Circular RNA Hsa_Circ_0007444 Inhibits Tumor Progression Through MiR-23a-3p/DICER1 in Ovarian Cancer

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Abstract

Background: Circular RNAs are a class of non-coding regulatory RNAs reported to be involved in cancer development and progression. Previous studies, including our own, have indicated that hsa_circ_0007444 was downregulated in ovarian cancer (OC) tissues. Herein, we demonstrated another mechanism of hsa_circ_0007444 in ovarian cancer.

Methods: The expression of hsa_circ_0007444, miR-23a-3p, and *DICER1* were determined by quantitative real-time PCR. Cell proliferation, invasion, migration, and apoptosis were examined by cell counting kit 8, transwell, and flow cytometry assays. The roles of hsa_circ_0007444 in tumor growth and metastasis were assessed *in vivo* using a nude mouse xenograft model. The bioinformatics tools were employed to predict the binding sites, which were then verified by dual-luciferase reporter assay and RNA immunoprecipitation (RIP) assays. *DICER1* protein level was measured by western blot.

Results: Hsa_circ_0007444 was downregulated in ovarian cancer cell lines compared with normal ovarian epithelial cell lines. Also, gain- and loss-of-function results indicated that hsa_circ_0007444 inhibited cell proliferation, invasion, migration, increased cell apoptosis of ovarian cancer cells *in vitro*, and impaired tumor growth and lung metastasis *in vivo*. Additionally, the results of the bioinformatics analysis, RIP, dual-luciferase reporter, and rescue assays confirmed that hsa_circ_0007444 could interact with AGO2 and sponge miR-23a-3p, thereby upregulating *DICER1* expression, which was an important tumor suppressor in ovarian cancer.

Conclusion: We found that overexpressed hsa_circ_0007444 could inhibit ovarian cancer progression through the hsa_circ_0007444/miR-23a-3p/*DICER1* axis.

1. Background

Ovarian cancer is the third most lethal cancer in gynecology and the second leading cause of worldwide gynecological cancer death (1). It is estimated that in 2021, there will be about 21,410 newly diagnosed cases of ovarian cancer and 13,700 ovarian cancer deaths in the United States (2). Over 95% of ovarian malignancies are epithelial ovarian cancer (3). The development of cancer of the ovaries, which starts in the deep pelvic cavity, is usually asymptomatic in the early stages of the disease. Symptoms like pelvic pain and abdominal swelling only become obvious in the late stages of OC. As a result, about 70% of patients are diagnosed at stage III/IV and present with extensive metastasis. With such advanced disease, surgery combined with platinum-based chemotherapy is the rudimentary treatment for OC patients. However, due to the widespread metastasis and chemotherapy resistance in late-stage OC, the 5-year survival rate of OC patients is only about 46% (4). Therefore, it is vital to further understand the mechanisms of OC development, progression, and metastasis.

Circular RNAs (circRNAs) are a class of endogenous non-coding RNAs with covalently closed structure and widely present in eukaryotic cells (5, 6). There are three main subtypes of circRNAs: exonic circRNAs (ecircRNAs) (7), which are mainly generated by back-splicing, predominantly cytoplasmic, may harbor

microRNA (miRNA) response elements (MREs) and serve as miRNA sponges; circular intronic RNAs (8) (ciRNAs), which are formed by intron cyclization directly; exonic-intronic circRNAs (9) (EicRNAs), which consist of both introns and exons. The circRNAs lack the 5' cap and 3' poly-A tail, and since they do not have 5' or 3' ends, they are resistant to exonuclease degradation, making circRNAs more stable than linear RNAs (10, 11). The rapid development of high-throughput sequencing has enabled the analysis and identification of numerous circRNAs and characterized many of their functions in eukaryotic cells. Accumulating evidence has revealed that circRNAs perform several biological functions, such as regulation of RNA splicing, chromatin remodeling, mRNA translation, and mainly act as miRNA sponges (12–14). In recent years, numerous studies of circRNAs have indicated that they have a crucial role in the progression of various cancers, including gastric cancer (15), colon cancer (16), lung cancer (17), liver cancer (18), breast cancer (19) and OC (20).

MiRNAs are a class of evolutionarily conserved, small, non-coding RNAs of 18 to 25 nucleotides, which regulate gene expression post-transcriptionally by promoting the degradation of target mRNAs or inhibiting the translation of target mRNAs (21). In addition, circRNAs in the cytoplasm also act as competing endogenous RNAs (ceRNAs) to antagonize miRNA function. Emerging evidence indicates that the circRNA/miRNA/mRNA axis is critically important in various human cancers (22). For example, hsa_circ_0051240 sponges miR-637 and thus induces KLK4 expression to promote proliferation, migration, and invasion of OC cells (23). In addition, circPLEKHM3 can function as a tumor suppressor in OC cells by targeting the miR-9/BRCA1/DNAJB6/KLF4/AKT1 axis (24). Also, circMUC16-mediated autophagy was found to promote epithelial OC cell invasion and metastasis by sponging miR-199a-5p, thereby regulating the expression of BECN1 and RUNX1 (25). Therefore, circRNAs may represent novel molecules for understanding OC pathogenesis and potential therapeutic target in OC.

Through high-throughput sequencing and qRT-PCR validation, hsa_circ_0007444 was downregulated in OC tissues in both studies from our previous research and Lang's group (26, 27). In addition, the expression of hsa_circ_0007444 was also found to be negatively correlated with lymphatic metastasis of OC patients (27). However, the mechanisms by which hsa_circ_0007444 regulates OC progression remain unclear.

2. Methods

2.1 Sample collection

The tissue samples analyzed in this study included 21 OC tissues collected from OC patients (43~77 years old) and 21 normal ovarian tissues obtained from patients with cervical cancer undergoing prophylactic removal of ovarian tissue at the Women's Hospital of Nanjing Medical University (Nanjing Maternity and Child Health Care Hospital) between 2015 and 2017, without any adjuvant chemotherapy or radiotherapy treatment before surgery. All samples were immediately frozen in liquid nitrogen and maintained at -80°C until further use. The study was approved by the Ethics Committee of the Women's Hospital of Nanjing Medical University, and all patients signed informed consent.

2.2 Cell Culture

The OC cell lines SKOV3, A2780, and HO8910 cells were purchased from KeyGEN Biotech. Co. Ltd. (Nanjing, China). SKOV3 cells were cultured in McCoy's 5A medium (KeyGEN Biotech. Co., Ltd.) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific Inc., Waltham, MA, USA), 100 ng/mL streptomycin, and 100 U/mL penicillin. A2780, HO8910, and 293T cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; KeyGEN Biotech Co., Ltd.) supplemented with 10% FBS, 100 ng/mL streptomycin, and 100 U/mL penicillin (Thermo Fisher Scientific Inc.). All cells were cultured at 37°C in an incubator with a humidified atmosphere containing 5% CO₂.

