Identification of Circular RNAs as a Novel Biomarker for Ovarian Endometriosis

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Abstract

Background: Endometriosis is a challenging disease with symptoms such as dysmenorrhea and infertility. However, its etiology is still vague and there is still no effective markers or treatment. This study aimed to profile the circular RNAs (circRNAs) expressed in eutopic endometrium from patients with ovarian endometriosis and explore potential clues to the pathogenesis of endometriosis, providing an evidence for clinical diagnosis and treatment.

Methods: A total of 63 clinical samples, including control endometrium ($n = 22$) and eutopic endometrium ($n = 41$), were collected from Peking Union Medical College Hospital between May 1, 2016, and December 31, 2016. Of them, four samples in each group were used for circRNA microarray. Then, four upregulated circRNAs were screened out for quantitative real-time polymerase chain reaction (qRT-PCR) validation. After that, bioinformatics analysis was performed to predict miRNAs targeted by validated circRNAs and investigate the circRNA-miRNA-mRNA interactions.

Results: Among 88 differentially expressed circRNAs, 11 were upregulated and 77 were downregulated in eutopic endometrium of patients with endometriosis. qRT-PCR validation results for two upregulated circRNAs (*circ_0004712* and *circ_0002198*) matched the microarray results. The area under the receiver operating characteristic curve of *circ_0002198* for distinguishing ovarian endometriosis was 0.846 (95% confidence interval [*CI*]: 0.752–0.939; $P < 0.001$) while that of *circ_0004712* was 0.704 (95% *CI*: 0.571–0.837; $P = 0.008$). On the basis of target prediction, we depicted the molecular interactions between the identified circRNAs and their dominant target miRNAs, as well as constructed a circRNA-miRNA-mRNA network.

Conclusions: This study provides evidence that circRNAs are differentially expressed between eutopic and normal endometrium, which suggests that circRNAs are candidate factors in the activation of endometriosis. *circ_0002198* and *circ_0004712* may be potential novel biomarkers for the diagnosis of ovarian endometriosis.

Key words: Circular RNA; Endometriosis; Microarray; miRNA; mRNA

INTRODUCTION

Endometriosis is considered as a challenging disorder. Over 176 million women worldwide have been tormented by endometriosis associated pain and infertility, which severely affects their quality of life.[1,2] Unfortunately, there are still no specific markers and therapies. The essential reason for such adversity is due to the elusive pathogenesis. It is to date widely assumed that ectopic lesions arise through retrograde endometrial fragments during menstruation. Not all the women suffered endometriosis despite most women undergoing retrograde menstruation. The affected women may have certain susceptible factors, which partially comes down to the eutopic endometrium.[3]

Evidences have indicated that gene expression level in eutopic endometrium of endometriosis is aberrantly altered by comparison with control endometrium.[4,5] These alterations of eutopic endometrium might be the source of the pathogenesis of endometriosis, which keep endometrial debris easier alive in the ectopic site. Recently, emerging

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Access this article online

Quick Response Code:

Website:
www.cmj.org

DOI:
10.4103/0366-6999.226070

evidences reveal that circular RNAs (circRNAs) are involved in regulating gene expression as competitive endogenous RNAs (ceRNAs).[6-8] Moreover, circRNAs are characterized by covalently linked terminals and high stability as well as abundant expression level.[9,10] All abovementioned characteristics make circRNAs become evaluate indicators for diagnosis, prognosis, and therapeutic-response prediction. However, the relationship between circRNAs and endometriosis is unknown.

Given their pivotal biological roles, we investigated and identified dysregulated circRNAs in eutopic endometrium to offering new idea for diagnosis and treatment.

Methods

Ethical approval
This study was approved by the Institutional Review Board and Hospital Local Ethics Committee (No. JS-875). All participants provided informed consent.

Study population
A total of 63 clinical samples were collected at the Department of Obstetrics and Gynecology of Peking Union Medical College Hospital between May 1, 2016, and December 31, 2016. Eutopic endometrium samples were collected from 41 women who underwent hysteroscopic and laparoscopic surgery for ovarian endometriosis (22–46 years old; proliferative phase: $n = 28$; secretory phase: $n = 13$; American Fertility Society [AFS] Stage III–IV). Tissue samples of control endometrium were acquired from another 22 women without endometriosis who underwent hysteroscopy and laparoscopy for other benign ovarian cysts, infertility or uterine septum (no endometriosis, 25–43 years old; proliferative phase: $n = 16$; secretory phase: $n = 6$), similarly with a previous study.[11] All patients met the criteria as follows: regular menstruation (25–32 days), no hormonotherapy for at least 6 months, and histopathology confirmation. All obtained samples were excluded from abnormalities of endometrium by the pathological diagnosis, and proliferative and secretory endometrium phases were distinguished by hematoxylin and eosin staining. Tissue collection and storage were conducted as described previously.[12] Four samples for each group were randomly selected for a microarray analysis. Besides, all the samples were validated by quantitative real-time polymerase chain reaction (qRT-PCR).

Total RNA isolation and quality control
Total RNA was isolated with TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA quantity and quality were measured using a NanoDrop ND-1000 (Thermo Fisher Scientific, Wilmington, DE, USA). RNA integrity and genomic DNA contamination were assessed by denaturing agarose gel electrophoresis.

Circular RNA microarray analysis
The Arraystar Human circRNA Array 8 × 15 K V2 Microarray (Arraystar, Rockville, MD, USA) contains 15,000

probes for 13,617 human circRNAs, all of which have been confirmed by Jeck et al.,[10] Salzman et al.,[13] Memczak et al.,[14] Zhang et al.,[15] Zhang et al.,[16] Guo et al.,[17] and You et al.[18] Total RNA was first digested with Rnase R (Epicentre, Madison, WI, USA) to remove linear RNAs and enrich for circRNAs. Then, the enriched circRNA was amplified and transcribed into fluorescent complementary RNA, utilizing random primers according to the Arraystar Super RNA Labeling protocol (Arraystar). After hybridization and washing, processed slides were scanned using an Agilent Scanner G2505C (Agilent Technology, Santa Clara, CA, USA). Thereafter, Agilent Feature Extraction software (version 11.0.1.1, Agilent Technology, Santa Clara, CA, USA) was utilized to analyze acquired array images. Quantile normalization and subsequent data were processed using the R software package (version 3.3.1; R Foundation Inc. Vienna, Austria).

