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ORIGINAL RESEARCH

The Regulatory Role of circRNA_101308 in Cervical Cancer and the Prediction of Its Mechanism

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Jun Jiao[1]
Xinlin Jiao[1]
Qingqing Liu[1]
Wenjie Qu[1]
Daoxin Ma[2]
Youzhong Zhang[1]
Baoxia Cui[1]

[1]Department of Obstetrics and Gynecology, Qilu Hospital, Shandong University, Jinan 250012, People's Republic of China; [2]Hematology Oncology Center, Qilu Hospital, Shandong University, Jinan 250012, People's Republic of China

Purpose: Accumulating evidence indicates that circular RNAs (circRNAs) are closely involved in canceration and cancer progression. However, the role of circRNAs in cervical cancer (CC) is largely unknown. Here, we characterized the role of circRNA_101308 in CC.

Materials and Methods: The expression of circRNA_101308 in CC tissues was measured by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Then, circRNA_101308 was overexpressed in CC cells to detect its function by proliferation and apoptosis assays, Transwell assays and animal experiments. The potential mechanism of circRNA_101308 in CC was explored by RNA pull-down, Gene Ontology (GO) and pathway analyses.

Results: CircRNA_101308 was significantly downregulated in CC tissues. The level of circRNA_101308 was much lower in CC patients with lymph node metastasis or deep myometrial invasion compared to those patients without lymph node metastasis and superficial myometrial invasion. CircRNA_101308 overexpression inhibited CC cell proliferation, invasion and migration. MiR-26a-5p, miR-196a-5p, miR-196b-5p, miR-335-3p, and miR-1307-3p were found to be sponged by circRNA_101308 in CC cells. Further, GO and pathway analyses predicted the potential functional processes and pathways of circRNA_101308 in CC.

Conclusion: CircRNA_101308 is downregulated and acts as a tumor suppressor in CC. CircRNA_101308 can participate in many different processes by sponging different miRNAs in CC cells. This exploration of circRNA_101308 provides new directions for research on cancer development and the clinical treatment of CC.

Keywords: circular RNA, cervical cancer, circRNA_101308, microRNA

Introduction

Cervical cancer (CC) ranks as the 4th most common malignancy diagnosed and the 4th leading cause of cancer death in women worldwide.[1] Squamous cell carcinoma (SCC) is the main pathological type of CC. Despite recent improvements in vaccination and screening, CC still carries high morbidity and mortality rates, especially in developing countries and areas, and is a global health crisis that is extremely harmful to social development.[2] China is one of the countries with increasing incidence and mortality rates of CC.[3,4] Therefore, improving our understanding of the underlying cellular basis of CC development is critical for improving CC prevention and treatment. In recent decades, numerous factors, including high-risk human papillomavirus (HPV) infection,[5] immune system disorders,[6] aberrant DNA methylation,[7] and noncoding RNA deregulation, have been found to be involved in the development and progression of CC.

Correspondence: Baoxia Cui
Tel +8618560081862
Email cuibaoxia@sdu.edu.cn

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As a type of noncoding RNA, circular RNAs (circRNAs) play a role in physiological and pathological processes. CircRNAs are indicated to be stable and highly conserved across species and to show stage-specific and tissue-specific features.[8] These characteristics comprise critical criteria for use as potential diagnostic biomarkers for cancer and predictive markers for patient outcomes.[9] Regarding the function of circRNAs, current studies have shown that circRNAs can serve as microRNA (miRNA) sponges,[10] endogenously compete with linear mRNAs,[11] regulate protein translation by interact with RNA binding proteins,[12] translate into proteins,[13] and so on. Sponging miRNAs by sequestering them and preventing them from binding their target genes is the most prominent and widely studied function of circRNAs. MiRNAs are another type of noncoding RNA that plays pivotal roles in different diseases. In cancers, miRNAs can function either as tumor suppressors or oncogenes depending on their targeted genes and pathways.[14,15] CircRNAs play an important role in the development of cancer by sponging miRNAs. For example, by sponging miR-203-3p to upregulate the expression of SOCS3, circTADA2As suppresses the proliferation, migration, invasion and clonogenicity of breast cancer cells.[16] CircIRAK3 was shown to promote the migration and invasion of breast cancer cells by regulating miR-3607 and its target, FOXC1.[17]

CircRNAs also play an important role in CC carcinogenesis and progression. Evidence indicates that circRNAs could be promising diagnostic and prognostic biomarkers in CC.[18] Accumulating evidence has shown that circRNAs are involved in cervical tumor progression as either tumor-suppressors or tumor-promoters via various mechanisms, among which miRNA sponging is the most important.[19,20] Hsa_circ_0007534 acts as a tumor suppressor by regulating the activity of miR-498/BMI-1 and inhibits the proliferation and invasion of cervical cancer cells;[21] while hsa_circ_0000515 and hsa_circ_0000263 regulate miR-326/ELK1 and miR-150-5p/MDM4, respectively, leading to promotion of cervical cancer proliferation and metastasis.[22,23] CircRNAs represent a new promising approach for diagnosing and treating CC; however, the roles of circRNAs in CC remain largely unknown.

