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RESEARCH ARTICLE

Overexpressed miR-9 promotes tumor metastasis via targeting E-cadherin in serous ovarian cancer

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Abstract **MicroRNAs (miRNAs) play critical roles in the development and progression in various cancers. Dysfunctional miR-9 expression remains ambiguous, and no consensus on the metastatic progression of ovarian cancer has been reached. In this study, results from the bioinformatics analysis show that the 3′-UTR of the E-cadherin mRNA was directly regulated by miR-9. Luciferase reporter assay results confirmed that miR-9 could directly target this 3′-UTR. miR-9 and E-cadherin expression in ovarian cancer tissue was quantified by qRT-PCR. Migration and invasion were detected by wound healing and Transwell system assay in SKOV3 and A2780. qRT-PCR and Western blot were performed to detect the epithelial–mesenchymal transition-associated mRNA and proteins. Immunofluorescence technique was used to analyze the expression and subcellular localization of E-cadherin, N-cadherin, and vimentin. The results showed that miR-9 was frequently upregulated in metastatic serous ovarian cancer tissue compared with paired primary ones. Upregulation of miR-9 could downregulate the expression of E-cadherin but upregulate the expression of mesenchymal markers (N-cadherin and vimentin). Overexpression of miR-9 could promote the cell migration and invasion in ovarian cancer, and these processes could be effectively inhibited via miR-9 inhibitor. Thus, our study demonstrates that miR-9 may promote ovarian cancer metastasis via targeting E-cadherin and a novel potential therapeutic approach to control metastasis of ovarian cancer.**

Keywords ovarian cancer; metastasis; miR-9; E-cadherin

Introduction

Epithelial ovarian cancer is the fourth most lethal cancer among women and the leading cause of gynecological cancer deaths worldwide. The five-year survival rate for epithelial ovarian cancer patients is only 44% in the USA [1], because most of these patients are diagnosed at an advanced stage with widely omental metastases [2,3]. However, the molecular mechanisms of metastasis remain ambiguous. A better understanding of these mechanisms is especially needed for the early diagnosis and enhanced treatment of ovarian cancer.

MicroRNAs (miRNAs) are a class of evolutionarily conserved small non-coding RNAs which negatively modulate gene expression by pairing with the 3′-UTR of target mRNAs to direct their posttranslational repression [4]. miRNAs play vital roles in numerous biological processes, such as the regulation of cell cycle, differentiation, apoptosis, and angiogenesis, as well as cancer initiation and progression [5–7]. Many studies have shown that miRNAs are highly dysfunctional and deregulated in various tumors, either as an onco-miRNA or as a suppressor miRNA. miR-9 acts as a metastasis-associated miRNA to promote many cancers to acquire malignant phenotypes, resulting in cancer progression and poor prognosis. These cancers include breast cancer [8], esophageal squamous cell carcinoma [9], hepatocellular cancer [10], colorectal cancer [11], and melanoma [12]. Other studies have shown that miR-9 was downregulated in endometriod and clear cell ovarian cancer [13] and recurrent ovarian serous cancer compared with the primary tumor [14]. We have previously demonstrated that upregulated miR-9 was associated with improved

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prognosis, longer survival rates, and cisplatin sensitivity [15]. These results demonstrated that the role of miR-9 remains unclear and needs further verification.

Epithelial–mesenchymal transition (EMT) is a multi-faced transdifferentiation program that enables tumor cells acquire malignancy-associated phenotypes and important to tumor aggression and metastasis [16]. Recently, numerous studies have documented that miRNAs may be involved in the EMT process.

In our present study, we investigated the function of miR-9 in primary ovarian cancer tissue compared with paired metastatic ones. miR-9 was upregulated in metastatic sites and had a reverse correlation with E-cadherin expression *in vitro* and patient samples. Therefore, we hypothesized that miR-9 may be involved in the metastasis of ovarian cancer via the regulation of E-cadherin expression.

Materials and methods

Tissue samples

A total of 25 paired formalin-fixed and paraffin-embedded ovarian serous tumor samples were collected at Tongji Hospital (Wuhan, Hubei, China). The participants provided informed consent between January 2015 and July 2015. All patients underwent debulking and subsequent platinum-centered chemotherapy. The protocol was approved by the Ethics Committee of Tongji Hospital.