Quantitative real-time polymerase chain reaction validation
During the qRT-PCR validation stage, we recruited 41 endometria from patients with endometriosis and 22 normal endometria for control. Total isolated RNA was reversely transcribed using SuperScript III Reverse Transcriptase (Invitrogen Life Technologies). Subsequently, qRT-PCR was performed by ViiA 7 RT-PCR System (Applied Biosystems, Foster City, CA, USA) in a 10-μl reaction volume, including 5-μl 2 × PCR Master Mix (Arraystar), 0.5 μl/10 μmol/L forward/reverse primers, 2 μl cDNA, and 2 μl RNAase-free H_2O. The cycling program was initiated from 95°C for 10 min, followed by 40 cycles of 95°C for 10 s and 60°C for 60 s. Divergent primers were designed and optimized for circRNAs. Supplementary Table 1 listed all the PCR primers, the specificity of which was verified by a single-peak on the melting curve. The threshold cycle method ($2^{-\Delta\Delta CT}$) was used to calculate relative expression levels, which were normalized to β-actin levels.

Bioinformatics analysis
To further elucidate the role of circRNAs in endometriosis, we first used the Arraystar target prediction software based on TargetScan[19] and miRanda[20] to predict the targeted miRNAs of each circRNA, which described interactions between circRNAs and miRNAs. According to the miRNA support vector regression (mirSVR) algorithm, we ranked the top five predicted miRNA targets for each circRNA. Then, we used TargetScan and miRDB to predict the related target genes according to the miRNAs targeted by circRNAs. Finally, the interaction network was depicted using the validated circRNAs and predicted miRNAs/mRNAs, preliminarily accounting for interactions of circRNAs-miRNAs-mRNAs in pathogenesis of endometriosis. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses further annotated the function and signaling pathways of these genes.

Statistical analysis
Data were first explored by the tests of normality. If the normal distribution was satisfied, Student's t-tests was

used; otherwise, nonparametric Mann-Whitney *U*-test was used. The thresholds for differentially expressed circRNAs were expressed as an absolute value of fold change (FC) >2 (EuE = 1) and *P* < 0.05. Above statistical analyses were processed with R software version 3.3.1 and GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA).

RESULTS

Clinical characteristics of the participants

In the study, a total of 63 endometrial biopsies were obtained from women with laparoscopically and histopathologically proven presence (*n* = 41) or absence (*n* = 22) of endometriosis. The clinical characteristics of the participants were presented in Supplementary Tables 2 and 3 where the mean (standard deviation) ages of patients in endometriosis and control group were 32 ± 6 years and 32 ± 5 years, respectively. There were no differences in age, cycle phase, parity, and infertility between two groups. Patients with endometriosis were more likely to gain moderate-to-severe dysmenorrhea (visual analogue scale [VAS] ≥4) and high level of CA125 by comparison with controls (*Z* = −2.627, *P* = 0.009 and *t* = 7.529, *P* < 0.001, respectively). All the patients with endometriosis were judged to be Stage III and IV according to the AFS staging system.

Circular RNA expression profiles in eutopic endometrium relative to those in normal endometrium

According to the circRNA microarray, a total of 88 circRNAs, 11 significantly upregulated and 77 significantly downregulated, were differentially expressed between eutopic endometrium and normal endometrium. The two types of endometrial tissue could be clearly distinguished using hierarchical clustering, scatterplot, and volcano plots [Figure 1]. Hierarchical clustering indicated the relative expression level of circRNAs in eutopic and normal endometrium. Scatter plot evaluated the variation in circRNA expression between the two groups. Volcano plot displayed the statistical significance of differentially expressed circRNAs between cases and controls.

Quantitative real-time polymerase chain reaction validation of selected circular RNAs

To confirm the differentially expressed circRNAs in the microarray, four circRNAs were selected for validation based on the following criteria: (1) upregulated in eutopic endometrium, (2) FCs >2, (3) *P* < 0.05, (4) raw intensity >200, (5) exonic-related circRNAs, and (6) length between 200 and 3000 bp. Of them, two circRNAs (*circ_0004712* and *circ_0002198*) matched the microarray results and met the statistical cutoff by comparison with control endometrium [*Z* = −2.653, *P* = 0.008; *Z* = −4.498, *P* < 0.001; Figure 2a]. *circRNA_0002503* was expressed at a higher level in the eutopic endometrium of patients with endometriosis; however, the difference was not statistically significant. And, *circ_0000141* could not be amplified by qPCR [Figure 2a]. Intriguingly, the two validated circRNAs were not affected by the menstrual cycle [Figure 2b]. We also performed a subgroup analysis between VAS and CA125, respectively, where patients with endometriosis were classified as two groups: one with moderate-to-severe dysmenorrhea (VAS ≥4) or CA125 >35 U/ml and the other with VAS <4 or CA125 ≤35 U/ml. As a result, no significant association between the two validated circRNAs and VAS or CA125 was found [Supplementary Figure 1]. Receiver operating characteristic curve analyses revealed that *circ_0004712* and *circ_0002198* were valuable biomarkers for distinguishing women with or without
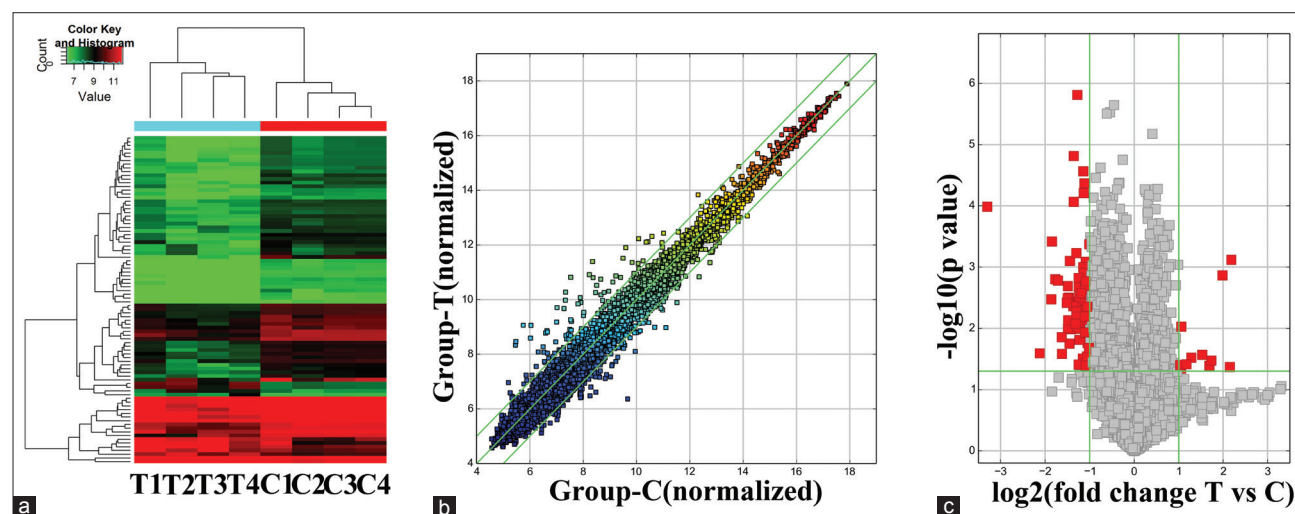

