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MiR-99a suppressed cell proliferation and invasion by directly targeting HOXA1 through regulation of the AKT/mTOR signaling pathway and EMT in ovarian cancer

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Abstract. – OBJECTIVE: Ovarian cancer (OC) is the third frequently tumor worldwide. MicroRNA-99a (miR-99a), acting as a tumor suppressor, has been reported to be downregulated in multiple tumors. We aimed at exploring the significant roles of miR-99a in ovarian cancer.

PATIENTS AND METHODS: Quantitative Real-time polymerase chain reaction (qRT-PCR) and Western blotting were applied to calculate the mRNA and protein levels of miR-99a and its target genes. Kaplan-Meier method was conducted to evaluate the overall survival of ovarian cancer patients. CCK8 and transwell assays were performed to measure the proliferative and invasive abilities.

RESULTS: miR-99a, acting as a prognosis predictor, was downregulated in ovarian cancer tissues and cell lines. miR-99a mediated the expression of homeobox A1 (HOXA1) through directly targeting to the 3'-untranslated region (3'-UTR) of its mRNA in ovarian cancer cell lines. miR-99a inhibited the proliferation of ovarian cancer by AKT/mTOR pathway *in vitro* and *in vivo*, and it suppressed the invasion-mediated epithelial-mesenchymal transition (EMT) through direct targeting to the 3'-UTR of HOXA1 mRNA.

CONCLUSIONS: miR-99a suppressed the proliferation through AKT/mTOR signaling pathway and the invasion-mediated EMT in ovarian cancer. The newly identified miR-99a/HOXA1/AKT/mTOR axis provides novel insight into the pathogenesis of ovarian cancer.

Key Words:
miR-99a, Proliferation, Invasion, EMT, Ovarian Cancer.

Introduction

Ovarian cancer (OC), a common tumor in women with high morbidity and mortality, is the highest mortality gynecological cancer around the world[1,2]. Although, the treatment of ovarian cancer has been greatly improved, the metastasis remained to be challenges for the treatment of ovarian cancer[3]. Unsatisfactory, the 10-year overall survival rate was less than 30%, although it has great advances in surgery and chemotherapy[4]. Therefore, it is urgent to explore the underlying molecular mechanisms of ovarian cancer and to identify new biomarkers for early diagnosis and treatment of ovarian cancer. MicroRNAs (miRNAs), a kind of small endogenous non-coding RNA that with 18-25 nucleotides in length, inhibits the expression of target genes through binding to the 3'-untranslated region (3'-UTR) of mRNA at post-transcriptional level[5,6]. Increasing evidence[7,8] indicated that miRNAs may critical regulate growth, differentiation and metastasis of tumor. MiR-99a has been reported to act as a tumor suppressor in multiple tumors that are including oral squamous cell carcinoma, bladder cancer, prostate cancer and osteosarcoma[9-12]. Tsai et al[13] found that miR-99a acted as a tumor suppressor to suppress the cellular viability. Besides, Zhang et al[14] indicated that miR-99a inhibited the proliferation, migration and dedifferentiation vascular smooth muscle[14]. Similarly, Wang et al[15] revealed that miR-99a suppressed the tumor aggressiveness and the proliferation and metastasis of breast cancer. Therefore, we conjectured that miR-99a may mediate the proliferation and metastasis through regulating AKT/mTOR pathway and epithelial-mesenchymal transition (EMT) in ovarian cancer.

The homeobox A1 (HOXA1) acted as a DNA-binding transcription factor and may mediate gene expression and cell differentiation[16,17].

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HOXA1 has been reported to play a vital role in multiple biological processes, and contributed to the tumorigenesis and development in non-small cell lung cancer[18]. Zha et al[19] indicated that loss of HOXA1 enhanced the proliferation and metastasis in hepatocellular carcinoma, and overexpression of HOXA1 was associated with poor outcome of HCC patients. Moreover, HOXA1 has been found to reinforce the growth, metastasis and elicits an invasion gene expression signature[20]. HOXA1 has been predicted to improve the proliferation, invasion and metastasis in prostate cancer cells[21]. Thus, we strongly believe that miR-99a may play a role in carcinogenesis through directly targeting to the 3'-UTR of HOXA1 mRNA in ovarian cancer. In this study, we elucidated that miR-99a was significantly downregulated in ovarian cancer tissues and cell lines, and downregulation of miR-99a predicted a lower 10-year overall survival rate. MiR-99a suppressed the proliferation through AKT/mTOR signal pathway and inhibited the invasion-mediated EMT in ovarian cancer. In addition, miR-99a suppressed the growth of ovarian cancer *in vivo*.

