MiR-449a functions as a tumor suppressor in endometrial cancer by targeting CDC25A

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Abstract. Accumulating evidence has demonstrated that microRNAs (miRNAs) play critical roles in cancer initiation and development by functioning either as oncogenes or as tumor suppressors. The role of microRNA-449a (miR-449a) in endometrial cancer remains unclear. We examined the levels of miR-449a and miR-449b in benign endometrium, type I and type II endometrial cancer tissues by quantitative real-time polymerase chain reaction. To further investigate the roles of miR-449a in regulating the behavior of endometrial cancer cells, we overexpressed miR-449a in the endometrial cancer cell line HEC-1B, which had low endogenous miR-449a expression. We analyzed the effects of miR-449a overexpression on CDC25 expression, proliferation, invasion and apoptosis of HEC-1B cells. We found that miR-449a and miR-449b levels were markedly reduced in type II endometrial cancer tissues but not in type I endometrial cancer tissues compared with normal endometrium. Overexpression of miR-449a significantly inhibited the proliferation, invasion and clonogenic survival of HEC-1B cells. MiR-449a overexpression also induced apoptosis in HEC-1B cells. In addition, real-time RT-PCR and western blot analysis showed that CDC25A expression was suppressed by miR-449a overexpression. Our results suggest that miR-449a may act as a tumor suppressor by targeting CDC25A in endometrial cancer.

Introduction

Endometrial cancer has become the most common female genital tract tumor in some western countries (1,2) and accounts for 70-80% of all primary uterine malignancies in the United States (3,4). The incidence of endometrial cancer is continuously rising worldwide. There are mainly 2 types of endometrial cancers based on their histopathological characteristics (5); type I endometrial cancers, which account for 80% of endometrial cancers, are the endometrioid endometrial adenocarcinomas. They are generally estrogen-related, low grade and with a relatively high survival rate (~85% at 5 years). Surgery remains the cornerstone treatment for patients with type I endometrial cancer (6). Type II endometrial cancers develop mainly in elderly and post-menopausal women. They are usually hormone-independent and belong to high grade endometrioid adenocarcinomas, papillary serous and clear cell carcinomas and carcinosarcomas. Compared with type I endometrial cancer, the prognosis of type II endometrial cancer is poorer due to its high invasiveness and metastasis. In addition, 50% of all relapses occur in patients with type II endometrial cancer (7). The molecular mechanism underlying the initiation and progression of type II endometrial cancer remains unclear.

MicroRNAs (miRNAs) are a class of non-protein-coding small RNAs containing ~18-24 nucleotides. MiRNAs have been shown to regulate gene expression by complementarily binding to the 3' untranslated region (UTR) of target mRNAs, leading to the degradation of target genes or the reduction of gene translation (8). Numerous studies have shown that miRNAs are involved in the regulation of several key biological processes, including proliferation, apoptosis, differentiation and metabolism. Emerging evidence suggests that miRNA dysregulation is associated with the initiation and progression of multiple human cancers, including lymphoma, gastric, prostate and pancreatic cancer (9,10). Depending on the downstream targets, miRNAs can function either as oncogenes or as tumor suppressors (11,12).

MicroRNA-449a (MiR-449a) has been demonstrated to inhibit cancer cell proliferation in multiple cancers, such as breast, prostate, bladder, liver, gastric and lung cancer, by targeting a broad spectrum of molecules involved in cell cycle regulation and apoptosis (13-18). MiR-449a expression in these tumor tissues is reduced or lost (13-18). However, the role of miR-449a in endometrial cancer has not been clarified. In this study, we examined the expression of miR-449 in normal endometrium, type I and type II endometrial cancer tissues and further investigated the effects of exogenous overexpression of miR-449a on the behavior of endometrial cancer cells.