Previously, we identified that circRNA_101308 was markedly downregulated in CC tissues compared to matched pancancerous tissues with human circRNA microarray analysis,[24] indicating that circRNA_101308 may be associated with CC development. The role of circRNA_101308 has not been reported in any diseases. CircRNA_101308 is a transcription product of the transmembrane and coiled-coil domain 3 (*TMCO3*) gene located on chromosome 13. The role of circRNA_101308 in the tumorigenesis and progression of CC attracts our interests. In this work, we verified the decreased expression of circRNA_101308 in CC tissues and demonstrated the effect of circRNA_101308 in the viability, migration and invasion of CC cells. Through detecting miRNAs sponged by circRNA_101308 and predicting their target genes, we preliminary explored the potential role and network of circRNA_101308 in CC. Our findings clarify the importance of circRNA_101308 in CC and provide insight into its role and mechanisms in the progression of CC.

Materials and Methods

Patient Samples

Seventy pairs of CC tissues and matched paracancerous tissues were collected from patients at Qilu Hospital of Shandong University, Jinan, China. All samples were macrodissected within 15 min after uterine resection, and the tissues in each pair were confirmed by a pathologist as squamous cell carcinoma of the cervix and nontumor cervical tissue. All samples were stored at −80°C after surgical resection. This study was approved by the Medical Ethics Committee of Qilu Hospital, Shandong University. Written informed consent was obtained from all patients.

Cell Culture

The CC cell lines SiHa, CaSki and HeLa were obtained from the gynecological laboratory at Qilu Hospital of Shandong University. CaSki and HeLa cells were cultured in RPMI 1640 medium (Gibco-BRL, Grand Island, USA), and SiHa cells were cultured in α-minimum essential medium (Gibco-BRL); both media were supplemented with 10% fetal bovine serum (FBS, Gibco-BRL) at 37°C in a 5% CO_2 atmosphere. The use of the cell lines was approved by the Medical Ethics Committee of Qilu Hospital, Shandong University.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) Analysis

Tissue samples were ground into powder in liquid nitrogen. Total RNA was extracted and purified using Trizol reagent (Invitrogen, Carlsbad, USA) following the manufacturer's protocol. RNA was quantified and assessed with a NanoDrop ND-1000 (Implen, Munich, Germany). Then 3 μg of total RNA was reverse transcribed to complementary DNA (cDNA) with 1 μL of random primers (0.5 μg/mL) using SuperScript III Reverse

Transcriptase kit (Invitrogen) in a total system of 20 μL. The PCR mixtures (10 μL) contained 2 μL of cDNA template, 5 μL of a 1× final concentration of SYBR Green (TOYOBO, Osaka, Japan) and 0.5 μL of forward or reverse primers (10 μM) specific for circRNA_101308 and GAPDH. The sequences of the primers, which were synthesized by Biosune (Shanghai, China), are shown in Table 1. The reactions were performed in a LightCycler 480 II instrument (Roche, Basel, Switzerland) via the following protocol: one cycle at 95°C for 5 min; 40 cycles at 95°C for 15 s, 62°C for 15 s, and 72°C for 40 s. Melting curves were acquired at 95°C for 15 s, 65°C for 1 min, and a final incubation at 95°C for 10 s. All tests were conducted in triplicate. The relative RNA level was normalized to the GAPDH mRNA level using the $2^{-\Delta\Delta Ct}$ method.

Cell Transfection

To obtain stable CC cells overexpressing circRNA_101308, circRNA_101308 cDNA was synthesized and inserted into the pLCDH-ciR lentiviral vector (Geneseed, Guangzhou, China). SiHa, HeLa and CaSki cells were infected with lentivirus at a multiplicity of infection of 2. Stably transfected cell clones were selected using puromycin (2 μg/mL, Sigma, St. Louis, USA). The overexpression efficiency was validated through qRT-PCR after selection.

