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Original Article

MiR-100-5p, miR-199a-3p and miR-199b-5p induce autophagic death of endometrial carcinoma cell through targeting mTOR

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Abstract: Objective: The study aimed to explore the association between three miRNAs (miR-100-5p, miR-199a-3p and miR-199b-5p) and mTOR induced autophagy in EEC cells. The expression of three miRNAs and autophagy-related genes *Beclin1* and *LC3* in Ishikawa and KLE cells were detected. Methods: The effects of three miRNAs on proliferation and apoptosis in EEC cells were analyzed in KLE and ISK cells transfected with miR-100-5p, miR-199a-3p and miR-199b-5p mimics and inhibitors. Quantitative real-time polymerase chain reaction (qRT-PCR), CCK-8 method, flow cytometry and luciferase reporter assay were used to assess the effects of three miRNAs on cell viability, proliferation, apoptosis, and autophagy. Results: We found an increased expression of *Beclin1* and *LC3* in Ishikawa and KLE cells transfected by miR-100-5p, miR-199a-3p and miR-199b-5p mimics compared with NC (P<0.05). Additionally, Ishikawa and KLE cells transfected with miR-100-5p, miR-199a-3p and miR-199b-5p mimics grew more slowly than mock and mimics control (P<0.05); we also found an increased apoptosis incidence of Ishikawa cell transfected with miR-100-5p, miR-199a-3p and miR-199b-5p mimics (P<0.05). Finally, luciferase reporter results showed miR-100-5p, miR-199a-3p and miR-199b-5p were all down-regulated the luciferase activity in cells transfected with miRNA mimics compared with mock. All results suggested that miR-100-5p, miR-199a-3p and miR-199b-5p may induce the autophagic death of EEC cell through targeting mTOR. Conclusions: Our results suggested that miR-100-5p, miR-199a-3p and miR-199b-5p may induce the autophagic death of EEC cell through targeting mTOR.

Keywords: mTOR, autophagy, microRNA (miRNA), *Beclin1*, *LC3B*, endometrial endometrioid adenocarcinoma (EEC)

Introduction

Endometrial carcinoma (EC) is the sixth most common cancer in women worldwide, and its incidence is rapidly increasing in China [1]. In some developed areas of China, the incident of EC has exceeded cervical cancer and is being the leading form of female reproductive system cancer [2]. Endometrial endometrioid adenocarcinoma (EEC) is the main form of EC, which accounts for more than 80% of all EC patients [3]. Alteration of some genes has been connected with etiology of EEC, and may also serve as targets for drug delivery and therapy [4, 5]. Further knowledge of the molecular signaling pathways actively involved in the occurrence and development of EEC may provide new targets for better therapeutic strategies than those that are currently available.

mTOR gene is located on chromosome 1 and encodes for the serine/threonine kinase, which was involved in many cellular pathways including autophagy signal pathway [6]. It has been proven that phosphorylation of mTOR kinase was associated with the grade and stage of EEC [7]. In recent years, the mTOR induced autophagy signal pathway have thus become of interest in research into the pathogenesis of EEC. It is of particular interest that deregulation of some micro RNAs (miRNAs) coexists with increased expression of mTOR kinase in EEC. miRNAs are small, non-coding RNA molecules composed of 19 to 25 nucleotides which could down-regulate gene expression [8]. Torres et al. has pointed out that miR-100, miR-99a and miR-199b were down-regulation in EEC tissues and plasma [9]. Wu et al. has further proven that miRNA-199a-3p could regulate prolifera-

tion of EEC by targeting mTOR [10]. Based on these results, we intend to identify the association between three miRNAs (miR-100-5p, miR-199a-3p, miR-199b-5p) and mTOR induced autophagy in EEC cells.

Autophagy is a very complicated metabolism process regulated by several autophagy-related genes. *Beclin1* is the first identified mammalian autophagy mediated gene involved in autophagic initiation [11]. *LC3* is ubiquitously distributed and essential to the formation of autophagosomes in mammalian cells [12]. In this study, we examined the expression of three miRNAs (miR-100-5p, miR-199a-3p and miR-199b-5p) and autophagy-related genes *Beclin1* and *LC3* in Ishikawa and KLE cells, through real-time quantitative PCR, CCK-8 assay, flow cytometer and luciferase reporter assay. To our knowledge, this study is the first to investigate the expression of miR-100-5p, miR-199a-3p, miR-199b-5p and their relationship with mTOR induced autophagy in EEC cells.

Materials and methods

Cell culture

Human EEC Ishikawa (well differentiated) and KLE (poorly differentiated) cell lines were purchased from Biofavor Biotech Co. Ltd, Wuhan, China. Both of cell lines were grown in Dulbecco modified Eagle medium (HyClone, Uath, USA) containing 10% fetal bovine serum (HyClone) and the penicillin-steptomycin solution (100 U/ml penicillin; 100 μg/ml streptomycin; HyClone) in a humidified incubator with 5% CO_2 at 37°C.