2.3 RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA). 200 ng total RNA was reversely transcribed into cDNA in a reaction volume of 20 µL using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc.). Bulge_loopTM miRNA qRT-PCR Primer Sets (one RT primer and a pair of qPCR primers for each set) specific for miRNA were designed by RiboBio Co., Ltd. (Guangzhou, China). cDNA amplification was performed and measured using the Applied Biosystems ABI Vii7 Real-Time PCR System (Applied Biosystems, Foster City, USA) with the AceQ Universal SYBR qPCR Master Mix (Vazyme Biotech. Co. Ltd., Nanjing, China). Samples of mRNA and circRNA were normalized to β-ACTIN, and samples of miRNA were normalized to U6 small nuclear RNA (U6 snRNA), and relative expression was calculated by the $2^{-\Delta\Delta C_t}$ method. All primers used were listed in Supplementary Table 1.

2.4 Western blot analysis

About 2×10^7 OC cells were lysed with RIPA lysis buffer (Beyotime, Shanghai, China), including 100 µL RIPA, 1 mM phenylmethanesulfonyl fluoride (PMSF), placed on ice for 30 min and then centrifuged for 30 min, 14,000rpm, at 4°C. The supernatant was collected and the protein concentration was calculated using the bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher Scientific Inc.). About 10 µg of protein was loaded and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene Fluoride (PVDF) membranes (Millipore Corp., Bedford, MA, USA). Subsequently, the membranes were soaked in 5% nonfat milk for 1 h, then incubated with specific primary antibodies overnight at 4°C, washed several times with tris-buffered saline-tween 20 (TBST), incubated with the corresponding secondary antibody for 2 h, washed three times with TBST and finally imaged using the FluoroChem M imaging system (Protein Simple Inc., San Jose, CA, USA). Antibodies used for Western Blot analysis were β-ACTIN (sc-81178, Santa Cruz Biotechnology, Santa Cruz, CA, USA), DICER1 (20567-1-AP, Proteintech Inc., Rosemont, IL, USA).

2.5 Cell Counting Kit-8 (CCK-8) assay

Cell proliferation was measured by the Cell Counting Kit-8 (CCK-8) kit (KeyGEN Biotech Co., Ltd.). Briefly, 2,000 cells per well of control and treated A2780 and SKOV3 cells were seeded in 96-well plates. After the cells were attached to the surface of the 96-well plate for 0, 24, 48 and 72 h, 10 µL of CCK-8 solution was added into each well and cells were continued to culture in the incubator at 37°C for 2 h, then the 0 h

absorbance or optical density (OD) was measured on a Synergy H4 Hybrid Reader (BioTek Instruments, Shanghai, China) at a wavelength of 450 nm.

2.6 Colony formation assay

About 3,000 OC cells were seeded in 6-well plates. After incubation for 10 days, the colonies were gently washed twice with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet (Sigma-Aldrich, Saint Louis, MO, USA) for 15 min. Then, the number of colonies was counted and calculated. All of the experiments were performed in triplicate.

2.7 Wound healing assay

About 6×10^5 control and hsa_circ_0007444-overexpressing A2780 and SKOV3 cells were seeded into one well of 6-well plates. Then, the medium was changed every three days, and when the cell confluence reached 100%, the cells were scratched with a 200- μ L pipette tip, washed three times with PBS and incubated in serum-free medium for 48 h. The images of the plates were captured at 0, 24, and 48 h after scratching. Eventually, the wound healing area was analyzed by Image J (NIH, Bethesda, MD, USA).

2.8 Transwell assay

The cell migration and invasion abilities were analyzed using the Transwell system (Corning Inc., Corning, NY, USA) with or without Matrigel (BD Biosciences, San Jose, CA, USA) following the manufacturer's instructions. Briefly, first, the upper chamber was coated with Matrigel (1:7 dilution with serum-free medium) for 24 h. Then, 200 μ L of cell suspension (5×10^5 cells) in serum free medium of that transfected with control or hsa_circ_0007444-overexpressing SKOV3 cells for 24 h were seeded in the upper chamber with or without Matrigel, and 600 μ L of medium with 20% FBS was added in the lower chamber. After 24~72 h of culture, the cells were fixed in 4% paraformaldehyde for 1 h and stained with 0.1% crystal violet for 0.5 h. Then, the Matrigel and /or the cells on the upper surface of the top chamber were gently removed with a cotton swab. Images were captured under a microscope (Nikon Corp., Tokyo, Japan). The cells on the lower surface of the upper chambers were lysed with RIPA lysis buffer and the protein concentration was determined by measuring the OD at 560 nm on a Synergy H4 Hybrid Reader (BioTek Instruments).

2.9 Flow cytometry analysis

Apoptosis in A2780 and SKOV3 cells was assessed using a phycoerythrin (PE)-Annexin V Apoptosis Detection kit (BD Biosciences). The A2780 and SKOV3 cells were first cultured in six-well plates and transfected with an hsa_circ_0007444-overexpression vector or a control vector or siRNAs against hsa_circ_0007444 for 48 h with Lipofectamine 2000 (Invitrogen). After 24 h, 1 μ L of apoptosis inducer A and B from the Apoptosis Inducers Kit (Beyotime) was added to each well. After 48 h, we suspended the cells in Annexin V binding buffer and double-stained with fluorescein isothiocyanate (FITC) and propidium iodide (PI) for 25 min in the dark, and cell apoptosis was measured using a BD FACS Calibur™ flow cytometer (BD Biosciences.).

2.10 Vector construction and cell transfection

Both the control and hsa_circ_0007444 overexpression vectors were constructed by Genomeditech Co., Ltd. (Shanghai, China). Briefly, first, after designing and synthesizing the hsa_circ_0007444 primers, PCR amplification was performed and the amplification products were cloned into hsa_circ_0007444 overexpression vector GM-7183 or control vector PGMLV-6395. After separation of the PCR amplification products by agarose gel electrophoresis, the DNA fragments were recovered and purified by using a DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA), and double restriction enzyme digestion. Eventually, the product was transferred to competent *Escherichia coli* cells, and the bacteria were evenly applied to a 10-cm culture agar plate with ampicillin, which selected for ampicillin resistant cells (successfully transfected bacterial cells). Bacteria were cultured in a humidified atmosphere containing 5% CO₂ at 37°C. After 18 h, a single bacterial colony was picked and inoculated into 15-mL sterilized ethylene-polypropylene (EP) tubes using a 10 µL pipette tip for large scale production of the vectors. Vector constructs were confirmed by sequencing. siRNAs used in this study were synthesized by RiboBio CO., Ltd. OC cells were plated in one well of a six-well plates until cell confluence reached 80-90%. For transfection, 3 µg of hsa_circ_0007444 overexpression vector or control vector or 10 µL siRNA were transfected using Lipofectamine 2000 (Invitrogen) following to instruction provided by the manufacturer.

