

Analysis of the circular RNA transcriptome in the grade 3 endometrial cancer

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Background/aims: Circular RNAs (circRNAs), a class of newly discovered endogenous non-coding RNAs, have shown large capabilities in gene regulation. Patients with the grade 3 endometrial cancer (EC) have a generally poor prognosis, and the specific role of circRNAs in the grade 3 EC remains unclear. This study aims to investigate the roles of circRNAs in the grade 3 EC.

Methods: In the current study, we screened the expression profiles of circRNAs taken from two women with the grade 3 EC and adjacent non-cancerous endometrial tissue using circRNAs sequencing. Bioinformatic analyses were applied to study these differentially expressed circRNAs. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) of six dysregulated circRNAs was performed to validate the sequencing results. Bioinformatic analyses, including the negative correlation network analyses of circRNAs–microRNAs (miRNAs)–messenger RNAs (mRNAs) and the Cytoscape, were used to delineate the interaction of circRNAs/miRNAs of the entire network.

Results: Data of circRNA sequencing showed a significant change in 75,928 unique circRNAs ($P < 0.05$). The upregulated hsa_circ_0039569 and hsa_circ_0001610 and down-regulated hsa_circ_0000437, hsa_circ_0001776, and hsa_circ_0009043 were validated by qRT-PCR analysis. Using bioinformatical methods, we found that hsa_circ_0039569 has the MRE of hsa-miR-542-3p and hsa-let-7c-5p. Hsa-miR-542-3p and hsa-let-7c-5p were down-regulated in the grade 3 EC validated by qRT-PCR analysis. In the clinicopathological parameters, the expression level of hsa_circ_0039569 was significantly correlated with tumor differentiation ($P = 0.001$).

Conclusion: This is the first study demonstrated that there were a lot of differences between the tissue of the grade 3 EC and adjacent non-cancerous endometrial in circRNA expression and may offer novel molecular candidates for diagnosis and clinical treatment of the grade 3 EC.

Keywords: grade 3 endometrial cancer, circRNAs, transcriptome, RNA-Seq

Introduction

Endometrial cancer (EC) is the most common gynecological malignancy of the female reproductive system. Traditionally, EC was divided into type 1 and type 2,¹ grades 1 and 2 are considered “type 1” EC, while grade 3 is considered “type 2”. Type 1 may arise from atypical hyperplasia and is associated with excess progesterone and estrogen receptor. In addition, the prognosis is relatively good. Type 2 may arise from atrophic endometrium and is associated with a lack of estrogen and progesterone receptor. However, it shows the worse prognosis than type 1 because of the aggressive cancerous tissue.

Non-coding RNAs (ncRNAs) play an important role in epigenetics increasingly. NcRNAs can be divided into circular non-coding RNAs (circRNAs) and linear

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ncRNAs. However, in recent years, we have observed the explosive growth of published research on various aspects of circRNA biology. It indicates that this molecular plays an important role in normal cellular differentiation, tissue homeostasis as well as in the development of the disease. As it is known that circRNAs arise from precursor mRNAs, their biogenesis remains unspecific. circRNAs differ from other RNAs in their extraordinary continuous closed loop structure, which is covalently linked by free 3' and 5' ends.² It is also called a “back-splicing” structure,³ and is generated from the joining of an upstream 3' splice site to a downstream 5' splice site, which prevents them from degradation by RNA exonucleases.⁴ There are many biological functions of circRNA, and the most important function is to regulate gene expression. An increasing number of studies have found that many circRNAs as miRNA sponges or competitive endogenous RNAs (ceRNAs) can regulate the expression of miRNA targets, hsa_circ_0072309 inhibits proliferation and invasion of breast cancer cells via sponging miR-492,⁵ hsa_circZNF609 promotes breast cancer cell growth, migration, and invasion via sponging miR-145-5p.⁶ In addition to acting as miRNA sponges, circRNAs can also regulate the transcription and alternative splicing. The experiment of co-localization indicated that circRNAs could interact with RBPs. It has been reported the interaction among circ-Foxo3 with E2F1, ID-1, HIF α , and FAKs played a part in anti-aging and anti-stress.⁷ Besides the non-coding function of circRNAs, scientists have also studied the capacity of translation of circRNAs.⁸ Based upon the functions of circRNAs, researchers have studied the role of circRNAs in pathology and physiology. The evidence show that circRNAs exert a regulatory function in different diseases via different mechanisms. Moreover, circRNAs have the potential to serve as clinical diagnosis markers and therapeutic targets.⁹

To date, only one study has shown that circRNAs may play roles in EC, with grade (1–2) type I EC.¹⁰ However, it was known little about the underlying regulatory mechanisms of circRNAs with the grade 3 EC. To our knowledge, this was the first study of circRNAs transcriptome analysis of the grade 3 EC in the worldwide. We made a description about the factor of circRNAs transcriptome analysis of the grade 3 EC, detected the differentially expressed circRNAs, and made a further analysis of the related microRNAs of the differentially expressed circRNAs. In short, the differentially expressed circRNAs might become the diagnosis, treatment, and the potential biomarker of the prognosis of the grade 3 EC.

Materials and methods

Ethics statement

The project was approved and supervised by the Ethics Committee of the First Affiliated Hospital of Jinan University, Guangzhou, People's Republic of China. All samples were collected from the patient who had undergone surgery in the First Affiliated Hospital of Jinan University. All the enrolled patients have signed the informed consent. This study was conducted in accordance with the Declaration of Helsinki.

EC and normal tissue samples

Tissues were collected from the patients with EC undergone surgery at the Department of Surgery at the First Affiliated Hospital of Jinan University, and the nontumorous tissue was collected at the site 10 mm away from the affected region at the same time. The tumor specimen was staged and classified by pathological result. Two pairs of snap-frozen endometrial tissue with the grade 3 EC and adjacent non-cancerous endometrial tissue were collected for circRNA sequencing analysis (Tables 1 and 2) and another 60 samples (15 pairs of the grade 3 EC and adjacent non-cancerous endometrial tissue, 15 pairs of the grade 1–2 EC and adjacent non-cancerous endometrial tissue) were collected for qRT-PCR validation.

