Circular RNA circMAN1A2 promotes ovarian cancer progression through the microRNA-135a-3p/IL1RAP/pTAK1 pathway

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Research Article

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Abstract

Background

Ovarian cancer (OC) is the most lethal malignancy in women owing to its diagnosis only at the advanced stage. Elucidation of its molecular pathogenesis could reveal new tumor markers and targets for therapy. Circular RNAs (circRNAs) are stable, conserved, and functional biomolecules that can be used as effective biomarkers for various cancers. In this study, a potential circRNA related to early diagnosis of OC, circMAN1A2, was analyzed.

Methods

Overexpression/knockdown of circMAN1A2 in OC cells was used to decipher its effects on cell proliferation with CCK8, EdU, cell cycle, clone formation and wound healing assay. RNA pull-down, RIP and Dual luciferase assay were used to explain the underlying mechanism of circMAN1A2 regulation of OC cell proliferation. In vivo, the effect of circMAN1A2 in OC was evaluated using nude mouse xenograft experiments.

Results

circMAN1A2 was highly expressed in OC and promoted proliferation, clone formation, and tumorigenicity of OC cells. In addition, we found that circMAN1A2 acts as a sponge for microRNA (miR)-135a-3p; miR-135a-3p directly targets the 3' untranslated region of IL1RAP in OC cells, thereby regulating the phosphorylation of TAK1, which results in promotion of OC cell growth. Therefore, circMAN1A2 promotes OC cell proliferation by inhibiting the miR-135a-3p/IL1RAP/pTAK1 axis.

Conclusion

circMAN1A2 may be a biomarker for early detection of OC and a target for subsequent therapy.

Background

Ovarian cancer (OC) is the leading cause of death in women diagnosed with gynecological cancer [1]. Since OC symptoms are nonspecific, most cases are diagnosed at an advanced stage, which results in a poor prognosis for this disease [2]. Early screening strategies do not have a significant beneficial effect on OC morbidity or mortality [3], mainly because of the lack of diagnostic markers for OC. CA-125 levels are globally elevated in most epithelial OCs, but only in half of early-stage epithelial OCs [4].

Several circular RNAs (circRNAs), which are stably expressed noncoding RNAs, have been reported as potential diagnostic biomarkers of OC. Among them, circ-ABCB10, circMAN1A, and circEPSTI1 can be
used as diagnostic biomarkers and therapeutic targets for OC [5–7]. circMAN1A2 expression is significantly upregulated in the sera of OC patients and has a promising clinical application as a good diagnostic marker [6]. Cytoplasmically expressed circRNAs can regulate tumor cell proliferation and migration by adsorbing microRNAs (miRNAs, miRs) to regulate target genes.

miRNAs are highly conserved small noncoding RNAs (18–25 bp in length) that can curb protein expression by binding to the 3' untranslated region (3'UTR) of target mRNAs. During the occurrence and development of many cancers, such as OC, miRNAs can regulate tumor growth and metastasis [8]. In recent years, noncoding RNAs have become a hot topic for research as competitive endogenous RNAs (ceRNAs) that can adsorb miRNAs to regulate tumor development and metastasis. Among them, circRNAs can also act as ceRNAs to regulate the activity of miRNAs. circ-ITCH acts as a ceRNA sponge for miR-145, increasing the protein level of RASA1 and inhibiting the malignant progression of OC cells in vitro and in vivo through the circ-ITCH–miR-145–RASA1 axis, which contributes to the occurrence and development of cancer [9]. circCDR1as exerts a tumor suppressor effect by sponging miR-135b-5p, whereas miR-135b-5p as a tumor-promoting factor targets HIF1AN in OC [10].

In this study, we analyzed circRNAs related to the early diagnosis and prognosis of OC using OC cells and screened out the circRNA with the most significant differential expression: circMAN1A2 (circBase ID: hsa_circ_0000119). circMAN1A2 is highly expressed in OC cells, and in vivo and in vitro experiments have shown that it promotes cell growth and metastasis. In terms of the mechanism of action, we demonstrated that circMAN1A2 adsorbs miR-135a-3p to upregulate the expression of IL1RAP and promote the phosphorylation of TAK1, thus promoting the growth of OC. Furthermore, circMAN1A2 is significantly elevated in the sera of OC patients [6]; therefore, circMAN1A2 may be a marker for early diagnosis and target for treatment of OC patients.