2.11 Dual luciferase reporter assay

The binding sites of miR-23a-3p in hsa_circ_0007444, and those of miR-23a-3p in DICER1 were predicted by STARBASE (<http://starbase.sysu.edu.cn/>). Wild type (hsa_circ_0007444-WT or DICER1 3'UTR-WT) and mutant type (hsa_circ_0007444-MUT or DICER1 3'UTR-MUT) containing miR-23a-3p binding sites or not were subcloned into the pGL3-basic vector (Promega Corp., Madison, WI, USA) by Genomeditech Co., Ltd. About 250 ng of dual-luciferase reporter plasmids and 2.5 µL of miR-23a-3p mimics or miR-mimic-NC were co-transfected into A2780 and SKOV3 cells using Lipofectamine 2000 (Invitrogen) for 24 h. Afterward, the luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega Corp.). All the experiments were performed in triplicate.

2.12 RNA-binding protein immunoprecipitation (RIP) assay

The RNA-binding protein immunoprecipitation (RIP) assays were performed using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore Corp.) following the manufacturer's instructions. Briefly, about 2×10^7 hsa_circ_0007444-overexpressing SKOV3 cells were dissolved in 210 µL cell lysis buffer. After 24 h, a 100-µL aliquot of cell lysis buffer was incubated with AGO2 RIP beads or IgG RIP beads and rotated overnight. RIP beads were pre-incubated with 5 µg AGO2 antibody (Abcam, Cambridge, UK) and 5 µg IgG antibody (Millipore Corp.). The next day, the immunoprecipitated RNA molecules were purified from these mixtures following the RIP kit protocol. Eventually, the hsa_circ_0007444 level was measured by qRT-PCR analysis using the primers provided above.

2.13 Lentivirus infection and stable cell line generation

Lentivirus packaging system was constructed using a lentivirus packaging kit (Genomeditech Co., Ltd.) and lentivirus was infected into HEK293T cells. Eventually, the infected lentivirus was collected, and

lentivirus concentration was determined. The titer of hsa_circ_0007444-overexpressing lentivirus and control lentivirus was about 2×10^9 TU/mL and 1×10^9 TU/mL, respectively. Lentivirus was diluted to 1×10^7 TU/mL with complete culture medium. According to the multiplicity of infection (MOI) values (MOI values = lentivirus concentration $\times$ lentivirus volume/cells number), 20 μ L of diluted lentivirus and 2 μ L of 1 mg/mL Polybrene were used to infect 5×10^4 cells per well on 6-well plates. The cells were cultured in an incubator at 37°C for 48 h with a humidified atmosphere containing 5% CO₂. Stable cells lines were established after 4 μ g/mL puromycin selection for 48 h.

2.14 *In vivo* animal experiment

The 46 female 4-6-week-old BALB/c nude mice (13-18 g) used in this study were purchased from Model Animal Research Center of Nanjing University (Nanjing, China). All mice were housed in a standard individually ventilated cage (IVC) system laboratory animal room.

Sixteen mice were randomly divided into two groups, 8 mice in each group, and then the control and hsa_circ_0007444-overexpressing SKOV3 cells (1×10^7 in 200 μ L PBS) were subcutaneously injected into the left armpit of the mice. Mouse weight and tumor volume (volume (mm³) = $0.5 \times \text{length} \times \text{width}^2$) were monitored every 2~3 days.

The other 30 BALB/c nude mice were randomly divided into two groups, 15 mice in each group, one group was tail-vein injected with control SKOV3 cells (2×10^6 cells in 200 μ L PBS) and the other group was tail-vein injected with hsa_circ_0007444-overexpressing SKOV3 cells (2×10^6 cells in 200 μ L PBS). Eight weeks later, mice were sacrificed by spinal cord dislocation to collect the mouse lungs and record the number of metastatic lesions in the lungs (Diameter > 1 mm). Lung metastatic nodules were stained with hematoxylin-eosin staining and counted.

2.15 Cell nuclear and cell cytoplasmic separation assay

The nuclear and cytoplasmic fractions of cells were isolated using the PARIS™ kit (Invitrogen). First, 10×10^6 OC cells were washed twice with 10 mL of pre-cooled PBS. Then, the cells were digested with 1 mL trypsin and collected into 15 mL EP tube containing 3 mL of medium with 10% FBS to stop digestion of cells. Cell pellets were re-suspended in 500 μ L cell fraction buffer, incubated on ice for 10 min, and then centrifuged at 500g and 4°C for 5 min to separate the nuclear and cytoplasmic cell fractions. Nuclear pellets were homogenized with 1 mL cell disruption buffer. Eventually, RNA concentration was measured, genomic DNA was removed, reverse transcription was performed, and the results were verified by qRT-PCR analysis.

2.16 Agarose gel electrophoresis and Sanger sequencing

Electrophoresis was performed with a mixture of 10 μ L PCR products and 2 μ L loading buffer in a 1.5% agarose gel. The target gene products were recycled, purified before Sanger sequencing on an ABI3730

DNA Analyzer Instrument (Applied Biosystems). The sequencing results were analyzed by Chromas system (Technelysium Pty Ltd., South Brisbane, Australia).

2.17 Immunohistochemistry (IHC) analysis and hematoxylin and eosin (H&E) staining

Immunohistochemistry (IHC) analysis was performed using an IHC kit (PK10006; Proteintech Inc.) according to the manufacturer's protocol. Control or hsa_circ_0007444-overexpressing tumor paraffin sections were stained with Ki67 (Proteintech Inc.) and DICER1 (Proteintech Inc.), respectively. Lung metastatic nodules were stained with hematoxylin and eosin (H&E), following the instructions in a reported study (28).

2.18 Statistical Analysis

The statistical analysis of the results was performed using the SPSS 23.0 statistical analysis software (IBM Corp., Armonk, NY, USA). The experimental values are expressed as the mean $\pm$ SD. The differences between two groups were examined using the Student's t-test. Pearson correlation analysis was performed to confirm the correlation between the hsa_circ_0007444 and DICER1 expression. P -value < 0.05 was considered statistically significant.