Patients and Methods

Sample Collection

Cancer tissue samples were obtained from 47 ovarian cancer patients that sought treatment at Qilu Hospital, meanwhile, the matched controls were gathered from non-necrotic ovarian tissues. After the surgical operation, all the samples were immediately frozen in liquid nitrogen and stored at -80°C for backup. Neither radiotherapy nor chemotherapy therapy were used in any patients before surgery. Informed consent was obtained from all individual participants and this (samples collection and examining) study was approved by the Ethics Committee of Qilu Hospital of Shandong University.

Cell Culture

Two ovarian cancer cell lines IGROV-1 and HO-8910 and a normal ovarian epithelial cell line IOSE80, were collected from American Type Culture Collection (ATCC, Manassas, VA, USA). Dulbecco's Modified Eagle's Medium (DMEM) (HyClone, South Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), was conducted to culture all the cells in a 37°C in a humidified atmosphere consisting of 5% CO_2.

Transfection

The miR-99a mimic and the miR-99a inhibitor, as well as corresponding negative control (NC), were purchased from GenePharma (Shanghai, China). IGROV-1 cells were transfected with the miR-99a mimic or the miR-99a inhibitor to up- or down-regulate the miR-99a expression by Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

According to the manufacturer's instructions, the miRNeasy Mini Kit (Qiagen, Hilden, Germany) was used to extract miRNA from tissues or cell lines. The first complementary deoxyribose nucleic acid (cDNA) chain was synthesized by the TaqMan miRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA); followed the miRNA-specific TaqMan MiRNA Assay Kit (Applied Biosystems, Foster City, CA, USA) was employed to perform the qPCR. The relative levels of miRNA were derived using $2^{-\Delta\Delta Ct}$ method and the U6 small nuclear RNA was used as the normalization. For the mRNAs, total RNA was extracted by using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. The first cDNA chain was synthesized by using the Omniscript Reverse Transcription Kit (Qiagen, Hilden, Germany) from total RNA. The QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany) was conducted to perform the qRT-PCR using a QuantiTect SYBR Green PCR System (Qiagen, Hilden, Germany). The $2^{-\Delta\Delta Cq}$ method was used to analyze the relatively quantitative data, which was normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH). PCR cycling conditions were as follows: an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of denaturing at 95°C for 10 s, annealing and synthesis at 60°C for 60 s. The primers are as follows: miR-99a: forward, 5'-AACCCGTAGATCCGATCTTGTG-3' and reverse, 5'-TGGTGTCGTGGAGTCG-3'; U6: forward, 5'-GCTTCGGCAGCACATATACTAAAAT-3' and reverse, 5'-CGCTTCACGAATTTGCGTGTCAT-3'. HOXA1: forward, 5'-TCCTGGAATACCCCATACTTAGC-3' and reverse, 5'-GCACGACTGGAAAGTTGTAATCC-3'; GAPDH: forward, 5'-GACTCATGACCACAGTCCATGC-3', and reverse, 5'-AGAGGCAGGGATGATGTTCTG-3'.

Western Blotting

The Radioimmunoprecipitation Assay (RIPA) Buffer (Beyotime, Shanghai, China) was used to extract their total proteins on ice. After half an hour of hydrolysis on ice, the proteins were centrifuged and the supernatant was collected. And then the protein concentration was tested by using a Bicinchoninic Acid protein Assay (BCA) (Pierce, Rockford, IL, USA). Equal amounts of proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed the blots were transferred onto a polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland). After blocking in 5% nonfat dried milk for 1 h, the membrane was incubated with the primary antibodies at 4°C overnight. The primary antibodies were HOXA1 (1:1000; Abcam, Cambridge, MA, USA), E-cadherin (1:1000; Abcam, Cambridge, MA, USA), N-cadherin (1:1000; Abcam, Cambridge, MA, USA), Vimentin (1:1000; Abcam, Cambridge, MA, USA), p-AKT (1:1000, Cell Signaling, Danvers, MA, USA), AKT (1:1000, Cell Signaling, Danvers, MA, USA), p-mTOR (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mTOR (1:1000; Abcam, Cambridge, MA, USA) and GAPDH (1:3000, Cell Signaling, Danvers, MA, USA). Subsequently, the membranes were incubated the secondary horseradish peroxidase (HRP)-conjugated antibody (Beyotime, Shanghai, China) about 2 h at room temperature after washing with TBST buffer (Tris-buffered saline and Tween-20, pH 8.0) three times. Finally, protein signal was determined with Enhanced Chemiluminescence (ECL) Kit (Pharmacia Biotech, Arlington, MA, USA) using a Bio-Rad Gel Doc XR instrument (Bio-Rad, Hercules, CA, USA).

