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Original Article

Effect of microRNA-199a-5p in proliferation and apoptosis through directly action of NF-κB1 on esophageal cancer

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Abstract: Aberrant expression of microRNA-199a-5p (miR-199a-5p) has been frequently reported in some cancers excluding esophageal cancer (EC). The role and its molecular mechanism of miR-199a-5p in EC have not been reported. In this study, we explored the effects of miR-199a-5p overexpression on apoptosis and invasion in EC cells. The mRNA level of miR-199a-5p in EC cell lines was determined by real-time PCR. The miR-199a-5p mimic was transiently transfected into BC cells using Lipofectamine™ 2000 reagent. Subsequently, the Brdu-ELISA results showed that introduction of miR-199a-5p inhibited cell proliferation. Our data also demonstrated that miR-199a-5p mimic arrested cell cycle progression and promoted apoptosis of EC109 and TE-1 cells. In addition, miR-199a-5p overexpression could also inhibit invasion and EMT of EC109 and TE-1 cells, qRT-PCR were used to determine the expressions of matrix metalloproteinase-2 and -9 (MMP-2 and -9) in EC cells. Next, we found that NF-κB1 expression was evidently reduced by up-regulation of miR-199a-5p. Bioinformatics analysis predicted that the NF-κB1 was a potential target gene of miR-199a-5p. Luciferase reporter assay further confirmed that miR-199a-5p could directly target the 3'UTR of NF-κB1. Moreover, overexpression of NF-κB1 in EC cells transfected with miR-199a-5p mimic partially reversed the inhibitory of miR-199a-5p mimic. In conclusion, miR-199a-5p induced cell apoptosis and inhibited metastasis in EC cells by down-regulation of NF-κB1.

Keywords: MicroRNA-199a-5p, esophageal cancer, proliferation, invasion, EMT, NF-κB1

Introduction

Esophageal cancer (EC) is still a major cause of morbidity and mortality, with about 380,000 new cases and 150,000 deaths per year and little change in survival rates in the recent 30 years [1]. Since most clinical trials of chemotherapeutics for advanced BC have shown limited benefits, new prognostic markers and effective treatment strategies based on current cancer-genome analyses are necessary. Recently, numerous transcriptome sequencing between EC patients and normal have demonstrated that several genes are closely related to human esophageal cancer [2-4]. However, the precise molecular mechanisms of BC are unclear. As far as we know, the mechanisms of EC are revealed by focusing on known genes, but focusing on unknown microRNAs (miRNAs) may also lend insight into the biology of EC.

The discovery of non-coding RNA in the human genome was an important conceptual breakthrough in the post-genomic sequencing era [5-7]. miRNAs constitute a class of small, non-coding RNA molecules, 19-22 nucleotides in length, that modulate gene expression. As a new layer of gene-regulation mechanism, regulation is achieved through imperfect pairing with target messenger RNAs (mRNAs) of protein-coding genes and transcriptional or post-transcriptional [8]. Moreover, miRNAs can regulate cellular proliferation, differentiation and apoptosis, as well as cancer initiation and progression [9]. Actually, many studies have showed that miRNAs were differentially regulated in diverse cancer types such as colorectal cancer [10], breast cancer [11, 12], lung cancer [13, 14]. Recently, a lot of reports showed that many miRNAs are aberrantly expressed in multiple cancers such as EC. The miR-17-5p was overexpressed in EC cells, and up-regulation of miR-17-5p significantly promoted proliferation, migration and invasion of EC cells by targeting PTEN signaling [15]. MiRNA-1207-5p promoted

proliferation of EC cells by targeting stomatin-like protein 2 [16]. These miRNAs act as oncogene, whereas some tumor suppressor miRNAs were also studied in EC. For example, miRNA-183 suppresses apoptosis and promotes proliferation in esophageal cancer by targeting PDCD4 [17]. The level of miR-144 was markedly down-regulated in EC tissues.

