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Original Article

miR-135a acts as a tumor suppressor by targeting ASPH in endometrial cancer

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Abstract: Endometrial cancer (EC) ranks as the fourth most commonly diagnosed cancer type in women worldwide. MicroRNAs (miRNAs) are important regulators with crucial roles in regulating diverse biologic processes, including tumor initiation and progression. Previous studies have demonstrated that miR-135a was correlated with tumorigenesis in various cancers. However, its expression and biologic role in EC remained to be determined. This study aimed to clarify whether miR-135a acts as a tumor suppressor in EC by regulating the expression of aspartate-β-hydroxylase (ASPH). Expression of miR-135a was measured by qRT-PCR and the results demonstrated that miR-135a was downregulated in EC cell lines compared to a normal cell line. Cell counting kit-8 (CCK-8) and wound-healing assays demonstrated that overexpression of miR-135a significantly inhibited cell proliferation and migration. Online prediction algorithm and dual luciferase activity reporter assay revealed that ASPH acts as a direct target of miR-135a. ASPH expression was downregulated in EC cell lines when miR-135a was overexpressed. Collectively, our results indicate that miR-135a targets ASPH to inhibit EC cell proliferation and migration, suggesting a tumor suppressive role of miR-135a in EC.

Keywords: miR-135a, ASPH, endometrial cancer, proliferation, migration

Introduction

Endometrial cancer (EC) is the most commonly diagnosed gynecologic cancer and is estimated to result in about 45,000 deaths worldwide per year [1, 2]. The improvement in understanding of molecular mechanisms related to EC progression has helped us to develop molecular targeted drugs for potential clinical use with the aim to extend the overall survival of EC patients [3, 4]. Therefore, increased understanding of the mechanisms related to EC development and progression is essential for the development of prognostic and therapeutic targets for EC.

microRNAs (miRNAs) were identified in 1993 and their biologic roles regulating almost allcell behaviors including differentiation, apoptosis, and proliferation have become evident [5, 6]. The development and progression of tumor is accompanied by abnormal cell behaviors [7]. Therefore, studies have been conducted to identify miRNAs as potential biomarkers for the progression of tumors including EC [8]. Chung et al. investigated miRNA expression in EC cells and a normal cell line and found up to thirty miRNAs were upregulated in EC [9]. Ma et al. found that miR-302a-5p/367-3p-mediated high mobility group AT-hook 2 (HMGA2) expression regulated the malignant behavior of EC cells and revealed the miR-302a-5p/367-3p-HMGA2 axis may be a predictive biomarker for EC metastasis and overall survival [10].

miR-135a is a miRNA found to be downregulated in several human cancers. For instance, miR-135a was found decreased in gastric cancer and correlated with poor overall survival of gastric cancer patients [11]. In addition, overexpression of miR-135a could suppress gastric cancer cell growth by targeting TRAF5 [11]. Furthermore, miR-135a was able to induce prostate cancer cell apoptosis by targeting STAT6 [12]. However, it is uncertain whether miR-135a has a role in the development and progression of EC.

Aspartate β-hydroxylase (ASPH) is a type II transmembrane protein that belongs to the α-ketoglutarate-dependent dioxygenase family [13]. Recent studies have demonstrated that ASPH plays crucial roles in human cancer. For instance, ASPH was found overexpressed in pancreatic cancer and involved in the proliferation and metastasis of pancreatic cancer patients, implicating a potential to validate ASPH as a therapeutic target [14]. In addition, ASPH also could alter hepatocellular carcinoma cell proliferation, colony formation, and tumor cell senescence by targeting glycogen synthase kinase 3β and p16 expression [15]. Futhermore, ASPH was regulated by miR-200a to promote hepatoma cell malignancy by targeting ERK and PI3K/Akt pathways [16].

In our study, we aimed to investigate whether miR-135a may regulate the expression of ASPH in EC. We found that miR-135a was downregulated, while ASPH was upregulated in EC cell lines. Importantly, we found miR-135a could negatively regulate ASPH expression in EC. Further, luciferase activity reporter assay and a series of *in vitro* functional assays including western blot, cell proliferation assay, and cell migration assay demonstrated ASPH was a direct target of miR-135a.

