



Original article

CircRNA PLOD2 enhances ovarian cancer propagation by controlling miR-378

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ABSTRACT

It has been confirmed that circular RNA participates in tumorigenesis through a variety of ways, so it may be used as a molecular marker for tumor diagnosis and treatment. In this study, the expression of circ-LOPD2 in ovarian cancer tissues and cell lines was detected by qRT-PCR and Western blot. The dual luciferase report was used to verify the target of circ-LOPD2, and the silencing and overexpression of circ-CSP1 in cell lines was used to explore its relationship with miRNA-378. The cell proliferation was detected by CCK8 method, and the expression level of miRNA-378 was detected by qRT-PCR. The results showed that circ-LOPD2 was highly expressed in ovarian cancer (OC) tissues, circ-LOPD2 expression levels were higher in OVCAR3 and A2780, and circ-LOPD2 expression levels in CAOV3 were lower. After silencing circ-LOPD2, the growth ability of OVCAR3 and A2780 cells decreased, while overexpression of circ-LOPD2 led to the opposite result. We also found that miR-378 is a target of circ-LOPD2. Silencing circ-LOPD2 will increase the expression of miR-378, and overexpression of circ-LOPD2 will decrease the expression of miR-378. In summary, our results show that circ-LOPD2 as a miR-378 sponge promotes the proliferation of ovarian cancer cells, which may in turn promote the development of OC.

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1. Introduction

In worldwide, Ovarian cancer (OC) is one of the diseases that threatens female lives and health, and at the same time, it is the second most common cause of death from female gynecological cancer (Bray et al., 2018). According to reports, in 2018, there were 300,000 newly diagnosed OC patients worldwide and more than 180,000 deaths from OC (Siegel et al., 2018). Ovarian cancer accounts for 2.5% of all malignancies in women. Most serious ovarian cancer is already found as stage III (51%) or stage IV (29%) (Torre et al., 2018), and most are diagnosed at a late stage with extensive peritoneal metastasis, so the 5-year survival rate is only about 30%. Ovarian cancer accounts for 5% of all cancer deaths due to low survival rates due to late diagnosis, so improving early diagnosis and prognosis is a research focus (Li et al., 2019).

Circular RNA (circRNA) is a type of endogenous non-coding RNA. They are usually stable, abundant and conserved RNA molecules with complex tissue and stage-specific expression patterns, its covalent closed loop structure does not have a 5' cap or 3' poly a tail, which improves its tolerance to exonuclease. At present, several studies have shown that circRNA can participate in tumorigenesis through a variety of ways, and has certain research value for tumor diagnosis and treatment (Fu et al., 2018; Yang et al., 2017; Yang et al., 2018a,b; Han et al., 2017; Du et al., 2016). Lysyl hydroxylase (LH) is a protein encoded by procollagen-lysine 2-oxoglutarate 5-dioxygenase (PLOD), which is a key enzyme mediating collagen-mediated oxygenase. PLOD gene families can be divided into three subtypes PLOD1, PLOD2, PLOD3, each subtype of the corresponding lysine hydroxylase gene encoding, the family belongs to 2-keto glutaric acid dependent two-plus oxygen members of the family of enzymes, with qi acid hydroxylase activity (Shao et al., 2018), can be in Fe²⁺ and 2 - keto glutaric acid is formed under the condition of homologous dimers, catalytic single former collagen lysine hydroxyl, lysine hydroxylation of collagen can be cross linked after secrete extracellular form stable structure of hydroxyl lysine pyridine chain (Maria de Castro-Sobrinho et al., 2017). When the expression of PLOD2 is significantly increased in cells, the generation of hydroxylysine pyridine chain is increased, which will lead

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to excessive deposition of collagen fiber, damage of ECM structure, and further cause tumor progression and metastasis. At present, studies have found that the expression of PLOD2 in breast cancer (Esbona et al., 2018), biliary duct cancer (Niu, et al., 2012), gastric cancer (Li et al., 2020), colorectal cancer (Zhang et al., 2013), glioma (Song et al., 2017), liver cancer (Samczuk et al., 2018), endometrial cancer (Wan et al., 2020) and other tumor tissues is higher than that in normal tissues, and its expression level is closely related to tumor stage, differentiation, metastasis and prognosis. However, the circular structure of PLOD2 (circ-PLOD2) and its function in tumor development remains unclear.