Table 1 Patient and tissue sample chart

Sample No.	Condition	Patient No.
A1	EC	1
A2	Normal	
B1	EC	2
B2	Normal	
E1	A1+B1	
E2	A2+B2	

Table 2 Detailed clinical pathological parameters of patients being sequenced

Clinicopathological parameters	Patient 1	Patient 2
Age (years old)	72	67
Histopathologic grades	G3	G3
FIGO stage	Ia	Ia
Myometrial invasion	<1/2	<1/2
Lymph node metastasi	Negetive	Negetive
BMI	24.56	25.67
Diabetes	No	No
Hypertension	No	No

RNA isolation

We used TRIzol (Invitrogen, Carlsbad, CA, USA) to extract total RNA from each frozen specimen and treating them with DNase (Takara, Dalian, People's Republic of China) according to the manufacturer's instructions. The RNA integrity was assessed using an Agilent Bioanalyzer2100 (Agilent Technologies, Santa Clara, CA, USA). The quantity (ng/mL) and purity (260/280 and 260/230 ratios) of total RNAs were assessed using NanoDrop 2,000 spectrophotometer (Thermo, Waltham, MA, USA) and denaturing agarose gel electrophoresis. Each RNA sample had an A260:A280 ratio above 1.9 and an A260:A230 ratio above 1.8. The values of RIN of A1, A2, B1, and B2 were 8.8, 8.7, 8.9, and 8.6.

Circrnas sequencing analysis

RNA library preparation and circRNA sequencing were performed by the Beijing Genomics Institute (Shengzheng, People's Republic of China). The sample preparation and microarray hybridization were performed according to the Arraystar's standard instructions. In short, total RNA samples were digested with RNase R (Epicenter Inc, San Francisco, CA, USA) to remove linear RNAs and enrich circRNAs. The rRNA-depleted RNA was used to build up the RNA libraries with TruSeq Stranded Total RNA Library Prep Kit (Illumina, San Diego, CA, USA). Quality of libraries were checked with BioAnalyzer 2100 system (Agilent Technologies, Richardson, TX, USA). The RNA libraries were denatured as single-stranded DNA molecules. The cDNAs were captured on Illumina Flow Cells (Illumina, San Diego, CA, USA) amplified in situ as clusters and finally sequenced with 150-bp paired reads on HiSeq 4000 sequencing system (Illumina, San Diego, CA, USA).

We used Cluster and Tree View software to perform hierarchical clustering analysis, in order to analyze the differentially expressed circRNAs between the grade 3 EC and adjacent non-cancerous endometrial tissue. The predicted functions of the differentially expressed circRNAs between the grade 3 EC and adjacent non-cancerous endometrial tissue were obtained by GO (<http://www.geneontology.org/>) and KEGG pathway analysis (<http://www.genome.jp/kegg/>).

Delineation of circrna/mirna interactions

The circRNA/microRNA interaction was predicted using Arraystar's home-made miRNA target prediction software based on TargetScan and miRanda.^{11,12} We searched MREs

on circRNAs using the software and then selected the miRNAs according to seed-matching sequence to establish circRNA-miRNA network. Then, we used Cytoscape (version 3.6.0, USA) to draw the graph of the circRNA/miRNA network.

Quantitative real-time polymerase chain reaction (qRT-PCR) validation

MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) was used for synthesizing cDNA according to manufacturer's instructions after RNA extraction. The expression level of the circRNAs was evaluated by SYBR Green qPCR. Specific divergent primers were designed to amplify the circular transcripts, only primers achieving a single peak in the melting curve were considered proper for qRT-PCR validation. PCR was performed in 10 µL reaction volume, including 2 µL of cDNA, 5 µL 2×Master Mix, 0.5 µL of Forward Primer (10 µM), 0.5 µL of Reverse Primer (10 µM), and 2 µL of double-distilled water. The reaction was set at 95°C for 10 mins for pre-denaturation, then at 95°C for 10 s and at 60°C for 60 s repeating 40 cycles. Housekeeping gene U6 was used as an internal control. Both target and reference were amplified in triplicate

Table 3 Primers allowed for amplification of genes in this work

Gene	Primer sequences
hsa_circ_0039569	F:AAAATAGTGCCCTACGGCG R:GGCAGACGGTAACGGACGTA
hsa_circ_0009043	F:TGGTGGACAACATCCCCAAG R:ACTTGATTTTGCTTCATGGCAGT
hsa_circ_0001523	F:GTGTCTTGGGCCCTGTGAAC R:GGGGGTCAACCTTCTTCTGT
hsa_circ_0001776	F:TCAAACCTCGACAAGGTGCT R:CCTTAGAACACCCGGAAGGT
hsa_circ_0001610	F:AATGGCCATGCTTGGATTCTT R:ATGGCGGTATGTGCCAATGA
hsa_circ_0000437	F:ATCCCCGTACGTCCACTACC R:CCTGCATATTTTCTGGCAATCTCA
hsa-let-7c-5p	F:CGGGCTGAGGTAGTAGGTTGT R:CAGTGCGTGTCGTGGAGT
hsa-miR-542-3p	F:CGGGCTGTGACAGATTGATAA R:CAGTGCGTGTCGTGGAGT
U6	F:CTCGCTTCGGCAGCACATATACT R:ACGCTTCACGAATTTGCGTGTC

Abbreviations: F, forward primer; R, reverse primer.

wells. The relative expression of candidate circRNAs was normalized to U6 and then calculated using the $2^{-\Delta\Delta Ct}$ method. Primers are designed using PRIMER5 software (Table 3).

Statistical analysis

All statistical analyses in this study were accomplished by the GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA) and the SPSS 23.0 software package (IBM, Chicago, IL, USA). Paired *t* test, independent *t* test, and chi-square test were used in this study accurately. *P*-value of 0.05 or less was considered statistically significant.

Results

Identification of differentially expressed circrna profiles

High throughput sequencing analysis facilitated our research on circRNA expression signatures in the grade 3 EC. A total of 75,928 circRNA targets were detected by microarray probes in two pairs of samples. In the initial stage of experiments, circRNA expression profile of two pairs of the grade 3 EC and adjacent non-cancerous tissues were performed. We confirmed the differentially expressed circRNA with statistical significance between the two groups by using Scatter plot filtering and Volcano Plot filtering (Figure 1A and B). Box

