MicroRNA-21 regulates the proliferation and apoptosis of cervical cancer cells via tumor necrosis factor-α

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Abstract. The proliferation and apoptosis of tumor cells are regulated by a variety of microRNAs (miRs). miR-21 can inhibit the apoptosis of cancer cells *in vitro*. Tumor necrosis factor α (TNF-α) serves an important role in the induction of proliferation of cervical cancer cells. Previous studies have demonstrated that the expression level of miR-21 is associated with TNF-α expression in alveolar macrophages. However, to the best of our knowledge, whether miR-21 regulates TNF-α in cervical cells has not been reported. The present study was designed to investigate whether miR-21 regulates TNF-α expression, proliferation and apoptosis of cervical cancer cells. miR-21, miR-21 inhibitor and control miRNA were synthesized and transfected into HeLa cervical cancer cells. Reverse transcription-quantitative polymerase chain reaction was used to measure the expression levels of miR-21 and TNF-α at the mRNA level. Western blotting was used to measure the expression levels of TNF-α at the protein level. MTT assay and Hoechest-33342 staining were used to measure the proliferation and apoptosis of HeLa cells. miR-21 was identified to upregulate the mRNA and protein expression levels of TNF-α. Furthermore, upregulation of TNF-α enhanced the proliferation capability of HeLa cells. Changes in the expression levels of miR-21 and TNF-α did not significantly affect the apoptosis of Hela cells. In conclusion, the present study demonstrated that miR-21 regulates the expression of TNF-α in HeLa cells. Additionally, the expression level of TNF-α was positively associated with the proliferation capability of Hela cells, but not apoptosis. Therefore, miR-21 regulates the proliferation of HeLa cells through regulation of TNF-α. These results provide novel potential therapeutic targets for the treatment of cervical cancer.

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Introduction

Cervical cancer has a high incidence in females and poses a serious health risk. The number of new patients of cervical cancer worldwide of each year is nearly 50 million (1). At present, there is no effective treatment for patients with advanced cervical cancer. Therefore, discovery of targets has great significance in the targeted treatment and prognosis cervical cancer (2,3).

Tumor necrosis factor-α (TNF-α) serves an important role in a wide range of biological processes and regulates cell proliferation, differentiation and apoptosis (4). Previous studies have demonstrated that TNF-α presents in a variety of tumor tissues, and not only can inhibit tumor cell growth then induce tumor necrosis, but also improve the proliferation, migration and invasion capacity of tumor cells (2,5). It has been demonstrated that TNF-α has certain associations with cervical cancer in older women (6).

MicroRNAs (miRNAs/miRs) are involved in the regulation of cellular functions of many genes, and are widely presented in eukaryotes. miRNAs function mainly through complementary binding to the target gene which can induce the degradation of its mRNA, or suppress gene transcription to reduce the expression levels of its target genes (7,8). One protein may be regulated by a variety of miRNAs. It has been demonstrated that miRNAs serve an important regulatory role in many human diseases. Some miRNAs have been used as markers for the clinical detection of early tumors, and some have been used as therapy targets (9). Studies have revealed that altered expression of miRNAs is correlated with the proliferation, apoptosis and migration of cervical cancer cells, and certain miRNAs including miR-27b, miR-24 and miR-21, have been identified to be overexpressed in cervical cancer (10,11). To the best of our knowledge, the association between miR-21 and TNF-α in cervical cancer has not been reported.

Therefore, in the present study, TNF-α was chosen as a downstream target of miR-21. The effect of miR-21 on the expression of TNF-α and the association between TNF-α and proliferation and apoptosis of cervical cancer cells was investigated.

Materials and methods

Materials. HeLa human cervical cancer cells were purchased from Shanghai Beinuo Biological Technology Co., Ltd.

(Shanghai, China). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum and trypsin digestion enzymes were purchased from Hyclone; GE Healthcare (Logan, UT, USA). PBS and antibiotics were purchased from Beyotime Institute of Biotechnology (Haimen, China). A liposome cell transfection kit was purchased from Invitrogen; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). An RNA extraction kit (TRIzol) and a cDNA synthesis kit were purchased from Beyotime Institute of Biotechnology. Anti-human TNF-α antibody was purchased from Shanghai Xin Le Biotechnology Co., Ltd. (Shanghai, China). Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) amplification reagents were purchased from Omega Bio-Tek, Inc. (Norcross, GA, USA). Hoechest-33,342 stain was purchased from Beijing Suolaibao Biotechnology Co., Ltd. (Beijing, China). Primers were synthesized by Shanghai Shenggong Co., Ltd. (Shanghai, China).

