JBUON 2021; 26(5): 1768-1774
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
Email: editorial_office@jbuon.com

ORIGINAL ARTICLE

miR-34a-5p blocks cervical cancer growth and migration by downregulating CDC25A

Tao Jiang, Hongyan Cheng

Department of Obstetrics and Gynecology, Suizhou Hospital, Hubei University of Medicine, Suizhou, Hubei Province, China.

Summary

Purpose: *To define mechanisms underlying the regulation of CDC25A by miR-34a-5p in cervical cancer (CC).*

Methods: *Quantifications of miR-34a-5p and CDC25A levels were performed in 68 CC tissues and matched controls. CC cell lines HeLa and SiHa were transfected to monitor their biological behavior changes.*

Results: *Low-expressed miR-34a-5p and up-regulated CDC25A were noted in CC. Either overexpression of miR-34a-5p or inhibition of CDC25A blocked the growth and migration of HeLa and SiHa cells, resulting in an increase in apoptosis ($p<0.05$), while inhibition of miR-34a-5p or overexpression of CDC25A promoted tumor growth. Dual-luciferase reporter (DLR) assay and cell transfection identified the targeting relationship between miR-34a-5p and CDC25A, and correlation analysis revealed a negative association between them in CC. Co-transfection assays showed that overexpression of CDC25A reversed the inhibition of miR-34a-5p on CC growth and migration.*

Conclusion: *miR-34a-5p mediates growth, migration and other malignant behaviors in CC by regulating CDC25A.*

Key words: *miR-34a-5p, CDC25A, cervical cancer, migration*

Introduction

Cervical cancer (CC) is a globally prevalent gynecological malignancy with the second highest incidence [1]. An estimated 570,000 new CC cases and 311,000 female deaths were reported in 2018 [2]. Owing to technological advancements, the diagnosis and treatment of CC have been greatly improved in developed countries, however, the mortality in developing countries remains 18-fold higher than that in developed countries [3,4]. Accumulating evidence supports the application of biomarkers for pre- and early identification and timely treatment of CC, thereby improving the prognosis of patients [5,6]. Nevertheless, since molecular mechanisms involved in CC tumorigenesis have not been fully clarified, there is still a lack of sustained and effective treatments [7]. Therefore, elucidating the mechanisms lays a foundation for the development of new treatment schemes.

MicroRNAs (miRNAs or miRs) are endogenous non-coding RNAs that mediate biological processes of various tumors and regulate protein production by interacting with mRNAs [8,9]. miR-34a-5p is known as a typical tumor suppressor mRNA in breast cancer, ovarian cancer, and oral cancer [10-12]. Besides, it is a promising therapeutic tool against metastasis, chemical resistance and recurrence. Over-expressed miR-34a-5p hinders the growth of CC cells and accelerates apoptosis through suppressing Bcl-2 [14]. Only roles of miR-34a-5p in CC cells have been described, the regulatory mechanism remains unknown. Therefore, it is necessary to explore its mechanism of action

Corresponding author: Hongyan Cheng, MM. Department of Obstetrics and Gynecology, Suizhou Hospital, Hubei University of Medicine, #8 Wendi Rd, Zengdu District, Suizhou 441300, Hubei Province, China.
Tel: +86 17786250527; Email: hqdxg3@163.com
Received: 18/02/2021; Accepted: 15/03/2021

in CC. Regulated by various miRNAs, CDC25A is involved in tumor cell metastasis and radiation resistance [15]. Ding et al believe that miR-122-5p regulates the radiosensitivity of CC cells by targeting CDC25A [16]. However, whether there is a regulatory relationship between miR-34a-5p and CDC25A remains to be confirmed. Therefore, we aimed to identify the regulatory relationship and inspire a novel target for CC.

Methods

Sample collection

Sixty-eight CC tissues and matched controls were sampled and stored in a liquid nitrogen tank. The patients were 52.87±5.34 years old and diagnosed with CC, with intact clinical data, no other malignant tumors, and no surgical contraindications. The present study was performed after approval from the Ethics Committee, and all participants signed the informed consent form.

Cell treatment

Human CC cells (HeLa, SiHa, ME180, C-33 A) and normal cervical epithelial cells (Ect1/E6E7) (Shanghai Yaji Biotechnology Co., Ltd.) were placed in RPMI 1640 medium (Amyjet Scientific Co., Ltd., Hubei, China) comprising 10% fetal bovine serum (FBS) (5% CO_2, 37°C, saturated humidity). HeLa and SiHa cells were selected for transfection. After reaching 80-90% confluence, they were intervened by blank controls (Vector, miR-NC), inhibitors (miR-34a-5p inhibitor, siRNA CDC25A) and mimics (miR-34a-5p mimics, pcDNA CDC25A) by a Lipofectamine™2000 transfection kit (Invitrogen, USA).

qRT-PCR

In this test, total RNAs were isolated first by TRIzol reagent (Shanghai Meiyan Biological Technology Co., Ltd.). Afterwards, cDNAs were obtained from RNA samples with a reverse transcription kit (GeneCopoeia). PCR amplification was conducted by a SYBR Green PCR kit (Takara Biotechnology Co., Ltd) on a PCR instrument (ABI, USA, 7500) under conditions of 95°C for 5 min, 95°C for 10 s, 60°C for 20 s, for a total of 40 cycles. U6 and GAPDH served as internal references of miR-206 and SNHG14, respectively. And the data were calculated by $2^{-\Delta\Delta CT}$.

