miR-636 Inhibits EMT, Cell Proliferation and Cell Cycle of Ovarian Cancer by Directly Targeting the Transcription Factor Gli2 of Hedgehog Pathway

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Primary research

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Abstract

Objective: Ovarian cancer (OVC) is the fifth leading cause of cancer-related deaths in women and has a significant impact on physical and mental health of women. This study explores the molecular mechanism of miR-636 acting as a tumor suppressor in OVC in vitro and in vivo, and provides new insight into the treatment of OVC.

Methods: Protein-protein interaction (PPI) analysis was performed to identify the hub gene in Hedgehog (Hh) pathway. TargetScan database was used to predict the upstream regulatory miRNAs of Gli2 to obtain the target miRNA. qRT-PCR was performed to test the expression of miR-636, while Western blot were conducted to detect the expression of Hh and EMT (epithelial-mesenchymal transition) related genes in OVC cell lines. MTT assay and wound healing assay were used to measure the effect of miR-636 on OVC cell proliferation and migration. Flow cytometry was carried out to examine the effect of miR-636 on cell cycle, and Western blot was used for identification of changes in expression of Hh and EMT related proteins. Dual-luciferase reporter gene assay was implemented to detect the targeted relationship between miR-636 and Gli2. The xenotransplantation model was used to detect the effect of miR-636 on OVC cell proliferation in vivo.

Results: PPI interaction analysis found that Gli2 was the hub gene in Hh pathway. Based on TargetScan and GEO databases, Gli2 was found to be targeted regulated by the upstream miR-636. In vitro experiments discovered that miR-636 was significantly lowly expressed in OVC cell lines. Overexpressing miR-636 significantly inhibited HO-8910PM cell proliferation and migration abilities as well as induced cell cycle arrest in G0/G1 phase, while the inhibition of miR-636 promoted cell proliferation and migration abilities. Dual-luciferase reporter gene assay revealed that Gli2 was a target gene of miR-636. Besides, overexpressing miR-636 decreased protein expression of Gli2, while the inhibition of miR-636 increased protein expression of Gli2. Furthermore, the overexpression and inhibition of miR-636 both affected the expression of proteins related to Hh signaling pathway and EMT. Rescue experiments verified that overexpression of Gli2 reversed the inhibitory effect of miR-636 on HO-8910PM cell proliferation and migration abilities, and attenuated the blocking effect of miR-636 on HO-8910PM cell cycle. The xenotransplantation model suggested that miR-636 inhibited cell growth of OVC by decreasing Gli2 expression. Besides, overexpressing Gli2 potentiated the EMT process in OVC via decreasing E-cadherin protein expression and increasing Vimentin protein expression, and it reversed the inhibitory effect of miR-636 on OVC cell proliferation and migration abilities in vivo.

Conclusion: miR-636 inhibits the Hh pathway activation via targeted binding to Gli2, thus inhibiting EMT, cell proliferation and migration in OVC.

Introduction

Ovarian cancer (OVC) belongs to gynecological malignancies, and most OVC cases are diagnosed at an advanced stage owing to the concealed lesion sites. According to the latest data revealed in A Cancer
Journal for Clinicians, OVC takes up 2.5% of all malignancies among females. Due to its relatively higher mortality which accounts for 5% of all cancer-related deaths, OVC has become the fifth leading cause of cancer-related deaths in women\(^1\). OVC can be classified as epithelial ovarian cancer, non-epithelial ovarian cancer and metastatic ovarian cancer, among which epithelial ovarian cancer is the most common type in females of all races and nationalities, accounting for 90% of all OVC cases\(^2\). At present, the effective treatment for OVC is surgery combined with chemotherapy, but up to 80% of patients will experience disease recurrence after chemotherapy\(^3\). Thus, it is urgent to develop molecular targeted treatment for patients with OVC, which requires an in-depth understanding on cell signaling pathways and molecular functions related to the occurrence and development of OVC. Current researches have mainly focused on studying cell proliferation and migration of OVC and finding novel molecular targets, so as to improve clinical therapeutic effect and prognosis of patients.