Cell cultures and transfection. HeLa cells were conventionally cultured in DMEM containing 10% fetal bovine serum. The third generation of cells were collected, counted and seeded at a density of $10x10^4$ cells/well and cultured for 18 h before transfection. miR-21 mimic (forward, UAGCUUAUCAGACUGAUG UUGA and reverse, AACAUCAGUCUGAUAAGCUAUU), miR-21 inhibitor (UCAACAUCAGUCUGAUAAGCUA) and negative control (forward, UUCUCCGAACGUGUCACGUTT and reverse, ACGUGACACGUUCGGAGAATT for miR-21 mimic; CAGUACUUUUGUGUAGUACAA for miR-21 inhibitor) (Dharmacon; GE Healthcare Life Sciences, Little Chalfont, UK) were diluted with 50 μl Opti-minimum essential medium (MEM; Thermo Fisher Scientific, Inc.) to a final concentration of 100 nM. Liposome (2 μl) was added to 50 μl Opti-MEM. A total of 5 min later, the two diluted liquids were mixed and incubated at room temperature for 30 min, and then added to the culture media. The culture media was replaced 4 h later. Each experiment was repeated three times.

RT-qPCR. Total RNAs of cells before transfection, and 36 or 48 h after transfection were extracted and then reverse transcribed into cDNA according to the manufacturer's protocol. cDNA was used as template to perform PCR with the following profile: An initial 5-min incubation at 95°C, 30 cycles of (45 sec at 95°C, 45 sec at 50°C and 40 sec at 72°C), and a final extension of 3 min at 72°C. Reaction mixtures contained the following ingredients at the given final concentrations: 2 ng/μl DNA template; 10 mM Tris-HCl (pH: 8.3); 1.5 mM MgCl2; 200 μM each of dNTP; 0.2 units of Taq DNA polymerase; and 0.5 μM of each primer. The primer sequences for TNF-α, miR-21 and GAPDH (internal control) are presented in Table I. mRNA expression was quantified as normalization to the internal control GAPDH using the $2^{-\Delta\Delta Cq}$ method (12).

Western blot analysis. Cells were collected at 36 or 48 h after transfection, washed with PBS, and lysed using radioimmunoprecipitation assay lysis buffer (Thermo Fisher Scientific, Inc.) followed by centrifugation at 5,000 x g for 20 min at 37°C for protein extraction. Proteins were quantified using a Bicinchoninic Acid kit, according to the manufacturer's protocol. Equal amounts of protein (20 μg/lane) were separated

Table I. Primer sequences.

Gene	Sequence (5'-3')
TNF-α-F	CCCGCATCCCAGGACCTCTCT
TNF-α-R	CGGGGGACTGGCGA
miR-21-F	CTAAGACCTGTGGAATGGC
miR-21-R	CTCAAAGATGTCATTGCC
GAPDH-F	CGGAGTCAACGGATTTGGTCGTA
GAPDH-R	AGCCTTCTCCATGGTGGTGAAG

F, forward; R, reverse; TNF-α, tumor necrosis factor-α; miR, microRNA.
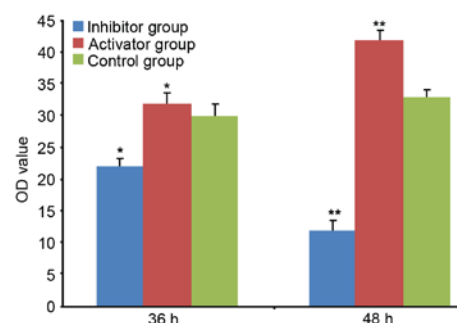

Figure 1. miR-21 levels at 36 and 48 h after transfection. HeLa cells were transfected with miR-21 or miR-21 inhibitor. Data are expressed as the mean ± standard error. *P<0.05 and **P<0.01 vs. other two groups at the same time-point. OD, optical density; miR-21, microRNA-21.
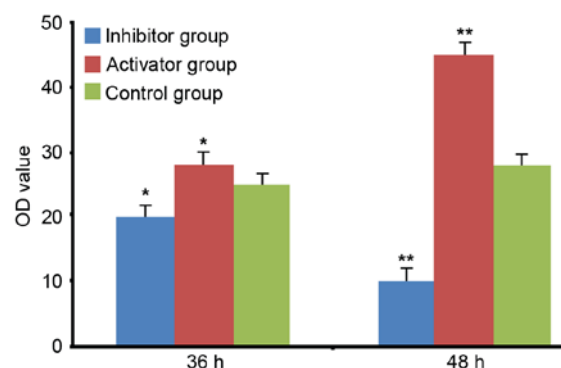

Figure 2. Tumor necrosis factor-α levels at 36 and 48 h after transfection. HeLa cells were transfected with miR-21 or miR-21 inhibitor. Data are expressed as the mean ± standard error. *P<0.05 and **P<0.01 vs. other two groups at the same time-point. OD, optical density; miR-21, microRNA-21.

