European Review for Medical and Pharmacological Sciences 2019; 23: 8219-8229

MicroRNA-15a-5p down-regulation inhibits cervical cancer by targeting TP53INP1 *in vitro*

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Abstract. – OBJECTIVE: An increasing number of reports have shown that microRNAs (miRNAs) play a vital role in the occurrence and development of cancer by acting as tumor inhibitors or oncogenes. The purpose of this research was to explore whether the expression level of microRNA-15a-5p (miR-15a-5p) was related to TP53 regulated inhibitor of apoptosis 1 (TP53INP1) in cervical cancer, and to explore the role of miR-15a-5p in cervical cancer *in vitro*.

PATIENTS AND METHODS: Human cervical cancer tissues and adjacent normal tissues were obtained from 30 cervical cancer patients. Firstly, we carried out the quantitative Real Time-PCR (qRT-PCR) assay to evaluate the level of miR-15a-5p in cervical cancer tissues and cell lines. The TargetScan and the Dual-Luciferase Reporter Assay were used to confirm the relationship between TP53INP1 and miR-15a-5p. Besides, the Cell Counting Kit-8 (CCK-8) and the flow cytometry analysis were performed to detect the effect of miR-15a-5p on cell proliferation and apoptosis in cervical cancer cells.

RESULTS: Our results showed that the expression of miR-15a-5p was enhanced in cervical cancer tissues and cells lines. The data from the Dual-Luciferase Reporter Assay demonstrated that TP53INP1 was a direct target of miR-15a-5p. We also found that TP53INP1 was down-regulated in the cervical cancer tissues and cell lines compared with the adjacent normal tissues and normal cervical cells. Besides, the down-regulation of miR-15a-5p depressed cervical cancer cell proliferation and enhanced cell apoptosis. Our results clearly suggested that the down-regulation of TP53INP1 successfully impaired the tumor-inhibition effects of miR-15a-5p inhibitor in cervical cancer cells.

CONCLUSIONS: Our findings indicated that miR-15a-5p functioned as a tumor-promoting gene in cervical cancer by directly targeting TP53INP1, indicating that miR-15a-5p might be a potential treatment target for cervical cancer patients.

Key Words:
Cervical cancer, Apoptosis, MicroRNA-15a-5p, TP53INP1.

Introduction

Cervical cancer, one of the most common malignancies in females[1,2], has become the main health issue of women for high morbidity and mortality[3], especially in young women[4,5]. Combined treatments have been a standard therapeutic method for cervical cancer patients, including chemotherapy and radiotherapy[6]. Also, radiotherapy alone has been used for cervical cancer at the early stage of cervical cancer[7]. However, low radiosensitivity, hypoxia, cellular glutathione, or other elements may result in the re-occurrence of cervical cancer[8,9]. Thus, the basic research on the pathogenesis of cervical cancer is very necessary to lessen the recurrence rate and advance the clinical treatment of cervical cancer. Particularly, the biological functions and the regulatory effects of the relative genes in cervical cancer need to be explored. Besides, the specific targets or genes for cervical cancer remain largely unknown. In this research, we investigated the molecular mechanism correlated with the tumorigenesis and development of cervical cancer and seek novel cure targets for cervical cancer. MicroRNAs (miRNAs), a group of small non-coding RNAs with 20-22 nucleotides, can be directly combined with the 3′-untranslated region (3′-UTR) of target genes to regulate the expression of the target genes[10]. In various cancers, miRNAs can act as a part of oncogenic or tumor inhibitors to regulate the tumor formation and the expression of their target genes[11]. As we all know, many studies have revealed that miRNAs

