

Original Article

Down regulation of p19ARF by microRNA218 induces apoptosis of EC9706

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Received September 7, 2016; Accepted October 28, 2016; Epub December 1, 2016; Published December 15, 2016

Abstract: microRNAs are a variety of non-coding small RNAs which involve in various biological process including cell signaling transduction and cell cycle regulation. This study is designed to investigate the regulatory role of microRNA218 and p19ARF in esophageal cancer. microRNA218 and scramble miRNA (miRNA) were synthesized and transfected into esophageal squamous cell carcinoma EC9706 cells using liposomal transfection techniques. MTT assay, caspase-3 activity assay, and flow cytometry were used to investigate the effect of microRNA218 on EC9706 cells. siRNA of cycle inhibitor p19ARF or p19ARF plasmids were transfected into EC9706 cells. microRNA218 was then transfected into p19ARF-silenced or p19ARF-overexpressed EC9706 cells. Western blot was used to measure the expression levels of p19ARF and apoptosis of each type of cells were measured. Transfection of microRNA218 reduced the growth of EC9706 cells, increased phosphatidylserine eversion, activated caspase-3, and decreased the expression levels of p19ARF. Silence of p19ARF enhanced microRNA218-induced apoptosis of EC9706 cells. Overexpression of p19ARF inhibited microRNA218-induced apoptosis of EC9706 cells. microRNA218 induces apoptosis of EC9706 cells via down-regulation of p19ARF.

Keywords: microRNA218, p19ARF, EC9706 cells, apoptosis

Introduction

Esophageal squamous cell carcinoma (ESCC) threatens the lives of patients [1, 2]. Currently, treatment of ESCC is individualized comprehensive treatment appropriate to the different stages, primarily chemotherapy and surgery [3, 4]. Targeted Molecular therapy has been a hot spot for treatment of ESCC [5, 6]. Only a few targets are being used for treatment of ESCC [7, 8]. The effect of targeting inhibitor of apoptosis proteins such as Bcl-2 and the AIPs for the treatment of ESCC is poor [9-11]. More effective molecular targets for treatment of ESCC are needed. MicroRNA has not been used as a target to treat ESCC [12]. microRNAs are non-coding small RNAs that involve in cell signaling transduction and cell cycle regulation. For example, microRNA-143 inhibits the growth of ESCC, and microRNA-34a is associated with tumor metastasis [13, 14], suggesting that microRNA may also be involved in the occurrence and development of ESCC [14, 15]. Levels of microRNA143 in esophageal cancer

tissues were significantly higher than that of adjacent tissues, suggesting microRNA143 may be involved in the development of esophageal cancer [16, 17]. Anti-tumor strategy is to kill tumor cells without affecting normal cells, and cell apoptosis is regulated by anti-apoptotic proteins and pro-apoptotic proteins [18, 19]. Cell cycle inhibitor protein, p19ARF, is a widely studied anti-apoptotic molecule [20, 21]. Although there are many drugs targeting p19ARF, none of them can reduce the levels of p19ARF effect into ideal levels [22]. This study uses EC9706 cell as a cell model to explore the regulatory role of microRNA218 on p19ARF and EC9706 cells to provide a theoretical basis for targeted molecular therapy of esophageal cancer.

Materials and methods

Reagents and cells

EC9706 cells were purchased from ATCC. Fetal bovine serum and cell culture medium were

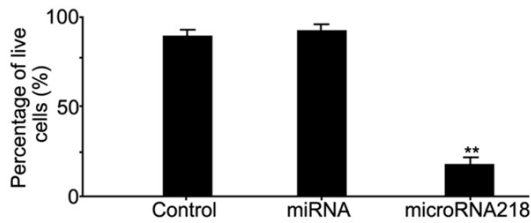


Figure 1. Transfection of microRNA218 decreased the viability EC9706 cells. Note: MTT assay was used to analyze the viability of EC9706 cells transfected with control miRNA or microRNA218 or non-transfected EC9706 cells, ** $P < 0.01$, compared with miRNA group.

purchased from Hualan Biological Co. (Beijing, China). Liposome transfection kit was purchased from Invitrogen. Apoptosis detection reagents, caspase-3 assay kit, protein extraction kit, and BCA protein assay kit were purchased from Biyuntian Biotechnology Institute (Haimen, Jiangsu, China). MTT assay reagents were purchased from Beijing Dingguo Co. (Beijing, China). Antibodies purchased from Santa Cruz, USA (North California, United States). microRNA218, control miRNA, and p19ARF plasmids were purchased from Jima Biotechnology Co., Ltd. (Suzhou, China). Sequences for microRNA218, control miRNA, and p19ARF are as follows: 5'-CATCTCGTCAGACTGCAAGTAT-3' and 5'-AAGTAGGGACAAAGTAAAGCA-3'; 5'-CTTCAACTCTGAGGGCTAGC-3' and 5'-TTATCACGTCGATTGACAT-3'; 5'-CGCTACCATTAGCGTGATGC-3' and 5'-TTATCACTCTACGATAGGT-3'.

