5-Aminolevulinic acid photodynamic therapy in human cervical cancer via the activation of microRNA-143 and suppression of the Bcl-2/Bax signaling pathway

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Received December 17, 2014; Accepted December 22, 2015

DOI: 10.3892/mmr.2016.5248

Abstract. 5-Aminolevulinic acid photodynamic therapy (ALA-PDT) is a method using a photosensitizer and light radiation for disease treatment, and is currently used for the treatment of skin cancers, precancerous lesions and viral warts. The present study aimed to investigate the effect of ALA-PDT on human cervical cancer through the regulation of microRNA-143 (miR-143) and the Bcl-2/Bax signaling pathway. The results demonstrated that ALA-PDT reduced proliferation, increased cytotoxicity and induced apoptosis in HeLa human cervical carcinoma cells. Reverse transcription-quantitative polymerase chain reaction analysis demonstrated that the expression levels of miR-143 were increased following ALA-PDT treatment. Western blotting indicated that the expression levels of Bcl-2 and Bax were significantly reduced and increased, respectively, by ALA-PDT treatment. In addition, upregulation of miR-143 expression reduced Bcl-2 expression and increased Bax expression in HeLa cells. However, downregulation of miR-143 expression inhibited the effect of ALA-PDT on Bcl-2/Bax protein expression. In conclusion, the current study demonstrated that ALA-PDT affected human cervical cancer via the activation of miR-143 and the suppression of the Bcl-2/Bax signaling pathway.

Introduction

Cervical cancer is a key threat to women's health, with its incidence second to breast cancer in women worldwide (1). According to a report from the World Health Organization there are approximately 500,000 new cases of cervical cancer worldwide each year, of which 80% occur in developing countries (2,3).

MicroRNAs (miRNAs) are endogenous, single-stranded RNA molecules, with an approximate length of 22 nucleotides. miRNAs are widely present in eukaryotic cells and function to regulate gene expression, in addition to serving roles in a variety of important physiological and pathological processes (4). Previous studies have indicated that miRNAs are associated with the occurrence and development of various tumors, however there have been few reports about the role of miRNAs in cervical cancer (2,5).

The occurrence of cervical cancer is a complex process with multiple stages and steps, and is associated with the expression of various oncogenes and tumor suppressor genes, in addition to the regulation of apoptosis (6). Bcl-2 protein is predominantly localized in the mitochondria and rough endoplasmic reticulum, where it functions as an anti-apoptotic protein by inhibiting the oligomerization of Bax and Bak, thereby prolonging the life cycle of cells (7). Overexpression of Bcl-2 inhibits apoptosis, thereby inducing immortality in damaged cells and promoting the development of tumors, acting in combination with genes regulating proliferation and inhibiting growth (8).

Divaris *et al* (9) first used 5-aminolevulinic acid photodynamic therapy (ALA-PDT) for the treatment of skin diseases in 1990. At present, topical ALA-PDT is predominantly used for the treatment of benign and malignant skin tumors, including solar keratoses, seborrheic keratoses, basal cell epithelioma, Bowen's disease and squamous cell carcinoma (10). In addition, topical ALA-PDT has been demonstrated to exhibit a unique effect on certain infections of the skin, such as acne vulgaris and warts caused by human papillomavirus (HPV) infection (11). Thus, due to the high selectivity and broad application prospects of ALA-PDT, it has received increased attention from clinicians (12,13). In the current study, the potential anticancer effect of ALA-PDT on HeLa human cervical cancer cells was investigated, by assessing its effects on the proliferation, cytotoxicity and apoptosis of HeLa cells. In addition, the mechanisms underlying the effect of ALA-PDT on HeLa cells were investigated.

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Key words: 5-aminolevulinic acid photodynamic therapy, human cervical cancer, microRNA-143, Bcl-2/Bax

Materials and methods

Reagents. Gibco Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma-Aldrich (St. Louis, MO, USA). An Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) Apoptosis Detection kit was purchased from Bestbio, Co. (Shanghai, China). TRIzol® reagent and TaqMan miRNA Real-Time Quantitative PCR assays were purchased from Thermo Fisher Scientific, Inc (Invitrogen). A bicinchoninic acid (BCA) Protein Assay kit was purchased from Beyotime Institute of Biotechnology (Nanjing, China).