MiR-199a-5p has been involved in regulation of multiple biological prBCesses such as cell proliferation, apoptosis, migration and invasion [20-25]. In recent years, miR-199a-5p was considered as a tumor suppressor and down-regulated in prostate, gastric and non-small cell lung cancers [25-27]. Guo and his colleague found that miR-199a-5p also functioned as a tumor suppressor through inhibiting proliferation and inducing apoptosis in liver cancer cells by targeting hexokinase 2 [21].

In this paper, down-regulation of miR-199a-5p was frequently observed in BC cell lines. Up-regulation of miR-199a-5p inhibited cell proliferation arrested cell cycle and induced apoptosis of EC cells. In addition, miR-199a-5p overexpression could also inhibit invasion and epithelial-mesenchymal transition EMT of EC cells. Moreover, we found that NF-κB1 was the direct target of miR-199a-5p in EC and confirmed that miR-199a-5p functioned as a tumor suppressor by down-regulation of NF-κB1. Therefore, our results showed critical roles for miR-199a-5p in the pathogenesis of EC and suggested its potential application in tumor treatment.

Material and methods

Cell culture and miRNA transfection

Human EC cell lines such as EC109 and TE-1, and an immortalized normal human fallopian tube epithelial cell line FTE187 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). All cell line were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco Co., USA) including 10% fetal bovine serum (FBS) (Gibco Co., USA), 100 U/ml penicillin and 100 ug/ml streptomycin. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 on 0.1% gelatin-coated culture flasks. To enhance the expression of miR-199a-5p in cell lines, miR-199a-5p mimic and miR-negative control were respectively transfected into EC109 and TE-1 cells. One day before transfection, cells at about 40 to 60% confluency were changed to the antibiotic-free media. After 24 h, cells were transfected with 50 nM miR-199a-5p mimic using Lipofectamin™ 2000 reagent (Invitrogen, USA) following protocol.

Quantitative real-time PCR assay

The expression of miR-199a-5p and selected four genes (E-cadherin, Vimentin, MMP-2 and MMP-9) were measured with an opticon fluorescence (SYBR Green II) quantitative real-time PCR system (MJ Research, USA). Briefly, total RNA of EC109 and TE-1 cells was separated with using Trizol reagent (Life Technologies, CA). Two microgram RNA was used for gene-specific reverse transcription polymerase chain reaction (RT-PCR) using one-step RT-PCR kit (Qiagen, Venlo, the Netherlands) following the manufacturer's protBCols. And then the RT product (cDNA) subjected to 40 cycles of amplification with corresponding PCR primers, in which SYBR Green II was uesd as the signal fluorescence and ROX as the control fluorescence. The mRNA reverse transcription took place on an Applied Biosystems' GeneAmp PCR system 9700 and real-time PCR was carried out using the ABI7500 Real-Time PCR machine (Applied Biosystems). All samples were processed at the same time to avoid inter-experiment variance. Each sample experiment was performed triplicate to confirm the data, and the mean value was used in the analysis. The expression level was analyzed by the double delta CT (ΔΔCT) method. Delta CT (ΔCT) values represent normalized target genes levels with respect the internal control. Delta CT (ΔΔCT) values were calculated as the ΔCT of each test sample minus the mean ΔCT of the calibrator samples for each target gene. U6 snRNA and GAPDH mRNA were used to normalize. The fold change was calculated using the equation $2^{(-\Delta\Delta CT)}$.

Cell proliferation and cycle assay

To study the role of miR-199a-5p mimic in proliferation of EC109 and TE-1 cells, 5×10^4 cells were seeded in 96-well plate and allowed to grow for 24 h in complete medium. The medium was then removed and the cells were transfected with miR-199a-5p mimic or miR-NC for 24 h at 37°C. Cell Proliferation ELISA-BrdU (colorimetric) Kit was used to detect the cells proliferation according to the manufacturer's protBCols. Following, the EC109 and TE-1 cells were transfected with miR-199a-5p mimic for 24 h to detect cell cycle distribution. After transfection,

