CircRNA expression profiles in decidual tissue of patients with early recurrent miscarriage

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Abstract
Circular RNAs (circRNAs) are a novel class of endogenous noncoding RNAs that play important roles in gene expression regulation. This study aimed to evaluate the potential role of circRNAs in decidual tissue of patients with early recurrent miscarriage (RM). We constructed circRNA expression profiles in decidual tissue using microarray data. A total of 123 differentially expressed circRNAs, including 78 upregulated and 45 downregulated circRNAs were detected in the early RM group compared with the control group (\( P < 0.05 \)). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis also revealed the enrichment of specific circRNAs. The verified circRNA-targeted miRNA-
CircRNA expression profiles in decidual tissue of patients

Introduction

Early recurrent miscarriage (RM), defined as three or more consecutive pregnancy losses before 12 weeks of gestation with or without previous live births, occurs in approximately 1–3% of couples. In recent years, progress in the fields of cytogenetics and immunogenetics as well as improved understanding of implantation and maternal embryo interactions have provided new insights into the possible causes of this condition. Previous studies have demonstrated that infectious diseases, uterine pathologies, parental predisposition, endocrine dysfunctions, embryonic genetic disorders, autoimmune diseases, acquired and inherited thrombophilia, and environmental factors are at least partly responsible for RM. Nevertheless, in at least 50% of RM patients no cause can be identified. Recent studies have revealed the relevance of noncoding RNAs in the pathogenesis of RM. However, the roles of circular RNAs (circRNAs) in RM are unknown.

CircRNAs are a novel class of endogenous noncoding RNAs that play important roles in gene expression regulation. They are single RNA molecules with covalently linked ends, and are highly stable compared with their linear counterparts. Previously, circRNAs were considered to have uncertain biological importance and thought to be generated by splicing errors. However, recently, an explosion of research into all aspects of circRNA biology points to vital roles of these molecules in cellular homeostasis and disease development. Previous research revealed that the human circRNA circRS-7 is resistant to miRNA-mediated target destabilization and strongly suppresses miR-7 activity, which is involved in different diseases. A more recent study showed that circRNA_100290 may function as a competing endogenous RNA to regulate CDK6 expression by sponging miR-29b family members, and represents a potential target for human oral squamous cell carcinoma therapy. However, the potential role of circRNAs in RM remains unclear.

In this study, we analyzed the expression profiles of circRNAs in the decidual tissue from patients with early RM. Target genes of the selected circRNAs were predicted and assessed using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses. This work will help understand the potential biological functions of circRNAs and their pathophysiological mechanisms in early RM.

Materials and methods

Clinical specimens and ethics statements

Uterine decidual tissues were collected at the Department of Family Planning at Chongqing Health Centre for Women and Children (Chongqing, China) from December 2016 to September 2017. The decidual tissues from 4 women aged 25–35 years were obtained by dilation and curettage after spontaneous abortion at 6–10 weeks of gestation. Normal decidual tissues were obtained from 4 women aged 26–34 years during early pregnancy (6–10 weeks) by dilatation and curettage after termination of pregnancy. The clinical data are presented in Table 1. Inclusion criteria for early recurrent miscarriage were (1) two or more consecutive pregnancy losses and (2) no history of successful pregnancy. Exclusion criteria for early miscarriage were infections, endocrine or metabolic disorders, anatomic abnormalities, autoimmune diseases, paternal or maternal chromosomal abnormalities and abnormal karyotyping of the miscarriage product. Inclusion criteria for the normal controls were (1) elective termination of normal pregnancy, (2) with at least 2 live births and (3) no history of miscarriage, preeclampsia, ectopic pregnancy, preterm delivery and systemic diseases. The anatomic, hormonal and disease profiles of patients in both groups are summarized in Table 1. After dilation and curettage, decidual tissues were immediately separated from the products of conception, washed thoroughly with sterile normal saline and finally stored in liquid nitrogen for future use. All karyotypes were normal for the miscarriage products obtained (Table 2).