Cell culture. HeLa human cervical carcinomas cells were obtained from the experimental center of Qilu Hospital (Jinan, China). HeLa cells were cultured in DMEM containing 10% FBS, 100 U/ml penicillin and 100 U/ml streptomycin (both Beyotime Institute of Biotechnology) at 37˚C and 5% CO_2. Culture media was replaced every 2-3 days.

Cell treatments and cell proliferation assay. HeLa cells ($1.5x10^4$ cells/well) were seeded in a 96-well plate and cultured for 24 h. Subsequently, HeLa cells were treated with various concentrations of ALA (0, 0.1, 0.25, 0.5, 1.0, 2.0 and 4.0 μM; Sigma-Aldrich) for 24 h as previously described (14). Subsequently, PDT was conducted using a Aila laser generator apparatus (XD-635AB; Shanghai Fudan-Zhangjiang Bio-Pharmaceutical, Co., Ltd., Shanghai, China), with a dose of 5 J/cm^2 HeLa cells. A total of 20 μl MTT was added to each well and incubated for 4 h. Subsequently, 150 μl dimethyl sulfoxide (Shanghai Macklin Biochemical Co., Ltd., Shanghai, China) was added to each well followed by gentle agitation for 20 min. The absorbance of each well was measured at a wavelength of 570 nm using the LabSystems Multiskan MS Microplate Reader (Thermo Fisher Scientific, Inc.).

Lactate dehydrogenase (LDH) assay for cytotoxicity. HeLa cells ($1.5x10^4$ cells/well) were seeded in a 96-well plate and cultured for 24 h. Subsequently HeLa cells were treated with different concentrations of ALA (0, 0.25, 0.5 and 1.0 μM) for 24 h, after which PDT was conducted using a dose of 5 J/cm^2 of HeLa cells. A total of 100 μl LDH solution (Shanghai Enzyme-linked Biotechnology, Co., Ltd., Shanghai, China) was added to each well and incubated for 30 min. The absorbance was read at a wavelength of 490 nm using the LabSystems Multiskan MS Microplate Reader.

Annexin V-FITC/PI apoptosis assay. HeLa cells ($1x10^6$ cells/well) were seeded in a 6-well plate and cultured for 24 h. Subsequently, HeLa cells were treated with different concentrations of ALA (0, 0.25, 0.5 and 1.0 μM) for 24 h, after which PDT was conducted using a dose of 5 J/cm^2 of HeLa cells. A total of 10 μl Annexin-V FITC was added to each well and incubated for 10 min in the dark. Then, 10 μl PI was added to each well and incubated for 30 min in the dark. Flow cytometry was conducted using a FACSCalibur flow cytometer and CellQuest™ Pro software, version 5.1 (BD Biosciences, San Jose, CA, USA) to analyze apoptosis.

Reverse transcription-quantitative PCR analysis of miR-143 expression. HeLa cells ($1x10^6$ cells/well) were seeded in a 6-well plate and cultured for 24 h at 37˚C and 5% CO_2. Subsequently, HeLa cells were treated with various concentrations of ALA (0, 0.25, 0.5 and 1.0 μM) for 24 h, after which PDT was conducted using a dose of 5 J/cm^2 HeLa cells. Total RNA was extracted from HeLa cells using TRIzol® reagent, and 1 ng RNA was converted to cDNA using the SuperScript III First-Strand Synthesis System for RT-PCR (Thermo Fisher Scientific, Inc.). The miRNAs were measured using TaqMan miRNA Real-Time PCR assays and the Rotor-Gene 3000 thermal cycler (Qiagen China Co., Ltd., Shanghai, China). The PCR cycling conditions were as follows: 95˚C for 1 min, followed by 40 cycles at 95˚C for 30 sec, 60˚C for 30 sec, 95˚C for 45 min, 55˚C for 1 min and 55˚C for 10 sec. The primers used were as follows miR-143, forward 5'-UGAGAUGAAGCA CUGUAGCUC-3' and reverse 5'-TGAGATGAAGCACTG TAGCTCT-3'; U6 small nuclear (sn)RNA, forward 5'-CGC TTCGGCAGCACATATACTA-3' and reverse 5'-CGCTTC ACGAATTTGCGTGTCA-3' (Sangon Biotech Co., Ltd., Shanghai, China). The relative expression levels of miR-143 were calculated using the $2^{-\Delta\Delta Cq}$ method, following normalization to U6 snRNA.