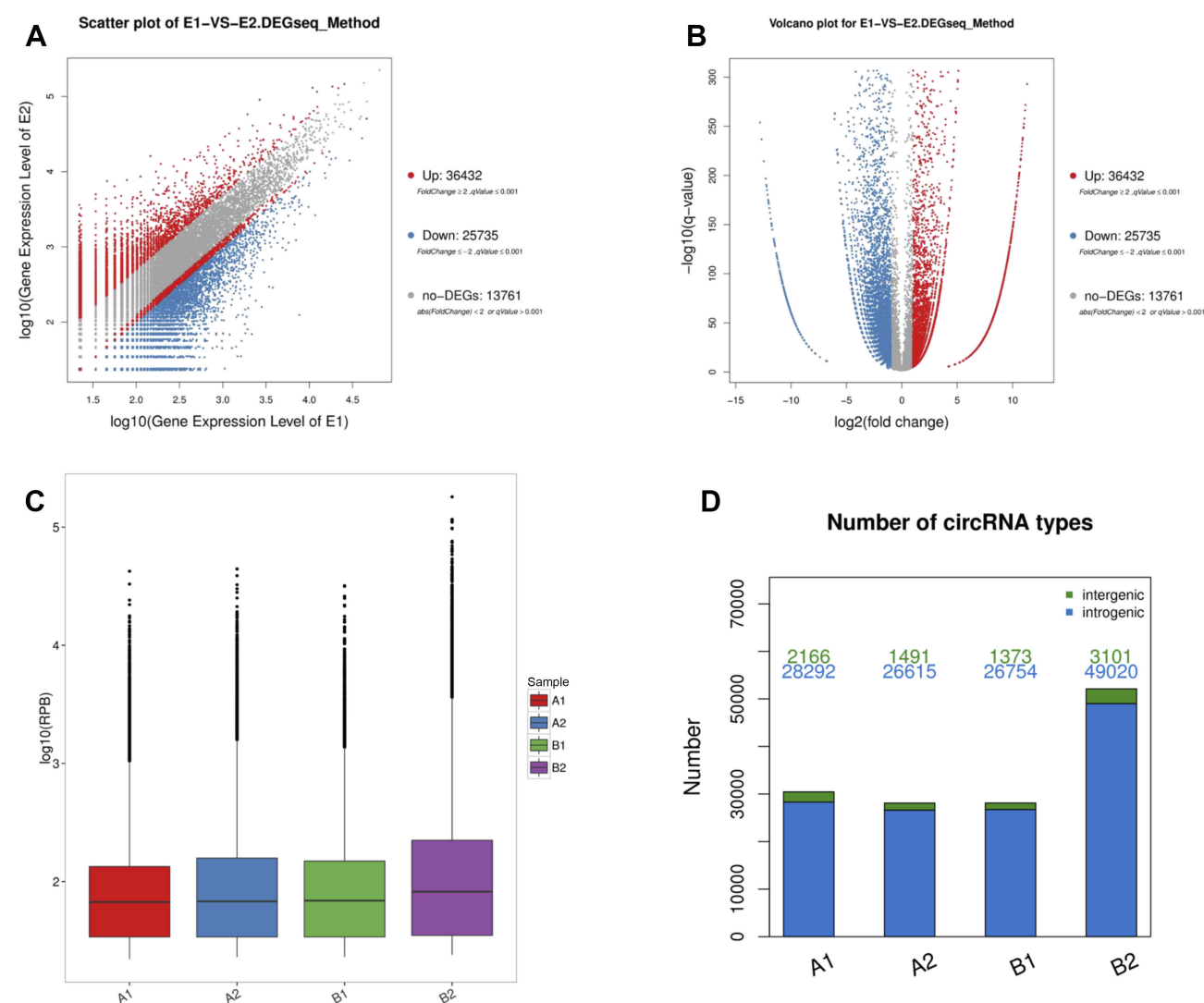


Figure 1 Bioinformatics analysis of differentially expressed circRNAs in patients with the grade 3 EC and adjacent non-cancerous endometrial tissue.

Notes: (A) Scatter plot shows the variation of circRNA between the adjacent non-cancerous endometrial (Y-axis) versus the grade 3 EC tissue (X-axis). The upper red squares to the left indicate 2.0 fold changes of circRNA, while the bottom blue squares indicate negative fold changes of 2.0. (B) Volcano plot shows the differential circRNA expression in the grade 3 EC and adjacent non-cancerous endometrial tissue. (C) Box plot shows the distributions of circRNAs in more direct way. (D) The bar diagram shows the circRNA category. Most of them are transcribed from the intragenic, some are from intergenic.

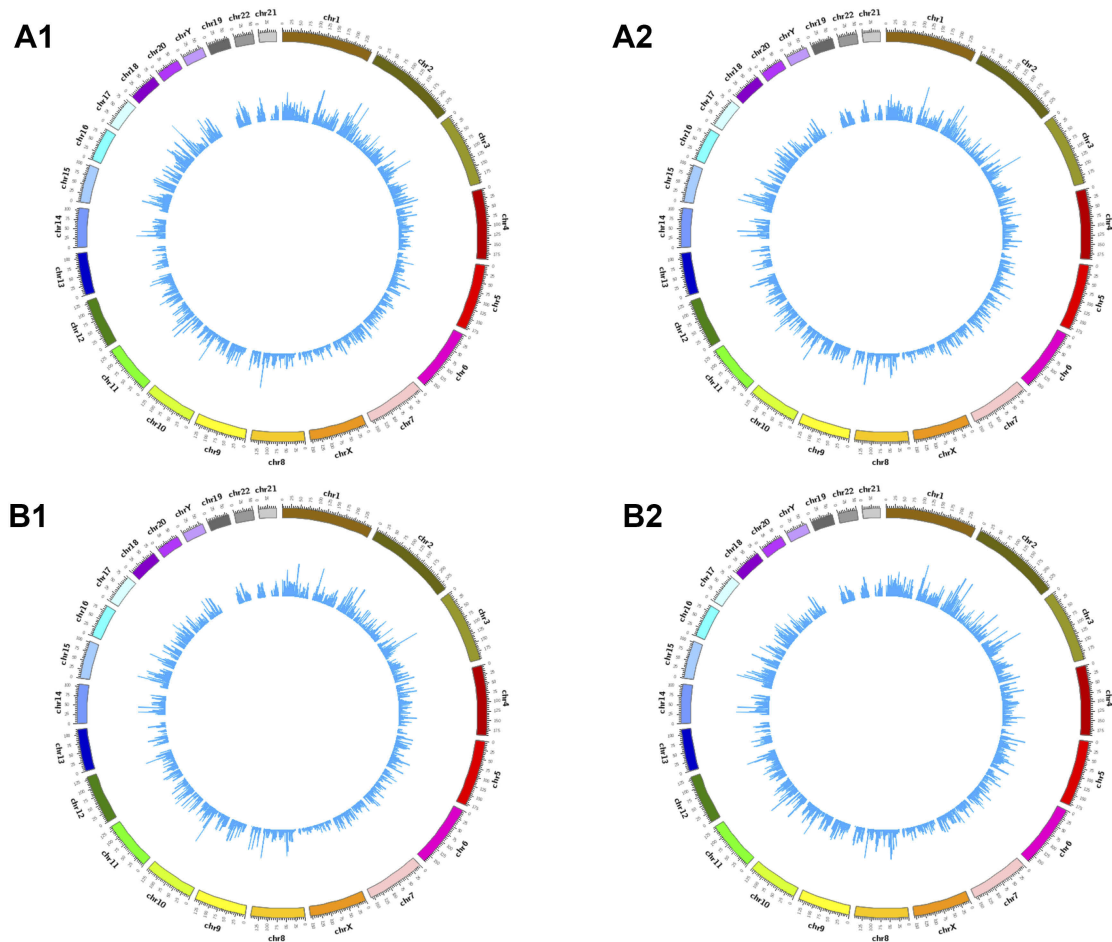


Figure 2 The distribution of differentially expressed circRNAs in human chromosomes (A1, A2, B1, and B2).