Assessment of Cell Proliferation and Apoptosis

Stably transfected cells were seeded into 96-well plates (2×10^3 cells per well) and cultured for 0, 24, 48, and 72 h. Cell proliferation was assessed using a Cell Counting Kit-8 (CCK-8, Solarbio, Beijing, China). Ten microliters of CCK-8 reagent was added to each well at each time point and incubated at 37°C for 2 h. The optical density (OD) was measured at 450 nm in a Model 680 microplate reader (Bio-Rad, Hercules, USA). Each experiment was performed in at least triplicate.

After transfection, cells (about 10^7 cells) were collected and washed with PBS. Cells were then incubated with Annexin V-fluorescein isothiocyanate (FITC, Sigma) and propidium iodide (PI, Sigma) in accordance with the manufacturer's instructions. The double-stained cells were detected using a Beckman Gallios flow cytometer (Beckman, Brea, USA) to measure the apoptosis rate.

Table 1 The Sequences of Primers for qRT-PCR

RNA	Sequence
hsa_GAPDH forward sequence	5′-GGGAAACTGTGGCGTGAT-3′
hsa_GAPDH reverse sequence	5′GAGTGGGTGTCGCTGTTGA3′
circRNA_101308 forward sequence	5′CACCTCCATCGAACCCATC3′
circRNA_101308 reverse sequence	5′TGGAAATGAAGACGCTCTGC3′

Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; qRT-PCR, quantitative reverse transcription polymerase chain reaction.

Cell Migration and Invasion Detection

Transwell units with 8-μm pores (Corning, Corning, USA) were coated with Matrigel matrix (Corning) for the invasion assay and were used without the Matrigel coating for the migration assay. Transfected cells (5×10^4 cells for invasion and 2.5×10^4 cells for migration) were suspended in 200 μL of serum-free medium and seeded in the upper chamber of the Transwell unit. Medium supplemented (700 μL) with 20% FBS was added to the lower chamber. After 24 h, the upper surface of the chamber inserts was wiped with a cotton swab to remove nonmigrated or noninvaded cells. Cells located on the lower surface of the inserts were fixed, stained and counted under a microscope (Olympus, Tokyo, Japan).

Xenograft Nude Mouse Model

Ten female BALB/c nude mice (5 weeks of age) were randomly divided into two groups (n=5). Transfected CaSki cells (10^7) were subcutaneously injected into the right flanks of the mice. The tumor volumes were monitored every 3 days by measuring the width (W) and length (L) of the tumors. After 4 weeks, the mice were sacrificed, and tumor tissues were excised and assessed. All animal care and experimental procedures were performed in accordance with the guidelines of the National Institutes of Health and approved by the Animal Care and Use Committee of Shandong University.

RNA Pull-Down and miRNA Sequencing

CaSki cells (8×10^7) were lysed and incubated with a biotin-conjugated probe for circRNA_101308. The circRNA_101308 probe and the negative control probe were synthesized by Cloud-Seq (Shanghai, China). Streptavidin magnetic beads were added, and the mixture was incubated. Then, the target RNAs bound to the beads were isolated. The extracts were used for high-throughput miRNA sequencing (Cloud-Seq, Shanghai, China).

miRNA Target Prediction and Pathway Enrichment Analysis

We selected the top 5 miRNAs binding to circRNA_101308 for further analysis. Interactions between the 5 miRNAs and their target mRNAs were predicted based on the TargetScan, miRTarBase, and miRBD databases. Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes

(KEGG) pathway analysis were performed to generate meaningful annotations of the target genes in order to determine the potential functional pathways of circRNA_101308 in CC.

Statistical Analysis

Data are presented as the means ± SDs. Comparisons between different groups were performed with Student's *t*-test. $P < 0.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed with SPSS 18.0.

Results

circRNA_101308 Is Downregulated in CC Tissues

We used qRT-PCR to measure the expression of circRNA_101308 in CC tissues and matched nontumor tissues. CircRNA_101308 was found to be significantly downregulated in CC tissues compared to paracancerous tissues (P<0.0001, Figure 1A). The result was consistent with the report of our previous circRNA microarray analysis. In addition, we analyzed the relationships of circRNA_101308 with the prognostically relevant risk factors in CC patients, including the clinical stage, tumor differentiation grade, tumor size, depth of myometrial invasion, vascular/lymphatic invasion, and lymph node metastasis status. Compared to CC patients without lymph node metastasis, patients with lymph node metastasis exhibited much lower levels of circRNA_101308 (P=0.038, Figure 1B). Patients with deep myometrial invasion exhibited lower expression of circRNA_101308 than patients with superficial myometrial invasion (P=0.004, Figure 1C). CircRNA_101308 expression was not significantly correlated with the other prognostic factors in patients with CC (Table 2). The clinical stages of the CC patients were determined according to the criteria established by the International Federation of Gynecology and Obstetrics (FIGO).