Figure 1: circRNA expression patterns in eutopic endometrium (Group T) relative to those in normal endometrium (Group C). (a) Hierarchical clustering of circRNAs. Each group included four individuals. circRNAs are represented by single rows and samples by single columns. The color scale indicates relative expression, upregulation (red), and downregulation (green). Fold change >2 and *P* < 0.05 were regarded as the differentially expressed circRNAs. (b) Scatter plot of circRNAs. The values corresponding to the X- and Y-axes are the normalized signal values. (c) Volcano plot of circRNAs. The values on the X- and Y-axes represent normalized fold changes and *P* values, respectively. The red points represent significantly differentially expressed circRNAs. circRNAs: Circular RNA.
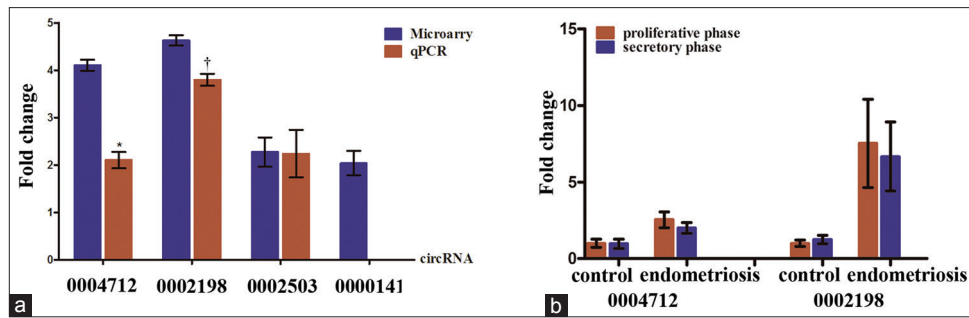
Figure 2: qRT-PCR validation of the four selected circRNAs. (a) The results of microarray and qPCR are shown as blue and red columns. Data that coincided with the microarray results and met the statistical cut-off are marked with *P < 0.05 and †P < 0.001. (b) Effect of menstrual cycle on the two identified circRNAs. Data are expressed as fold changes relative to the values for the proliferative phase group of controls. Both the circRNAs show no significant changes between proliferative (red columns) and secretory phases (blue columns). qRT-PCR: Quantitative real-time polymerase chain reaction; qPCR: Quantitative polymerase chain reaction; circRNAs: Circular RNAs.

endometriosis, with area under curve (AUC) value of 0.704 (95% confidence interval [CI]: 0.571–0.837; P = 0.008) and 0.846 (95% CI: 0.752–0.939; P = 0.000), respectively [Figure 3]. The diagnostic power of *circ_0004712* achieved notable improvement when the *circ_0004712* and *circ_0002198* were combined, while that of *circ_0002198* was not improved (AUC = 0.819; 95% CI: 0.713–0.925; P = 0.000).

Prediction of target miRNAs and mRNAs

To explore the interactions between circRNAs and miRNAs, we predicted the target miRNAs of each circRNA. Moreover, the dominant miRNAs targeted by top 10 up- and downregulated circRNAs were ranked based on mirSVR scores [Supplementary Table 4]. Of them, miR455-3p, miR876-3p, miR661, and miR323a-5p were found to be the common targets of both *circ_0004712* and *circ_0002198*. The details of the molecular interactions between these two circRNAs and above target miRNAs are depicted in Figure 4. To further study how the two circRNAs regulate gene expression as ceRNAs, we also predicted the related target genes according to the miRNAs targeted by circRNAs. On basis of above prediction, a total of 29 miRNAs and 62 mRNAs were recognized as downstream targets of *circ_0004712* and *circ_0002198*. Thereafter, the circRNA-miRNA-mRNA network was constructed, which clarified the gene regulatory relationships in endometriosis [Figure 5].

Enrichment analysis of circular RNA-targeted genes

GO and KEGG analysis was applied to enrich the function of circRNA-targeted genes [Figure 6]. The data indicated that the target genes of these circRNAs were mainly involved in the biological processes of creatine metabolic process, glutamine family amino acid metabolic process, and negative regulation of striated muscle cell [Figure 6a]. The main cell component which target genes participated in were mitochondrial inner membrane, organelle inner membrane, and mitochondrion, respectively [Figure 6a]. With regard to the molecular function, the circRNA-targeted genes play a role in phosphotransferase activity, SNAP receptor activity, and
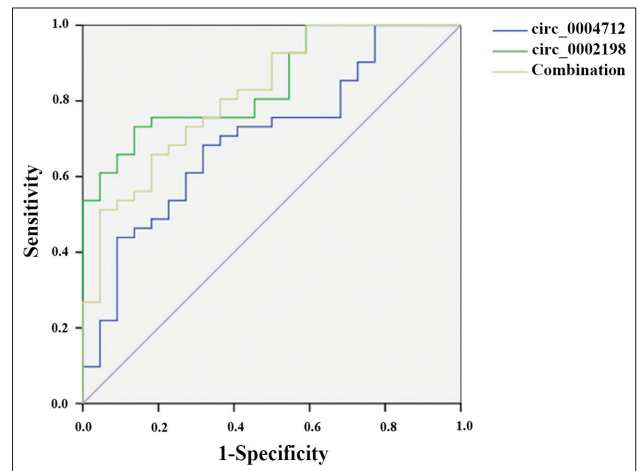

Figure 3: The receiver operating characteristic curve of specific circRNAs in distinguishing endometriosis. *circ_0004712* and *circ_0002198* were valuable biomarkers for distinguishing endometriosis, with AUC value of 0.704 (95% CI: 0.571–0.837; P = 0.008) and 0.846 (95% CI: 0.752–0.939; P < 0.001), respectively. The diagnostic power of circ_0004712 achieved notable improvement when the *circ_0004712* and *circ_0002198* was combined, while that of *circ_0002198* was not improved (AUC = 0.82; 95% CI: 0.71–0.93; P < 0.001). AUC: Area under the curve; circRNAs: Circular RNAs; CI: Confidence interval.

carboxylic ester hydrolase activity [Figure 6a]. In addition, KEGG analysis revealed that target genes might participate in arginine/proline metabolism and cytokine–cytokine receptor interaction [Figure 6b].