Materials and methods

Cell culture and transfection

We purchased three human EC cell lines: HEC-1B, KIe, and AN3CA and one normal cell line: HEC-251 from the Shanghai Institute of Cell Biology of Chinese Academy of Sciences (Shanghai, China). Dulbecco's Modified Eagle's Medium (DMEM) (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, Inc.) was used to culture the cell lines at a humidified atmosphere incubator containing 5% CO_2 at 37°C. miR-135a mimic and corresponding negative control (NC) were purchased from GenePharma (Shanghai, China). ASPH expression vector (pASPH) and empty vector were purchased from Genechem (Shanghai, China). Transfection was conducted using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated from the tissues and cells using TRIzol reagent (Beyotime, Jiangsu, China) according to the provided protocol. cDNA was synthesized with the one-step PrimeScript RT Reagent Kit (Takara, Dalian, China). qRT-PCR was carried out using SYBR Premix Ex Taq kit (Takara) on an ABI 7500 system (Applied Biosystems, Foster City, CA, USA). The primers used were as follows: miR-135a, forward 5'-TTTTCAGACTCC-3' and reverse 5'-CTCTT-GTCCTTCATTCCACC-3'; U6 snRNA, forward 5'-AGGGGCCGGACTCGTCATACT-3' and reverse 5'-GGCGGCACCACCATGTACCCT-3'. The following procedures were used: 1 cycle of denaturation at 95°C for 5 min followed by 35 cycles of amplification at 95°C for 10 s, 58°C for 30 s, and 72°C for 30 s. Relative expression levels were calculated using $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

Total protein was extracted from tissues and cells using RIPA Lysis Buffer (Beyotime) with standard protocols. Protein concentration was measured using BCA protein concentration determination kit (Beyotime) according to the manufacturer's protocol. The same amounts of protein (50 μg) were subjected to 10% SDS-polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride membranes. After blocking with 5% fat-free milk at room temperature for 1 h, the membranes were incubated with primary antibodies (ASPH: #ab172475, GAPDH: #ab181602; Abcam, Cambridge, MA, USA) at 4°C for overnight. The membranes were incubated with secondary antibody (#ab6721, Abcam) at room temperature for 1 h after washing three times with PBS. The protein signals were developed using enhanced ECL Kit (Beyotime).

Cell proliferation assay

Cell Counting Kit-8 (CCK-8, Beyotime) was used to measure cell proliferation. The cells were seeded into 96-well plates (3×10^3 cells/well) and cultured for 48 h before the addition of 10 μL of CCK-8 solution. After further incubation for 4 h, we measured the optical density at 450 nm with enzyme linked immunosorbent assay reader (Bio-Rad Laboratories, Hercules, CA, USA).

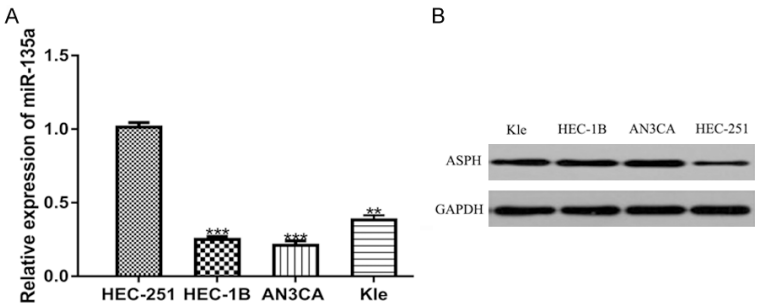

Figure 1. The expression of miR-135a and ASPH in EC. Expression of (A) miR-135a and (B) ASPH in EC cell lines (HEC-1B, Kle, and AN3CA). EC: endometrial cancer; miR-135a: microRNA-135a; ASPH: aspartate-β-hydroxylase.

Wound healing assay

Wound healing assay was performed to examine the migration capacity. Cells were seeded in 6-well plates and grown to about 90% confluence. The wound was artificially created using a 100 μl sterile pipette tip and washed with serum-free medium. Cell images were acquired at 0, and 24 h after the wound was created.

