

# MicroRNA-143 regulates the proliferation and apoptosis of cervical cancer cells by targeting HIF-1 $\alpha$

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**Abstract. – OBJECTIVE:** To investigate the effects of miR-143 on proliferation and apoptosis of cervical cancer HeLa cells and its target hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ).

**PATIENTS AND METHODS:** The expression levels of miR-143 in 30 cases of normal cervical tissues, 30 cases of cervical intraepithelial neoplasia tissues, and 30 cases of cervical cancer tissues, were detected via Real-time quantitative polymerase chain reaction (qPCR). Cervical cancer HeLa cells were transfected with miR-143 mimics, and the transfection effect was detected via Real-time qPCR. In the experiment, HeLa cells were divided into three groups: non-transfection group, miR-143 control group and miR-143 transfection group. The cell proliferation in each group was detected via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, and the cell apoptosis in each group was detected by flow cytometry. Moreover, the messenger RNA (mRNA) and protein expression levels of HIF-1 $\alpha$  in each group, were detected by qPCR and Western blotting, and the relationship between miR-143 and HIF-1 $\alpha$  was verified by dual-luciferase reporter gene assay.

**RESULTS:** The level of miR-143 in miR-143 transfection group was higher than those in non-transfection group and miR-143 control group; the differences were statistically significant ( $p < 0.05$ ). The proliferation rate was decreased, but the apoptosis rate was increased in miR-143 transfection group compared with those in the other two groups; the differences were statistically significant ( $p < 0.05$ ). The transient overexpression of miR-143 could decrease the mRNA and protein levels of HIF-1 $\alpha$  in HeLa cells ( $p < 0.01$ ). Dual-luciferase reporter gene assay proved that HIF-1 $\alpha$  was a direct target of miR-143.

**CONCLUSIONS:** MiR-143 can regulate the proliferation and apoptosis of cervical cancer HeLa cells, and HIF-1 $\alpha$  is a direct target of miR-143.

*Key Words:*

MiR-143, HIF-1 $\alpha$ , Proliferation, Apoptosis, Cervical cancer.

## Introduction

Micro ribonucleic acid (miRNA) is a kind of non-coding small molecular RNA. More and more evidence has proved that miRNA has a certain regulating effects in cell proliferation or apoptosis<sup>1</sup>, differentiation<sup>2</sup> and reproduction<sup>3</sup>, and pathogenesis of tumor<sup>4</sup>. There are increasingly more experiments demonstrating that the specific expression of miRNA exists in the occurrence and development of tumors, among which some miRNAs are involved in the regulation of occurrence and development of tumors. Cervical cancer is one of the female malignant tumors that frequently occur in the world. However, the pathogenesis of cervical cancer is still not clear at present. For improving the survival rate and prolonging the survival time of cervical cancer patients, early diagnosis, targeted therapy and accurate efficacy evaluation of cervical cancer were considered as an urgent problem to be solved. Recent studies have found that miRNA plays an important role in various diseases, such as tumor<sup>5</sup>, cardiovascular diseases<sup>6</sup> and diabetes mellitus<sup>7</sup>, whose mechanism may be related to the expressions of some disease-related genes regulated by miRNA. Moreover, the role of miRNA in cervical cancer has also been widely reported. Studies have shown that miRNA plays a role as proto-oncogene or tumor suppressor gene, and the increased expression of miR-143 can inhibit the cell proliferation<sup>8</sup>. However, the specific regulation mechanism of miR-143 in cervical cancer remains unclear. Therefore, this study aimed to verify the correlation of miR-143 with its downstream target gene, hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ), and its effects on proliferation and apoptosis of cervical cancer cells, so as to preliminarily investigate the possible mechanism of miR-143 in cervical cancer.