Western blotting (WB)

Cultured tissues and cells were lysed by RIPA buffer (Shifeng Biotechnology Co., Ltd., Shanghai, China), and the protein concentration and quantification were examined by a BCA kit (Thermo Fisher Scientific, USA). Electrophoresis (10% SDS-PAGE) and membrane transfer (PVDF, Millipore, USA) were performed. Following sealing with skim milk powder at 37°C for 30 min, the membrane was incubated with primary antibodies (1: 1000, Abcam, Cambridge, MA, USA) at 4°C overnight. Next, those antibodies were removed, and goat anti-rabbit secondary antibody (HRP) was adopted for a 1-2 h incubation (37°C). After blots were developed with ECL solution, band analyses were performed.

Determination of cell growth

HeLa and SiHa cells in logarithmic growth phase were placed overnight in a 96-well plate (5×10^3 cells/well). CCK-8 solution (10 μL, Sigma-Aldrich, St. Louis, Missouri, USA) was put in each well after transfection. Next, absorbances were measured at 450nm on a Multiskan™ GO reader (Thermo Scientific™).

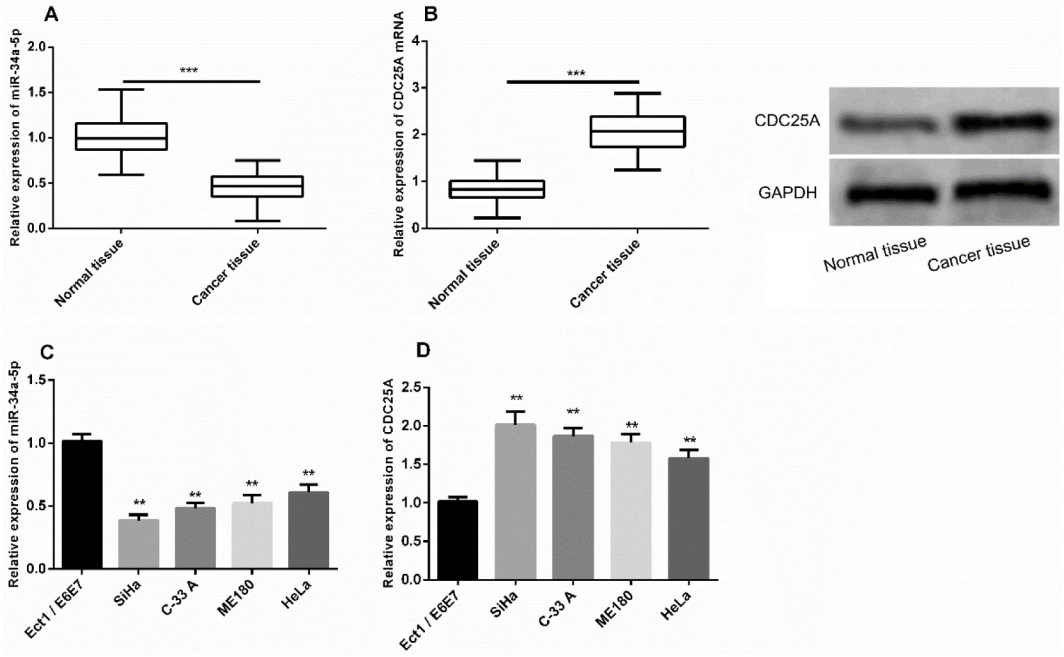

Figure 1. miRNA and protein levels in CC. **A:** miR-34a-5p expression in CC tissues. **B:** CDC25A mRNA and protein levels in CC tissues. **C:** miR-34a-5p expression in CC cell lines. **D:** CDC25A expression in CC cell lines. **p<0.01, ***p<0.001 *vs.* Ect1/E6E7 cells.