Bioinformatics prediction and luciferase activity assay

We employed TargetScan (www.targetscan.org/) to predict the potential targets of miR-135a. Cells were plated in 96-well plates and co-transfected with miR-135a mimic or NC and wild-type (wt) or mutant (mut) ASPH 3'-UTR using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.). After 48 h of transfection, luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Statistical analysis

Data are presented as mean ± SD and analyzed using GraphPad Prism 6.0 (La Jolla, CA, USA). Differences were measured with one-way analysis of variance (ANOVA) for three-group comparisons and Student's t-tests for two-group comparisons. *P* value less than 0.05 was considered significant.

Results

Expression of miR-135a and ASPH in EC

To determine whether miR-135a expression was elevated in EC, we measured miR-135a expression in EC cell lines. We found miR-135a expression levels were significantly reduced in EC cell lines compared with the normal cell line (**Figure 1A**). Then, we measured the protein levels of ASPH in EC cell lines and the normal cell line. ASPH level in EC cell lines was higher than that in the normal cell line (**Figure 1B**). We therefore selected AN3CA and HEC-1B cell lines for the following *in vitro* experiments according to the expression of miR-135a and ASPH.

miR-135a regulates proliferation and migration in EC cells

Considering that miR-135a expression was elevated in EC, we then examined the effects of miR-135a on the behavior of EC cells. We transfected miR-135a mimic and NC-mimic to EC cell lines to manipulate the expression of miR-135a (**Figure 2A**). CCK-8 assay disclosed that overexpression of miR-135a decreases EC cell proliferation (**Figure 2B**). Wound-healing assay revealed that cell migration was decreased by miR-135a overexpression (**Figure 2C**).

miR-135a directly targets and regulates ASPH in EC cells

We searched the potential target gene of miR-135a using the available database TargetScan and found that ASPH contains a miR-135a binding site in its 3'-UTR (**Figure 3A**). Luciferase reporter assay revealed that miR-135a overexpression reduced the luciferase activity of the cells transfected wt ASPH 3'-UTR (**Figure 3B**). But miR-135a mimic did not affect the luciferase activity of mut ASPH 3'-UTR (**Figure 3B**). The levels of ASPH were downregulated by miR-135a mimic in EC cells (**Figure 3C**). Thus, ASPH was a direct target of miR-135a in EC.

ASPH overexpression leads to cell proliferation and migration stimulation

Next, EC cell lines were transfected with pASPH. The overexpression of ASPH in EC cell lines was confirmed by western blot (**Figure 4A**). Cell proliferation was notably stimulated by ASPH overexpression (**Figure 4B**). Furthermore, we found

