Pathology - Research and Practice 215 (2019) 594–599

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: www.elsevier.com/locate/prp

miR-495 promotes apoptosis and inhibits proliferation in endometrial cells via targeting PIK3R1

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ARTICLE INFO

Keywords:
Endometrial cancer
miR-495
PIK3R1
VEGF
Apoptosis
Proliferation

ABSTRACT

Endometrial cancer (EC) is a huge threat to women's health. The aims of this study were to investigate the role of microRNA (miR)-495 in the proliferation and apoptosis of EC cells *in vitro*. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed to detect the mRNA levels. In addition, dual-luciferase reporter assay was used to verified that PIK3R1 was a target of miR-495. After transfection with miR-495 mimics, Cell Counting Kit 8 (CCK-8) assay was performed to evaluate the cell viability of EC cells. The protein expression of PIK3R1, vascular endothelial growth factor (VEGF), Bcl-2, Bax, caspase 3 after transfection was analyzed using western blotting. Furthermore, cell apoptosis rate of EC cells was evaluated by flow cytometry. These results showed that miR-495 was significantly down-regulated in tumor tissues compared with the adjacent normal tissues, while PIK3R1 was up-regulated. The proliferation of the EC cells that were transfected with miR-495 mimics was markedly inhibited, and apoptosis was significantly promoted. In addition, down-regulated expression of PIK3R1, Bcl-2, VEGF expression and upregulated expression of Bax and caspase 3 expression were observed after transfected with miR-495 mimic. Together these findings indicated that miR-495 acts as a tumor suppressor gene by directly targeting PIK3R1 at the post-transcriptional level in EC cells *in vitro*.

1. Introduction

Endometrial cancer (EC) is of the most prevalent malignancies among females [1]. The past few years witnessed the increasing incidence and younger trend [2]. In 2016, the newly diagnosed cases augmented to 1.2 million [3]. Moreover, EC incidence paralleling with mortality may increase 50–100% in 2025 [4]. Although the prognosis of EC are good, the expected survival time is just 12–15 months [5]. Thence, investigating the mechanism of the occurrence and development of EC may provide new ideas for the diagnosis and treatment of EC.

MicroRNAs, the family of non-coding RNAs, play a crucial role in the pathology of multi-tumors including endometrial cancer [6–8]. For instance, miR-613 plays an active role in the proliferation and migration of colon cancer cells [9]. miR-200a and miR-200b promoted the cell proliferation of endometrial cancer [10]. miR-326 inhibits epithelial-mesenchymal transition (EMT) and metastasis of EC [11]. miR-495 plays as an oncogene and induces the proliferation and invasion of bladder cancer cells [12]. Meanwhile, miR-495 inhibited the cell growth and migration of EC [13]. Many reports demonstrated that miRNAs regulating gene founctions participated in cell proliferation, migration, and angiogenesis of cancers [14,15]. MiRNAs regulates gene expression via downregulating the mRNA levels of its target genes or posttranscriptionally suppressing via targeting the binding site of 3′-untranslated region (3′-UTR) [16]. miR-495 suppressed the proliferation and increased the apoptosis rate of gastric cancer cells via targeting Twist1 [17]. However, even though the roles of miR-495 have been investigated in various cancers [18–20], the potential mechanisms of miR-495 in regulating the proliferation and apoptosis of EC has not been fully elucidated.

In our study, the objective was to investigate the correlation between miR-495 and the proliferation and apoptosis of EC cells via PIK3R1. Here, we demonstrated that miR-495 expression in EC tumor was low, and miR-495 was able to inhibit cell proliferation and promote apoptosis in human EC HEC-1 A cells by suppressing PIK3R1 expression.