circRNA_101308 Overexpression Inhibits CC Cell Proliferation in vitro and in vivo

To explore the role of circRNA_101308 in CC, we overexpressed the level of circRNA_101308 in SiHa, CaSki, and HeLa cells by transfection with a circRNA_101308 overexpression vector. The overexpression efficiency of circRNA_101308 was identified by qRT-PCR (Figure 2A). CCK-8 assays showed that compared to the control treatment, circRNA_101308 overexpression obviously suppressed the proliferative ability of SiHa, CaSki and HeLa cells (Figure 2B–D). Flow cytometric analysis was conducted to assess the influence of circRNA_101308 overexpression on apoptosis. These results showed that changes in the expression of circRNA_101308 did not affect the apoptosis of SiHa, CaSki and HeLa cells compared to the si-NC cells (P>0.05, Figure S1).

To further evaluate the effect of circRNA_101308 on the proliferation of CC cells, we performed xenograft experiments. The role of circRNA_101308 in inhibiting CC cell proliferation was validated by establishing a subcutaneous tumor model in nude mice. The tumor volume was calculated as follows: Tumor Volume=Length×Width×Height×π/6. The volume of tumors in the circRNA_101308 overexpression group was markedly smaller than that of tumors in the control group (P<0.001; Figure 2E), indicating that circRNA_101308 overexpression obviously suppressed CC cell proliferation in vivo.

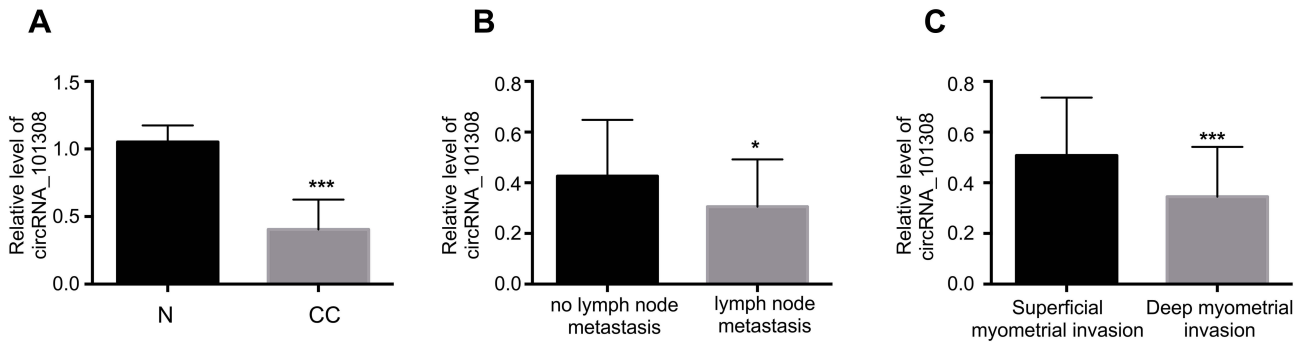

Figure 1 CircRNA_101308 is downregulated in cervical cancer. (**A**) The level of circRNA_101308 in cervical cancer tissue (CC) and paracancerous tissues (N). (**B**) The level of circRNA_101308 in cervical cancer patients with or without lymph node metastasis. (**C**) The level of circRNA_101308 in cervical cancer patients with deep myometrial invasion or superficial myometrial invasion.
Notes: **P*<0.05, ****P*<0.001.

Table 2 Relationships Between circRNA_101308 and the Clinicopathologic Features of Patients with Cervical Cancer

Characteristics	No. of Patients	Mean ± SD	*P* value
FIGO stage			
I	54	0.383 ± 0.236	0.470
II	16	0.429 ± 0.146	
Tumor differentiation			
Moderate/High	37	0.353 ± 0.254	0.098
Low	33	0.440 ± 0.162	
Tumor size			
<4 cm	50	0.407 ± 0.212	0.433
≥4 cm	20	0.361 ± 0.236	
Myometrial invasion			
superficial	21	0.507 ± 0.229	0.004**
deep	49	0.345 ± 0.167	
Vascular/Lymphatic invasion			
no	32	0.390 ± 0.225	0.899
yes	38	0.397 ± 0.216	
Lymph node metastases			
no	51	0.427 ± 0.222	0.038*
yes	19	0.306 ± 0.187	

Notes: *P<0.05, **P<0.01.
Abbreviations: FIGO, International Federation of Gynecology and Obstetrics; SD, standard deviation.