MicroRNAs (miRNAs) have been proven to be a type of endogenous non-coding small RNAs, consisting of 18–25 nucleotides, which can mediate the down-regulation of post-transcriptional protein expression through specific recognition of the sequence in the 3'UTR of the target mRNA (Filipowicz et al., 2008). It negatively regulates the expression of target genes, directly participates in various physiological processes such as cell proliferation, differentiation, and apoptosis, and promotes tumorigenesis (Malik et al., 2014; Duyu et al., 2014; Wang et al., 2014; Mei et al., 2014; Tong et al., 2014). As a tumor miRNA, MiR-378 is overexpressed in solid tumors, by improving cell survival rate, reducing apoptosis *in vitro*, promoting tumor growth, angiogenesis and metastasis (Ma et al., 2014; Yu et al., 2014). Through bioinformatics analysis, we found that miR-378 is a target of circ-LOPD2. Therefore, in this study, we have analyzed the role of circ-LOPD2 in OC and the development of OC through miR-378.

2. Materials and methods

2.1. Clinical specimens

The normal ovarian tissue, benign tumor, borderline tumor and ovarian cancer tissue were collected from ovarian cancer patients undergoing gynecological surgery in our hospital. The collected tissue was immediately stored in liquid nitrogen and the tumor specimens were independently identified by the pathologist. The specimens were used accordance with regulations and ethical standards. None of the OC patients in this study had received chemotherapy or radiation before surgery. All subjects were informed and agreed to participate in this study, and was approved by the ethics committee.

2.2. Cell culture and transfection

Human OC cell lines CAOV3, A2780 and OVCAR3 were purchased. A2780 was cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT, USA), and OVCAR3, CAOV3 were cultured in RPMI-1640 (HyClone). Both DMEM and RPMI-1640 were supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (100 U/mL). The cell culture conditions were 5% CO₂ and 37 °C.

2.3. Western blot

In the ultra-clean workbench, the collected tissue samples were cut into pieces and fully ground, and harvested immediately into the EP tube. It was washed with ice, followed by phosphate buffered saline (PBS) to remove the remaining medium. It was lysed with RIPA lysate, and kept in ice cold condition for 30 min. The sample was centrifuged (16,000 g, 10 min), and the supernatant is the source of cell lysate. Using the BCA protein quantification kit (Beyotime) total protein content was calculated. Sample was prepared by adding the loading buffer and mixed well, boiled for 10 min, and centrifuged at 12,000 g, for 2 min. SDS-PAGE with

80 V for 20–30 min, 180 V voltage for about 60 min; partially dried at 15 V, transferred to PVDF membrane (Millipore) for 1 h. Then blocked at room temperature using blocking solution (5% skimmed milk powder, and 5% BSA for phosphorylated samples) for 1 h; discarded the blocking solution, and washed the membrane with 1X TBST, 5 min/ 3 times. Then primary antibody was added incubated for overnight. Recovered the primary antibody, washed the membrane with 1X TBST for 5 min (3 times), then added secondary antibody, and shaken for 1 h at room temperature. Further, discard the secondary antibody, washed the membrane with 1X TBST, 5 min (3 times) and chemiluminescence substrates (equal amounts of liquid A and liquid B, currently used, enhanced chemiluminescence, Thermo Fisher Scientific) were added. A Chemi-Scope mini imaging system (Clinx Science) was used to expose and image. Western blot sample preparation of cells can be directly added lysate (Duyu et al., 2014).