2. Materials and methods

2.1. Human tissue samples

Human EC tissues and adjacent normal tissues were collected from

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https://doi.org/10.1016/j.prp.2019.01.020
Received 21 November 2018; Received in revised form 21 December 2018; Accepted 12 January 2019
0344-0338/

30 patients diagnosed with EC at Renmin Hospital of Wuhan University from March 2016 to June 2017. All the patients received no chemotherapy and radiotherapy before. All patients have signed and offered the informed content. The study has been approved by the Ethics Committee of Renmin Hospital of Wuhan University. The samples were collected and quickly placed in liquid nitrogen and stored at −80 °C.

2.2. Cell culture

The human EC cell line (HEC-1 A) and normal uterine epithelial cells were obtained from the Shanghai Institute of Cell Biology of the Chinese Academy of Sciences (Shanghai, China). HEC-1 A and normal uterine epithelial cells were treated with DMEM (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Takara, Shiga, Japan), and 1% penicillin/streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) in the incubator at 37 °C under 5% CO_2.

2.3. Luciferase reporter assays

The target gene of miR-495 predicted in reference to TargetScan 7.1 (http://www.targetscan.org/vert_71/). Luciferase reporter assays was conducted to determine the luciferase activity. The fragments of PIK3R1 were cloned into pMIR-REPORT vector (Ambion; Thermo Fisher Scientific, Inc.). After this, cells were transfected with miR-495 mimics or miR-495 NC mimics. Then the cells were collected at 48-hour trnasfection. Subsequently, Dual-Luciferase Reporter Assay System (Promega, Fitchburg, WI, USA) was performed to evaluated the luciferase activity, which was normalized by *Renilla*.

2.4. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

mirVana miRNA Isolation kit (Ambion; Thermo Fisher Scientific, Inc.) was used to extract total RNA. NanoDrop2000 (Thermo Fisher Scientific, Inc.) was applied to evaluate the purity and concentration of RNA accroding to its manufacturer's instruction. RT-qPCR was performed to determine the mRNA levels with TaqMan microRNA PCR kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) under the following conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 1 min, 60 °C for 40 s, 72 °C for 30 s and 72 °C for 1 min. U6 and GAPDH respectively served as internal control of MicroRNA and PIK3R1, VEGF, Bcl-2, Bax and Caspase3. The primers were synthetized and purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Subsequently, the mRNA levels were determined and analyzed with the $2^{-\Delta\Delta Ct}$ method [21]. The sequences of the primers were listed in Table 1.

Table 1
Oligonucleotide primers used for Q-PCR.

Name	Primer sequence
miR-495	Forward 5'-TCCGATTCTTCACGTGGTAC-3'
	Reverse 5'-GTGCAGGGTCCGAGGT-3'
U6	Forward 5'-CTCGCTTCGGCAGCACA-3'
	Reverse 5'-AACGCTTCACGAATTTGCGT-3'
PIK3R1	Forward 5'-GCTTTGCCGAGCCCTATAA-3'
	Reverse 5'-ACATTGAGGGAGTCGTTGTG-3'
Bax	Forward 5'-CACCAGCTCTGAACAGATCATGA-3'
	Reverse 5'-TCAGCCCATCTTCTTCCAGATGT-3'
VEGF	Forward 5'-AACTTTCTGCTGTCTTGGGT-3'
	Reverse 5'-TCTCGATTGGATGGCAGTA-3'
Bcl-2	Forward 5'-CACCCCTGGCATCTTCTCCTT-3'
	Reverse 5'-AGCGTCTTCAGAGACAGCCAG-3'
Caspase3	Forward 5'-AACTGGACTGTGGCATTGAG-3'
	Reverse 5'-ACAAAGCGACTGGATGAACC-3'
GAPDH	Forward 5'-GGAGCGAGATCCCTCCAAAAT-3'
	Reverse 5'-GGCTGTTGTCATACTTCTCATGG-3'

2.5. miRNA transfection

Cells were seeded in 24-well plates and cultured to reach 70% confluence in the atmosphere humidified with 5% CO_2 at 37 °C. miR-495 mimics and miR-495 NC mimics were purchased from GenePharma Co. (Shanghai, China). Lipofectamine® 3000 transfection reagent (Invitrogen, Inc.) was applied to transfect cells with miR-495 mimics and miR-495 NC mimics. The cells were divided into three groups: i) control group (CON); ii) negative control group (NC) and iii) miR-495 mimics group (mimics). Then cells were collected and used for other experiment.