Transfection

Before transfection, Ishikawa and KLE cells were seeded in 24-well plates and grown to 80% confluence. Using Lipofectamine 2000 Transfection Reagent (Thermo Fisher, Waltham, USA), the cells were transfected with miR-100-5p, miR-199a-3p, miR-199b-5p miRNA mimics, inhibitor and NC mimics/inhibitors (Shanghai GenePharma Co. Ltd, Shanghai, China), as well as normal control, in accordance with the instructions provided by Thermo Fisher. The Ishikawa cells were treated with 10-ng/mL rapamycin (Sigma-Aldrich, St Louis, MO). Cells were collected at different time points for analysis after transfection.

RNA isolation and reverse transcription of total RNA

Isolation of total RNA was used the TRIzol Plus RNA Purification Kit (Invitrogen) according to manufacturer protocol. Total RNA (500 ng) was reverse transcribed using Precision nanoScript Reverse Transcription kit (Primer Design) according to manufacturer protocol.

Real-time qPCR for Beclin1e and LC3 expression

Amplification of *Beclin1e*, *LC3* and reference gene *GADPH* was performed in 20 μL reactions. Each reaction consisted of: 1 μL template, 10 μL SuperReal PreMix Plus (SYBR Green), 0.6 μL Forward primer, 0.6 μL Reverse primer and 7.8 μL ddH_2O. Reactions were carried out in Rotor Gene 6000 2-plex HRM thermo cycler (Corbett Research) using the following protocol: 95°C for 15 min and 45 quantification cycles of 95°C for 10 sec, 60°C for 20 sec, and 72°C for 30 sec.

miRNA reverse transcription and real-time qPCR

miRNA was reversed using miRcute Plus miRNA First-Strand cDNA Synthesis Kit (TIANGEN, Beijing, China) according to manufacturer protocol. qPCR reactions for miRNA expression analysis were prepared as follows: 1 μL miRNA first strand cDNA, 5 μL 2× miRute miRNA premix, 0.2 μL Forward primer, 0.2 μL Reverse primer and 3.6 μL Rnase free ddH_2O. All qPCR reactions were performed in duplicates in ViiA7 Real-Time PCR System (Applied Biosystems) using the following PCR protocol: 94°C for 2 min, 5 cycles of 94°C for 20 sec, 65°C for 30 sec, and 72°C for 34 sec and 40 cycles of 94°C for 20 sec and 60°C for 34 sec.

Cell viability assay

Cell viability was performed using the Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Japan). The MC3T3-E1 cells (1×10^4 per well) were plated in 96-well plates and incubated overnight, then they were treated with DEX at various concentrations for 24 h. After treatment, 10 μl of CCK-8 solution were added to 100 μl of culture medium and the plate was incubated for 2 h to induce the water-solution production of formazan dye. The absorbance values at 450 nm were measured using a microplate reader (Bio-Rad, Hercules, CA, USA).