2.4. qRT-PCR

Total RNA was extracted from the tissue and cell samples using TRIzol reagent (Takara, Shiga, Japan). According to the manufacturer's instructions, the appropriate amount of chloroform was added to TRIzol. The upper aqueous phase was added to a new centrifuge tube after centrifugation, and the same volume of isopropyl alcohol was added to precipitate the RNA. After centrifugation, the supernatant was discarded, and the RNA pellet was washed with 75% ethanol. The precipitate was then dried and dissolved in diethyl pyrocarbonate (DEPC)-treated water. The ratio of absorbance at 260 nm (OD) was then measured using an ultraviolet spectrophotometer (Unico, Shanghai, China) to calculate RNA concentration. Then, 2 µg of RNA was reverse-transcribed to complementary DNA (cDNA) using avian myeloblastosis virus transcriptase and random primers (Takara). Then, real-time quantitative PCR amplification of cDNA was conducted using the Sybr PrimeX EX-TAQ Patent II Kit (Takara, Shiga, Japan). Finally, the threshold period (Ct) of the target gene and 18S rRNA (18 s)/U6 was compared using the 2- $\Delta\Delta$ Ct method to determine relative expression levels of the target gene.

2.5. CCK8 assay

In logarithmic growth phase cells, trypsin digestion (0.25% trypsin) was performed to make it as the single cell. CCK8 method was used to detect the cell vitality.

2.6. Dual-luciferase reporter assay

HEK293 T cells were resuspend and seeded into 24-well plates. After 24 h, HEK293 T cells were transfected with wild-type clones containing the binding site sequence of circ-LOPD2 and miR-378, and the mutant control clone was used as the control group. The transfection control was the psiCHECK2 vector expressing renal cell luciferase. The cells were harvested 48 h after transfection, and the luciferase activity in the cell extracts was detected with the dual luciferase reporter gene system (Promega, USA). The firefly activity was normalized according to the ratio of firefly to renal cell luciferase signal, reflecting the transfection efficiency of this experiment.

2.7. Statistical analysis

SPSS 22.0 software was used to perform statistical analyses. Each group of data uses the mean \pm SD to reflect the overall parameters. Results were verified by three replicate experiments. All p-values were two-sided; p < 0.05 was considered statistically significant.

3. Result

3.1. The expression level of circ-LOPD2 in ovarian cancer tissue

qRT-PCR and Western blot were used to detect the expression level of circ-LOPD2 in normal ovarian tissues, benign tissues, borderline tumor tissues and ovarian cancer tissues (Fig. 1A and B). The results showed that the protein expression and mRNA expression levels of circ-LOPD2 in borderline tumors and OC were significantly higher than those in normal and benign ovarian tissues ($P < 0.05$).

3.2. The expression level of circ-LOPD2 and miRNA-378 in OC cells

In addition, we also compared the expression levels of circ-LOPD2 (Fig. 2A) and miRNA-378 (Fig. 2B) in various ovarian cancer cell lines OVCAR3, A2780 and CAOV3. The results showed that the expression levels of circ-LOPD2 in OVCAR3 and A2780 were significantly increased compared with CAOV3 ($P < 0.05$). Compared with CAOV3, the expression level of miR-1236-3p in OVCAR3 and A2780 was significantly reduced ($P < 0.05$).

3.3. The role of circ-CSPP1 in OC cells

In our study, we down-regulated the expression of circ-LOPD2 in OVCAR3 and A2780, and up-regulated the expression of circ-LOPD2 in CAOV3 to detect the role of circ-LOPD2 in OC cells. After transfection, the qRT-PCR results showed that the transfection successfully reduced the expression level of circ-LOPD2 in OVCAR3 and A2780, and the circ-LOPD2 in CAOV3 was successfully down-regulated (Fig. 3A–C).

The MTT method was used to detect the proliferation of OC cells after transfection. The results showed that after silencing circ-LOPD2 in OVCAR3 and A2780, cell growth was significantly slowed down. The overexpression of circ-LOPD2 in CAOV3 significantly promoted cell growth and increased cell proliferation.

A B C

3.4. Target of circ-LOPD2

The bioinformatics prediction software showed that miR-378 is the target of circ-LOPD2. We verified it with dual luciferase reporter gene (Fig. 4). The results showed that compared with the other three groups, it contained circ-LOPD2, miR-378 and miR-378. The

luciferase activity in the group co-transfected with the wild-type clone of the mimic binding site sequence was doubled. This indicated that circ-LOPD2 directly bound with miR-378.