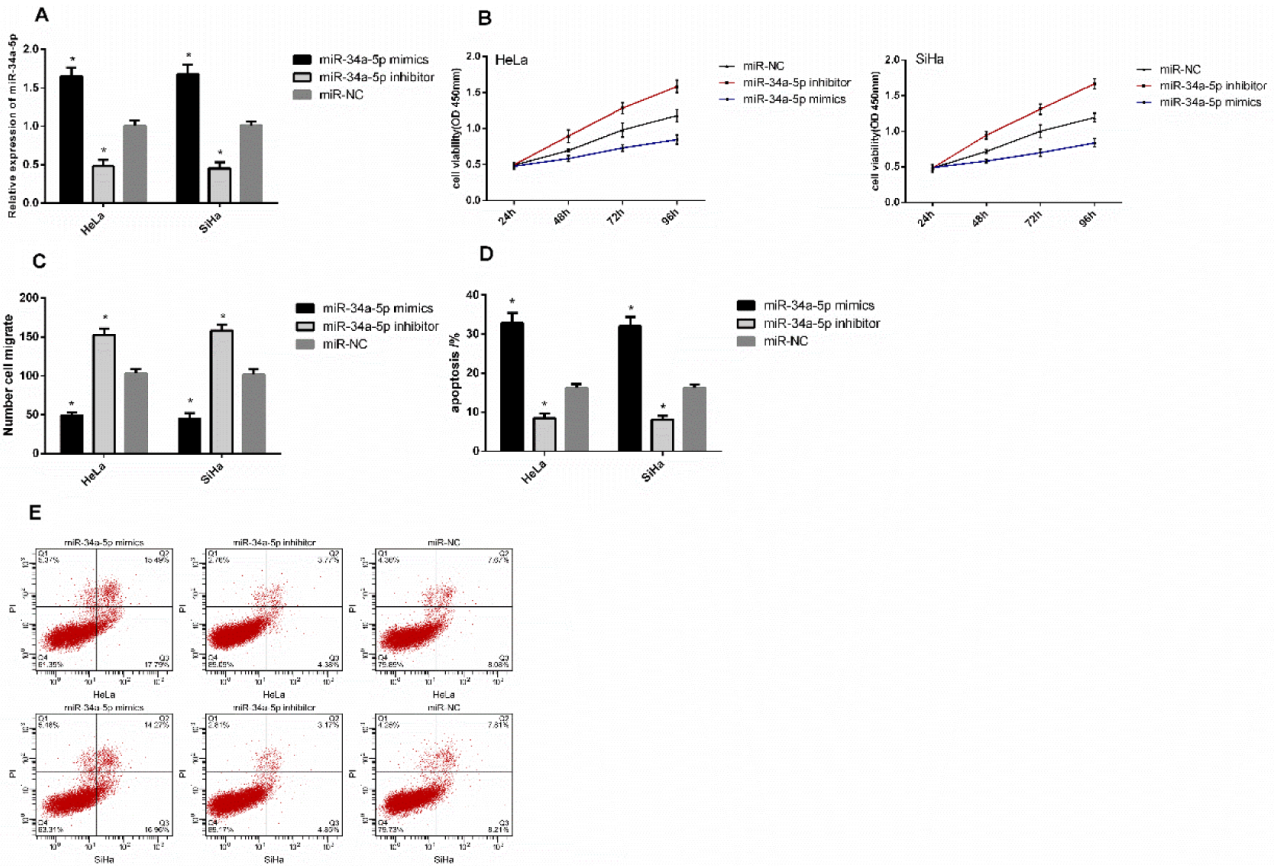

Figure 2. Regulation of miR-34a-5p on CC cells. **A:** Changes of miR-34a-5p after different transfections. **B:** Changes of cell viability after different transfections. **C:** Changes of cell migration after different transfections. **D:** Changes of cell apoptosis after different transfections. **E:** Flow cytometry. *p<0.05 *vs.* miR-NC.

