miR-187 induces apoptosis of SiHa cervical carcinoma cells by downregulating Bcl-2

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ABSTRACT. Cervical carcinoma is a life-threatening illness posing considerable danger to women’s health. microRNAs (miRNAs) have been shown to regulate multiple cellular events, including growth and proliferation, and miR-187 is thought to regulate the growth and apoptosis of certain cell types. Our study focused on the influence of miR-187 on the growth, proliferation, and apoptosis of SiHa cervical carcinoma cells, and explored the mechanism behind its pro-apoptotic effect. miR-187 and control (scrambled) miRNA were synthesized with a standard protocol and lipofected into SiHa cells. Thiazolyl blue tetrazolium bromide assays and tests of caspase-3 activity were then performed to examine growth, proliferation, and apoptosis by flow cytometry. Small interfering RNA (siRNA) and an expression plasmid were synthesized for inhibition and overexpression of Bcl-2, respectively, and following their transfection, western blotting was used to examine Bcl-2 protein levels. Compared to transfection with control
miRNA, miR-187 significantly reduced SiHa cell growth and decreased Bcl-2 expression. Increased translocation of phosphatidylserine and activation of caspase-3 were observed in miR-187-transfected cells. Moreover, inhibition of Bcl-2 enhanced the pro-apoptotic effect of this miRNA, while Bcl-2 overexpression had the opposite effect. miR-187 inhibits the growth and proliferation of SiHa cells, and induces their apoptosis via downregulation of Bcl-2. Bcl-2 represents a potential therapeutic target for cervical carcinoma.

Key words: miR-187; Bcl-2; Cervical cancer; Apoptosis

INTRODUCTION

Cervical carcinoma is a malignancy of the female genital system with a relatively high mortality rate of approximately 12% (Chang et al., 2014). Despite its complicated pathogenesis, recent studies have demonstrated that viral infection, sexual behavior, and number of births are major cervical carcinoma determinants (Yuan et al., 2014). Recent clinical trials have shown that cervical carcinoma represents a significant threat to women’s health (Pedroza-Torres et al., 2014; Wen et al., 2015).

Early detection and treatment are the principle strategies of cervical carcinoma therapy. Although chemotherapy, radiotherapy, and surgery have been used to treat this disease with promising efficacy, certain disadvantages impede their further development, including clinical complications such as bleeding and toxic side effects (Huang et al., 2013; Zhang et al., 2013; Yu et al., 2014). Thus, the current emphasis and challenge in cervical carcinoma clinical practice is the improvement of treatment efficacy.

A precision medicine approach has recently been considered for cervical carcinoma treatment, with specific therapies being explored (Liu et al., 2013; Chen et al., 2014; Yang et al., 2014). This strategy includes the identification of more accurate targeting molecules (Luo et al., 2013), the efficacies of which need to be improved to enhance the effectiveness of cervical carcinoma therapy (Luo et al., 2013; Phuah et al., 2013). microRNAs (miRNAs) show promise as novel therapeutic targets for the treatment of this malignancy (Phuah et al., 2013).

miRNAs represent a category of short, non-coding RNAs with multiple biological functions. For example, miR-218 has been shown to inhibit the growth of cervical carcinoma cells, while miR-34a is associated with tumor metastasis (Xu et al., 2013; Li et al., 2015; Cong et al., 2016). This suggests that miRNAs are involved in the occurrence and development of cervical carcinoma (Cong et al., 2016). In addition, preliminary experiments have demonstrated that in cancer cells, expression of miR-187 is significantly higher than that in normal tissues (Bonetta et al., 2015; Song et al., 2015). The present study explored the regulatory effect of miR-187 on the SiHa cervical carcinoma cell line.