DISCUSSION

In our study, we identified 88 differentially expressed circRNAs, 11 upregulated and 77 downregulated, in eutopic endometrium of patients with endometriosis compared with those in normal endometrium. Two of four upregulated circRNAs were confirmed by qPCR following a microarray screening. The two circRNAs, *circ_0004712* and *circ_0002198*, identified in the present study have not been reported in other diseases. However, we found that the increased level of *circ_0004712* and *circ_0002198* can help identify the patients with endometriosis.
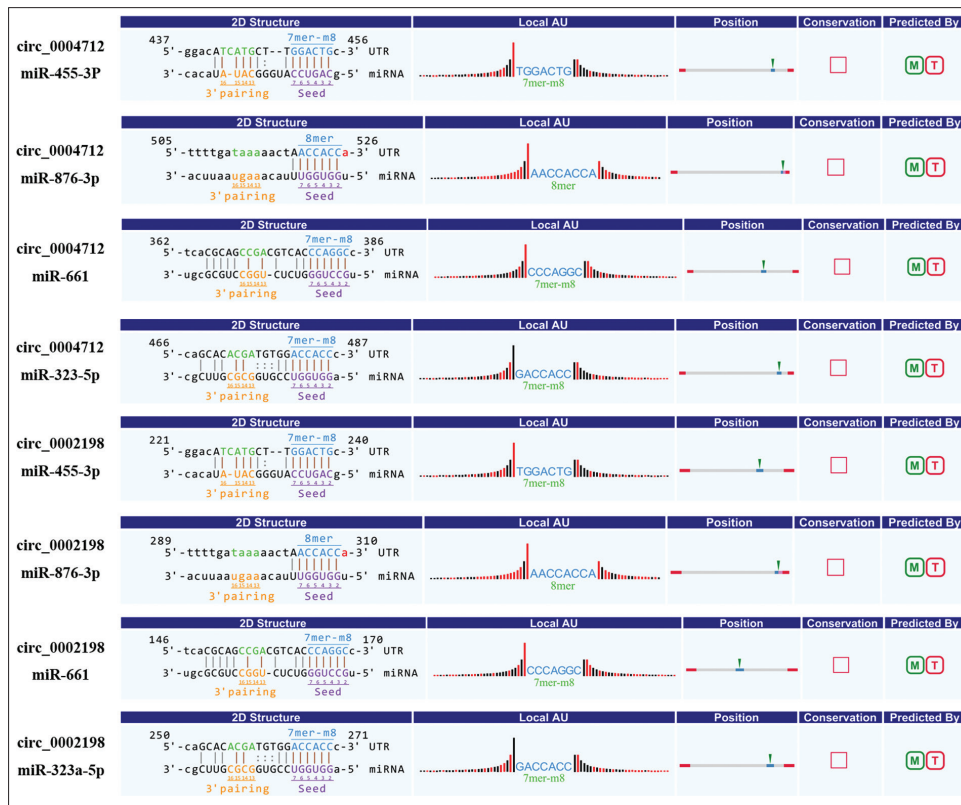
Figure 4: The molecular interactions between the two circRNAs and their dominant target miRNAs. The common and dominant target miRNAs of both *circ_0004712* and *circ_0002198* were miR455-3p, miR876-3p, miR661 and miR323a-5p, respectively. circRNAs: Circular RNAs.

To explore the role of circRNAs in endometriosis, we first performed prediction of target miRNAs for circRNAs. In the rank of top 5 target miRNAs, it was found that *circ_0004712* and *circ_0002198* act together to target miR455-3p, miR876-3p, miR661, and miR323a-5p, which have not been reported in endometriosis yet. Nevertheless, in preeclampsia patients, miR455-3p was significantly downregulated and was linked to the suppression of hypoxia signaling.[21] In atherosclerosis, miR876 can induce endothelial cell apoptosis.[22] Moreover, miR-661 was able to activate *p53* to inhibit cell cycle progression.[23] As regards the miR-323a-5p, in patients with refractory epilepsy caused by focal cortical dysplasia, its elevated level was positively correlated with the duration of epilepsy, seizure frequency, and poor prognosis.[24]

Subsequently, we also predicted the related target genes according to the miRNAs targeted by circRNAs. On basis of above prediction, a total of 29 miRNAs and 62 mRNAs were recognized as downstream targets of *circ_0004712* and *circ_0002198*. Among them, 4 targeted miRNAs and 2 mRNAs have been reported to be associated with endometriosis. For example, miR-503, repressed in endometriosis, induces apoptosis and cell cycle arrest and inhibits cell proliferation, angiogenesis, and contractility of ovarian endometriotic stromal cells.[25] miR-196a, overexpressed in eutopic endometrium, activates the MEK/ERK signal and represses the progesterone receptor and decidualization from women with endometriosis.[26]

miR-196b, downregulated in endometriotic stromal cells, inhibits proliferation and induces apoptosis by targeting *c-myc* and *Bcl-2* expression.[27] Hypoxia-coordinated AUF1/miR-148a was reported to destabilize DNA methyltransferase 1 mRNA during the pathogenesis of endometriosis.[28] Besides, *TNFRSF6B* (*NM_003823*) and *PGC-1α* (*NM_002630*) have been found to participate in pathogenesis of endometriosis. For instance, decoy receptor 3 *(DcR3)/TNFRSF6B*, a pleiotropic immunomodulator regulated by estrogen, promotes cell adhesion and enhances endometriosis development by activating focal adhesion kinase (FAK).[29] *PGC-1α*, highly expressed in ovarian endometrioma, promotes local estrogen biosynthesis by stimulating aromatase expression and activity.[30]