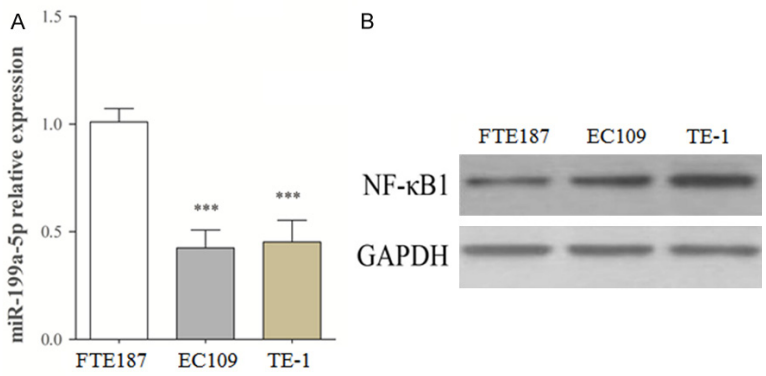


Figure 1. The altered expression of miR-199a-5p and NF-κB1 in EC cell lines. A. The relative level of miR-199a-5p in EC cell lines and FTE187 cell line by RT-PCR. B. NF-κB1 protein level in in EC109 and TE-1 cell lines compared with FTE187 cell were determined by WB. All data are presented as mean ± SEM, n=4, **P*<0.05, ***P*<0.01, ****P*<0.001.

EC109 and TE-1 cells were collected by trypsinization, washed with ice-cold PBS, and fixed in ice-cold 70% methanol overnight. Then, cells were centrifuged, resuspended in ice-cold phosphate buffer saline (PBS), and incubated with RNase (Sigma, USA) for 30 min at 37°C, and then were incubated with propidium iodide at room temperature of 30 min. The analyses of cell cycle distribution were performed by FACS can flow cytometer (BD Biosciences, USA).

Annexin V-FITC/PI analysis

EC109 and TE-1 cells were transfected with miR-199a-5p mimic for 24 h. After transfection, cells were harvested and washed twice in PBS and double-stained with Annexin V-FITC and PI by using the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, USA) following the manufacturer's protBCols. Then, each sample was quantitatively analyzed at 488 nm emission and 570 nm excitation by FACS Calibur flow cytometer (BD Biosciences, USA).

Western blot analysis

To extract the proteins, EC109 and TE-1 cells were washed twice in cold PBS, and then lysed in RIPA lysis buffer (Beyotime Institute of Biotechnology, China) with protease inhibitor cBCktail (Merk, Germany). The protein concentration of cell lysates was quantified by BCA Kit (Beyotime Institute of Biotechnology, China), and equal quantities (50 μg) of proteins were separated by SDS-PAGE on 10% gels, and then transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, USA). The membranes were blBCked in 5% shimmed milk diluted with Tri Buffered Saline Tween-20 (TBST) at room temperature for 1 h and incubated overnight at 4°C with primary anti-NF-κB1 antibody (1:1000; Cell Signaling Technology Inc, USA). The membranes were then incubated with a goat anti-rabbit IgG conjugated to horseradish peroxidase secondary antibody (1:2000; Cell Signaling Technology Inc, USA) for 2 h. The proteins were visualized using ECL-plus reagents (Beyotime Institute of Biotechnology, China). The density of the bands was measured using the Image J software (USA), and values were normalized to the densitometric values of GAPDH (1:1000; Cell Signaling Technology Inc, USA) in each sample.

Luciferase reporter assay

EC109 and TE-1 cells (2×10^6/well) were seeded in 24-well plates and incubated overnight before transfection. Cells were co-transfected with pMIR-EGFR-3'UTR wild-type or mutant reporter plasmid, miR-199a-5p mimic or miR-NC, and pRL-SV40 renilla plasmid (Promega, USA) using Lipofectamine 2000. At 48 h after co-transfection, both firefly and renilla luciferase activities were quantified using a dual luciferase reporter system (Promega, USA) following the manufacturer's protBCols. Each treatment was performed in triplicate in three independent experiments.