Cell Counting Kit-8 (CCK-8) Assay

The ability of cell proliferation was ascertained by using CCK-8 Assay. In brief, 3000 cells were hatched in 96-well plates. Meanwhile, the absorbance was evaluated after cultured 24 h, 48 h, 72 h or 96 h using CCK-8 (Dojindo, Kumamoto, Japan), which was measured by a Model 680 Microplate Reader (Bio-Rad, Hercules, CA, USA). The optical density value in each well was determined at a wavelength of 450 nm.

Transwell Assay

Cells were resuspended in serum-free media and were added in the upper chamber that was coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). In the lower chamber, we added normal Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) with 15% fetal bovine serum (FBS) to make it act as an attractant. After cells were incubated for about 24 h at 37°C, the invasive cells were fixed and stained by 4% paraformaldehyde and 10% crystal violet, respectively. Finally, we photographed and counted the invasive cells under a microscope.

Dual-Luciferase Reporter Assay

TargetScan (www.targetscan.org) was used to foretell the potential downstream target genes of miR-99a, and HOXA1 was found to be a candidate target. To verify our conjecture that miR-99a directly targeting to HOXA1, the predicted binding sequences on the 3'-UTR of HOXA1 mRNA were mutated from 5'-UACGGGU-3' to 5'-AUGCCCA-3' in ovarian cancer cells. Subsequently, both the wild type and the mutant 3'-UTR of HOXA1 mRNA were inserted into the dual-luciferase reporter vectors, which were designated as WT or MUT. For the luciferase assay, we applied Lipofectamine 3000 Reagent (Invitrogen, Carlsbad, CA, USA) to co-transfected with the miR-99a mimic and WT or MUT plasmid into IGROV-1 cells. Next, luciferase activity was measured by using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Xenograft Tumor Formation Assay

The nude mice with four weeks of age were purchased from Charles River Laboratories (Beijing, China). Transfected IGROV-1 cells were injected subcutaneously into the axilla of one side of the nude mice to establish the xenograft model. The length and width of the xenograft tumor were evaluated and recorded every 3 days after the transplant tumor model was completed. The volume of the xenografts was calculated as length multiply by the square of width divided by two. The experiment was terminated after 26 days of culture, the mice were sacrificed and the xenografts were dissected out. All animal experiments were performed in Animal Laboratory Center of Shandong University and approved by the Shandong University Animal Care and Use Committee.

Statistical Analysis

All the data are presented as mean±standard deviation (SD) from at least three independent experiments. Statistical Product and Service Solutions (SPSS) 16.0 software (SPSS Inc., Chi-

cago, IL, USA) was employed to analyze data. Student's *t*-tests or one-way analysis of variance (ANOVA) was used to compare the differences between two or more groups. One-way ANOVA test was followed by post-hoc test LSD (Least Significant Difference). The association between the expression of miR-99a and the overall survival of ovarian cancer patients were assessed by Kaplan-Meier curve and log-rank test. Results were considered to be statistically significant if $p<0.05$.

Results

Downregulation of miR-99a Predicted Poor Prognosis of Ovarian Cancer

We found that, by calculating the mRNA level of miR-99a in 47 pairs of ovarian cancer and adjacent normal tissues, the expression of miR-99a was significantly lower in ovarian cancer tissues in comparison to the corresponding adjacent normal tissues ($p<0.0001$) (Table I, Figure 1A). In addition, we discovered that low expression of miR-99a was associated with poor 10-year overall survival of ovarian cancer patients ($p=0.0475$) (Figure 1B).

miR-99a Suppressed the Proliferation and Invasion in Ovarian Cancer Cells

Apart from tissues, the expression of miR-99a was assessed in two ovarian cancer cell lines (IGROV-1 and HO-8910) and a normal epithelial cell line IOSE80. Similar with the findings in tissues, the expression of miR-99a was higher in normal cell line IOSE80 cells than that in ovarian cancer cell lines IGROV-1 ($p=0.0005$) and HO-8910 ($p=0.0011$) (Figure 2A). To explore the functions of miR-99a on proliferation and invasion in ovarian cancer, the miR-99a mimic or the miR-99a inhibitor was transfected to up- ($p<0.0001$) or down-regulated ($p=0.0023$) miR-99a in ovarian cancer cell line IGROV-1 (Figure 2B). CCK8 assay showed that the proliferation was inhibited ($p=0.0062$ and 0.0021 at 72 and 96) by the miR-99a mimic, while it exactly showed the opposite in the cells that transfected with the miR-99a inhibitor (Figure 2C). Transwell assay showed that the invasive ability was eliminated ($p=0.0092$) by the miR-99a mimic while enhanced by the miR-99a inhibitor ($p=0.0027$) (Figure 2D). All the findings demonstrated that the miR-99a suppressed the capacities of proliferation and invasion in ovarian cancer cell line IGROV-1.