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Materials and methods

Patient tissue specimen. This study was approved by the Institutional Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University. All patients signed the informed consent form. The patients with endometrial cancers underwent abdominal hysterectomy in our department. Surgical tissue specimens from 2 patients with type I endometrial cancer, 2 patients with type II endometrial cancer and 2 patients undergoing uterine fibroids hysterectomy were obtained. The diagnosis for endometrial cancer was confirmed by histopathological examination. The benign endometrial tissue specimens were confirmed to be free of malignant lesions. The endometrium was dissected. The cancerous lesions from endometrium were removed and used for the determination of miR-449 levels. Small pieces of the lesions were sectioned and stained with H&E to further confirm that >90% of the cells in the lesions were cancer cells.

Immunohistochemical staining for estrogen and progesterone receptor. Endometrial tissues were sectioned and fixed in 4% PFA. After blocking overnight, tissue sections were then incubated with primary antibodies to estrogen receptor (ER; 1:100, Abcam, USA) or progesterone receptor (PR; 1:100, Abcam, USA) overnight, then washed and incubated with secondary antibody. Tissue sections stained with secondary antibody only were used as the negative control. The stained tissue section was observed under a microscope at a magnification of x100.

Determination of miR-449 level in tissue specimens. Total RNA was extracted from tissues by the TRIzol method and 100 ng total RNA was used for reverse transcription (RT). The primer sequences for reverse transcription of miR-449a and miR-449b are listed in Table I. The total volume of the RT reaction was 20 μl. The RT conditions were: incubation at 16˚C for 10 min, 37˚C for 30 min and 65˚C for 5 min. The cDNA products were used as the templates for quantitative PCR. The primer sequences for PCR are shown in Table I. U6 was used as the housekeeping gene. The forward primer for U6 was: 5'-CTCGCTTCGGCAGCACA-3'; the reverse primer for U6 was: 5'-AACGCTTCACGAATTTGCGT-3'. The volume of PCR reaction was 20 μl. The PCR conditions were: incubation at 95˚C for 10 min, 95˚C for 15 sec, 60˚C for 1 min, 40 cycles. The PCR instrument was ABI 7900 HT Fast Real-Time PCR system. Cycle threshold of each sample was determined.

Cell culture. The endometrial cancer cell line (HEC-1B) was purchased from Shanghai Institute of Cell Biology (Shanghai, China). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) in a humidified incubator at 37˚C with 5% CO_2.

miRNA transfection. The sense sequence of hsa-miR-449a mimics was: 5'-UGGCAGUGUAUUGUUAGCUGGU3' and the antisense sequence was: 5'-CAGCUAACAAUACACUGC CAUU-3'. The sequences for miRNA negative control (NC) were: 5'-UUCUCCGAACGUGUCACGUTT-3' for sense and 5'-ACGUGACACGUUCGGAGAATT-3' for antisense. All the RNA oligonucleotides were purchased from GenePharma (Shanghai, China). HEC-1B cells were seeded into 6-well plates at a density of $3x10^5$ cells/well or into 96-well plates at a density of $2.5x10^3$ cells/well 24 h before transfection. After reaching 30-50% confluence, the cells were transfected with miRNA duplexes using Lipofectamine 2000 (Invitrogen, Shanghai) at a final concentration of 50 nM. The hsa-miR-449a mimics labeled with 6-FAM at the 5'- end were used to determine the transfection efficiency.

Cell proliferation assay. HEC-1B cells were seeded into a 96-well plate at a density of $2.5x10^3$ cells/well and transfected the next day. At 48 h after transfection, cell proliferation was analyzed using the Cell Counting Kit-8 (CCK-8) assay (Beyotime, China). Briefly, 10 μl CCK-8 solution was added to each well and incubated at 37˚C for 1-2 h. The absorbance at 450 nm was measured using a microplate reader.

Colony formation assay. HEC-1B cells were harvested 24 h after transfection, re-suspended in DMEM supplemented with 10% FBS and seeded in 6-well plates at a density of 800 cells/well. The cells were cultured under standard culture conditions for 10 days. The cells were then fixed with methanol for 15 min and stained with crystal violet for 10-30 min.