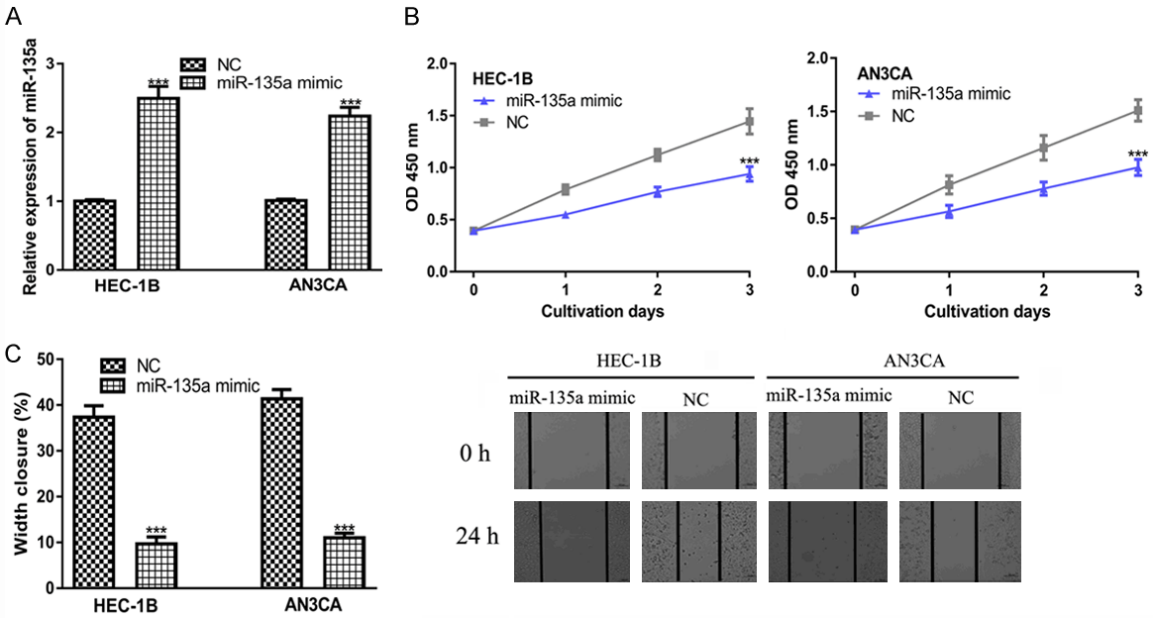

Figure 2. miR-135a overexpression inhibits cell proliferation and migration. A. Expression of miR-135a in cells transfected with miR-135a mimic or NC-mimic. B. CCK-8 assays revealed that miR-135a overexpression inhibited cell proliferation. C. Wound-healing assays revealed that miR-135a overexpression inhibited cell migration. EC: endometrial cancer; miR-135a: microRNA-135a; NC: negative control; CCK-8: cell counting kit-8.

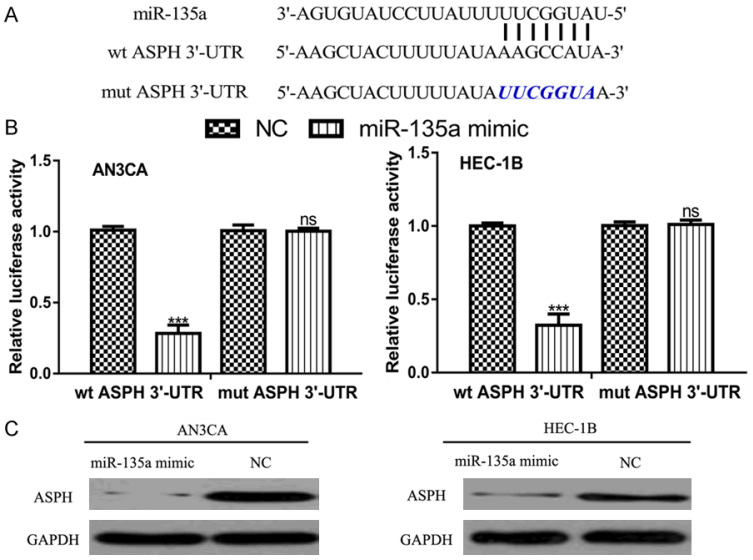

Figure 3. miR-135a targets ASPH in EC. A. ASPH contains a putative binding site for miR-135a in its 3'-UTR as predicted by TargetScan. B. Overexpression of miR-135a decreased the luciferase activity of cells transfected with wt ASPH 3'-UTR. C. miR-135a overexpression decreased ASPH in EC cells. EC: endometrial cancer; miR-135a: microRNA-135a; ASPH: aspartate-β-hydroxylase; NC: negative control; wt: wild-type; mut: mutant; UTR: untranslated region.

cell migration was also enhanced by ASPH overexpression (**Figure 4C**). Importantly, we found the effects of miR-135a mimic on ASPH expression, cell proliferation, and migration could be restored by ASPH overexpression (**Figure 4A-C**).

Discussion

Although the improvements in treatment have greatly improved the overall survival of EC patients, the prognosis is still disappointing [17]. To date, the significance of miRNAs in the progression and development of tumors including EC is increasingly appreciated [6, 8]. Therefore, an enhanced understanding of the regulatory mechanisms miRNAs in EC may provide new targets for EC diagnosis or therapy [8].