3. Results

3.1 Hsa_circ_0007444 is downregulated in ovarian cancer

Our previous study showed that hsa_circ_0007444 was significantly downregulated in OC tissues compared with normal ovarian tissues (26). Hsa_circ_0007444 is generated by circularization of back-spliced exons from the RHOBTB3 gene on the forward strand of chromosome 5 (from 95,091,099 - 95,099,324, 479 bp, hg19 genome build) (Figure 1A). We designed junction primers of hsa_circ_0007444 to validate its existence and amplified hsa_circ_0007444 junction expression in cDNA from A2780 cells (Figure 1B) followed by Sanger sequencing (Figure 1C). In this study, we performed qRT-PCR analysis to further confirm the expression of hsa_circ_0007444 in normal tissues, ovary cancer tissues, and cell lines. Compared to normal ovarian tissues, para-tumor tissues, and normal ovarian epithelial cell line (IOSE80), hsa_circ_0007444 was downregulated in OC tissues and OC cell lines (Figure 1D-E). These results suggested that hsa_circ_0007444 is probably involved in the progression of OC.

3.2 Downregulation of hsa_circ_0007444 significantly promoted ovarian cancer progression *in vitro*

Considering its downregulation in cancer tissues and cell lines, we hypothesized that hsa_circ_0007444 might serve as a tumor suppressor in OC. Two siRNAs were designed to target the back-splice junction site of hsa_circ_0007444, which did not affect the expression of its parental gene RHOBTB3 (Figure 2A). Then, cell proliferation, migration, invasion, and apoptosis of the OC cells A2780 and SKOV3 were analyzed by the CCK-8 assay, colony formation assay, transwell assay with or without Matrigel, wound

healing assay, and flow cytometry analysis. The obtained results indicated that knockdown of hsa_circ_0007444 significantly promoted cell proliferation (Figure 2B-C), invasion (Figure 2D), migration (Figure 2D-E), and inhibited apoptosis (Figure 2F) of both A2780 and SKOV3 cells.

3.3 Up-regulation of hsa_circ_0007444 significantly inhibited ovarian cancer progression *in vitro*

To further confirm the function of hsa_circ_0007444 in OC progression, we first transfected A2780 and SKOV3 cells with control or hsa_circ_0007444-overexpression vector, and qRT-PCR analysis revealed that hsa_circ_0007444 was significantly upregulated after transfecting the hsa_circ_0007444 expression vector in A2780 and SKOV3 cells (Figure 3A). As a result, enforced expression of hsa_circ_0007444 significantly inhibited cell proliferation (Figure 3B, 3C), migration and invasion (Figure 3D-F). Additionally, Western blot analysis showed that enforced expression of hsa_circ_0007444 increased the expression of cleaved caspase 3, indicating that its upregulation enhanced cell apoptosis (Figure 3G).

3.4 Hsa_circ_0007444 functions as a sponge for miR-23a-3p in OC cells

CircRNAs in the cytoplasm mainly functions as sponges for miRNAs, thus regulating their target genes' expression. Accordingly, we first analyzed the hsa_circ_0007444 distribution in SKOV3 cells, and our results revealed that hsa_circ_0007444 was most abundant in the cytoplasm (Figure 4A). Thus, we further analyzed the binding ability of hsa_circ_0007444 with AGO2 on the Circinteractome database (29), which resulted in 8 AGO2 binding sites in the hsa_circ_0007444 circRNA (Supplementary Table 2), RIP-qPCR analysis was performed to confirm the interaction between AGO2 and hsa_circ_0007444, and the results indicated that hsa_circ_0007444 could directly bind to AGO2 (Figure 4B). Then, the prediction of the miRNAs that can bind hsa_circ_0007444 by the PITA and miRanda bioinformatic tools revealed 1,448 and 973 miRNA targets, respectively. Among them, 547 miRNAs were found by both tools (Supplementary Table 3-7). It was also found that hsa_circ_0007444 has at least two complementary sequences with the miR-23a-3p, miR-103a-3p, miR-107, miR-1270 and miR-550a-5p seed region (Figure 4C). In addition, the analysis of the expression of these miRNAs in hsa_circ_0007444-knockdown and overexpressing OC cells revealed that the expression of miR-1270 was undetectable in both A2780 and SKOV3 cells and only miR-23a-3p was consistently upregulated in hsa_circ_0007444-silenced A2780 and SKOV3 cells, and downregulated in hsa_circ_0007444-overexpressing A2780 and SKOV3 cells (Figure 4D-E). Additionally, miR-23a-3p was also significantly upregulated in OC tissues, as shown in the GEO dataset GSE65819 (Figure 4F). In fact, the binding site of miR-23a-3p in hsa_circ_0007444 was predicted by RNAhybrid (30) (Figure 4G). Transfection of miR-23a-3p mimics decreased the transcriptional activity of the luciferase reporter containing miR-23a-3p binding sites, but it did not affect the transcriptional activity of the luciferase reporter containing the mutant miR-23a-3p binding sites, suggesting that miR-23a-3p can directly target hsa_circ_0007444 (Figure 4H). All these results mentioned above suggested that hsa_circ_0007444 might function as a sponge for miR-23a-3p.

3.5 The inhibitory effect of hsa_circ_0007444 on OC cell proliferation, migration and invasion are reversed by miRNA-23a-3p

Previous studies have shown that miR-23a-3p acts as an oncogene in various cancers such as liver, colorectal and pancreatic cancers (31–33), and it is upregulated in OC tissues (34). However, its function in OC has not been reported to date. To investigate whether hsa_circ_0007444 exerts its anti-cancer effects by sponging miR-23a-3p, we conducted rescue experiments in OC cells. Our results showed that miR-23a-3p expression was significantly reduced in A2780 and SKOV3 cells transfected with the hsa_circ_0007444-overexpression vector, while miR-23a-3p was significantly upregulated in the cells transfected with miR-23a-3p mimics alone or the cells transfected with both the hsa_circ_0007444-overexpression vector and miR-23a-3p mimics (Figure 5A). Moreover, the rescue experiments showed that transfection of miR-23a-3p mimics recovered the inhibitory effects of hsa_circ_0007444 overexpression on proliferation (Figure 5B), migration, and invasion (Figure 5C-F) in A2780 and SKOV3 cells.