miR-99a Regulated the Expression of HOXA1 Through Binding to the mRNA 3'-UTR of HOXA1

HOXA1 was predicted to be a target gene of miR-99a by TargetScan and the binding site was located at 1088-1094 on the 3'-UTR of HOXA1 mRNA. To validated miR-99a binding to the potential binding site of HOXA1, the binding sequences were mutated from 5'-UACGGGU-3' to 5'-AUGCCCA-3', and then inserted into the luciferase vectors (Figure 3A). The experiment results revealed that compared to the negative control, the miR-99a mimic reduced ($p=0.0008$) the luciferase activity of IGROV-1 cells that transfected with the wild type of HOXA1 3'-UTR; however, the luciferase activity of cells transfected with the mutated HOXA1 3'-UTR had no alteration

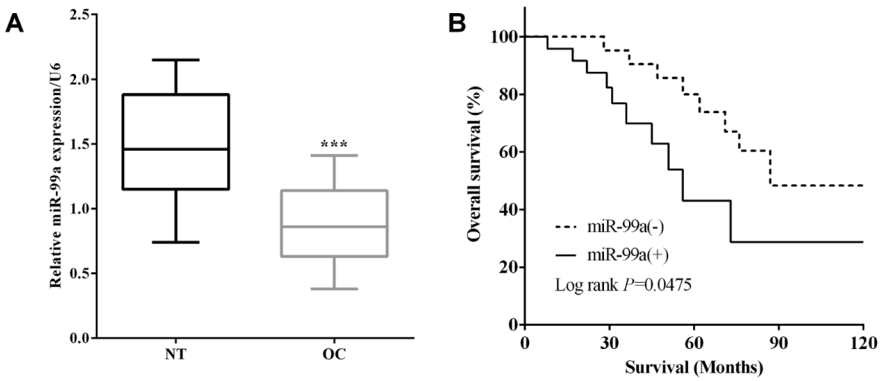

Figure 1. Downregulation of miR-99a predicted poor prognosis of ovarian cancer. ***A,*** The expression of miR-99a was lower in ovarian cancer tissues than that of corresponding adjacent normal tissues. ***B,*** Low expression of miR-99a predicted a poor 10-year survival rate of ovarian cancer patients.

Table I. miR-99a expression and clinicopathological features in 47 ovarian cancer.

Clinicopathological features	Cases (n=47)	miR-99a expression		p-value*
		Low (n=24) (%)	High (n=23) (%)	
Age (years)				
< 60	25	14 (53.8)	11 (46.2)	0.470
≥ 60	22	10 (40.0)	12 (50.0)	
Tumor size (mm)				
≤ 5.0	23	15 (65.2)	8 (34.8)	0.057
> 5.0	24	9 (37.5)	15 (62.5)	
FIGO stage				
I-II	21	14 (66.7)	7 (33.3)	0.054
III-IV	26	10 (38.5)	16 (61.5)	
Lymph-node metastasis				
Yes	24	16 (66.7)	8 (33.3)	0.029*
No	23	8 (34.8)	15 (65.2)	
Tumor grade				
Moderately/high differentiated	27	14 (55.6)	9 (44.4)	0.188
Poorly differentiated	20	10 (45.0)	14 (55.0)	
Invasion				
No	23	13 (61.9)	8 (38.1)	0.181
Yes	24	11 (42.3)	15 (57.7)	
HOXA1				
Low expression	22	7 (31.8)	15 (68.2)	0.013*
High expression	25	17 (68.0)	8 (32.0)	

*p-values are calculated with Chi-square test.