Determination of apoptosis by flow cytometry. HEC-1B cells were harvested 48 h after transfection, washed twice with cold phosphate-buffered saline (PBS) and re-suspended in 100 μl Annexin-binding buffer at the concentration of $2x10^5$-$1x10^6$cells/ml. Annexin V and PI double-staining was performed according to the manufacturer's protocol. The Annexin V-FITC apoptosis detection kit was purchased from Invitrogen. Cell apoptosis was analyzed within 1 h after staining using the flow cytometer system with FACSDiva software (FACSCalibur).

Transwell invasion assay. HEC-1B cells were harvested 24 h after transfection and re-suspended in serum-free DMEM medium at the concentration of $1.5x10^5$/ml. A total of 200 μl cell suspension was added into the Matrigel-coated Transwell chambers (BD Biosciences, USA) and 500 μl DMEM supplemented with 10% FBS was added into the lower chamber of each well. The number of penetrating cells was determined after 24 h incubation.

RNA extraction and real-time quantitative PCR (qPCR). Total RNA was extracted from HEC-1B cells 48 h after transfection using TRIzol reagent (Invitrogen, Shanghai) according to the manufacturer's instructions. Briefly, 500 ng of total RNA containing miRNA was polyadenylated by poly(A) polymerase and then reverse transcribed to cDNA using miRNA First-Strand cDNA Synthesis and qRT-PCR kits (Invitrogen). The cDNA was then used as the template for SYBR real-time PCR using SYBR-Green PCR Master mix (Toyobo, Osaka, Japan). The hsa-miR-449a specific qPCR reverse primer was provided by the cDNA Synthesis and qRT-PCR kits. The specific forward primer was designed for qPCR according to the manufacturer's protocol. Human U6 was used as the housekeeping control. For CDC25A mRNA qPCR, cDNA was synthesized by using qPCR RT kit (Toyobo) and human

Table I. RT and PCR primer sequences for miR-449a and miR-449b.

	miR-449a	miR-449b
Mature sequence	TGGCAGTGTATTGTTAGCTGGT	AGGCAGTGTATTGTTAGCTGGC
Stem-loop	GTCGTATCCAGTGCAGGGTCCG	GTCGTATCCAGTGCAGGGTCCG
RT primer	AGGTATTCGCACTGGATACGACACCAGC	AGGTATTCGCACTGGATACGACGCCAGC
Universal sense primer	GTGCAGGGTCCGAGGT	GTGCAGGGTCCGAGGT
Specific antisense primer	CGCTGGCAGTGTATTGTTAGCT	TGGGAGGCAGTGTATTGTTAGC

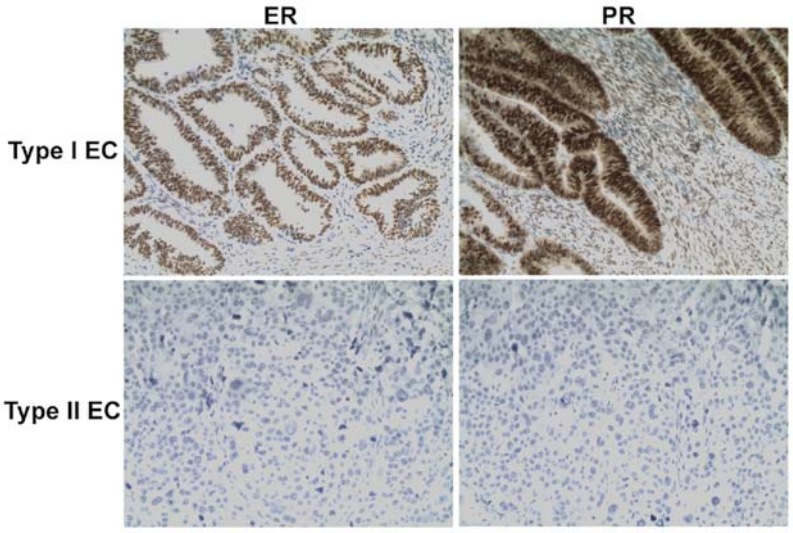

Figure 1. Immunohistochemical staining for ER and PR. Paraffin-embedded endometrial tissue specimens were sectioned and fixed. The tissue sections were then stained with anti-ER (1:100, Abcam, USA) and anti-PR antibodies (1:100, Abcam). The tissue sections were observed by microscopy.

