

ORIGINAL ARTICLE

MiR-200a promotes proliferation of cervical cancer cells by regulating HIF-1 α /VEGF signaling pathway

Xinlu Su, Cuihong Lang, Aiai Luan, Peng Zhao

Department of Gynecology, Weifang Maternal and Child Health Hospital, Weifang, China.

Summary

Purpose: To explore the biological function of micro ribonucleic acid (miR)-200a in cervical cancer (CC).

Methods: HeLa cells were transfected with miR-200a inhibitor or negative control (NC). Next, the effects of miR-200a down-regulation on the proliferation of CC cells were detected via methyl thiazolyl tetrazolium (MTT) assay and colony formation assay. Subsequently, flow cytometry was performed to determine the role of miR-200a in regulating the apoptosis of CC cells. Finally, Western blotting was conducted to detect the effects of miR-200a on the hypoxia-inducible factor 1- α (HIF-1 α)/vascular endothelial growth factor (VEGF) signaling pathway.

Results: RT-qPCR results confirmed that miR-200a was significantly down-regulated in HeLa cells transfected with miR-200a inhibitor compared with those transfected with

NC ($p < 0.05$). Besides, in comparison with cells in NC group, HeLa cells with down-regulated miR-200a showed weakened proliferation ability ($p < 0.05$) and a remarkably decreased colony number ($p < 0.05$). Moreover, flow cytometry results manifested that the down-regulation of the miR-200a expression level obviously promoted cell apoptosis ($p < 0.05$). Furthermore, western blotting analyses also confirmed that the protein expression levels of HIF-1 α and VEGF were increased after miR-200a overexpression.

Conclusions: MiR-200a facilitates the proliferation of CC cells and activates the HIF-1 α /VEGF signaling pathway by targeting EGLN1.

Key words: miR-200a, cervical cancer, EGLN1 gene, HIF-1 α /VEGF signaling pathway, proliferation

Introduction

Cervical cancer (CC) ranks third in terms of incidence and fourth in terms of cancer-related mortality. It is estimated that more than 500,000 new CC cases and 260,000 deaths resulting from CC occur every year [1]. In developing countries, the incidence and mortality rates of CC are gradually increasing, which are mainly due to the lack of screening procedures, diagnostic methods and effective treatment methods [2]. High-risk human papillomavirus (HPV) infection has been identified as a key attribute of many risk factors related to CC. However, HPV infection alone is not enough to cause cancer [3]. Extensive fundamental and clinical research has greatly facilitated the development

of new diagnostic and therapeutic technologies for CC patients [4]. Unfortunately, the prognosis is still unsatisfactory, and the overall survival rate of patients with advanced-stage disease is about 30-50% [5]. Therefore, it is crucial to conduct further research to understand the molecular mechanism of CC occurrence and development, so as to improve the prognosis of patients with this malignant tumor.

Micro ribonucleic acids (miRs) are a group of short (about 22 nucleotides in length) RNA molecules that cannot encode proteins, and they are known for their negative effects on gene expression through post-transcriptional regulation [6].

Corresponding author: Peng Zhao, BM. Department of Gynecology, Weifang Maternal and Child Health Hospital, 407 Qingnian Rd, Weicheng District, Weifang, 261011 Shandong, China.
Tel: +86 013792657768, Email: 275298903@qq.com
Received: 20/02/2020; Accepted: 22/03/2020

MiRs effectively reduce the stability or inhibit the translation of certain genes under physiological or pathological conditions by base pairing with specific target sites in the 3' untranslated regions (3'-UTRs) of these genes [7,8]. Due to the widespread distribution of these target genes, miRs exert a vital effect in tumorigenesis by influencing key processes such as cell proliferation, apoptosis, differentiation, angiogenesis and metastasis [9,10]. Under different cancer types or cell environments, the same miR may have opposite biological functions, either as an oncogene or as a cancer suppressor [11]. During the occurrence of CC, various miRs exhibit abnormal expressions. It is reported that their imbalance has potential diagnostic and clinical prognostic value [12]. Wang et al [13] evaluated the expressions of miR-93 and miR-200a in invasive CC and analyzed their clinical significance. They found that compared with benign lesion tissues, CC tissues have up-regulated miR-93 and miR-200a, and miR-93 and miR-200a are related to CC invasion and metastasis. However, there are no reports on the function and role of miR-200a. Therefore, cell assays were conducted to explore the function and potential mechanism of miR-200a in CC in this study.