Cell culture

EC9706 cells were cultured in DMEM according to previous method published by Wang et al. [9].

Transfection

microRNA218 and control microRNA were transfected into EC9706 cells according to the manufacturer's instruction. Transfection was performed when cells reached 68% confluence. 1 μ l (1 μ g/ μ l) of microRNA218 or control miRNA was mixed with lipo2000 and transfected into cells.

MTT assay of cell viability

MTT test was performed according to conventional methods [10]. MTT solution was added to

each well and incubated for 5 h. DMSO (100 μ l) was added to each well to stop reaction. Optical density at 420 nm was recorded using a microplate reader to draw a growth curve of EC9706 cells [11].

Flow cytometry measurement of apoptosis

Flow cytometry was performed to detect the apoptosis of EC9706 cells according to conventional methods. Briefly, EC9706 cells were transfected with either microRNA218 or control miRNA and cultured for 48 hours. Cells were then collected and resuspended. 100 μ l of reaction buffer and 2 μ l of Annexin-V-FITC were added to 500 μ l of cell suspension, mixed, and incubated for 17 min in dark for flow cytometry analysis with 426 nm and 628 nm [14].

Western blot

EC9706 cells were collected and proteins were extracted and quantified by BCA kit according to manufacturer's instruction. Equal amounts of proteins (15 μ g) were electrophoresed, transferred to membranes, and blocked. After washing with TBST, membranes were incubated with first antibody (1:800) over night at 4°C. After washing with TBST, membranes were incubated with secondary antibodies (1:1500) for 2 h at 37°C, and developed with ECL. Expression levels of p19ARF were analyzed [15].

Caspase-3 activity assay

Caspase-3 activity was detected according to kit instructions. Briefly, EC9706 cells were transfected with either microRNA218 or control miRNA and cultured for 48 hours. Cells were then collected, lysed, mixed with chromogenic substrate, and incubated at room temperature. Absorbance of each sample was recorded by a microplate reader [17]. Caspase-3 relative activity was calculated as follows: absorption value of cells transfected with microRNA218-absorption value of cells transfected with control miRNA.

Effect of up-regulation or down-regulation of p19ARF on microRNA218-transfected EC9706 cells

EC9706 cells were transfected with either p19ARF plasmids or siRNA of p19ARF. microRNA218 or control miRNA was further transfected