GAPDH was used as housekeeping gene for normalization. The sequence of the forward primer for hsa-miR-449a was: 5'-TGG CAG TGT ATT GTT AGC TGG T-3', and for U6 was: 5'-CGC AAG GAT GAC ACG CAA ATT C-3'. The sequence of the forward primer for CDC25A mRNA was: 5'-TAC CTA CTG ATG GCA AGC GTG T-3', and the sequence for the reverse primer was: 5'-GAC TGG CAT TTC ATA AAG AAC TCC T-3'. The sequence of GAPDH forward primer was: 5'-AGA AGG CTG GGG CTC ATT TG-3', and the sequence of GAPDH reverse primer was: 5'-AGG GGC CAT CCA CAG TCT TC-3'. The qPCR was performed on the Applied Biosystems 7500 detection system. The 10 μl PCR system contained 1 μl template (diluted 1:10), 5 μl SYBR-Green I Master Mix, 1 μl specific forward primer of miR-449a/CDC25A mRNA (1 μM), 1 μl reverse primer (1 μM), 1 μl plus solution and 1 μl RNase and DNase free water. The PCR reaction conditions were: incubation at 95˚C for 3 min, 95˚C for 15 sec, 60˚C for 1 min and 40 cycles. Cycle threshold (Ct) values for all samples were determined. The $\Delta\Delta$Ct method was used to compare the expression of miR-449a and CDC25A mRNA in each group.

Western blot analysis. Forty-eight hours after transfection, total protein was extracted using a cell lysing buffer containing 1 mol/l Tris-HCl (PH 6.8), glycerin, SDS, DTT (Sangon Biotech, China) and ddH_2O. Fifty-microgram of protein was loaded in the gel. After transfer, the blot was probed with CDC25A (1:500) and GAPDH (1:1000) antibody (Abcam, USA) and visualized by the secondary antibodies.

Statistical analysis. Statistical analysis of all data was performed using the software SPSS 17.0 (SPSS, Inc.). The differences between groups were analyzed using the Student's t-test when two groups were analyzed or assessed by one-way analysis of variance (ANOVA) when three or more groups were compared. All test results were two-sided and the significance level was set at $P<0.05$. The data are presented as the mean ± standard error (SE).

Results

The levels of miR-449a and miR-449b are markedly reduced in type II endometrial cancer tissues. Immunohistochemical staining for ER and PR showed that the type I endometrial cancer tissues were ER^+ and PR^+ and the type II endometrial cancer tissues were ER^- and PR^- (Fig. 1). Our RT-PCR results demonstrated that miR-449a and miR-449b levels in type II endometrial cancer tissues were markedly reduced by 288 and 424-fold, respectively, compared with benign endometrial tissues and type I endometrial cancer tissues (Table II). Type I endometrial cancer tissues had similar levels of miR-449a and miR-449b as the benign tissues.