2.6. Cell counting Kit 8 (CCK-8) assay for cell viability

The trnasfected cells were planted in 96-well plates (2 × 103 cells/well) and incubated for 0, 12, 24 and 48 h. Then the plates were supplemented with 10 μl CCK-8 solution (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). 2 h later, the absorbance at the wavelength of 490 nm was detected Microplate Reader (Epoch; BioTek Instruments, Inc., Winooski, VT, USA).

2.7. Flow cytometry

Cells were digested, collected with 0.25% trypsin (Takara Bio, Inc., Otsu, Japan), and centrifuged at 1000 rpm for 5 min. Later, cells were stained with 5 μl annexin V-fluorescein isothiocyanate (FITC) and 10 μL of propidium iodide (PI) in shade at 25 °C for 5 min. Then the stained cells were checked with FACS Calibur system (BD Biosciences,USA) and evaluated with FlowJo software (TreeStar, Ashland, OR, USA). All independent experiments performed in triplicate.

2.8. Western blot analysis

RIPA lysis buffer (Thermo Fisher Scientific, Inc.) was used to extraxted the total protein from HEC-1 A. The concentration and purity of protein were examined with Bradford assay (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The protien was isolated with SDS-PAGE and transfered to polyvinylidene fluoride (PVDF) membrane (EMD Millipore, Billerica, MA, USA). Then the membrane were blocked with 5% non-fat milk. After this, the membranes were incubated with anti-PIK3R1 (SAB1306396, 1: 1000), anti-vascular endothelial growth factor (VEGF; SAB1306008, 1: 1000), anti-Bcl-2 (SAB4500003, 1: 1000), anti-Caspase3 (C9598, 1: 3000), and anti-Bax (C8487, 1: 500) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Then the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (ab97051, 1: 2,000, Abcam, MA, USA). The protein level was captured with ECL kit and anylyzed with ImageJ.

2.9. Data analysis

The data were presented as the mean ± SD. All data were statistically analyzed using Graphpad Prism 4.0 (GraphPad Software, Inc., La Jolla, CA, USA). Student's *t*-test was used to anylyze the differences between two groups. One way analysis of variance followed by the Student-Newman-Keuls test was applied to evaluate the differences among multi groups. $P < 0.05$ was considered as statistical significance.

3. Results

3.1. miR-495 expression is downregulated in tumor tissues

As miR-495 has been reported to be down-regulated in EC [13], RT-qPCR was first performed to analyze the miR-495 expression level in EC tumor and normal tissues. In Fig. 1, miR-495 expression in tumor tissues was significantly lower than the normal tissues ($P < 0.05$). The

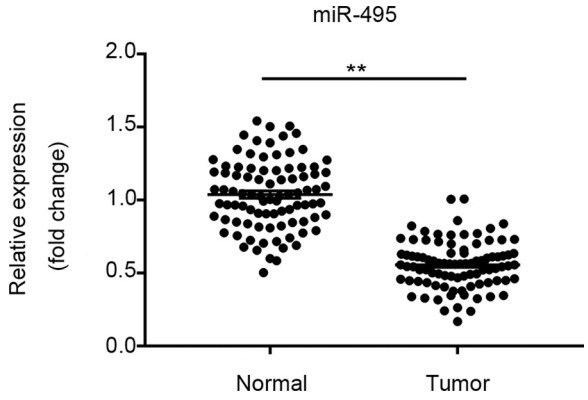

Fig. 1. Relative miR-495 expression in tumor and normal tissue.
Reverse transcription-quantitative polymerase chain reaction was performed to calculate the expression of miR-495. The results showed that miR-495 expression level was significantly lower in tumor tissues than that in the normal. Data are presented as the mean ± standard deviation. **P < 0.01.

values are expressed as fold changes from the indicated control. These results demonstrated that miR-495 is a miRNA that is involved in the progression of endometrial cancer. The mechanism underlying the regulation of EC by miR-495 requires to be further investigated.