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play important roles in various cancer types, including colorectal cancer[12], breast cancer[13,14], cervical cancer[15-18], hepatic carcinoma[19], and so on. Moreover, miRNAs are involved in cellular proliferation, differentiation, apoptosis, and progression. Thus, focusing on the unknown miRNAs could be a new insight into the treatment of cancer. Among plentiful tumors inhibiting miRNAs, miR-15a-5p belongs to miR-15a family and was found to regulate many biological behaviors, for example, cell proliferation, apoptosis, migration, and invasion in various human cancer cell lines[20-23]. TP53INP1, TP53 regulated inhibitor of apoptosis 1, was down-regulated in various cancers, correlates with poor prognosis and cancer relapse[24,25]. However, the functional roles of miR-15a-5p and TP53INP1 in human cervical cancer are still unknown. Therefore, the aim of this study was to investigate the effect of miR-15a-5p and TP53INP1 on the progression of cervical cancer and to explore the potential mechanism. In this paper, we found that miR-15a-5p was aberrantly up-regulated both in cervical cancer cell lines and clinical cervical cancer tissues. Then, we conducted molecular biology and bioinformatics methods to suggest that TP53INP1 was the direct target of miR-15a-5p in cervical cancer. Besides, we observed that the down-regulation of miR-15a-5p suppressed cell proliferation and induced cell apoptosis in cervical cancer cells by targeting TP53INP1. In addition, miR-15a-5p down-regulation could also affect the relative gene expression, such as TP53INP1, Bcl-2, Bax, and Bcl-2, while its effects could be reversed by TP53INP1-siRNA. Therefore, our results demonstrated important roles of miR-15a-5p in the pathogenesis of cervical cancer and indicated its potential functions in cervical cancer therapy.

Patients and Methods

Clinical Specimens Collection

The human cervical cancer tissues and the adjacent normal tissues were obtained from 30 cervical cancer patients from the Women's Hospital of Nanjing Medical University. These specimens were stored in liquid nitrogen or at -80°C for future use. The experiment was approved by the Ethics Committee of Women's Hospital of Nanjing Medical University. All patients were notified that their specimens would be used in this research.

Cell Culture

The human cervical cancer cell lines HeLa, SiHa, and the human normal cervical cell line H8 were provided by the American Type Culture Collection (ATCC, Manassas, VA, USA). The cell lines were cultivated in the Roswell Park Memorial Institute-1640 (RPMI-1640; Gibco, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), 1% penicillin/ streptomycin, and were incubated at 37°C in a humidified chamber supplemented with 5% CO_2.

Cell Transfection

HeLa and SiHa cells were seeded in the 6-well plates at a density of 2.0×10^6 per well and transfected with the TP53INP1-siRNA, control-siRNA, inhibitor control, miR-15a-5p inhibitor, or miR-15a-5p inhibitor+TP53INP1-siRNA for 48 h by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's manual. After transfection, the cells were collected for further experiments. The efficiency of cell transfection was detected by the qRT-PCR and Western blot assay.

CCK-8 Assay

The Cell Counting Kit-8 (Sigma-Aldrich, St. Louis, MO, USA) was carried out to measure cell proliferation following the manufacturer's protocols. In brief, the cells were seeded in 96-well plates and cultured for 24 h. Then, the cells were transfected with the inhibitor control, miR-15a-3p inhibitor, or miR-15a-3p inhibitor+TP53INP1-siRNA for 48 h and then, we added 10 µl CCK-8 solution to each well and incubated them for another hour at 37°C. The microplate reader (Eon, Bio-Teck, Winooski, VT, USA) was used to measure the optical density (OD) at 450 nm.

Dual-Luciferase Reporter Assay

The TargetScan Release 7.1 (www.targetscan.org/vert_71) was used to predict the relationship between miR-15a-5p and TP53INP1. The results suggested that TP53INP1 was the potential target of miR-15a-5p. Then, TP53INP1 3'-UTR DNA segments containing the target sequence of miR-15a-5p were inserted into a pmirGLO vector (Promega, Madison, WI, USA) to form the reporter vector TP53INP1-wild-type (TP53INP1-WT). Also, the mutant 3'-UTRs of TP53INP1 was inserted into a Luciferase reporter vector (pmiR-REPORT, Ambion, Austin, TX, USA) to form TP53INP1-mutated-type (TP53INP1-MUT)

constructs. Then, the mimic control or miR-15a-5p mimic and TP53INP1-WT or TP53INP1-MUT were co-transfected into 293 T cells with Lipofectamine-2000 (Invitrogen, CA, USA) for 48 h. The Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) was performed to detect the Luciferase activity according to the manufacturer's instructions. The experiments were carried out at least in triplicate.