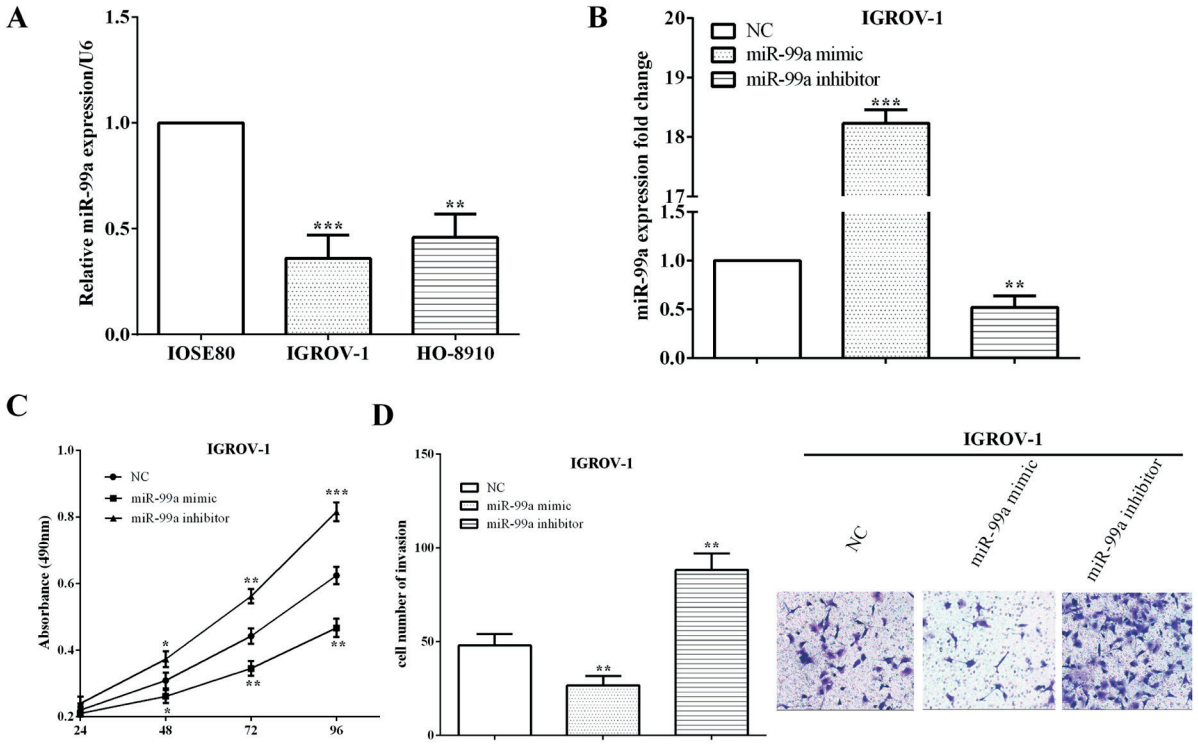

Figure 2. MiR-99a suppressed the proliferation and invasion in ovarian cancer cells. ***A,*** miR-99a was downregulated in ovarian cancer cell lines IGROV-1 and HO-8910 compared to normal epithelial cell line IOSE80. ***B,*** The miR-99a mimic or the miR-99a inhibitor was transfected in IGROV-1 cells to up- or down-regulate miR-99a. ***C,*** CCK8 assay revealed that the miR-99a mimic inhibited the proliferation in IGROV-1 cells. ***D,*** Transwell assay illuminated that the miR-99a mimic suppressed the invasive ability in ovarian cancer cell line IGROV-1.