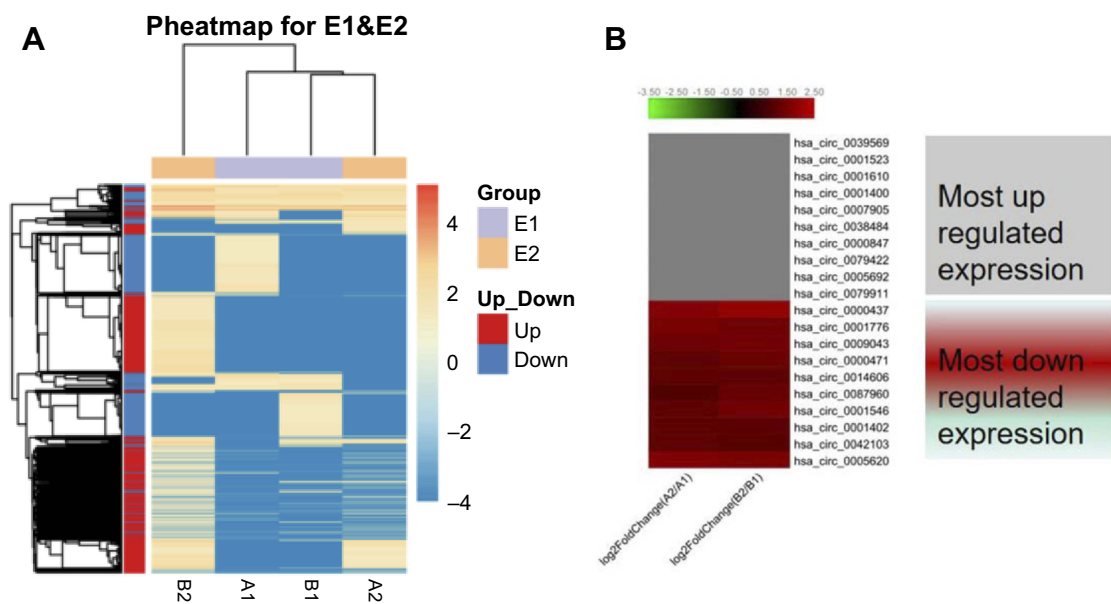


Figure 3 Heat map and hierarchical clustering showing the expression values of all target circRNAs and the most up and downregulated circRNAs. Each column represents a sample and each row represents a circRNA.

Notes: (A) Hierarchical cluster analysis of all target circRNAs. (B) Hierarchical cluster analysis of the most 10 up and downregulated circRNAs. A2/A1 means A2 divided A1. B2/B1 means B2 divided B1.

plots show the normalized intensities from the two groups (Figure 1C). What is more, we analyzed the category of differentially expressed circRNAs. Most of them are transcribed from the intragenic, some are from intergenic (Figure 1D). Furthermore, we analyzed the distribution of the differential expression of circRNAs on all the human chromosomes (Figure 2).

Identification of differentially expressed circRNAs in patients with the grade 3 EC and adjacent non-cancerous endometrial tissue

Hierarchical cluster was performed to analyze all the circRNAs and shown the top 10 up and downregulated circRNAs between the grade 3 EC and adjacent non-cancerous endometrial tissues (Figure 3A and B). The data suggested that the expression of circRNAs in the grade 3 EC tissues was different from the matched non-tumor tissue. A total of 62,167 unique circRNAs were significantly altered in women with the grade 3 EC and adjacent non-cancerous

endometrial tissue ($P < 0.05$). Among them, 25,735 were significantly upregulated and 36,432 were down-regulated. The top 10 up and downregulated circRNAs between the two groups are listed in Tables 4 and 5, respectively. Among these, the expression levels of hsa_circ_0039569, hsa_circ_0001523, hsa_circ_0001610, hsa_circ_0001400, and hsa_circ_0007905 were up-regulated, and hsa_circ_0000437, hsa_circ_0001776, hsa_circ_0009043, hsa_circ_0000471, and hsa_circ_0014606 were downregulated as top 5, respectively.

Construction of the circRNA/miRNA interaction network and prediction of circRNA-miRNA associations

Considered that the circRNA had the potential to serve as miRNA sponges to regulate gene expression, so we made a further exploration about the function of ceRNA of circRNA. We used TargetScan and miRanda database software to predict the target miRNAs of circRNAs based on the base pairing rule in Arraystar. All the differentially expressed

Table 4 Comparison of the grade 3 EC with the adjacent non-cancerous endometrial tissue for the top 10 upregulated circRNAs (fold change > 2 and $P < 0.05$) ranked by fold changes in microarray data

CircRNA ID	CircRNA type	Chrom	P-value	Symbol	Fold change
hsa_circ_0039569	intronic	chr 16	4.07867E-06	CCL22	21.76285
hsa_circ_0001523	intronic	chr 5	5.86834E-05	ZNF608	20.98622
hsa_circ_0001610	intronic	chr 6	0.00065652	TNFRSF21	18.81641
hsa_circ_0001400	intronic	chr 4	0.00014689	RELI	16.98614
hsa_circ_0007905	intronic	chr 1	6.78686E-5	STX6	16.86283
hsa_circ_0038484	intronic	chr 16	0.00057861	VWA3A	15.28875
hsa_circ_0000847	intronic	chr 18	0.00576386	SMAD2	14.28628
hsa_circ_0079422	intronic	chr 7	0.00581689	ICAI	13.32534
hsa_circ_0005692	intronic	chr 16	0.0063233	GLIS2	14.97822
hsa_circ_0079911	intronic	chr 7	0.0034546	POU6F2	12.27567