Quantitative Real Time-PCR (qRT-PCR) Assay

The total RNA was extracted from cervical cancer cell lines or tissues using TRIzol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instruction. Then, the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) was applied to assess the RNA concentrations at 260 and 280 nm (A260/280). RNA was transcribed into cDNA by PrimeScript™ RT-PCR Kit (TaKaRa, Otsu, Shiga, Japan). Subsequently, the levels of miR-15a-5p and TP53INP1 were detected by ABI 7500 Real Time-PCR system (Applied Biosystems, Foster City, CA, USA) with the SYBR Green PCR kit (TaKaRa, Otsu, Shiga, Japan). U6 and GAPDH were used to normalize the expression of miR-15a-5p and TP53INP1. The amplification conditions were as follows: denaturing for 35 cycles for 1 min at 94°C, annealing for 1 min at 60°C, and chain extension for 60 s at 72°C, the last extension step for 10 min at 72°C. The primer sequences for PCR were listed in Table I. The relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method. All experiments were carried out at least 3 times.

Western Blot Assay

The cervical cancer cells were washed with ice-cold PBS and then lysed with RIPA buffer (Beyotime Biotechnology, Shanghai, China), centrifuged at 12.000 rpm for 30 min at 4°C. The Total Protein Extraction Kit (Phygene Life Sciences, Shanghai, China) was employed to extract the total protein. The protein concentration was evaluated by a BCA protein kit (Beyotime Biotechnology, Haimen, China). Then, 40 μg of protein were loaded onto 10% SDS-PAGE and then transferred into polyvinylidene difluoride (PVDF) membrane. Then, the membrane was blocked with 5% non-fat milk in PBST at room temperature for 1 h and incubated at 4°C overnight, respectively, in the primary antibody (TP53INP1, Bcl-2, Bax, p21, and β-actin). After that, we washed the membranes with PBST four times and then incubated them with the secondary antibody (Bosis, Beijing, China) for 1 h at room temperature. Finally, the protein of interest was visualized using the enhanced chemiluminescence (ECL) Western blotting detection kits (Merck Millipore, Billerica, MA, USA) according to the manufacturer's instructions.

Flow Cytometry

The HeLa and SiHa cells were transfected with the inhibitor control, miR-15a-3p inhibitor, or miR-15a-3p inhibitor+TP53INP1-siRNA for 48 h. The transfections were performed in triplicate in 24-well plates. After 48 h, we used Annexin V-FITC/propidium iodide (PI) cell apoptosis detection kit (Cat No. 70-AP101-100; MultiScienc-

Table I. Primer sequences for qPCR.

Gene	Direction	Sequences (5'-3')
miR-15a-5p	Forward	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGTAAA
	Reverse	ACGTAGCAGCACATCATGGTTT
TP53INP1	Forward	GACTCACGGGCACAGAAGTGGAAGC
	Reverse	CCACTGGGAAGGGCGAAAG
p21	Forward	GACTTTGTCACCGAGACACC
	Reverse	GACAGGTCCACATGGTCTTC
Bax	Forward	CGTCCACCAAGAAGCTGAGCG
	Reverse	CGTCCACCAAAGCTGAGCG3
Bcl-2	Forward	TTGGATCAGGGAGTTGGAAG
	Reverse	TGTCCCTACCAACCAGAAGG
GAPDH	Forward	CTTTGGTATCGTGGAAGGACTC
	Reverse	GTAGAGGCAGGGATGATGTTCT
U6	Forward	GCTTCGGCAGCACATATACTAAAAT
	Reverse	CGCTTCACGAATTTGCGTGTCAT