Finally, the circRNA/miRNA/mRNA network was constructed. Functional analyses and prediction of the target genes of circRNAs were carried out in eutopic and control endometrium for the first time. The two identified circRNAs were predicted to mediate arginine and proline metabolism via *CKMT1A* and *CKMT1B*. Both genes are effective modulators of ATP synthase-coupled respiration and belongs to mitochondrial creatine kinases (MtCK), which were found to be implicated in several tumors with poor prognosis.[31-34] Overexpressed MtCK may be part of a metabolic adaptation of cancer cells and could sustain high energy turnover, but would be also protective against stress situations like hypoxia and possibly protect cells from
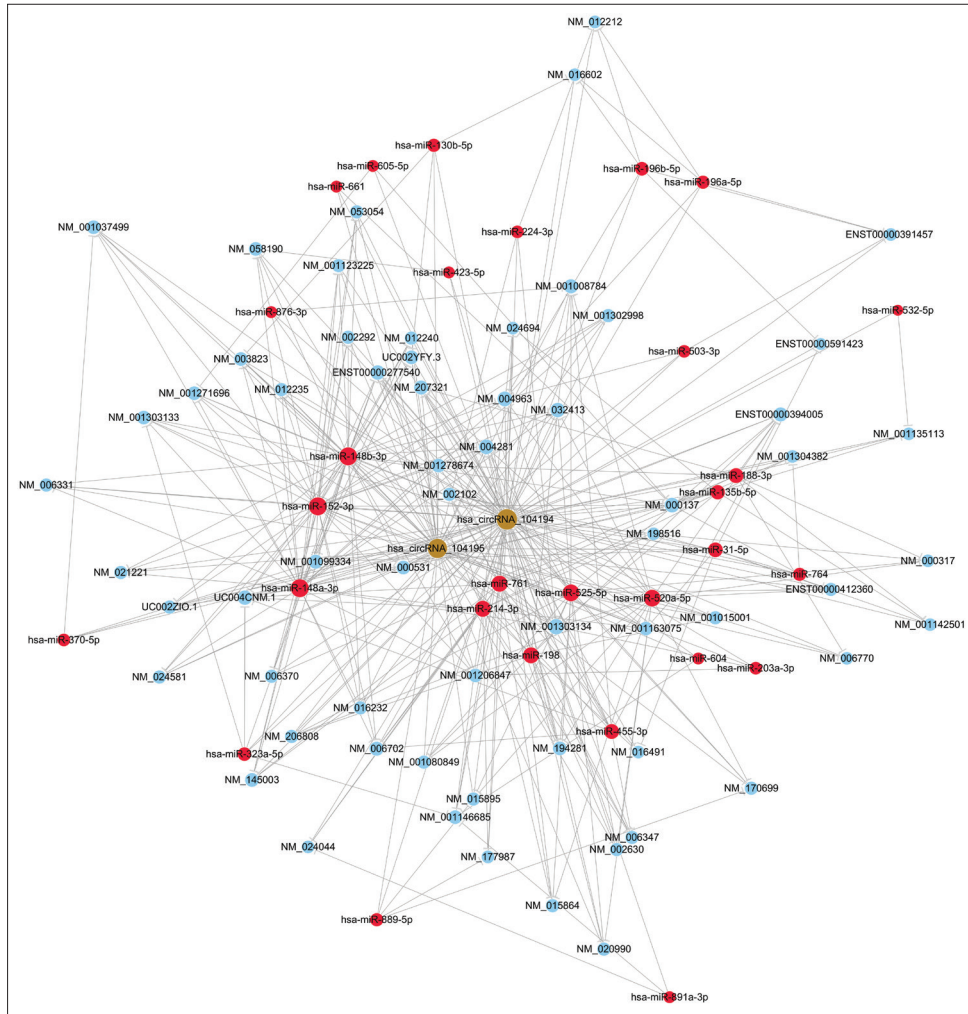
Figure 5: The circRNA-miRNA-mRNA network. The two identified circRNAs are denoted by yellow circle nodes. The red circle nodes represent target miRNAs, and blue circle nodes are target mRNAs. circRNAs: Circular RNAs.

apoptosis.[35] Although endometriosis is a benign disease, it exhibits malignant-like biological behaviors, including adhesion, aggression, and angiogenesis. Consequently, we speculated that *CKMT1A*- and *CKMT1B*-mediated metabolic pathways are involved in endometriosis progression. In addition, cytokine–cytokine receptor interaction signaling pathways were predicted to have strong relationships with the target genes, such as *CCL23* (*ENST00000591423*)*, CCR10* (*NM_016602*), and *TNFRSF6B (NM_003823)*. *CCL23* has been reported to enhance endothelial cell migration, invasion, adhesion, and angiogenesis,[36,37] which were also the pathophysiologic processes involved in endometriosis. *CCR10* and its receptors regulated tissue-specific migration, maintenance, and functions of immune cells. Increasing evidence also found that *CCR10*/ligands were frequently exploited by epithelium-originated cancer cells for their survival, proliferation, and evasion from immune surveillance.[38] Moreover, the evidence revealed that *TNFRSF6B* stimulated endometriosis development by activating FAK.[29]

The findings highlight the relationship between circRNAs and ovarian endometriosis, which will provide a novel biomarker for screening ovarian endometriosis and help explore the role of circRNAs in the activation of endometriosis. However, this study had several limitations. First, the sample size was small and all cases were revised AFS Stage III–IV; thus, further validation involving in larger cohorts of patients with early stages of this disease is warranted. Second, noninvasive biomarkers are more likely to evaluate the diagnostic value of circRNA in clinical applications. Further studies will be needed to assess the level of circRNA in peripheral blood or menstrual blood samples. Third, the molecular mechanism and function of the present circRNAs should be tested by associated experiments in the future.