3.2. miR-495 reduces PIK3R1 expression by binding to its 3′-UTR

PIK3R1 was a potential target for miR-495 according to TargetScan (Fig. 2A, http://www.targetscan.org/vert_71/). The dual-luciferase reporter assay was performed to elucidate whether miR-495 directly regulate PIK3R1. As shown by TargetScan, miR-495 was able to bind to nucleotide positions 3687–3693 of the 3′-UTR of PIK3R1. In Fig. 2B, transfection with miR-495 mimics significantly reduced luciferase activity in the cells that were transfected with the wild-type constructs compared with the negative control (P < 0.05). By contrast, there was no significant change observed in the cells that were transfected with the mutant reporters compared with the negative control. This result demonstrated that miR-495 could directly target PIK3R1 via binding to its 3'-UTR.

In addition, the overexpression of miR-495 significantly decreased PIK3R1 expression at the levels of mRNA (P < 0.05, Fig. 6A) and protein (P < 0.05, Fig. 6F and G). It was further indicated that miR-495 induced a decrease in PIK3R1 mRNA and protein expression

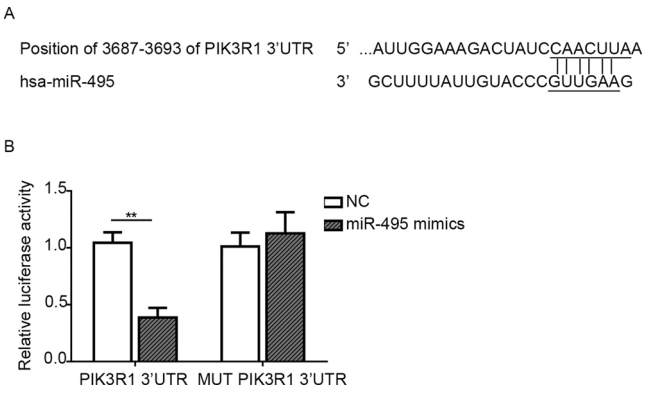

Fig. 2. Relative luciferase activity in the dual-luciferase reporter assays.
A: miR-495 regulated PIK3R1 via the binding site of miR-495 in PIK3R1 3′UTR. B: The luciferase activity in the miR-495 mimics transfection group was significantly lower than the negative control. In the mutant group, there were no apparent changes. It was indicated that miR-495 was able to target PIK3R1 by complementary binding with its 3′-untranslated region. Data are presented as the mean ± standard deviation. **P < 0.01. PIK3R1, phosphoinositide-3-kinase regulatory subunit 1.