Western blotting of Bcl-2 and Bax protein expression. HeLa cells ($1x10^6$ cells/well) were seeded into a 6-well plate and cultured for 24 h at 37˚C in 5% CO_2. Subsequently, HeLa cells were treated with various concentrations of ALA (0, 0.25, 0.5 and 1.0 μM) for 24 h, after which PDT was conducted using a dose of 5 J/cm^2 of HeLa cells. HeLa cells were then lysed on ice using radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology) and cultured for 30 min at 4˚C. The protein concentration was determined using a BCA Protein Assay kit. Equivalent volumes of protein were separated by 10% SDS-PAGE (Beyotime Institute of Biotechnology) and transferred onto polyvinylidine difloride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked for 1 h in 5% nonfat dried milk in Tris-buffered saline-Tween-20 (TBST; Jiancheng Bioengineering institute, Nanjing, China). The membranes were then incubated with mouse anti-Bcl-2 (1:1,000; cat. no. sc-7382; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-Bax (1:1,000; cat. no. sc-23959; Santa Cruz Biotechnology, Inc.) and anti-β-actin (1:500; cat. no. AF0003; Beyotime Institute of Biotechnology) monoclonal antibodies overnight at 4˚C. Subsequently, the membranes were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG secondary antibodies (1:5,000; cat. no. A0258; Beyotime Institute of Biotechnology), and observed using an Enhanced Chemiluminscence Advanced Western Blot Detection kit (cat. no. P0202; Beyotime Institute of Biotechnology). The proteins were analyzed using ImageJ 1.37 software (National Institutes of Health, Bethesda, MA, USA).

Transfection of miR-143 and anti-miR-143. miR-143, anti-miR-143 and negative plasmids were obtained from Sangon Biotech Co., Ltd. HeLa cells ($1x10^6$ cells/well) were seeded into a 6-well plate and cultured for 24 h at 37˚C in 5% CO_2. Subsequently, the plasmids (100 nmol/l) were transfected using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) into HeLa cells.

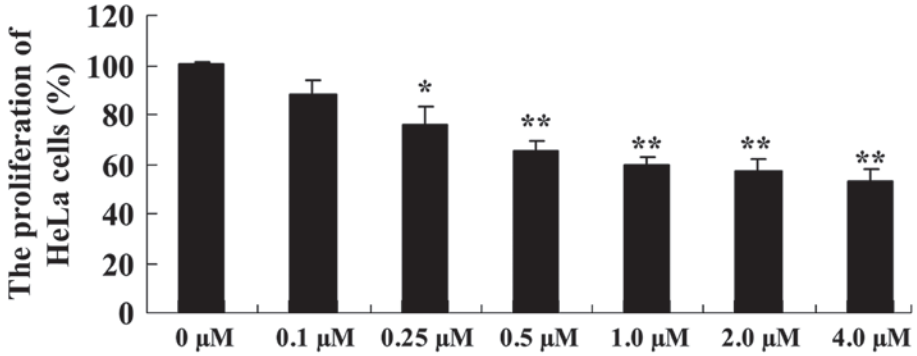

Figure 1. ALA-PDT inhibits the proliferation of HeLa cells. *P<0.05 vs. 0 μM ALA-PDT treatment group; **P<0.01 vs. 0 μM ALA-PDT treatment group. ALA-PDT, 5-aminolevulinic acid photodynamic therapy.