Cell culture

Human ovarian cancer cell lines SKOV3 and A2780 were purchased from the American Type Culture Collection (Manassas, VA, USA). SKOV3 and A2780 cells were cultured in Macoy's 5A medium or RPMI 1640 medium, respectively (Gibco, USA), supplemented with 10% fetal bovine serum (Gibco, USA), penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere containing 5% CO_2.

Reagents and miRNA transfections

miR-9 mimics or inhibitor was purchased from Ribobio Co. Ltd, Guangzhou, China. miR-9 mimics (50 nmol/L) or miR-9 inhibitor (100 nmol/L) was transfected into cells with Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions.

Plasmid construction and luciferase assay

The full-length 3′-UTR of E-cadherin from the genomic DNA of a normal patient was amplified using PCR, and the potential miR-9 binding site in the 3′-UTR of E-cadherin was mutated by the overlap extension PCR. Wild-type and mutant 3′-UTRs of E-cadherin were ligated into the Psi-Check2 plasmid (Promega) at the *Xho*I and *Not*I sites directly downstream from the renilla luciferase coding sequence. The authenticity and orientation of the inserts were confirmed by sequencing as previously described [15]. SKOV3 cells were seeded at 1.0×10^4/well in a 96-well plate at 24 h before transfection. Cells were co-transfected with miR-9 mimics or NC and wild-type or mutant E-cadherin 3′-UTR plasmids using Lipofectamine™ 3000 (Invitrogen). After 48 h, luciferase assay was performed using the Dual-Luciferase Reporter assay system (Promega).

miRNA target prediction

Algorithms, such as Pictar (http://pictar.mdc-berlin.de/), Targetscan (http://www.targetscan.org/), miRanda (http://www.microrna.org/microrna/home.do), RNA22 (http://cbcsrv.watson.ibm.com/rna22.html), and miRWalk (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/), were used to identify the target genes to predict miRNA potential targets [17,18].

RNA and miRNA extraction

Total RNAs including miRNAs were extracted from primary ovarian cancer tissue or formalin-fixed and paraffin-embedded tumor samples using miRNeasy FFPE kit (Qiagen Inc., Valencia, CA, USA) following manufacturer's instructions. The levels of mature miR-9 were determined by Bulge-Loop™ miRNA qRT-PCR Primer Set (Ribobio) with SYBR Green quantitative real-time PCR. The miR-9 levels were normalized to those of U6 snRNA. Gene mRNA was extracted using RNAprep pure cell kit (TIANGEN Biotech CO., LTD, China), and the expression of mRNA was determined by qRT-PCR using the PrimeScript RT reagent kit and SYBR Premix EX Taq (TaKaRa) according to the manufacturer's instructions. The expression of gene mRNA was normalized to that of β-actin by $2^{-\Delta\Delta Ct}$ method.

Wound healing assay

SKOV3 cells were first transfected with miR-9 mimics or inhibitor for 12 h, and then wound healing assay was performed according to the standard protocol [19].

Migration and invasion assay

Migration assay was performed using a migration chamber (Corning, New York, NY, USA) following the manufacturer's instructions. Matrigel invasion assay was performed using membranes coated with Matrigel matrix (BD Science, Sparks, MD, USA). First, the cells were transfected with miR-9 mimics or inhibitor. After 12 h, the

transfected cells were digested, and single cell suspensions (1×10^5 cells/well) were seeded into the upper chambers and allowed to invade for another 12 h at 37 °C in a CO_2 incubator. Then, cells on the lower side of the chamber were fixed, stained with 0.1% crystal violet for 15 min and counted using a light microscope according to the published criteria [20].

Immunofluorescence (IF) assay

After cells were transfected with miR-9 mimics for 48 h, cells were fixed with paraformaldehyde 4% and incubated with the primary antibodies E-cadherin (1:50, Cell Signaling Technology, Danvers, MA, USA), N-cadherin (1:50, Cell Signaling Technology, Danvers, MA, USA), and vimentin (1:100, Cell Signaling Technology, Danvers, MA, USA) overnight at 4 °C. The next day, cells were first washed with PBS and incubated with secondary antibody conjugated to FITC or CY3 (Invitrogen, Carlsbad, CA, USA). The nuclei were stained with DAPI. Images were acquired using a laser scanning confocal microscope (Olympus).