## Patients and Methods

### Patient Samples

A total of 30 cases of cervical squamous carcinoma tissues were taken from patients receiving operation from January 2014 to December 2016 in our hospital. Patients were aged 32-76 years old without undergoing radiotherapy and chemotherapy. In terms of pathological grading, there were 22 cases in grade 2 and 8 cases in grade 3. In terms of clinical staging, according to the International Federation of Gynecology and Obstetrics (FIGO) staging criteria, there were 20 cases in stage I and 10 cases in stage II. 30 cases of cervical intraepithelial neoplasia (CIN) tissues were taken from patients aged 30-50 years old, including 10 cases of grade-I CIN and 20 cases of grade-II-III CIN. Another 30 cases of normal cervical tissues from patients aged 23-54 years old receiving uterine myomas operation during the same period were selected as controls. All specimens were stored in the liquid nitrogen for a long time within 30 min after separation from the body, and definitely diagnosed by pathological examination. All patients and their families signed the informed consent. This study was approved by the Ethics Committee of First Affiliated Hospital of Chinese PLA General Hospital.

### Detection of miR-143 via Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Cervical tissues stored at  $-80^{\circ}\text{C}$  were taken to extract the RNA using RNAiso Plus reagent (TaKaRa, Otsu, Shiga, Japan). RNA obtained was dissolved using diethylpyrocarbonate (DEPC) water and denatured using 1% formaldehyde. The integrity of RNA was detected via agarose gel electrophoresis, and the concentration and purity of RNA were detected using an ultraviolet spectrophotometer. RNA with the absorbance 260 (A<sub>260</sub>)/A<sub>280</sub> of 1.8-2.0 was used for subsequent experiments. Reverse transcription was performed using the miRNA reverse transcription kit (TaKaRa, Otsu, Shiga, Japan). 20  $\mu\text{L}$  reaction system included 10  $\mu\text{L}$  2-fold miRNA Reaction Buffer Mix, 2  $\mu\text{L}$  0.1% bull serum albumin (BSA), 2  $\mu\text{L}$  miRNA Prime Script RT Enzyme Mix, 1  $\mu\text{L}$  total RNA, and 5  $\mu\text{L}$  RNase-free dH<sub>2</sub>O. Reaction conditions:  $37^{\circ}\text{C}$  for 60 min;  $85^{\circ}\text{C}$  for 5 s; products were frozen at  $20^{\circ}\text{C}$ . PCR amplification was performed using SYBR Premix Ex Taq<sup>TM</sup> II. 20  $\mu\text{L}$  reaction system included 10  $\mu\text{L}$  SYBR Premix Ex Taq<sup>TM</sup> II, 0.8  $\mu\text{L}$  PCR forward primer (10  $\mu\text{mol}$ ), 0.8  $\mu\text{L}$  PCR reverse primer (10  $\mu\text{mol}$ ),

0.4  $\mu\text{L}$  ROX reference data II (50 $\times$ ), 2.0  $\mu\text{L}$  DNA template, and 6.0  $\mu\text{L}$  dH<sub>2</sub>O. Reaction conditions:  $95^{\circ}\text{C}$  for 30 s,  $95^{\circ}\text{C}$  for 3 s,  $60^{\circ}\text{C}$  for 30 s, a total of 40 cycles. MiR-143 reverse transcription primer sequences: F: 5'-TGTAGTTTCGGAGTTAGT-GTCGCGC-3', R: 5'-CCTACGATCGAAAAC-GACGCGAACG-3'. U6 primer sequences: F: 5'-GTTTTGTAGTTTTTGGAGTTAGTGTGTGT-3', R: 5'-CTCAACCTACAATCAAAA-CAACACAAACA-3'. U6 was used as an internal reference when the amplification efficiencies of target gene and internal reference gene were the same. The cycle threshold (Ct) value was calculated;  $\Delta\text{Ct} = \text{Ct}_{\text{miR-143}} - \text{Ct}_{\text{U6}}$ , and the relative expression level of miR-143 was presented as  $2^{-\Delta\text{Ct}}$ .

### Cells and Reagents

Human embryonic kidney (HEK) 293 cell lines and cervical cancer HeLa cell lines were purchased by Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). miR-143 mimics and control vectors were obtained by Shanghai GenePharma Biotech Co., Ltd., (Shanghai, China); pGL3 luciferase detection system was from Promega (Madison, WI, USA).