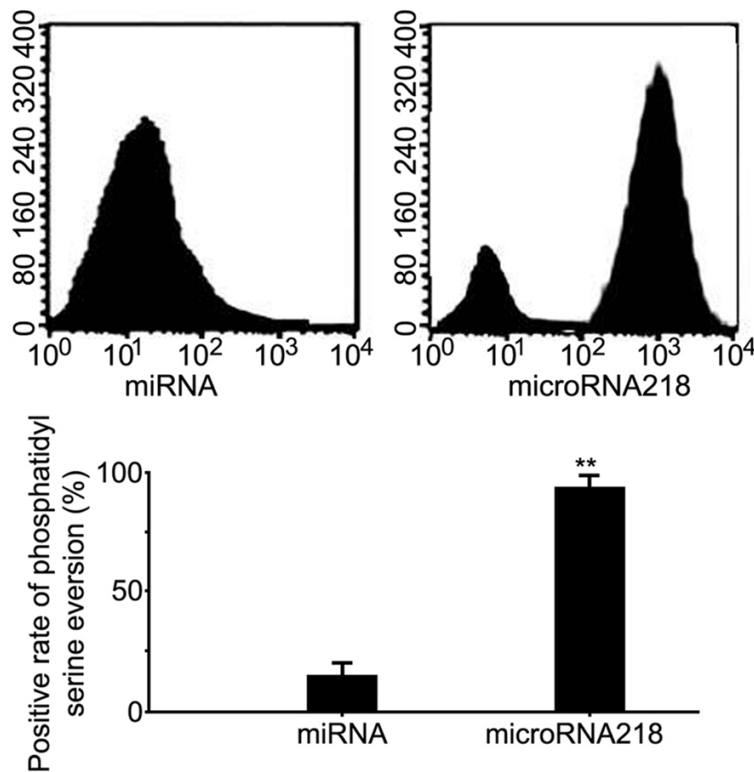


Figure 2. Transfection of microRNA218 induced apoptosis of EC9706 cells. Note: Flow cytometry was used to analyze the apoptosis of EC9706 cells transfected with control miRNA or microRNA218 or non-transfected EC9706 cells, ** $P < 0.01$, compared with miRNA group.

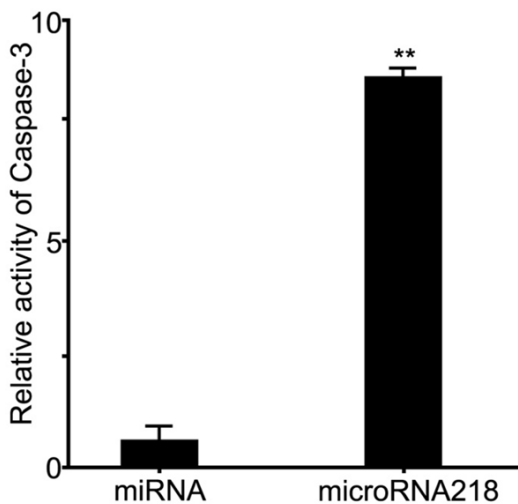


Figure 3. Transfection of microRNA218 activated caspase-3 in EC9706 cells. Note: Caspase-3 activity assay was used to analyze caspase-3 activity of EC9706 cells transfected with control miRNA or microRNA218 or non-transfected EC9706 cells, * $P < 0.05$, compared with miRNA group.

ed into EC9706 cells transfected with either p19ARF plasmids or siRNA of p19ARF using

lipo2000. 2 μ l (1.6 μ g/ μ l) of siRNA of p19ARF or p19ARF plasmid was suspended in lipo2000. 2 μ l (0.6 μ g/ μ l) of control microRNA or microRNA2181 was suspended with lipo2000. DNA and liposome mixtures were then drop-wisely added to cell culture medium [9].

Statistical analysis

SPSS 14.0. was used for statistical analysis. All data are expressed as mean $\pm$ standard error. Differences between groups were analyzed by t-test. $P < 0.05$ was considered statistically significant.

Results

Transfection of microRNA218 reduced viability of EC9706 cells

MTT assay results showed that transfection of microRNA218 significantly reduced the viability of EC9706 cells, compared with that of EC9706 cells transfected with control miRNA ($P = 0.0029$) (**Figure 1**). No significant difference was found in EC9706 cells transfected with control miRNA and non-transfected EC9706 cells, so EC9706 cells transfected with control miRNA were used as controls in this study.

Transfection of microRNA218 induced apoptosis of EC9706 cells

Flow cytometry analysis showed (**Figure 2**) that transfection of microRNA218 significantly increased the expression of phosphatidylserine in EC9706 cells, compared with that of EC9706 cells transfected control miRNA ($P = 0.017$).

Transfection of microRNA218 activated caspase-3 in EC9706 cells

Caspase-3 activity assay results showed (**Figure 3**) that transfection of microRNA218 significantly activated caspase-3 in EC9706 cells, compared with that of EC9706 cells transfected control miRNA ($P = 0.031$).

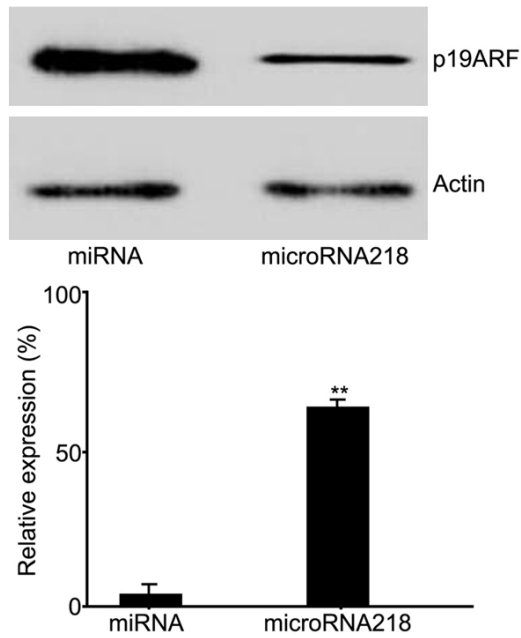


Figure 4. Transfection of microRNA218 reduced p19ARF expression in EC9706 cells.