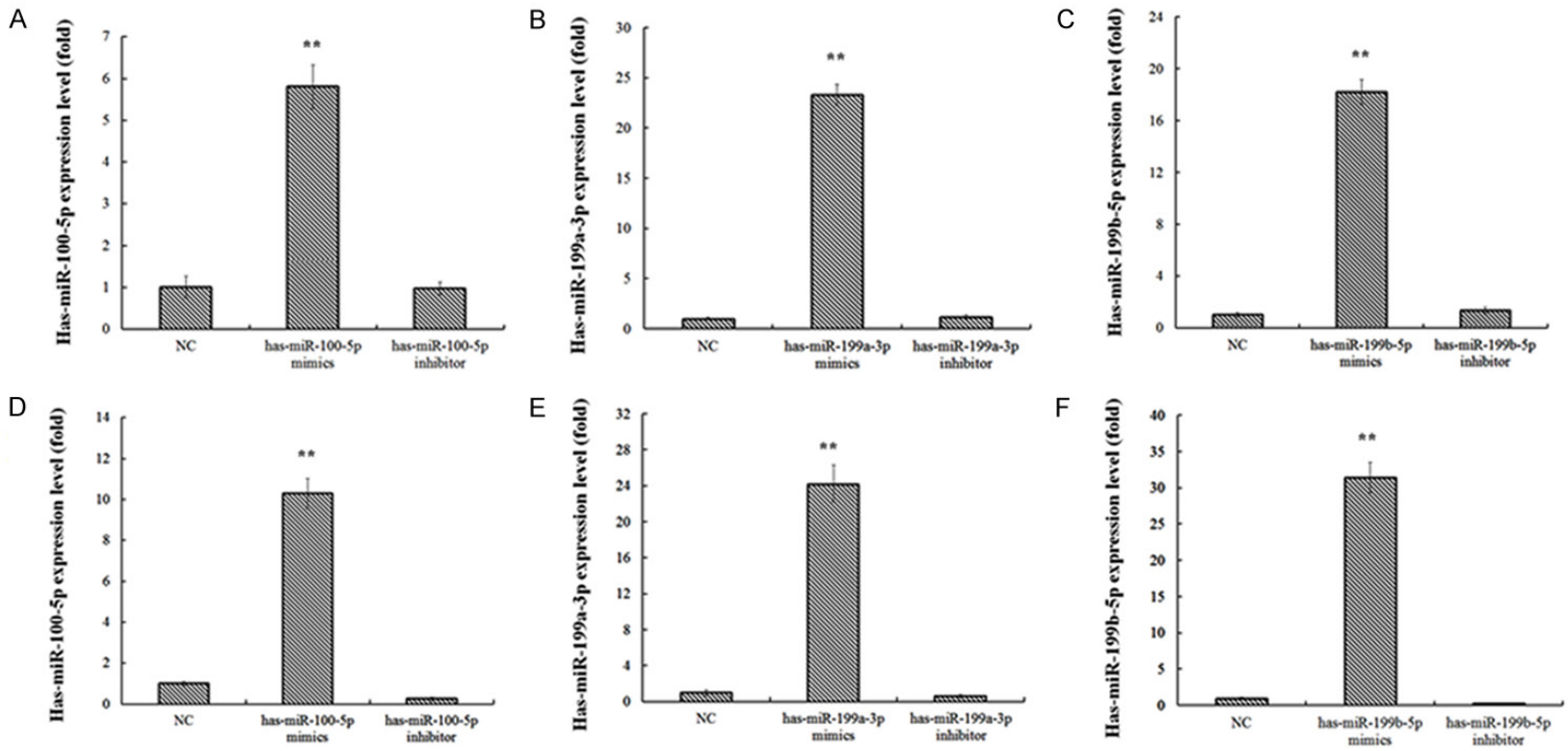


Figure 1. Overexpression of miR-100-5p, miR-199a-3p and miR-199b-5p in Ishikawa and KLE cells. A-C. Ishikawa cell; D-F. KLE cell, $^{**}P<0.01$.