### Cell Culture, Transfection and Grouping

After resuscitation, HeLa cells were incubated using the Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin mixture in an incubator with 5% CO<sub>2</sub> at  $37^{\circ}\text{C}$ , followed by digestion using trypsin and passage. At 1 d before transfection, cells were inoculated into a 6-well plate at a density of  $1 \times 10^5$ /well until the cell density reached 80%. HeLa cells were divided into three groups: non-transfection group, miR-143 control group and miR-143 transfection group. MiR-143 control group and miR-143 transfection group were transfected with random sequence and miR-143 mimics, respectively, while non-transfection group received no transfection. MiR-143 mimics and a negative control (NC) with fluorescent tags were synthesized by Shanghai GenePharma (Shanghai, China). HeLa cells were transfected with miR-30 mimics and negative control, respectively using Lipofectamine 3000 according to the instructions of transfection kit (Thermo Fisher Scientific, Waltham, MA, USA). 4-6 h post-transfection, cell culture media was changed.

### MTT Cell Proliferation Assay

At 48 h after transfection, 20  $\mu\text{L}$  3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

(MTT) (R&D Systems, Minneapolis, MN, USA, 5 mg/mL) were added into each well, followed by incubation for 4 h. The absorbance (A) value at 570 nm of each well was detected using a microplate reader to determine the cell proliferation.

#### Detection of Apoptosis via ELISA

The apoptosis ELISA kit was obtained from Hoffmann-La Roche Company (Basel, Switzerland). At 48 h after transfection, the apoptosis rate of HeLa cells was detected according to the manufactures' protocol.

#### Western Blotting

At 48 h after transfection, cells were collected and added with protein lysis buffer. After standing at room temperature for 30 min, cells were centrifuged at 12000 rpm for 10 min. The supernatant was taken and the protein concentration was detected via bicinchoninic acid (BCA). 20  $\mu$ L protein was loaded into each well, followed by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Then, the protein on the gel was transferred onto polyvinylidene difluoride (PVDF) membrane using the wet transfer method, sealed with 5% skim milk powder at room temperature for 2 h, and incubated with HIF-1 $\alpha$  and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primary antibodies Cell Signaling Technology (CST, Danvers, MA, USA) diluted at 1:100 at 4°C overnight. On the next day, horseradish peroxidase (HRP)-labeled secondary antibody was added for incubation at room temperature for 1 h and washed with Tris buffered saline Tween (TBST) for 3 times. The absorbance of each band was analyzed after ECL staining.

#### Dual-Luciferase Reporter Gene Assay

Bioinformatics software was used to predict that HIF-1 $\alpha$  was a target of miR-143. The reporter gene plasmids of wild-type and mutant-type HIF-1 $\alpha$ -3'-untranslated region (UTR) were constructed, and the wild-type, mutant-type and pRL-TK plasmids and miR-143 mimics and its control vector were transfected into HEK 293 cells. The activities of firefly luciferase and Renilla luciferase in different groups were detected by the microplate reader after 48 h according to the instructions of dual-luciferase assay kit (Promega, Madison, WI, USA). The relative fluorescence intensity of each group was presented as the ratio of intensity of firefly fluorescence/intensity of Renilla fluorescence.

#### Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA) was used for statistical processing. Data were presented as mean  $\pm$  standard deviation. Comparison between groups was done using One-way ANOVA test followed by Least Significant Difference (LSD).  $p < 0.05$  suggested that the difference was statistically significant.