Western blot

SKOV3 or A2780 cells were seeded in a 6-cm culture plate. After cell density of approximately 30%–50% was reached, miR-9 mimics or inhibitor was transfected and continued to be cultured for another 48 h. Then, the cells were collected and lysed by radioimmunoprecipitation assay buffer. The primer antibody was E-cadherin (1:1000, item number 3195, Cell Signaling Technology, Danvers, MA, USA). The primer antibody was GAPDH (1:5000, item number 60004, Proteintech Group, Inc., Wuhan, China). The subsequent procedure was performed as described previously [21]. The chemiluminescence was detected by a Bio-Rad Imaging system (Bio-Rad, Hercules, CA, USA).

Statistical analysis

All data are expressed as mean ± SD. Each experiment was repeated at least thrice independently. Statistical significance of differences was analyzed by two-tailed Student's *t*-test or one-way ANOVA. *P* value less than 0.05 was considered statistically significant. All statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

Results

miR-9 is upregulated in metastatic ovarian cancer tissue

The expression of miR-9 at the metastatic sites in ovarian cancer patients was compared with the paired primary site tissue. A total of 25 paired formalin-fixed and paraffin-embedded ovarian serous tumor samples were collected to quantify the expression of miR-9 using qRT-PCR. The average expression of miR-9 was significantly higher at the metastatic sites than at their paired primary sites ($P < 0.05$) (Fig. 1A). Furthermore, five paired fresh patient-derived ovarian cancer cell lines diagnosed with high-grade serous ovarian cancer were also detected by qRT-PCR. The results showed miR-9 expression of the metastatic sites was upregulated by at least 6-fold (Fig. 1B).

miR-9 level may be negatively correlated with the E-cadherin expression but positively correlated with vimentin expression

The different expression levels of E-cadherin and vimentin between ovarian cancer metastatic site tissue and paired primary tissue was confirmed. qRT-PCR was performed, and the results showed that the E-cadherin expression was generally downregulated at the metastatic sites than at the primary sites (Fig. 1C). By contrast, vimentin expression was remarkably upregulated at the metastatic sites (Fig. 1D), in accordance with miR-9 expression between the metastatic and primary sites. These results indicated that miR-9 was upregulated and may have a negative correlation with E-cadherin.

E-cadherin is a direct target of miR-9

Bioinformatics analysis tools were used to search for the potential target of miR-9. The results show that E-cadherin was a putative candidate. SKOV3 and A2780 cells were used to detect E-cadherin expression after miR-9 mimics or inhibitor transfection to test whether E-cadherin was regulated by miR-9. SKOV3 and A2780 cells were transfected with miR-9 mimics for 36 h. qRT-PCR results showed that miR-9 expression was significantly upregulated compared with transfection with miR-9 NC (Fig. 2A). Meanwhile, the E-cadherin expression was found to be considerably downregulated when miR-9 mimics was transiently transfected into SKOV3 and A2780 cells after 36 h of detection through qRT-PCR and 48 h through Western blot separately (Fig. 2B and 2C). By contrast, miR-9 expression was significantly downregulated when transfected with miR-9 inhibitor (data not shown), and the above effects were also restored as shown by qRT-PCR and Western blot results (Fig. 2D and 2E). Next, a luciferase reporter assay was performed to confirm whether E-cadherin was a direct target of miR-9. Wild-type or mutant-type E-cadherin 3′-UTR sequence was cloned into the psi-check 2 vector. SKOV3 cells were co-transfected with miR-9 mimics and the psi-check 2 plasmid (wild-type or mutant) for 48 h. The dual-luciferase reporter assay