Table 5 Comparison of the grade 3 EC versus the adjacent non-cancerous endometrial tissue for the top 10 downregulated circRNAs (fold change > 2 and $P < 0.05$) ranked by fold changes in microarray data

CircRNA ID	CircRNA type	Chrom	P-value	Symbol	Fold change
hsa_circ_0000437	intronic	chr 12	4.07975E-06	CORO1C	20.86782
hsa_circ_0001776	intronic	chr 7	5.26868E-05	ESYT2	20.75637
hsa_circ_0009043	intronic	chr2	6.19738E-05	EXOC6B	20.56233
hsa_circ_0000471	intronic	chr 4	6.76723E-05	N4BP2L2	18.82738
hsa_circ_0014606	intronic	chr 1	7.78686E-5	YYIAP1	16.87628
hsa_circ_0087960	intronic	chr 9	0.00032761	LPAR1	15.86223
hsa_circ_0001546	intronic	chr 5	0.00056386	FAM114A2	15.86119
hsa_circ_0001402	intronic	chr 4	0.00041863	TBC1D1	15.07822
hsa_circ_0042103	intronic	chr 17	0.0063233	MYOCD	14.97822
hsa_circ_0005620	intronic	chr 10	0.0087572	SH3PXD2A	14.87672

circRNAs were predicted according to the complementary miRNA matching sequence. There were a total of 451 miRNAs, the top 10 upregulated and downregulated circRNAs, could be combined with circRNAs. Then, used Cytoscape delineated an entire network of circRNA/miRNA interaction (Figure 4).

Validation for the differential expression level of circRNAs

To confirm the data analysis expression of circRNA microarray, six circRNAs (hsa_circ_0039569, hsa_circ_0001523, hsa_circ_0001610, hsa_circ_0000437, hsa_circ_0001776, and hsa_circ_0009043) were validated out using qRT-PCR in 30 pairs of samples including 15 pairs of the grade 3 EC and adjacent non-cancerous endometrial tissue, 15 pairs of the grade 1–2 EC and adjacent non-cancerous endometrial

tissue. The results showed that circRNAs were significantly differentially expressed between the grade 3 EC and the grade 1–2 EC.

The qRT-PCR results are presented in Figure 5. It showed that the expression of the circRNAs hsa_circ_0039569 and hsa_circ_0001610 were upregulated at the grade 3 EC and the grade 1–2 EC tissue versus the adjacent non-cancerous endometrial tissue, compared with the grade 1–2 EC tissue, hsa_circ_0039569 and hsa_circ_0001610 have a higher level of expression in the grade 3 EC. The hsa_circ_0001523 qPCR results were inconsistent with the microarray analysis. The hsa_circ_0009043, hsa_circ_0000437, and hsa_circ_0001776 were significantly downregulated at the grade 3 EC and the grade 1–2 EC tissue versus the adjacent non-cancerous endometrial tissue. And the hsa_circ_0009043 and hsa_circ_0001776 had a higher level of expression in the

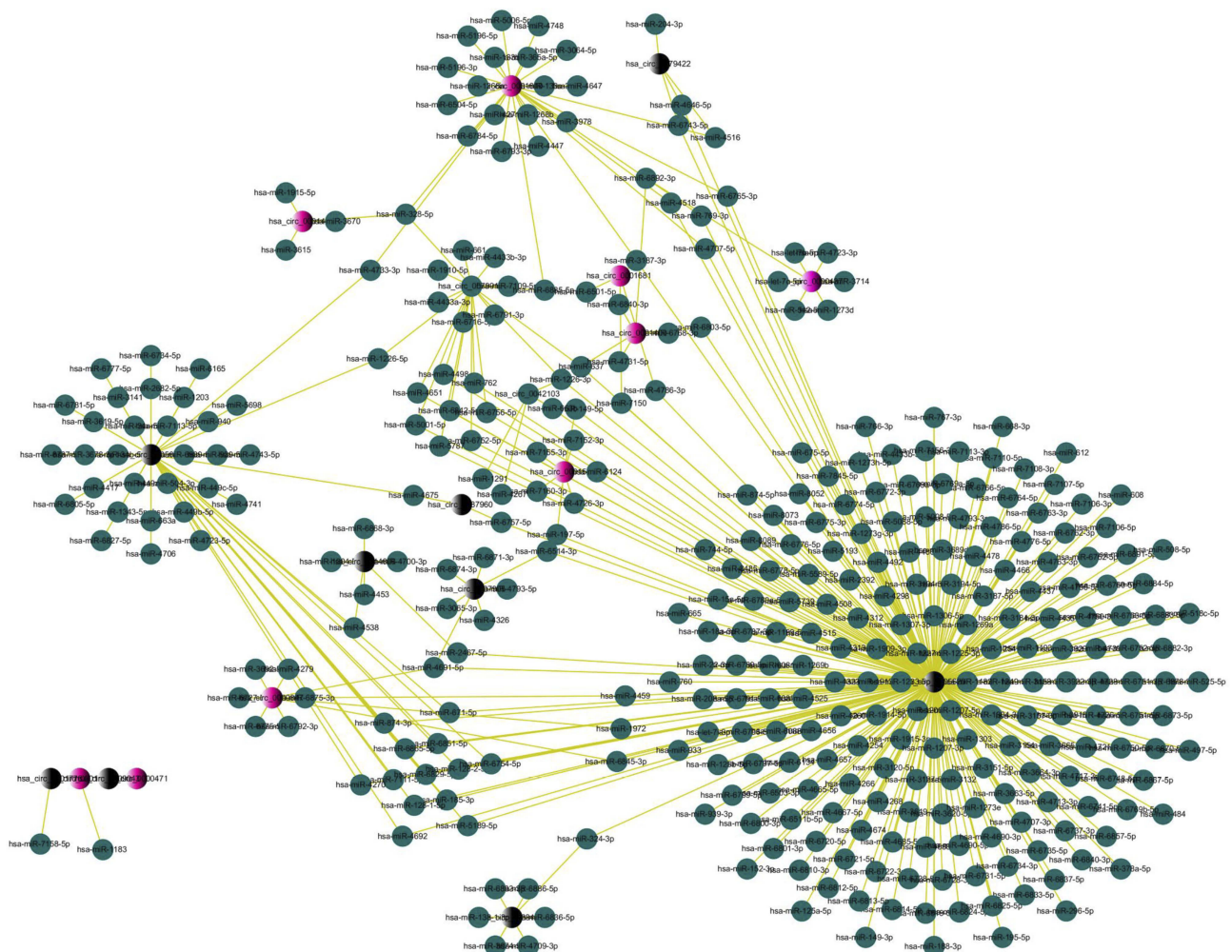


Figure 4 circRNAs-miRNAs network diagram.