Apoptosis is a form of programmed cell death regulated by several proteins with anti- and pro-apoptotic effects. Anti-tumor strategies aim to kill cancer cells while leaving normal cells undamaged. One potential mechanism to achieve this involves inducing apoptosis by regulating the proteins that promote and inhibit this process (Bumrungthai et al., 2015; Kong et al., 2015). Bcl-2 is an extensively studied inhibitor of apoptosis, and as such, drugs targeting it have been designed. However, the efficacy of such molecules in inhibiting Bcl-2 is limited (Deng et al., 2015; Jia et al., 2016). Furthermore, recent research has demonstrated that Bcl-2
is regulated by multiple miRNAs (Liu et al., 2015). Our study aimed to establish an effective way to decrease Bcl-2 levels using miR-187.

In summary, the current investigation focused on the influence of miR-187 on the growth, proliferation, and apoptosis of SiHa cervical carcinoma cells, and explored the mechanism by which it induces apoptosis via Bcl-2.

**MATERIAL AND METHODS**

**Reagents and cell model**

Fetal bovine serum and Dulbecco’s modified Eagle’s medium (DMEM) were purchased from Hualan Biological Engineering, Inc. (Beijing, China). The following sequences were synthesized for RNA interference experiments: miR-187, 5'-ATC ACT GCA ATC GTC AGG TCA T-3' and 5'-AAG AAC GGT AAG AAG TAA GCA-3'; control miRNA, 5'-CCT GAG GGT TCA ACT CTA GC-3' and 5'-TTA CGA TTG TCA CGT ACA T-3'; Bcl-2 small interfering RNA (siRNA), 5'-GCT ACA GTC CAC TAT GGT GC-3' and 5'-TAC GAT TCG ATC AGT ACT T-3'.

The following materials were used in this study: Bcl-2 overexpression plasmid (Suzhou GenePharma, Suzhou, China), liposome transfection kit (Lipofectamine 2000; Invitrogen, Carlsbad, CA, USA), thiazolyl blue tetrazolium bromide (MTT) assay kit (Beijing Dingguo Biotechnology, Beijing, China), annexin V-fluorescein isothiocyanate (FITC) and caspase-3 kits (Beyotime, Haimen, China), and anti-Bcl-2 (catalog No.: N-19) and anti-actin antibodies (catalog No.: I-19) (Santa Cruz, Dallas, TX, USA).

SiHa cervical carcinoma cells were purchased from the American Type Culture Collection (Manassas, VA, USA). The following treatment groups were used: control miRNA; miR-187; control miRNA+Bcl-2 siRNA plasmid, miR-187+Bcl-2 siRNA; and control miRNA+Bcl-2 overexpression plasmid, miR-187+Bcl-2 overexpression plasmid.

**Cell culture**

SiHa cells were revived and cultured in high-glucose DMEM according to a standard protocol (Chen et al., 2014).

**Transfection**

Separately, miR-187 and control miRNA were transfected into SiHa cells using routine methods (Chen et al., 2014) and Lipofectamine 2000. Cells were cultured until 80% confluent, then miR-187 or control microRNA at a final concentration of 10 nM was suspended in Lipofectamine 2000 and transfected into cells.

**MTT assay**

The MTT assay was performed following a previously published protocol (Liu et al., 2013). Specifically, 1 x 10⁶ SiHa cells were seeded in each well of a six-well plate and cultured for 8 h. MTT (2 mg/mL) was added to each well and incubated with cells for 4 h. Cells were then exposed to DMSO for 5 min, before absorption at 560 nm was tested on a
microplate reader. Optical density was plotted on the ordinate, with different groups on the abscissa, to assess the effect of treatments on cell proliferation. Untransfected cells and miR-187-transfected cells were treated as negative and positive controls, respectively.

### Flow cytometry

Flow cytometry was performed to examine apoptosis with the annexin V-FITC staining method (Li et al., 2015). Excitation and absorption wavelengths of 488 and 625 nm, respectively, were used. Cells were stained with annexin V-FITC for 20 min. The untransfected and miR-187-transfected groups were treated as negative and positive controls, respectively. All experiments were repeated three times.

