MiR-198 inhibits proliferation, invasion and migration of ovarian cancer cells by regulating the PI3K/Akt signaling pathway

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Objective: The specific objective of this investigation is to explore the impact of miR-198 on proliferation, migration as well as invasion of ovarian cancer (OC) cells.

Methods: OC tissue and adjacent normal tissue samples from OC patients were collected, and normal human ovarian epithelial cell IOSE80 and OC cell lines SKOV3, Caov3, A2780 and OVCAR3 were selected in this study for investigation. MiR-198 expression level was assessed using RT-qPCR. MTT, colony formation assay, Transwell and wound healing assay, and flow cytometry were adopted to analyze the role of miR-198 in OVCAR cell proliferation, invasion, migration, as well as apoptosis.

Meanwhile, the levels of PI3K/Akt signaling pathway-related proteins were determined by western blotting.

Results: A significant decrease in miR-198 level was revealed in the OC tissues and cells, contributing to the promotion of OVCAR3 cells in terms of proliferation, migration, invasion, and inhibition of apoptosis. MiR-198 overexpression had an opposite effect on these biological processes of OVCAR3 cells. Further study found that down-regulation of miR-198 caused a significant increase in the activity of PI3K/Akt signaling pathway in the OVCAR3 cells. In contrast, overexpressed miR-198 led to inhibition of this pathway’s activity.

Conclusion: MiR-198 may possess an ability to inhibit activation of the PI3K/Akt pathway, thus suppressing the OC cell proliferation, migration, as well as invasion.

Key words: ovarian cancer; miR-198; PI3K/Akt signaling pathway; proliferation; invasion; migration

INTRODUCTION

Ovarian cancer (OC) is a common cancer and the fifth leading cause of death from malignant tumors in females (Siegel et al., 2018). OC subtypes are mainly divided into epithelial OC and non-epithelial OC. Epithelial OC is the most common and its histological types include serous (62%), mucinous (5%), clear cell (8%), endometrioid (20%), and other types (5%) (Program, 2005, Ramalingam, 2016). Among them, most serous OC are diagnosed at an advanced stage and are highly malignant, namely high-grade serous OC (Köbel et al., 2010). The serous subtype accounts for 80% of OC deaths, and in patients with advanced serous OC the 5-year survival rate (15%) is low (Sopik et al., 2015). Although aggressive treatment is currently possible with surgery and adjuvant chemotherapy, the 5-year survival rate (35%-40%) remains unsatisfactory for patients at Stage III or IV. What’s worse, most patients relapse after treatment and eventually become chemoresistant (Waldmann et al., 2013). The results of clinical trials have shown no significant increase in the overall survival rate of OC patients after conventional treatment, which suggests that the conventional treatment may have reached a plateau (Odlushi, 2017). Therefore, it is of great significance to find new therapeutic strategies for the diagnosis, treatment and prognosis of OC patients.

MicroRNAs (miRNAs) participate in mediating gene expression in a range of developmental and physiological processes, and their dysregulation is associated with all types of cancer. Interaction of miRNAs and their target mRNAs induces degradation and/or translational repression of mRNA (Svoronos et al., 2016). Oncogenic miRNAs (oncomirs) usually refer to miRNAs that target coding transcripts of tumor suppressor proteins, showing up-regulation in different cancers (Frixia et al., 2015). In contrast, miRNAs with tumor suppressor functions can down-regulate oncogenes and are frequently lost in cancer (Almeida et al., 2011). Clinically, miRNAs pose important effects on various cancers, such as OC (Svoronos et al., 2016). MiRNAs have a regulatory impact upon OC invasion and metastasis. For example, miR-7 can cause a decrease in epithelial growth factor receptor expression to inhibit activity of the AKT-ERK1/2 pathway and reverse the epithelial-mesenchymal transition (EMT), thus eventually inhibiting tumor metastasis (Loganathan et al., 2012). MiR-150 suppresses a tendency of OC cells to invade and metastasize by negatively regulating the zinc finger E-box binding homeobox 1 (ZEB1) (Jin et al., 2014). In patients with epithelial OC, low miR-146a and miR-198 expression is considered to be a marker predicting poor prognosis (Wilezyski et al., 2017, Xu et al., 2020). In addition, in other human malignancies, such as the non-small cell lung cancer (Wang et al., 2019), prostate cancer (Ray et al., 2019) and breast cancer (Niu et al., 2019), miR-198 was found to participate in their development and progression. However, the biological function of miR-198 in OC and its potential mechanism are still unclear.