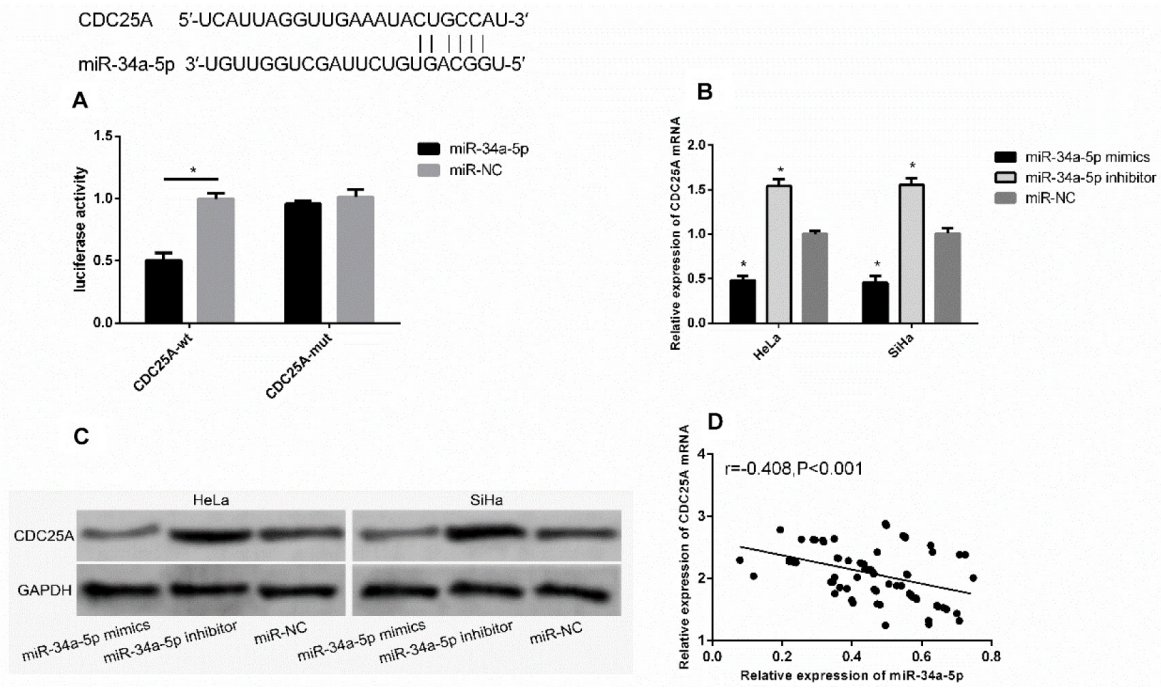

Figure 3. Verification of targeted regulatory relationship. **A:** Binding loci between miR-34a-5p and CDC25A; DLR assay. **B:** Changes of CDC25A mRNA level after different transfections. **C:** Changes of CDC25A protein level after different transfections. **D:** Correlation analysis. *p<0.05 *vs.* miR-NC.

Determination of cell migration

Cells were inoculated in Transwell inserts (BD Biosciences, USA) comprising fibronectin-coated polycarbonate filters for 24 h. Migrated cells were immobilized in 1% paraformaldehyde, dyed with hematoxylin, and monitored under a BX43 vertical microscope (Olympus Corporation).

Determination of cell apoptosis

Annexin V-FITC/PI kit (Abnova) was used for determination of cell apoptosis. After two phosphate buffered saline (PBS) washes, digested cells were prepared into suspension (1×10^6 cells/mL) with 100μL binding buffer. The suspension was incubated with AnnexinV-FITC and propidium iodide (PI) for 5 min at room temperature in the dark, and then it was tested in a flow cytometry system.

Dual-luciferase reporter (DLR) assay

Prediction of downstream target genes of miR-34a-5p was carried out through an online website. pmirGLO reporter vectors comprising CDC25A-3'UTR wild type (Wt) and CDC25A-3'UTR Mutant (mut) were transfected into CC cells along with miR-34a-5p inhibitor, miR-34a-5p mimics, and miR-NC, separately. A Dual luciferase (DLR) system was used for luminescence intensity determination 48 h after transfection.

Statistics

GraphPad 7 was employed for graphing. T-test was used for between-group comparisons, one-way ANOVA for multi-group comparisons, LSD-t test for *post-hoc* tests, and Pearson test for correlation analyses. Significance was determined when probability (P) values were <0.05.

Results

miRNA and protein levels in CC

Total RNAs were extracted from 68 CC tissues and matched controls to quantitate miR-34a-5p and CDC25A levels in CC tissues. Downregulation of miR-34a-5p and upregulation of CDC25A were noted (both p<0.05). Afterwards, we evaluated their levels in CC cell lines, and found that miR-34a-5p was lower and CDC25A was higher than those in normal cervical epithelial cells (Ect1/E6E7) (Figure 1).

Regulation of miR-34a-5p on CC cells

HeLa and SiHa cells were treated with overexpressed and inhibited miR-34a-5p, as shown in Figure 2. miR-34a-5p mimics upregulated miR-34a-5p level, and miR-34a-5p inhibitor downregulated it. Cytological behavior analyses revealed miR-34a-5p