3.5. circ-CSPP1 acts as a sponge for miR-1236-3p

In addition, we also found that silencing circLOPD2 increased the expression of miR-miR-378 in OVCAR3, and silencing circ-LOPD2 increased the expression of miR-378 in A2780. Overexpression of circ-LOPD2 reduced the expression of miR-378 in CAOV3 (Fig. 5).

4. Discussion

Circular RNA has attracted much attention in the field of non-coding RNA in recent years. In recent years, many studies have shown that circRNA participates in tumor development through regulation of transcription (Yang et al., 2017), as a scaffold for enzymes, as a template for translation, and causing interactions between circRNA and proteins (Du et al., 2016). The role of circular RNA as a miRNA sponge in tumor development has also been confirmed. For example, Han's report showed that circ-MTO1 as a miRNA-9 sponge plays a role in inhibiting the progression of hepatocellular carcinoma (Han et al., 2017); Wang's report showed that circ-NT5E acts as a miRNA-422a sponge to promote the occurrence of glioblastoma (Wang et al., 2018); Yang et al. stated in the report that circ-ITCH acts as a miR-17/miR-224 sponge to inhibit the progression of bladder cancer (Yang et al., 2018a,b). Several studies have shown that there is an association between circRNA and the invasion and metastasis of ovarian cancer (Sanger et al., 1976). circRNA can participate in the regulation of a variety of tumor-related signaling pathways to affect the occurrence and development of tumors, such as nuclear factor kappa B (NF- κ B) signaling pathway, transforming growth factor β (TGF- β) signaling pathway, and these pathways can affect the development of ovarian cancer. Proliferation, invasion, migration and epithelial-mesenchymal transition (EMT) process, etc. Studies have performed high-throughput sequencing analysis on the primary peritoneal and lymph node metastases of EOC and found that it is associated with NF- κ B, phosphatidylinositol 3 kinase/protein kinase B (PI3K/AKT) and TGF (both in metastatic cancer). Contains multiple mi R-24/let-7 binding sites corresponding to up-regulation of mRNA and down-regulation of circRNA. It shows that circRNA can use its circularization to competitively inhibit the lin-

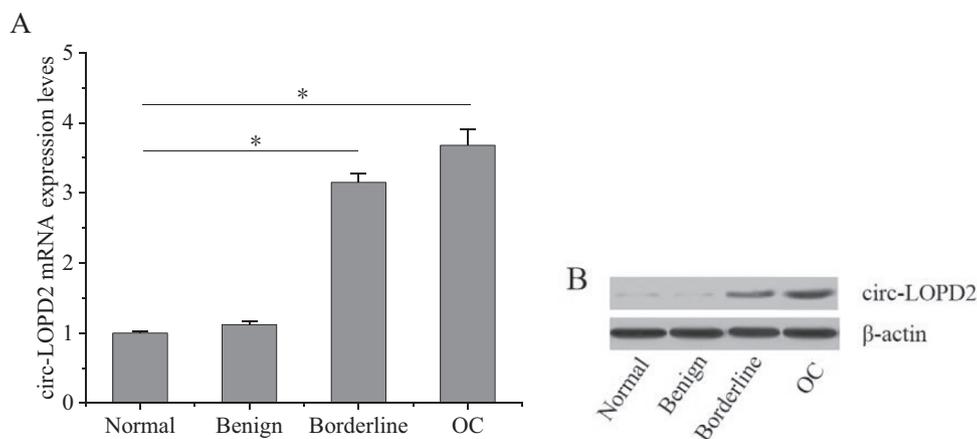


Fig. 1. The expression level of circ-LOPD2 in ovarian cancer tissue (A was the expression level of circ-LOPD2 mRNA in each tissue; B was the expression of circ-LOPD2 protein in each tissue).