The study presented here found low expression of miR-198 in OC. Furthermore, the impacts of miR-198 upon OC cell proliferation, invasion, migration, and apoptosis were determined by loss-of-function and gain-of-function experiments. Additionally, we examined the effects of miR-198 on the PI3K/Akt pathway activity. Presented results provide a theoretical reference for further understanding of the molecular mechanism of OC progression, and also propose a new therapeutic target for OC.
MATERIALS AND METHODS

Clinical sample collection
The OC tissues and corresponding non-tumor adjacent tissues were obtained from 60 OC patients who received no radiotherapy and chemotherapy before surgery between 2018 and 2019. The clinical tissues were stored in liquid nitrogen immediately after surgical collection for subsequent RT-qPCR to detect expression of related genes. All patients had given written informed consent for publication. An approval was obtained from the Ethics Committee of Changzhou No. 2 People's Hospital.

Cell culture and transfection
Human normal ovarian epithelial cells IOSE80, OC cell lines OVCAR3, SKOV3, Caov3, and A2780 were purchased from the Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China). All cells were cultured in RPMI-1640 medium (Solarbio, Beijing, China), in a humidified incubator with 5% CO₂ at 37°C. The media were supplemented with 10% fetal bovine serum (FBS, Bioind, Kibbuiz, Israel) and 1% penicillin/streptomycin (Gibco, USA). All cells were divided into five groups: control group (control), miR-198 inhibitor NC group (in-NC), miR-198 inhibitor group (in-miR-198), miR-198 mimics NC group (NC), as well as miR-198 mimics group (miR-198). Before transfection, OVCAR3 cells were seeded in 6-well plates (1.0×10⁵ cells/well). When confluency reached 80–90%, OVCAR3 cells were transfected with mimics and inhibitor using the HiperFect transfection reagent (GIAGEN, Germany). Upon transfection completion, RT-qPCR was employed to analyze the transfection efficiency.

RT-qPCR
Trizol reagent (Invitrogen, Carlsbad, USA) was utilized for isolation of total cellular RNA. MiRNA First Strand cDNA Synthesis Kit (Vazyme, Nanjing, China) was then adopted to reverse transcript RNA into cDNA. Subsequently, RT-qPCR measurements were performed using SYBR Green PCR Master Mix Kit with U6 as an endogenous reference. The primer sequences used in our study were as follows: U6, forward: 5'-CGCTTCAGAATTTGCGTGTCAT-3', reverse: 5'-TCAAGTGCCTCAACCCATCT-3'; miR-198, forward: 5'-ATCTCCTCTGTCTGGGT-3'. The invasive ability of the cells was analyzed by using matrigel-coated transwell culture inserts (pore size: 8 μm). After rinsing three times with PBS, the transfected cells were plated into the upper chamber, while 600 μL of 10% FBS medium was added to the lower chamber. Upon completion of 24-hour incubation, non-invasive cells were wiped off, while the invasive cells were stained with 10% crystal violet upon completion of the fixation step with 4% paraformaldehyde. Finally, the number of invasive cells was observed under an inverted microscope.

Wound healing assay
Cells were plated in 12-well plates after 24-hour incubation at 37°C. A wound gap was created in the center of the plate by using a sterile 10 μl pipette tip. Prior to addition of a serum-free medium, PBS was employed to gently rinse the cells for three times. Cell migration was observed by an inverted microscope at 0 hour and 24 hours, respectively. The scratched area was measured by the Image J software.

Flow cytometry
OVCAR3 cells were plated into 12-well plates overnight for cell adhesion. Subsequently, the cells were dissociated into single cells by digestion with EDTA-free trypsin and were collected. PBS was then used to rinse the collected cells to prepare single-cell suspensions (1×10⁶ cells/ml). PE Annexin V Apoptosis Detection Kit with 7-ADD (BD biosciences, USA) was employed to stain the cells. Eventually, a flow cytometer (BD FACSVersa, USA) and the FlowJo software were adopted for apoptosis detection.