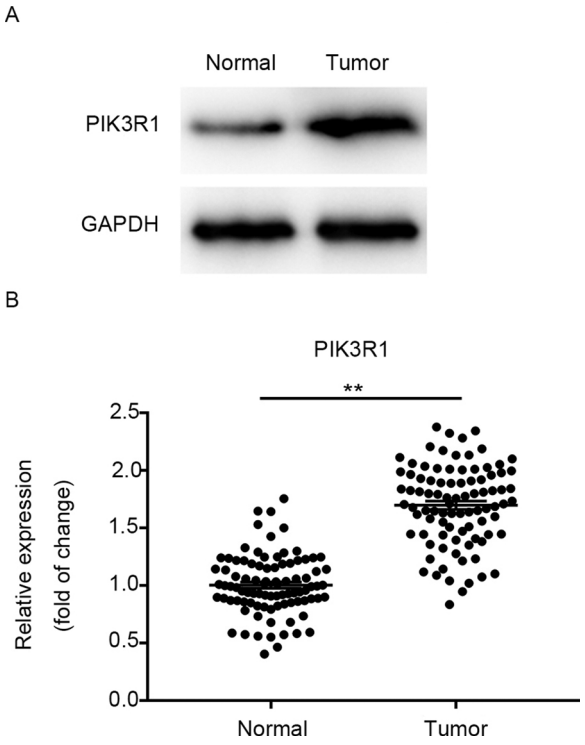

Fig. 3. Relative PIK3R1 expression in normal and tumor tissues was assayed using reverse transcription-quantitative polymerase chain reaction and western blotting.
A: In tumor tissues, the protein level of PIK3R1 was significantly higher than that in normal tissues. B: the mRNA and protein expression were significantly upregulated compared with the adjacent normal tissues. The values are expressed as fold changes from the normal tissues. Data are presented as the mean ± standard deviation. **P < 0.01. PIK3R1, phosphoinositide-3-kinase regulatory subunit 1.

through binding to the PIK3R1 3′-UTR.

3.3. PIK3R1 expression is upregulated in tumor tissues

PIK3R1 expression level in normal and tumor tissues was assayed by RT-qPCR and western blotting, respectively. In tumor tissues, PIK3R1 mRNA and protein expression (P < 0.01, Fig. 3A and B) were significantly upregulated compared with the adjacent normal tissues.

3.4. Up-regulated miR-495 expression suppresses the cell viability of EC tumor cells

To elucidate the biological roles of miR-495 in endometrial cancer, miR-495 mimic was constructed, and transfection was performed to over-express miR-495. After transfection, CCK-8 assay was used to detect cell viability. As shown in Fig. 4, the cell viability in the miR-495 mimics group was significantly reduced compared with the negative control and control group (P < 0.05). There was no significant difference between the control group and negative group. These findings indicated that miR-495 had the potential to repress the viability of EC tumor cells.

3.5. Overexpression of miR-495 leads to apoptosis of HEC-1 A cells

To investigate the effect of miR-495 on the apoptosis of HEC-1 A EC cells, annexin V-FITC/PI staining was performed. As shown in Fig. 5A and B, the apoptosis rate of the miR-495 mimic group was 18.91%. The apoptosis rates of the control group and the negative control group were 7.97% and 7.73%, respectively. Compared with the control and

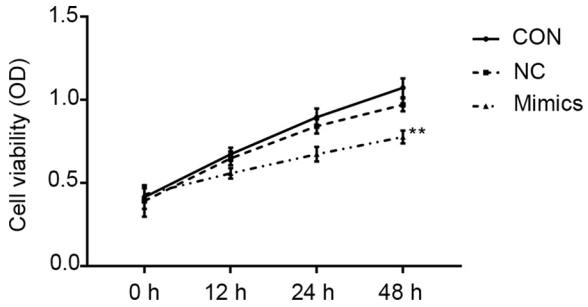

Fig. 4. Viability of HEC-1A cells after transfection with miR-495 mimics.
Cell Counting Kit-8 assay was used to detect cell viability. miR-495 mimic was able to dramatically inhibit the proliferation of HEC-1 A cells. There was no significant difference between the negative control group and control group. Therefore, the results indicated that miR-495 was able to inhibit the proliferation of HEC-1 A endometrial cancer cells. Data are presented as the mean ± standard deviation. **P < 0.01. miR, microRNA.

NC, the total apoptosis rate was increased significantly in the miR-495 mimic group (P < 0.05). By contrast, there was no marked difference between control and NC. These findings indicated that miR-495 might be involved in the apoptosis of EC cells.

3.6. Elevated Bax and caspase 3, and inhibited Bcl-2, VEGF and PIK3R1 expression after miR-495 mimic transfection

To assess the expression of apoptosis-related proteins, western blotting was performed, and the expression of PIK3R1, Bcl-2 and VEGF was analyzed. PIK3R1 has been indicated to be the target of miR-495 (http://www.targetscan.org/vert_71). Bcl-2, Bax, Caspase3 participate in apoptosis, and VEGF is involved in the formation of blood vessels [22].