Methods

Materials

The following materials were used in this study: Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) (Hyclone, South Logan, UT, USA), Opti-minimum essential medium (MEM) (Gibco, Rockville, MD, USA), Lipofectamine™ 2000 and TRIzol reagents (Invitrogen, Carlsbad, CA, USA), All-in-One™ miRNA real-time quantitative polymerase chain reaction (RT-qPCR) detection kit (GeneCopoeia, Rockville, MD, USA), PrimeScript RT kit and SYBR Premix Ex Taq™ kit (TaKaRa, Dalian, China), methyl thiazolyl tetrazolium (MTT) solution, dimethyl sulfoxide (DMSO) and bicinchoninic acid (BCA) protein assay kit (Beyotime Biotechnology, Shanghai, China), pMIR-REPORT Luciferase vector, dual luciferase detection system and enhanced chemiluminescence (ECL) solution (Thermo, Waltham, MA, USA), apoptosis detection kit, BCA protein concentration determination kit (Beyotime Biotechnology, Shanghai, China), hypoxia-inducible factor 1- α (HIF- α), vascular endothelial growth factor (VEGF), Egl-9 family hypoxia inducible factor 1 (EGLN1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies (Cell Signaling Technology, Danvers, MA, USA) and a flow cytometer (BD, Franklin Lakes, NJ, USA).

Cell culture and transfection

CC HeLa cells were preserved in this laboratory and cultured in DMEM containing 10% FBS, 100 U/mL penicillin and 100 μ g/mL streptomycin in a humidified

incubator with 5% CO₂ at 37°C. MiR-200a mimic, miR-200a inhibitor and their corresponding negative controls (NCs) were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). Then the cells were seeded in 6-well plates at a density of 2×10^5 /well. After 24 h, they were transfected with Lipofectamine 2000. In the Opti-MEM without antibiotics, the nucleotides of miR-200a mimic and miR-200a inhibitor were used at the final concentrations of 40 and 80 nM, respectively. Six hours later, the medium was replaced with DMEM containing 10% FBS.

RT-qPCR analysis

Total RNA was isolated from HeLa cells using TRIzol kit, and the expression of miR-200a was detected using the All-in-One™ miRNA RT-qPCR detection kit. Thermal cycling procedures: at 95°C for 10 min, denaturation at 95°C for 15 s and annealing/elongation at 60°C for 15 s for 45 cycles. In order to quantify the expression of EGLN1 messenger RNA (mRNA), the total RNA was reversely transcribed into cDNA using PrimeScript RT kit. Thermal cycling conditions for reverse transcription: at 37°C for 15 min and 85°C for 5 s. Then, SYBR Premix Ex Taq™ kit was applied to detect the EGLN1 mRNA expression in the synthesized cDNA. Thermal cycling conditions for qPCR: at 95°C for 5 min, 95°C for 30 s and 65°C for 45 s. U6 and GAPDH were used as internal references for the mRNA levels of miR-200a and EGLN1, respectively. Finally, the relative gene expression was analyzed and normalized using the $2^{-\Delta\Delta C_q}$ method. The primer design is as follows: miR-877: forward: 5'-GGC TAA CAC TGT CTG GTA ACG ATG-3' and reverse: 5'-GTG CAG GGT CCG AGG T-3', U6: forward: 5'-CTC GCT TCG GCA GCA CA-3' and reverse: 5'-AAC GCT TCA CGA ATT TGC GT-3', EGLN1: forward: 5'-GAA GGC GAA CCT GTA CCC C-3' and reverse 5'-TTC ATG CAC GGC ACG ATG TA-3', and GAPDH: forward: 5'-CGG AGT CAA CGG ATT TGG TCG TAT-3' and reverse: 5'-AGC CTT CTC CAT GGT GGT GAA GAC-3'.