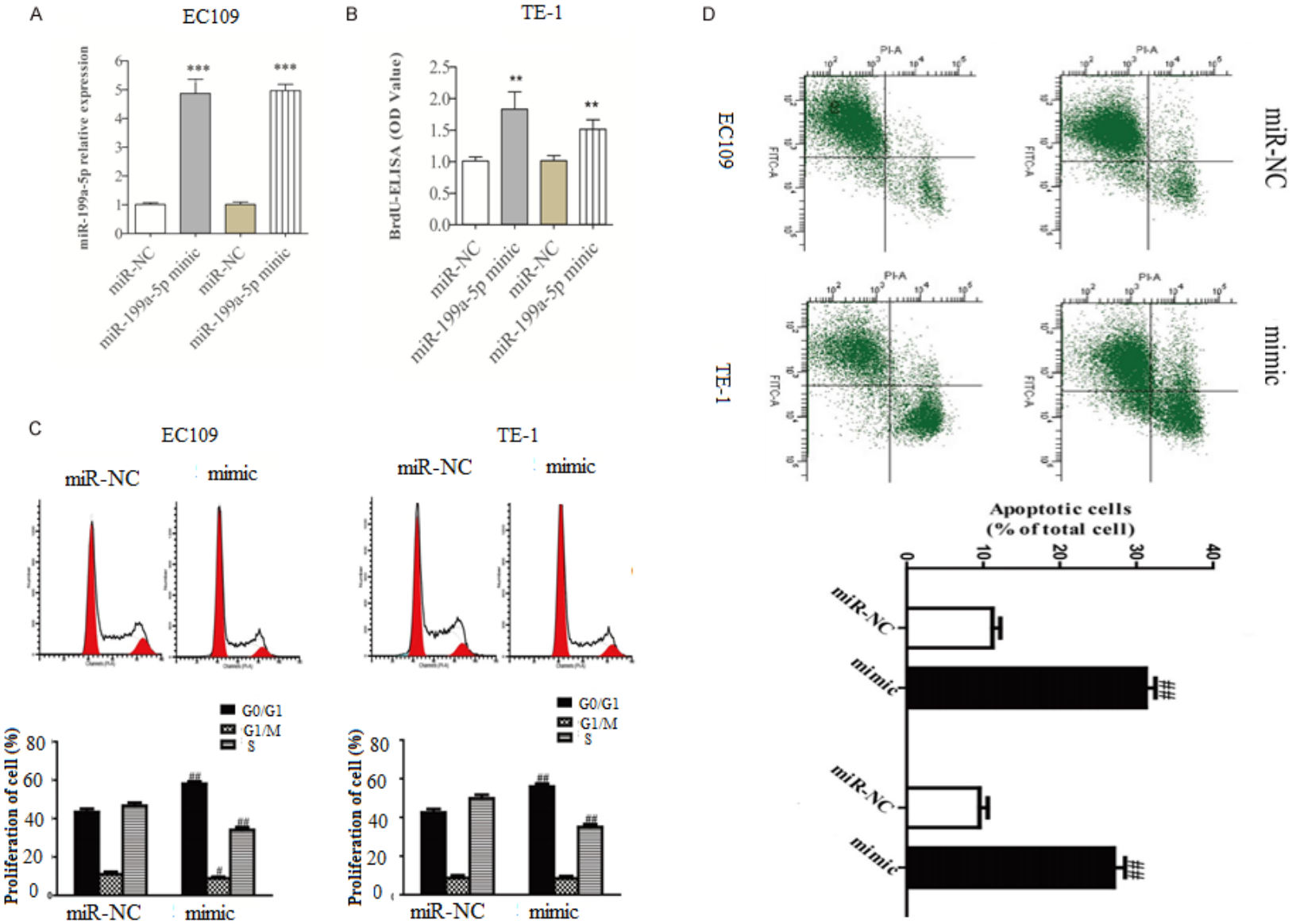
Statistical analysis

All statistical analyses were performed using SPSS 18. Data from each group were expressed as mean ± standard error of the mean (S.E.M.) and statistically analyzed by Student's t test. Differences were considered statistically significant at a p value of <0.05.