**Methods**

**Cell lines and culture**

The OC cell lines SKOV3 and A2780 and the normal ovarian cell line ISOE-80 were purchased from ATCC. 293T cell lines were maintained in our laboratory. The SKOV3, A2780, and ISOE-80 cells were cultured in RPMI-1640 (Gibco, USA), and 293 cell lines were cultured in DMEM (Gibco, USA) at 37 °C in 5% CO₂. The media were supplemented with 10% fetal bovine serum (FBS; Hyclone), 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Gibco, USA).

**Plasmid construction and cell transfection**

To construct the circMAN1A2 overexpression vector, full-length human circMAN1A2 was subcloned into the pLO5ciR vector (Geenseed Biotech, China). To knockdown circMAN1A2, siRNAs targeting the back splice junction of circMAN1A2 (siRNA1, siRNA2, siRNA3) and siRNA-NC were synthesized (GenePharma Biotech, China). The most effective siRNA1 and si-NC molecules were subcloned into the lentivirus vector (pLKO.1) to construct sh-circMAN1A2 and sh-NC vectors. The circMAN1A2 vector, sh-circMAN1A2, and
their control plasmids were transfected into 293T cells for lentivirus preparation, and the viruses were collected to infect A2780 and SKOV3 cells, followed by selection on puromycin. miR-139a-3p mimics, mimics-NC, inhibitor, and inhibitor-NC were purchased from GenePharma (Shanghai, China). All transfections were performed using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer’s instructions. The sequences of siRNAs, shRNAs, miR-139a-3p mimics, mimics-NC, inhibitor, and inhibitor-NC are listed in Table S1.

**Polymerase chain reaction (PCR) and real-time quantitative polymerase chain reaction (RT-qPCR)**

Total RNA from the tumor tissue samples or cell lines with or without 4 U/µg of RNase R (ab286929, Abcam) was extracted using TRIzol reagent (Thermo Fisher, USA). Nuclear and cytoplasmic RNA from the OC cells was separated using NE-PER™ Kit (Thermo Fisher, USA) following the manufacturer’s instructions. Subsequently, the RNA was reverse transcribed into cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher, USA), Genomic DNA (gDNA) is extracted with DNA extraction kit (TIANGEN, China). For PCR, cDNA and gDNA were used as templates to amplify the target sequence according to PCR Amplification Kit (Takara, Japan), and 2% agarose gel was used to detect the amplification results. For RT-qPCR, the target genes were amplified in a 20 µL reaction using GoTaq® qPCR Master Mix (A6002, Promega). The relative gene expression levels were quantified using the 2 – ΔΔCt method. Primer sequences are listed in Table S2.

**Western blotting**

Total protein was extracted from cell lysates (Beyotime, China). Equal amounts of denatured proteins (30 µg) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The resolved proteins were transferred to polyvinylidene fluoride membranes (Millipore, USA) and probed using IL1RAP (1:1,000; 73070, Cell Signaling Technology), HGF (1:1,000; 52445, Cell Signaling Technology), TAK1 (1:1,000; 5206, Cell Signaling Technology), and p-TAK1 (1:1,000; 9339, Cell Signaling Technology) antibodies for 1.5 h. The membranes were then incubated with goat anti-rabbit antibody (1:5,000; SA00001-2, Proteintech) for 1 h. The blots were visualized after chemical development using an electrochemiluminescence reagent (Thermo Scientific, Massachusetts, USA). GAPDH was used as an internal reference antibody (1:5,000; 10494-1-AP, Proteintech).

**Fluorescence in situ hybridization (FISH)**

FITC-labeled circMAN1A2 (5-CTTCCTCTTCTCAAATTTCAC-3) and Cy3-labeled hsa-miR-135a-3p probes (5-CGCCACGCUCCCAAUCCCUUA-3) (Geneseed, Guangzhou, China) were used to observe colocalization of circMAN1A2 and hsa-miR-135a-3p in OC cells. FISH assay was performed using Fluorescent in Situ Hybridization Kit (GenePharma, China), according to the manufacturer’s instructions. The cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; Beyotime, China). Images were photographed using a fluorescence microscope (Leica, Germany).