Cell proliferation analysis

Transfected cells were harvested and seeded into 96-well plates at a density of 2×10^3 cells/well. Then the cells were continuously cultured in an incubator for 24, 48 and 72 h. Next, cell proliferation was measured by MTT assay at designated time points by adding 20 μ L of MTT solution to each well. After incubation at 37°C for 4 h, the DMEM with 10% FBS was carefully removed, and 150 μ L of DMSO was added to each well to dissolve formazan crystals. Subsequently, the absorbance at the wavelength of 490 nm of each sample was detected using the microplate reader. During colony formation assay, the transfected cells were counted and seeded in 6-well plates with 300 cells/well. The original medium was replaced with fresh complete medium every 3 days. After 14 days of culture at 37°C, the number of viable colonies was determined. Then the colonies were fixed with 100% methanol for 10 min and stained with crystal violet for 15 min at room temperature. Finally, images of colonies were captured using a digital camera and colonies were counted.

Cell apoptosis analysis

At 48 h after transfection, the apoptosis rate was quantified by gating propidium iodide (PI) and Annexin V-positive cells on a fluorescence activated cell sorting flow cytometer according to the instructions of the kit.

Detection via Western blotting

Total proteins were isolated from the cultured cells using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) and quantified according to the BCA protein assay kit. Then the proteins of equal quality (30 µg) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% gel), imprinted on a polyvinylidene fluoride (PVDF) membrane (Roche, Basel, Switzerland), and sealed at room temperature for 1 h in a sealing solution supplemented with 5% skimmed milk powder. Subsequently, the membrane was incubated with primary antibody at 4°C overnight and then with secondary antibody at room temperature for 2 h. After washing with tris buffered saline-tween (TBST), the immune complexes on the membrane were visualized using ECL solution.

Detection of luciferase activity

TargetScanHuman 7.2 was applied to predict the potential targets of miR-200a. Wild type (wt) and mutant (mut) 3'-UTRs of EGLN1 were amplified by Shanghai GenePharma Co., Ltd. and cloned into pMIR-REPORT Luciferase vectors to form pMIR-EGLN1-3'-UTR-wt and pMIR-EGLN1-3'-UTR-mut, respectively. Next, the cells were paved in a 24-well plates at a density

of 1.0×10^5 cells/well and co-transfected with wt or mut luciferase plasmid and miR-200a mimic or NC using Lipofectamine™ 2000. After 48 h of incubation, the cells were harvested, and the luciferase activity was evaluated using the dual luciferase reporter gene assay system. Besides, firefly luciferase activity was standardized as sea kidney luciferase activity.

Statistics

Data were expressed as mean \pm standard deviation and analyzed by SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Differences between two groups were analyzed by using the Student's t-test. Comparison between multiple groups was done using one-way ANOVA test followed by *post hoc* test (least significant difference). $P < 0.05$ indicated the differences were statistically significant.

Results

Effects of miR-200a down-regulation on HeLa cell proliferation

In order to study the potential function of miR-200a in CC malignant phenotypes, miR-200a inhibitor or NC was transfected into HeLa cells. RT-qPCR results confirmed that miR-200a was significantly down-regulated in HeLa cells transfected with miR-200a inhibitor compared with those transfected with NC ($p < 0.05$, Figure 1A). Next, the effects of miR-200a down-regulation on the proliferation of CC cells were detected *via* MTT assay. It was