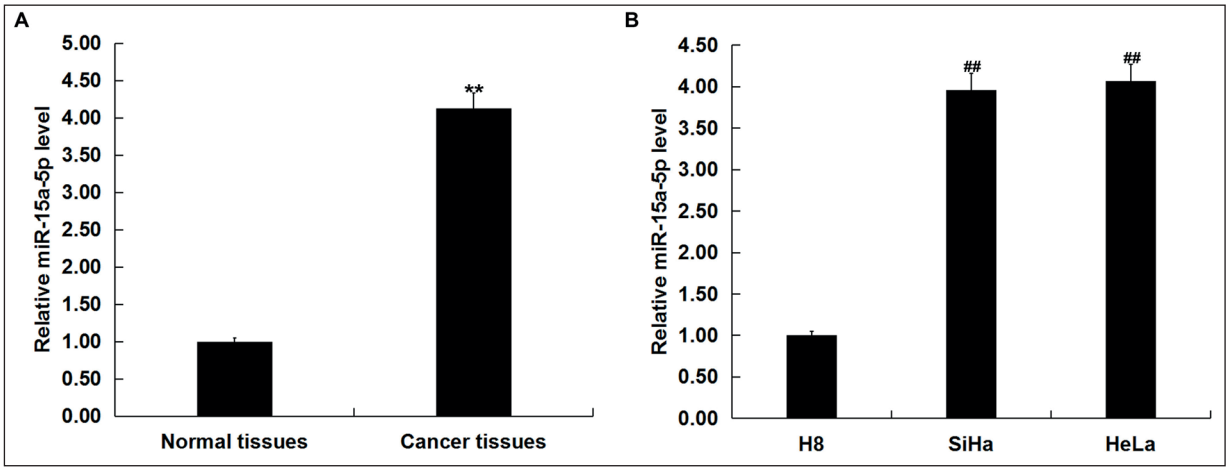

Figure 1. MiR-15a-5p was up-regulated in cervical cancer tissues and cells. **A,** MiR-15a-5p expression was higher in the cervical cancer tissues than in the adjacent normal tissues (as determined by qRT-PCR). **B,** qRT-PCR was used to measure the miR-15a-5p expression in cervical cancer cell lines (HeLa, SiHa) and normal cervical cells H8. The data were expressed as the mean ± SD; $^{**}p<0.01$ *vs.* normal tissues; $^{\#\#}p<0.01$ *vs.* H8.

es, Hangzhou, Zhejiang, China) to analyze cell apoptosis by FACScan flow cytometer (Becton Dickinson, Franklin Lake, NJ, USA). The data were analyzed with WinMDI 2.8.

Statistical Analysis

The data were presented as the mean ± SD (standard deviation) of at least three independent experiments carried out in triplicate. The significant differences between the groups were evaluated by the Student's *t*-test or One-way ANOVA with a Tukey's post-hoc test. *p*-values < 0.05 were considered significant.

Results

MiR-15a-5p Expression Was Up-Regulated in Cervical Cancer Tissues and Cell Lines

First of all, the qRT-PCR assay was performed to detect the levels of miR-15a-5p in 30 cervical cancer tissues and 30 normal tissues, and our results indicated that miR-15a-5p expression was markedly increased in cervical cancer tissues compared with the normal tissues (Figure 1A). The relative expression of miR-15a-5p was also largely increased in the cervical cancer cells (HeLa and SiHa) compared with the human normal cervical cells H8 (Figure 1B). These data demonstrated that miR-15a-5p was up-regulated in cervical cancer.

TP53INP1 Was a Target of MiR-15a-5p

To explore the molecular mechanisms of the role of miR-15a-5p in cervical cancer cell lines, the putative miR-15a-5p targets were predicted by using bioinformatics tool. The results revealed that TP53INP1 was a potential target of miR-15a-5p (Figure 2A). Moreover, the results from the Luciferase reporter analysis suggested

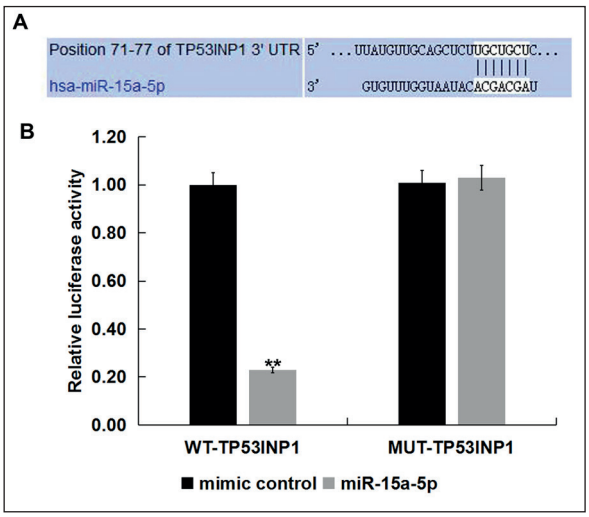

Figure 2. TP53INP1 was a direct target of miR-15a-5p. **A,** Schematic representation of TP53INP1 3'UTRs indicating putative miRNA target site. **B,** The relative Luciferase activities were detected using Dual-Luciferase Reporter Assay. All results were shown as mean ± SD, $^{**}p<0.01$ *vs.* mimic control.