### Western blot

Proteins were extracted from transfected cells for western blotting according to standard methods (Cong et al., 2016). Extraction was carried out by lysing 1 x 10^5 cells. Proteins were quantified using a microplate reader and separated by centrifugation. Protein suspensions (15 mg protein) were electrophoresed and transferred to a nitrocellulose membrane, which was then blocked with 5% skim milk. The membrane was exposed to anti-Bcl-2 primary antibody (1:800) at 4°C for 12 h and anti-mouse immunoglobulin G secondary antibody (1:1500) at 37°C for 2 h, being washed three times after each incubation. Horseradish peroxidase activity was used to visualize specific protein bands with a gel imaging system. Protein quantification was based on grayscale intensity.

### Examination of caspase-3 activity

Caspase-3 activity was tested with a caspase-3 kit (Song et al., 2015). Transfected SiHa cells (1 x 10^5) were treated with a chromogenic substrate on a six-well plate and incubated at room temperature for 20 min. The chromophore p-nitroaniline was then detected by light absorbance using a microplate reader (Thermo Scientific, Waltham, MA, USA). All experiments were repeated three times. Relative caspase-3 activity was estimated with a routine protocol (Song et al., 2015).

### Alteration of Bcl-2 expression

Bcl-2 siRNA and expression plasmid (at a final concentration of 10 nM) (pcDNA3.1 with CMV as the promoter) were transfected separately into SiHa cells using Lipofectamine 2000. miR-187 or control miRNA (at a final concentration of 10 nM) were then transfected into the same cells.

### Statistical analysis

The SPSS 14.0 software (SPSS Inc., Chicago, IL, USA) was used for data analysis. Normally distributed measurement data are reported as means ± standard deviations. The t-test was performed to assess statistical significance, with P values < 0.05 being considered statistically significant. All experiments were repeated three times.
RESULTS

miR-187 transfection decreased cell viability and reduced cell growth

Compared to control miRNA, transfection with miR-187 significantly decreased the viability of SiHa cells, as measured by MTT assays (P < 0.01). No difference was observed between untransfected SiHa cells and those treated with control miRNA (Figure 1; P > 0.05). Therefore, the latter were used as a negative control in subsequent experiments.

Figure 1. Analysis of cell viability in three treatment groups. **P < 0.01 versus control microRNA (miRNA).

miR-187 transfection induced apoptosis of SiHa cells

miR-187 transfection significantly increased phosphatidylserine translocation compared to the control miRNA according to annexin V-FITC double staining (Figure 2; P = 0.0031). This suggests that apoptosis was induced by miR-187.

Figure 2. miR-187 transfection enhanced translocation of phosphatidylserine. **P < 0.01 versus control microRNA (miRNA). A. A representative histogram of annexin-V expression (phosphatidylserine translocation) analyzed by flow cytometry. B. Percentage of phosphatidylserine translocation in SiHa cells transfected with miRNA or miR-187.

miR-187 transfection activated caspase-3 in SiHa cells

Compared with the control miRNA construct, miR-187 transfection significantly increased caspase-3 activity in SiHa cells (Figure 3; P < 0.05), implying that this miRNA promoted apoptosis via activation of this enzyme.
miR-187 transfection increased caspase-3 activity in SiHa cells. *P < 0.05 versus control microRNA (miRNA).

miR-187 transfection decreased Bcl-2 protein expression in SiHa cells

In comparison to SiHa cells transfected with control miRNA, those treated with miR-187 exhibited significantly decreased expression of Bcl-2 protein (Figure 4), as observed by western blotting.

Bcl-2 inhibition enhanced the pro-apoptotic effect of miR-187

As shown in Figure 5A, Bcl-2 siRNA successfully inhibited expression of its target, and enhanced the decrease in Bcl-2 levels induced by miR-187. Moreover, the Bcl-2 siRNA+miR-187 group demonstrated the highest levels of caspase-3 activity (Figure 5B), suggesting that Bcl-2 inhibition increased the pro-apoptotic effect of miR-187. However, other BCL-2 independent effects might also be involved in miR-187-mediated cell apoptosis as miR-187 also has BCL-2-independent activities.