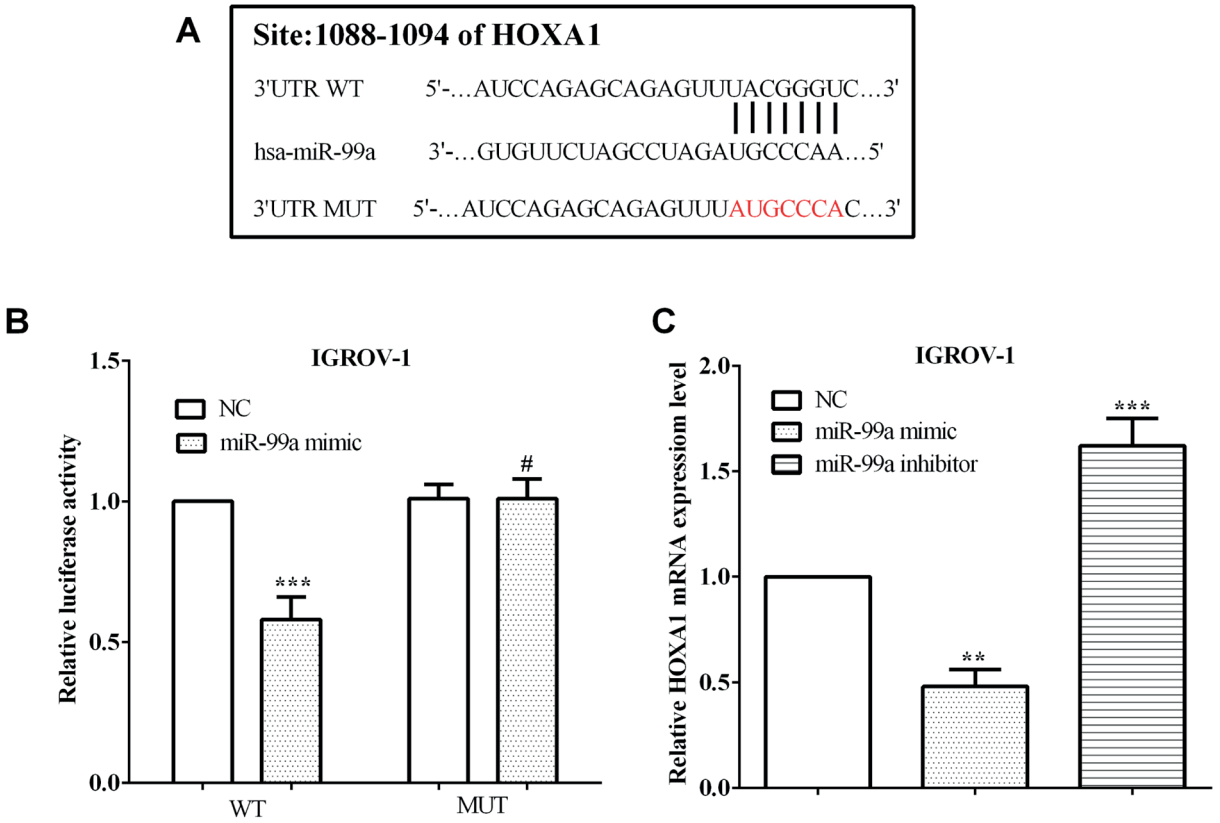

Figure 3. MiR-99a regulated the expression of HOXA1 through binding to its mRNA 3'-UTR. ***A,*** TargetScan predicted HOXA1 was a direct target gene of miR-99a and the binding site was located at its mRNA 3'-UTR. ***B,*** The luciferase reporter assay indicated that miR-99a targeted to the 3'-UTR of HOXA1 mRNA in IGROV-1 cells. ***C,*** Overexpression of miR-99a inhibited the mRNA level of HOXA1, while knockdown of miR-99a enhanced the expression of HOXA1 in IGROV-1 cells.

(p=0.7078) (Figure 3B). After transfected with the miR-99a mimic or the miR-99a inhibitor in IGROV-1 cells, mRNA levels of HOXA1 were measured by qRT-PCR. As expected, overexpression of miR-99a inhibited (p=0.0004) the mRNA level of HOXA1, while knockdown of miR-99a promoted (p=0.0012) it in IGROV-1 cells (Figure 3C). The results showed that the miR-99a adjusted the expression of HOXA1 in ovarian cancer cells IGROV-1.

miR-99a Inhibited the Invasion-Mediated EMT and Proliferation Through AKT/mTOR Signal Pathway

The expression of HOXA1 in tissues and cell lines were calculated by qRT-PCR, and we discovered that the expression of HOXA1 was significantly higher in ovarian cancer tissues as compared with the corresponding non-tumor tissues (p<0.0001) (Figure 4A). Similarly, the expression of HOXA1 was assessed to be up-regulated in ovarian cancer cell lines IGROV-1 (p=0.0002) and HO-8910 (p=0.0011) compared with the normal epithelial cell line IOSE80 (Figure 4B). Moreover, western blot was conducted to assess the expression of the proteins that associated with EMT and AKT/mTOR pathway in IGROV-1 cells. We discovered that the miR-99a mimic suppressed the expression of HOXA1 and E-cadherin, while the expression of N-cadherin and Vimentin was improved in IGROV-1 cells (Figure 4C), indicating that the miR-99a inhibited the EMT through regulating the expression of HOXA1. Meanwhile, the miR-99a mimic eliminated the expression of p-PI3K and p-AKT in IGROV-1 cells (Figure 4D), which elucidated that miR-99a inhibited the proliferation through HOXA1/AKT/mTOR pathway. All the results revealed that miR-99a targeted to HOXA1 inhibited the proliferation and invasion through regulation of the AKT/mTOR signaling pathway and EMT of ovarian cancer.

miR-99a Suppressed the Xenograft Growth in Vivo

To further uncover the important roles of miR-99a in the growth of ovarian cancer *in vivo*, the IGROV-1 cells that stably expressing with the miR-99a mimic or its control plasmid was conducted to inject into the nude mice at subcutaneous. We calculated the xenograft tumors' volume every 3 days and found that the group of transfecting with the miR-99a mimic had a slower growth rate than that of the control group (Figure 5A). In addition, the mice were euthanized after 26 days, and the volume of the resulting tumors was measured. As expected, we discovered that the tumor volumes of the control group were bigger than that of overexpressed miR-99a (p=0.0001), which indicated that miR-99a inhibited the growth of ovarian cancer *in vivo* (Figure 5B).