3.6 hsa_circ_0007444 upregulates DICER1 expression by sponging miR-23a-3p

The prediction of the potential target genes of miR-23a-3p by miRwalk revealed 1391 target genes (Supplementary Table 8). Meanwhile, RNA-sequencing transcriptome analysis of control and hsa_circ_0007444 knockdown OC cells found that a total of 211 mRNAs were significantly upregulated in hsa_circ_0007444-silenced SKOV3 cells (Supplementary Table 9 and Supplementary Figure 1). In addition, 14 predicted target genes were significantly downregulated in the hsa_circ_0007444-silenced OC cells (Figure 6A). As shown in Supplementary Figure 2A, the qRT-PCR analysis indicated that only five predicted target genes (*KLF6*, *TRMT9B*, *ZNF107*, *ZNF512*, and *DICER1*) were significantly downregulated in the hsa_circ_0007444-silenced SKOV3 cells, whereas only *DICER1* was significantly upregulated in the hsa_circ_0007444-overexpressing SKOV3 cells. Western blot analysis confirmed the upregulation of DICER1 in the hsa_circ_0007444-overexpressing SKOV3 cells and downregulation of DICER1 in the hsa_circ_0007444-silenced SKOV3 cells (Figure 6B). Recent studies showed that DICER1 was downregulated in various tumors and associated with a poor prognosis (35, 36). Moreover, it has been reported that DICER1 expression was decreased in OC tissues and downregulation of DICER1 promotes OC progression (37). Compared to normal ovarian tissues, in this study, DICER1 expression was downregulated in OC tissues (Figure 6C). Transfection of miR-23a-3p mimics could attenuate the upregulation of DICER1 by the overexpression of hsa_circ_0007444 (Figure 6D, Supplementary Figure 2B). Luciferase reporter assay showed that DICER1 is a downstream target of miR-23a-3p since its activity decreased after co-transfection with miR-23a-3p mimics and the DICER1-WT luciferase reporter (Figure 6E). Importantly, we found that the expression level of hsa_circ_0007444 was positively correlated with the abundance of DICER1 mRNA transcript in OC tissues, not in para-cancer tissues (Supplementary Figure 2C-D). These results showed that hsa_circ_0007444 acts as a miR-23a-3p sponge to regulate DICER1 expression.

3.7 Overexpression of hsa_circ_0007444 suppresses ovarian cancer progression *in vivo*

To further confirm the tumor-suppressive role of hsa_circ_0007444 in OC *in vivo*. We first established a SKOV3 cell line stably overexpressing hsa_circ_0007444 or control by infecting SKOV3 cells with hsa_circ_0007444-overexpression or control lentivirus. Subsequently, the hsa_circ_0007444-overexpressing or control SKOV3 cells were subcutaneously injected into BALB/c nude female mice (n = 8 in each group), and the tumor volume was measured every two to three days. The size of the tumors in the hsa_circ_0007444-overexpressed group was significantly smaller than that in the control group (Figure 7A-B). IHC results showed that the expression of Ki67 in the hsa_circ_0007444-overexpressing group was decreased as compared to the control group, indicating that hsa_circ_0007444 could inhibit the growth of OC *in vivo* (Figure 7C-D).

To investigate the role of hsa_circ_0007444 in tumor metastasis, hsa_circ_0007444-overexpressed and control SKOV3 cells were tail-vein injected into the BALB/c nude female mice (n = 15 in each group). Eight weeks later, H&E staining analysis of the lung of these mice showed that the hsa_circ_0007444-overexpressing group had more lung metastatic colonies than the control group (Figure 7E-F). These results indicated that hsa_circ_0007444 might serve as a tumor suppressor in OC progression.

4. Discussion

Our previous study identified one upregulated and five downregulated circRNAs, including hsa_circ_0007444 (circRHOBTB3), which was significantly downregulated in OC tissues compared with normal ovarian tissues (26). Also, notably, it has been reported that hsa_circ_0007444 expression was negatively correlated with lymphatic metastasis in OC patients (27). Accordingly, in this study, we further investigated the function and mechanism of hsa_circ_0007444 in OC growth and metastasis.

Consistent with the results reported by Deng's and Sun's group in 2020, which indicated that hsa_circ_0007444 could inhibit gastric cancer growth by sponging miR-654-3p (38) and inhibit OC progression by regulating the PI3K/AKT signaling pathway (39), our study also demonstrated the tumor-suppressive function of hsa_circ_0007444 in OC growth and metastasis both *in vitro* and *in vivo*. Both our study and Sun's group demonstrated that hsa_circ_0007444 could inhibit ovarian tumor growth *in vivo*. Besides, by using the tail vein injection model, our study also demonstrated that hsa_circ_0007444 could significantly inhibit the lung metastasis of OC cells *in vivo*, which is consistent with the negative correlation between hsa_circ_0007444 expression and lymphatic metastasis, further indicating that hsa_circ_0007444 may be the potential therapeutic target of OC.

Both our study and that of Sun's group found that hsa_circ_0007444 is mainly located in the cytoplasm of OC cells. Also, the results of our RIP-qPCR analysis also showed that hsa_circ_0007444 could bind to AGO2 and may act as a miRNA sponge. Additionally, our further study indicated that hsa_circ_0007444 binds miR-23a-3p and thus regulates the expression of miR-23a-3p and its target gene *DICER1*. *DICER1* is

the key enzyme in miRNA production and also a tumor suppressor. Silencing of DICER1 can promote cancer cell invasion and metastasis and is associated with poor prognosis (36). It has been reported that DICER1 is downregulated in OC tissues, and downregulation of DICER1 can promote OC metastasis (37). In addition, several miRNAs were found to be downregulated in cancer due to the downregulation of DICER1. However, miR-23a was found to be upregulated in several cancers, such as OC (40), nasopharyngeal carcinoma (41), and gastric cancer (42). Additionally, miRNA could also act as the upstream regulator of DICER1, which promoted the degradation of DICER1 (35, 43). Furthermore, it has been reported that despite having a reduced level of DICER1, Treg cells significantly overexpressed several miRNAs, including miR-23a (44), which indicated that miR-23a might also act as the upstream regulator of DICER1. Our study first demonstrated the involvement of the hsa_circ_0007444/miR-23a-3p/DICER1 axis in OC development.

Notably, the previous studies have shown that miR-23a-3p can inhibit PTEN and thus elevate the expression of PD-L1 and promote the phosphorylation of AKT in macrophages (45). Also, miR-23a-3p was found to regulate the PTEN/PI3K/AKT signaling pathway in osteosarcoma cells, hepatic stellate cells, and hepatic fibrosis tissues (46, 47). Hotspot mutations in DICER1 were found to result in the activation of PI3K/AKT/mTOR signaling by modulation of specific miRNA population (48). These results indicated that hsa_circ_0007444 might regulate the PI3K/AKT pathway by directly sponging miR-23a-3p.

In addition, we also noticed that the circRNA could regulate the same cancer progression process through a different mechanism. For example, Zeng, *et al.* reported that circHIPK3 promotes colorectal cancer growth and metastasis by sponging miR-7 (49), while Yan, *et al.* showed that circHIPK3 promotes colorectal cancer cell proliferation and metastasis through the circHIPK3/miR-1207-5p/FMNL2 axis (50). Gao, *et al.* demonstrated that the circRNA MYLK promotes hepatocellular carcinoma progression by sponging miR29a and thus regulates KMT5C (51). Furthermore, Li *et al.* showed that circular RNA MYLK sponges miR-362-3p and regulates Rab23 expression to promote hepatocellular carcinoma progression(52). These results further demonstrated the multi-function of circRNAs in the disease regulatory network.