circRNA_101308 Overexpression Inhibits CC Cell Migration and Invasion

SiHa, CaSki and HeLa cells showed decreased migration through a Transwell insert after upregulation of circRNA_101308 expression (Figure 3A); the results of the invasion experiments showed the same trend (Figure 3B). These findings demonstrated that ectopic expression of circRNA_101308 influences the migration and invasion of CC cells and that the migratory and invasive abilities of CC cells were greatly inhibited after overexpression of circRNA_101308.

circRNA_101308 Sponges Multiple miRNAs

Given that circRNAs can bind to different miRNAs and regulate their downstream genes, we investigated the miRNAs that bind to circRNA_101308 via a pulldown assay with a biotin-conjugated probe in order to explore the molecular mechanism of circRNA_101308. We found 178 different miRNAs bounding circRNA_101308 with a fold change of >2.0 compared to the negative control. The five miRNAs with the highest fold changes were miR-26a-5p, miR-196a-5p, miR-196b-5p, miR-335-3p, and miR-1307-3p. All the miRNAs candidates are shown in Table S1.

Functional Pathway Analysis of circRNA_101308

We predicted the molecular mechanism of circRNA_101308 in CC through GO enrichment analysis of the top five target miRNAs and their target genes. The target genes of circRNA_101308 were mainly involved in biological processes represented by terms such as regulation of cellular biosynthetic process, positive regulation of metabolic process, and neurotransmitter transport (Figure 4A); enriched in molecular function terms such as protein binding, RNA binding, lipid binding, and heterocyclic compound binding (Figure 4B); and involved in the cellular components represented by the terms organelle membrane and intracellular organelle (Figure 4C). KEGG pathway analysis revealed that the target genes were enriched in the TNF signaling pathway, estrogen signaling pathway, endocytosis, sphingolipid signaling pathway, and other such pathways (Figure 4D).

Discussion

Previously mistaken as a by-products of transcription, circRNAs have attracted more attention for their roles in diseases in recent years. With the widespread application of RNA sequencing and microarray, circRNAs have been studied and discussed in many diseases, including cancers. An association between abnormal expression of circRNAs and the development of cancer has been established. Some characteristics and functions of circRNAs have been clarified. Additionally, the function of circRNAs in cancers has changed our perception of cancer, especially in carcinogenesis and cancer development. CircRNAs are reported to be involved in different important biological processes, including the proliferation, apoptosis, infiltration, and metastasis of cancer cells.[22,25] Understanding the role and mechanism of circRNAs provides new insights into tumorigenesis and tumor progression, and thus provides the basis for new potential biomarkers and treatment therapy.

Despite some progress in prevention and treatment, CC is still an important health problem.[2] Some studies have shown the ectopic expression of noncoding RNAs such as circRNAs and miRNAs in CC. Recent studies have revealed the ectopically expressed circRNA profiles associated with HPV 16 infection[26] and radioresistance.[27] CircRNAs with dysregulated expression are found to be associated with CC carcinogenesis and progression