Table 1 Clinical Characteristics of the subjects.

| Characteristics          | Normal     | Unexplored recurrent miscarriage | P value |
|--------------------------|------------|---------------------------------|---------|
| Mean age (years)         | 30.5 ± 3.11| 30.75 ± 3.09                    | >0.05   |
| Smoking (%)              | 0%         | 0%                              | NA      |
| Mean BMI(kg/m2)          | 20.8 ± 1.04| 20.9 ± 1.06                     | >0.05   |
| Menarche (years)         | 13.25 ± 0.96| 13.5 ± 1.29                    | >0.05   |
| Gestational age          | 7.5 ± 0.85 | 7.75 ± 0.50                     | >0.05   |
| Number of pregnancies    | 2.5 ± 0.58 | 4.5 ± 1.91                      | <0.01   |
| Miscarriage              | 0 ± 0      | 4.25 ± 1.5                      | <0.01   |

Statistical analysis was performed by Student’s t-test. The results are presented as mean ± SEM. Boldface data indicate significant differences with the control.
Centrations were determined by OD260 reading on a NanoDrop ND-1000 spectrophotometer according to the manufacturer’s instructions with the following parameters: OD A260/A280 ratio, ~2.0 for pure RNA (ratios between 1.8 and 2.1 were acceptable); OD A260/A230 ratio, >1.8. RNA integrity was assessed by standard denaturing agarose gel electrophoresis.

**CircRNA microarray analysis**

Sample preparation and microarray hybridization were performed with Array star (Aksomics, Rockville, MD, USA) according to the manufacturer’s instructions. CircRNAs were enriched by removing linear RNAs with RNase R (Epicentre, Madison, WI, USA), and amplified and labeled with Array star Super RNA Labeling Kit (Array star). The labeled circRNAs were hybridized to the Array star Human circRNA Array V2 (8 x 15K, Array star). After slide washing, the arrays were scanned on an Agilent G2505C Microarray Scanner. The Agilent Feature Extraction software (version 11.0.1.1) was used to analyze the acquired array images. Quantile normalization and subsequent data processing were performed using the R software limma package. CircRNAs demonstrating fold changes >1.5 and P-values >0.05 were regarded as significantly differentially expressed. Volcano plot and hierarchical clustering were performed to show the distinguishable expression patterns between the two groups. CircRNAs microarray analysis was performed by Kangchen Bio-tech (Shanghai, China).

**Real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR)**

Total RNA was extracted using TRIzol reagent (Invitrogen). Reverse transcription was performed with Super Script TM III Reverse Transcriptase (Invitrogen). Primer sequences are listed in Table 3. Quantification of circRNAs, miRNA and mRNA was performed on an ABI PRISM 7500 system (Applied Biosystems, Carlsbad, CA, USA). Data were analyzed by the \(-\Delta\Delta^{CT}\) method, with \(\beta\)-actin used to normalize the relative circRNA, miRNA and mRNA expression levels. All samples were evaluated in triplicate with negative and positive controls on each plate.

**GO and KEGG analyses.** GO, which describes genes from any organism (http://www.geneontology.org) was used, covering the domains of Biological Process (BP), Cellular

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### Table 2: Anatomic, hormonal and disease profiles of patients.