**Cell Counting Kit-8 (CCK8) assay**
Briefly, a single-cell suspension was prepared using trypsin and inoculated in 96-well plates at a density of $3 \times 10^3$ cells/well. Next, 10 µL of CCK8 reagent (Solarbiox) was added and the cells incubated for 60 min at 72 h. The optical density (OD) at 490 nM was measured using a microplate reader (51119670DP, Thermo Scientific). All the experiments were performed in triplicate.

**EdU staining**

A single-cell suspension was prepared using trypsin and inoculated in six-well plates at a density of $5 \times 10^5$ cells/well. EdU staining was performed using an EdU staining kit (BeyoClick™ EdU-594, Beyotime) according to the manufacturer’s instructions. Images were photographed using a fluorescence microscope (Leica, Germany).

**Clone formation assay**

Cells were digested using 0.25% trypsin and resuspended by centrifugation to obtain a single-cell suspension; 200 cells/well were inoculated in a six-well plate. The cells were then fixed for 15 min in 5 ml of 4% paraformaldehyde, an appropriate amount of 1X GIMSA dye solution (G1015, Solarbiox) was applied, and the cells were incubated for 10–30 min at room temperature. The dishes were air-dried, and the clones were counted directly with the naked eye.

**Cell cycle assay**

Cells were harvested and fixed in pre-cooled 70% ethanol at 4°C overnight, stained with propidium iodide (PI), and measured using flow cytometry (Becon Dickinson FACS Calibur, USA).

**Wound healing assay**

Approximately $1 \times 10^6$ cells were added to a six-well plate, and the next day, the pipette tip was used to scratch parallel lines on the cell layer. The cells were then washed three times with phosphate-buffered saline, the streaked cells were removed, and serum-free medium was added. The remaining cells were then placed in a 37°C, 5% CO$_2$ incubator and cultivated. Images were acquired after 0 and 24 h.

**Dual luciferase assay**

The sequences of circMAN1A2 or IL1RAP 3'UTR containing the wild-type or mutant binding site of hsa-miR-135a-3p were designed and synthesized by GenePharma (Shanghai, China). These sequences were transfected into the psiCHECK2 vector (Promega, USA). 293T cells were co-transfected with the psiCHECK2 plasmids containing Insertion sequence and hsa-miR-135a-3p mimics/mimics-NC or hsa-miR-135a-3p inhibitors/inhibitor-NC using Lipofectamine 2000 (Invitrogen, USA). After 48 h of incubation, the activities of firefly and Renilla luciferase were measured using Dual Luciferase Reporter Assay Kit (Promega, USA), Firefly fluorescence as an internal reference.

**RNA pull-down assay**

a biotinylated circMAN1A2 probe (5-CTTCCTCTTCTCAAATTTCAC-3) synthesized by RiboBio (Guangzhou, China) was used; an oligo probe (5-CCCCACCTTTATCTATACCT-3) was used as a control.
Approximately $1 \times 10^7$ OC cells were transfected with the biotin-labeled circMAN1A2 probe. Subsequently, the biotin-coupled RNA complex was purified through streptavidin-coated magnetic bead adsorption. The enriched circMAN1A2 and miR-135a-3p were analyzed using qRT-PCR.

**Animals**

Four-week-old female BALB/c nude mice were chosen for the xenograft experiments. All procedures were approved by Hainan Women and Children’s Medical Center Animal Care and Use Committee. The mice were subcutaneously inoculated with SKOV3 cells infected with the circMAN1A2 overexpression/mock plasmid or A2780 cells infected with lentiviruses carrying sh-circMAN1A2/sh-NC plasmid ($2.5 \times 10^6$ cells; 200 ml). After 28 days, the mice were sacrificed and photographed. Tumors are extracted and photographed. The tumor volume was calculated in accordance with the formula $(\text{length} \times \text{width}^2)/2$, and the volume was measured weekly. The tumor weight was also determined.