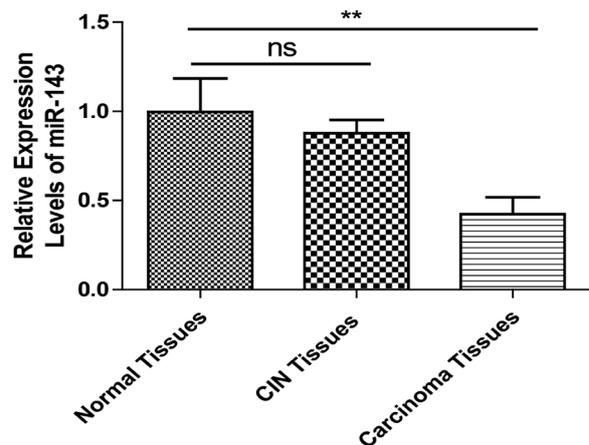
## Results

#### MiR-143 Expression Levels in Different Cervical Tissues

The relative expression levels of miR-143 in normal cervical tissues, CIN tissues and cervical squamous cell carcinoma tissues were shown in Figure 1, respectively; the differences were statistically significant ( $p < 0.05$ ). The results of pairwise comparisons showed that there was no statistically significant difference between normal cervical tissues and CIN tissues ( $p > 0.05$ ); there were statistically significant differences in normal cervical tissues compared with CIN tissues and cervical squamous cell carcinoma tissues ( $p < 0.05$ ).

#### Detection of miR-143 Transfection Efficiency

The results of qPCR showed that the miR-143 level in miR-143 transfection group at 48 h after transfection was higher than those in non-transfection group and miR-143 control group; the



**Figure 1.** The expression of hsa-miR-143 measured by qRT-PCR in normal cervical tissues, CIN tissues and cervical squamous cell carcinoma tissues. NS: none significant; \*\* $p < 0.01$ .

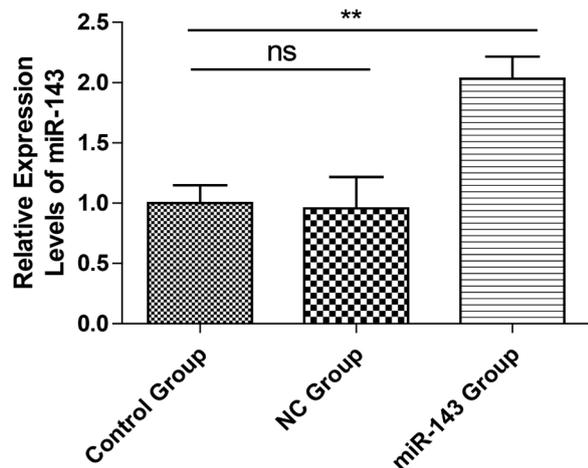
differences were statistically significant ( $p < 0.05$ ). There was no statistically significant difference in the miR-143 level between non-transfection group and miR-143 control group ( $p > 0.05$ ) (Figure 2).

**Effect of miR-143 Overexpression on HeLa cell Proliferation**

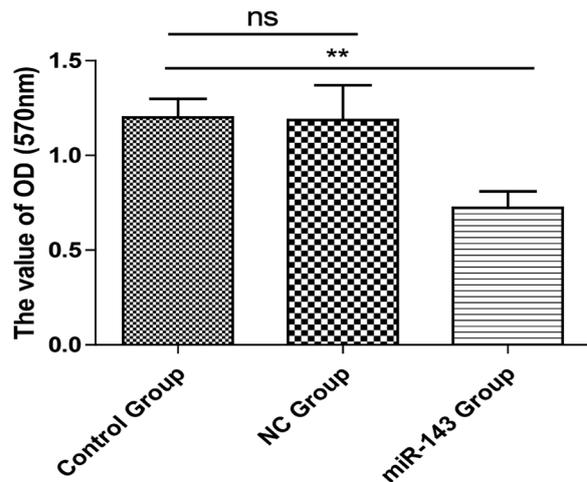
MTT cell proliferation assay revealed that compared with those in non-transfection group and miR-143 control group, HeLa cell proliferation rate in miR-143 transfection group was decreased at 48 h after transfection, and the differences were statistically significant ( $p < 0.05$ ). There was no statistically significant difference in the HeLa cell proliferation rate between non-transfection group and miR-143 control group ( $p > 0.05$ ) (Figure 3).

**Effect of miR-143 Overexpression on HeLa Cell Apoptosis**

Compared with those in non-transfection group and miR-143 control group, the HeLa cell apoptosis rate in miR-143 transfection group was increased at 48 h after transfection; the differences were statistically significant ( $p < 0.05$ ). There was no statistically significant difference in the HeLa cell apoptosis rate between non-transfection group and miR-143 control group ( $p > 0.05$ ) (Figure 4).