Transfection microRNA218 decreased p19ARF protein expression in EC9706 cells

Western blot results showed that transfection of microRNA218 significantly reduced p19ARF expression at protein levels in EC9706 cells, compared with that of EC9706 cells transfected control miRNA ($P = 0.0065$) (**Figure 4**).

Silence of p19ARF enhanced microRNA218-induced apoptosis of EC9706 cells

p19ARF was silenced to investigate its role in microRNA218-induced apoptosis of EC9706 cells. Western blot result (**Figure 5**) showed that expression of p19ARF was significantly decreased by siRNA; the expression level of p19ARF in EC9706-microRNA218-si-p19ARF group was significantly lower than that of EC9706-microRNA218-siControl group. Flow cytometry results showed that decreased p19ARF expression by siRNA significantly increased microRNA218-induced apoptosis of EC9706 cells, compared to that of EC9706 cells transfected with control siRNA and microRNA218 ($P = 0.014$) (**Figure 5**).

Overexpression of p19ARF inhibited microRNA218-induced apoptosis of EC9706 cells

In order to examine the effect of p19ARF on microRNA218-induced apoptosis of EC9706

cells, p19ARF was over-expressed in EC9706 cells. As shown in **Figure 6**, western blot showed that p19ARF was significantly over-expressed in microRNA218 + p19ARF group, compared to that of microRNA218 group. Caspase-3 activity assay results showed that overexpression of p19ARF significantly inhibited microRNA218-induced apoptosis of EC9706 cells ($P = 0.0072$) (**Figure 6**).

Discussion

In this study, EC9706 cells were used as models to study the role of microRNA218 in esophageal cancer at molecular and protein levels. The purpose of this study is to explore the possible regulatory mechanism of microRNA on esophageal squamous cells. Data showed that transfection of microRNA218 reduced the viability and induced apoptosis of EC9706 cells, which is not contradictory to previous studies that microRNA is involved in cell growth and survival [13, 14]. So far, the regulation of microRNA218 on EC9706 remains to be elucidated [3]. microRNA-143 can inhibit the growth of ESCC cells, and microRNA-34a is associated with tumor metastasis [13, 14], suggesting that microRNA may also be involved in the occurrence and development of esophageal cancer [13-15].

Cell cycle inhibitor protein p19ARF belongs to inhibitors of apoptosis proteins [23]. Whether p19ARF is regulated by microRNA218 and its role in EC9706 cells remains unclear [24, 25]. The study showed that transfection of microRNA218 reduced the levels of p19ARF. Overexpression of microRNA218 or reduction of p19ARF increased apoptosis. In contrast, overexpression of p19ARF inhibited microRNA218 induced apoptosis of EC9706 cells.

However, there is no clue how microRNA218 regulates p19ARF. According to the current results, we speculate that microRNA218 may regulate the level of p19ARF through direct or indirect regulation. Promoter analysis should be performed in future research to investigate how microRNA218 regulates p19ARF. Immunoprecipitation technique should also be applied to check if p19ARF and microRNA218 can be co-immunoprecipitated.

Three different results uncovered the role of p19ARF in microRNA218 induced EC9706 cells apoptosis. Western blot results showed that