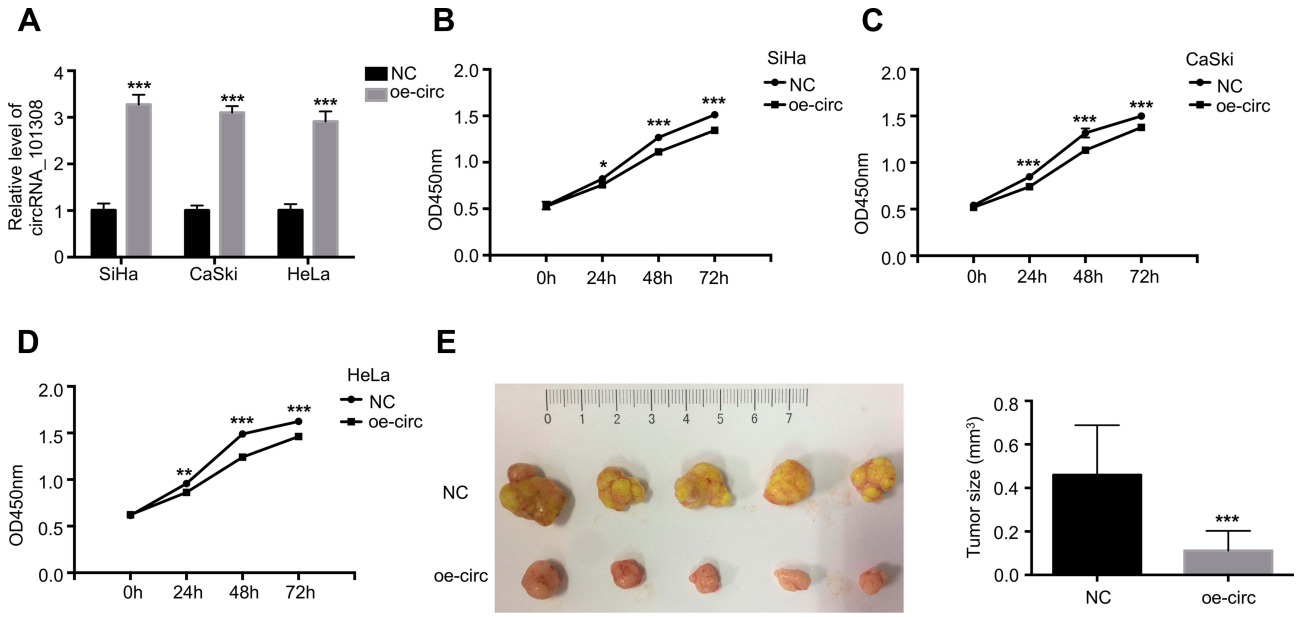

Figure 2 CircRNA_101308 overexpression inhibits cell proliferation in vitro and in vivo. (**A**) The efficiency of overexpression was identified by qRT-PCR. (**B**–**D**) Cell viability of SiHa, CaSki and HeLa cells was detected by CCK-8. (**E**) CaSki cells with or without circRNA_101038 overexpression were subcutaneously inoculated into mice, and the tumor size was calculated.
Notes: *P<0.05, **P<0.01, ***P<0.001.

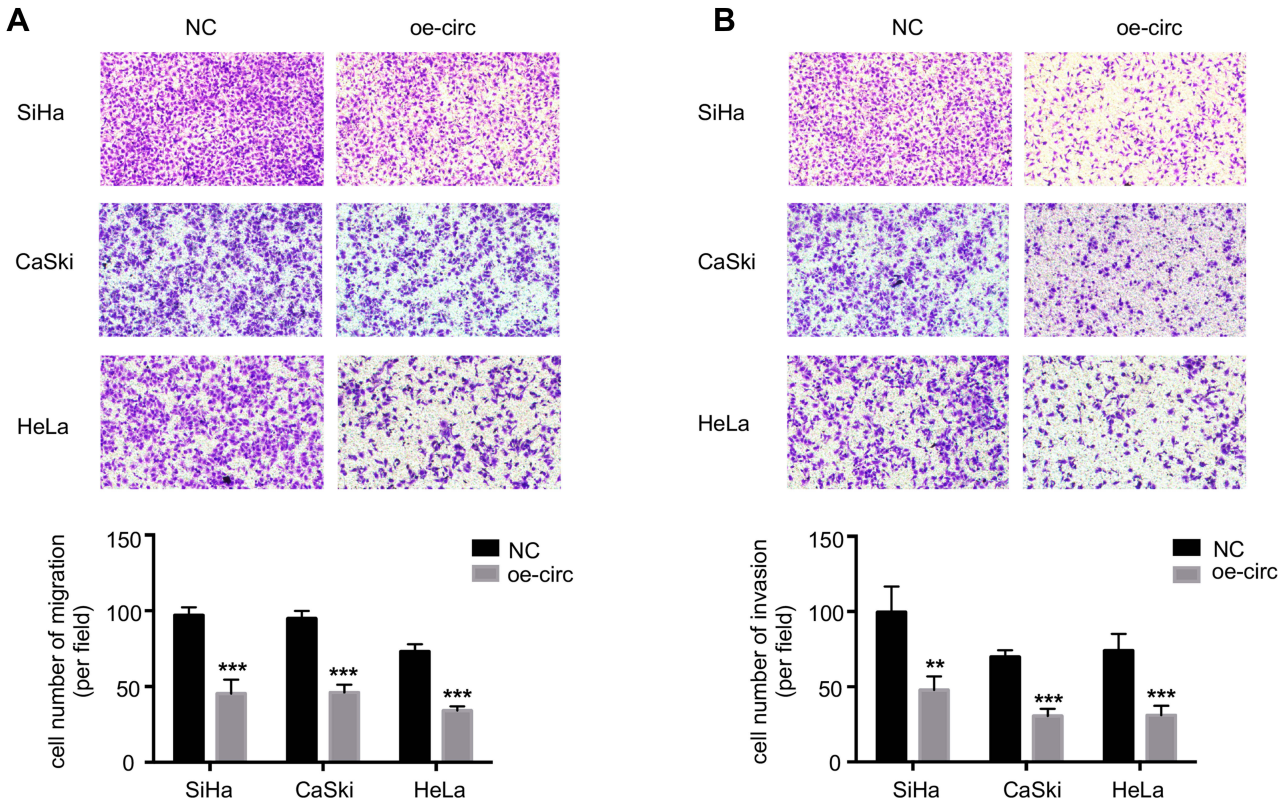

Figure 3 CircRNA_101308 overexpression inhibits cell migration and invasion in vitro. (**A**) Migration ability of SiHa, CaSki and HeLa cells with or without circRNA_101308 overexpression. (**B**) Invasion ability of SiHa, CaSki and HeLa cells with or without circRNA_101308 overexpression.
Notes: **P<0.01, ***P<0.001.