**Figure 2.** The expression of hsa-miR-143 measured by qRT-PCR in HeLa cells. HeLa cells were transfected with miR-143 mimics and negative control (NC) miRNA respectively. 48 h after transfection, qRT-PCR was used to detect the expression of miR-143 between three groups: control group, NC group and miR-143 group. NS: none significant; \*\* $p < 0.01$ .



**Figure 3.** MTT analysis of the cell proliferation at 48 h in HeLa cells. HeLa cells were transfected with miR-143 mimics and negative control (NC) miRNA respectively. 48 h after transfection, MTT assay was used to detect cell viability between three groups: control group, NC group and miR-143 group. NS: none significant; \*\* $p < 0.01$ .

**Effects of miR-143 Overexpression on Messenger RNA (mRNA) and Protein Levels of HIF-1α in HeLa Cells**

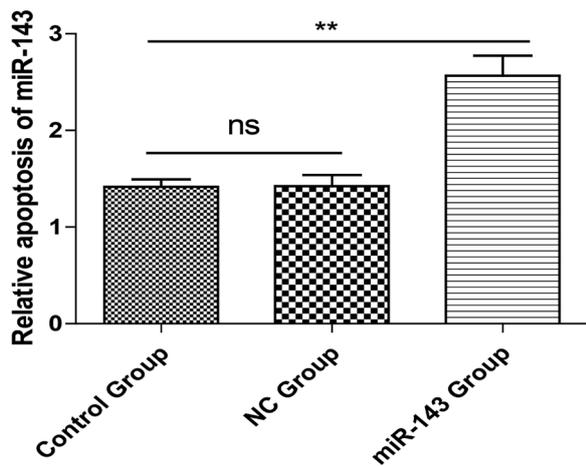
Compared with those in non-transfection group and miR-143 control group, the transient overexpression of miR-143 could reduce the mRNA and protein levels of HIF-1α in HeLa cells; the differences were statistically significant ( $p < 0.01$ ). There were no statistically significant differences in mRNA and protein levels of HIF-1α between non-transfection group and miR-143 control group ( $p > 0.05$ ) (Figure 5).

**Validation of Correlation Between miR-143 and HIF-1α**

The results of dual-luciferase reporter gene assay showed that miR-143 could significantly inhibit the luciferase activity of cells transfected with wild-type HIF-1α-3'UTR plasmid, but had no effect on the luciferase activity of cells transfected with mutant-type HIF-1α-3'UTR plasmid, confirming that HIF-1α is a direct target of miR-143, and miR-143 negatively regulates the HIF-1α expression (Figure 6).

**Discussion**

MiRNAs are abnormally expressed in a variety of malignant tumors. Studies have also found that the changes in miRNA expression in cervical