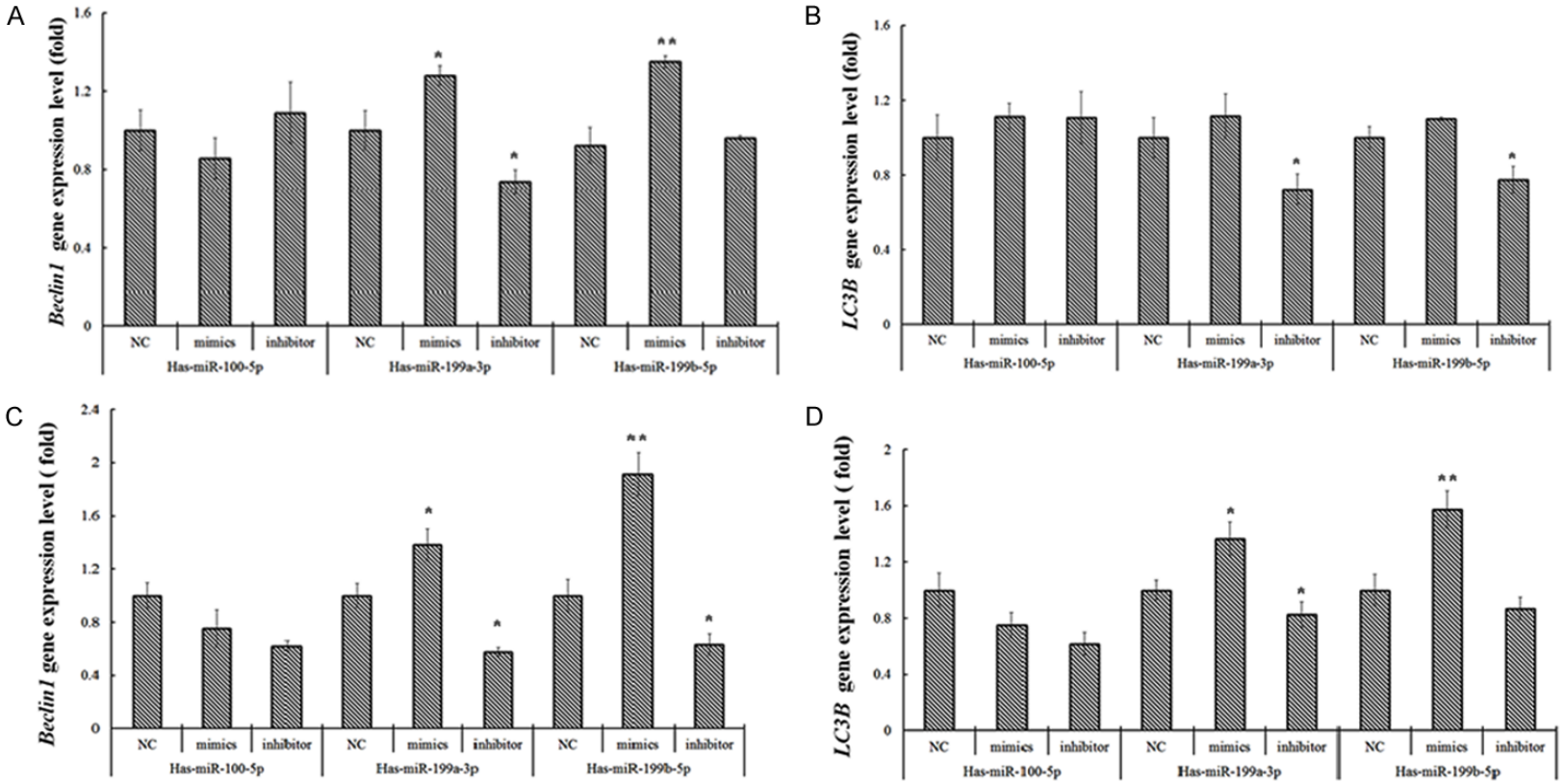


Figure 2. *Beclin1* and *LC3B* mRNA expression in Ishikawa and KLE cells. A, B. Ishikawa cell; C, D. KLE cell, **P*<0.05; ***P*<0.01.

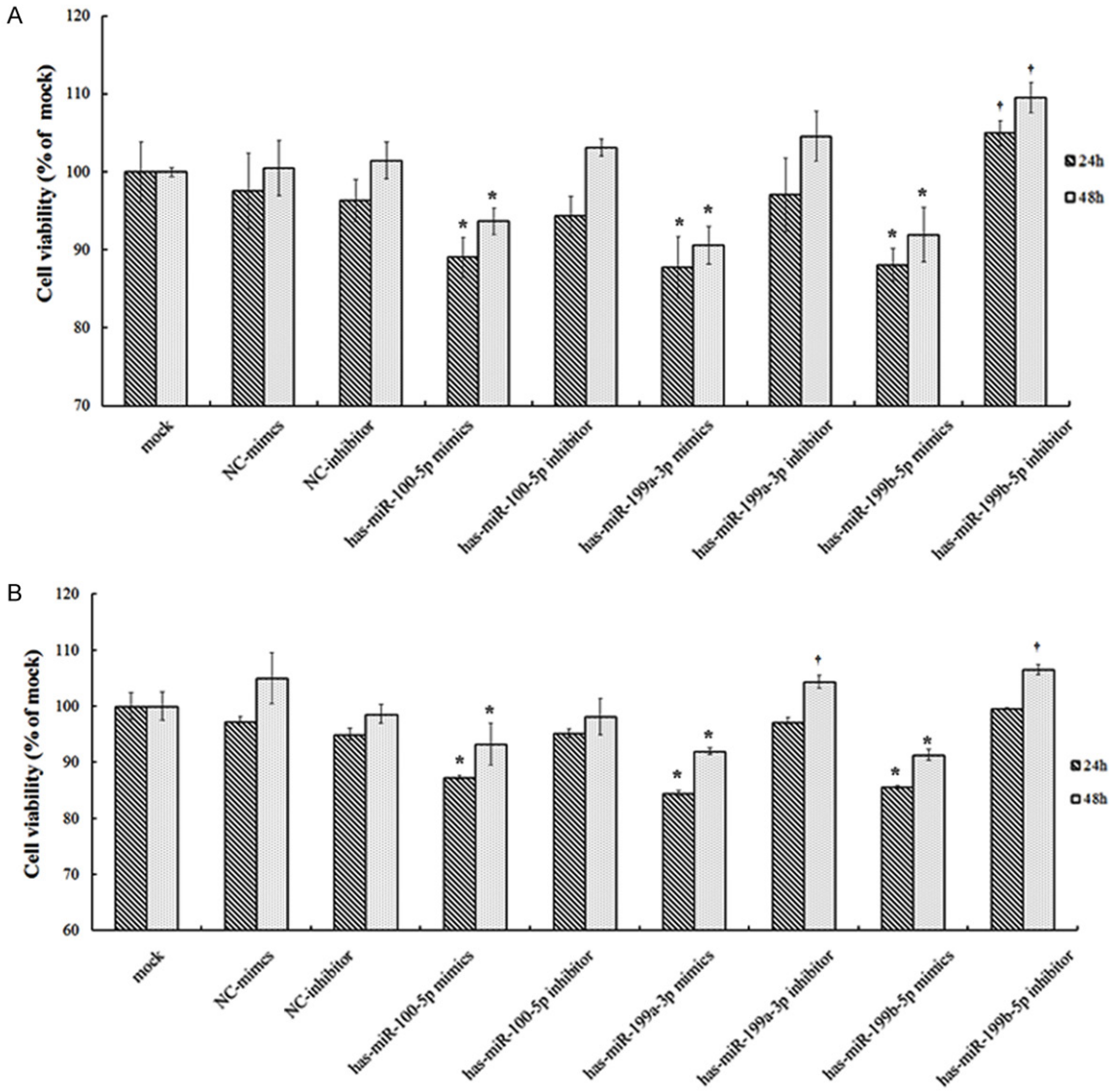


Figure 3. Proliferation of Ishikawa and KLE cells were inhibited by miR-100-5p, miR-199a-3p and miR-199b-5p. (A) Ishikawa cell (B) KLE cell. **P*<0.05 for 24 h group; †*P*<0.05 for 48 h group.