Overall, we revealed for the first time, the circRNA expression patterns and an associated circRNA-miRNA-mRNA network between women with and without endometriosis. *circ_0004712* and *circ_0002198* were confirmed to be novel biomarkers in discriminating endometriosis from controls. Our findings lay the foundation for in-depth mechanistic studies on endometriosis, which can strengthen the reliability of the two identified circRNAs as diagnostic and therapeutic targets.
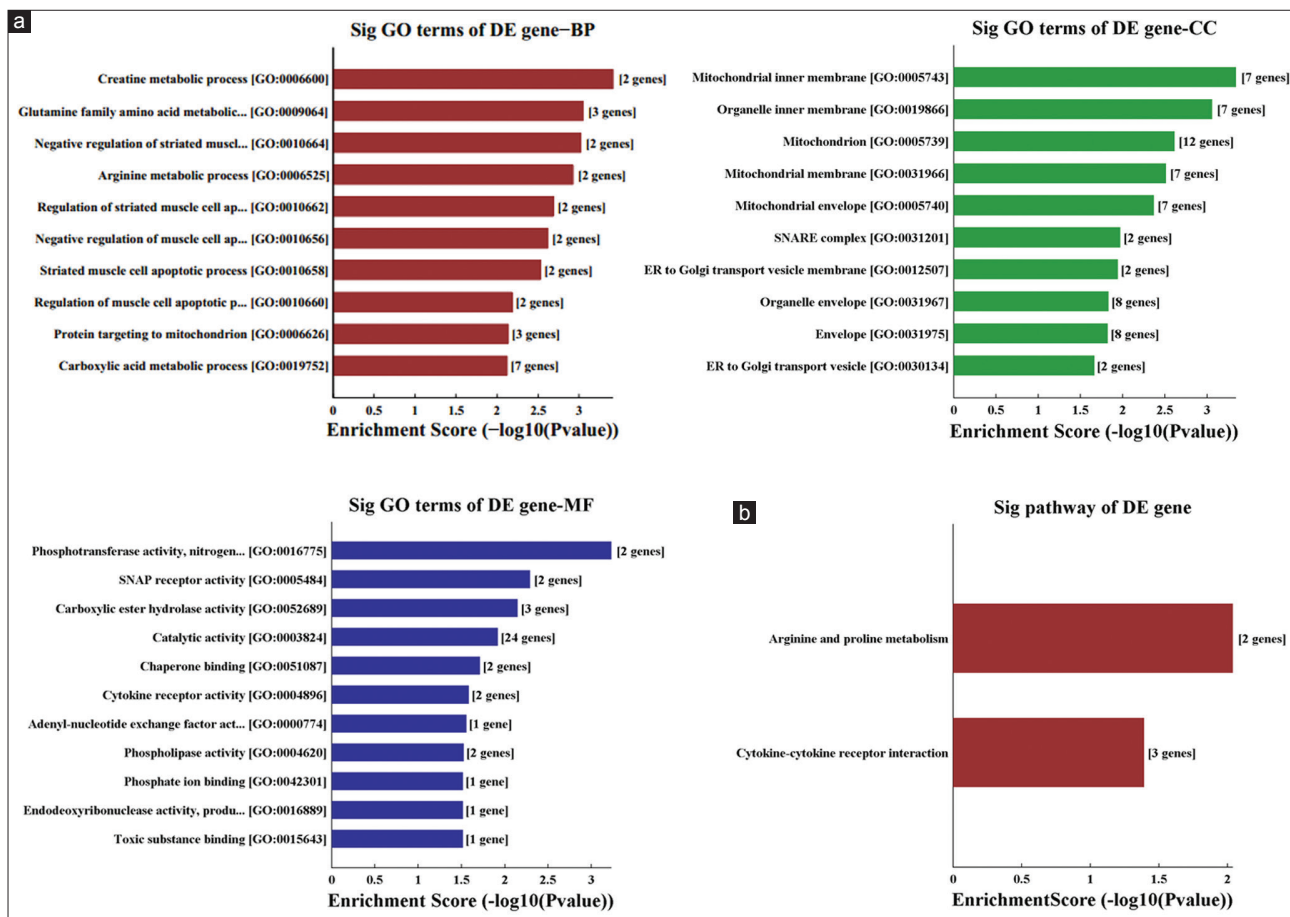
Figure 6: GO and KEGG analysis for the circRNA-targeted mRNAs. (a) GO enrichment for the target mRNAs, including biological processes, cell component, and molecular function. (b) Annotated pathways for the target mRNAs. GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; circRNAs: Circular RNAs.

Supplementary information is linked to the online version of the paper on the Chinese Medical Journal website.

Conflicts of interest

There are no conflicts of interest.

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环状RNA可作为鉴定卵巢子宫内膜异位症的一种新型生物标志物

摘要

背景： 子宫内膜异位症是一种极具挑战性的妇科疾病，主要症状为痛经和不孕。目前，它的发病机制尚不清楚，也没有有效的标记物和治疗办法。本研究的主要目的是分析卵巢子宫内膜异位症患者在位内膜中环状RNAs（circRNAs）的表达；探索子宫内膜异位症的潜在发病机制，为临床诊断和治疗提供依据。

方法： 收集2016年5月1日至2016年12月31日北京协和医院的标本共63例，其中，卵巢子宫内膜异位症患者的在位内膜41例，对照组内膜22例。每组各4例标本用于circRNAs的芯片分析，从中选取4个上调明显的circRNAs用于大样本qRT-PCR验证。之后采用生物信息学分析预测验证出的环状RNA的靶向miRNA及circRNA-miRNAs-mRNAs作用网络。

结果： circRNAs芯片结果显示卵巢子宫内膜异位症患者的在位内膜较对照组内膜相比，共88个差异表达的circRNAs，其中11个上调、77个下调。qRT-PCR验证结果显示*circ_0004712*和*circ_0002198*与芯片结果一致，其受试者特征曲线下的面积分别为0.704 (95%*CI*: 0.571 - 0.837; *P* = 0.008)、0.846 (95%*CI*: 0.752 - 0.939; *P* < 0.001)。根据靶基因预测，我们还描述了*circ_0004712*、*circ_0002198*与其主要靶miRNAs的相互作用，同时构建了一个circRNA-miRNA-mRNA作用网络。