Results

The level of miR-199a-5p was decreased in EC cell lines

Our findings showed that the miR-199a-5p expression level was evidently reduced in EC109


A
EC109
miR-199a-5p relative expression
***
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miR-NC
miR-199a-5p mimic
miR-NC
miR-199a-5p mimic
B
TE-1
BrdU-ELISA (OD Value)
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miR-NC
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mimic
PI-A
FITC-A
Apoptotic cells
(% of total cell)
miR-NC
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Figure 2. Effects of miR-199a-5p overexpression on proliferation, cell cycle and apoptosis of EC109 and TE-1 cells. EC109 and TE-1 cells were transfected with miR-199a-5p mimic or miR-NC. A. The mRNA levels of miR-199a-5p in EC109 and TE-1 cells were detected by RT-PCR. B. Cell proliferation was assessed by BrdU-ELISA assay. C. Cell cycle was detected by flow cytometry. D. Apoptosis of EC109 and TE-1 cells was measured by flow cytometric analysis of cells labeled with Annexin-V/PI double staining. All data are presented as mean ± SEM, n=4, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. miR-NC.

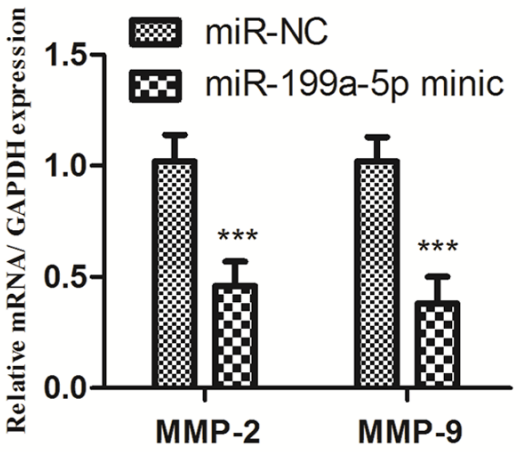

Figure 3. Overexpression of miR-199a-5p decreased expressions and secretions of MMP-2 and-9. The mRNA levels of MMP-2 and -9 were examined by qRT-PCR. All data are presented as mean ± SEM, n=4. $^{***}P<0.001$ vs. miR-NC.

and TE-1 cell lines compared to that of FTE187 (**Figure 1A**). Furthermore, we also found NF-κB1 was predicted to be a direct target of miR-199a-5p by using the four online prediction software (RNA22, TargetScan 6.2, PITA and Micro.org). Then, the protein expression level of miR-199a-5p target gene was further assessed among EC109 and TE-1 cell lines. Our results showed that the protein level of NF-κB1 in EC109 and TE-1 cells was significantly increased compared with FTE187 cell (**Figure 1B**).

MiR-199a-5p inhibited proliferation, arrested cell cycle and induced cell apoptosis

Our data indicated that the level of miR-199a-5p was evidently up-regulated after transfection with miR-199a-5p mimic compared to miR-NC group (**Figure 2A**). To investigate the role of miR-199a-5p in proliferation of EC cells, EC109 and TE-1 cell were transfected with miR-199a-5p mimic or miR-NC. Results from Brdu-ELISA assay showed that overexpression of miR-199a-5p significantly suppressed the viabilities of EC109 and TE-1 cells (**Figure 2B**). These findings indicated that up-regulation of miR-199a-5p could effectively inhibit proliferative of EC cell lines. Next, we further evaluate whether miR-199a-5p could arrest cell cycle tentative of EC cells by flow cytometry. Our findings suggested that up-regulation of miR-199a-5p dramatically enhanced the percentage of cells in the G1/G0 peak and reduced the percentage of cells in the S peak in both EC109 and TE-1 cells compared with cells transfected with miR-NC (**Figure 2C**). Thus, overexpression of miR-199a-5p might inhibit BC cell proliferation by impeding the G1/S cell cycle transition. Furthermore, we use flow cytometry analysis to detect the total apoptosis rates of EC109 and TE-1 cells. We found that the number of apoptotic EC109 and TE-1 cells was significantly higher in miR-199a-5p group than that in miR-NC group (**Figure 2D**).