**Statistical analysis**

Data are expressed as mean ± standard deviation. Multiple comparisons were performed using one-way analysis of variance (ANOVA) followed by Dunnett’s post-hoc test. All statistical analyses were performed using SPSS (version 19.0; IBM Corp.). Statistical significance was set at $P < 0.05$.

**Results**

**circMAN1A2 is overexpressed in OC cells**

We investigated the circRNAs circABCB10(hsa_circ_0112343), circMAN1A2(hsa_circ_0000119), circEPSTI1(hsa_circ_0000479), circ-ITCH(Hsa_circ_0001141), circHIPK3(hsa_circ_0000284), and circLARP4(hsa_circ_0003222), which are all associated with the early diagnosis and poor prognosis of OC. These circRNAs were detected in SKOV3, A2780 human OC, and ISOE-80 human normal ovarian epithelial cells. Among them, circMAN1A2 expression in OC cells showed the highest difference (Fig. 1a). PCR revealed that only circMAN1A2 was detected in cDNA. The looping site of circMAN1A2 was formed by splicing instead of gDNA rearrangement, and circMAN1A2 was more stable in RNase R-treated OC cells (Fig. 1b, 1c). Nucleocytoplasmic isolation and FISH experiments showed that circMAN1A2 was mainly localized to the cytoplasm of OC cells (Fig. 1d, 1e).

**circMAN1A2 promotes OC cell proliferation**

To explore the effect of circMAN1A2 on OC cell proliferation, three siRNAs targeting circRNA were designed for the junction of circMAN1A2. circMAN1A2 overexpression and knockdown plasmids were constructed (Fig. 2a). The up- and downregulation efficiency of circMAN1A2 in the OC cells was measured using qPCR (Fig. 2b, 2c). circMAN1A2 overexpression and knockdown had no effect on transcription of the maternal gene MAN1A2 (Fig. 2d). Among the three siRNAs, the siRNA with the most significant silencing efficiency was selected for subsequent experiments. The effects of circMAN1A2 on OC cell proliferation were detected using CCK8, EdU, and colony formation assays. Upregulation of
circMAN1A2 promoted whereas its downregulation inhibited the proliferation of OC cells (Fig. 2e–2g). Furthermore, upregulation of circMAN1A2 increased the proportion of cells in the S phase and decreased that of cells in the G1 phase of the cell cycle. This meant that the upregulation of circMAN1A2 promoted DNA replication in and proliferation of OC cells. However, circMAN1A2 downregulation had the opposite effect on the cell cycle (Fig. 2h). The wound healing experiments also showed that upregulation of circMAN1A2 promoted the proliferation and outward migration ability of OC cells, and upregulation of circMAN1A2 inhibited this ability (Fig. 2i).

**circMAN1A2 directly binds to miR-135a-3p**

To further explore the molecular mechanism by which circMAN1A2 regulates OC proliferation, we first used miRanda (www.miranda.org), regRNA2.0 (regrna2.mbc.nctu.edu.tw), and miRcancer (http://mircancer.ecu.edu/) to analyze the potential binding of circMAN1A2 with the OC-related miR-449a and miR-135a-3p (Fig. 3a). We detected the expression of miR-449a and miR-135a-5p in both normal ovarian epithelial \ and OC cells. The expression of miR-135a-5p was significantly downregulated in the OC cells, whereas that of miR-449a was not significantly changed. (Fig. 3b). Next, the adsorption capacity of circMAN1A2 for miR-449a and miR-135a-3p was measured using dual luciferase assay; circMAN1A2 significantly adsorbed miR-135a-3p (Fig. 3c). The miR-135a-3p-binding site in circMAN1A2 was mutated for dual luciferase assay, and the resulting mutant circMAN1A2 could not bind to miR-135a-3p (Fig. 3d). RNA pull-down experiments showed that circMAN1A2 could bind to miR-135a-3p in OC cells (Fig. 3e). Immunofluorescence analysis showed that circMAN1A2 co-localized with miR-135a-3p in OC cells (Fig. 3f).