As shown in Fig. 6A–C, the mRNA expression of VEGF, Bcl-2 and PIK3R1 in miR-495 mimics group decreased significantly compared with the negative control group and control group (P < 0.05), while the expression of Caspase3 and Bax increased dramatically compared (Fig. 6D and E, P < 0.05). The protein expression levels of PIK3R1, VEGF, Bcl-2,Bax, Caspase3 was inconsistence with the mRNA levels (Fig. 6F and G) These results suggested that miR-495 might affect EC progression by regulating the expression of apoptosis-related proteins.

4. Discussion

Endometrial cancer has attracted attention due to its burdens on families and the society [23]. Conventional treatments, including chemotherapy and radiotherapy, are associated with adverse side effects [24]. The prognosis of endometrial carcinoma is dependent on many factors, including tumor stage and grade [25]. Recently, miRNAs have received increasing attention via its widely diagnosis and therapy usage. miR-495, as a family of miRNAs, was reported to have a low expression in endometrial cancer tissues [26].

PIK3R1 is the most frequently mutated canonical cancer-related genes [27]. PIK3R1 is a member of phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway, which is constitutively activated in almost all cancer types [28]. PIK3R1 expression was higher in non-serous ovarian cancer than that in the serous epithelial ovarian cancer [29]. Forkhead Box A1 could interfere the development of hepatocellular carcinoma via inhibiting PIK3R1 expression [28].

In the present study, a low expression of miR-495 results in high expression of PIK3R1 and high PI3K/Akt signaling activity in HEC-1 A cells. We hypothesized whether up-regulating miR-495 expression using miR-495 mimics could reverse this effect and lead to a low PIK3R1 expression. Then we found that the expression of PIK3R1 was significantly decreased after the treatment of miR-495 mimics. Moreover, up-regulated miR-495 inhibited the proliferation of HEC-1 A