MicroRNAs (miRNAs) refer to a class of single-stranded non-coding RNA molecules in the length of 20–24 nucleotides that are widely existed in eukaryotes. miRNAs are highly conservative in evolution with the capacity of binding to the 3'-untranslated region (3'-UTR) of target genes to induce mRNA degradation or inhibit mRNA translation, ultimately regulating cell differentiation, proliferation and apoptosis\(^4\)–\(^6\). Hedgehog (Hh) is one of the important signaling pathways in embryonic period and plays a role in regulating cell proliferation, differentiation, epithelial-mesenchymal transition (EMT) and stem cell maintenance to make the development of tissues and organs normal. The abnormal activation of Hh signaling pathway in adult tissues is associated with the formation, self-renewal and drug-resistance of various tumors\(^7\). Increasing evidence has showed that EMT in malignant tumors is responsible for the increased migration and invasion abilities of tumor cells, and helps the tumor cells to develop drug-resistance to conventional treatment\(^8\)–\(^10\). Studies have shown that miRNAs can regulate tumor cell proliferation and migration by regulating Hh signaling pathway. miR-214 has been found to inhibit SuFu (Hh pathway inhibitor) protein expression in breast cancer to activate Hh signaling pathway\(^11\). miR-7-5p and miR-506 have been seen to play an inhibitory role in bladder cancer and human cervical cancer by regulating the Hh pathway transcription factor Gli3\(^12\),\(^13\).

In this study, we found that miR-636 exhibited a significant low expression in OVC with the ability of targeted regulating Hh pathway factor Gli2, but the molecular mechanism of miR-636 regulating Hh pathway in OVC remains elusive. Therefore, we investigated the mechanism of miR-636 regulating OVC cell proliferation and metastasis by \textit{in vitro} and \textit{in vivo} experiments to gain more insight into the pathogenesis of OVC and provide new thought for further clinical diagnosis and treatment.

1 Materials And Methods

1.1 Cell lines and patients

OVC cell lines (HO-8910, HO-8910PM, CoC1, Caov-3, Caov-4) were purchased from cell resource center of Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (3111C0001CCC000280;
Human normal ovarian cell line HOSEpiC was accessed from the cell bank of Chinese Academy of Sciences (Shanghai, China). All cell lines were grown in RPMI 1640 (Gibco, 11875093) medium containing 10% fetal bovine serum (FBS; Gibco, 10099141C) in 5% CO₂ at 37 °C.

Paired clinical OVC tumor (n = 30) and adjacent normal tissue samples (n = 30) were collected from OVC patients in The Second Affiliated hospital of Zhejiang University School of Medicine, and the tissue samples were immediately frozen in liquid nitrogen at -80 °C after surgical excision. All patients had not received preoperative chemotherapy or radiotherapy, and had signed the informed consent. This study was approved by Ethics Committee of The Second Affiliated hospital of Zhejiang University School of Medicine.

1.2 Bioinformatics Analysis

Hh signaling pathway related genes were obtained from Kyoto Encyclopedia of Genes and Genomes database (KEGG, https://www.kegg.jp/kegg/pathway.html). Associated analysis was performed on the identified Hh-related genes by means of constructing a PPI network on STRING database (https://string-db.org/). TargetScan database (http://www.targetscan.org/vert_71/) was used to predict the upstream regulatory miRNAs of Gli2. OVC miRNA expression microarray GSE58517 (5 normal tissue samples and 5 OVC tissue samples) was downloaded from Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/). Normal tissue samples were used as control, and differential analysis was performed by using R package “limma”, with the threshold set as |logFC|>2 and p value < 0.05.