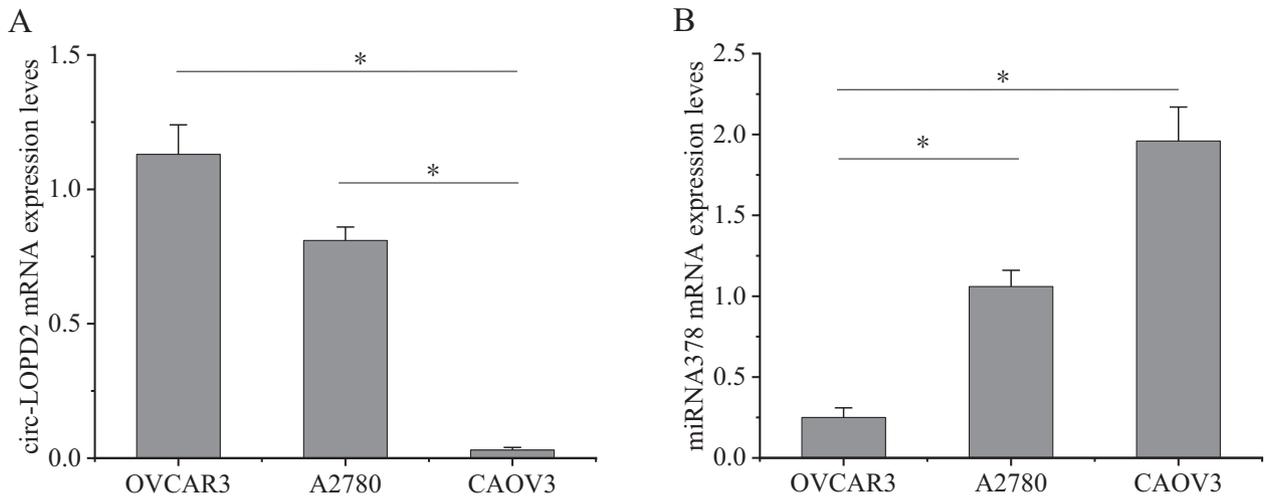


Fig 2. The mRNA expression level of circ-LOPD2 and miRNA-378 in OC cells (A was the expression level of circ-LOPD2 in OC cells; B was the expression of miRNA-378 in OC cells).

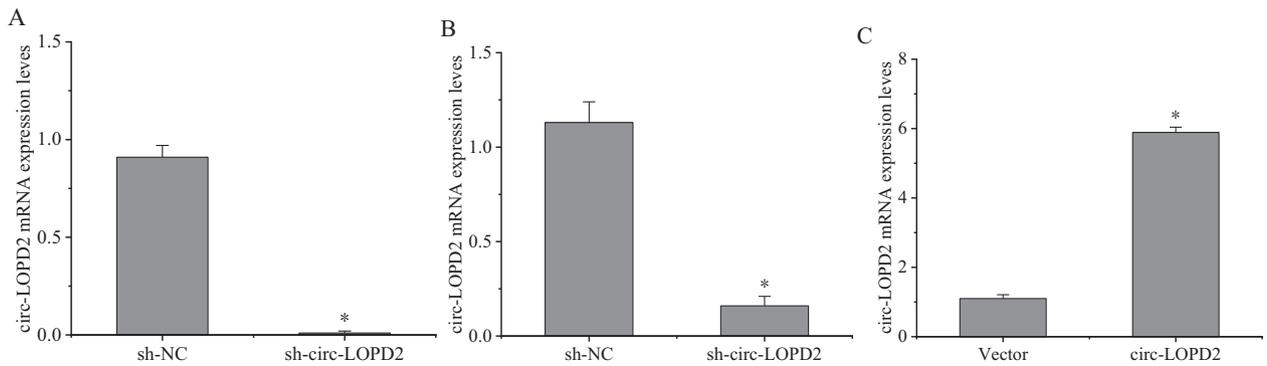


Fig 3. The qRT-PCR results after transfection (A was OVCAR3; B was A2780; C was CAOV3).