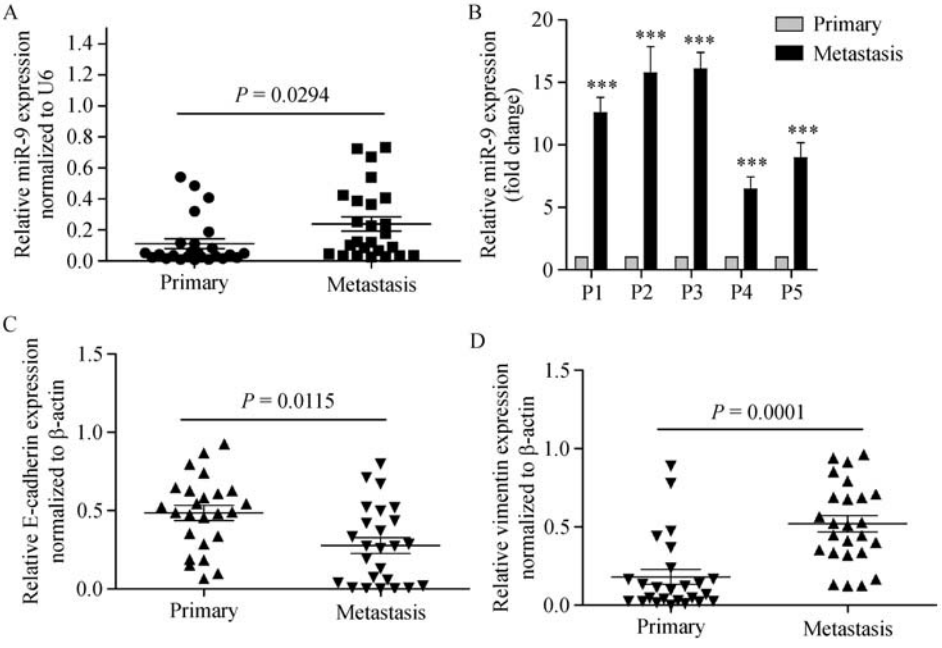

Fig. 1 miR-9 was upregulated and correlated with the expression of E-cadherin and vimentin in metastatic ovarian cancer tissue compared with primary tissue. (A) qRT-PCR shows that miR-9 was frequently upregulated in the 25 metastatic site tissue samples compared with their paired primary tissue ($P < 0.05$). (B) Upregulated miR-9 was also detected in five paired patient-derived serous ovarian tumor cell lines. U6 was used as an endogenous control. (C and D) The expression of E-cadherin and vimentin was detected by qRT-PCR at 25 metastatic sites tissue samples compared with their paired primary tissue. The relative expression was normalized to β-actin.

showed that the luciferase activity was significantly reduced in the wild-type E-cadherin 3′-UTR but not the mutant type (Fig. 2F and 2G). These results demonstrated that E-cadherin was directly targeted by miR-9.

miR-9 promotes ovarian cancer cell migration and invasion

SKOV3 and A2780 cells were transfected with miR-9 mimics for 24 h to elucidate the function of miR-9 in cancer cell metastasis. The cells became scattered and displayed a spindle-like or fibroblast morphology, but these effects were not observed when the cells were transfected with miR-9 inhibitor (Fig. 3A and 3B). These phenomena implied that miR-9 can promote cell motility. Wound healing assay showed that the ability of migration in SKOV3 cells was enhanced after miR-9 mimic transfection for 24 h (Fig. 3C). Then, we used Transwell assay to evaluate the migration and invasion capacities of the SKOV3 cells. Fig. 3D shows that miR-9 mimics could significantly enhance the migration and invasion of ovarian cancer cells. By contrast, the migration and invasion ability of SKOV3 cells were decreased when transfected with miR-9 inhibitor. Migration and invasion assay was also conducted in A2780 cells (data not shown). These results revealed that miR-9 may promote ovarian cancer cell motility.

miR-9 regulates EMT-related genes to promote tumor cell metastasis

SKOV3 and A2780 cells were transfected with miR-9 mimics for 36 h to further confirm whether miR-9 could modulate the EMT-related genes to enhance ovarian cancer metastasis. Then, qRT-PCR showed that E-cadherin expression was downregulated, while mesenchymal markers, such as N-cadherin, vimentin, β-catenin, and MMP-9, were inversely upregulated (Fig. 4A and 4B). Furthermore, IF was detected to explore the molecular mechanism and subcellular localization of miR-9-E-cadherin axis in promoting metastasis. IF results indicated that the intensity of E-cadherin was reduced while the intensity of N-cadherin or vimentin was increased after miR-9 mimics were transfected in A2780 or SKOV3 cells for 48 h, respectively (Fig. 4C and 4D). EMT is a vital molecular change in tumor mobility and metastasis [22,23]. These results demonstrated that miR-9 may regulate EMT-related genes to promote ovarian cancer cell metastasis.