1.3 Overexpression and knockdown of genes

miR-636 mimic, mimic NC, miR-636 inhibitor, inhibitor NC, agomiR-636 and agomiR-NC were all purchased from Shanghai GenePharma Co., Ltd. Short hairpin RNA (shRNA) targeting Gli2 was synthesized by Sangon Biotech Co., Ltd (Shanghai). pEGFP1 overexpression vector was used to establish pEGFP1-Gli2 recombinant plasmid. Transfection was carried out by Lipofectamine®3000 (Invitrogen company, USA) according to instructions.

1.4 Real-Time fluorescence quantitative PCR (qRT-PCR)

Total RNA was extracted from tissues and cells using Trizol (Invitrogen), and then cDNA was synthesized by reverse transcription kit (Invitrogen). qRT-PCR was performed on ABI 7900HT instrument (Applied Biosystems, USA) with miScript SYBR Green PCR Kit (Qiagen, Germany) under the following thermal cycling conditions: pre-denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C 2 min, 95 °C 5 s and 60 °C 30 s. Smo, Gli2, Snail, Vimentin, Tgfβ, E-cadherin were normalized with GAPDH as internal reference, and miR-636 was normalized with U6 as internal reference. The expression differences of target genes in control group and test group were compared by $2^{-\Delta\Delta Ct}$ value. The experiment was performed three times. All primers used were shown in Supplement Table 1.
1.5 Western blot

After transfection for 48 h, cells in different treatment groups were washed with precooled Phosphate Buffered Saline (PBS, Thermo fisher, USA) 3 times. Transfected cells were lysed on ice with whole cell lysate for 10 min, and protein quantitation was determined using BCA protein assay kit (Thermo Fisher, USA). The protein samples were then treated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V after boiled for 10 min at 95 °C with 10 µl loading buffer, after which the proteins were transferred onto nitrocellulose membrane at 100 mA for 120 min. After being blocked with 5% bovine serum albumin (BSA) or Tris-Buffered Saline Tween (TBST) for 60 min, the membrane was incubated overnight at 4 °C with primary antibodies. The membrane was washed with 1 × TBST (Solarbio, Beijing, China) on a shaking table three times with 5 min per time and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody goat anti-rabbit IgG for 120 min at room temperature. TBST was used to wash the membrane three times, and an electrochemiluminescence kit (ECL; Solarbio, Beijing, China) was employed to visualize the protein bands. All antibodies used were detailed in Supplement Table 2.

1.6 Cell proliferation assay

MTT method was implemented for the detection of cell proliferation. HO-8910PM cells were seeded into 96-well plates at a density of 5 × 10^3 cells/well, and each treatment was run in triplicate. After 1, 2, 3, 4 and 5 d, sterile MTT solution (Beyotime) was added to cells for assessment of cell proliferation according to instructions. The absorbance at 490 nm was measured by an enzyme-labeled instrument (Molecular Devices, Sunnyvale, CA, USA).

1.7 Wound healing assay

For wound healing assay, HO-8910PM cells (1 × 10^6) were planted into 6-well plates, and then cell monolayers were wounded with a 200 µl sterile pipette tip when cells grew to 80% in confluence. Isolated cells were removed with mediums, and the cells remained were cultured in fresh mediums for 24 h. Images were photographed at 0 h and 24 h and the wound closure rate was measured.

1.8 Flow cytometry

For cell cycle detection, treated cells (48 h after transfection) of different groups were collected and digested with 0.25% trypsin. Cells were washed with PBS and re-suspended in 70% ice-cold ethanol (1 ml) for 24 h at 4 °C. Then the cells were stained with propidium iodide (PI) and ribonuclease in dark for 30 min (4 °C). Flow cytometry was used according to standard procedure. The results were analyzed using ModFit.

1.9 Dual-Luciferase Assay

To verify whether miR-636 can directly targeted bind to Gli2 3'UTR, wild-type (WT) and mutant-type (MUT) Gli2 3'UTR were inserted into psiCHECK luciferase reporter vector (Sangon Co., LTD, Shanghai, China). Subsequently, HO-8910PM cells were seeded into 48-well plates for 24 h of incubation, and miR-636
mimic/mimic NC and psiCHECK-WT/MUT were then co-transfected into cells. Finally, the luciferase activity was determined by luciferase assay kit (Promega, Fitchburg, WI, USA).