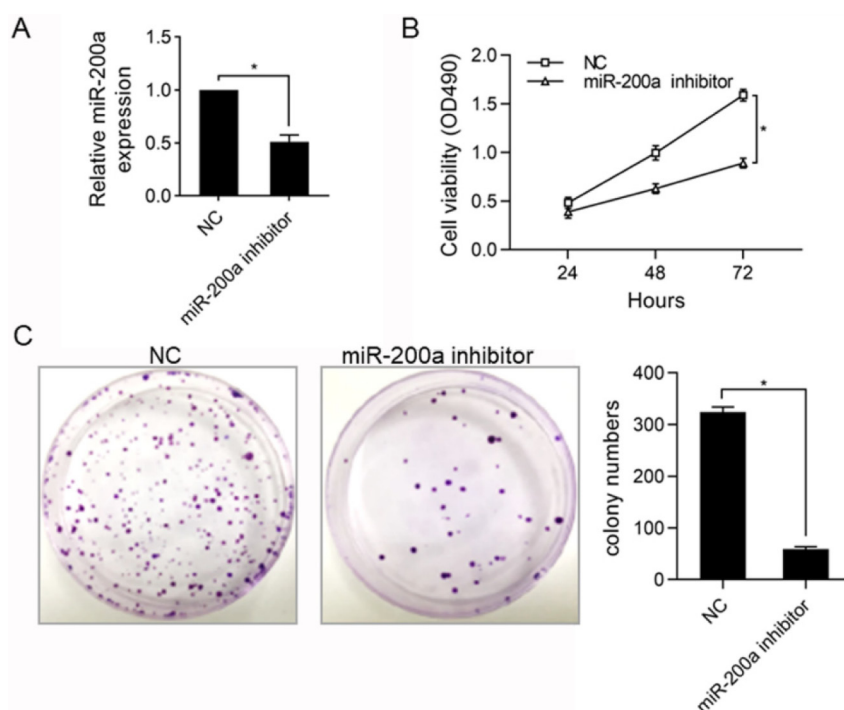


Figure 1. Effects of miR-200a down-regulation on HeLa cell proliferation. **A:** Detection results of the effects of miR-200a down-regulation *via* RT-qPCR. **B:** Detection results of the effects of miR-200a down-regulation on CC cell proliferation *via* MTT assay. **C:** Analysis results of the effects of miR-200a down-regulation on the cloning ability of CC cells *via* colony formation assay. (magnification: 10×) (* $p < 0.05$).

found that in comparison with cells in NC group, HeLa cells with down-regulated miR-200a showed weakened proliferation ability ($p < 0.05$ Figure 1B). At the same time, the effects of miR-200a down-regulation on CC cell proliferation were further analyzed and detected by colony forming assay. According to the results, in comparison with that in NC group, the clone number of HeLa cells with down-regulated miR-200a in miR-200a inhibitor group was markedly decreased ($p < 0.05$, Figure 1C). The above results suggest that miR-200a may play a tumor promoting role in the development of CC.

Effects of miR-200a down-regulation on HeLa cell apoptosis

Next, the role of miR-200a in regulating the apoptosis of CC cells was determined. Data from

flow cytometry revealed that the down-regulation of the miR-200a expression level obviously promoted cell apoptosis ($p < 0.05$, Figure 2).

EGLN1 was a direct target gene of miR-200a in HeLa cells

To further explore the molecular mechanism of miR-200a in CC, especially to understand its connection with some classical signaling pathways, TargetScan Release 7.2 (http://www.targetscan.org/vert_72/) was utilized to search for the putative target of miR-200a. Interestingly, highly conserved potential binding sites were observed in the 3'-UTR of EGLN1 mRNA (Figure 3A). It is well known that EGLN1 is an important regulatory factor of HIF, which is closely correlated with tumor proliferation, metastasis and invasion, so it was selected as

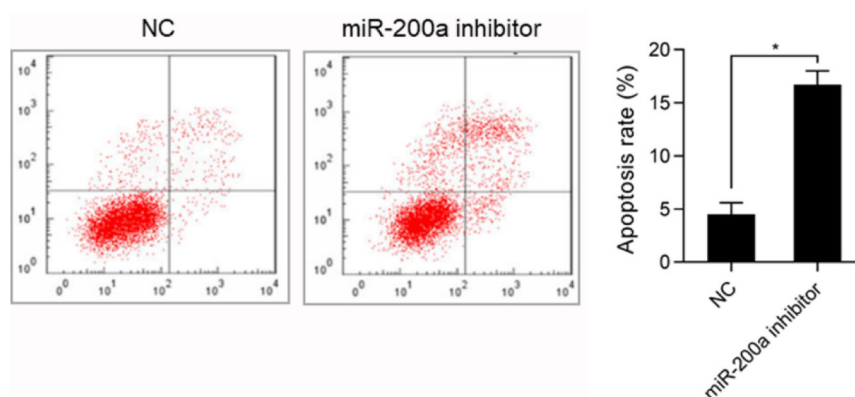


Figure 2. Effects of the down-regulation of miR-200a on HeLa cell apoptosis (* $p < 0.05$).