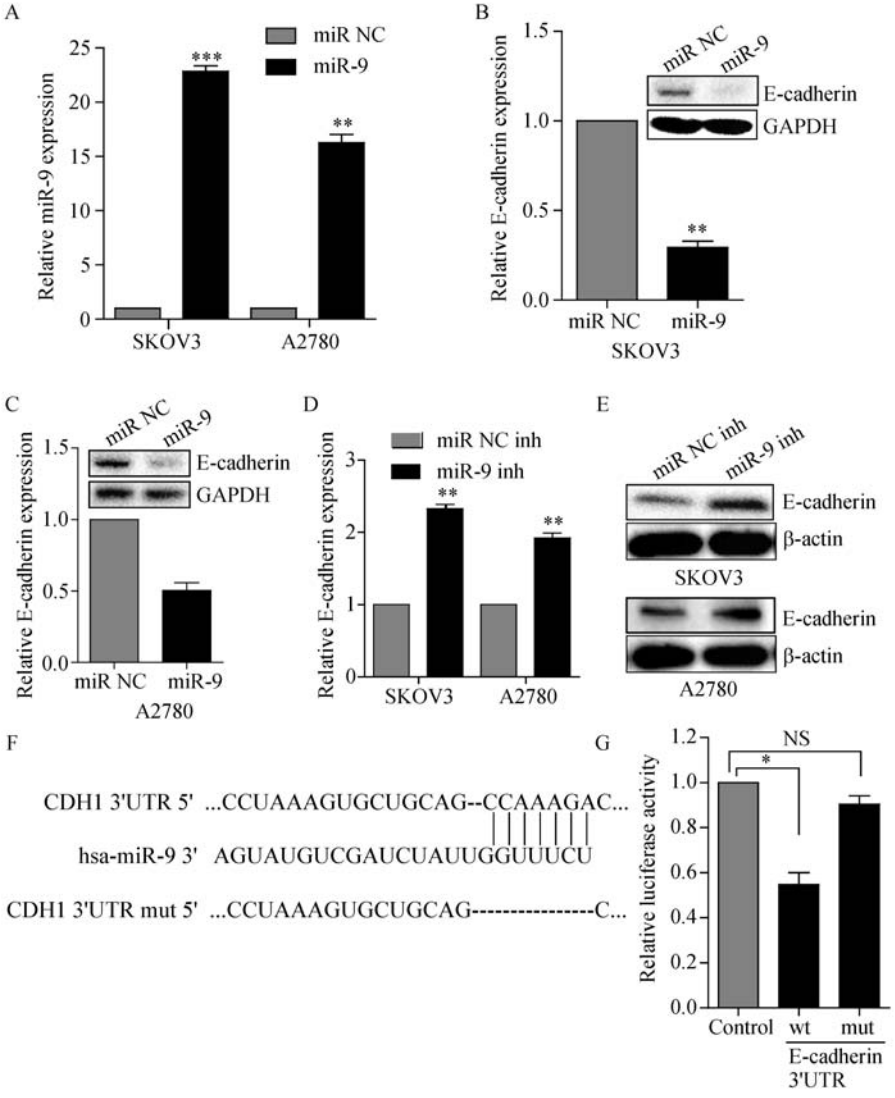

Fig. 2 miR-9 directly targets E-cadherin in ovarian cancer cells. (A) Upregulated miR-9 was detected by qRT-PCR using miR-9 mimics in SKOV3 and A2780 cells. (B and C) The relative E-cadherin expression was downregulated after miR-9 mimics transfection for 36 or 48 h as detected by qRT-PCR or Western blot, respectively. (D and E) Relative E-cadherin expression was upregulated after miR-9 inhibitor transfection for 36 or 48 h as detected by qRT-PCR or Western blot, respectively. (F) Potential binding sites of miR-9 in the 3′-UTR of E-cadherin. (G) Plasmid containing wild-type or mutant-type 3′-UTR of E-cadherin was co-transfected in SKOV3 cells with miR-9 mimics. Dual-luciferase reporter assay showed that transfected miR-9 could reduce the luciferase activity in wild-type 3′-UTR (wt-3′-UTR) of E-cadherin but not in mutant-type 3′ UTR (mut-3′ UTR) of E-cadherin. Data are expressed as mean ± SD of three independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. NS represents no significance.