Finally, through bioinformatics analysis using available databases (riboCIRC:

http://www.ribocirc.com/rna_calling.html, and circRNADb:

<http://reprod.njmu.edu.cn/circrnadb/circRNADb.php>), we also found that hsa_circ_0007444 has the potential to encode a peptide. Therefore, further studies are needed to completely understand the function and mechanism of hsa_circ_0007444 encoded peptide in cancer and to develop new therapeutic drugs for the treatment of cancer.

Conclusions

We found that downregulated hsa_circ_0007444 in OC could interact with AGO2 to sponge miR-23a-3p to induce DICER1 expression, and its overexpression could effectively impair the cell proliferation, invasion,

migration, and apoptosis in vitro. It can also inhibit growth and lung metastasis in vivo. This newly discovered ceRNA network could offer a possible therapeutic target in the treatment of OC.

Abbreviations

circRNA
circular RNA
qRT
quantitative real-time
RIP
RNA immunoprecipitation
CCK-8
cell counting-kit 8
ceRNAs
competing endogenous RNAs
SDS-PAGE
sodium dodecyl sulfate polyacrylamide gel electrophoresis
PVDF
polyvinylidene Fluoride
OD
optical density
PBS
phosphate-buffered saline
BCA
bicinchoninic acid
FITC
fluorescein isothiocyanate
PI
propidium iodide
IHC
immunohistochemistry
H&E
hematoxylin and eosin
miRNA
microRNA
MREs
miRNA response elements
WB
Western Blot

Declarations

Ethics Approval and Consent to Participate

This study has been approved by the Ethics Committee of Nanjing Maternity and Child Health Care Hospital (approval number NFKSL-022). Written consent was obtained from the patients involved in this study.

Consent for publication: The authors affirm that human research participants provided informed consent for publication of the images and tables.

Availability of data and materials

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Competing interest

The authors declare that they have no conflict of interest.

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Authors' Contributions

ZM and SY performed the experiments and drafted the manuscript; TF, XJ and JXM performed development of methodology and writing, review and revision of the paper; SYQ, XHZ and SR provided acquisition, analysis and interpretation of data. All authors read and approved the final paper.

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References

1. Siegel RL, Miller KD, Jemal A. Cancer Statistics. 2017. CA Cancer J Clin. 2017;67(1):7-30.
2. Siegel RL, Miller KD, Fuchs HE, Jemal A. Cancer Statistics. 2021. CA: a cancer journal for clinicians. 2021;71(1).
3. Lheureux S, Braunstein M, Oza AM. Epithelial ovarian cancer: Evolution of management in the era of precision medicine. CA Cancer J Clin. 2019;69(4):280–304.

4. Lheureux S, Gourley C, Vergote I, Oza AM. Epithelial ovarian cancer. *Lancet*. 2019;393(10177):1240–53.
5. Salzman J, Gawad C, Wang PL, Lacayo N, Brown PO. Circular RNAs are the predominant transcript isoform from hundreds of human genes in diverse cell types. *PLoS One*. 2012;7(2):e30733.
6. Hsu MT, Coca-Prados M. Electron microscopic evidence for the circular form of RNA in the cytoplasm of eukaryotic cells. *Nature*. 1979;280(5720):339–40.
7. Chen I, Chen C-Y, Chuang T-J. Biogenesis, identification, and function of exonic circular RNAs. 2015;6(5):563–79.
8. Zhang Y, Zhang X-O, Chen T, Xiang J-F, Yin Q-F, Xing Y-H, et al. Circular Intronic Long Noncoding RNAs *Molecular Cell*. 2013;51(6):792–806.
9. Jeck WR, Sorrentino JA, Wang K, Slevin MK, Burd CE, Liu J, et al. Circular RNAs are abundant, conserved, and associated with ALU repeats. *Rna*. 2012;19(2):141–57.
10. Qu S, Liu Z, Yang X, Zhou J, Yu H, Zhang R, et al. The emerging functions and roles of circular RNAs in cancer. *Cancer Lett*. 2018;414:301–9.
11. Nigro JM, Cho KR, Fearon ER, Kern SE, Ruppert JM, Oliner JD, et al. Scrambled exons *Cell*. 1991;64(3):607–13.
12. Qu S, Yang X, Li X, Wang J, Gao Y, Shang R, et al. Circular RNA: A new star of noncoding RNAs. *Cancer Lett*. 2015;365(2):141–8.
13. Li X, Yang L, Chen LL. The Biogenesis, Functions, and Challenges of Circular RNAs. *Mol Cell*. 2018;71(3):428–42.
14. Cortes-Lopez M, Miura P. Emerging Functions of Circular RNAs. *Yale J Biol Med*. 2016;89(4):527–37.
15. Shi P, Wan J, Song H, Ding X. The emerging role of circular RNAs in gastric cancer. *American journal of cancer research*. 2018;8(10):1919–32.
16. Xu XW, Zheng BA, Hu ZM, Qian ZY, Huang CJ, Liu XQ, et al. Circular RNA hsa_circ_000984 promotes colon cancer growth and metastasis by sponging miR-106b. *Oncotarget*. 2017;8(53):91674–83.
17. Hu W, Bi ZY, Chen ZL, Liu C, Li LL, Zhang F, et al. Emerging landscape of circular RNAs in lung cancer. *Cancer Lett*. 2018;427:18–27.
18. Qin M, Liu G, Huo X, Tao X, Sun X, Ge Z, et al. Hsa_circ_0001649: A circular RNA and potential novel biomarker for hepatocellular carcinoma. *Cancer Biomark*. 2016;16(1):161–9.
19. Wang X, Fang L. Advances in circular RNAs and their roles in breast Cancer. *J Exp Clin Cancer Res*. 2018;37(1):206.
20. Chen Q, Zhang J, He Y, Wang Y. hsa_circ_0061140 Knockdown Reverses FOXM1-Mediated Cell Growth and Metastasis in Ovarian Cancer through miR-370 Sponge Activity. *Molecular therapy Nucleic acids*. 2018;13:55–63.
21. Ha M, Kim VN. Regulation of microRNA biogenesis. *Nature reviews Molecular cell biology*. 2014;15(8):509–24.
22. Chan JJ, Tay Y. Noncoding RNA:RNA Regulatory Networks in Cancer. *Int J Mol Sci*. 2018;19(5).