Analysis of cell apoptosis

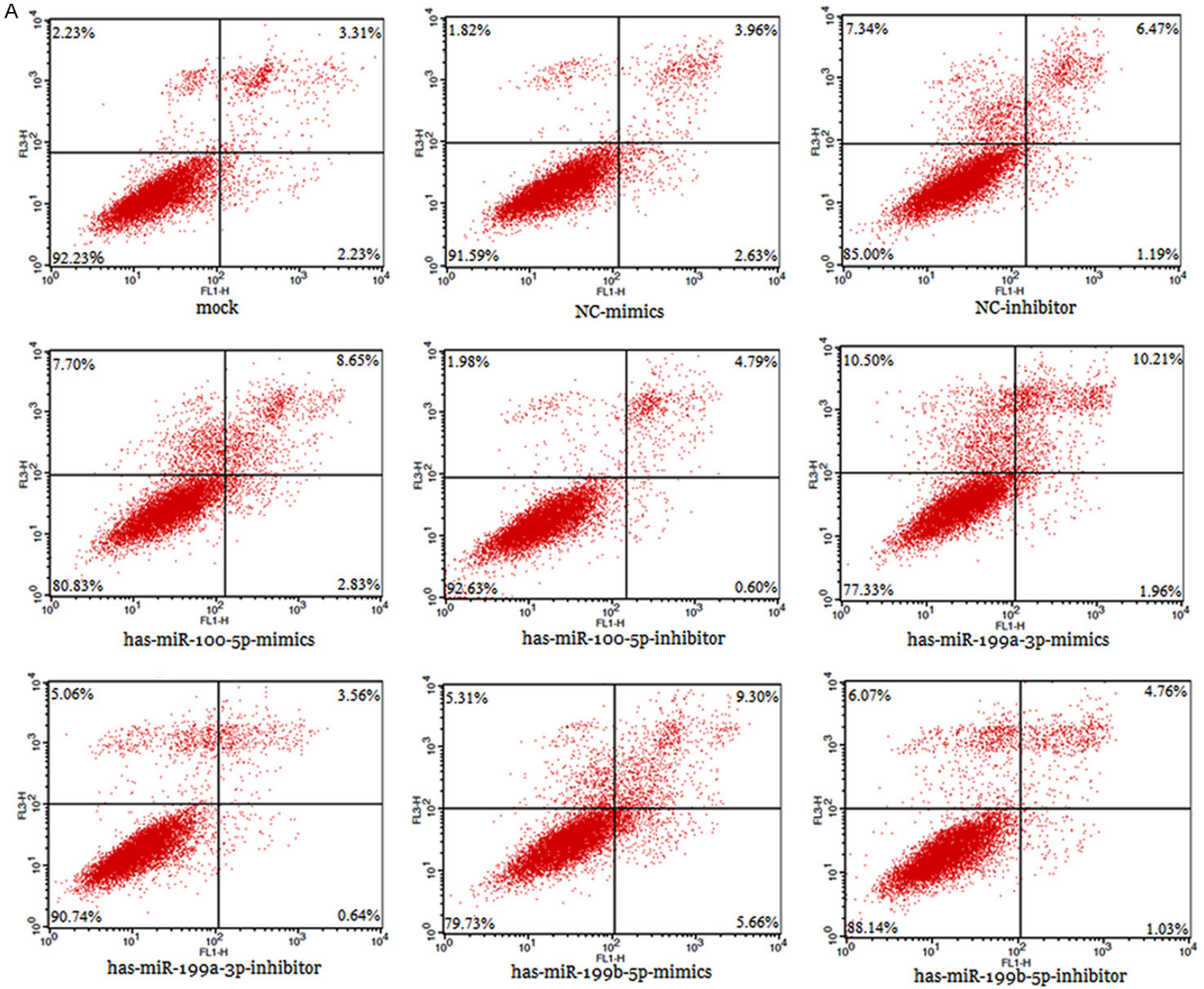
Annexin V-FITC apoptosis Detection kit (KeyGEN BioTECH, Jiangsu, China) was used to determine the cellular apoptosis. Ishikawa cells (4×10^6 per well) were cultured in 6-well plate for overnight, and treated respectively. According to manufacturer's instruction, cells were collected by trypsinization, then washed twice with phosphate-buffered saline (PBS) and centrifuged at 1000 rpm 5 min. Finally, cells were suspended in 500 μl of 1× binding buffer, and incubated in the dark with 5 μl Annexin V-fluorescein isothiocyanate (FITC) and 5 μl propidium iodide (PI) for 10 min at room temperature. Flow cytometer (FACSCalibur Becton Dickinson, Franklin Lakes, NJ, USA) was used to analyze cellular apoptosis.