Effects of miR-199a-5p overexpression on expressions of MMP-2 and -9 in BC cells

MMPs may be responsible for the impaired invasion of miR-199a-5p mimic-transfected cells. To confirm this hypothesis, we further detected the levels of MMP-2 and -9 at the mRNA levels by qRT-PCR. The overexpression of miR-199a-5p operated a distinct reduction in MMP-2 and -9 expressions at the mRNA (**Figure 3**) levels. Our results suggested that up-regulation of miR-199a-5p suppressed the migratory ability of EC109 and TE-1 cells by down-regulation of MMP-2 and -9.

NF-κB1 is a direct target of miR-199a-5p in EC cells

Because NF-κB1 was a binding target of miR-199a-5p predicted by the RNA22, TargetScan 6.2, PITA and Micro.org, the protein expression of NF-κB1 was determined by Western blotting inEC109 and TE-1 cells transfected with miR-199a-5p mimic. Our results demonstrated that NF-κB1 expression was apparently decreased after up-regulation of miR-199a-5p (**Figure 4A**). To further confirm whether a direct target of miR-199a-5p was NF-κB1, 3'-UTR of NF-κB1 was cloned into a luciferase reporter vector and the putative miR-199a-5p binding site in the NF-κB1 3'-UTR was mutated. Luciferase

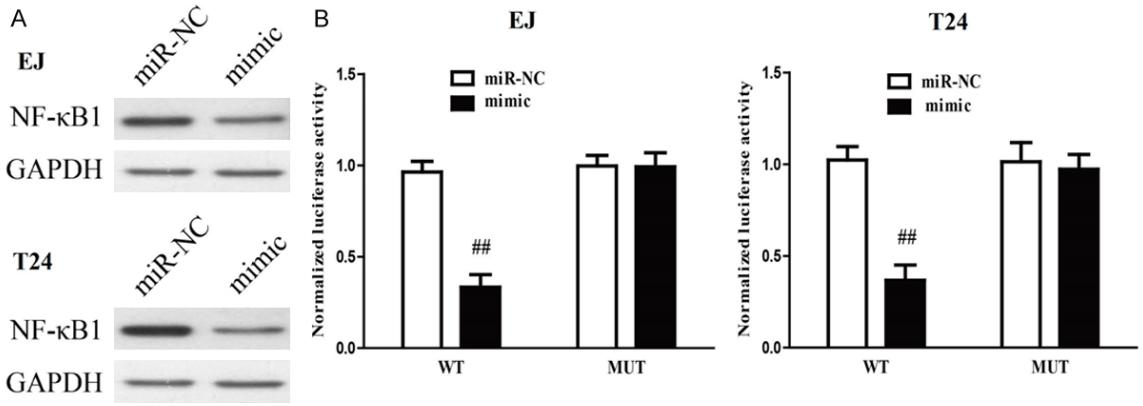

Figure 4. NF-κB1 was a direct target of miR-199a-5p. EC109 and TE-1 cells were transfected with miR-199a-5p mimic or miR-NC. A. The protein expression of NF-κB1 was determined by Western blot. GAPDH was detected as a loading control. B. The analysis of the relative luciferase activities of NF-κB1-WT, NF-κB1-MUT in EC cells. All data are presented as mean ± SEM, n=6. $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. miR-NC.

reporter assay was used to determine the effect of miR-199a-5p mimic. Our data displayed that introduction of miR-199a-5p dramatically suppressed the luciferase activity of pMir-NF-κB1 3'-UTR WT (**Figure 4B**). The effect of miR-199a-5p was abolished by mutation of the miR-199a-5p-binding site in the NF-κB1 3'-UTR, which suggested that miR-199a-5p directly and negatively regulated NF-κB1.

Discussion

In recent years, many reports have shown the miRNAs are dysregulated in multiple types of cancers [28-30]. It is critical for identification of cancer-specific miRNAs and their targets to understand their role in oncogenesis and define novel therapeutic targets [31-33]. In this paper, we focused on the function of miR-199a-5p in the pathogenesis of EC. Firstly, we detected miR-199a-5p level in EC cell lines by real-time RT-PCR assay. We found that the level of miR-199a-5p was significantly reduced in cancer cells compared to the normal human fallopian tube epithelial cell line FTE187. Our results are in line with others reports which have demonstrated that miR-199a-5p was also evidently down-regulated in colorectal cancer, breast cancer, glioblastoma and oral squamous cell carcinoma [34, 35]. Therefore, aberrant regulation of miR-199a-5p was altofrequent of in multiple types of cancer cells and tissues, indicating that decreased miR-199a-5p might play a critical role in tumorigenesis.