by 10% SDS-PAGE, transferred to a polyvinylidene fluoride membrane (Thermo Fisher Scientific, Inc.), and blocked with 5% nonfat milk at 37°C for 1 h. After washing with TBS with Tween-20 (TBST), membranes were incubated with primary mouse anti-human TNF-α antibody (1:1,000; RayBiotech, Inc., Norcross, GA, USA; cat. no. 119-1633) overnight at 4°C. After washing with TBST, membranes were incubated with horseradish peroxidase-conjugated anti-mouse secondary antibodies (1:2,500; Abcam, Cambridge, UK; cat. no. ab97046.) for 3 h at 37°C, and developed with enhanced chemiluminescence
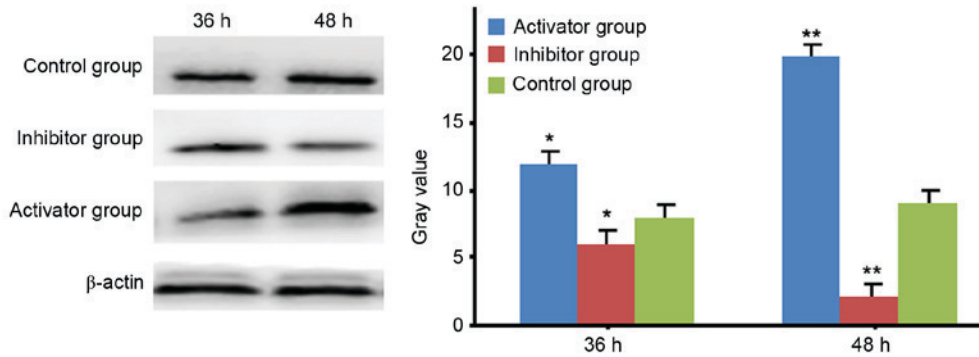
Figure 3. Western blot analysis of levels of tumor necrosis factor-α after HeLa cells were transfected with miR-21 or miR-21 inhibitor. β-actin served as an internal control. Data are expressed as the mean ± standard error. *P<0.05 and **P<0.01 vs. control. miR-21, microRNA-21.
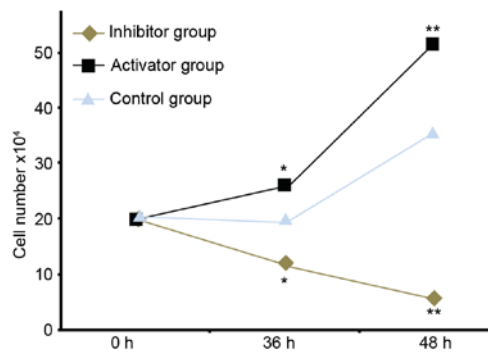

Figure 4. Effect of TNF-α on HeLa cell proliferation. HeLa cells were transfected with miR-21 or miR-21 inhibitor. Data are expressed as the mean ± standard error. *P<0.05 and **P<0.01 vs. control. miR-21, microRNA-21.

Figure 5. Apoptosis of HeLa cells following transfection with miR-21 or miR-21 inhibitor (magnification, x200). Data are expressed as the mean ± standard error. P>0.05. miR-21, microRNA-21.

(Thermo Fisher Scientific, Inc.). Expression levels of TNF-α were analyzed with β-actin (1:2,500; Abcam; cat. no. ab8227) serving as an internal control.

MTT assay. The cell suspension was counted after transfected cells were seeded into 96-well plates at a density of $1x10^4$ cells/well. MTT solution (20 μl) was added to each well at 36 or 48 h after transfection, and incubated for 4 h. Dimethyl sulfoxide (150 μl) was added to each well to stop reaction. The optical density at 560 nm was recorded using a microplate reader to calculate the proliferation rate of HeLa cells.

Hoechest-33,342 staining. Transfected cells were transferred into 6-well plates and cultured for 36 or 48 h. After removal of the cell culture media and washing with PBS for 3-5 times, 200 μl 4% paraformaldehyde was added to each well and kept at 4°C for 20 min to fix the cells. After washing with PBS for 3-5 times, 1 ml Hoechest-33,342 solution was added to each well and kept at 37°C for 20 min, rinsed again with PBS, and observed under an inverted fluorescence microscope.