Discussion

Ovarian cancer is the fourth frequently tumor and one of the most important female tumors wordlwide[22,23]. Therefore, it is urgent to identify new biomarkers for the early diagnosis and metastasis of ovarian cancer. Increasing evidence indicated that miRNAs play vital roles as diagnostics biomarkers and therapeutic targets for human cancers. MiRNAs have been validated to repress the protein degradation and translation through binding to the mRNA 3'-UTR of target genes at post-transcriptional level[24]. MiR-99a has been reported to be low expressed and acted as a predictor of poor prognosis in breast cancer and oral squamous cell carcinoma[9,25]. Consistent with the findings, we discovered that miR-99a was downregulated in ovarian cancer tissues

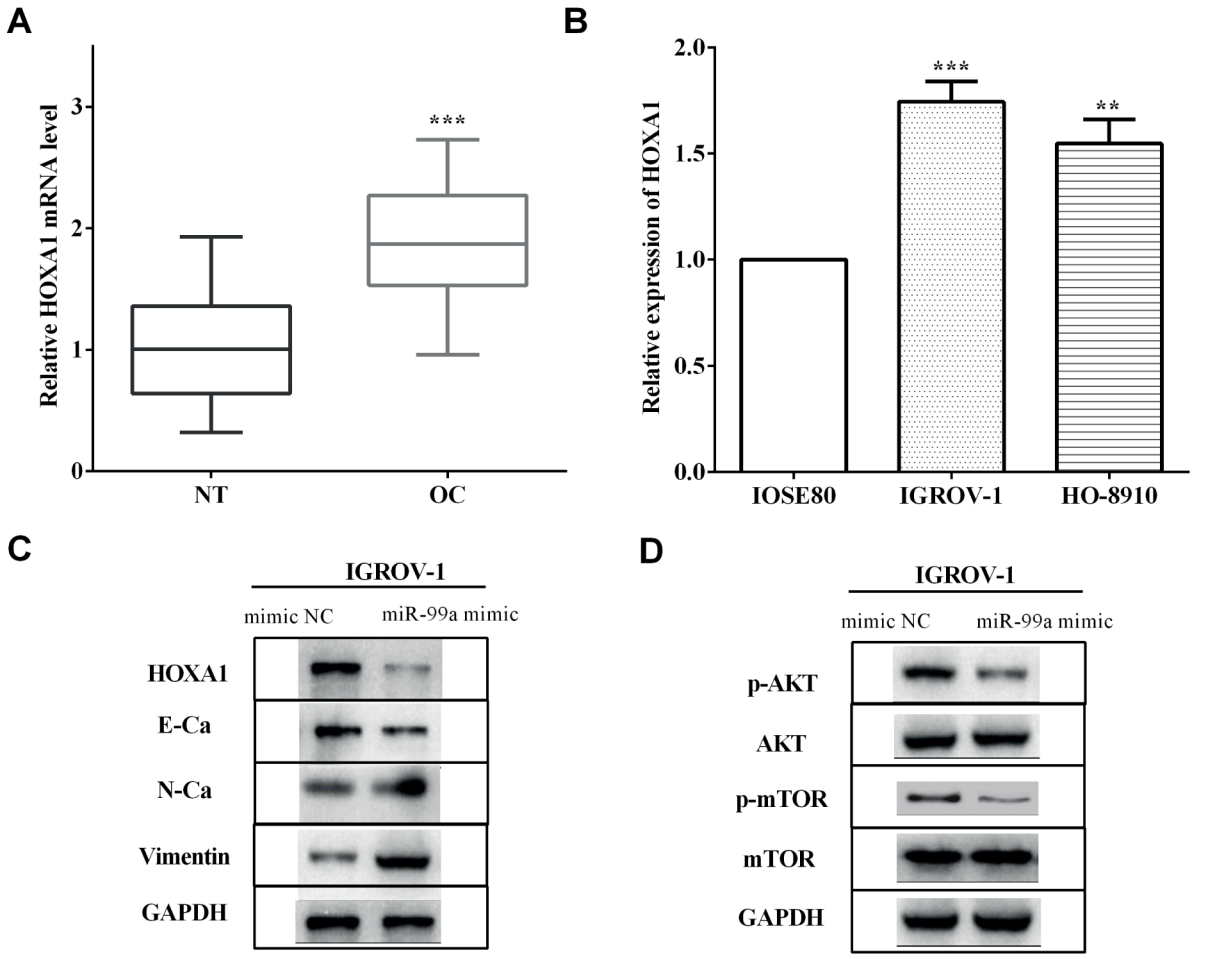

Figure 4. MiR-99a suppressed the invasion-mediated EMT and proliferation through AKT/mTOR signal pathway. ***A,*** HOXA1 was overexpressed in ovarian cancer tissues in compared to the corresponding adjacent normal tissues. ***B,*** The expression of HOXA1 was higher in ovarian cancer cell lines than that of normal cells. ***C,*** Western blot illuminated that miR-99a inhibited the EMT through HOXA1. ***D,*** MiR-99a inhibited the proliferation through HOXA1/PI3K/AKT pathway.

compared to the adjacent normal tissues. Moreover, we found that miR-99a acted as a prognosis marker and that low expression of miR-99a predicted poor 10-year overall survival of ovarian cancer patients. Mei et al[26] demonstrated that miR-99a suppressed the proliferation and migration in esophageal squamous cell carcinoma. In adenocarcinoma, miR-99a eliminated the ability of proliferation and invasion in cervical cancer[27]. Our results were consistent with all the findings above. We found that miR-99a inhibited the proliferation and invasion, while downregulation of miR-99a showed the opposite results in ovarian cancer. In addition, we also found that miR-99a inhibited the xenograft growth *in vivo*, which was consistent with the findings in osteosarcoma[12].