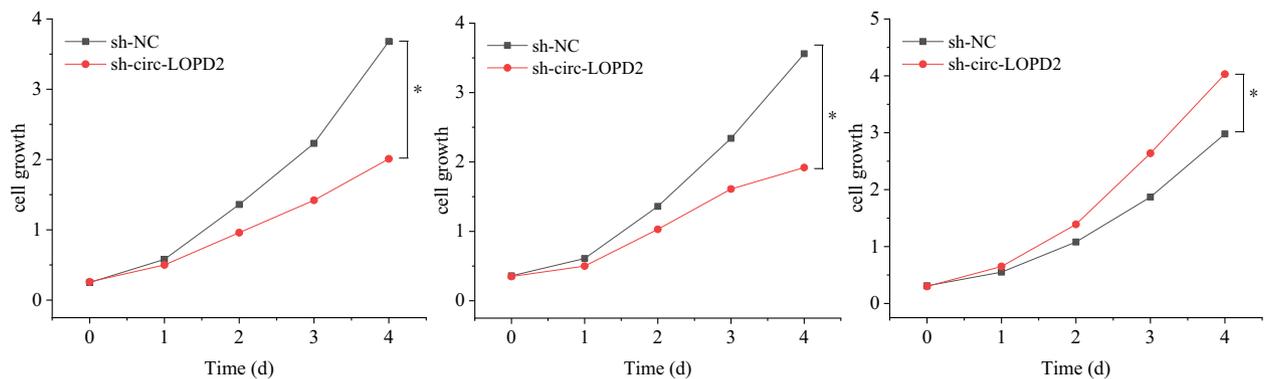


Fig 4. Cell proliferation curve after transfection (A was OVCAR3; B was A2780; C was CAOV3).

ear splicing and sponge function of miRNA, and regulate the expression of genes related to tumor metastasis, which ultimately leads to the widespread metastasis of ovarian cancer (Ahmed et al., 2016). This differential expression pattern may make these circRNAs become highly heterogeneous cancer transcriptome biomarkers.

MiRNAs are widely present in the human body and can be isolated from urine, plasma, semen and saliva, or encapsulated in microvesicles (Lawrie et al., 2008; Bar et al., 2010; Gallo et al., 2012; Hanson et al., 2009). They have also been expressed in differ-

ent organs including liver, adipose tissue and muscle. Accumulated evidence shows that miRNAs regulate a variety of key regulatory biological functions, including cell growth and development, apoptosis, metabolism, stress response and hematopoietic differentiation (Xue et al., 2013). A single miRNA may regulate the function and expression of various target genes, and amplification or inhibition of miRNA signals by regulating feedback mechanisms may lead to significant changes in miRNA expression, leading to various diseases including ovarian cancer, endometriosis, cardiovascular disease and ovarian underresponse (Marí-Alexandre et al., 2018;