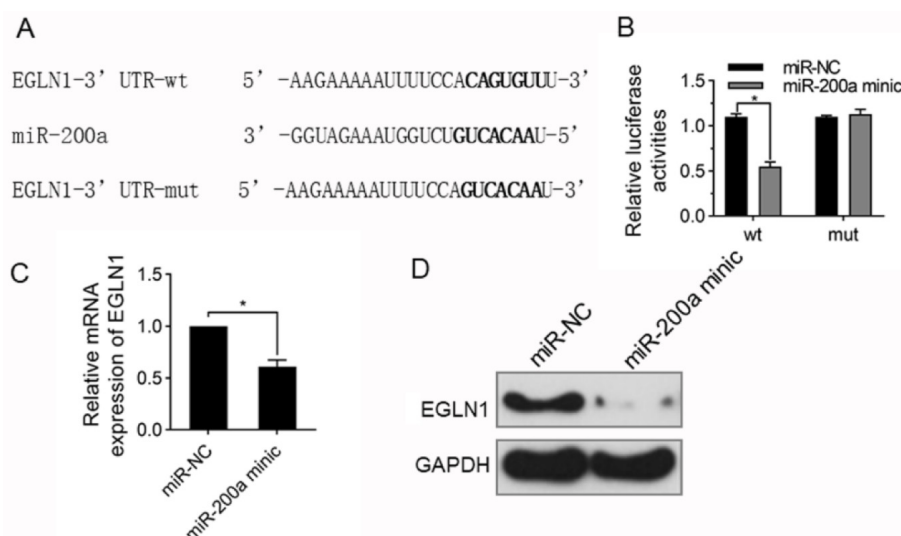


Figure 3. EGLN1 is a direct target gene of miR-200a in HeLa cells. **A:** Sequences of miR-200a binding site with EGLN1 3'-UTR-wt and EGLN1 3'-UTR-mut. **B:** HeLa cells are co-transfected with EGLN1 3'-UTR-wt/mut sequence of the miR-200a binding site with miR-200a mimic or miR-NC. After 48 h of transfection, the luciferase activity is measured. **C:** RT-qPCR analysis of the EGLN1 mRNA expression after HeLa cells are transfected with miR-200a mimic or miR-NC. **D:** Western blotting analysis of the EGLN1 mRNA expression after HeLa cells are transfected with miR-200a mimic or miR-NC (* $p < 0.05$).

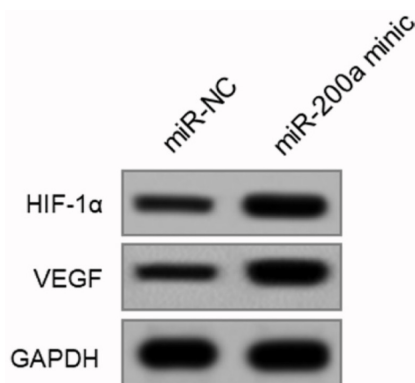


Figure 4. Effects of miR-200a on the HIF-1 α /VEGF signaling pathway detected *via* Western blotting. The results manifested that the protein expression levels of HIF-1 α and VEGF were increased after miR-200a overexpression

a potential target gene. Firstly, an EGLN1-3'-UTR-wt luciferase reporter gene containing a miR-200a putative binding site was constructed and it was found that the overexpression of miR-200a resulted in a prominent decrease in the luciferase activity of the wt reporter gene (Figure 3B). However, when the binding site of miR-200a was mutated by the reporter gene (mut), the overexpression of miR-200a could not exert its inhibitory role in HeLa cells. In addition, RT-qPCR and western blotting analyses also confirmed that the EGLN1 expression was inhibited in cells overexpressing miR-200a (Figure 3C-3D). Therefore, the target verification experiment validated that EGLN1 was the target of miR-200a in HeLa cells.

Effects of miR-200a on the HIF-1 α /VEGF signaling pathway

To explore the mechanism of miR-200a in inhibiting CC cell proliferation, the correlation between miR-200a and the HIF-1 α /VEGF pathway was mainly explored. Then western blotting was performed to detect the changes in the protein levels of HIF-1 α and VEGF. The results manifested that the protein expression levels of HIF-1 α and VEGF were increased after miR-200a overexpression (Figure 4), indicating that miR-200a activates the HIF-1 α /VEGF signaling pathway.