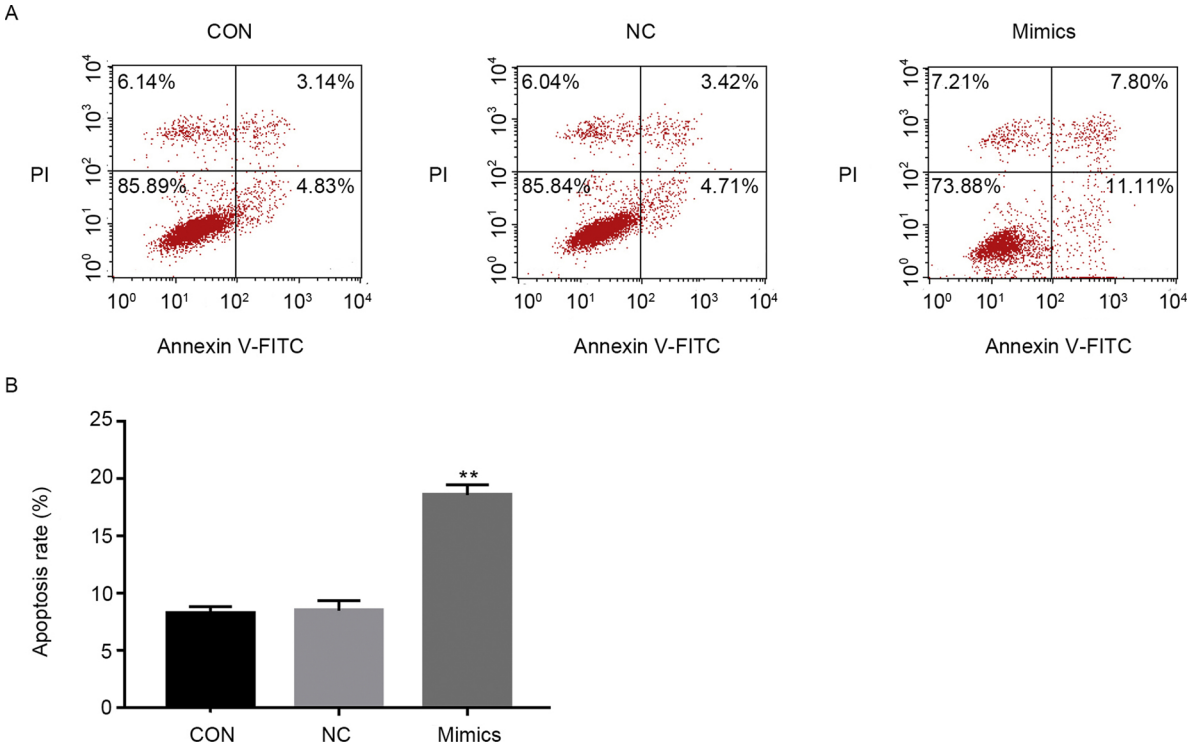

Fig. 5. Detection of apoptosis rate of HEC-1 A cells using annexin V- fluorescein isothiocyanate/propidium iodide staining assay.
A-B: After transfection, the apoptosis rate in the mimics group was up-regulated significantly. By contrast, there was no significant difference between the negative control group and control group. It was indicated that miR-495 was able to promote the apoptosis of HEC-1 A endometrial cancer cells. The apoptosis rate of the treatment groups are as follows: control group, 7.97%; negative control, 8.13%, and mimics group, 18.91%. Data are presented as the mean ± standard deviation. **P < 0.01.