Statistical analysis. SPSS version 11.3 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. All data are expressed as the mean ± standard error. Differences between groups were analyzed by two-way analysis of variance with the Bonferroni post hoc test. P<0.05 was considered to indicate a statistically significant difference.
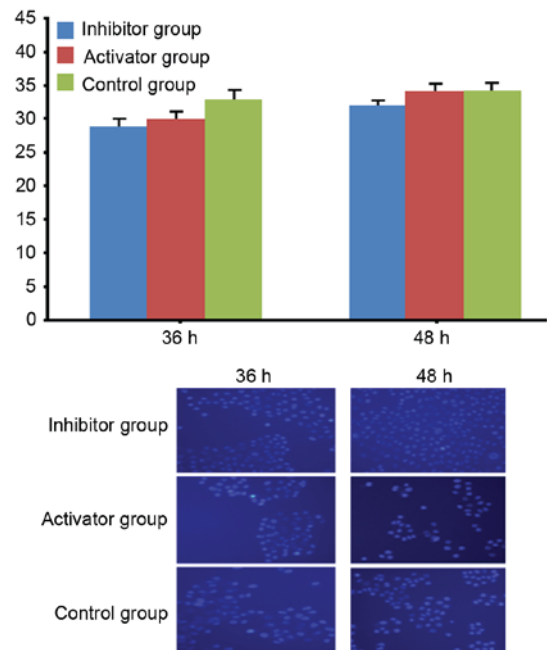
Results

RT-qPCR assay of miR-21 mRNA expression. The RT-qPCR results demonstrated that the expression levels of miR-21 were significantly upregulated at 36 and 48 h after transfection of miR-21, compared with the control group (P<0.05; Fig. 1). Transfection of an miR-21 inhibitor resulted in the down-regulation of miR-21 at both time points after transfection of miR-21, compared with the control group (P<0.01; Fig. 1), suggesting that manipulation of miR-21 levels through transfection of miR-21 and miR-21 inhibitor was successful.

RT-qPCR assay of TNF-α mRNA expression. The RT-qPCR results revealed that the mRNA expression levels of TNF-α were significantly upregulated at 36 and 48 h after transfection of miR-21, compared with the control group (P<0.05; Fig. 2). Transfection of the miR-21 inhibitor resulted in the downregulation of TNF-α at 36 and 48 h after transfection of

miR-21, compared with the control group (P<0.05 and P<0.01, respectively; Fig. 2), suggesting that the expression of TNF-α is positively associated with miR-21.

Western blot analysis of TNF-α. Western blot analysis demonstrated that the expression levels of TNF-α were significantly increased in a time-dependent manner after transfection of miR-21, compared with the control (P<0.05; Fig. 3). In contrast, the expression levels of TNF-α were significantly decreased in a time-dependent manner after transfection of miR-21 inhibitor, compared with the control (P<0.05; Fig. 3).

MTT assay of HeLa cell proliferation. The MTT results demonstrated that at 36 and 48 h after transfection, upregulation of TNF-α enhanced HeLa cell proliferation in a time-dependent manner. Conversely, inhibition caused a decrease in cell proliferation in a time-dependent manner (Fig. 4).

Hoechest-33,342 staining. Hoechest-33,342 staining results demonstrated that no significant difference was found in the apoptosis of HeLa cells among different groups.

Discussion

Cervical cancer is a complex pathological process in which proliferation and apoptosis of cervical cancer cells serves an important role. The proliferation and apoptosis of cervical cancer cells are regulated by multiple signaling pathways, including p53, phosphatase and tension homolog (PTEN) and TNF-α pathways. TNF-α not only can inhibit proliferation to induce apoptosis of cervical cancer cells by regulating the downstream nuclear factor-κB, receptor activating protein kinase 3, epidermal growth factor receptor and other proteins, but also can induce the proliferation, migration and invasion of cancer cells. Therefore, assessing the association between TNF-α and proliferation and apoptosis of cervical cancer cells is of great significance (13-16). Although studies have confirmed that miRNAs serve an important role in the proliferation and apoptosis of cancer cells, the study of miRNA regulation of TNF-α is still relatively rare. Previous studies have demonstrated that miR-29a regulates the incurrence of atherosclerosis via regulation of TNF-α, indicating the existence of a regulatory relationship between TNF-α and miRNA (17,18). It has been demonstrated that miR-21 serves an important role in the regulation of cancer cell proliferation and apoptosis. Inhibition of miR-21 promotes the migration of cervical cancer cells through RAS P21 protein activator 1. miR-21 could be used as a potential marker for detection of metastasis of cervical cancer (19). Overexpression of miR-21 in cervical cancer cells affects cell migration and proliferation via inhibition of PTEN expression (20).

The present study demonstrated that the expression levels of TNF-α were associated with expression levels of miR-21. Up- or down-regulation of TNF-α promoted or inhibited the proliferation of cervical cancer cells, respectively, but had no effect on the apoptosis of cervical cancer cells. These results indicated that miR-21/TNF-α signaling serves a regulatory role in the proliferation of cervical cancer cells.

In conclusion, there was an association between TNF-α and miR-21 in HeLa cervical cancer cells. The expression of TNF-α was positively associated with the proliferation of cervical cancer cells, but had no effect on apoptosis. Therefore, miR-21 regulates the proliferation of cervical cancer HeLa cells via downregulation of TNF-α. These results provide novel potential therapeutic targets for the treatment of cervical cancer.

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