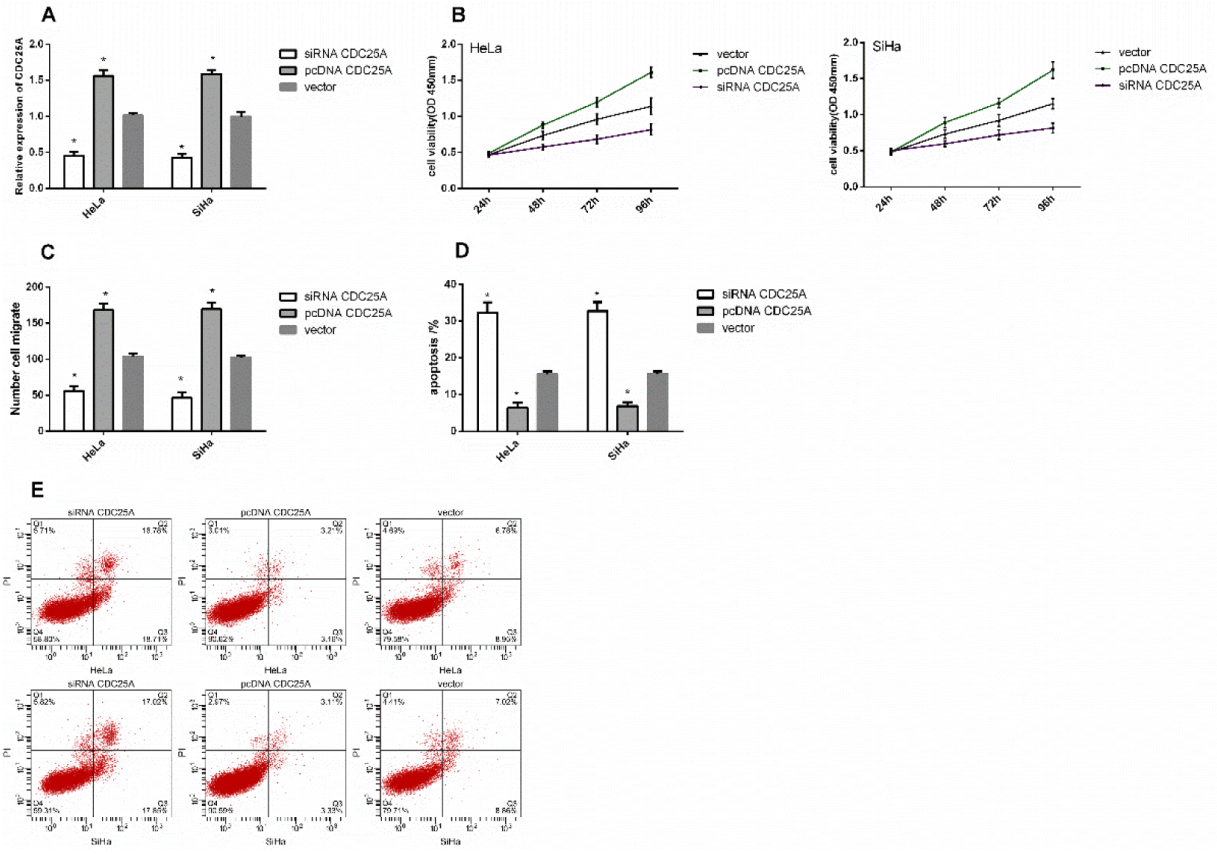

Figure 4. Regulation of CDC25A on cervical cancer cells. **A:** Changes of CDC25A level after different transfections. **B:** Changes of cell viability after different transfections. **C:** Changes of cell migration after different transfections. **D:** Changes of cell apoptosis after different transfections. **E:** Flow cytometry. *p<0.05 *vs.* Vector.

mimics blocked the growth and migration of HeLa and SiHa cells, resulting in an increase in apoptosis ($p<0.05$ *vs.* miR-NC). Quite the opposite, miR-34a-5p inhibitor induced growth and migration, and blocked apoptosis.

Validation of targeting relationship

Through an online website, we noticed that miR-34a-5p shared target binding loci with CDC25A. Thus, we carried out DLR assays and found inhibited luciferase activity of CDC25A-Wt after miR-34a-5p mimics treatment ($p<0.05$). miR-34a-5p mimics resulted in decreased CDC25A level, and miR-34a-5p inhibitor caused increased CDC25A level ($p<0.05$). Moreover, Pearson test revealed a negative association between miR-34a-5p and CDC25A in CC ($r=-0.408$, $p<0.001$;Figure 3).

Regulation of CDC25A on CC cells

HeLa and SiHa cells were transfected to further verify the regulatory effect of CDC25A on CC cells, as shown in Figure 4. CDC25A level decreased after siRNA treatment, but increased after pcDNA treatment. Furthermore, siRNA CDC25A blocked the growth and migration of HeLa and SiHa cells and enhanced apoptosis ($p<0.05$ *vs.* Vector). Conversely, pcDNA CDC25A induced cell growth and migration.

Cell co-transfection

miR-34a-5p mimics+pcDNA CDC25A induced no significant changes in growth, migration and apoptosis of CC cells ($p>0.05$ *vs.* NC), while compared with miR-34a-5p mimics group, growth and migration were enhanced and apoptosis was slowed down ($p<0.05$). These findings indicated that pcDNA CDC25A reversed all the effects of miR-34a-5p mimics on the behaviors of CC cells (Figure 5).