Discussion

Currently, CC is one of the most common gynecologic malignant tumors and its morbidity and mortality rates are increasing every year. High-risk HPV is the main factor leading to initial cervical cancer [2], but the exact mechanism in controlling the pathogenesis of CC is still unclear. Current treatment options for CC include surgery,

chemotherapy and radiotherapy, which, however, have low therapeutic value for patients with CC in advanced stages, maintaining their 5-year adverse survival rate at 30-50% [14]. In fact, recurrence and metastasis are the two most worrying causes leading to poor prognosis of CC patients. Therefore, it is urgent to further increase the information about the potential molecular mechanisms, so as to develop attractive targets for treating this disease.

MiRs are a group of endogenous conservative small non-coding RNAs (ncRNAs). They regulate the expression of target genes by binding to the 3'-UTR of target genes, thus inducing their degradation or translation to inhibit these genes [15]. More and more studies have shown that miRs act as crucial regulators of biological activities related to different cancers. Ji et al [16] researched the significance of miR-200a in the progression of CC and found that the expression of miR-139-5p in CC tissues and cell lines was decreased, and its lower level was related to lymph node metastasis. Bioinformatics analysis revealed that transcription factor 4 (TCF4) is a new target of miR-139-5p, and the repair of TCF4 is demonstrated to attenuate the tumor inhibitory activity of miR-139-5p in CC progression and restore normal Wnt/ β -catenin signaling, proving that miR-139-5p inhibits the Wnt/ β -catenin signaling by targeting TCF4 and is a crucial tumor suppressor in the pathogenesis of CC. The miR-200 family comprises a total of five members, namely miR-200a, miR-200b, miR-200c, miR-429 and miR-141, of which three members (miR-200a, miR-200b and miR-429) are located on chromosome 1p36 and the other two (miR-200c and miR-141) are located on chromosome 12p13 [17]. MiR-200a has been established as a key regulator of HSC activation in liver fibrosis. Ji et al [16] explored the expression of SIRT1 gene in miR-200a in controlling liver fibrosis and discovered that miR-200a regulates SIRT1/Notch1 expression during the activation and fibrosis of hepatic stellate cells. In this study, it was discovered that HeLa cells with miR-200a down-regulation showed weakened proliferation and clone formation abilities. Flow cytometry manifested that the down-regulation of the miR-200a expression level significantly promoted cell apoptosis. The above results suggest that miR-200a may play a tumor promoting role in the development of CC, and that miR-200a is extremely crucial to the proliferation of CC cells.

EGLN1 is an important regulatory factor of HIF, and HIF-1 α can induce tumor cells to become more aggressive and better exert its effects in tumor development [18]. According to reports, HIF-1 α regulates tumor angiogenesis by modulating the expression of various angiogenic factors

(including VEGF) under hypoxic stress [19]. VEGF contributes to the growth and metastasis of pancreatic cancer. Sun et al [20] investigated the role of miR-21 in the development of pancreatic cancer and proved that knocking down miR-21 will lead to a decrease in HIF-1 α expression, thus inhibiting VEGF expression. Besides, miR-21 inhibition also down-regulates other VHL downstream molecules including MMP-2 and MMP-9. The above results indicate that miR-21 regulates the malignant phenotype of pancreatic cancer at least in part by regulating VHL expression, so as to modulate the HIF-1 α /VEGF pathway or other downstream molecules, and suppressing miR-21 can increase the expression of VHL, thus regulating the HIF-1 α /VEGF pathway and the expressions of MMP-2 and MMP-9 and further inhibiting the proliferation, migration and invasion of pancreatic cancer cells. In this study, the molecular mechanism of miR-200a in CC was explored, and the putative target of miR-200a was searched using TargetScan. Inter-

estingly, highly conserved potential binding sites were observed in the 3'-UTR of EGLN1 mRNA. The luciferase reporter gene assay confirmed that EGLN1 was the direct target gene of miR-200a. Emphasis was put on the correlation between miR-200a and the HIF-1 α /VEGF pathway in the further exploration of the mechanism of miR-200a in inhibiting CC cell proliferation. The results suggest that miR-200a activates the HIF-1 α /VEGF signaling pathway.