1.10 Mice experiment

A total of 20 male nude mice (6-week-old) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd, and then housed in sterile conditions (12 h/12 h, dark/light; 25°C; 60%-70% humidity). HO-8910PM cells (1 × 10^6) were inoculated into the abdomen of mice. The mice were divided into two groups with 10 mice in each group when tumor volume reached to 70 mm^3. Thereafter, the mice were injected with agomiR-636 (10 nmol/50 ml) or agomiR-NC twice a day, and once a week for 4 weeks. Tumor volume measurement method: Volume (cm^3) = (length × width^2) / 2. The nude mice were euthanized after 4 weeks, and the tumors were isolated, weighed and photographed. Mice care and laboratory procedures were approved by the ethics committee for laboratory animals.

1.11 Immunohistochemistry (IHC)

Transplanted tumor tissues from mice were placed in 4% paraformaldehyde for fixation in a refrigerator at 4 °C. After gradient dehydration with different concentration of ethanol, the tissues were transparently treated with dimethylbenzene and embedded in paraffin. Immunochemical staining was performed after the tissue blocks were cut into slices. Thereafter, the slices were treated with dimethylbenzene and finally sealed using neutral balsam for slides preparation. All antibodies used were detailed in Supplement Table 2.

1.12 Statistical analysis

All data were processed using SPSS 22.0 software. Mean ± standard deviation (SD) was used to express measurement data, while t test was used for comparison between two groups and one-way ANOVA was performed for comparison among more than two groups. * P < 0.05 was considered lowly significant, ** P < 0.01 was considered median significant, while *** P < 0.001 was considered highly significant.

2 Results

2.1 The expression of miR-636, Hh and EMT related genes in OVC cell lines

Many literatures have found that Hh signaling pathway is abnormally activated in tumor and affects tumorigenesis and development. Gli gene is an important player in Hh signaling pathway. In this study, we obtained Hh signaling pathway related genes by using KEGG database (Supplement Table 3). 37 genes were obtained and subjected to association analysis in an interaction network (Fig. 1A). It was found that Gli2 was in the center of the network, while our further retrieval of the location of Gli2 in the Hh
signaling pathway revealed that Gli2 gene was in the center as well (Fig. 1B). To learn about the upstream regulatory miRNAs of Gli2, miRNA expression chip GSE58517 of OVC was obtained from GEO database. Differential analysis was performed on normal samples and tumor samples of the chip. And then 23 differentially expressed miRNAs (DEmiRNAs) were screened (Fig. 1D), among which 16 miRNAs were considerably down-regulated in tumor samples. Meanwhile, the upstream regulatory miRNAs of Gli2 were predicted by TargetScan database, and four candidate miRNAs were obtained from the intersection between down-regulated DEmiRNAs and predicted miRNAs (Fig. 1C). The four candidate miRNAs were further analyzed in GSE58517 dataset (Table 1), finding that miR-636 was down-regulated in the greatest level in OVC (logFC=-5.88). qRT-PCR was performed on clinical OVC cancer tissues (n=30) and adjacent normal tissues (n=30), showing that miR-636 was notably down-regulated in cancer tissues (Fig. 1E), which implied that miR-636 may play a crucial role in the occurrence of OVC.

| Symbol     | logFC      | P Value     |
|------------|------------|-------------|
| hsa-miR-3170 | -5.832962968 | 0.015331487 |
| hsa-miR-466  | -5.30360532  | 0.026900184 |
| hsa-miR-636  | -5.884162127 | 0.036453732 |
| hsa-miR-3591-3p | -4.465207808 | 0.040734882 |