Western blot
Total proteins were extracted from the cells lysed with the RIPA lysis buffer and quantified by BCA assay. After separation using 12% SDS-PAGE, the proteins were then electrophoretically transferred onto nitrocellulose membranes. Upon completion of the blocking step with skim milk powder for 2 hours at room temperature, the membranes were incubated overnight with rabbit anti-p-PI3K (ab182651, 1:1000), anti-PI3K (ab32089, 1:2000), anti-p-Akt (ab38449, 1:2000), anti-Akt (ab8805, 1:2000), or anti-GAPDH (ab9485, 1:2000) antibodies at 4°C. After three subsequent washes with PBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature and then the membranes were washed again with PBST, and immunolabeling was detected with the ECL chemiluminescence kit. ImageJ software was used to determine the gray values of the protein bands.

Statistical analysis
Data processing was performed via SPSS 22.0 software. The results were expressed as the mean ± standard deviation (S.D.). Comparisons between two groups were performed using t-test, while multiple comparison testing was done by using one-way analysis of variance (ANOVA) with LSD post-hoc test. P<0.05 indicates a significant difference.

MITT and colony formation assay
OVCAR3 cells were seeded into 96-well plates (2.0×10³ cells/well). Cell proliferation was measured by using an MTT assay kit, and the absorbance values were measured at 450 nm with a spectrophotometric plate reader. For the colony formation assay, OVCAR3 cells were plated into 6-well plates (5.0×10³ cells/well) and cultured in 10% FBS medium at 37°C for 14 days. Then, following the fixation step using 10% methanol, 0.5% crystal violet was employed to stain the cells. Colonies with >50 cells were counted under an inverted microscope. Each experiment was repeated at least three times.
RESULTS

Low miR-198 expression in ovarian cancer

It could be seen from the results of RT-qPCR in Fig. 1A that there is a significantly lower expression of miR-198 in the OC tissues when compared to the normal tissues ($P<0.01$). At the cellular level, a markedly decreased miR-198 expression was found in the SKOV3, Caov3, and A2780 cell lines when compared to the IOSE80 cell line ($P<0.05$) (Fig. 1B). Among these, OVCAR3 cells with the lowest expression of miR-198 were selected for subsequent studies. Furthermore, miR-198 expression level in OVCAR3 was intervened through transfection of miR-198 mimics or inhibitors. According to the RT-qPCR results, a markedly lower miR-198 expression was shown in the in-miR-198 group when compared to the in-NC group ($P<0.05$), while the miR-198 group had a significantly higher level of miR-198 than the NC group ($P<0.05$) (Fig. 1C).

MiR-198 inhibits OVCAR3 cell proliferation, migration and invasion, and promotes apoptosis

To further investigate the functional impact of miR-198 on OC cells, cell proliferation, migration and invasion were examined after intervention with miR-198 expression. Figure 2A–B shows that down-regulation of miR-198 expression led to a marked promotion of...
OVCA3 cell proliferation and colony formation when compared to the in-NC or NC groups \( (P<0.05) \), while miR-198 overexpression led to an inhibition of those processes \( (P<0.05) \). The results of wound healing assay (Fig. 2C) and transwell assay (Fig. 2D) showed that down-regulation of miR-198 expression has significantly promoted migration and invasion of the OVCA3 cells when compared to the in-NC or NC groups \( (P<0.05) \), while miR-198 overexpression inhibited OVCA3 cell migration and invasion \( (P<0.05) \). In addition, a decrease in apoptosis was found in the in-miR-198 group \( (P<0.05) \), while an increase in the miR-198 group \( (P<0.05) \). Taken together, OC cell proliferation, migration, as well as invasion could be inhibited, while apoptosis was promoted by miR-198. Therefore, we reasoned that miR-198 could act as a tumor suppressor gene of OC.

MiR-198 inhibits activation of the PI3K/Akt pathway in OVCA3 cells

The PI3K/Akt pathway has a pivotal role in OC metastasis and recurrence, which is considered to be an important target for the OC treatment (Rodon et al., 2013). As can be seen in the KEGG pathway enrichment analysis based on the RNA sequencing data in the Cancer Genome Atlas (TCGA), the PI3K/Akt pathway shows a high activation in advanced OC (stage III and IV) when compared to early OC (stage II) (Xiao et al., 2020). Impact of miR-198 upon this pathway in OVCA3 cells was further examined. Figure 3 shows upregulation of the p-PI3K and p-Akt expression in the in-miR-198 group \( (P<0.05) \), while there is inhibition of these genes in the miR-198 group \( (P<0.05) \). Collectively, miR-198 plays an inhibitory role in OC by inhibiting activation of the PI3K/Akt pathway.