| Item                                    | RM group | Control group |
|-----------------------------------------|----------|---------------|
| Parental chromosomes                    | No       | No            |
| FSH (U/L)                               | 3–8      | 3–8           |
| LH (U/L)                                | 3–8      | 3–8           |
| Troponin (ng/ml)                         | <0.016   | <0.016        |
| Antiphospholipid antibodies              | Negative | Negative      |
| Lupus anticoagulant (s)                 | 33–40    | 33–40         |
| Anti-cardiolipin antibodies              | Negative | Negative      |
| Karyotyping of the miscarriage product  | No       | No            |
| Karyotyping of both partners             | No       | No            |
| Activated protein C resistance           | Negative | Negative      |
| Factor V Leiden                         | Negative | Negative      |
| Prothrombin mutations                   | Negative | Negative      |
| Prolactin (ng/ml)                       | 6–26     | 6–26          |
| Fasting plasma glucose (mmol/L)         | 4.0–5.9  | 4.0–5.9       |
| Thyroid stimulating hormone (mIU/L)     | 0.35–4.5 | 0.35–4.5      |
| Free thyroxin T4 (pmol/L)               | 10–19    | 10–19         |
| Free triiodothyronine (pmol/L)          | 3–5.7    | 3–5.7         |
| Thyroid peroxidase antibody (IU/ml)     | <5.6     | <5.6          |
| Toxoplasmosis IgG (IU/ml)               | <1.6     | <1.6          |
| Toxoplasmosis IgM (S/CO)                | <0.8     | <0.8          |
| Cytomegalovirus IgG (AU/ml)             | <6.0     | <6.0          |
| Cytomegalovirus IgM (S/CO)              | <0.85    | <0.85         |
| Rubella IgG (IU/ml)                     | <5.0     | <5.0          |
| Rubella IgM (S/CO)                      | <0.75    | <0.75         |
| HIV-ag/ab (S/CO)                        | <1.0     | <1.0          |
| Group B Streptococci-DNA                | Negative | Negative      |
| Chlamydia trachomatis-DNA               | Negative | Negative      |
| Hepatitis B-Sag (IU/ml)                 | <0.05    | <0.05         |
| Hepatitis C-Ab (S/CO)                   | <1.0     | <1.0          |
| Bacterial vaginosis                     | Negative | Negative      |
| Uterine cavity                          | No       | No            |
| Hysterosalpingography                   | No       | No            |
| serial ultrasound                       | No       | No            |

All patients included in the current study provided written consent before surgery, and the Institutional Review Board for Ethics of Chongqing Medical University and Chongqing Health Centre for Women and Children approved the study protocol.

**RNA isolation and quality control**

Total RNA was isolated using TRIzol (Invitrogen). RNA concentrations were determined by OD260 reading on a NanoDrop reagent according to the manufacturer’s instructions with the following parameters: OD A260/A280 ratio, ~2.0 for pure RNA (ratios between 1.8 and 2.1 were acceptable); OD A260/A230 ratio, >1.8. RNA integrity was assessed by standard denaturing agarose gel electrophoresis.
Component (CC) and Molecular Function (MF). Pathway analysis was carried out for functional analysis to map genes to KEGG pathways (http://www.genome.jp/kegg). Fisher’s exact test, the chi-square test and the false discovery rate (FDR) were used for significance detection, where the P-value denotes the significance of the GO term and pathway correlated with the condition. A small FDR indicates a small error in judging the P-value.

CircRNA-miRNA-mRNA network construction

CeRNA hypothesis RNA transcripts can crosstalk by competing for common microRNAs, with microRNA response elements (MREs) as the foundation of this interaction. Any RNA transcript with MREs might act as ceRNA, and ceRNAs include pseudogene transcripts, lncRNAs, circRNAs and mRNAs, these transcripts can compete for the same microRNA response elements (MERS) to regulate mutually. To find potential target of microRNAs, the target/microRNAs is predicted with home-made miRNA target prediction software based on TargetScan & miRanda.

Statistical analysis

Mean ± standard deviation (SD) was used to present quantitative data. Significant differences between two groups with normal distribution were estimated by t-test, while two groups with abnormal distribution were estimated by nonparametric test. CircRNAs/mRNAs with fold changes ≥1.5 and P-values <0.05 were considered to be differentially expressed, corrected by Bonferroni method.