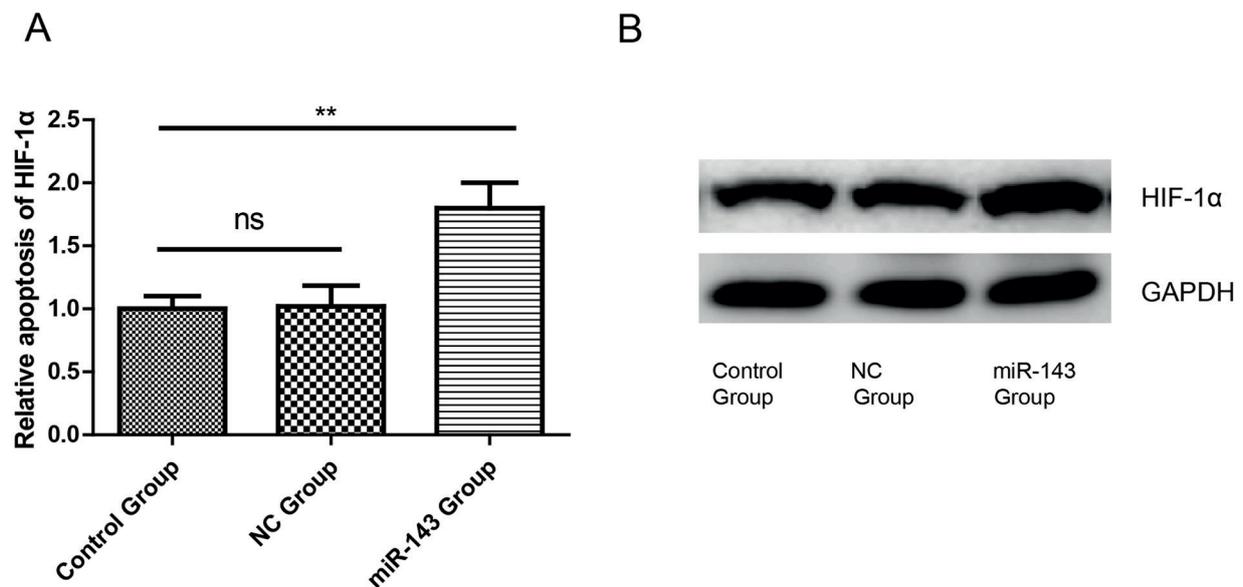


**Figure 4.** Detection of HeLa cell apoptosis by ELISA in HeLa cells. HeLa cells were transfected with miR-143 mimics and negative control (NC) miRNA respectively. 48 h after transfection, ELISA assay was used to detect cell apoptosis between three groups: control group, NC group and miR-143 group. NS: none significant; \*\* $p < 0.01$ .

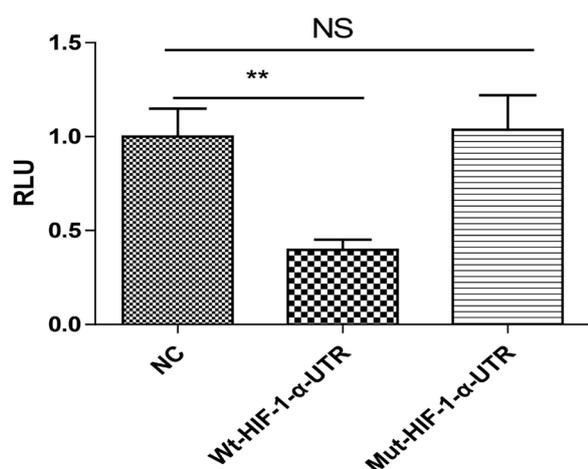
cancer are helpful in regulating the cell cycle regulatory pathway, and the activation of cell pathway inhibits other target genes<sup>1,2,4</sup>. Therefore, the expression pattern of miRNA can be used as a potential marker of cervical cancer. At present, miRNAs associated with cervical cancer include miR-126, miR-143, miR-145, miR-21, miR-15b,

miR-16, miR-146a, miR-155, miR-34a, miR-218, miR-127 and miR-200a<sup>5</sup>.

Various studies have identified that miR-143 has a certain regulating effect in tumorigenesis. For example, miR-143 is lowly expressed in colon cancer<sup>9</sup>, lung cancer<sup>10</sup> and prostate cancer<sup>11</sup>, but highly expressed in liver cancer<sup>12</sup>. Among them, miR-143 is involved in the regulation of MYO6 expression in prostate cancer<sup>11</sup>. ERK5, as a direct target of miR-143, can inhibit the cell proliferation<sup>13</sup>. In colon cancer, the high expression of miR-143 reduces the cell proliferation and clone formation abilities. Moreover, DNMT3A is also a direct target of miR-143, whose expression is regulated by miR-143<sup>14</sup>. In recent years, studies have proved that miRNA has a certain regulating effect in cervical cancer. Cheng et al<sup>15</sup> found that miR-218 can inhibit the proliferation and induce the apoptosis of HeLa cells. The overexpression of miR-214 in HeLa cells can down-regulate the protein and mRNA expressions of MEK3 and JNK1, and significantly inhibit the cell proliferation. Further studies have shown that miR-214 inhibits the expressions of MEK3 and JNK1 through targeting their 3'UTR, and the results suggested that miR-214 may inhibit the proliferation of HeLa cells by targeting the non-coding regions of MEK3 and JNK1 mRNA<sup>16</sup>. It was found in this



**Figure 5.** HeLa cells were transfected with miR-143 mimics and negative control (NC) miRNA respectively. 48 h after transfection, qRT-PCR and Western blot were used to detect mRNA and protein expressions of HIF-1 $\alpha$  between three groups: control group, NC group and miR-143 group. (A) Detection of HIF-1 $\alpha$  mRNA expression by qRT-PCR. (B) Detection of HIF-1 $\alpha$  protein expression by Western blot in HeLa cells. NS: none significant; \*\* $p < 0.01$ .