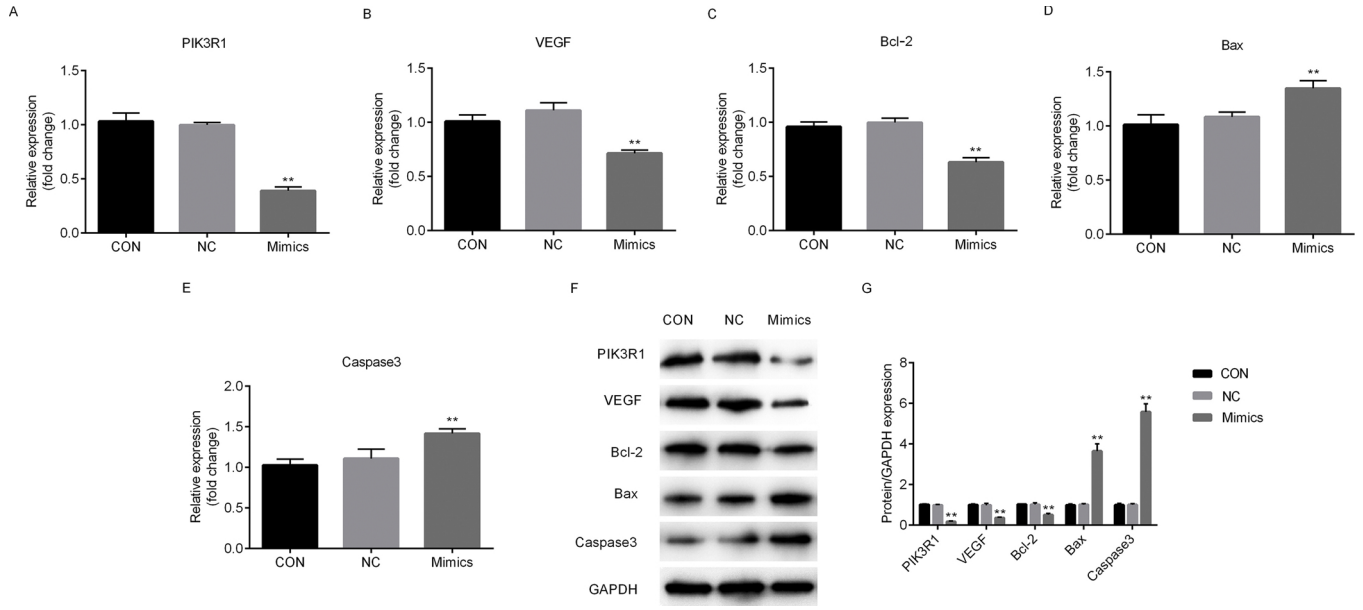

Fig. 6. Western blotting of the expression of apoptosis-related proteins.
A-E: transfection with miR-495 mimics significantly downregulated the mRNA levels of PIK3R1, Bcl-2 and VEGF and upregulated the mRNA levels of Bax and caspase 3. F: The protein levels of PIK3R1, Bcl-2 and VEGF were significantly decreased after the treatment of miR-495 mimics compared with CON and NC groups, while the protein levels of Bax and caspase 3 were increased. G: Quantitative analysis of F. **P < 0.01 vs. CON and NC. Values are expressed as fold changes from the indicated control and negative control. Data are presented as the mean ± standard deviation. CON, control; NC, negative control. PIK3R1, phosphoinositide-3-kinase regulatory subunit 1; VEGF, vascular endothelial growth factor.

endometrial cancer cell HEC-1 A and promoted cell apoptosis *in vitro*. This finding was in accordance with previous studies, which demonstrated miR-495 as a tumor suppressor gene that was down-regulated in human endometrial carcinoma [26].

Tumor treatment is being directed toward the proliferation and apoptosis of tumor cells. Investigating tumor cell apoptosis provides new ideas for further exploring the mechanism of endometrial cancer and options for treatment [30,31]. Numerous enzymes and proteins play important roles during cell apoptosis. Bcl-2 could promote the survival and inhibit the apoptosis of tumor cells [32]. On the contrary, the proapoptotic protein Bax combined with Caspase3 could promote the apoptosis of tumor cells.

The expression level of anti-apoptotic and pro-apoptotic proteins determines whether apoptosis is initiated [33]. Under apoptotic stimuli, free Bax forms oligomers, resulting in the release of cytochrome C from the intermembrane space of the mitochondria into the cytoplasm. Taken this into consideration, we hypothesize that transfection of miR-495 could promote the production of proapoptotic proteins, Bax and Caspase3, and reduce the production of antiapoptotic Bcl-2 proteins, which results in the initiation of apoptosis.

Based on the literature, VEGF is an important factor in the formation of new blood vessels in tumor cells [34]. At present, many studies attempt to reduce VEGF expression and receptor function, thereby blocking or inhibiting tumor growth [35]. The overexpression of VEGF has been observed in many types of tumors, which may indicate their involvement in pathological angiogenesis [36]. Taken together, our findings indicated that the upregulation of miR-495 decreased VEGF expression and inhibited tumor growth, which is essential for tumor treatment.

The aim of the paper was to investigate whether miR-495 inhibits the proliferation of endometrial cancer cells by targeting PIK3R1 *in vitro*. Further research should examine the mechanism of miR-495 in the regulation of cell proliferation and apoptosis in other EC cell lines. Additionally, the associated signaling pathways that participate in the development of EC, including PI3K/Akt, should also be further investigated.

In conclusion, this study demonstrated that miR-495 expression was low in EC tumor tissues, while its target PIK3R1 was elevated at the levels of mRNA and protein. In addition, up-regulated miR-495 expression inhibited cell proliferation and promoted apoptosis in HEC-1 A cells. Furthermore, the overexpression of miR-495 leads to increase in Bax and caspase 3 expression and decreases in Bcl-2 and VEGF expression. These findings indicated that miR-495 might be a novel candidate for the treatment of EC.

Conflict of interest

There is no conflict of interest in this study.

Acknowledgment

The present study was funded by National Natural Science Foundation of China (81801421).

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