that, compared with the control group, miR-15a-5p mimic significantly decreased the Luciferase activity in TP53INP1-WT reporter, while the Luciferase activity in TP53INP1-MUT reporter had no evident effect compared with the control group (Figure 2B). These results suggested the direct targeting relationship between miR-15a-5p and TP53INP1.

TP53INP1 Was Down-Regulated in Cervical Cancer Tissues and Tumor Cells

Then, the mRNA expression of TP53INP1 in cervical cancer tissues was detected by qRT-PCR analysis, and the results indicated that, compared with the normal control tissues, the mRNA level of TP53INP1 was significantly decreased in the cervical cancer tissues (Figure 3A). Besides, the relative lower mRNA and the protein expression of TP53INP1 were observed in the cervical cancer cell lines (HeLa and SiHa), compared with H8 cells (Figures 3B and 3C). In summary, our data elucidated that TP53INP1 may play a vital role in cervical cancer.

TP53INP1-SiRNA Reversed the Incremental Effects of MiR-15a-5p Inhibitor on TP53INP1 Expression in Cervical Cancer Cells

To study the functional relevance of TP53INP1 in miR-15a-5p-mediated effects in cervical cancer cells, the miR-15a-5p inhibitor, TP53INP1-siRNA, the corresponding negative control, or the miR-15a-5p inhibitor+TP53INP1-siRNA were transfected into the HeLa and SiHa cells. The transfection efficiency was detected by qRT-PCR assay or/and Western blot assay. As presented in Figure 4A, the miR-15a-5p expression levels were suppressed in the HeLa and SiHa cells when transfected with the miR-15a-5p inhibitor. Meanwhile, the mRNA level and the protein expression of TP53INP1 were suppressed when transfected with the TP53INP1-siRNA into HeLa (Figure 4B) and SiHa cells (Figure 4C). On the other hand, the mRNA and protein expression of TP53INP1 was enhanced in HeLa (Figure 4D) and SiHa (Figure 4E) cells transfected with the miR-15a-5p inhibitor, and these enhancements were partially restored by TP53INP1-siRNA. We found that TP53INP1 could interfere with miR-15a-5p expression.

MiR-15a-5p Inhibitor Efficiently Suppressed Proliferation and Promoted Apoptosis in Hela and SiHa Cells

To investigate the effect of miR-15a-5p in the Hela and SiHa cells, the cells were transfected with the inhibitor control, miR-15a-5p inhibitor, or miR-15a-5p inhibitor+TP53INP1-siRNA and the cell viability was determined. We found that miR-15a-5p inhibitor significantly inhibited the cell viability of HeLa (Figure 5A) and SiHa (Figure 5B) cells compared with the control group, while the cell viability was significantly increased in the miR-15a-5p inhibitor and TP53INP1-siRNA co-transfected cells. In addition, the apoptosis assay by flow cytometry indicated that the miR-15a-5p inhibitor contributed to cell apoptosis much more than the control. Besides, the inhibi-