**Figure 6.** Detection of the target sites of miR-143 by dual-luciferase reporter gene assay in HeLa cells. Luciferase reporter assays in HeLa cells, with co-transfection of WT or Mut HIF-1 $\alpha$  and miR-143 as indicated. Transfection of miR-143 mimics markedly weakened luciferase activity of WT- HIF-1 $\alpha$ -3'-UTR. NS: none significant; \*\* $p < 0.01$ .

experiment that miR-143 could inhibit the proliferation and promote the apoptosis of HeLa cells. Combined with other experiments, it was found that miR-143 was differentially expressed in many kinds of tumors, suggesting that miR-143 may become a new target for tumor diagnosis, treatment and prognosis. At present, there are few studies on the role and mechanism of miR-143 in cervical cancer. In this study, the expressions of miR-143 in normal cervical tissues and cervical cancer tissues, and its relationship with pathological features of cervical cancer were studied via fluorescence qPCR<sup>17</sup>. The expression level of miR-143 in cervical cancer tissues was decreased compared with the normal level, and the difference was statistically significant. Therefore, it is believed that miR-143 may inhibit the growth of tumor cells, playing a similar role to a tumor suppressor, which is consistent with the literature. In this experiment, it was found that the transient overexpression of miR-143 in HeLa cells could inhibit the cell proliferation and promote apoptosis. The above results indicated that miR-143 may be involved in the occurrence and development of cervical cancer. HIF is a kind of heterodimeric protein complex composed of two subunits (HIF-1 $\alpha$  and HIF-1 $\beta$ ), and the activity of HIF-1 is mainly regulated by HIF-1 $\alpha$ <sup>18</sup>. Human HIF-1 $\alpha$  gene is located on chromosome 14, and the protein consists of 826 amino acids, mainly existing in the nucleus, some in the cytoplasm.

In normal aerobic environment, HIF-1 $\alpha$  is regulated by the ubiquitin-protease system, so its expression in normal cells is not stable. In tumor microenvironment, hypoxia leads to the continuous accumulation of HIF-1 $\alpha$ , making cancer cells continuously adapt to the hypoxia microenvironment, also facilitating the proliferation, migration, invasion and other activities of cancer cells<sup>19</sup>. In recent years, a large number of studies have confirmed that the accumulation of HIF-1 $\alpha$  in cancer cells is closely related to the occurrence and development of many malignant tumors, such as gastric cancer, liver cancer, breast cancer, ovarian cancer and lung cancer<sup>20</sup>. In this study, it was found for the first time that both mRNA and protein levels of HIF-1 $\alpha$  in HeLa cells were significantly decreased after transient overexpression of miR-143 compared with those in control group; the differences were statistically significant. In addition, dual-luciferase reporter gene assay further confirmed that HIF-1 $\alpha$  was a direct target of miR-143 and miR-143 negatively regulated the expression of HIF-1 $\alpha$ . The above results demonstrate that miR-143 may play a role as a cancer suppressor gene in cervical cancer, and the abnormally low expression of miR-143 in cervical cancer may affect its function of inhibiting HIF-1 $\alpha$ , leading to the further accumulation of HIF-1 $\alpha$  and accelerating the proliferation of cancer cells. However, the mechanism of miR-143 in inhibiting the proliferation of cervical cancer cells by targeting HIF-1 $\alpha$  and relevant molecular signaling pathways still need to be further investigated in subsequent studies.

## Conclusions

We showed for the first time in our study that miR-143 may regulate the proliferation and apoptosis of HeLa cells through targeting HIF-1 $\alpha$ , suggesting the value of miR-143 as a potential new target for the treatment of cervical cancer.

## Conflict of Interest

The Authors declare that they have no conflict of interest.

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