To further explore the effect of miR-636 on OVC cell lines, we first verified the expression of miR-636, Hh signaling pathway and EMT-related genes in OVC cell lines. The results of qRT-PCR showed that the expression levels of miR-636 and E-cadherin in OVC cell lines were significantly lower than those in normal ovarian cell line, while the expression levels of Hh signaling pathway related genes (Smo, Tgfb, Gli2, Snail) and EMT-related gene Vimentin were remarkably higher than those in normal ovarian cell line (Fig. 1F). Furthermore, Western blot found that E-cadherin protein expression was evidently lower in OVC cells than that in normal ovarian cells, while Smo, Gli2 and Vimentin were significantly up-regulated (Fig. 1G), and it was consistence with the results of qRT-PCR. Taken together, these findings elucidated that the Smo-Gli2-miR-636 axis in OVC may inhibit tumor growth by EMT.

2.2 miR-636 inhibits OVC cell proliferation and migration and induces cell cycle arrest in G0/G1 phase

To investigate the effect of miR-636 on the growth of OVC cells in vitro, miR-636 mimic and miR-636 inhibitor were transfected into OVC cell line HO-8910PM. The results of qRT-PCR showed that the expression of miR-636 in HO-8910PM cells was significantly increased and decreased after transfection of miR-636 mimic and miR-636 inhibitor, respectively (Fig. 2A). Moreover, MTT assay and wound healing
assay were implemented to examine the effect of miR-636 on proliferation and migration of HO-8910PM cells, finding that overexpression of miR-636 markedly suppressed OVC cell proliferation and migration in vitro, while inhibition of miR-636 posed an opposite effect (Fig. 2B-C). Since miR-636 could significantly inhibit cell proliferation, we further investigated the role of miR-636 in OVC cell cycle. Flow cytometry was performed on cells with overexpression or inhibition of miR-636, showing that overexpression of miR-636 blocked HO-8910PM cell cycle in G0/G1 phase, while inhibition of miR-636 remarkably decreased the number of HO-8910PM cells in G0/G1 phase (Fig. 2D). Collectively, these findings validated that miR-636 acted as a tumor suppressor gene to inhibit OVC cell proliferation, migration in vitro and block OVC cell cycle in G0/G1 phase.

2.3 miR-636 inhibits the activation of Hh signaling pathway and EMT process by directly targeting Gli2

We had found that miR-636 could targeted bind to Gli2 by TargetScan database as described in 2.1. To further investigate the molecular mechanism of miR-636 regulating OVC cell cycle, cell proliferation and migration, Western blot and dual-luciferase assay were employed to determine whether miR-636 could directly target Gli2. Based on the TargetScan database, potential targeted binding sites of miR-636 on Gli2 3'UTR were predicted, and the corresponding mutation sites were designed (Fig. 3A). The results of western blot suggested that overexpressing miR-636 inhibited the expression of Gli2, whereas inhibiting miR-636 expression led to the up-regulation of Gli2 (Fig. 3B). Besides, dual-luciferase assay demonstrated that overexpression of miR-636 inhibited the luciferase activity of Gli2-WT, but had no influence on that of Gli2-MUT (Fig. 3C).

qRT-PCR also revealed that overexpression of miR-636 in HO-8910PM cell line remarkably decreased the expression of Smo, Tgfb, Gli2, Snail, Vimentin and increased E-cadherin expression, while the opposite results could be observed after miR-636 was inhibited (Fig. 3D). Moreover, Western blot indicated that overexpression of miR-636 decreased the expression of Hh signaling pathway related proteins (Smo, Gli2), Vimentin and increased E-cadherin expression, while opposite results could be observed after the inhibition of miR-636 (Fig. 3E). To sum up, we found that miR-636 could directly targeted bind to Gli2 to inhibit the activation of Hh signaling pathway and EMT process, thereby suppressing HO-8910PM cell proliferation and migration.