In order to identify such a therapeutic or prediction biomarker, we focused on investigating the miRNAs that were abnormally expressed in EC. We found that miR-135a expression was downregulated in EC cell lines compared with the normal cell line. Moreover,

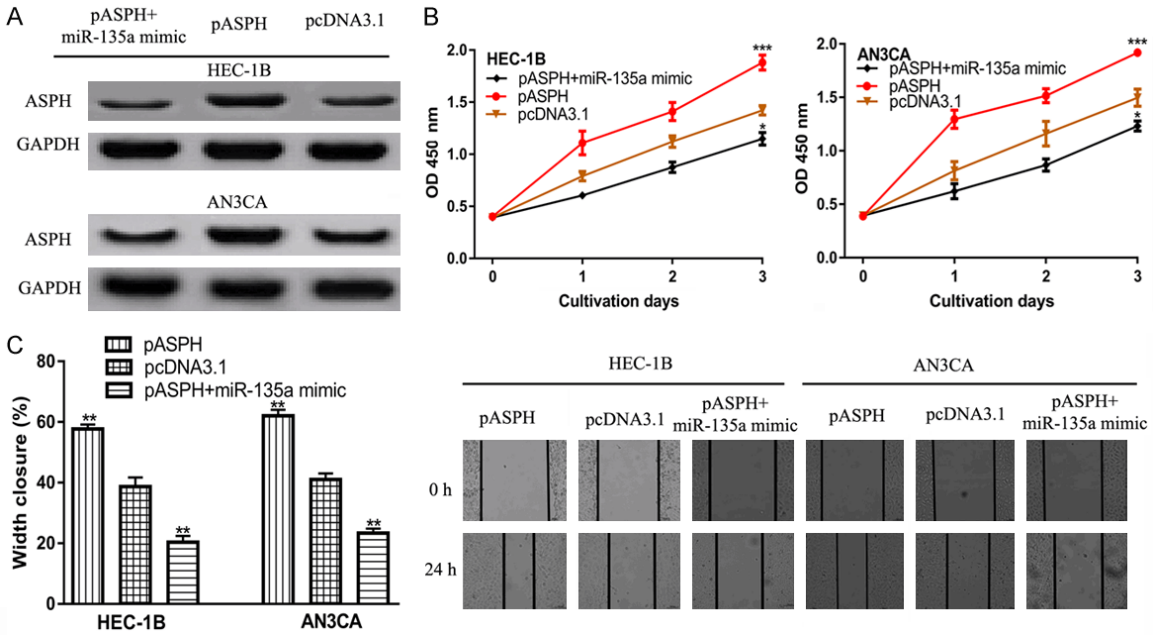

Figure 4. ASPH overexpression promoted cell proliferation and migration. (A) Expression of ASPH, (B) Cell proliferation, and (C) Cell migration in cells transfected with pASPH, pcDNA3.1, or pASPH and miR-135a mimic. EC: endometrial cancer; miR-135a: microRNA-135a; ASPH: aspartate-β-hydroxylase.

to address the biologic role of miR-135a in the progression of EC, we analyzed the effects of miR-135a on EC cell proliferation and migration. We found that miR-135a overexpression inhibited cell proliferation and migration *in vitro*. Therefore, our results revealed that miR-135a plays a crucial role in the progression of EC and might be a therapeutic target for EC.

We have now understood that miRNAs exert tumor suppressive or oncogenic roles in the development and progression of tumor by regulating the expression of specific targets [8]. We therefore explored the potential target gene mediating the oncogenic role of miR-135a in EC. Luciferase reporter assay revealed that miR-135a mimic could decrease the luciferase activity of cells transfected with wt ASPH 3'-UTR. Furthermore, inverse correlation between miR-135a and ASPH expression was observed in EC tissues. We also observed ASPH expression could be negatively regulated by miR-135a. Collectively, these data revealed that ASPH was a direct downstream target of miR-135a in EC. The overexpression of ASPH promoted cell proliferation and migration *in vitro* and restored the inhibitory effects of miR-135a mimic on the above-mentioned cell behaviors. Taken together, ASPH is a strong candidate mediator for the oncogenic role of miR-135a in EC.

In conclusion, these results demonstrated the expression of miR-135a was reduced in EC. In addition, we found miR-135a inhibited cell proliferation and migration through regulating the expression of ASPH. Our results provided new insights on the progression mechanism of EC and novel therapeutic targets for treatment.

Disclosure of conflict of interest

None.

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