Results

CircRNA expression profiles in early RM

Hierarchical clustering was performed to assess the various circRNA expression patterns among samples (Fig. 1A). In total, 123 significantly expressed circRNAs were identified, including 78 upregulated and 45 downregulated circRNAs (P < 0.05). The top 6 upregulated and 6 downregulated circRNAs with fold changes above 1.5 are listed in Table 4. For these circRNAs, box (Fig. 1B), scatter (Fig. 1C) and volcano plots (Fig. 1D) were produced to show variations in

Figure 1  CircRNA expression profiles in early RM. (A) A cluster heat map showing the differentially expressed circRNAs with over a 1.5-fold change. Red indicates a high expression level, and green indicates a low expression level. (B) The box plot shows the distributions of circRNAs in a direct way. (C) The scatter plot was used for assessing the variation in circRNA expression between the test and control samples. The values of the x- and y-axes in the scatter plot are the log2 normalized signal values of the samples (log2 scaled). The green lines are fold-change lines. The circRNAs above the top green line and below the bottom green line indicate a more than 1.5-fold change in circRNA expression between the two compared samples. (D) The volcano plot was constructed using fold-change values and P-values. The vertical lines correspond to a 1.5-fold change up and down, and the horizontal line represents a P-value of 0.05. The red point in the plot represents the significantly differentially expressed circRNAs. (E) The classification of circRNA types among upregulated and downregulated circRNAs.
circRNA expression between the RM and normal groups in a more direct way. Approximately 92% of all upregulated circRNAs were exonic; 2% and 4% were intronic and sense overlapping, respectively. In contrast, 69% of the downregulated circRNAs were exonic and 18% intronic; 7%, 4% and 2% were sense overlapping, antisense and intergenic, respectively (Fig. 1E). These data revealed distinctive circRNA expression profiles in RM samples.

| circRNA              | Regulation | Symbol   | FC(abs)    | P-value   |
|----------------------|------------|----------|------------|-----------|
| hsa_circRNA_103092   | Up         | CYP24A1  | 4.9284325  | 0.037623179|
| hsa_circRNA_062557   | Up         | CHCHD10  | 4.4542227  | 0.031336967|
| hsa_circRNA_000479   | Up         | EPSTI1   | 3.1997295  | 0.01541564 |
| hsa_circRNA_103091   | Up         | CYP24A1  | 2.9721579  | 0.023352465|
| hsa_circRNA_103093   | Up         | CYP24A1  | 2.890573   | 0.006884006|
| hsa_circRNA_100917   | Up         | PICALM   | 1.8940515  | 0.031945817|
| hsa_circRNA_048977   | Down       | ZNF358   | 2.9098372  | 0.024454049|
| hsa_circRNA_043944   | Down       | VAT1     | 2.721594   | 0.030690389|
| hsa_circRNA_091761   | Down       | BCAP31   | 2.4802884  | 0.043924533|
| hsa_circRNA_037366   | Down       | NDUFB10  | 2.1525704  | 0.045942371|
| hsa_circRNA_104179   | Down       | ZUFSP    | 1.9384605  | 0.02309318565|
| hsa_circRNA_100468   | Down       | TMEM63A  | 1.6481241  | 0.02534861166|

Figure 2 Validation of four randomly selected differential circRNAs. (A) The relative expression levels of four randomly selected differential circRNAs, between early RM and control group. (B) Comparison between qRT-PCR and microarray results reveal a good correlation of such two methods. circRNAs: circular RNAs; qRT-PCR: quantitative reverse transcription polymerase chain reaction.
Validation of circRNA expression in the decidual tissue by qRT-PCR

Four differentially expressed circRNAs were randomly chosen to verify the microarray results in the decidual tissue. Expression of hsa_circRNA_103092 and hsa_circRNA_103093 were significantly upregulated while that of hsa_circRNA_104179 and hsa_circRNA_100468 were downregulated in the RM group (Fig. 2A). Their expression patterns were similar to those observed in the microarray analysis results, which indicated that our microarray analysis results were reliable (Fig. 2B).

Recent evidences have demonstrated that circular RNAs play a crucial role in fine tuning the level of miRNA mediated regulation of gene expression by sequestering the miRNAs. Their interaction with disease associated miRNAs indicates that circular RNAs are important for disease regulation.19 To facilitate researcher’s study, the circRNA/microRNA interaction was predicted with Arraystar’s homemade miRNA target prediction software based on Targetscan (18) & miRanda.20 According to our data, miR-224 and miR-522-3p interact with multi-circRNAs. So, we focused on circRNAs interaction with miR-224 and miR-522-3p. Three upregulated circRNAs (hsa_circRNA_103091, hsa_circRNA_103092, hsa_circRNA_103093) were related with miR-224 (Fig. 3) and miR-522-3p (Fig. 4).