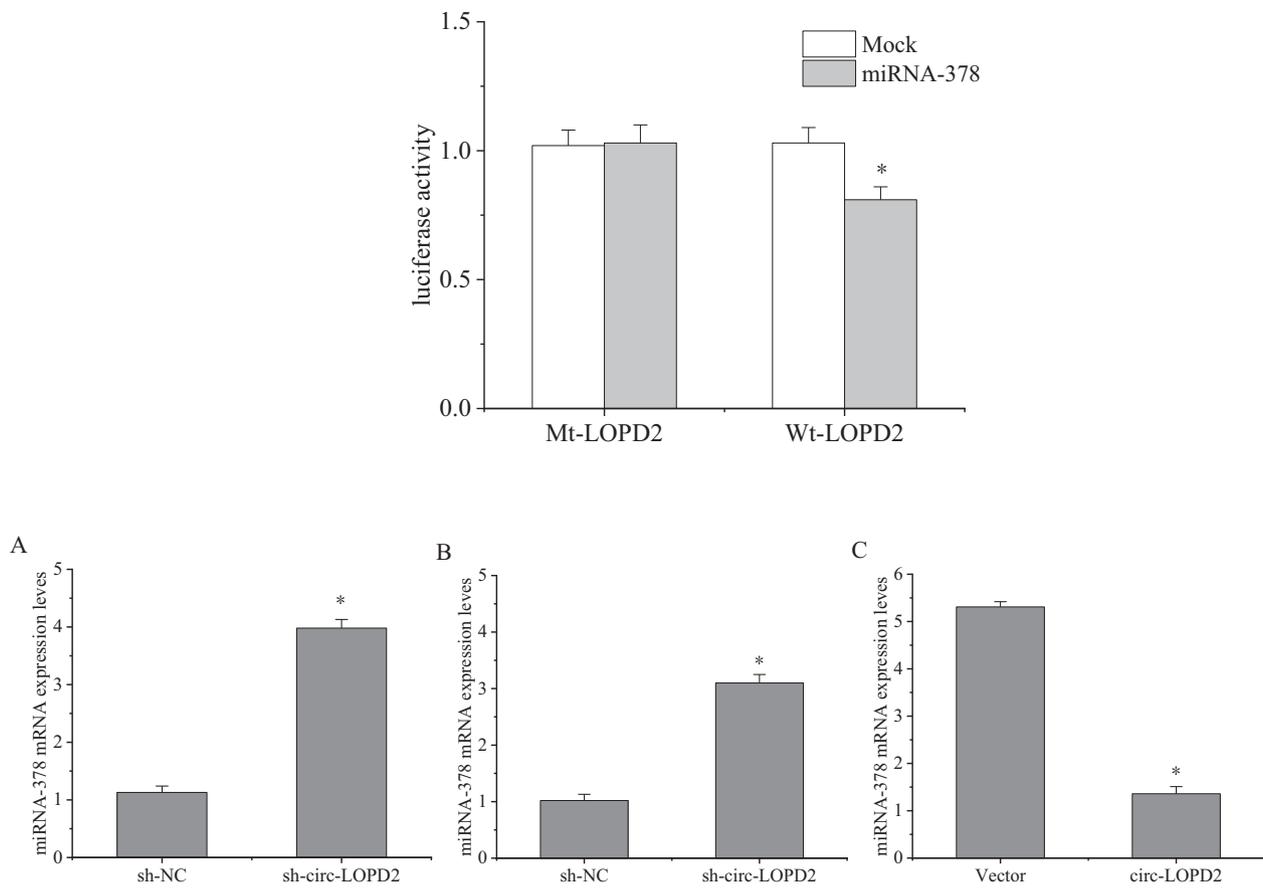


Fig 5. Dual-luciferase reporter assay.

Romakina et al., 2018). In this study, firstly, by collecting tissue samples and using qRT-PCR and Western blot, it was proved that circ-LOPD2 expression is significantly different in normal ovarian and OC tissues. And had a cancer-promoting effect at the cell level. Bioinformatics and dual luciferase reporter gene experiments proved that miR-378 was a target of circ-LOPD2. Currently, the identified MiR-378 was associated with a number of diseases including cancer, and previous studies revealed different molecular pathways confirmed the carcinogenic effects of miR-378 in a number of solid tumors, such as glioblastoma (Lee et al., 2007), Non-small cell lung cancer (Chen et al., 2011), breast cancer, nasopharyngeal carcinoma, acute myeloid leukemia (Qian et al., 2012), etc. In our study, after silencing circ-LOPD2, the growth ability of OVCAR3 and A2780 cells decreased, while overexpression of circ-LOPD2 led to the opposite result. We also found that miR-378 is a target of circ-LOPD2. Silencing circ-LOPD2 will increase the expression of miR-378, and overexpression of circ-LOPD2 will decrease the expression of miR-378. A number of studies have shown that the expression level of this gene is down-regulated in tissue, and it is believed that it is involved in prostate cancer, liver fibrosis, bladder cancer (Zaravinos et al., 2011) and colorectal cancer (Wang et al., 2014) as a tumor suppressor. In addition, Wang et al. (2014) found that miR-378 has the potential as a biomarker for early diagnosis in a meta-analysis study.

5. Conclusions

circ-LOPD2 acts as a miR-378 sponge to promote the occurrence and development of OC. This study reveals the function of circ-

LOPD2 in OC and aims to provide a new direction for the study of early diagnosis and targeted therapy of OC.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors' contribution

This study was done by the authors named in this article, and the authors accept all liabilities resulting from claims which relate to this article and its contents.

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