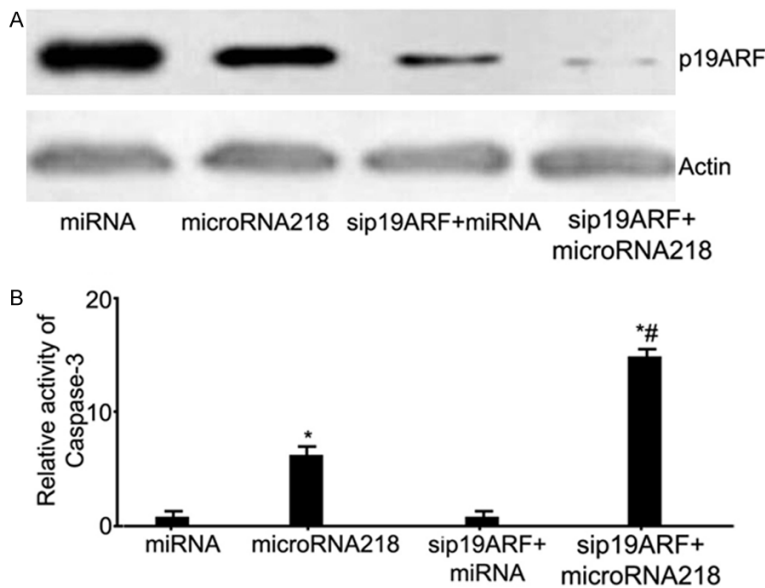


Figure 5. Silence of p19ARF enhanced microRNA218-induced apoptosis of EC9706 cells. Note: Flow cytometry was used to analyze apoptosis of EC9706 cells transfected with control miRNA, microRNA218, microRNA218 + sip19ARF, or control miRNA + sip19ARF, * $P < 0.05$, compared with control miRNA group, # $P < 0.05$, compared with control miRNA + sip19ARF group.

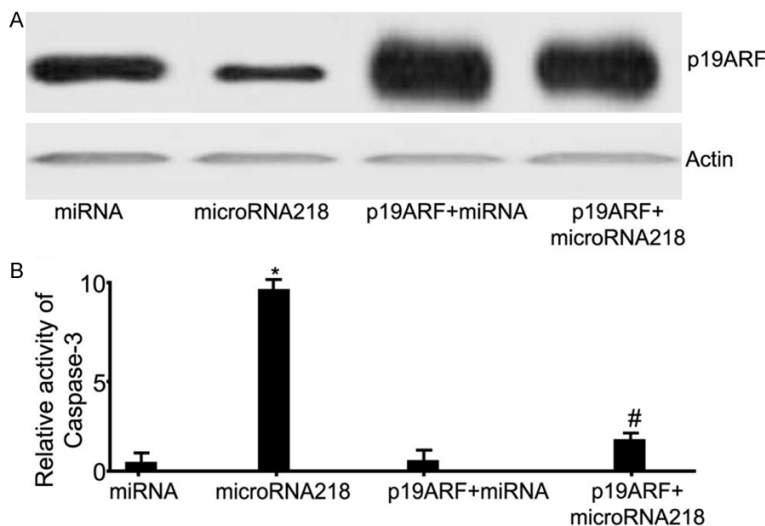


Figure 6. Overexpression of p19ARF inhibited microRNA218-induced apoptosis of EC9706 cells. Note: Caspase-3 assay was used to analyze apoptosis of EC9706 cells transfected with control miRNA, microRNA218, or microRNA218 + p19ARF group. A. Western blot assay of p19ARF expression. B. Caspase-3 activity. * $P < 0.05$, compared with control miRNA group, # $P < 0.05$, compared with microRNA218 + p19ARF group.

overexpression of microRNA218 decreased p19ARF in EC9706 cells. Secondly, silence of p19ARF enhanced microRNA218 induced EC9706 cells apoptosis. Thirdly, overexpression of p19ARF inhibited microRNA218 induced EC9-

706 cells apoptosis. All these data suggested that p19ARF played a very important role in microRNA218 induced EC9706 cells apoptosis, indicating that p19ARF protein may be used as a target for targeted molecular therapy of esophageal cancer [26]. It has been shown that p19ARF inhibits apoptosis of other cancer cells [28]. This is the first study of p19ARF, microRNA218, and esophageal cancer.

This study does not include esophageal cancer tissues and adjacent tissues, so it is impossible to measure the levels of p19ARF protein in tumor tissues and adjacent tissues which will provide us the relationship between p19ARF and esophageal cancer from a clinical point of view. This study also lacks of an animal model of esophageal cancer, which will allow us to test the efficacy of targeting microRNA218 in treatment of esophageal cancer. How exactly microRNA218 and p19ARF regulates EC9706 cells is still not fully understood. All these shortcomings will be overcome in future study.

Conclusion: Transfection of microRNA218 inhibited the viability of EC9706 cells. microRNA218 induced apoptosis of esophageal cancer EC9706 cells by suppression of p19ARF expression, suggesting that p19ARF might be a potential therapeutic target for esophageal cancer and providing a useful theoretical basis for treatment of esophageal cancer.

Acknowledgements

Research supported by the Projects of medical and health program in Zhejiang Province (NO. 2016KYB024).

Disclosure of conflict of interest

None.

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