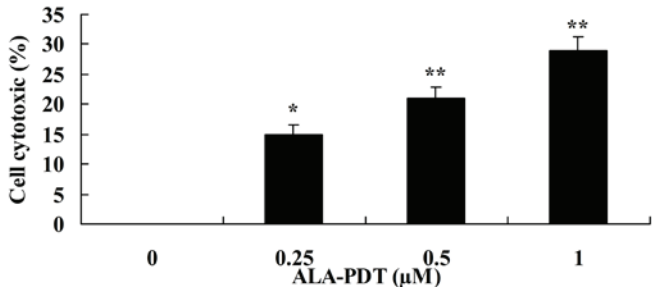

Figure 2. ALA-PDT induces cell cytotoxicity of HeLa cells. *P<0.05, **P<0.01 vs. 0 μM ALA-PDT treatment group. ALA-PDT, 5-aminolevulinic acid photodynamic therapy.

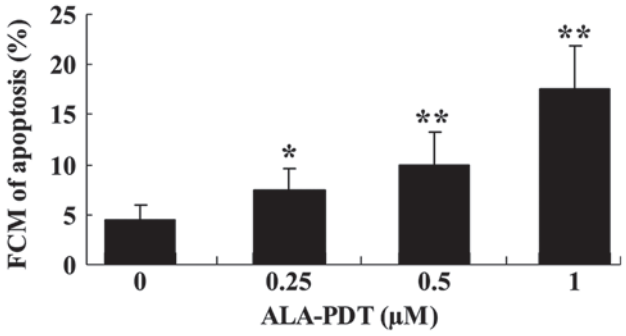

Figure 3. ALA-PDT induces apoptosis in HeLa cells. *P<0.05, **P<0.01 vs. 0 μM ALA-PDT treatment group. ALA-PDT, 5-aminolevulinic acid photodynamic therapy; FCM, flow cytometry.

Statistical analysis. Statistical analysis was conducted using SPSS software, version 17.0 (SPSS, Inc., Chicago, IL, USA). All experiments were performed three times and data were presented as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Results

ALA-PDT inhibits the proliferation of HeLa cells. As presented in Fig. 1, the proliferation of HeLa cells was inhibited following treatment with ALA-PDT in a dose-dependent manner. Following treatment with ALA-PDT for 24 h, the proliferation of HeLa cells was significantly reduced, compared with the 0 μM ALA-PDT treatment group (0.25 μM, P<0.05; 0.5-4.0 μM, P<0.01; Fig. 1).

ALA-PDT induces cytotoxicity in HeLa cells. To investigate the effect of ALA-PDT on HeLa cells, the level of cytotoxicity was measured using an LDH assay. Treatment with ALA-PDT increased the level of cytotoxicity observed in HeLa cells, compared with the 0 μM ALA-PDT treatment group (0.25 μM, P<0.05; 0.5 and 1.0 μM, P<0.01; Fig. 2).

ALA-PDT induces apoptosis in HeLa cells. To investigate whether apoptosis is promoted following treatment with ALA-PDT, the level of apoptotic HeLa cells was assessed using an annexin V-FITC/PI apoptosis assay. Treatment with ALA-PDT for 24 h induced apoptosis in HeLa cells, compared with the 0 μM ALA-PDT treatment group (0.25 μM P<0.05; 0.5 and 1.0 μM, P<0.01; Fig. 3).

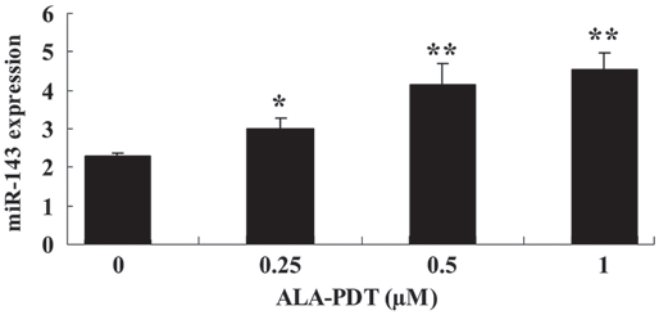

Figure 4. ALA-PDT increases miR-143 expression in HeLa cells. *P<0.05, **P<0.01 vs. 0 μM ALA-PDT treatment group. ALA-PDT, 5-aminolevulinic acid photodynamic therapy; miR-143, microRNA-143.