Luciferase reporter assay

The influence of miR-100-5p, miR-199a-3p miR-199b-5p miRNA mimics to mTOR gene was performed used Dual-Glo Luciferase Assay System (Promega, Madison, USA).

Statistical analysis

Nonparametric one-way analysis of variance and Student t test were performed, with $P<0.05$ considered statistically significant.


A
2.23%
3.31%
92.23%
2.23%
mock
1.82%
3.96%
91.59%
2.63%
NC-mimics
7.34%
6.47%
85.00%
1.19%
NC-inhibitor
7.70%
8.65%
80.83%
2.83%
has-miR-100-5p-mimics
1.98%
4.79%
92.63%
0.60%
has-miR-100-5p-inhibitor
10.50%
10.21%
77.33%
1.96%
has-miR-199a-3p-mimics
5.06%
3.56%
90.74%
0.64%
has-miR-199a-3p-inhibitor
5.31%
9.30%
79.73%
5.66%
has-miR-199b-5p-mimics
6.07%
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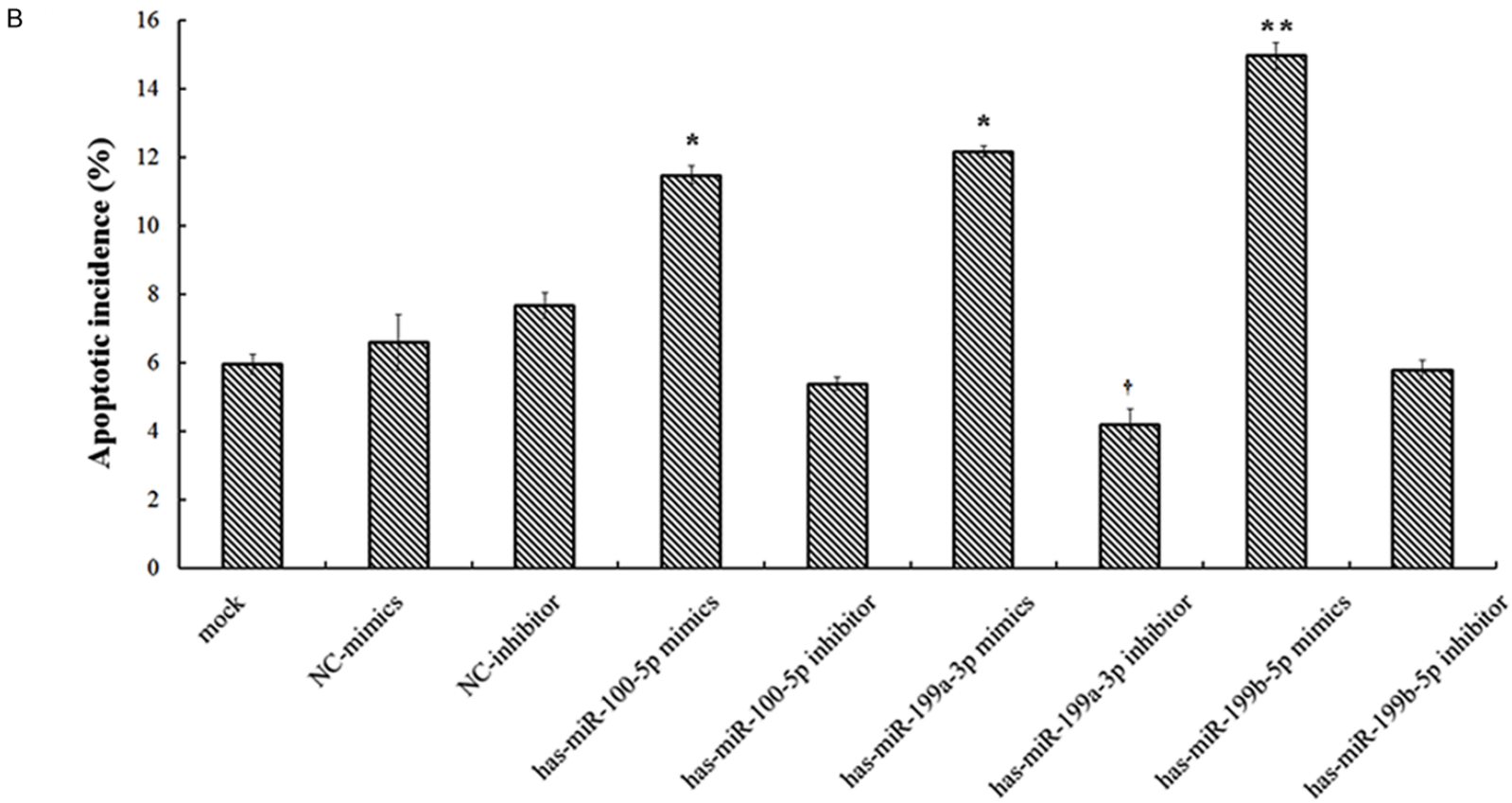