Bcl-2 overexpression negated the pro-apoptotic effect of miR-187

As shown in Figure 6A, the Bcl-2 plasmid effectively elevated Bcl-2 expression, and attenuated the reduction of this protein caused by miR-187. In addition, the Bcl-2 plasmid+miR-187 group exhibited lower caspase-3 activity than the miR-187 group (Figure 6B). This indicates that Bcl-2 overexpression diminished the pro-apoptotic influence of miR-187.
miR-187-induced cervical carcinoma cell apoptosis

DISCUSSION

Our study explored the effect of miR-187 on cervical cancer in a SiHa cell model. Furthermore, specific mechanisms were identified, furthering our understanding of its role in this malignancy. We found that miR-187 reduced the viability of cervical cancer cells,
inhibited their growth, and induced their apoptosis, consistent with the findings of a previous study (Pedroza-Torres et al., 2014).

Prior research has offered limited data concerning the mechanisms underlying the effect of miR-187 on cervical cancer (Pedroza-Torres et al., 2014). Recent study has demonstrated that miR-218 significantly inhibits cervical cancer growth, and miR-34a is associated with tumor metastasis (Xu et al., 2013; Li et al., 2015). This implies that miRNAs are involved in the progression of cervical cancer.

Bcl-2 is an anti-apoptosis protein widely expressed in different cell types (Lin et al., 2015), but its status as a target regulated by miR-187 remains unclear. In addition, the specific mechanisms responsible for the functions of Bcl-2 warrant further investigation (Bhattacharjya et al., 2015; Honegger et al., 2015). The present study revealed that miR-187 does indeed decrease Bcl-2 expression. Moreover, apoptosis of SiHa cells was enhanced as a result of miR-187-induced Bcl-2 inhibition. Furthermore, overexpression of Bcl-2 negated the pro-apoptotic effect of miR-187. Bcl-2 is recognized as an inhibitor of apoptosis. This study explored its impact on tumor progression through overexpression and RNA interference.

There were three key findings in the present investigation that demonstrate the pivotal role played by Bcl-2 in the pro-apoptotic effect of miR-187 on SiHa cells: 1) Bcl-2 protein expression was significantly reduced in SiHa cells transfected with miR-187; 2) Bcl-2 siRNA enhanced the pro-apoptotic influence of miR-187; and 3) plasmid-based Bcl-2 overexpression attenuated apoptosis induced by miR-187. Together, these observations suggest that miR-187 affects apoptosis of SiHa cells via downregulation of Bcl-2. Previous studies have shown Bcl-2 to be involved in other cancers, enhancing cancer cell proliferation and exacerbating lesions (Lao et al., 2014; Geng et al., 2015; Kogo et al., 2015). However, none of these have addressed the relationship between Bcl-2 and cervical cancer. Our study is the first to demonstrate that Bcl-2 also inhibits apoptosis in cervical cancer, identifying it as a potential target for molecular therapy based on regulation by miR-187 (Zhou and Wang, 2015). However, we cannot exclude other BCL-2 independent effects during miR-187-mediated cell apoptosis and requires further investigation.

Three limitations to our study should be considered: 1) we did not test tumor tissue and adjacent normal tissue from cervical cancer patients; 2) our study did not assess long-term prognosis in relation to Bcl-2 expression; and 3) animal models are needed to explore the effect of miR-187 on cervical cancer in vivo.

**CONCLUSION**

miR-187 induces apoptosis of cervical cancer cells via downregulation of Bcl-2. Bcl-2 is a potential drug target for treatment of this disease, and its inhibition could improve the efficacy of clinical therapy.

**Conflicts of interest**

The authors declare no conflict of interest.

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