Discussion

Despite a decline in mortality of CC due to advances in diagnostic technologies, the prognosis remains disappointing for the complex triggering factors [17,18]. Alteration in genes is the funda-

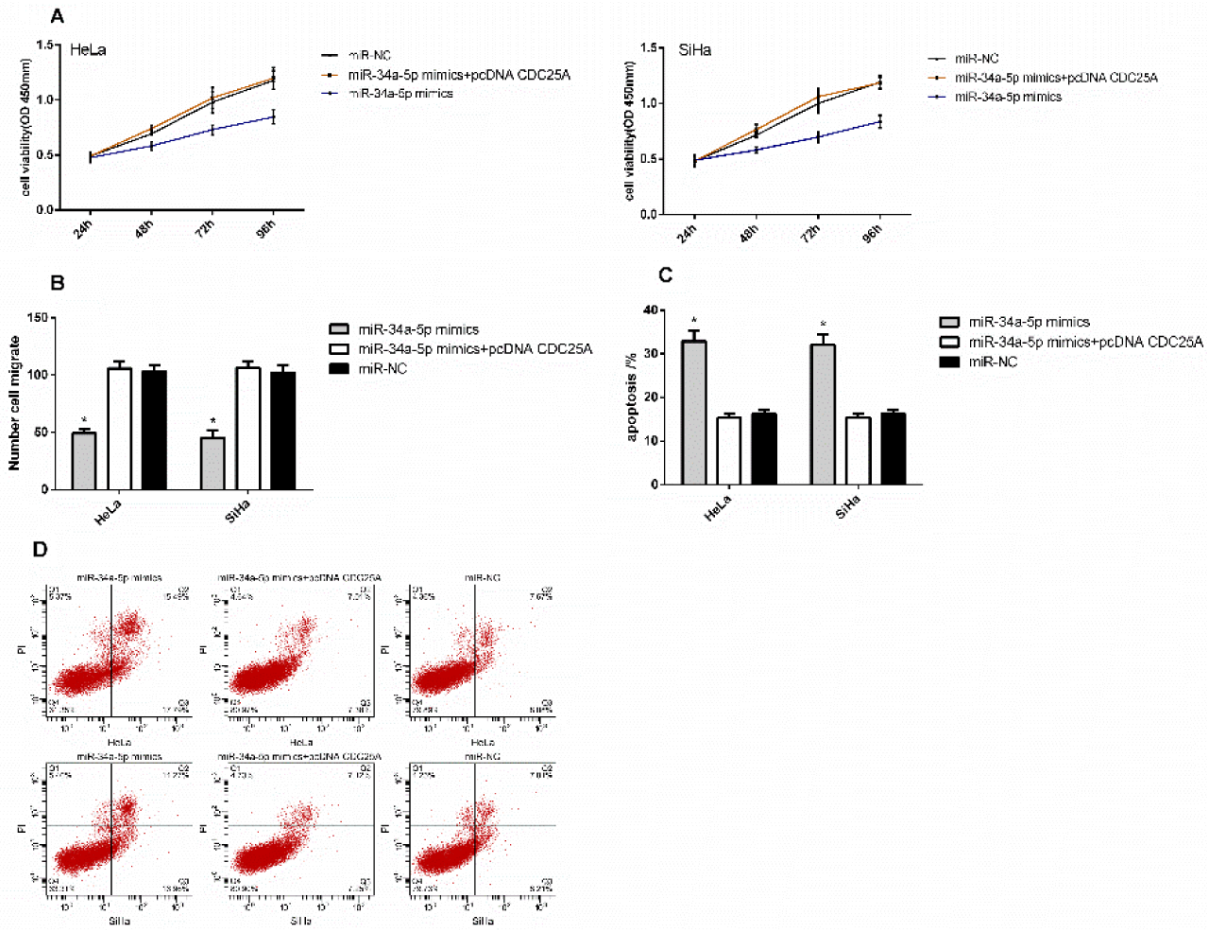

Figure 5. Cell co-transfection. **A:** Changes of cell viability after different transfections. **B:** Changes of cell migration after different transfections. **C:** Changes of cell apoptosis after different transfections. **D:** Flow cytometry. miR-34a-5p mimics. *$p<0.05$ *vs.* the other two groups.

mental cause of cancer, so comprehending the pathogenesis from the gene level is beneficial to the diagnosis and treatment of CC [19]. Growth and migration of tumor cells, the biological characteristics of cancer, are closely related to the occurrence and development of tumors [20]. As tumor suppressors or oncogenes regulating cellular behaviors, miRNAs are considered to be diagnostic markers and therapeutic targets for various cancers [21-24].

miR-34a-5p functions as a therapeutic target for many diseases. As previously reported, sevoflurane mediates glioma progression by regulating miR-34a-5p [25]. Furthermore, miR-34a-5p exists in colon carcinoma, whose abnormal expression is correlated to the recurrence and disease-free survival of patients [26]. We quantitated miR-34a-5p expression in CC tissues and cells by qRT-PCR to determine its changes. It turned out that it was lowly expressed in CC tissues compared with normal ones, further validating the tumor-suppressing role of miR-34a-5p. HeLa and SiHa cells were treated with overexpressed and inhibited miR-34a-5p. Overexpressed miR-34a-5p blocked the growth and migration of cells, resulting in an increase in apoptosis. However, inhibited miR-34a-5p showed a tumor-promoting effect. miR-34a-5p significantly alleviates cell resistance and controls its resistance behavior by mediating AGTR1 in osteosarcoma [27]. Moreover, it targets BACE1 in Alzheimer's disease to reduce apoptosis and oxidative stress [28]. These collectively indicate differential expression and regulatory roles of miRNAs in different diseases. The specific regulatory mechanism of miR-34a-5p in CC has been imperfectly clarified.