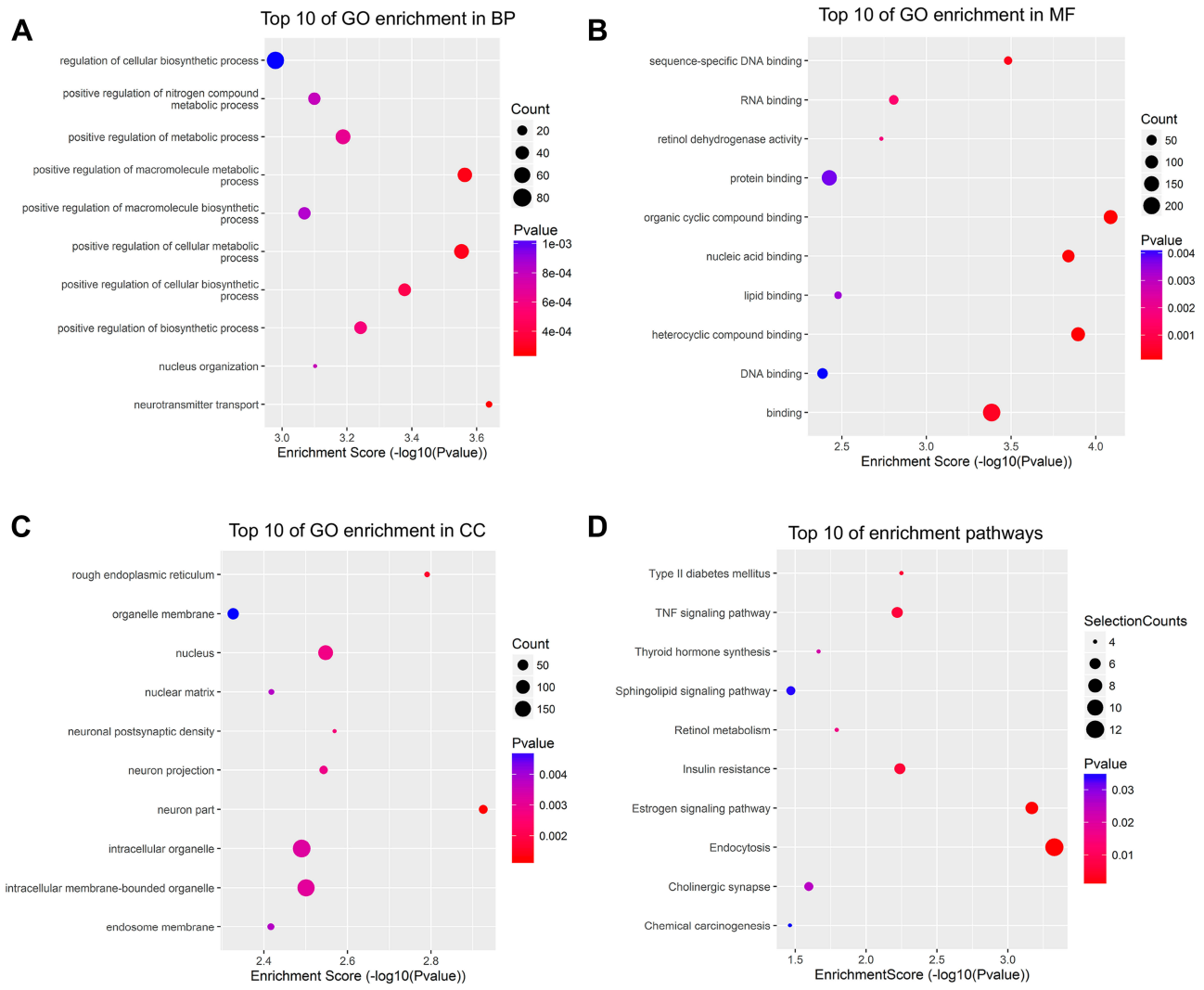

Figure 4 GO and pathway analysis of circRNA_101308 in cervical cancer. (**A**–**C**) The top ten processes of circRNA_101308 enrichment in biological process (BP), molecular functions (MF), and cellular component (CC) respectively by GO analysis. (**D**) The top ten pathways of circRNA_101308 enrichment predicted by pathway analysis.