ALA-PDT increases the expression of miR-143 in HeLa cells. The aim of the investigation was to determine whether treatment with ALA-PDT alters the expression of miR-143 in HeLa cells. Following treatment with ALA-PDT for 24 h, the expression levels of miR-143 were increased in HeLa cells, compared with the 0 μM ALA-PDT treatment group (0.25 μM, P<0.05; 0.5 and 1.0 μM, P<0.01; Fig. 4).

ALA-PDT inhibits the Bcl-2/Bax signaling pathway in HeLa cells. To investigate the effect of ALA-PDT treatment on the Bcl-2/Bax signaling pathway in HeLa cells, the expression levels of Bcl-2 and Bax were analyzed by western blotting (Fig. 5A and B). The results indicated that Bcl-2 and Bax

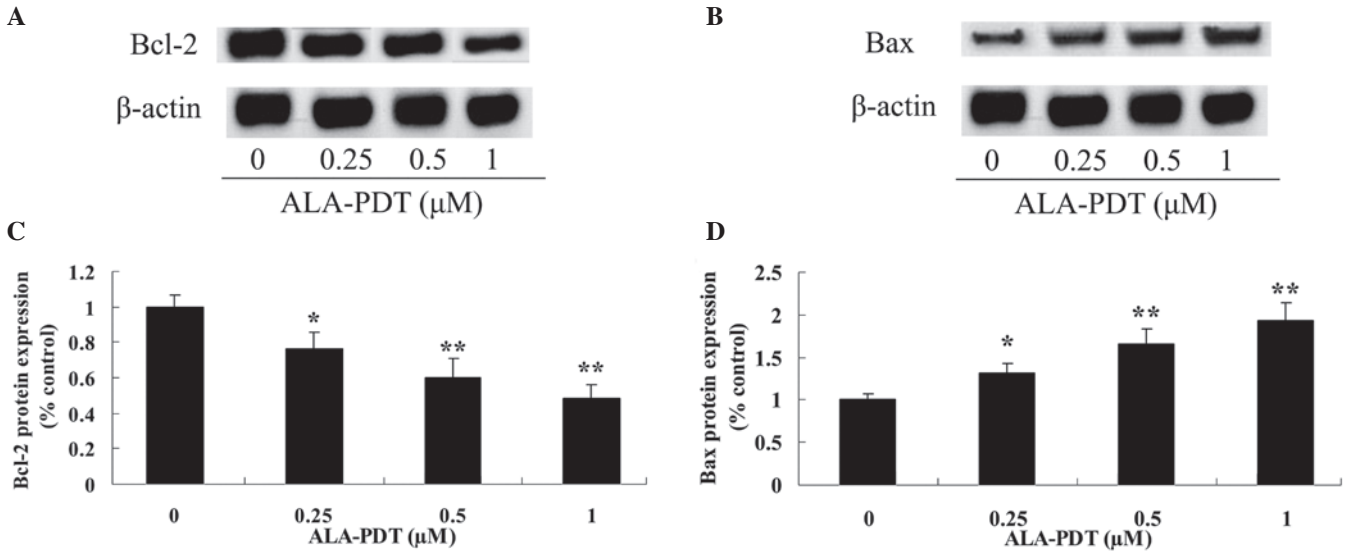

Figure 5. ALA-PDT inhibits the Bcl-2/Bax signaling pathway in HeLa cells. The effect of ALA-PDT on (A) Bcl-2 and (B) Bax protein expression by western blotting and the quantification of (C) Bcl-2 and (D) Bax protein levels. *P<0.05 vs. 0 μM ALA-PDT treatment group; **P<0.01 vs. 0 μM ALA-PDT treatment group. ALA-PDT, 5-aminolevulinic acid photodynamic therapy.