Notes: The red and black nodes represent circRNA, while the blue nodes represent target miRNAs. Red and black nodes represent upregulation and downregulation, respectively.

grade 3 non-cancerous endometrial tissue versus the grade 1–2 non-cancerous endometrial tissue, whereas hsa_circ_0000437 had a lower level of expression.

The relationship between the expression of hsa_circ_0039569 and clinicopathological parameters of the 30 patients

The qPCR results showed that hsa_circ_0039569 was higher expression of the grade 3 EC versus the grade 1–2 EC. To analyze the relationship between the expression of hsa_circ_0039569 and clinicopathological parameters of the 30 patients, the patients were divided into low expression group (n=15) and high expression group (n=15) with the hsa_circ_0039569 relative expression level $2^{-\Delta\Delta C_T}$, median is 2.985. The correlation between the hsa_circ_0039569 and clinicopathological data was analyzed. The results showed that the expression level of hsa_circ_0039569 was not correlated with age, lymph

node metastasis, tumor size, FIGO stage, and myometrial invasion ($P>0.05$), and was significantly correlated with tumor differentiation ($P=0.001$). The difference was statistically significant (Table 6).

Prediction of circRNA-miRNA associations

In order to explore the mechanism of circRNAs, the circRNA-miRNA axis in cancer-related pathways was predicted. DIANA-miRPath determined all the candidate miRNAs that were involved in possible pathways by p -value cutoff at 0.05. The data showed that hsa_circ_0039569 was associated with cancer-related pathways for the grade 3 EC with the greatest potential. Base on TargetScan and miRanda, the hsa_circ_0039569/microRNAs interaction was predicted using Arraystar's home-made miRNA target prediction software. KEGG analysis of all microRNAs showed that miR-542-3p and -let-7c-5 was associated with cancer-related pathways for

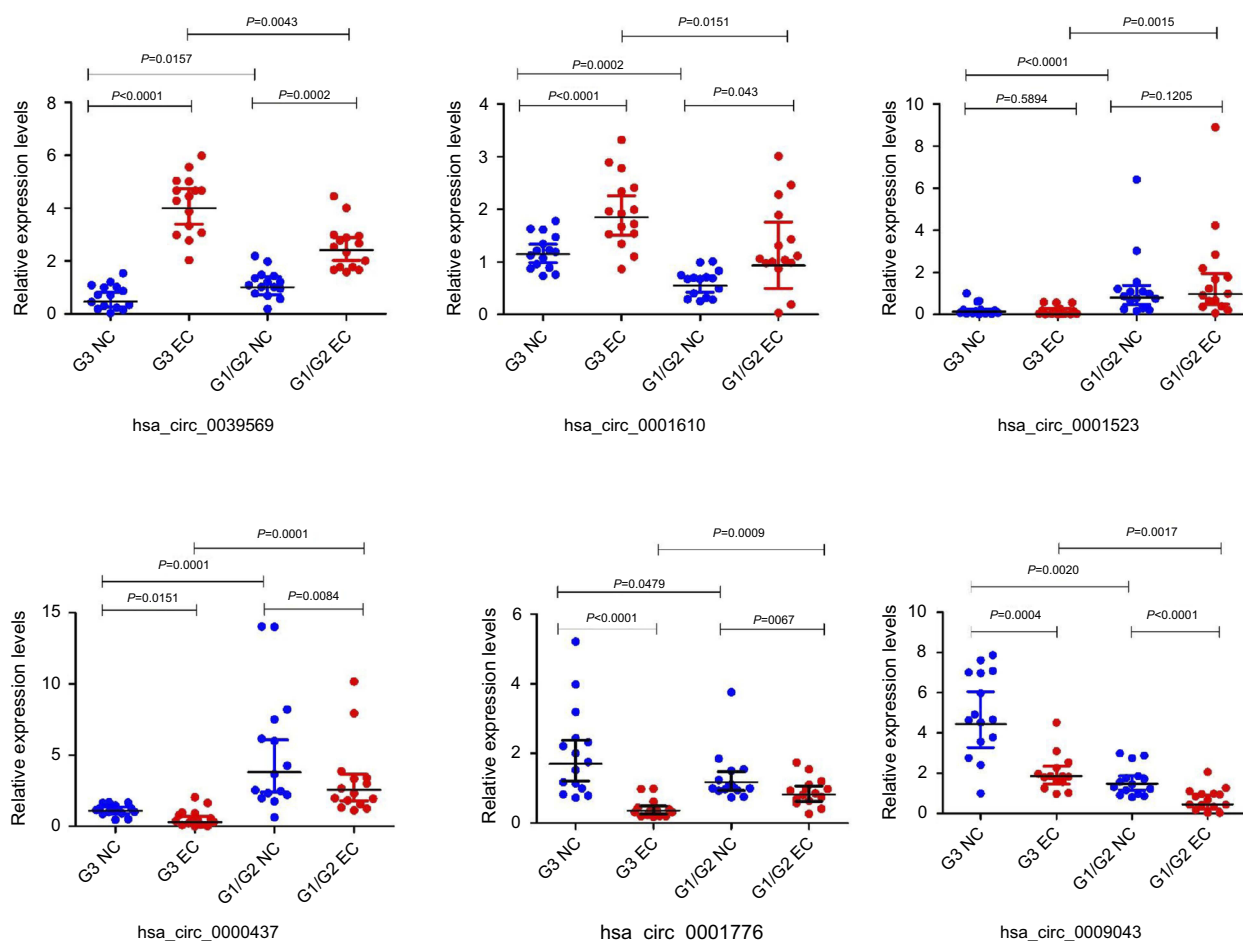


Figure 5 Quantitative real-time PCR validation for the expression of six circRNAs.

Notes: The expression levels of six circRNAs were validated by qPCR in 30 patients. G3 NC, grade 3 non-cancerous endometrial tissue. G1/G2 NC, grades 1–2 non-cancerous endometrial tissue. G3 EC, grade 3 EC. G1/G2 EC, grades 1–2 EC.