2.4 Gli2 plays a major role in the miR-636-mediated Hh pathway in HO-8910PM cells

Gli2 is a transcription factor with highly conserved C2H2-Zn finger DNA-binding domains. Gli2 is an effector molecule or a major activating transcription factor in the downstream of Hh pathway, and studies have shown that Gli2 is also a key regulator in various malignancies. In order to verify the effect of Gli2 on OVC cells, qRT-PCR and Western blot were first conducted to examine the Gli2 expression after overexpressing or silencing Gli2. Gli2 mRNA exhibited an upward trend after Gli2 was overexpressed, while its expression was significantly decreased after silencing Gli2 (Fig. 4A). Besides, similar trends could be observed in protein expression as judged by Western blot (Fig. 4B). Furthermore, MTT assay was
performed to assess the effect of overexpression or silencing of Gli2 on proliferation of HO-8910PM cells, finding that overexpression of Gli2 significantly facilitated proliferation of HO-8910PM cells \textit{in vitro}, while opposite effect was produced by silencing Gli2 (Fig. 4C). The results of wound healing assay indicated that overexpressing Gli2 significantly enhanced the migration ability of HO-8910PM cells, while silencing Gli2 noticeably suppressed cell migration (Fig. 4D). Flow cytometry was performed to detect the cell cycle of HO-8910PM, and it was found that overexpressing Gli2 noticeably decreased the number of HO-8910PM cells in G0/G1 phase, whereas silencing Gli2 made HO-8910PM cell cycle arrest in G0/G1 phase (Fig. 4E). Taken together, these findings elucidated that Gli2 could promote cell proliferation and migration of OVC cells \textit{in vitro}, and played an important regulatory role in miR-636-mediated Hh signaling pathway in HO-8910PM cells.

2.5 \textbf{Rescue experiments confirm that miR-636 inhibits cell proliferation, migration and EMT by regulating Gli2}

To further study the mechanism by which miR-636 targeted Gli2 to regulate cell proliferation and EMT, Gli2 and miR-636 were co-transfected into HO-8910PM cells. qRT-PCR and Western blot showed that the expression of Gli2 in miR-636 mimic + oe-Gli2 group was similar to that in mimic-NC + oe-NC group (Fig. 5A-B). MTT assay and wound healing assay suggested that the suppression of HO-8910PM cell proliferation and migration induced by miR-636 overexpression was reversed when Gli2 and miR-636 were simultaneously overexpressed (Fig. 5C-D). Flow cytometry was performed to evaluate the change of HO-8910PM cell cycle after Gli2 and miR-636 were simultaneously overexpressed, finding that the blocking effect of miR-636 on HO-8910PM cell cycle was attenuated by the overexpression of Gli2 (Fig. 5E). Western blot was employed to detect the expression of Hh signaling pathway related proteins (Smo, Gli2) and EMT-related proteins (Vimentin, E-cadherin), showing that the overexpression of Gli2 reversed the inhibitory effect of miR-636 on Hh signaling pathway and promoted EMT of HO-8910PM cells (Fig. 5F). Collectively, these findings illustrated that miR-636 inhibited cell proliferation, migration, blocked cell cycle and suppressed Hh signaling pathway and EMT process in OVC by targeted inhibiting Gli2 expression, while the effects could be reversed by the overexpression of Gli2.

2.6 \textbf{In vivo experiments prove that miR-636 inhibits EMT and occurrence of OVC in mice by targeting Gli2}

To explore the effect of miR-636 on OVC occurrence, HO-8910PM cells were used to construct xenograft tumor mouse models. Subsequently, agomiR-636 or agomiR-NC was inoculated into the tumors of the models for twice a week. The results showed that agomiR-636 significantly retarded the growth of OVC in mouse models (Fig. 6A-B). Additionally, qRT-PCR was employed to detect miR-636 expression in transplanted tumors, finding that miR-636 was markedly up-regulated in agomiR-636 treatment group relative to that in agomiR-NC treatment group (Fig. 6C). The results of IHC on xenograft tumor tissues revealed that Ki-67, Gli2 and Vimentin were obviously down-regulated in agomiR-636 treatment group, while the protein expression level of E-cadherin was remarkably up-regulated (Fig. 6D). Taken together,
these findings validated that miR-636 inhibited the EMT and growth of OVC tumor in mice by silencing Gli2, and injection of agomiR-636 could effectively suppress tumor growth in the xenograft model.