Discussion

Epithelial ovarian cancer is a lethal and highly metastatic disease with the highest mortality and morbidity in all gynecologic cancers in the developed world with approximately 14 030 deaths from the disease in 2013 [1]. The majority of epithelial ovarian cancer patients will eventually relapse at metastatic sites. EMT plays a key role in the metastasis of tumor cells, and this process possesses a multi-faceted transdifferentiation program that enables tumor cells to acquire malignancy-associated phenotypes [16]. Numerous studies have indicated that miRNAs, which are important gene regulators acting as oncomiRs or anti-oncomiRs, are increasingly involved in regulating the malignant tumor progression. EMT was also found to be regulated by metastamiRs [24], which exert important roles in various processes of metastasis. In this study, miR-9 was identified to be a metastamiR in ovarian serous cancer. miR-9 is selectively expressed in neural tissue under normal conditions and exhibits a regulator and

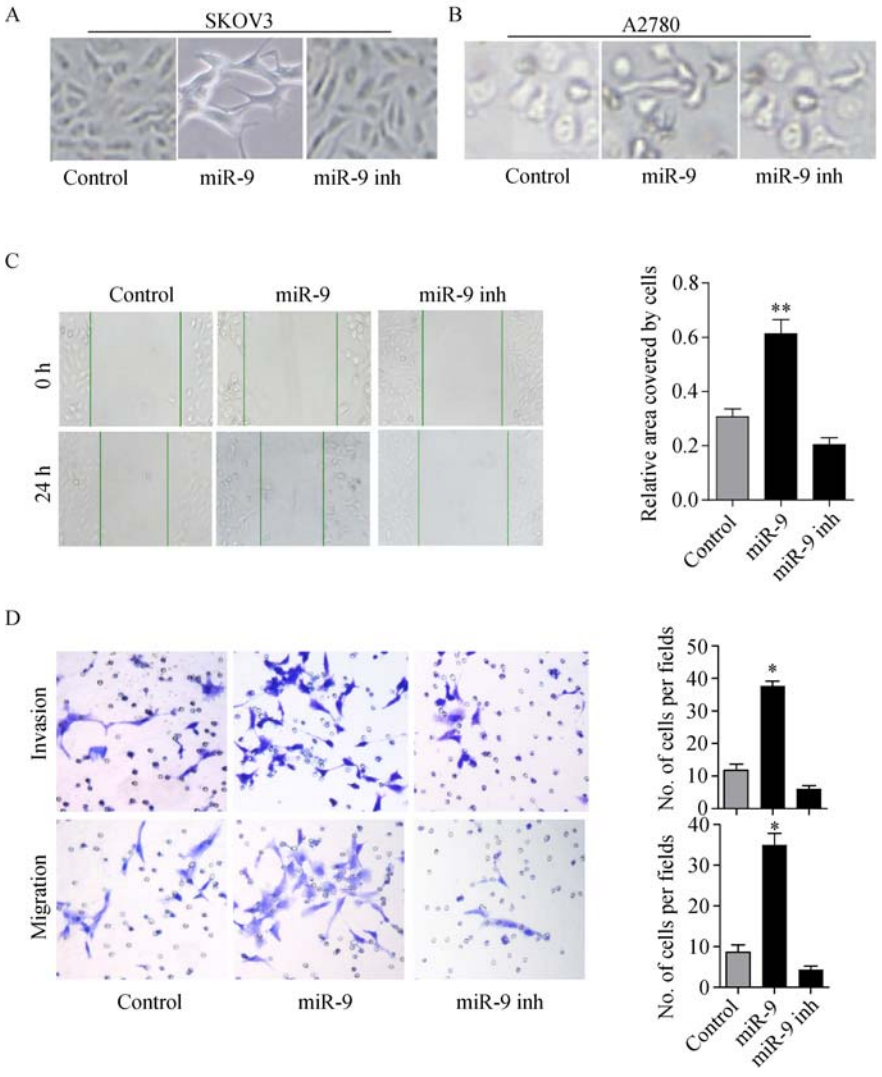

Fig. 3 miR-9 promotes ovarian cancer cell metastasis. (A and B) Phase contrast images of SKOV3 and A2780 cells transfected with miR-9 mimics or inhibitor. Images showed that these cells become scattered and displayed spindle-like or fibroblast morphology. Transfected miR-9 inhibitor could reverse this morphology. (C) Wound healing assay showed that transfected miR-9 mimics promoted SKOV3 cell migration compared with transfection with miR-9 inhibitor or miR-9 NC. Images were taken at 0 and 24 h after wound application. (D) Images and summary of SKOV3 cell invasion and migration