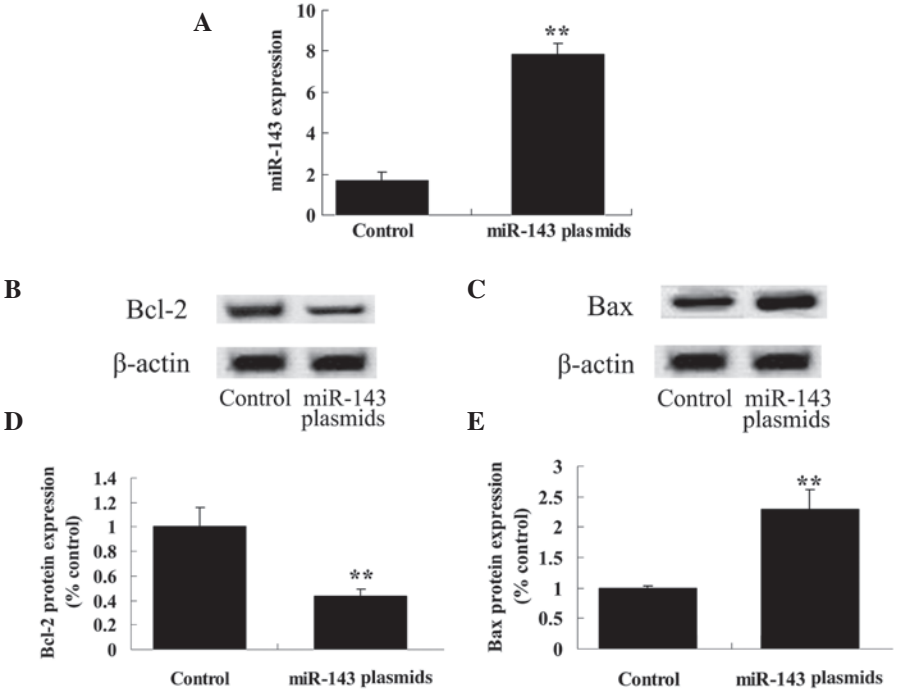

Figure 6. Upregulation of miR-143 expression downregulates the Bcl-2/Bax signaling pathway in HeLa cells. (A) Upregulation of miR-143 increased miR-143 expression, inhibited (B) Bcl-2 and (C) Bax protein expression by western blotting assays. Quantification of (D) Bcl-2 and (E) Bax protein levels. **P<0.01 vs. control group. miR-143, microRNA-143.

protein expression levels were reduced and increased, respectively, following treatment with ALA-PDT for 24 h (0.25 μM, P<0.05; 0.5 and 1.0 μM, P<0.01; Fig. 5C and 5D).

Upregulation of miR-143 expression downregulates the Bcl-2/Bax signaling pathway in HeLa cells. To further investigate the potential association between miR-143 expression levels and the effect of ALA-PDT on HeLa cells, the effect of ALA-PDT on HeLa cells following miR-143 plasmid transfection was analyzed. The results demonstrated that the miR-143 plasmid markedly increased the expression levels of miR-143 in HeLa cells (P<0.01; Fig. 6A). In addition, the miR-143 plasmid downregulated the Bcl-2/Bax signaling pathway in HeLa cells (P<0.01; Fig. 6B-E).

Downregulation of miR-143 expression reverses the effect of ALA-PDT on HeLa cells and upregulates the Bcl-2/Bax signaling pathway in HeLa cells. To investigate the mechanism underlying the effect of ALA-PDT treatment on apoptosis in HeLa cells, anti-miR-143 plasmids were transfected into