3 Discussion

With the development of sequencing technology, numerous miRNAs have been discovered. As in-depth researches on miRNAs continue to emerge, miRNAs have been found to play a critical role in occurrence and development of various cancers. Besides, miRNAs can act as a biomarker or therapeutic target, which provide novel treatment strategies for cancers. miR-636 is a class of non-coding small RNAs that locates on chromosome 17q25.1. Recent studies have reported that miR-636 is significantly up-regulated in hepatocellular carcinoma (HCC), and the up-regulated miR-636 may facilitate the occurrence of HCC by decreasing RAS expression, while adenine nucleotide translocase 2 (ANT2) suppression by shRNA can restore miR-636 expression and plays an anti-cancer role in HCC. miR-636 is found to be one of the biomarkers in patients with pancreatic cancer and urothelial carcinoma, and can be used in early diagnosis of patients with pancreatic cancer. Currently, studies on the role of miR-636 in OVC remain scarce. In this study, miR-636 was found to be extremely down-regulated in OVC cells. We speculated that miR-636 may function as a tumor suppressor in OVC. Bioinformatics analysis predicted that miR-636 could targeted bind to Gli2 in Hh signaling pathway, so as to suppress the activation of Hh signaling pathway and EMT in OVC.

Hh signaling pathway is highly conserved in evolution in insects and mammals and plays an essential role in embryonic development and morphogenesis. In mammals, Hh signal transduction happens primarily in cilia structure of cells. Inadequate Hh signal can result in congenital developmental abnormalities (cyclops, holoprosencephaly, etc.), while excessive Hh signal can lead to basal cell carcinoma of the skin, medulloblastoma and other tumors. Gli2 is one of zinc finger transcription factors in Hh signaling pathway. Several studies have found that abnormal activation of Gli2 results in the occurrence of various tumors, such as basal cell carcinoma, prostate cancer, breast cancer and HCC. Similarly, there are also multiple researches indicating that Gli2 is abnormally highly expressed in various tumors, which facilitates the development of EMT.

Here we found that miR-636 was extremely lowly expressed in OVC tissues and cells, and was associated with the expression of Hh signaling pathway and EMT-related genes. Overexpressing miR-636 markedly suppressed OVC cell proliferation and migration, and induced cell cycle arrest in G0/G1 phase. While the inhibition of miR-636 significantly promoted cell proliferation and migration ability as well as increased the protein expression of Vimentin and decreased E-cadherin expression, thereby potentiating the EMT process. In the meantime, xenotransplantation experiments validated that miR-636 inhibited tumor growth and EMT of OVC. Dual-luciferase assay predicted that miR-636 could directly targeted regulate Gli2. In OVC cells, overexpressing miR-636 decreased Gli2 expression, while Gli2 expression was increased by inhibiting miR-636. In order to further investigate the effect of Gli2 on the occurrence and development of OVC, Gli2 was overexpressed or silenced in OVC cells. The overexpression of Gli2 was
found to remarkably promote cell proliferation and migration, which was in consistent with the results of inhibition of miR-636. While Gli2 silencing was found to significantly inhibit cell proliferation and migration and block cell cycle in G0/G1 phase, which was in line with the results of the overexpression of miR-636. Also rescue experiments revealed that the regulatory role of miR-636 in OVC cells could be partially reversed by Gli2 after miR-636 and Gli2 were simultaneously overexpressed. Collectively, these findings illustrated that miR-636 could inhibit the EMT, cell proliferation and cell cycle by directly targeting the transcription factor Gli2 of Hh pathway in OVC.

In summary, we found that miR-636 was lowly expressed in OVC, and overexpressing miR-636 inhibited cell proliferation and migration as well as blocked cell cycle in G0/G1 phase. miR-636 regulated Hh signaling pathway by targeting Gli2, so as to suppress proliferation, migration, cell cycle and EMT of OVC cells. The results not only allow us to gain more insight into the effect of miR-636 on OVC, but also lay a foundation for searching new approaches on targeted therapy.