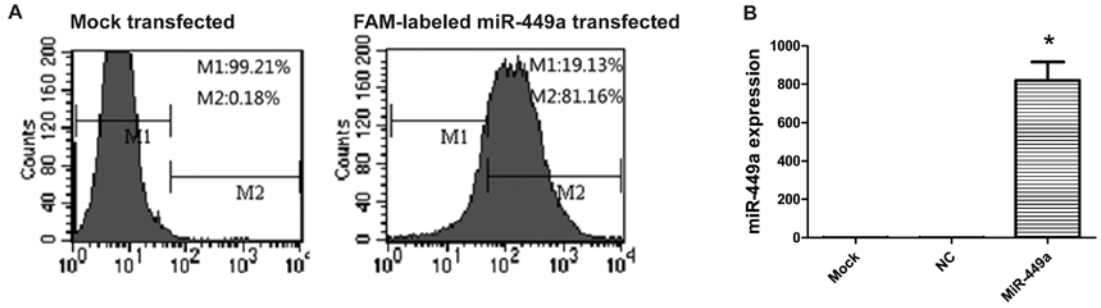

Figure 2. Overexpression of miR-449a in HEC-1B cells. (A) The transfection efficiency of FAM-labeled miR-449a into HEC-1B cells was 88.7%. Cells were harvested 24 h after transfection and analyzed on a flow cytometer. (B) The level of miR-449a was significantly increased in miR-449a-transfected cells. Total RNA was extracted from miR-449a, NC, or mock-transfected cells 48 h after transfection. The level of miR-449a was determined by RT-PCR. The fold-change of miR-449a level was determined by the $2^{-\Delta\Delta Ct}$ method. The data are presented as mean ± SE (n>3). *P<0.01, miR-449a transfected cells vs. mock or NC-transfected cells.

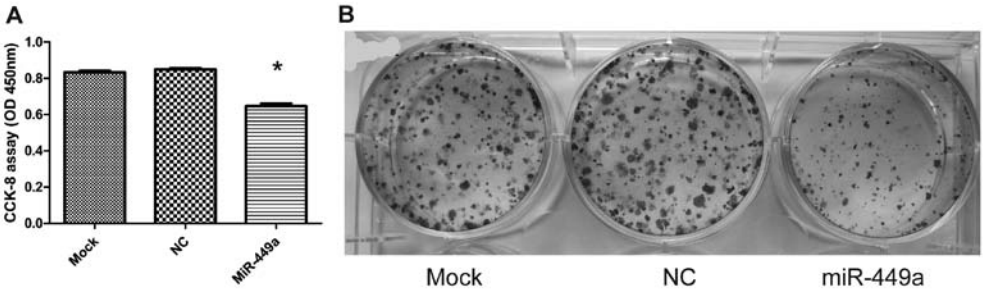

Figure 3. Overexpression of miR-449a significantly inhibits HEC-1B cell proliferation. (A) Overexpression of miR-449a significantly inhibited HEC-1B cell proliferation. Cell proliferation was analyzed by CCK-8 according to the manual of the CCK-8 kit 48 h after transfection. The data are presented as mean ± SE (n>3). *P<0.01, miR-449a-transfected cells vs. mock or NC-transfected cells. (B) Overexpression of miR-449a significantly suppressed clonogenicity of HEC-1B cells. HEC-1B cells were seeded in 6-well plates at a density of 800 cells/well. The cells were cultured for 10 day and stained with crystal violet for 10-30 min.

Table II. Cycle threshold of RT-PCR for miR-449a and miR-449b amplification.

Sample type	miR-449a	miR-449b	U6
Benign	21.97±0.59	21.80±0.53	18.73±0.50
Type I endometrial cancer	22.17±0.90	22.15±0.70	19.20±0.36
Type II endometrial cancer	30.39±0.03	30.78±0.30	18.98±0.77

Overexpression of miR-449a in HEC-1B cells inhibits cell proliferation and invasion. To further investigate the role of miR-449a in regulating the behavior of endometrial cancer cells, we overexpressed miR-449a in HEC-1B cells. We first determined the miR-449a transfection efficiency. Flow cytometry showed 88.7% of cells carrying FAM-labeled miR-449a 24 h after transfection (Fig. 2A). The expression of miR-449a was further confirmed by RT-PCR. The level of miR-449a increased significantly in cells transfected with miR-449a compared with NC or mock-transfected cells (Fig. 2B). The endogenous miR-449a level in HEC-1B cells was very low. Transfection of miR-449a reduced cell proliferation significantly (0.834±0.016 or 0.850±0.015 for mock or NC transfection vs. 0.648±0.028 for miR-449a transfection, P<0.01; Fig. 3A). To further confirm the inhibitory effect of miR-449a on HEC-1B cell proliferation, we performed colony formation assay. Consistently, our results showed that overexpression of miR-449a suppressed clonogenicity of HEC-1B cells (Fig. 3B). In addition to inhibiting cell proliferation, overexpression of miR-449a also significantly reduced HEC-1B cell invasion. The Transwell invasion assay revealed that the invasion of miR-449a-transfected cells was markedly reduced compared to the mock or NC-transfected-cells (P<0.01; Fig. 4A and B).