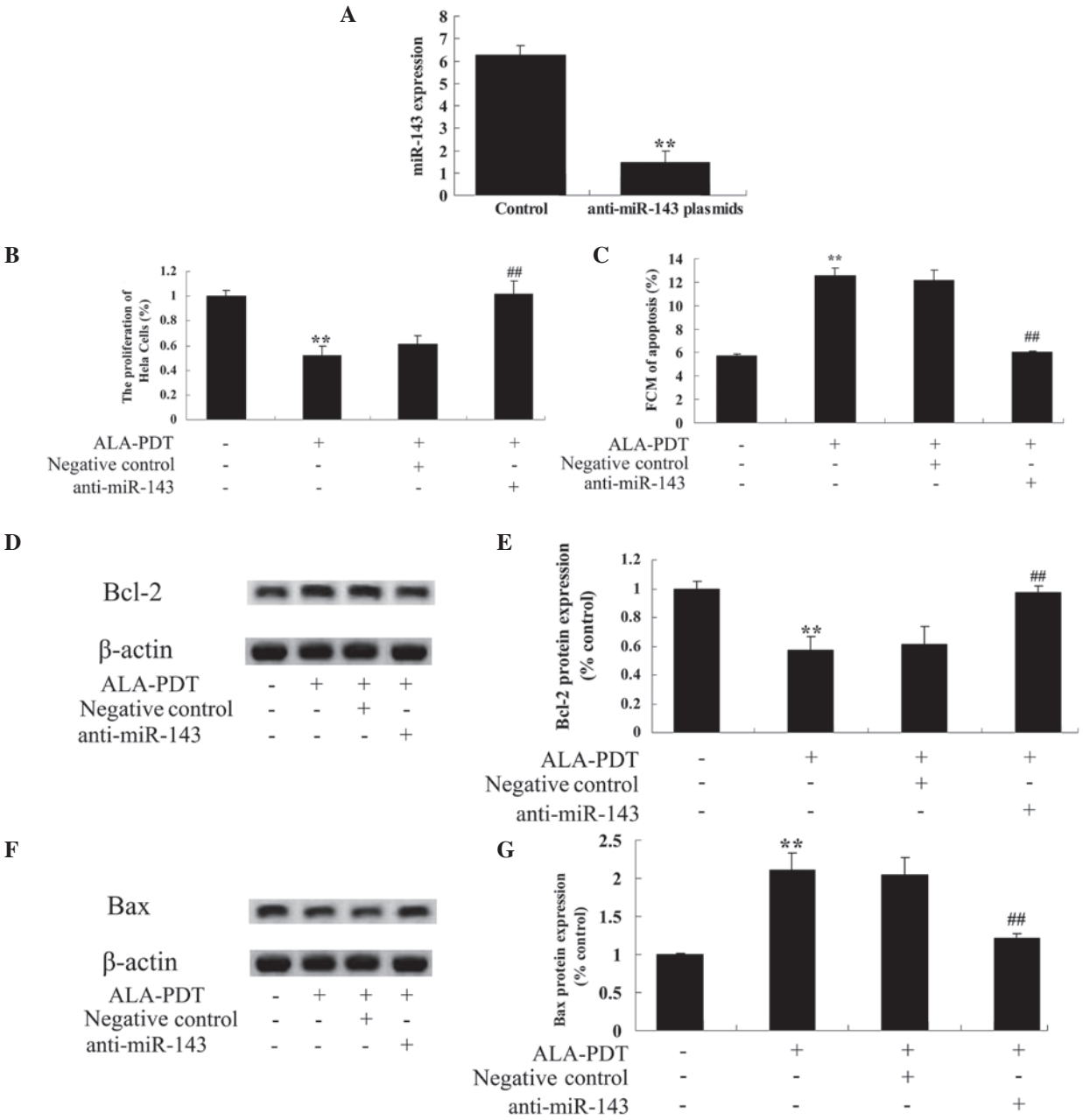

Figure 7. Downregulation of miR-143 expression inhibits the effect of ALA-PDT on HeLa cells and upregulates the Bcl-2/Bax signaling pathway in HeLa cells. (A) Downregulation of miR-143 reduced miR-143 expression, (B) inhibits the effect of ALA-PDT on the proliferation and (C) cellular apoptosis in HeLa cells. Expression of (D) Bcl-2 and (F) Bax by western blotting and quantification of (E) Bcl-2 and (G) Bax protein levels. **P<0.01 vs. 0 μM ALA-PDT treatment group; $^{\#\#}$P<0.01 vs. ALA-PDT treatment group transfected with negative control. miR-143, microRNA-143; ALA-PDT, 5-aminolevulinic acid photodynamic therapy; FCM, flow cytometry.

HeLa cells. The results demonstrated that the anti-miR-143 plasmid significantly reduced the miR-143 expression levels in HeLa cells (P<0.01; Fig. 7A). In addition, the anti-miR-143 plasmid significantly inhibited the effect of ALA-PDT on proliferation (P<0.01, Fig. 7B) and apoptosis (P<0.01; Fig. 7C) in HeLa cells. Furthermore, the anti-miR-143 plasmid significantly blocked the effect of ALA-PDT on Bcl-2 and Bax levels in HeLa cells (P<0.01; Fig. 7D-G).