GO and KEGG pathway analysis of the host genes of differentially expressed circRNAs

Reports revealed that circRNAs can regulate the expression of the host genes.21 Then GO and KEGG pathway analysis of the host genes of differentially expressed circRNAs were performed. The ontology covers three domains: Biological Process (BP), Cellular Component (CC) and Molecular Function (MF). The top 10 dysregulated GO processes and KEGG pathways are shown in Fig. 5. For upregulated circRNAs, the top 3 GO processes included multicellular organism development, system development and anatomical structure development in the BP group (Fig. 5A), intracellular, intracellular part, intracellular organelle in the CC (Fig. 5B) and protein binding, binding and ubiquitin-like protein ligase activity in the MF group (Fig. 5C). Axon guidance, signaling pathways regulating pluripotency of stem cells and breast cancer were the top 3 KEGG pathways (Fig. 5D).

Construction of the circRNA-miRNA-mRNA network

Considering that some circRNAs can act as “miRNA sponges” by efficiently binding and inhibiting miRNA transcription, consequently influencing downstream mRNA expression and contributing to various diseases.22 Through merging the common targeted miRNAs, we constructed ceRNA network (Fig. 6). The network involves three circRNAs (hsa_circRNA_104179, hsa_circRNA_103092, hsa_circRNA_103093), 27 microRNAs (miR-224-3p, miR-522-3p, hsa-miR-421, etc), 82 mRNAs (H2AFV, DIMT1, EXOSC8, etc).

Validation of identified circRNA and their network

To further confirm the ceRNA network, we randomly selected one circRNA-miRNA-mRNA pathway...
hsa-miR-522-3p vs hsa_circRNA_103091

| 2D Structure | Local AU | Position | Conservation | Predicted By |
|--------------|----------|----------|--------------|--------------|
| 363 5’-acACTCAGTGGAGAACACCATTTAC-3’ UTR 3’ pairing | ACCATT | 5 mer-8 | X | M T |
| 718 5’-ctttaccgggtgtACACATTAC-3’ UTR 3’ pairing | ACCATT | 8 mer-7 | X | M T |

hsa-miR-522-3p vs hsa_circRNA_103092

| 2D Structure | Local AU | Position | Conservation | Predicted By |
|--------------|----------|----------|--------------|--------------|
| 554 5’-acACTCAGTGGAGAACACCATTTAC-3’ UTR 3’ pairing | ACCATT | 5 mer-8 | X | M T |
| 980 5’-ctttaccgggtgtACACATTAC-3’ UTR 3’ pairing | ACCATT | 8 mer-7 | X | M T |

hsa-miR-522-3p vs hsa_circRNA_103093

| 2D Structure | Local AU | Position | Conservation | Predicted By |
|--------------|----------|----------|--------------|--------------|
| 172 5’-acACTCAGTGGAGAACACCATTTAC-3’ UTR 3’ pairing | ACCATT | 5 mer-8 | X | M T |

Figure 4  hsa_circRNA_103091, hsa_circRNA_103092 and hsa_circRNA_103093 contain complementary sequence and diverse binding sites with miR-522-3p.

Figure 5  GO analysis and 10 KEGG pathways of dysregulated circRNAs. (A,B,C) GO analysis of dysregulated circRNAs based on the values in the enrichment score under the theme of BP, CC and MF. (D)Ten KEGG pathways of dysregulated circRNAs.
Discussion

In recent years, circRNAs, novel noncoding RNAs with distinct properties, have gained much interest. circRNAs are an abundant class of endogenous RNAs, which have recently been rediscovered and re-evaluated for their important roles in the regulation of gene expression. circRNAs, which resist to exonuclease RNase R, are highly stable and highly conserved among the species. Recently, circRNAs emerged for many biological functions, especially their association with human diseases and in particular with cancer. As miRNA sponges, circular RNAs attract the most focus, can function as oncogenes or as tumor suppressor genes. However, the expression and potential functional roles of circRNAs in decidual tissues of RM remain largely unknown.