23. Zhang M, Xia B, Xu Y, Zhang Y, Xu J, Lou G. Circular RNA (hsa_circ_0051240) promotes cell proliferation, migration and invasion in ovarian cancer through miR-637/KLK4 axis. *Artificial cells, nanomedicine, and biotechnology*. 2019;47(1):1224–33.
24. Zhang L, Zhou Q, Qiu Q, Hou L, Wu M, Li J, et al. CircPLEKHM3 acts as a tumor suppressor through regulation of the miR-9/BRCA1/DNAJB6/KLF4/AKT1 axis in ovarian cancer. *Mol Cancer*. 2019;18(1):144.
25. Gan X, Zhu H, Jiang X, Obiegbusi SC, Yong M, Long X, et al. CircMUC16 promotes autophagy of epithelial ovarian cancer via interaction with ATG13 and miR-199a. *Mol Cancer*. 2020;19(1):45.
26. Teng F, Xu J, Zhang M, Liu S, Gu Y, Zhang M, et al. Comprehensive circular RNA expression profiles and the tumor-suppressive function of circHIPK3 in ovarian cancer. *Int J Biochem Cell Biol*. 2019;112:8–17.
27. Ning L, Long B, Zhang W, Yu M, Wang S, Cao D, et al. Circular RNA profiling reveals circEXOC6B and circN4BP2L2 as novel prognostic biomarkers in epithelial ovarian cancer. *Int J Oncol*. 2018;53(6):2637–46.
28. Feldman AT, Wolfe D. Tissue processing and hematoxylin and eosin staining. *Methods Mol Biol*. 2014;1180:31–43.
29. Dudekula DB, Panda AC, Grammatikakis I, De S, Abdelmohsen K, Gorospe M. CircInteractome. A web tool for exploring circular RNAs and their interacting proteins and microRNAs. *RNA Biol*. 2016;13(1):34–42.
30. Rehmsmeier M, Steffen P, Hochsmann M, Giegerich R. Fast and effective prediction of microRNA/target duplexes. *RNA*. 2004;10(10):1507–17.
31. Liu J, Fan L, Yu H, Zhang J, He Y, Feng D, et al. Endoplasmic Reticulum Stress Causes Liver Cancer Cells to Release Exosomal miR-23a-3p and Up-regulate Programmed Death Ligand 1 Expression in Macrophages. *Hepatology*. 2019;70(1):241–58.
32. Wang X, Gao X, Tian J, Zhang R, Qiao Y, Hua X, et al. LINC00261 inhibits progression of pancreatic cancer by down-regulating miR-23a-3p. *Arch Biochem Biophys*. 2020;689:108469.
33. Lee Y, Kim SJ, Choo J, Heo G, Yoo JW, Jung Y, et al. miR-23a-3p is a Key Regulator of IL-17C-Induced Tumor Angiogenesis in Colorectal Cancer. *Cells*. 2020;9(6).
34. Prahm KP, Høgdall C, Karlsen MA, Christensen IJ, Novotny GW, Høgdall E. Identification and validation of potential prognostic and predictive miRNAs of epithelial ovarian cancer. *PloS one*. 2018;13(11):e0207319.
35. Rupaimoole R, Ivan C, Yang D, Gharpure KM, Wu SY, Pecot CV, et al. Hypoxia-upregulated microRNA-630 targets Dicer, leading to increased tumor progression. *Oncogene*. 2016;35(33):4312–20.
36. Foulkes WD, Priest JR, Duchaine TF. DICER1: mutations, microRNAs and mechanisms. *Nature reviews Cancer*. 2014;14(10):662–72.
37. To SKY, Mak ASC, Eva Fung YM, Che CM, Li SS, Deng W, et al. β -catenin downregulates Dicer to promote ovarian cancer metastasis. *Oncogene*. 2017;36(43):5927–38.

38. Deng G, Mou T, He J, Chen D, Lv D, Liu H, et al. Circular RNA circRHOBTB3 acts as a sponge for miR-654-3p inhibiting gastric cancer growth. *J Exp Clin Cancer Res*. 2020;39(1):1.
39. Yalan S, Yanfang L, He C, Yujie T. Circular RNA circRHOBTB3 inhibits ovarian cancer progression through PI3K/AKT signaling pathway. *Panminerva medica*. 2020.
40. Yang Z, Wang XL, Bai R, Liu WY, Li X, Liu M, et al. miR-23a promotes IKK α expression but suppresses ST7L expression to contribute to the malignancy of epithelial ovarian cancer cells. *Br J Cancer*. 2016;115(6):731–40.
41. Bao L, You B, Shi S, Shan Y, Zhang Q, Yue H, et al. Metastasis-associated miR-23a from nasopharyngeal carcinoma-derived exosomes mediates angiogenesis by repressing a novel target gene TSGA10. *Oncogene*. 2018;37(21):2873–89.
42. Hu X, Wang Y, Liang H, Fan Q, Zhu R, Cui J, et al. miR-23a/b promote tumor growth and suppress apoptosis by targeting PDCD4 in gastric cancer. *Cell death disease*. 2017;8(10):e3059.
43. Martello G, Rosato A, Ferrari F, Manfrin A, Cordenonsi M, Dupont S, et al. A MicroRNA targeting dicer for metastasis control. *Cell*. 2010;141(7):1195–207.
44. Divekar AA, Dubey S, Gangalum PR, Singh RR. Dicer insufficiency and microRNA-155 overexpression in lupus regulatory T cells: an apparent paradox in the setting of an inflammatory milieu. *J Immunol*. 2011;186(2):924–30.
45. Ren W, Shen S, Sun Z, Shu P, Shen X, Bu C, et al. Jak-STAT3 pathway triggers DICER1 for proteasomal degradation by ubiquitin ligase complex of CUL4A(DCAF1) to promote colon cancer development. *Cancer Lett*. 2016;375(2):209–20.
46. Dong Z, Li S, Wang X, Si L, Ma R, Bao L, et al. lncRNA GAS5 restrains CCl(4)-induced hepatic fibrosis by targeting miR-23a through the PTEN/PI3K/Akt signaling pathway. *Am J Physiol Gastrointest Liver Physiol*. 2019;316(4):G539-g50.
47. Liu J, Chen M, Ma L, Dang X, Du G. lncRNA GAS5 Suppresses the Proliferation and Invasion of Osteosarcoma Cells via the miR-23a-3p/PTEN/PI3K/AKT Pathway. *Cell Transplant*. 2020;29:963689720953093.
48. Klein SD, Martinez-Agosto JA. Hotspot Mutations in DICER1 Causing GLOW Syndrome-Associated Macrocephaly via Modulation of Specific microRNA Populations Result in the Activation of PI3K/ATK/mTOR Signaling. *Microna*. 2020;9(1):70–80.
49. Zeng K, Chen X, Xu M, Liu X, Hu X, Xu T, et al. CircHIPK3 promotes colorectal cancer growth and metastasis by sponging miR-7. *Cell death disease*. 2018;9(4):417.
50. Yan Y, Su M, Qin B. CircHIPK3 promotes colorectal cancer cells proliferation and metastasis via modulating of miR-1207-5p/FMNL2 signal. *Biochem Biophys Res Commun*. 2020;524(4):839–46.
51. Gao J, Li E, Liu W, Yang Q, Xie C, Ai J, et al. Circular RNA MYLK Promotes Hepatocellular Carcinoma Progression Through the miR29a/KMT5C Signaling Pathway. *Onco Targets Ther*. 2020;13:8615–27.
52. Li Z, Hu Y, Zeng Q, Wang H, Yan J, Li H, et al. Circular RNA MYLK promotes hepatocellular carcinoma progression by increasing Rab23 expression by sponging miR-362-3p. *Cancer cell international*. 2019;19:211.