Overexpression of miR-449a induces apoptosis and suppresses CDC25A expression in HEC-1B cells. We also examined whether overexpression of miR-449a affected apoptosis in HEC-1B cells. Our results showed that the population of apoptotic cells in miR-449a-transfected HEC-1B cells was markedly increased compared with that in mock or NC-transfected cells (P<0.01; Fig. 5A and B). CDC25A is a member of the CDC25 family of phosphatases and is known to play a critical role in regulating cell cycle. It has been demonstrated that CDC25A is one of the targets of miR-449a in breast and bladder cancer (13,15). Thus, we tested if CDC25A was also affected by miR-449a in HEC-1B cells. Indeed, the RT-PCR and western blotting assay showed that both mRNA and protein levels were significantly down-regulated in miR-449a-transfected cells compared to NC or mock-transfected cells (P<0.05; Fig. 6A and B).

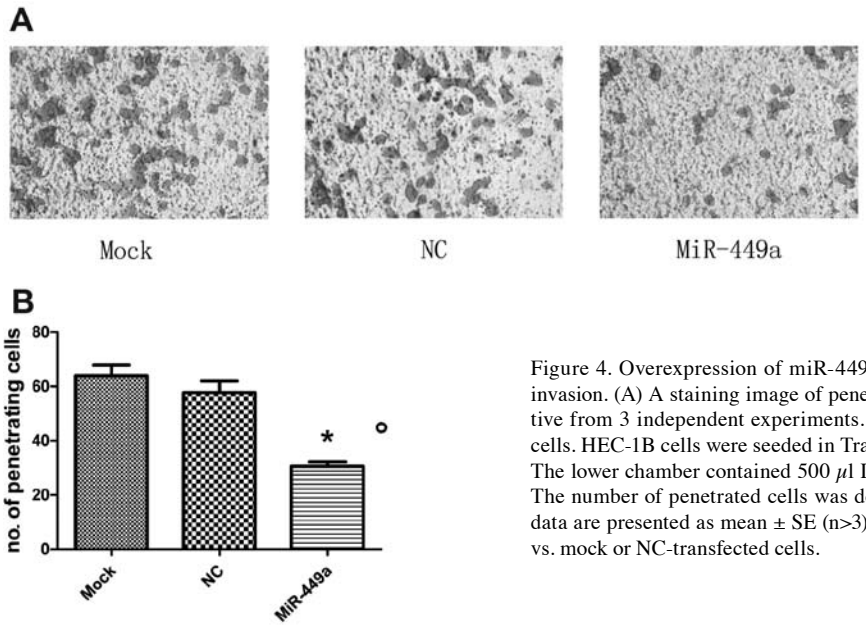

Figure 4. Overexpression of miR-449a significantly inhibits HEC-1B cell invasion. (A) A staining image of penetrated cells. The image is representative from 3 independent experiments. (B) The quantification of penetrated cells. HEC-1B cells were seeded in Transwell chamber in serum-free media. The lower chamber contained 500 μl DMEM supplemented with 10% FBS. The number of penetrated cells was determined 24 h after incubation. The data are presented as mean ± SE (n>3). *P<0.01, miR-449a-transfected cells vs. mock or NC-transfected cells.