It has been presumed that many miRNAs were decreased in cancers, suggesting that they may normally function as tumor suppressor genes. Hence, we hypothesized that miR-199a-5p was an inhibitory factor of growth in BC cells. Because level of miR-199a-5p was down-regulated in cancer cells and tissues, we expected that up-regulation of miR-199a-5p would lead to arresting cell growth. Using the Brdu-ELISA assay, we found that EC109 and TE-1 cells transfected with the miR-199a-5p mimic exhibited decreased growth compared to cells transfected with miR-NC. Cell cycle analyses also showed that the percentage of cells in the G1-phase was increased and the percentage of cells in the S-phase was decreased in cells transfected with miR-199a-5p mimic compared to cells transfected with miR-NC. Moreover, flow cytometry analysis demonstrated that miR-199a-5p mimic could evidently induced apoptosis of EC109 and TE-1 cells compared with miR-NC group. It has been well known that cell cycle progression and apoptosis are regulated by numerous proteins.

It has been reported that miR-199a-5p affects cell growth, metastasis and apoptosis of cancer cells possibly by targeting CDK6, RBCK1 and cMet [24, 25, 36-39]. Although bioinformatic tools may help to reveal putative mRNA targets of miRNAs, experimental procedures are required for their validation. In our paper, we show that miR-199a-5p targeted the NF-κB1 mRNA, thus revealing a possible mechanism assBCiated with ovarian oncogenesis. Actually, NF-κB1, a member of the Rel/NF-κB transcription factor family, plays critical roles in the regulation of immune responses, embryo

and cell lineage development, cell-cycle progression, cell apoptosis, and tumorigenesis [40-42]. Our data showed that NF-κB1 was a target of miR-199a-5p. First, using western blotting, we confirmed that introduction of miR-199a-5p could cause the significant decrease in NF-κB1 protein level. In addition, we found that the ability of miR-199a-5p to regulate NF-κB1 expression was direct, because it bound to the 3'UTR of NF-κB1 mRNA with complementarity to the miR-199a-5p seed region. Moreover, the luciferase activity of NF-κB1 3'-UTR was specifically responsive to miR-199a-5p up-regulation. However, mutation of the miR-199a-5p binding site abolished the effect of miR-199a-5p on the regulation of luciferase activity. In this study, overexpression of NF-κB1 could also rescue EC cells from inhibition of cell growth, invasion and EMT caused by miR-199a-5p.

NF-κB1 facilitates invasion and metastasis of cancer cells partly by transcriptional regulation of MMP-2 and MMP-9. Liu et al have demonstrated that miR-9 suppressed migration and invasion of uveal melanoma cells in part through direct targeting NF-κB1 expression and down-regulation of its downstream molecules MMP-2 and -9 [43, 44]. Another report has shown that miR-9 could inhibit metastasis of melanoma cells by suppression of E-cadherin expression through targeting NF-κB1 [45]. In our paper, we found that MMP-2 and MMP-9 altered in the same pattern as NF-κB1. Therefore, NF-κB1-regulated MMP-2 and MMP-9 may contribute to miR-199a-5p-suppressed invasion and metastasis of EC cells.

Conclusion

In summary, we showed that miR-199a-5p was evidently decreased in BC cells. Introduction of miR-199a-5p inhibited proliferation, invasion and induced apoptosis of BC cells through directly targeting NF-κB1. This novel miR-199a-5p/NF-κB1 axis might provide new insights into the molecular mechanisms underlying progression and metastasis of BC, and overexpression of miR-199a-5p might be a potential therapeutic strategy for the treatment of EC in the future.

Disclosure of conflict of interest

None.

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