Conclusions

To sum up, it can be concluded that miR-200a facilitates the proliferation of CC cells and activates the HIF-1 α /VEGF signaling pathway by targeting EGLN1.

Conflict of interests

The authors declare no conflict of interests.

References

- Hua F, Li CH, Gao YC, Li J, Meng F. Molecular mechanism and role of NF-kappaB in the early diagnosis of cervical cancer. *JBUON* 2019;24:720-8.
- Wei X, Zhou Y, Qiu J, Wang X, Xia Y, Sui L. Low expression of TUG1 promotes cisplatin sensitivity in cervical cancer by activating the MAPK pathway. *JBUON* 2019;24:1020-6.
- Hildesheim A, Wang SS. Host and viral genetics and risk of cervical cancer: a review. *Virus Res* 2002;89:229-40.
- Sakuragi N. Up-to-date management of lymph node metastasis and the role of tailored lymphadenectomy in cervical cancer. *Int J Clin Oncol* 2007;12:165-75.
- Wang N, Hou MS, Zhan Y, Shen XB, Xue HY. MALAT1 promotes cisplatin resistance in cervical cancer by activating the PI3K/AKT pathway. *Eur Rev Med Pharmacol Sci* 2018;22:7653-9.
- Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell* 2009;136:215-33.
- Lu J, Getz G, Miska EA et al. MicroRNA expression profiles classify human cancers. *Nature* 2005;435:834-8.
- Lorente-Cebrian S, Gonzalez-Muniesa P, Milagro FI, Martinez JA. MicroRNAs and other non-coding RNAs in adipose tissue and obesity: emerging roles as biomarkers and therapeutic targets. *Clin Sci (Lond)* 2019;133:23-40.
- Calin GA, Croce CM. MicroRNA signatures in human cancers. *Nat Rev Cancer* 2006;6:857-66.
- Ors-Kumoglu G, Gulce-Iz S, Biray-Avcı C. Therapeutic microRNAs in human cancer. *Cytotechnology* 2019;71:411-25.
- Yu T, Ma P, Wu D, Shu Y, Gao W. Functions and mechanisms of microRNA-31 in human cancers. *Biomed Pharmacother* 2018;108:1162-9.
- Li J, Liu Q, Clark LH, Qiu H, Bae-Jump VL, Zhou C. Deregulated miRNAs in human cervical cancer: functional importance and potential clinical use. *Fut Oncol* 2017;13:743-53.
- Wang L, Wang Q, Li HL, Han LY. Expression of MiR200a, miR93, metastasis-related gene RECK and MMP2/MMP9 in human cervical carcinoma--relationship with prognosis. *Asian Pac J Cancer Prev* 2013;14:2113-8.
- Tian Q, Wang L, Sun X, Zeng F, Pan Q, Xue M. Scopoletin exerts anticancer effects on human cervical cancer cell lines by triggering apoptosis, cell cycle arrest, inhibition of cell invasion and PI3K/AKT signalling pathway. *JBUON* 2019;24:997-1002.
- Hammond SM. An overview of microRNAs. *Adv Drug Deliv Rev* 2015;87:3-14.
- Ji X, Guo H, Yin S, Du H. miR-139-5p functions as a tumor suppressor in cervical cancer by targeting TCF4 and inhibiting Wnt/beta-catenin signaling. *Onco Targets Ther* 2019;12:7739-48.
- Feng X, Wang Z, Fillmore R, Xi Y. MiR-200, a new star miRNA in human cancer. *Cancer Lett* 2014;344:166-73.
- Semenza GL. Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. *Oncogene* 2010;29:625-34.
- Manalo DJ, Rowan A, Lavoie T et al. Transcriptional regulation of vascular endothelial cell responses to hypoxia by HIF-1. *Blood* 2005;105:659-69.
- Sun J, Jiang Z, Li Y, Wang K, Chen X, Liu G. Downregulation of miR-21 inhibits the malignant phenotype of pancreatic cancer cells by targeting VHL. *Onco Targets Ther* 2019;12:7215-26.