Figure 4. Apoptosis of Ishikawa cell was induced by miR-100-5p, miR-199a-3p and miR-199b-5p. A. Apoptosis rate of Ishikawa cell detected by flow cytometer; B. Quantification of apoptotic index. *P<0.05; **P<0.01.

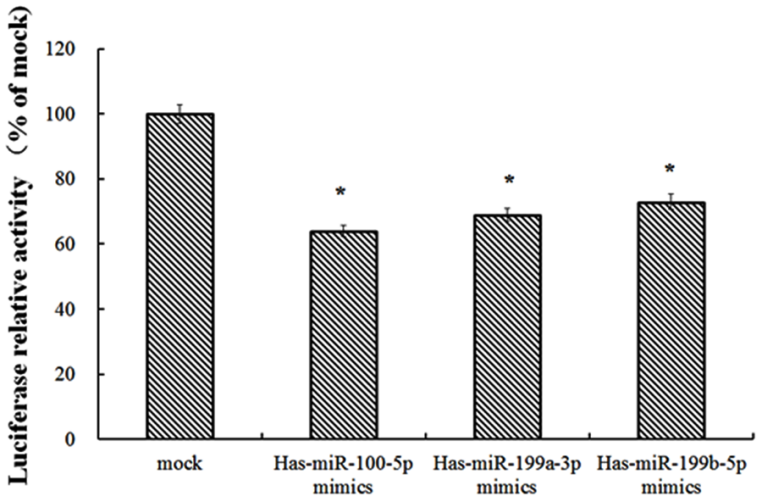

Figure 5. mTOR is a Target of miR-100-5p, miR-199a-3p and miR-199b-5p.

Results

Overexpression of miR-100-5p, miR-199a-3p and miR-199b-5p in Ishikawa and KLE cells

To analyze the potential role of miR-100-5p, miR-199a-3p and miR-199b-5p in EEC cells, we used miR-100-5p, miR-199a-3p, miR-199b-5p miRNA mimics, inhibitor and NC mimics/inhibitors, to transfect the Ishikawa and KLE cells, respectively. The expression levels of miR-100-5p, miR-199a-3p and miR-199b-5p in Ishikawa and KLE cells after transfection were determined by SYBR-Green stem-loop real-time PCR analysis. The results showed that miR-100-5p, miR-199a-3p and miR-199b-5p expression levels were all increased in the Ishikawa and KLE cells compared with NC (P<0.01). The expression levels of three miRNAs in miRNA inhibitor and NC, which were not significantly different (**Figure 1**).

Beclin1 and LC3 mRNA expression in Ishikawa and KLE cells

Beclin1 and *LC3* were autophagy related genes. To investigate the association between miR-100-5p, miR-199a-3p and miR-199b-5p and cell autophagy, we also detected the expression level of *Beclin1* and *LC3* mRNAs in Ishikawa and KLE cells after transfection. We found an increased expression of *Beclin1* and *LC3* mRNA in Ishikawa and KLE cells transfected by miR-100-5p, miR-199a-3p and miR-199b-5p mimics compared with NC (P<0.05), and the expression levels of *Beclin1* and *LC3* mRNA were lower in Ishikawa and KLE cells transfected by miR-199a-3p and miR-199b-5p miRNA inhibitors compared with NC (P<0.05) (**Figure 2**).

Proliferation of Ishikawa and KLE cells were inhibited by miR-100-5p, miR-199a-3p and miR-199b-5p

The impact of three miRNAs on Ishikawa and KLE cells proliferation was determined by CCK-8 assay. Compared with the mock and the miRNA mimics control, Ishikawa and KLE cells transfected with miR-100-5p, miR-199a-3p and miR-199b-5p mimics grew more slowly (P<0.05). Reduction of the intracellular miR-100-5p, miR-199a-3p and miR-199b-5p level by transfection with the miRNA inhibitor increased their growth rate (**Figure 3**), suggesting that overexpression of miR-100-5p, miR-199a-3p and miR-199b-5p could suppress the proliferation of Ishikawa and KLE cells.