through the regulation of cell migration, invasion, metastasis and recurrence in CC.[18,28] The roles of certain circRNAs in CC have been elucidated. CircRNAs participate in cancer progression by regulating the activity of target miRNAs, leading to inhibition or promotion of some important genes. Hsa_circ_0007534, a tumor suppressor in CC, regulates the viability and invasion of cancer cells by regulating the expression of miR-498 and its target BMI-1.[29] Hsa_circ_0000515, which is upregulated in CC, promotes CC progression by modulating miR-326 to increase the level of ELK1.[7] Hsa_circ_0000263, a tumor promoter in CC, promotes the expression of MDM4 by competitive binding with miR-150-5p to promote cancer cell proliferation and migration.[23]

In this work, we verified the role of circRNA_101308 in CC. Our pre-experimental results suggest that circRNA_101308 had significantly lower expression in CC tissues by circRNA microarray analysis. We confirmed the downregulation of circRNA_101308 in CC tissues. Moreover, circRNA_101308 was associated with certain clinicopathological factors of patients. CC patients with lymph node metastasis and/or deep myometrial invasion often exhibit much lower levels of circRNA_101308 than negative patients. Lymph node metastasis and deep myometrial invasion are high-risk factors for cancer recurrence. This indicated that the reduced expression of circRNA_101308 may be involved in the development of CC. It also suggested that CC patients with lower expression of circRNA_101308

may have more malignant tumors and worse prognosis. While all patients included in this study are patients in early stages, and the follow-up time is short, no recurrence or death has occurred yet. We will continue to follow-up to analyze the relationship between circRNA_101308 and patient prognosis. In this work, we collected specimens only from CC patients with stage I and II disease because patients with more advanced stages were treated with radiotherapy or chemotherapy rather than with surgery. Because we could not collect a sufficient number of fresh cancer tissues from individuals with stage III or stage IV disease, the correlation analysis between circRNA_101308 and the patient clinical stage was restricted to early stages.

The role of circRNA_101308 has not been studied in cancer. In this work, we demonstrated that circRNA_101308 overexpression markedly inhibited CC cell proliferation, migration and invasion. In addition, overexpression of circRNA_101308 inhibited tumor growth in mice. These results revealed the role of circRNA_101308 as a tumor suppressor in CC. As the major function of circRNAs is miRNA sponging, we explored the potential mechanisms of circRNA_101308 in CC by RNA pull-down and miRNA sequencing. MiR-26a-5p, miR-196a-5p, miR-196b-5p, miR-335-3p, and miR-1307-3p were demonstrated to be sponged by circRNA_101308 in CC cells. These five miRNAs have been proven to be involved in several malignant tumors but have not been studied in detail in CC. For example, miR-26a-5p inhibits tumor progression in papillary thyroid carcinoma by suppressing the expression of Wnt5a[30] but enhances lung cancer metastasis by activating the JAK2/STAT3 pathway.[31] Moreover, miR-26a-5p has been demonstrated to be an important target of several noncoding RNAs in cancers.[32,33] The other four above mentioned target miRNAs have also been shown to participate in different ways in different diseases.[34–36]

As noted previously, the same circRNA can play different or identical roles in different or identical tumors through multiple pathways.[37,38] This suggests that circRNA can function by adsorbing different miRNAs or through other pathways, forming a complex regulatory network. We systematically analyzed and predicted the anti-tumor mechanism of circRNA_101308 in CC by combining the target miRNAs and bioinformatics analysis. Here, we showed the potential processes and pathways in which circRNA_101308 participates, such as RNA binding, protein binding, TNF signaling pathway, Estrogen signaling pathway, endocytosis, and so on. These may be the pathways that circRNA_101308 participate in to play the tumor suppressive role in CC. The systematic analysis of circRNA_101308 provides comprehensive basic knowledge for exploring the role of circRNA_101308 in CC. However, given that these results are based solely on bioinformatics analysis, further in-depth studies are critical to verify the role of the potential circRNA_101308/miRNA axes in CC.

Conclusion

In the current study, we demonstrated that circRNA_101308 was significantly decreased in CC and could suppress the proliferation, invasion and migration of CC cells. This work explored the role and potential mechanisms of circRNA_101308 in CC. Our exploration of circRNA_101308 adds a new dimension to the study of its role in CC and provides new directions for oncogenesis, progression and treatment of CC.

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Disclosure

The authors report no conflicts of interest in this work.

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