HOXA1 was upregulated in melanocytic nevi and melanoma[28]. Bitu et al[29] demonstrated that HOXA1 was overexpressed in oral squamous cell carcinoma and that overexpression of HOXA1 was associated with worse outcome. Similarly, in gastric cancer Yuan et al[30] reported that HOXA1 reinforced the proliferation and that overexpression of HOXA1 predicted poor prognosis. We revealed that HOXA1 was overexpressed in ovarian cancer and that overexpression of HOXA1 predicted worse overall survival. HOXA1 has been found to be a direct target gene of several miRNAs including miR-433, miR-10a, miR-30b and miR-100[31-34]. As reported by Wang et al[35], HOXA1 was confirmed to be a direct target gene of miR-99a using a luciferase reporter system in ovarian cancer. The expression of HOXA1 was higher in ovarian cancer tissues than that in the adjacent normal tissues. Additionally, miR-99a targeting to the 3'-UTR of HOXA1 mRNA suppressed the proliferation, invasion and EMT by AKT/mTOR pathway of ovarian cancer.

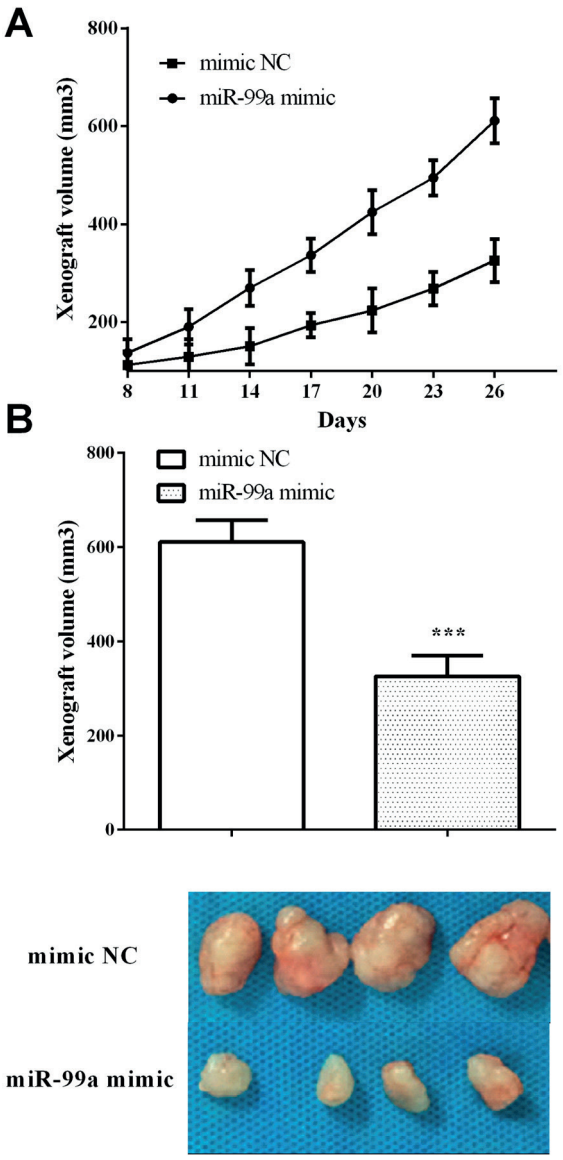

Figure 5. MiR-99a suppressed the xenograft growth *in vivo*. ***A,*** The tumor volumes of cells that transfected with miR-99a mimic were smaller than the control group. ***B,*** The miR-99a mimic inhibited the xenograft growth of ovarian cancer.

Conclusions

We have demonstrated that miR-99a acted as a prognosis predictor, which was downregulated in ovarian cancer tissues and cell lines. Furthermore, miR-99a mediated the expression of HOXA1 through directly targeting to its 3'-UTR of mRNA in ovarian cancer. Moroever, miR-99a inhibited the proliferation of ovarian cancer by AKT/mTOR pathway *in vitro* and *in vivo*, and suppressed the invasion-mediated EMT through targeting HOXA1.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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