Apoptosis of Ishikawa cell was induced by miR-100-5p, miR-199a-3p and miR-199b-5p

To clarify the association between three miRNAs and apoptosis of Ishikawa cell, we carried out flow cytometry. Compared with the mock and the miRNA mimics control, we found an increased apoptosis incidence of Ishikawa cell transfected with miR-100-5p, miR-199a-3p and miR-199b-5p mimics (P<0.05). In contrast, the apoptosis incidence of Ishikawa cell transfected with miR-199a-3p inhibitor was lower than NC inhibitor control (P<0.05) (**Figure 4**).

mTOR is a target of miR-100-5p, miR-199a-3p and miR-199b-5p

To directly investigate the relationship between three miRNAs and its targets, we constructed the luciferase reporter vectors in HEK 293 cell, miR-100-5p, miR-199a-3p and miR-199b-5p were all down-regulated the luciferase activity in cells transfected with miRNA mimics compared with mock (**Figure 5**). These results indicating that the mTOR 3'UTR was the target directly regulated by miR-100-5p, miR-199a-3p and miR-199b-5p.

Discussion

In the present study, we investigated the expression levels of three miRNAs (miR-100-5p, miR-199a-3p and miR-199b-5p) and autophagy-related genes *Beclin1* and *LC3* in Ishikawa and KLE cells. We found an increased expression of *Beclin1* and *LC3* in Ishikawa and KLE cells transfected by miR-100-5p, miR-199a-3p and miR-199b-5p mimics compared with NC. Additionally, Ishikawa and KLE cells transfected with miR-100-5p, miR-199a-3p and miR-199b-5p mimics grew more slowly; we also found an increased apoptosis incidence of Ishikawa cell transfected with miR-100-5p, miR-199a-3p and miR-199b-5p mimics. Finally, luciferase reporter results showed miR-100-5p, miR-199a-3p and miR-199b-5p were all downregulated the luciferase activity in cells transfected with miRNA mimics compared with mock. All results suggested that miR-100-5p, miR-199a-3p and miR-199b-5p may induce the autophagic death of EEC cell through targeting mTOR.

MicroRNAs usually bind to the 3'UTR of target mRNAs, which inhibit mRNA translation or induce mRNA degradation [8]. Several studies have found abnormal expression of miRNAs in tumors cells, suggesting that miRNAs may have similar functions like proto-oncogenes or tumor suppressor genes [13-15]. MiR-100-5p exhibited a low expression level in head and neck squamous cancer cell [16]. abnormal expression of miR-199a-3p has been found in several kinds of cancer [17]. MiRNA-199b-5p is involved in the Notch signaling pathway, and it has been found associated with risk of breast [18], ovarian [19], lung [20] and renal cancers [21]. In our study, we identified that overexpression of miR-100-5p, miR-199a-3p and miR-199b-5p in transfected cells significantly reduced EEC cell proliferation and accelerated cell apoptosis. Combined with the previous results, we suggested that miR-100-5p, miR-199a-3p, miR-199b-5p may play important roles in the development of human cancer, and the results identified here need to be confirmed in further studies.

Autophagy is a conserved, programmed response to metabolic and environmental stress, which could regulate the survival and death of cancer cells [22]. There are several important genes in different stages of autophagy, such as *Beclin1* and *LC3B*. *Beclin1* is involved in autophagosome formation at an early stage, rather than the expansion step [23]. Previous studies have found a lower expression level of Beclin1 in breast, ovarian and colorectal cancer cells [24, 25]. *LC3B* is closely associated with the number of autophagosomes, which play an indicator role in autophagosome formation [26]. In our present study, the expression level of *Beclin1* and *LC3* were both increased in Ishikawa and KLE cells transfected by miR-100-5p, miR-199a-3p and miR-199b-5p mimics. We speculated that miR-100-5p, miR-199a-3p and miR-199b-5p may have influence on autophagy by targeting mTOR, so we further investigated the association between three miRNAs and mTOR using luciferase reporter assay.

mTOR kinase is a major target in the autophagy signaling pathway, which regulates cell transcription, translation, and cytoskeletal organization [27]. An increased expression level of mTOR increases has been found in several types of cancers [28]. In our study, miR-100-5p, miR-199a-3p and miR-199b-5p were all downregulated the luciferase activity in cells transfected with miRNA mimics, which indicating that mTOR 3'UTR was the target directly regulated by miR-100-5p, miR-199a-3p and miR-199b-5p.

In conclusion, after Ishikawa and KLE cells transfected by mTOR targets miRNA (miR-100-5p, miR-199a-3p, miR-199b-5p), autophagy-related genes *Beclin1* and *LC3* were overexpressed, cell grow more slowly, and an increased apoptosis incidence were detected. Our results suggested that miR-100-5p, miR-199a-3p and miR-199b-5p may induce the autophagic death of EEC cell through targeting mTOR. These findings help us better understand the molecular mechanisms underlying the occurrence and development of EEC, and also implicate that miR-100-5p, miR-199a-3p and miR-199b-5p could be considered as promising biomarkers for early detection and prognosis of EEC.

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Disclosure of conflict of interest

None.

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