Table 6 The relationship between the expression of hsa_circ_0039569 and clinicopathological parameters of the thirty patients

Clinicopathological		Number of cases		
Parameters	(30)	Hsa_circ_0039569 expression Low (%)	High (%)	P-value
Age distribution (years old)				
<45	4	1 (25.00%)	3 (75.00%)	0.427
≥45	26	12 (46.15%)	14 (53.85%)	
Tumor size (cm)				
<4	25	16 (36.00%)	9 (64.00%)	0.317
≥4	5	2 (40.00%)	3 (60%)	
Histopathologic grades				
G1 and G2	15	12 (80.00%)	3 (20.00%)	0.001
G3	15	3 (20.00%)	12 (80.00%)	
FIGO stage				
Ia	24	15 (62.50%)	9 (37.5%)	0.368
Ib	4	1 (25.00%)	3 (75.00%)	
II-IIIa	2	1 (50.00%)	1 (50.00%)	
Myometrial invasion				
<1/2	24	14 (58.33)	10 (41.67%)	0.272
≥1/2	6	2 (33.33%)	4 (66.67%)	
Lymph node metastasis				
Positive	2	1 (50.00%)	1 (50.00%)	1.000
Negative	28	14 (50.00%)	14 (50.00%)	

the grade 3 EC with the greatest potential. Firstly, we further analyzed the binding sequence between hsa_circ_0039569 and hsa-miR-542-3p or hsa-let-7c-5p interaction by TargetScan and miRanda analysis. We found circ_0039569 had a perfect match sequence (7-mer-m8 seed type) to bind miR-542-3p (Figure 6A) or let-7c-5p (Figure 6B). To made a confirmation of the relationship between circRNA between hsa_circ_0039569 and hsa-miR-542-3p or hsa-let-7c-5p, hsa-miR-542-3p and hsa-let-7c-5p were validated by using qRT-PCR in 30 pairs of samples.

The qRT-PCR results are presented in Figure 7. It showed that the expression of the microRNAs hsa-miR-542-3p and hsa-let-7c-5p was upregulated at the grade 3 non-cancerous endometrial tissue and the grade 1–2 non-cancerous endometrial tissue versus the the grade1–3 EC tissue, compared with the grade 1–2 non-cancerous endometrial tissue, hsa-miR-542-3p, and hsa-let-7c-5p have a higher level of expression in the non-

cancerous endometrial tissue. So, the hsa_circ_0039569-hsa-miR-542-3p/hsa-let-7c-5p axis was predicted as one of the circRNA-miRNA axis. Spearman correlation test was used to analyze the correlation between hsa_circ_0039569 and hsa-miR-542-3p and hsa-let-7c-5p in the grade 3 EC. The correlation coefficient R results are -0.8923 and -0.9027 (Figure 8). So, hsa_circ_0039569 has a negative correlation with hsa-miR-542-3p and hsa-let-7c-5p.

Discussion

Presently, the mechanism and role of circRNAs in cancer progression haven't been well identified.¹³ Emerging evidence have found that circRNAs played an important part role in multiple pathological and physiological functions. The available evidence showed that circRNAs are associated with proliferation,¹⁴ cell cycle,¹⁵ apoptosis,¹⁶ and autophagy,¹⁷ involving nervous system disease, cardiovascular system disease, and series of tumors.^{18,19} Especially, more and more studies have found that circular RNA plays an increasingly important role in malignant tumors which were related to sex hormones. Accumulating evidence indicates that hsa_circ_006054, hsa_circ_100219 hsa_circ_0006528, and circ-ABCD10 might also play roles in breast cancer.^{20–22} Furthermore, hsa_circ_0001982 promotes breast cancer cell carcinogenesis by targeting miR-143.²³ Additionally, the expression of circ-Foxo3 was downregulated in mouse breast tumor tissues compared with normal breast, lipid tissues, and skin, and as an inhibitor of breast cancer tumor cells inhibits tumor cell proliferation and survival.²⁴ It has been proved that circ-SMARCA5 acts as an oncogene in prostate cancer by promoting the cell cycle and inhibiting apoptosis.²⁵

In the field of EC, up to the present time, just one study has shown that circRNAs may play roles in EC, with grade (1–2) type I EC, it reported the circular transcriptome specific for EC as determined by RNA sequencing and found that the abundance of circRNAs was lower in the grade 1–2 EC than in adjacent non-cancerous endometrial tissue. What is more, they found that numerous “hotspot” genes from which circRNAs were transferred that may account for alterations in circRNA expression between the non-cancerous endometrial tissue and the grade 1–2 EC. Most importantly, they have also identified circRNAs which were differentially expressed between the grade 1–2 EC and normal endometrial tissue. More and more researchers

A

TargetSite	Alignment	Class
chr16:57397619-57397640[+]	Target: 5' cuccaucccuguggCUGUCACc 3' miRNA : 3' aaagucaauaguuaGACAGUGu 5'	7mer-m8
chr16:57397640-57397664[+]	Target: 5' ccUUGGUCACCUCCGUGCUGUCACu 3' :: : miRNA : 3' aaAGUCAAU--AGUUA-GACAGUGu 5'	7mer-m8

B

TargetSite ↕	Alignment ↕	Class ↕
chr16:57397542-57397561[+]	Target: 5' ggcucaggAGCC--CUACCUCc 3' ↑ : miRNA : 3' uugguaugUUGGAUGAUGGAGu 5'	7mer- m8

Figure 6 The interaction between hsa_circ_0039569 and hsa-miR-542-3p or hsa-let-7c-5p was predicted by TargetScan and miRanda.

Notes: (A) The interaction between hsa_circ_0039569 and hsa-miR-542-3p. (B) The interaction between hsa_circ_0039569 and hsa-let-7c-5p.

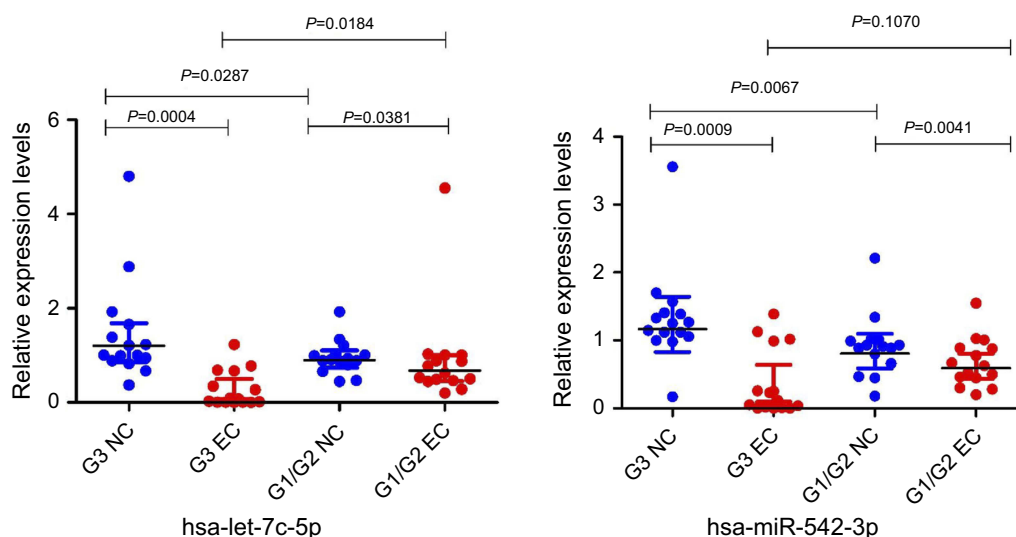


Figure 7 Quantitative real-time PCR validation for the expression of two miRNAs.