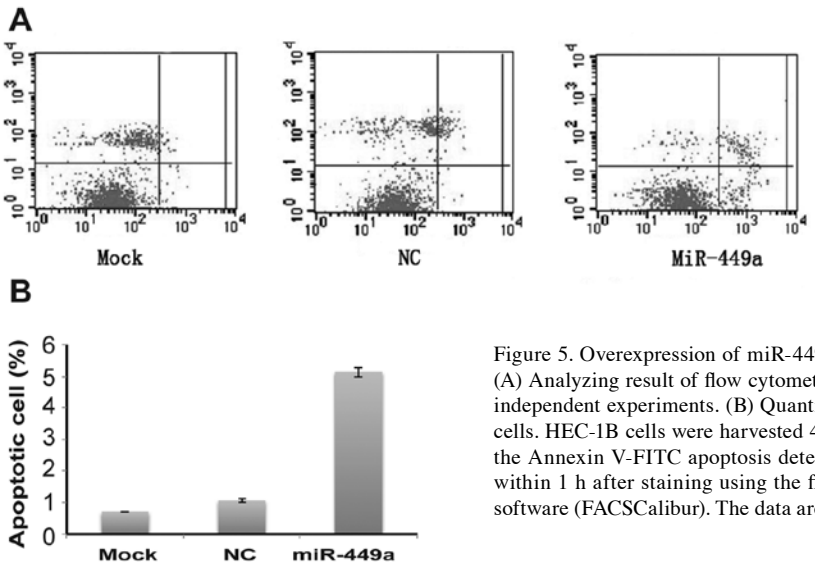

Figure 5. Overexpression of miR-449a induces apoptosis in HEC-1B cells. (A) Analyzing result of flow cytometry. The image is representative from 3 independent experiments. (B) Quantification of the population of apoptotic cells. HEC-1B cells were harvested 48 h after transfection and stained with the Annexin V-FITC apoptosis detection kit. Cell apoptosis was analyzed within 1 h after staining using the flow cytometer system with FACSDiva software (FACSCalibur). The data are presented as mean ± SE (n>3).

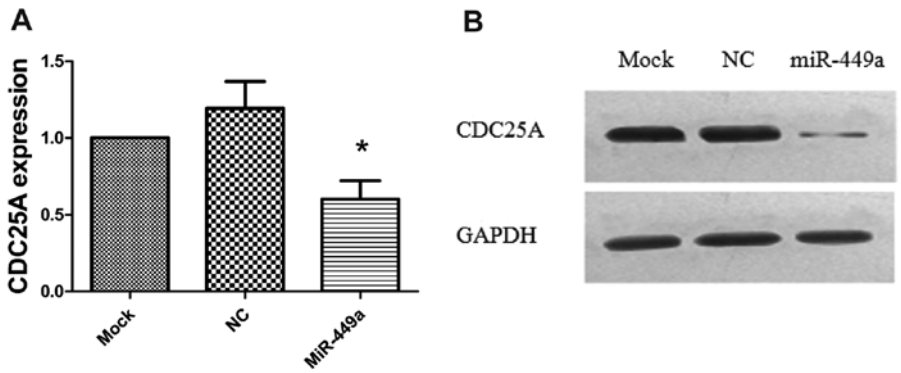

Figure 6. Overexpression of miR-449a reduces CDC25A expression in HEC-1B cells. (A) mRNA level of CDC25A was reduced in miR-449a-transfected cells. Total RNA was extracted from miR-449a, NC, or mock-transfected cells 48 h after transfection. The fold change of CDC25 mRNA level was determined by the $2^{\Delta\Delta Ct}$ method. GAPDH was used as the housekeeping gene. The data are presented as mean ± SE (n>3). *P<0.01, miR-449a-transfected cells vs. mock or NC-transfected cells. (B) Protein level of CDC25A was reduced in miR-449a-transfected cells. Forty-eight hours after transfection, total protein was extracted. Fifty-microgram protein was loaded in gel. After transfer, the blot was probed with anti-CDC25A and anti-GAPDH antibody. The image is representative from 3 independent experiments.