Our study was the first to screen the differential expression profiles circRNAs in decidual tissues of women with early RM. There were 78 upregulated and 45 downregulated circRNAs in the decidual tissue compared with the normal controls. Four circRNAs (hsa_circRNA_103092, hsa_circRNA_103093, hsa_circRNA_100468) were verified by qRT-PCR. The results

tissues of RM as compared to those in the controls (Fig. 7A and B). PRLR, a potential target mRNA, was significantly increased (Fig. 7C).
showed that the expression patterns of circRNAs were similar to those observed in the microarray analysis.

Studies have showed that circRNAs play an important role in regulation of the host genes expression.27,29 Then we performed GO and KEGG pathway analyses. The top 10 KEGG pathways were Axon guidance, signaling pathways regulating pluripotency of stem cells, breast cancer, pathway in cancer, ubiquitin mediated proteolysis, TGF-beta signaling pathway, Hippo signaling pathway, neurotrophin signaling pathway, Wnt signaling pathway and Endocytosis. Those pathways almost participate in immune response and inflammatory mediation, which were verified a significant interaction with recurrent miscarriage.30 Therefore, we speculate that circRNAs may involve in recurrent miscarriage by regulating their host genes.

Meanwhile, circRNAs can regulate miRNA-targeted gene expression, transcription and protein synthesis as ceRNA molecules or efficient miRNA sponges.8,11 In this study, we found that some dysregulated circRNAs harbored one or more binding sites for miRNAs. For example, miR-224 and miR-522-3p were predicted to interact with hsa_circRNA_103091, hsa_circRNA_103092, hsa_circRNA_103093. A number of studies have identified aberrant expression of miR-224 in different types of neoplasms. For example, elevated miR-224 expression levels were noted in cervical cancer,31 ovarian cancer,32 breast cancer,33 lung cancer34 and glioma.35 MiR-224 can target different genes to promote cell proliferation, to control cell invasion and expression of metastasis. MiR-522-3p can promote tumorigenesis in human colorectal cancer.36 A study found that miR-522-3p might be involved in abnormal resolution of inflammation.37

Acting as miRNA sponges, circRNAs can bind miRNAs through miRNA response element (MRE) and negatively regulate their activity.38 We constructed circRNA-miRNA-mRNA network. The network involves three circRNAs (hsa_circRNA_104179, hsa_circRNA_103092, hsa_circRNA_103093), 27 microRNAs (miR-224-3p, miR-522-3p, hsa-miR-421, etc), 82 mRNAs (H2AFV, DMT1, EXOSC8, etc). The target genes revealing in the network, which participate in kinds of pathways are associated with miscarriage. For instance, TGF-beta is important in the maternal support of embryo development and participates in modulating the extent of decidual invasion.39

One circRNA-miRNA-mRNA pathway was selected for network validation by qRT-PCR. While the expression levels of both hsa_circRNA_103092 and PRLR were significantly increased, miR-224 expression was significantly decreased. PRLR was previously deemed as an essential component for endometrial receptivity, and a possible association was suggested between PRLR and recurrent miscarriage.39 Based on our initial results, hsa_circRNA_103092 may participate in recurrent miscarriage through upregulating the activity of PRLR via its binding to miR-224.

Overall, this study shows for the first time the circRNA profiles in decidual tissues from patients with early RM. Bioinformatics analysis further predicted the potential effects of these differentially expressed circRNAs and their possible interactions with miRNAs and mRNAs. The present study suggested that it is meaningful to further investigate the differential expression of circRNAs as miRNA sponges and their potential functions in the pathogenesis of or therapy for early RM. Further researches on the detailed mechanisms of those pathways are underway.

Conflict of interest

The authors declare no conflict of interest.

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