Notes: The expression levels of two miRNAs were validated by qPCR in 30 patients. G3 NC, grade 3 non-cancerous endometrial tissue. G1/G2 NC, grades 1–2 non-cancerous endometrial tissue. G3 EC, grade 3 EC. G1/2 EC, grades 1–2 EC.

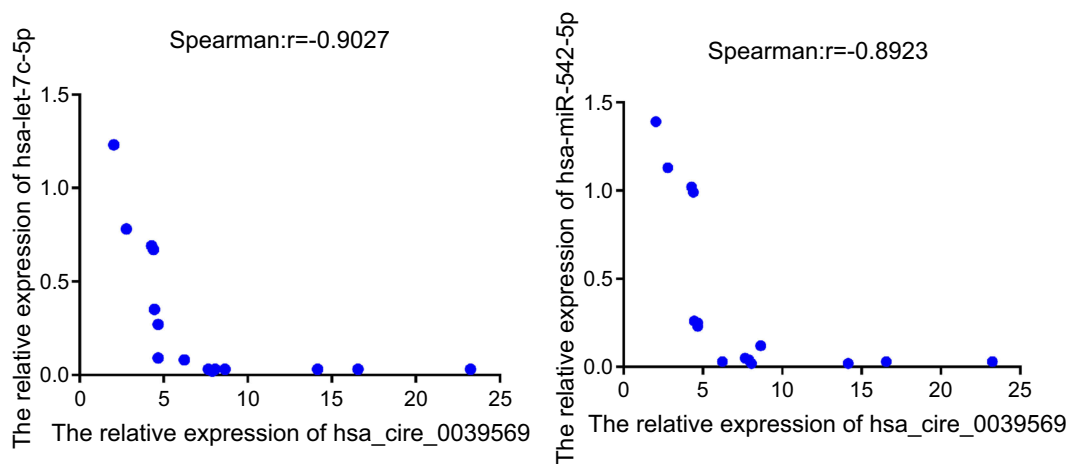


Figure 8 The result of spearman correlation test between hsa_circ_003956 and hsa-miR-542-3p/hsa-let-7c-5p in the grade 3 EC.

acknowledge that the patients with the grade 3 EC have a generally poor prognosis,²⁶ and the specific role of circRNAs in the grade 3 EC remains unclear.

In this study, the differentially expressed profiles of circRNAs in the grade 3 EC tissue and adjacent non-cancerous endometrial tissue were analyzed and validated. Up to now, this is the first study to evaluate the differential expression of circRNAs in the grade 3 EC patients. It indicated that the grade 3 EC-associated circRNAs could be exploited as new candidates for diagnosis and treatment of the grade 3 EC.

Base on the above study, there were 25,735 circRNAs significantly upregulated and 36,432 circRNAs significantly downregulated in the grade 3 EC tissue compared with adjacent non-cancerous endometrial tissue. Among these, the expression levels of hsa_circ_0039569, hsa_circ_0001523, hsa_circ_0001610, hsa_circ_0001400, and hsa_circ_0007905 were upregulated, and hsa_circ_0000437, hsa_circ_0001776, hsa_circ_0009043, hsa_circ_0000471, and hsa_circ_0014606 were downregulated as top 5, respectively. The upregulated hsa_circ_0039569 and hsa_circ_0001610 and downregulated hsa_circ_0000437, hsa_circ_0001776, and hsa_circ_0009043 were validated by qRT-PCR analysis. Hsa_circ_0039569 were selected for further analysis, in the clinicopathological parameters of the 30 patients, the expression level of hsa_circ_0039569 was significantly correlated with tumor differentiation ($P=0.001$), and was not correlated with age, lymph node metastasis, tumor size, FIGO stage and myometrial invasion ($P>0.05$). Furthermore, using Cytoscape to delineate an entire network of circRNAs/miRNAs interaction. This was the first time of our study which predicted

hundreds of circRNAs/miRNAs interaction. An interaction network of circRNAs/miRNAs was constructed, which provided valuable information for ceRNA research of circRNAs in the grade 3 EC. In addition, the results showed that the hsa_circ_0039569 was highly expressed in the grade 3 EC compared to control. With bioinformatical methods, we found that hsa_circ_0039569 had MREs of hsa-miR-542-3p and hsa-let-7c-5p. Hsa-miR-542-3p and hsa-let-7c-5p were downregulated at the grade 3 EC were validated by qRT-PCR analysis. Predicting that hsa_circ_0039569-hsa-miR-542-3p/hsa-let-7c-5p was one of the circRNA-miRNA axis. Our results indicated that it was worthwhile to investigate these novel dysregulated circRNAs as miRNA sponges and their potential biological functions in the grade 3 EC.

All in all, for the first time, our study revealed the circRNA expression signatures of the grade 3 EC. Furthermore, the regulation of hsa_circ_0039569-hsa-miR-542-3p/hsa-let-7c-5p axis was initially predicted in the grade 3 EC. Next, our laboratory is doing further research on the mechanism of hsa_circ_0039569. The result of our study emphasized that hsa_circ_0039569 could be an important prediction of the grade 3 EC. This study has laid the foundation for the functional studies, further diagnosis, treatment of circRNAs of the grade 3 EC.

Conclusion

This study demonstrated that lots of differentially expressed circRNAs in endometrial tissue of the grade 3 EC patients compared with adjacent non-cancerous endometrial tissue. Therefore, it may offer novel molecular candidates for diagnosis and clinical treatment of the grade 3 EC.

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Author contributions

Fei Ye, Xiao Yu Wang, and Xue Feng Jiang conceived and designed the experiments. Fang Ma and Qiong Lan Tang performed the experiments. Lei Cai, Ming Chen, and Xiao Xia Ran analyzed the data. Fei Ye prepared the figures and wrote the manuscript. All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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