**Declarations**

**Ethics approval and consent to participate**

The experimental protocol was established, according to the ethical guidelines of the Helsinki Declaration and was approved by the Human Ethics Committee of The Second Affiliated hospital of Zhejiang University School of Medicine. Written informed consent was obtained from individual or guardian participants.

**Consent for publication**

Not applicable.

**Availability of data and materials**

The data and materials in the current study are available from the corresponding author on reasonable request.

**Competing interests**

The authors declare that they have no potential conflicts of interest.

**Funding**

Not applicable

**Authors’ contributions**

Jiong Ma contributed to the study design, conducted the literature search and performed data analysis. Chunxia Zhou acquired the data and wrote the article. Xuejun Chen revised the article and gave the final
approval of the version to be submitted. All authors read and approved the final manuscript.

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**Figures**
Figure 1

Differential expression of miR-636, Hh and EMT related genes in OVC (A) Association analysis of Hh signaling pathway related genes (each circle represents a gene and the edges between circles indicate associations); (B) Hh signaling pathway in KEGG database (red represents the location of Gli2 gene in the signaling pathway); (C) The intersection between genes predicted by TargetScan database and down-regulated DEmiRNAs; (D) Heat map showed the DEmiRNAs in GSE58517 dataset; (E) The expression of
miR-636 in clinical cancer tissues and adjacent normal tissues; (F) The differential expression of miR-636, Hh and EMT related genes in OVC cells and normal ovarian cells were detected by qRT-PCR; (G) The protein expression of Hh and EMT related genes were examined by Western blot.

Figure 2

miR-636 inhibits OVC cell proliferation and migration and makes cell cycle arrest in G0/G1 phase Relative expression of miR-636 in OVC cell line HO-8910PM upon the overexpression or inhibition of miR-636; (B) MTT assay, (C) Wound healing assay and (D) flow cytometry showed the effect of miR-636 overexpression/silencing on cell proliferation, migration and cell cycle, respectively.
miR-636 directly targeted binds to Gli2 and inhibits the activation of Hh signaling pathway and EMT (A) The binding sites of miR-636 on Gli2 3’UTR were predicted by TargetScan database; (B) Relative expression of Gli2 after miR-636 was overexpressed or inhibited; (C) The directly targeted relationship between miR-636 and Gli2 was verified by dual-luciferase assay; (D) qRT-PCR was performed to detect the expression of miR-636, Hh and EMT related genes in OVC cells with the overexpression or inhibition of miR-636, while (E) Western blot was conducted to examine the protein expression of Hh and EMT related genes.
Figure 4

Gli2 plays an important role in cell proliferation, migration and cell cycle of OVC. The relative level of Gli2 mRNA (A) and protein (B) expression after overexpressing or silencing Gli2 were detected by qRT-PCR and Western blot; The effects of Gli2 overexpression or inhibition on cell proliferation (C), migration (D) and cell cycle (E) were detected by MTT assay, wound healing assay and flow cytometry, respectively.
Rescue experiments confirm that miR-636 inhibits cell proliferation and EMT by regulating Gli2. Relative levels of Gli2 mRNA (A) and protein (B) expression were detected by qRT-PCR and Western blot after miR-636 mimic and oe-Gli2 were transfected into cancer cells; (C) Cell viability, (D) migration, (E) cell cycle and (F) the expression of Hh, EMT-related proteins were detected by MTT assay, wound healing assay, flow cytometry and Western blot in each treatment group.
Figure 6

Xenotransplantation experiment proves that miR-636 inhibits the EMT and development of OVC by targeting Gli2. Tumor growth (A) and tumor volume (B) of mice after the injection of agomir-636; (C) Relative expression of miR-636 in each treatment group was detected by qRT-PCR; (D) Expression of Gli2, Ki-67, Vimentin and E-cadherin in mice tumors were examined by immunochemistry.
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