Previous studies have suggested that miRNAs influence the biological behaviors of cells by regulating mRNA expression. So we employed a biological database to study the mechanism of miR-34a-5p in CC, and binding loci miR-34a-5p and CDC25A were discovered. DLR assay, cell transfection and correlation analysis demonstrated a negative regulation between miR-34a-5p and CDC25A, suggesting that miR-34a-5p can regulate CDC25A. CDC25A, a member of CDC25 family, is a dual specificity protein phosphatase that hydrolyzes tyrosine and serine/threonine residues for dephosphorylation [29]. It presents a high expression in various malignant tumors because of its protooncogene characteristics, which portends a poor prognosis of patients [30]. By suppressing CDC25A, miRNA-99a-5p slowed down cell-cycle pathway and progression of breast cancer [31], while CDC25A regulates tumor growth and migration [32,33]. HeLa and SiHa cells were transfected to further verify the regulatory effect of CDC25A on CC cells. Silencing CDC25A evidently blocked the growth and migration of CC cells and induced apoptosis, while overexpression of CDC25A enhanced cell growth and migration. Therefore, CDC25A functions as an oncogene in CC. Subsequently, as shown by co-transfection assays, overexpression of CDC25A eliminated the inhibition of miR-34a-5p on the growth and migration and the promotion on the apoptosis of tumor cells. These findings suggest that miR-34a-5p is able to mediate malignant behaviors of CC cells by regulating CDC25A.

The present study explored the regulatory relationship between miR-34a-5p and CDC25A to provide a clinical reference. However, there are several limitations because *in vivo* experiments in nude mice have not been carried out to observe the growth changes of the tumor. Moreover, there are numerous network mechanisms regulating cell growth, so the specific regulation of downstream pathways needs to be detailed.

To sum up, miR-34a-5p mediates malignant cellular behaviors in CC by regulating CDC25A.

Conflict of interests

The authors declare no conflict of interests.

References

1. Finocchario-Kessler S, Wexler C, Maloba M, Mabachi N, Ndikum-Moffor F, Bukusi E. Cervical cancer prevention and treatment research in Africa: a systematic review from a public health perspective. BMC Womens Health 2016; 16:29.
2. Canfell K, Kim JJ, Brisson M et al. Mortality impact of achieving WHO cervical cancer elimination targets: a comparative modelling analysis in 78 low-income and lower-middle-income countries. Lancet 2020; 395:591-603.
3. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018; 68:394-424.
4. Balasubramaniam SD, Balakrishnan V, Oon CE, Kaur G. Key Molecular Events in Cervical Cancer Development. Medicina (Kaunas) 2019; 55:384.
5. Nahand JS, Taghizadeh-Boroujeni S, Karimzadeh M, et al. microRNAs: New prognostic, diagnostic, and thera-

peutic biomarkers in cervical cancer. J Cell Physiol 2019; 234:17064-99.