Figures

Figure 1

Hsa_circ_0007444 expression in ovarian cancer cell lines and tissues. A. Schematic diagram of hsa_circ_0007444 generated by back-splicing from the exon 6 and exon 7 of RHOB TB3. B. Divergent primers detected circRNAs in complementary DNA (cDNA). C. Sanger sequencing revealed the back-splice junction of hsa_circ_0007444. D. qRT-PCR analysis of hsa_circ_0007444 expression in normal ovarian cells (IOSE80) and ovarian cancer (OC) cell lines (A2780, SKOV3, HO8910). E. qRT-PCR analysis of hsa_circ_0007444 expression in normal ovarian tissues and ovarian cancer tissues (n = 21). *p < 0.05, **p < 0.01, ***p < 0.0001

Figure 2

Down-regulation of hsa_circ_0007444 increases the proliferation, migration and invasion of ovarian cancer (OC) cells. A. hsa_circ_0007444 and RHOBTB3 level were measured by qRT-PCR analysis in small interfering (si)RNA-NC, si-hsa_circ_0007444-1 or si-hsa_circ_0007444-2 transfected cells. B. Evaluation of the cell viability by cell the CCK-8 assay in cells transfected by siRNA-NC or si-hsa_circ_0007444. D. Colony formation assay to analyze the invasion ability of A2780 and SKOV3 cells transfected with siRNA-

NC, siRNA1, siRNA2. C. Transwell migration, invasion assay, and E. wound healing assay, were used to determine the migration and invasion of A2780 cells and SKOV3 cells following the transfection. F. The apoptosis rate of OC cells was measured by flow cytometry analysis after transfection with siRNA-NC, siRNA1, siRNA2. *p < 0.05, **p < 0.01, ***p < 0.0001

Figure 3

Overexpressed hsa_circ_0007444 hindered the proliferation, migration and invasion of ovarian cancer (OC) cells. A. The relative abundance of hsa_circ_0007444 in OC cells transfected with hsa_circ_0007444 and control plasmids was determined by qRT-PCR analysis. B. Proliferation of A2780 and SKOV3 cells transfected with hsa_circ_0007444 and control vectors evaluated using the CCK-8 kit. C. Colony

formation assay to evaluate the proliferation of A2780 cells and SKOV3 cells transfected with hsa_circ_0007444 or control vectors. D-F. Migration and invasion abilities of A2780 and SKOV3 cells transfected with hsa_circ_0007444 and control vectors measured by Transwell assay with or without Matrigel and wound healing assay. G. Immunoblotting of cleaved caspase-3 in A2780 and SKOV3 cells transfected with hsa_circ_0007444 and control vectors. *p < 0.05, **p < 0.01, ***p < 0.0001

Figure 4

Hsa_circ_0007444 targets miR-23a-3p. A. qRT-PCR analysis of hsa_circ_0007444 expression in nuclear and cytoplasmic fractions of SKOV3 cells. The RNA ribosomal 45S and GAPDH were used as quality control. B. RNA-binding protein immunoprecipitation (RIP) assay was used to measure the amount of hsa_circ_0007444 pull down by AGO2 and IgG antibodies in SKOV3 cells. C. Schematic diagram showing that hsa_circ_0007444 has two complementary sequence to the miR-23a-3p, miR-103a-3p and miR-1270 and miR-550a-5p seed region. D-E. qRT-PCR analysis of miRNAs expression in ovarian cancer (OC) cells transfected with siRNA , hsa_circ_0007444 and control vectors. F. Relative expression of miR-23a-3p in normal ovarian tissues and OC tissues (GSE65819). G. The RNA binding structure between hsa_circ_0007444 and miR-23a-3p predicted by RNAhybrid. H. The binding sites between hsa_circ_0007444 and miR-23a-3p predicted by StarBase v.3.0. The relative luciferase activity in 293T cells co-transfected with hsa_circ_0007444-WT or hsa_circ_0007444-MUT and miR-23a-3p mimics or miR-NC was detected by the dual-luciferase reporter assay.

Figure 5

miR-23a-3p rescued the inhibitory effect of hsa_circ_0007444 on ovarian cancer cell progression. A. The relative expression of miR-23a-3p in A2780 and SKOV3 cells transfected with control vectors, hsa_circ_0007444 vectors, hsa_circ_0007444 vectors + miR-23a-3p or control vectors + miR-23a-3p was measured by qRT-PCR analysis. B. The proliferation of A2780 and SKOV3 cells was assessed by the CCK-8 assay. C-F. The migration and invasion of A2780 and SKOV3 cells were measured by Transwell assay with or without Matrigel. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$

Figure 6

miR-23a-3p directly targeted DICER1 in ovarian cancer cells. A. Overlapping data from high-throughput sequencing and miRWalk database showed that 14 genes could bind to miR-23a-3p. B. qRT-PCR analysis of DICER1 expression in normal ovarian tissues and ovarian cancer tissues (n = 21). C. qRT-PCR analysis and immunoblotting of DICER1 expression in SKOV3 transfected with siRNA-NC, siRNA, control vectors and hsa_circ_0007444 vectors. D. Immunoblotting of DICER1 in A2780 and SKOV3 transfected with

control vectors, hsa_circ_0007444 vectors, control vectors + miR-23a-3p mimics, hsa_circ_0007444 vectors + miR-23a-3p mimics. E. The putative binding sites between miR-23a-3p and 3' UTR of DICER1 were predicted by Starbase v.3.0. F. The relative luciferase activity in 293T cells co-transfected with DICER1-WT or DICER1-MUT and miR-23a-3p or miR-NC mimics was assessed by the dual-luciferase reporter assay. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$

Figure 7

Overexpression of hsa_circ_0007444 suppressed the progression of ovarian cancer growth in vivo. A-B. Tumor volume was measured in xenografts. C-D. Immunohistochemical analysis of Ki67. E. Overexpression of hsa_circ_0007444 suppressed lung metastasis. The arrow indicates the tumor in the lung. F. The number of lung metastatic nodules was calculated. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$

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