assay. Transfected miR-9 mimics enhanced the invasion and migration capabilities of SKOV3 cells compared with those of transfected miR-9 NC cells, which could be rescued by transfection of miR-9 inhibitor. Data are expressed as mean ± SD of three independent experiments. $*P < 0.05$, $**P < 0.01$.

prodifferentiation function [25]. miR-9 was first found to be upregulated in brain tumors than in tumors of other histological types [26]. The enhancement of metastasis of miR-9 overexpression has also been found in hepatocellular carcinoma [10], head and neck squamous cell carcinoma [27], colon cancer [28], esophageal squamous cell carcinoma [9], breast cancer [8,29], and cervical cancer [30]. This result suggests the role of a promoter or onco-miR in tumor development and progression. However, miR-9 is also downregulated in gastric cancer [31], pancreatic cancer [32], and ovarian cancer [14], indicating that a role of anti-miR in tumor progression. Interestingly, Ma *et al.* reported that miR-9 acted as a metastamiR to promote breast cancer metastasis [8], while Lwhmann *et al.* noted that miR-9 was also transcriptionally downregulated in early breast cancer development [33]. Moreover, in gastric tumor, Inoue *et al.* reported that miR-9 was upregulated [34], while Zheng *et al.* indicated that miR-9 functioned as a suppresser miRNA and was downregulated in gastric tumor progression [31]. The heterogeneity of these tumors or the limited specimens may have caused the highly diverse miR-9 expression even in similar tumors. Whether miR-9 functions as a promoter or suppressor in the progression of various tumors remains obscure. A