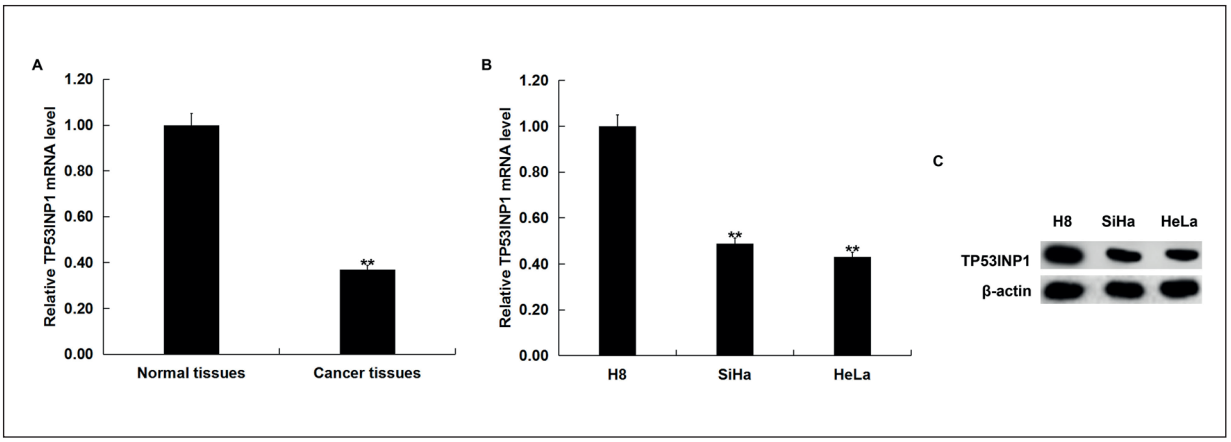

Figure 3. TP53INP1 was down-expressed in cervical cancer tissues and cells. **A,** qRT-PCR assay was carried out to detect the mRNA level of TP53INP1 in cervical cancer tissues and adjacent normal tissues. **B,** and **C,** qRT-PCR assay and Western blot assay were used to measure the TP53INP1 mRNA and protein expressions in cervical cancer cells (HeLa, SiHa) and in human normal cells H8. The data were expressed as the mean ± SD; $^{**}p<0.01$ *vs.* normal tissues; $^{\#\#}p<0.01$ *vs.*H8.