DISCUSSION

Ovarian cancer, particularly epithelial OC, is one of the most deadly gynecological malignancies. Identifying the molecular mechanism involved in OC progression contributes to providing better treatment options. Targeted therapy is effective in improving the overall survival of OC patients (Chen et al., 2019). MiR-198 is a therapeutic miRNA with effective therapeutic effects in lung cancer, colorectal cancer, hepatocellular carcinoma, as well as breast cancer (Gu et al., 2019). MiR-198 presents low expression in papillary thyroid carcinoma (Marotta et al., 2016), osteosarcoma (Luila et al., 2011) and hepatocellular carcinoma (Tan et al., 2011). In addition, miR-198 is a novel inhibitor that negatively regulates invasion of hepatocellular carcinoma cells via the HGF/c-MET pathway (Tan et al., 2011). Chonglei and others (????) have found an under-expression of miR-198 in multiple myelomas, and a significant inhibition of multiple myeloma cell colony formation, proliferation, and migration by ectopic restoration of miR-198 (Bi et al., 2015). MiR-198 down-regulates the central signaling of the proliferative pathway in liver cancer. In contrast, genes mediating cell adhesion are significantly up-regulated by miR-198 (Elfimova et al., 2013). What’s more, miR-198 promotes apoptosis in human prostate cancer, as well as in lung cancer cell lines (Li et al., 2018; Zhu et al., 2018). In conclusion, miR-198 exhibits inhibitory effects in cell proliferation, invasion, and migration, and it can also induce apoptosis, thus suppressing cancer cell growth. In our study, up-regulation of miR-198 expression caused inhibition of OC cell proliferation, migration and invasion and promoted apoptosis, suggesting that miR-198 has the potential to inhibit OC progression.

The PI3K/Akt signaling pathway participates in various cellular processes, such as cell metabolism, growth, and apoptotic death. Dysregulation of this pathway is linked to cancer occurrence and progression (Thorpe et al., 2015; Noorolyai et al., 2019). PI3K mainly phosphorylates the phosphatidylinositol secondary messenger through cell surface receptor activation and acts as an important regulator of macrophage phagocytosis. Akt binds to the PIP product of PI3K via its pleckstrin homology domain for recruitment to the plasma membrane. Activated Akt translocates into the cytoplasm or nucleus, which phosphorylates the mammalian target of rapamycin (mTOR) to promote cell growth and protein synthesis (Chaisuparat et al., 2016). It has been demonstrated in previous studies that the OC cell proliferation, invasion, and metastasis are promoted, while apoptosis is suppressed by abnormal activation of the PI3K/Akt pathway (Zhang et al., 2019). Therefore, in our study, p-PI3K and p-Akt expression in OC cells was assessed, so as to confirm the involvement of this pathway in the anticancer mechanism of miR-198 in OC. The results revealed that miR-198 overexpression has significantly inhibited the p-PI3K and p-Akt expression, while down-regulation of miR-198 had an opposite effect. According to the existing studies, down-regulated miR-198 inhibits the PI3K/AKT pathway activation via targeting PTEN, thus eventually suppressing proliferation and invasion of retinoblastoma cells (Wei et al., 2018). The above results suggest a regulatory role of miR-198 in OC via inhibition of the PI3K/Akt pathway activation.

In summary, our findings show that miR-198 is expressed at a low level in the OC tissues, and confirm that miR-198 is crucial for inhibition of the OC cell proliferation, migration, and invasion, as well as for promoting apoptosis. Therefore, we further propose that miR-198 may act as a tumor suppressor by down-regulation of the PI3K/Akt pathway activity. The current study will provide a theoretical basis and new therapeutic strategies for targeted therapy of OC. This study also has short-
comings. The number of ovarian cancer tissues is small, and the correlation between the expression of miR-198 and clinicopathological information needs to be further explored.

**Declarations**

Ethics approval and consent to participate: This study was approved by the Ethics Committee of Changzhou No.2 People’s Hospital.

Consent for publication: Not Applicable

Competing interests: The authors have no conflicts of interest to declare.

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**Authors’ Contribution**

Study concept and design: HX, HS; Acquisition of data: YZ, JC; Analysis and interpretation of data: YZ, JC; Statistical analysis: HX, HS. All authors have read and approved the final version of the manuscript.

Availability of data and materials: Research data are available.

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**Footnotes**

1. ABSTRACT

**Title**

MicroRNAs in ovarian cancer

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