结论： 本研究发现circRNA在在位内膜及对照组内膜之间的确存在差异，这表明circRNAs在卵巢子宫内膜异位症的发病过程中可能起着关键作用。此外，*circ_0004712*及*circ_0002198*可能成为诊断卵巢子宫内膜异位症的新型生物标记物。
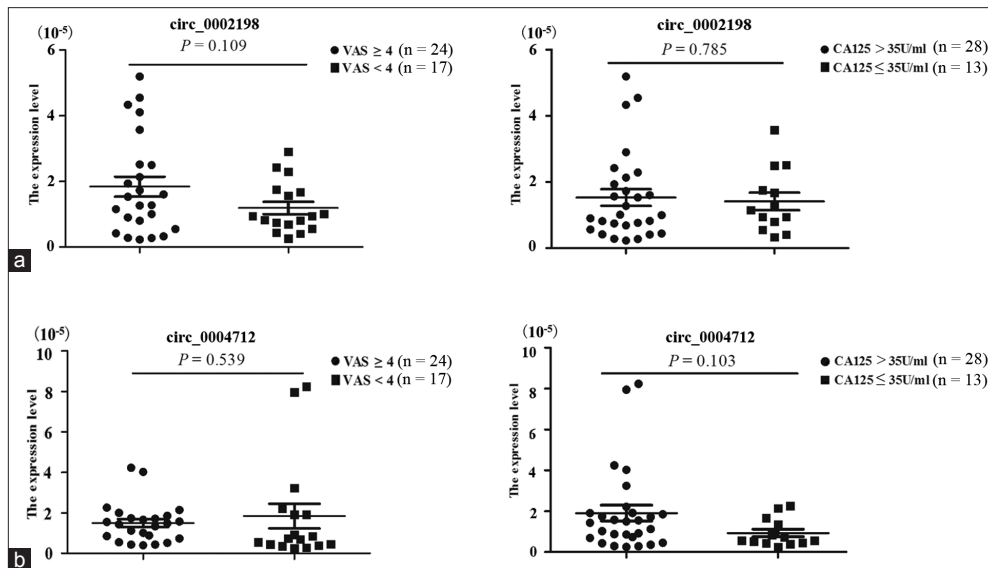
Supplementary Figure 1: The association between the two validated circRNAs and VAS or CA125. (a) The association between *circ_0002198* and VAS or CA125.(b) The association between *circ_0004712* and VAS or CA125. VAS: Visual analogue scale.

Supplementary Table 1: Primers used for qRT-PCR

Gene	Forward, 5'-3'	Reverse, 5'-3'
β-actin	GTGGCCGAGGACTTTGATTG	CCTGTAACAACGCATCTCATATT
circRNA_0004712	AGGGGTGAACCAGCCATTT	GCCAATCTCCCCTGAGTATGTT
circRNA_0002198	GCAAACCTATATCAGGAAACAGC	TTGAAGAGGTGGCACAACAGT
circRNA_0002503	CGTATTCACCTGCTCATCTCC	CTGGTGTTGGGATGATTTGA
circRNA_0000141	TCAGGCCCCATGCAGGTG	ACCATGCCACTCGGATCCTC

qRT-PCR: Quantitative real-time polymerase chain reaction.

Supplementary Table 2: Clinical characteristics of 41 patients with ovarian endometriosis

Patient	Cycle phase	Age (years)	Parity	Infertility	VAS	CA125	Diameter of cyst (cm)	rAFS stage	Indication for surgery
1	Proliferative	31	0	Yes	6.5	44.1	3.8	IV	Pain/infertility
2	Proliferative	33	0	Yes	8.0	81.8	2.5	IV	Pain/infertility
3	Proliferative	28	0	N/A	7.5	34.6	5.8	III	Pain
4	Proliferative	33	0	N/A	6.5	34.1	4.8	III	Pain
5	Secretory	45	2	No	0	54.1	7.6	IV	Cyst
6	Secretory	28	0	N/A	0	56.6	6.7	IV	Cyst
7	Secretory	28	1	No	4.5	28.1	4.6	III	Infertility
8	Proliferative	33	2	No	5.5	46.9	4.7	IV	Pain
9	Secretory	30	0	N/A	4.0	30.1	4.9	III	Pain
10	Proliferative	30	0	Yes	5.0	24.2	5.8	III	Pain/infertility
11	Proliferative	45	1	No	4.5	108.8	5.9	IV	Pain
12	Secretory	29	0	Yes	4.5	79.0	3.8	IV	Pain/infertility
13	Secretory	29	2	No	4.5	63.2	5.9	IV	Pain
14	Proliferative	32	2	No	0	1.9	6.4	III	Cyst
15	Secretory	27	0	N/A	7.0	60.5	5.3	IV	Pain
16	Proliferative	30	0	Yes	3.5	52.1	3.4	III	Pain/infertility
17	Proliferative	32	2	No	2.0	70.7	5.1	IV	Cyst
18	Proliferative	37	5	No	3.0	51.6	4.2	IV	Pain
19	Proliferative	32	1	No	4.0	23.1	7.0	III	Pain
20	Proliferative	28	0	N/A	0	51.0	5.0	IV	Cyst
21	Proliferative	39	2	No	5.5	37.1	6.5	III	Pain
22	Secretory	38	1	No	6.0	76.4	2.9	IV	Pain
23	Proliferative	25	0	N/A	0	60.5	7.3	IV	Cyst

Supplementary Table 2: Contd...

Patient	Cycle phase	Age (years)	Parity	Infertility	VAS	CA125	Diameter of cyst (cm)	rAFS stage	Indication for surgery
24	Proliferative	38	2	No	8.5	59.7	4.9	IV	Pain
25	Proliferative	24	0	N/A	4.0	40.3	6.7	III	Pain
26	Proliferative	25	0	N/A	7.5	30.2	5.9	IV	Pain
27	Proliferative	33	1	No	7.5	78.3	5.5	IV	Pain
28	Proliferative	30	0	N/A	0	37.4	5.3	III	Cyst
29	Secretory	25	0	N/A	2.0	42.0	8.3	IV	Cyst
30	Proliferative	34	3	No	0	185.2	6.6	III	Cyst
31	Secretory	36	0	Yes	0	29.3	4.5	IV	Infertility
32	Secretory	25	0	Yes	2.0	31.5	3.5	III	Infertility
33	Proliferative	31	0	N/A	5.5	56.6	4.8	IV	Pain
34	Proliferative	39	1	No	0	96.0	7.1	IV	Cyst
35	Secretory	46	1	No	0	22.4	3.6	III	Cyst
36	Proliferative	32	1	No	10.0	21.9	13.2	IV	Pain
37	Secretory	26	0	N/A	0	96.2	4.5	IV	Cyst
38	Proliferative	22	0	N/A	3.0	66.8	4.6	III	Pain
39	Proliferative	43	1	No	5.0	40.3	5.3	IV	Pain
40	Proliferative	29	0	N/A	5.0	24.4	7.0	IV	Pain
41	Proliferative	25	0	N/A	6.5	79.0	1.8	III	Pain

N/A: Never attempted pregnancy; VAS: Visual analogue scale; rAFS: Revised American Fertility Society.