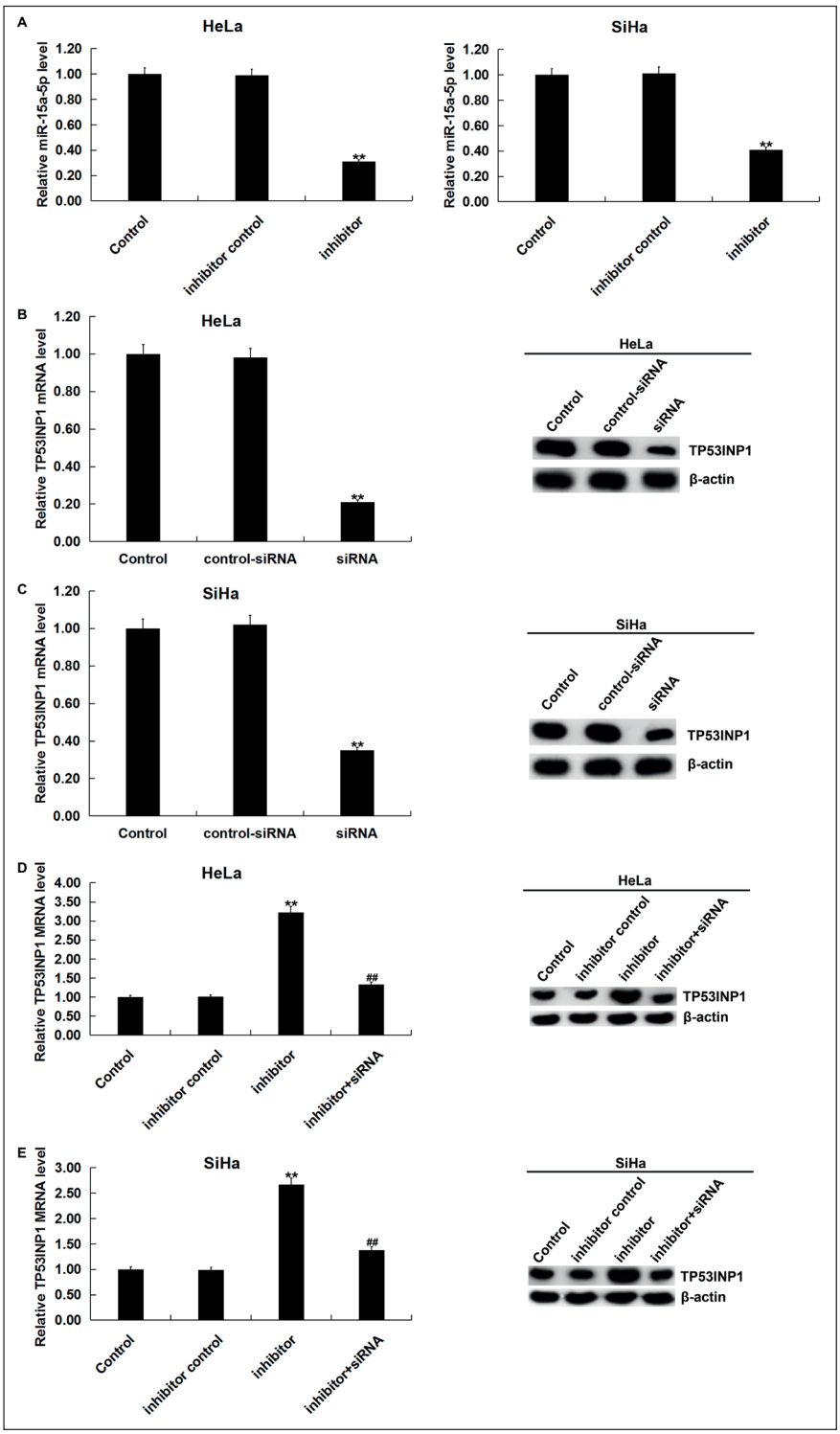

Figure 4. MiR-15a-5p regulates cervical cancer cells biological behaviour by targeting TP53INP1. **A,** HeLa and SiHa cells were transfected with the miR-15a-5p inhibitor or inhibitor control for 48 h, then, the miR-15a-5p level was measured by qRT-PCR. **B,** and **C,** HeLa and SiHa cells were transfected with the control-siRNA or TP53INP1-siRNA for 48 h, then, the qRT-PCR assays and Western blotting were used to evaluate the TP53INP1 mRNA and the protein level of HeLa and SiHa cells. **D,** and **E,** HeLa and SiHa cells were transfected with the inhibitor control, miR-15a-5p inhibitor, or miR-15a-5p inhibitor+TP53INP1-siRNA for 48 h, then, qRT-PCR assays and Western blotting were used to evaluate the TP53INP1 mRNA and the protein level of HeLa and SiHa cells. All experiments were performed in triplicate. The representative images are shown. The data were presented as mean ± SD; $^{**}p<0.01$ *vs.* control; $^{\#\#}p<0.01$ *vs.* inhibitor.