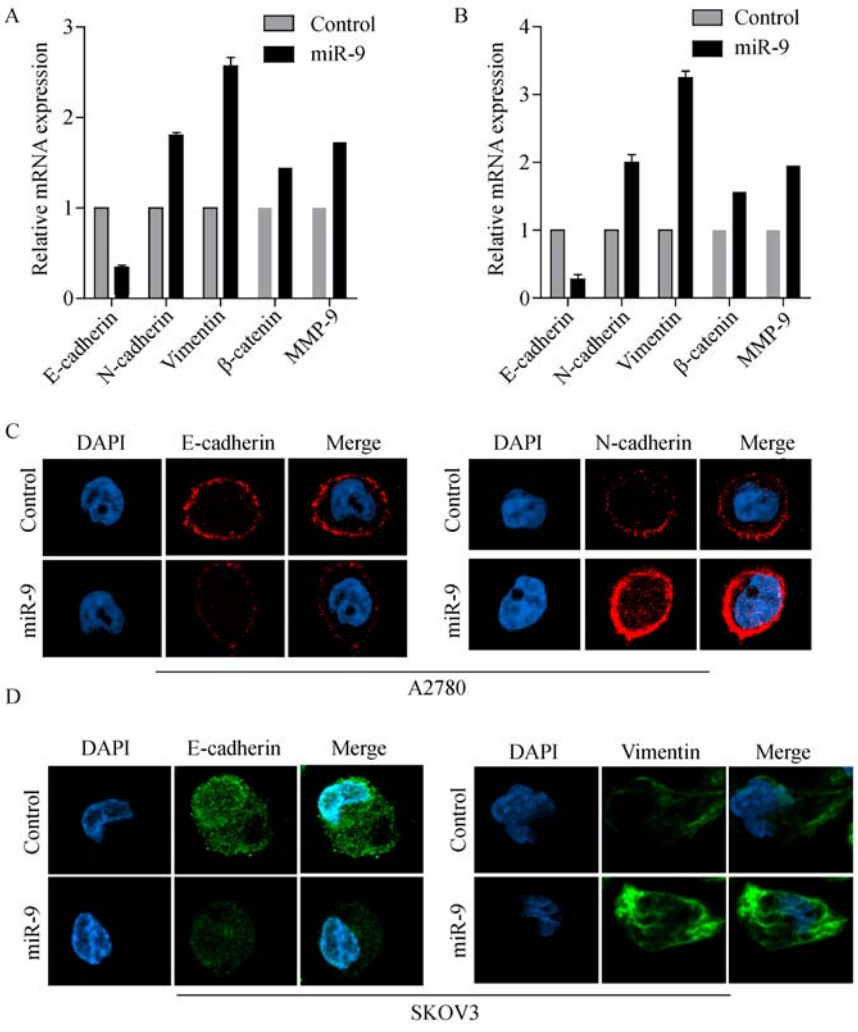

Fig. 4 miR-9 induces ovarian cancer cells epithelial−mesenchymal transition (EMT). (A and B) A2780 and SKOV3 cells were transfected with miR-9 mimics. The data showed that E-cadherin was downregulated, but mesenchymal markers were upregulated, respectively. (C) IF showed that the intensity of E-cadherin was decreased, while the intensity of N-cadherin was increased after miR-9 mimic transfection for 48 h in A2780 cells. (D) IF showed that the intensity of E-cadherin was decreased, while the intensity of vimentin was increased after miR-9 mimic transfection for 48 h in SKOV3 cells.

previous study has demonstrated that TGF-β upregulation of miR-182 expression may promote gallbladder cancer metastasis by targeting CADM1 [35]. Another study revealed that miR-182 may potentiate TGF-β-induced EMT and metastasis of cancer cells by directly targeting SMAD7 [36]. TGF-β signaling pathway plays vital roles in EMT and metastasis of cancer cells. However, the detailed mechanism of miR-9 in promoting the metastasis of the ovarian cancer cell should be further illuminated.

In this study, we demonstrated that the average level of miR-9 was significantly upregulated in 25 metastatic sites of ovarian cancer patients compared with paired primary sites. Furthermore, we also confirmed that miR-9 expression was negatively associated with E-cadherin expression but positively correlated with vimentin expression. Functional study indicated that miR-9 overexpression could increase ovarian cancer cell migration and invasion, which were confirmed by wound healing and Transwell assays. Similar to other studies which used informatics analysis tools, E-cadherin was predicted to be directly targeted by miR-9, which was further confirmed through luciferase reporter assay. qRT-PCR and Western blot experiment showed that E-cadherin expression was downregulated when cells were transfected with miR-9 mimics but was upregulated when endogenous miR-9 was transiently inhibited. IF results indicated that the intensity of E-cadherin was reduced in miR-9 overexpressed ovarian cancer cells, contrary to the enhanced intensities of N-cadherin and vimentin. These results confirmed that miR-9 was involved in the regulation of ovarian cancer cell

metastasis by directly targeting E-cadherin.

Thus, a high level of miR-9 was found at the ovarian cancer metastatic sites compared with their paired primary sites. We also demonstrated that miR-9 could promote metastasis in ovarian serous cancer by directly targeting E-cadherin 3′-UTR. Manipulation of miR-9 may regulate the migration and invasion of ovarian cancer cells. Thus, miR-9 may be a potential target for the prediction and treatment of serous ovarian cancer.

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Compliance with ethics guidelines

Bo Zhou, Hongbin Xu, Meng Xia, Chaoyang Sun, Na Li, Ensong Guo, Lili Guo, Wanying Shan, Hao Lu, Yifan Wu, Yuan Li, Degui Yang, Danhui Weng, Li Meng, Junbo Hu, Ding Ma, Gang Chen, and Kezhen Li declare that they have no conflict of interest. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (the ethical committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, China) and with the *Helsinki Declaration* of 1975, as revised in 2000. Informed consent was obtained from all patients included in the study.

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