Discussion

MiRNAs are considered new candidate therapeutic agents for endometrial cancer owing to their involvement in cancer initiation and progression (19). Boren *et al* demonstrated that the miRNA expression profile in endometrial cancer patients exhibits unique characteristics compared with that of healthy individuals (20). They found that 12 miRNAs are associated with endometrial cancer development, including 5 upregulated (let-7i, miR-221, miR-193, miR-152 and miR-30c) and 7 downregulated miRNAs (miR-185, miR-106a, miR-181a, miR-210, miR-103, miR-107 and let-7c) (20). In addition, miR-152 and miR-34b have also been demonstrated to play important roles in the development of endometrial carcinoma (21,22). In our study, we found that the expression of miR-449a and miR-449b was markedly reduced in type II endometrial cancer tissues but not in type I endometrial cancer tissues compared with normal endometrium, suggesting that miR-449 may be involved in the development of type II endometrial cancer. This result also indicates that different molecular mechanisms drive the initiation and progression of type I and type II endometrial cancer, resulting in distinct clinical characteristics of the two types of endometrial cancer. We also found that overexpression of miR-449a in endometrial cancer cells significantly reduced cell proliferation and invasion and induced apoptosis, suggesting that miR-449a may act as a tumor suppressor in endometrial cancer.

Our findings are consistent with studies on miR-449a in other types of malignancy, such as breast, prostate, bladder, gastric, liver and lung cancer (13-18). Yang *et al* reported that miR-449a/b expression leads to pRB dephosphorylation and cell cycle arrest at G1 phase in breast cancer cells by inhibiting oncogenic CDK6 and CDC25A (13). Noonan *et al* found that the expression of miR-449 in prostate cancer tissue is downregulated compared to patient-matched control tissue and overexpression of miR-449a in prostate cancer PC-3 cells results in cell cycle arrest, apoptosis and a senescence-like phenotype (14). Downregulation of miR-449a is also detected in human bladder cancer tissue (15). Exogenous miR-449a can significantly suppress the growth of bladder cancer cell T24 xenografts (15). In their study on gastric cancer, Bou Kheir *et al* found that miR-449 expression is reduced in mouse gastric cancer tissue and overexpression of miR-449 in gastric cancer cell line markedly inhibits cell growth and induces apoptosis. They further identified GMNN, MET, CCNE2, SIRT1 and CDK6 as the targets of miR-449 (17). Similarly, overexpression of miR-449 also inhibits hepatocellular carcinoma cell proliferation and induces apoptosis (16). In lung cancer, Ren *et al* showed that the level of miR-449a is significantly reduced in human lung cancer tissues and the low level of miR-449a correlates with cancer recurrence and survival of lung cancer patients (18). Consistent with other types of cancer, transient introduction of miR-449a causes cell cycle arrest and senescence in lung cancer cell line A549 and 95D (18).

The molecular mechanism underlying the miR-449a-mediated inhibition of cancer growth remains inconclusive. Several molecules appear to be the key targets of miR-449a. Our results demonstrated that CDC25A is a target of miR-449a in endometrial cancer. Similarly, CDC25A has also been shown to be a target of miR-449a in breast and bladder cancer cells (13,15). CDC25 is thought to be oncogenic and its expression is upregulated in various types of tumor tissues, including breast, ovarian, prostate, lung and endometrial cancer (23). CDC25 regulates cell cycle through the ATR/ATM-Chk1/Chk2-CDC25A-CDK2 signaling pathway (24). MiR-449a may inhibit cancer growth by blocking the expression and activity of the oncogene CDC25A. The loss of miR-449a leads to the upregulation of CDC25A, consequently resulting in malignant transformation. In addition to CDC25A, CD6, MET, p53 and p21 are also regulated by miR-449a (13-18).

In conclusion, we demonstrated that the levels of miR-449a and miR-449b in type II endometrial cancer tissues were markedly reduced compared to normal endometrium or type I endometrial cancer tissues and overexpression of miR-449a in endometrial cancer cells markedly reduced cell proliferation and invasion, induced apoptosis and suppressed CDC25A expression. Our findings suggest that miR-449a may act as a tumor suppressor in endometrial cancer and miR-449a may be a potential therapeutic agent for endometrial cancer.

Acknowledgements

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