**circMAN1A2 reverses the inhibitory effect of miR-135a-3p on OC cell growth**

To further investigate the interaction between circMAN1A2 and miR-135a-3p in OC cells, rescue experiments were performed by co-transfection with miR-135-3p mimics or a miR-135a-3p inhibitor and circMAN1A2 or si-circMAN1A2. CCK8, EdU, and colony formation assays displayed that miR-135a-3p repressed the proliferation of OC cells, and overexpression of circMAN1A2 significantly restored the inhibitory effect of miR-135a-3p on the proliferation of OC cells(Fig. 4a-4c). Furthermore, the miR-135a-3p inhibitor promoted the proliferation of OC cells. OC cell proliferation was significantly reduced upon circMAN1A2 knockdown (Figure S1a-S1c). Cell cycle analysis showed that overexpression of circMAN1A2 attenuated the arresting effect of miR-135a-3p at the G1 phase(Fig. 4d), and knockdown of circMAN1A2 rescued the cell cycle-promoting effect of the miR-135a-3p inhibitor at the G1-S phase (Figure S1d). Wound healing experiments also showed that overexpression of circMAN1A2 attenuated the inhibitory effect of miR-135a-3p (Fig. 4e), whereas circMAN1A2 knockdown reversed the effect of the miR-135a-3p inhibitor, on OC cell proliferation and outward migration (Figure S1e).

**IL1RAP is a direct target of miR-135a-3p**

miRNAs mainly bind to the 3'UTRs of their target mRNAs. We first used miRwalk (http://mirwalk.umm.uni-heidelberg.de/) to identify the target genes of miR-135a-3p; the threshold was set to 0.95, and the
predicted target genes were subjected to KEGG pathway analysis. The predicted target genes of miR-135a-3p were mostly found to be in the MAPK, PIK-Akt and RAS signaling pathway (Fig. 5a). miRDB (http://mirdb.org/) and TargetScan (https://www.targetscan.org/vert_72/) also predicted the target genes of miR-135a-3p to be in the MAPK signaling pathway. We identified five candidate target genes: HGF, PRLR, PPP2R2B, IL1RAP, PAK3 (Fig. 5b). The target genes of miR-135a-3p were screened by qPCR, and the results showed that miR-135a-3p mimics significantly silenced the expression of IL1RAP in OC cells (Fig. 5c, 5d). WB results also showed that miR-135a-3p mimics silenced the protein level of IL1RAP. miR-135a-3p inhibitor up-regulated the protein level of ILRAP (Fig. 5e). To determine the specific target site, the predicted target sequence of miR-135a-3p was mutated, and dual luciferase assay showed that miR-135a-3p could not bind to the 3’UTR of IL1RAP after mutation (Fig. 5f). TCGA database (http://gepia2. cancer-pku.cn/#index) analysis showed that IL1RAP was highly expressed in OC (Fig. 5g), in contrast to miR-135a-3p(https://www.biosino.org/dbDEMC/index), which was expressed at low levels in OC tissue and blood samples (Fig. 5h).

**miR-135a-3p promotes OC cell proliferation through the IL1RAP/p-TAK1 axis**

The effect of interaction between miR-135a-3p and IL1RAP on OC cell function was next confirmed. miR-135a-3p mimics were transfected into OC cells overexpressing IL1RAP and miR-135a-3p inhibitor were transfected into OC cells scilencing IL1RAP. The expression of IL1RAP was detected by qPCR(Fig. 6a), subsequently, CCK8, EdU, colony formation, cell cycle, and wound healing were detected OC cell function. miR-135a-3p mimics reversed the promoting effect of IL1RAP on OC cell proliferation (Fig. 6d–6f), and the miR-135a-3p inhibitor attenuated the inhibitory effect of IL1RAP downregulation on OC cell proliferation (Figure S2b–S2f). IL1RAP is the receptor protein of IL-1, which can autophosphorylate TAK1 through signal transmission and activate the downstream MAPK, NFKB, and other growth-related signaling pathways. In our subsequent western blotting experiments, we found that the circMAN1A2/miR-135a-3p/IL1RAP axis activates the phosphorylation of TAK1 (Fig. 6g, S2g).

