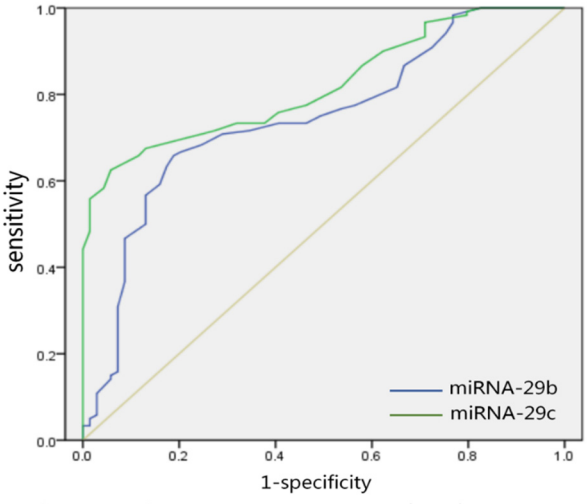

Figure 1. The ROC curve of miRNA-29b and miRNA-29c

4. Discussion

Endometriosis is the presence of endometrial tissue with growth function growing in areas other than the uterine body, according to its anatomical location, it can be divided into three categories: pelvic adhesion type, ovarian cyst type and deep infiltration type. Especially, ectopic endometrial cysts, localized in the ovarian parenchyma, can directly affect the female ovarian reserve [14]. An explanation for this phenomenon: Ovarian endometriotic cyst lesions involved ovarian inflammation, make it stimulate more should be dormant base follicles were raised into the growth track, and local lesions inflammation and ovarian fibrosis, affect its blood, into the growth stage of follicles is not enough nutritional support, to atresia. Therefore, the seemingly contradictory phenomenon increases the primary follicles entering the growth stage in the

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ovarian cortex of the affected side [15]. Such a vicious circle, accelerate the depletion of diseased follicle reserve. In recent years, lots of experimental results show that the follicle fluid, peritoneal irrigation fluid and blood IL-6, IL-8, IL-18, TNF-a, monocyte chemokine 1 and other inflammatory mediators content increased significantly, further confirmed the ovarian endometriosis cyst through inflammation to increase follicle recruitment to promote ovarian reserve consumption of scientific research hypothesis. MH deserved attention in the evaluation of ovarian reserve function, it is composed of two relative molecular mass of 72000 dimer monomer, through disulfide bond homodimeric glycoprotein, belongs to the transformation growth factor-superfamily members, these members play a very important role in tissue growth and differentiation. The synthesis of AMH originates from follicular granulosa cells located early in follicle development and is a crucial hormone for the regulation of follicle maturation [16], it is the gold standard for the prediction of ovarian reserve function [17]. In addition to cause dysmenorrhea, chronic pelvic pain, pelvic adhesion and other symptoms, EMS also has a great impact on infertility. Therefore, seeking specific molecular markers of great clinical significance for the diagnosis and treatment of EMS is very important, especially in women of childbearing age [18]. The results of this study shows that miRNA-29b, miRNA-29c and AMH in serum are inversely correlated, so the ovarian reserve function can be roughly evaluated according to the expression levels of miRNA-29b and miRNA-29c to expect early diagnosis and treatment of patients with fertility requirements and help them complete their fertility requirements.

The key to ectopic proliferation in the endometrium lies in the expression of genes in the site inner membrane, which is an intrinsic factor that triggers EMS [19].The miRNA can regulate various biological activities such as tumor cell proliferation, metastasis and invasion, and occupies a high position in the popular score of tumor biology research[20].The results of this study showed that miRNA-29b and miRNA-29c are highly expressed in the endometrial tissue of endometriosis patients and are associated with clinical stage. The sensitivity of miRNA-29b and miRNA-29c in endometrial tissues of endometriosis patients was 0.625 and 0.658, respectively, and the specificity was 0.771 and 0.800, respectively, suggesting the potential of these molecules as diagnostic biomarkers of EMS.

5. Conclusion

In conclusion, the expression levels of miRNA-29b and miRNA-29c are increased in the endometrial tissue of endometriosis patients compared with controls, and with the higher probability of endometriosis, these miRNAs are expected as potential biomarkers for the diagnosis of endometriosis. In addition, this study also found that the expression levels of miRNA-29b and miRNA-29c were negatively correlated with AMH, so it is expected to further evaluate the ovarian reserve function of endometriosis patients according to its expression level, and then should be therapied as early as possible. The disadvantage of this study is the need to take endometrial tissue for experiments. For patients with fertility needs, endometrial tissue biopsy is invasive examination. The next step is to study the expression levels of miRNA-29b andmiRNA-29c in serum, while considering the convenience to obtain more experimental evidence.

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