Discussion

Cervical cancer is the most prevalent type of malignant tumor of the female genital tract in Chinese women, with 140,000 new cases occurring annually in China, approximately 28.8% of the newly diagnosed cases of cervical cancer worldwide (15). In recent years, the increase in cervical cancer screening has led to significant reductions in its incidence and mortality. However, the increase in the of HPV infection rate in addition to alterations in lifestyle have resulted in an increase in the incidence, and a reduction in the age of onset of cervical cancer (16). The current study demonstrated that ALA-PDT inhibited proliferation and increased cytotoxicity and apoptosis in HeLa cells. Previous studies have demonstrated the cytotoxic effects of ALA-PDT against skin cancer cells (17,18). ALA-PDT has previously been demonstrated to be cytotoxic against HeLa cervical cancer cells, suppressing tumor growth

and inducing apoptosis (19). Therefore, ALA-PDT may represent a potential anticancer therapy for cervical cancer.

Numerous previous studies have indicated the potential role of miRNAs in cancer; studies in *C. elegans* and *Drosophila* demonstrated that miRNAs regulate cell proliferation and apoptosis, suggesting that miRNAs are associated with proliferation-associated diseases such as cancer (20,21). In addition, numerous miRNA genes are located in genomic regions which are frequently mutated, with variation in these regions commonly accompanied by the occurrence of cancer (22,23). Furthermore, compared with normal tissue, there is differential expression of miRNAs in malignant tumors and tumor cell lines (24,25). The current study demonstrated that ALA-PDT increased the expression levels of miR-143 in HeLa cells. Liu *et al* (26) demonstrated *in vitro* that overexpression of miR-143 significantly inhibited proliferation, promoted apoptosis and reduced the levels of Bcl-2 in HeLa cervical cancer cells. However, how ALA-PDT influences miR-143 expression remains to be fully elucidated.

Apoptosis, known as programmed cell death, is the automatic and orderly self-destruction of the cell, and is controlled by genes, involving a series of gene activation, expression and regulation. The Bcl-2 gene is a cancer-associated gene with interest in the study of apoptosis, in which Bcl-2/Bax is of particular importance (27). The predominant biological function of Bax is to promote apoptosis, thereby inhibiting tumorigenesis. Bcl-2 and Bax *in vivo* serve a role as a dimer protein (28). Homodimers of Bcl-2 inhibit apoptosis whilst homodimers of Bax promote apoptosis. When a Bcl-2/Bax heterodimer is formed, Bax is able to inhibit the anti-apoptotic function of Bcl-2 resulting in the promotion of apoptosis (29). In the current study, the results demonstrated that ALA-PDT reduces Bcl-2 and increases Bax protein expression in HeLa cells. He *et al* (30) demonstrated that ALA-PDT reduced proliferation and induced apoptosis of the Mel80 cervical cancer cell line via the suppression of Bcl-2 and the activation of Bax. Wei *et al* (14) additionally suggested that administration of ALA-PDT in combination with low-dose cisplatin was an effective and feasible therapy for cervical cancer though alterations in the expression of p21, Bcl-2 and Bax.

To further investigate the potential association of the effect of ALA-PDT on HeLa cells, miR-143 expression and the Bcl-2/Bax signaling pathway, the present study analyzed the anticancer effect of ALA-PDT on HeLa cells following transfection of miR-143 and anti-miR-143 plasmids. The data collected demonstrated that overexpression of miR-143 inhibited the Bcl-2/Bax signaling pathway. Furthermore, downregulation of miR-143 expression reduced the anticancer effect of ALA-PDT on HeLa cells through the upregulation of the Bcl-2/Bax signaling pathway. These data are supported by previous studies. Liu *et al* (26) demonstrated that overexpression of miR-143 significantly inhibited cell proliferation and promoted apoptosis, through the reduction in Bcl-2 levels, with downregulated expression of miR-143 increasing Bcl-2 levels in cervical cancer.

In conclusion, the results of the present study suggested that the anticancer effect of ALA-PDT may be used as a novel therapy for the treatment of human cervical cancer, by increasing the expression of miR-143 and downregulating the Bcl-2/Bax signaling pathway. Further studies are required to fully elucidate the precise anticancer effect of ALA-PDT in animal models and as a clinical treatment.

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