**circMAN1A2 accelerates xenograft tumor growth in vivo**

Next, to investigate whether circMAN1A2 affects tumor growth in vivo, a human OC cell xenograft tumor model was established, and circMAN1A2-overexpressing SKOV3 cells, circMAN1A2-knockdown A2780 cells, and their respective controls were subcutaneously injected into female nude mice. The tumor volume and weight in the circMAN1A2-overexpressing group were significantly higher than those in the control group, whereas the circMAN1A2 knockdown group exhibited significantly suppressed tumor growth (Fig. 7a–7c). In tumor tissue samples, the effects of circMAN1A2 on IL1RAP expression and TAK1 phosphorylation were analyzed using qPCR and WB; circMAN1A2 could activate IL1RAP and TAK1 phosphorylation to promote tumor growth (Fig. 7d-7g).

**Discussion**
With the advancement of RNA sequencing technology, numerous noncoding RNAs have been discovered, such as miRNAs, long noncoding RNAs, and circRNAs. These noncoding RNAs regulate tumor initiation and progression. circRNAs have certain features distinct from those of noncoding RNAs: they are circular in shape, lack 5' caps and 3' tails, and are relatively stable [11]. These distinguishing features, combined with their tissue-specific expression, make circRNAs more suitable in cancer than linear transcripts, as diagnostic markers of or therapeutic targets [12]. In recent years, multiple circRNAs have been identified as tumor markers of OC [13]. However, the expression and function of most circRNAs in OC development remain unclear.

Here, we analyzed circRNAs associated with the early diagnosis and poor prognosis of OC. Among them, circABCB10, circMAN1A2, and circEPSTI1 are considered early diagnostic markers of OC [5–7], and circITCH, circABCB10, circHIPK3, and circLARP4 are associated with its poor prognosis [5, 14–16]. The expression of these circRNAs was assessed in normal ovarian epithelial and OC cells; the expression of circMAN1A2 was significantly different in OC cells than in normal cells. Subsequent overexpression and knockdown experiments in OC cells confirmed that the increased expression of circMAN1A2 promoted the proliferation of OC cells, whereas a decreased expression of circMAN1A2 inhibited the proliferation of OC cells. In addition, in vivo xenograft experiments confirmed the pro-proliferative effect of circMAN1A2 on OC.

circRNAs can act as ceRNAs to regulate the expression of oncogenes or tumor suppressor genes at the post-transcriptional level. circ_0006528 can adsorb miR7-5p and regulate Raf1, involved in breast cancer proliferation and migration [17]. Under androgen regulation, circHIAT1 adsorbs miR-195-5p/29a-3p/29c-3p, regulates cyclin CDC42, and participates in renal clear cell cancer (ccRCC) development [18]. circRNA_000839 regulates hepatocellular carcinoma (HCC) invasion and migration through the miR-200b–RhoA axis [19]. In this study, we analyzed the binding sites of circMAN1A2 and miR-135a-3p, and FISH revealed that circMAN1A2 and miR-135a-3p show subcellular colocalization. Dual-luciferase and RNA pull-down assays further confirmed that circMAN1A2 directly binds to miR-135a-3p. Further, in the rescue experiment, only circMAN1A2 adsorbed miR-135a-3p to promote the growth of OC cells. In previous studies, miR-135a-3p was found to be downregulated in OC patients and tissues and thus could be considered a tumor suppressor, biomarker, and nucleic acid therapeutic agent in OC [20, 21]. Here, next, we analyzed potential targets of miR-135a-3p using miRDB, TargetScan, and miRwalk. WB and dual luciferase assays confirmed that miR-135a-3p could target the 3'UTR of IL1RAP, and functional experiments confirmed that miR-135a-3p could control the proliferation of OC cells by regulating the expression of IL1RAP. We also found that the circMAN1A2/miR-135a-3p/IL1RAP axis ultimately regulates the proliferation of OC cells by controlling the phosphorylation of TAK1. IL1RAP is a component of the IL-1 receptor complex. Generally, the expression of IL1RAP is low in normal cells, but it is highly expressed in various cancers such as gastric cancer, cervical cancer, and acute myeloid leukemia (AML). IL1RAP has been used as a therapeutic target in AML [22–24]. IL1RAP can also promote TAK1 phosphorylation through the MYD88/TRAF6 signaling axis to regulate multiple cancer growth-related pathways such as those involving JUN, P38, and NF-kB [25].
Conclusions

We found that circMAN1A2 is highly expressed in OC tissues, and Fan et al. confirmed that circMAN1A2 is highly expressed in the sera of patients with OC or other tumors [6]. These results confirmed that circMAN1A2 is regulated by the miR-135a-3p/IL1RAP/pTAK1 axis for OC growth; therefore, circMAN1A2 may be a biomarker for early diagnosis of OC.