6. Zheng M, Hou L, Ma Y et al. Exosomal let-7d-3p and miR-30d-5p as diagnostic biomarkers for non-invasive screening of cervical cancer and its precursors. Mol Cancer 2019; 18:76.
7. Chen M, Li L, Zheng PS. SALL4 promotes the tumorigenicity of cervical cancer cells through activation of the Wnt/β-catenin pathway via CTNNB1. Cancer Sci 2019; 110:2794-2805.
8. Nair VB, Manasa VG, Sinto MS, Jayasree K, James FV, Kannan S. Differential Expression of MicroRNAs in Uterine Cervical Cancer and Its Implications in Carcinogenesis; An Integrative Approach. Int J Gynecol Cancer 2018; 28:553-62.
9. Lv M, Ou R, Zhang Qs et al. MicroRNA-664 suppresses the growth of cervical cancer cells via targeting c-Kit. Drug Des Devel Ther 2019; 13:2371-9.
10. Engkvist ME, Stratford EW, Lorenz S, Meza-Zepeda LA, Myklebost O, Munthe E. Analysis of the miR-34 family functions in breast cancer reveals annotation error of miR-34b. Sci Rep 2017; 7:9655.
11. Zuo Y, Zheng W, Liu J, Tang Q, Wang SS, Yang XS. MiR-34a-5p/PD-L1 axis regulates cisplatin chemoresistance of ovarian cancer cells. Neoplasma 2020; 67:93-101.
12. Li YY, Tao YW, Gao S et al. Cancer-associated fibroblasts contribute to oral cancer cells proliferation and metastasis via exosome-mediated paracrine miR-34a-5p. EBioMedicine 2018; 36:209-20.
13. Wang X, Zhao Y, Lu Q et al. MiR-34a-5p Inhibits Proliferation, Migration, Invasion and Epithelial-mesenchymal Transition in Esophageal Squamous Cell Carcinoma by Targeting LEF1 and Inactivation of the Hippo-YAP1/TAZ Signaling Pathway. J Cancer 2020; 11:3072-81.
14. Wang X, Xie Y, Wang J. Overexpression of MicroRNA-34a-5p Inhibits Proliferation and Promotes Apoptosis of Human Cervical Cancer Cells by Downregulation of Bcl-2. Oncol Res 2018; 26:977-85.
15. Yanagida J, Hammiller B, Al-Matouq J et al. Accelerated elimination of ultraviolet-induced DNA damage through apoptosis in CDC25A-deficient skin. Carcinogenesis 2012; 33:1754-61.
16. Ding FN, Gao BH, Wu X, Gong CW, Wang WQ, Zhang SM. miR-122-5p modulates the radiosensitivity of cervical cancer cells by regulating cell division cycle 25A (CDC25A). FEBS Open Bio 2019; 9:1869-79.
17. Zhang Y, Guo X, Wang G et al. Real-World Study of the Incidence, Risk Factors, and Prognostic Factors Associated with Bone Metastases in Women with Uterine Cervical Cancer Using Surveillance, Epidemiology, and End Results (SEER) Data Analysis. Med Sci Monit 2018; 24:6387-97.
18. Kamineni A, Tiro JA, Beaber EF et al. Cervical cancer screening research in the PROSPR I consortium: Rationale, methods and baseline findings from a US cohort. Int J Cancer 2019; 144:1460-73.
19. Wu J, Zhao Y, Li F, Qiao B. MiR-144-3p: a novel tumor suppressor targeting MAPK6 in cervical cancer. J Physiol Biochem 2019; 75:143-52.
20. Yi Y, Tsai SH, Cheng JC et al. APELA promotes tumour growth and cell migration in ovarian cancer in a p53-dependent manner. Gynecol Oncol 2017; 147:663-71.
21. Wang R, Sun Y, Yu W et al. Downregulation of miRNA-214 in cancer-associated fibroblasts contributes to migration and invasion of gastric cancer cells through targeting FGF9 and inducing EMT. J Exp Clin Cancer Res 2019; 38:20.
22. Chen L, Sun H, Wang C, Yang Y, Zhang M, Wong G. miRNA arm switching identifies novel tumour biomarkers. EBioMedicine 2018; 38:37-46.
23. Sur D, Coza O, Havasi A et al. Exosomal miRNAs in colorectal cancer: the carriers of useful news. J BUON 2020; 25:23-34.
24. Lajos R, Braicu C, Jurj A et al. A miRNAs profile evolution of triple negative breast cancer cells in the presence of a possible adjuvant therapy and senescence inducer. J BUON 2018; 23:692-705.
25. Zhao H, Xing F, Yuan J, Li Z, Zhang W. Sevoflurane inhibits migration and invasion of glioma cells via regulating miR-34a-5p/MMP-2 axis. Life Sci 2020; 256:117897.
26. Gao J, Li N, Dong Y et al. miR-34a-5p suppresses colorectal cancer metastasis and predicts recurrence in patients with stage II/III colorectal cancer. Oncogene 2015; 34:4142-52.
27. Pu Y, Zhao F, Li Y et al. The miR-34a-5p promotes the multi-chemoresistance of osteosarcoma via repression of the AGTR1 gene. BMC Cancer 2017; 17:45.
28. Li P, Xu Y, Wang B, Huang J, Li Q. miR-34a-5p and miR-125b-5p attenuate Aβ-induced neurotoxicity through targeting BACE1. J Neurol Sci 2020; 413: 116793.
29. Shen T, Huang S. The role of Cdc25A in the regulation of cell proliferation and apoptosis. Anticancer Agents Med Chem 2012; 12:631-9.
30. Li H, Jiang M, Cui M et al. MiR-365 enhances the radiosensitivity of non-small cell lung cancer cells through targeting CDC25A. Biochem Biophys Res Commun 2019; 512:392-8.
31. Qin H, Liu W. MicroRNA-99a-5p suppresses breast cancer progression and cell-cycle pathway through downregulating CDC25A. J Cell Physiol 2019; 234:3526-37.
32. Zhao S, Wang Y, Guo T et al. YBX1 regulates tumor growth via CDC25a pathway in human lung adenocarcinoma. Oncotarget 2016; 7:82139-57.
33. Lau KM, Chan QK, Pang JC et al. Overexpression of HMGA1 deregulates tumor growth via cdc25A and alters migration/invasion through a cdc25A-independent pathway in medulloblastoma. Acta Neuropathol 2012; 123:553-71.