Supplementary Table 3: Clinical characteristics of 22 patients without ovarian endometriosis

Patient	Cycle phase	Age (years)	Parity	Infertility	Pathologic diagnosis	VAS	CA125	Diameter of cyst (cm)	Indication for surgery
1	Proliferative	27	0	N/A	Teratoma	0	14	4.9	Cyst
2	Proliferative	26	0	N/A	Serous cystadenoma	0	12.3	10.4	Cyst
3	Proliferative	30	1	No	Teratoma	0	12.7	8.0	Cyst
4	Proliferative	27	0	Yes	Simple cyst	3.0	13.7	3.1	Cyst infertility
5	Secretory	34	1	No	Secretory endometrium	2.5	15.2	–	Uterine diverticula
6	Proliferative	25	1	No	Proliferative endometrium	0	9.3	–	Uterine septum
7	Secretory	29	0	N/A	Teratoma	0	14.3	4.6	Cyst
8	Secretory	32	0	N/A	Paraovarian cyst	3.5	18.8	6.8	Cyst
9	Proliferative	30	0	N/A	Teratoma	6.5	20.4	5.0	Cyst
10	Proliferative	31	1	No	Proliferative endometrium	0	22.3	–	Uterine septum
11	Proliferative	34	1	No	Mesosalpinx cyst	0	18.6	6.7	Cyst
12	Proliferative	43	2	No	Simple cyst	0	19.3	–	Cyst
13	Proliferative	32	0	N/A	Mesosalpinx cyst	4.5	20.2	4.0	Cyst
14	Secretory	39	5	No	Serous cystadenoma	3.5	17.7	6.4	Cyst
15	Proliferative	39	3	No	Teratoma	6.5	8.5	4.0	Cyst
16	Secretory	26	0	N/A	Teratoma	5.0	9.2	5.5	Cyst
17	Proliferative	37	6	No	Teratoma	0	19.7	4.1	Cyst
18	Proliferative	36	5	No	Teratoma	0	13.4	3.7	Cyst
19	Proliferative	33	2	No	Teratoma	0	29.8	3.6	Cyst
20	Proliferative	31	0	N/A	Serous cystadenoma	0	17.2	5.1	Cyst
21	Secretory	25	0	N/A	Teratoma	3.5	11.5	5.9	Cyst
22	Proliferative	34	2	No	Proliferative endometrium	3.5	8.7	–	Uterine diverticula

N/A: Never attempted pregnancy; VAS: Visual analogue scale.

Supplementary Table 4: The dominant miRNAs targeted by top 10 up- and downregulated circRNAs

circRNA	Alias	FC	P	Top five targeted miRNAs				
				1	2	3	4	5
Upregulation								
hsa_circRNA_104195	hsa_circ_0002198	4.63	7.600E-04	miR-455-3p	miR-876-3p	miR-661	miR-323a-5p	miR-198
hsa_circRNA_405510		4.43	4.259E-02	miR-4307	miR-4506	miR-5100	miR-497-5p	miR-6832-3p
hsa_circRNA_104194	hsa_circ_0004712	4.11	1.359E-03	miR-455-3p	let-7g-5p	miR-876-3p	miR-661	miR-323a-5p
hsa_circRNA_404646		3.33	3.341E-02	miR-3918	miR-4726-5p	miR-4640-5p	miR-6762-5p	miR-423-5p
hsa_circRNA_101501	hsa_circ_0034953	3.22	4.051E-02	miR-146b-3p	miR-506-5p	miR-298	miR-873-5p	miR-185-5p
hsa_circRNA_406483		2.88	2.691E-02	miR-335-3p	miR-3612	miR-5002-5p	miR-4709-3p	miR-493-3p
hsa_circRNA_075503	hsa_circ_0075503	2.43	2.998E-02	miR-4673	miR-4645-5p	miR-1226-5p	miR-548b-3p	miR-383-5p
hsa_circRNA_002503	hsa_circ_0002503	2.27	4.520E-02	miR-6760-3p	miR-1182	miR-6837-5p	miR-4685-5p	miR-372-3p
hsa_circRNA_026462	hsa_circ_0026462	2.25	3.844E-02	miR-22-3p	miR-1908-3p	miR-1275	miR-6501-5p	miR-6829-3p
hsa_circRNA_103002	hsa_circ_0004816	2.08	9.380E-03	miR-330-5p	miR-449b-5p	miR-449a	miR-194-5p	miR-34c-5p
Downregulation								
hsa_circRNA_001062	hsa_circ_0001062	9.86	1.029E-04	miR-4307	miR-4753-3p	miR-6809-3p	miR-6873-3p	miR-607
hsa_circRNA_004183	hsa_circ_0004183	4.35	2.551E-02	miR-7162-5p	miR-6875-3p	miR-516b-3p	miR-516a-3p	miR-4687-3p
hsa_circRNA_083996	hsa_circ_0083996	3.64	3.337E-03	miR-581	miR-7161-3p	miR-6134	miR-7847-3p	miR-6780b-5p
hsa_circRNA_092547	hsa_circ_0001445	3.60	3.803E-04	miR-6740-3p	miR-4798-5p	miR-507	miR-1285-5p	miR-3664-5p
hsa_circRNA_406544		3.42	1.569E-03	miR-1285-5p	miR-335-3p	miR-17-3p	miR-4422	miR-627-3p
hsa_circRNA_001050	hsa_circ_0001050	3.27	1.657E-03	miR-3653-5p	miR-6758-5p	miR-4325	miR-4482-3p	miR-653-5p
hsa_circRNA_059914	hsa_circ_0059914	3.09	1.388E-02	miR-377-5p	miR-6086	miR-4756-3p	miR-103b	miR-767-3p
hsa_circRNA_101231	hsa_circ_0000467	3.08	2.611E-02	miR-153-5p	miR-382-5p	miR-520g-3p	miR-549a	miR-520h
hsa_circRNA_002082	hsa_circ_0002082	2.85	3.809E-03	miR-512-5p	miR-4773	miR-3611	miR-4742-3p	miR-6887-3p
hsa_circRNA_405210		2.83	3.729E-03	miR-6833-3p	miR-4659a-3p	miR-6809-3p	miR-4659b-3p	miR-4768-5p

circRNAs: Circular RNAs; FC: Fold change.