Abbreviations

GEO: Gene Expression Omnibus; KEGG: Kyoto Encyclopedia of Genes and Genomes; MAPK: mitogen-activated protein kinase; OC: ovarian cancer; IL1RAP: Interleukin 1 Receptor Accessory Protein; TAK1: Transforming Growth Factor-Beta-Activated Kinase 1; MAN1A2: Mannosidase Alpha Class 1A Member 2; RT-qPCR: real-time quantitative polymerase chain reaction; FC: Foldchange; OD: optical density; miRNA, miR: microRNA; ov: overexpression; NC: Negative control

Declarations

Ethics approval and consent to participate

This study were approved by a he Medical Ethics Committee of Hainan Women and Children's Hospital and conform to ARRIVE guidelines (https://arriveguidelines.org).

All animal procedures adhered to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1996).

Consent to publish

Not applicable.

Availability of data and materials

Data supporting the findings of this study are available from the corresponding author upon reasonable request. mRNA expression data from TCGA analysis website GEPIA2 (http://gepia2.cancer-pku.cn/#index) microRNA expression from dbDEMC website (https://www.biosino.org/dbDEMC/index).

Competing interests

The authors declare that no competing interests exist.

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Not applicable.

Authors’ contribution

BL and XW designed research ideas and planed experimental procedures. XW provided financial support. BL was a major contributor in writing the manuscript. CH and DA were participated in data collection and literature analysis. MN completed data analysis. All authors read and approved the final manuscript.

References


Figures
circMAN1A2 is overexpressed in ovarian cancer (OC) cells. a: Expression of OC-related circRNAs was detected in SKOV3, A2780 human ovarian cancer, and ISOE-80 human normal ovarian epithelial cells using qPCR. b: The looping site of circMAN1A2 was detected using agarose gel electrophoresis. c: Expression of circMAN1A2 in SKOV3 and A2780 human OC cells was detected using qPCR with or without RNase R treatment. d: After nucleocytoplasmic separation, nuclear and cytoplasmic expression...
circMAN1A2 was detected using qPCR. Localization of circMAN1A2 in cells was detected using immunofluorescence. * p<0.05, **p<0.01, ***p<0.01.

**Figure 2**

circMAN1A2 promotes ovarian cancer (OC) proliferation. a: Schematic diagram of circMAN1A2 overexpression and knockdown sites. b, c: circMAN1A2 overexpression and knockdown efficiency in OC
cells, as determined using qPCR. d: Effects of circMAN1A2 overexpression and knockdown on MAN1A2 expression, as determined using qPCR. e: Effect of circMAN1A2 overexpression and knockdown on OC cell proliferation, as determined using CCK8 assay. f: Effect of circMAN1A2 overexpression and knockdown on DNA replication rate of OC cells, as determined using EdU assay. g: Effect of circMAN1A2 overexpression and knockdown on single-cell proliferation ability of OC cells, as determined using colony formation assay. h: Promotion or blockade of the OC cell cycle at the G1/S phase under circMAN1A2 overexpression and knockdown, as determined using cell cycle assay. i: Effect of circMAN1A2 overexpression and knockdown on proliferation and outward migration of OC cells, as determined using wound healing experiments. * p<0.05, **p<0.01, ***p<0.001.
Figure 3

circMAN1A2 directly binds to miR-135a-3p. a: Venn diagram showing the miRNAs that can potentially bind to circMAN1A2 predicted using miRanda, regRNA2, and miRcancer. b: Expression of miR-449a and miR-135a-3p was detected using qPCR in SKOV3, A2780 human OC, and ISOE-80 human normal ovarian epithelial cells. c: Binding ability of circMAN1A2 toward miR-449a, miR-135a-3p, as determined using dual-luciferase assay. d: The direct binding site of circMAN1A2 for miR-135a-3p, as determined using dual-luciferase assay. e: Verification of the binding efficiency of circMAN1A2 toward miR-135a-3p, as determined using RNA pull down experiment. f: Colocalization of circMAN1A2 with miR-135a-3p was detected using immunofluorescence in OC cells. * p<0.05, **p<0.01, ***p<0.01.
circMAN1A2 reverses the inhibitory effect of miR-135a-3p on ovarian cancer (OC) cell growth. a: Effect of interaction between circMAN1A2 and miR-135a-3p on the proliferation of SKOV3 cells, as determined using CCK8 assay. b: Effect of interaction between circMAN1A2 and miR-135a-3p on DNA replication rate of SKOV3 cells, as determined using EdU assay. c: Effect of circMAN1A2 and miR-135a-3p interaction on single-cell proliferation ability of SKOV3 cells, as determined using colony formation assay. d: Effect of
interaction between circMAN1A2 and miR-135a-3p on the promotion or blockade of the SKOV3 cell cycle at the G1/S phase, as determined using cell cycle assay. e: Effect of circMAN1A2 and miR-135a-3p interaction on the proliferation and migration of SKOV3 cells, as determined using wound healing assay. * p<0.05, **p<0.01, ***p<0.01.

Figure 5
IL1RAP is a direct target of miR-135a-3p. a: miRwalk was used to predict the target genes of miR-135a-3p, and KEGG pathway analysis was performed on the target genes. b: Venn diagram showing intersection between the target genes of miR-135a-3p predicted using miRDB, TargetScan, and miRwalk and KEGG signaling pathway genes. c-e: Silencing effect of miR-135a-3p on the predicted target genes, as determined using qPCR and western blotting. f: Verification of the direct target sites of miR-135a-3p and IL1RAP using dual luciferase assay. g: IL1RAP is highly expressed in OC, as revealed by the GSEA database. h: miR-135a-3p is weakly expressed in OC tissues and blood, as revealed by the miRNA clinical database dbDEMC. * p<0.05, **p<0.01, ***p<0.01.
miR-135a-3p promotes proliferation of OC cells through the IL1RAP/p-TAK1 axis. 

a: qPCR detection of IL1RAP expression in SKOV3 cells transfected with miR-135a-3p mimics and ILRAP overexpression plasmids. 
b: Effect of interaction between miR-135a-3p and IL1RAP on the proliferation of SKOV3 cells, as determined using CCK8 assay. 
c: Effect of interaction between miR-135a-3p and IL1RAP on DNA replication rate of SKOV3 cells, as determined using EdU assay. 
d: Effect of interaction between miR-135a-3p and IL1RAP on cell cycle distribution of SKOV3 cells. 

Figure 6
135a-3p and IL1RAP on single-cell proliferation ability of SKOV3 cells, as determined using colony formation assay. e: Effect of interaction between miR-135a-3p and IL1RAP on promoting or blocking the SKOV3 cell cycle at the G1/S phase, as determined using cell cycle assay. f: Effect of miR-135a-3p and IL1RAP interaction on the proliferation and migration of SKOV3 cells, as determined using wound healing assay. g: Effect of the circMAN1A2/miR-135a-3p/IL1RAP regulatory axis on TAK1 phosphorylation in SKOV3 cells, as determined using western blotting. * p<0.05, **p<0.01, ***p<0.01.
Figure 7

circMAN1A2 accelerates xenograft tumor growth in vivo. a, b: Effects of circMAN1A2 overexpression and knockdown on ovarian cancer (OC) cell xenograft tumor volume and weight. c: Xenograft nude mice and tumors of each group are displayed (n = 5). d: Expression of circMAN1A2, miR-135a-3p, and IL1RAP in the transplanted tumors, as determined using qPCR. e: The protein level of IL1RAP and phosphorylation of TAK1 in the Xenograft tumors, as determined using western blotting. * p<0.05, **p<0.01, ***p<0.01.

Supplementary Files

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