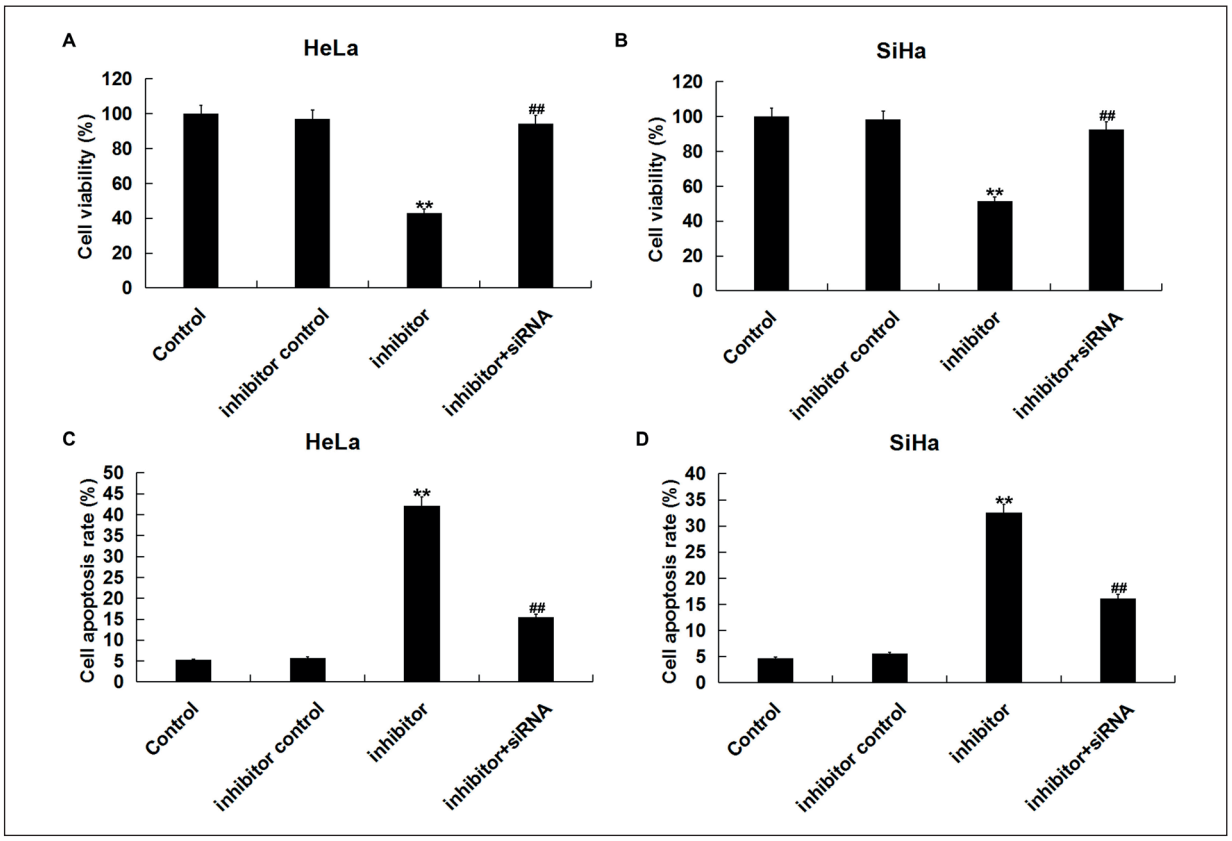

Figure 5. MiR-15a-5p regulates cervical cancer cells biological behaviour by targeting TP53INP1. **A,** HeLa and SiHa cells were transfected with the miR-15a-5p inhibitor or inhibitor control for 48 h, then, the miR-15a-5p level was measured by qRT-PCR. **B,** and **C,** HeLa and SiHa cells were transfected with the control-siRNA or TP53INP1-siRNA for 48 h, then, the qRT-PCR assays and Western blotting were used to evaluate the TP53INP1 mRNA and the protein level of HeLa and SiHa cells. **D,** and **E,** HeLa and SiHa cells were transfected with the inhibitor control, miR-15a-5p inhibitor, or miR-15a-5p inhibitor+TP53INP1-siRNA for 48 h, then, qRT-PCR assays and Western blotting were used to evaluate the TP53INP1 mRNA and the protein level of HeLa and SiHa cells. All experiments were performed in triplicate. The representative images are shown. The data were presented as mean ± SD; $^{**}p<0.01$ *vs.* control; $^{\#\#}p<0.01$ *vs.* inhibitor.

tion of TP53INP1 by siRNA-TP53INP1 clearly reversed the effects of the miR-15a-5p inhibitor on Hela (Figure 5C) and SiHa (Figure 5D) cell apoptosis.

Down-Regulation of MiR-15a-5p Regulated Bcl-2/Bax/p21 Expression

Finally, the apoptosis-related genes were determined in the present study. 48 h after that the Hela and SiHa cells were transfected with the inhibitor control, miR-15a-5p inhibitor, or miR-15a-5p inhibitor+TP53INP1-siRNA, we examined Bcl-2, Bax, and the p21 expression by qRT-PCR and Western blotting. Our results suggested that the down-regulation of miR-15a-5p enhanced the expression of Bax and p21, while decreased the level of Bcl-2 in HeLa (Figures 6A and 6D) and SiHa (Figures 6E and 6H), and these changes were notably eliminated by TP53INP1 silencing. Our results clearly suggested that the down-regulation of TP53INP1 successfully reversed the tumor inhibition effects of the miR-15a-5p inhibitor in cervical cancer cells.

Discussion

Inukai et al[26] have revealed that miRNAs play the role of tumor-inhibitor or tumor promoting in various cancers, depending on the special miRNA. It is necessary to identify the cancer-specific miRNAs and their targets to explore the roles in tumors and to confirm the original treatment targets[27-29]. In this study, we investigated the biomechanics of miR-15a-5p in cervical cancer. First of all, we evaluated the

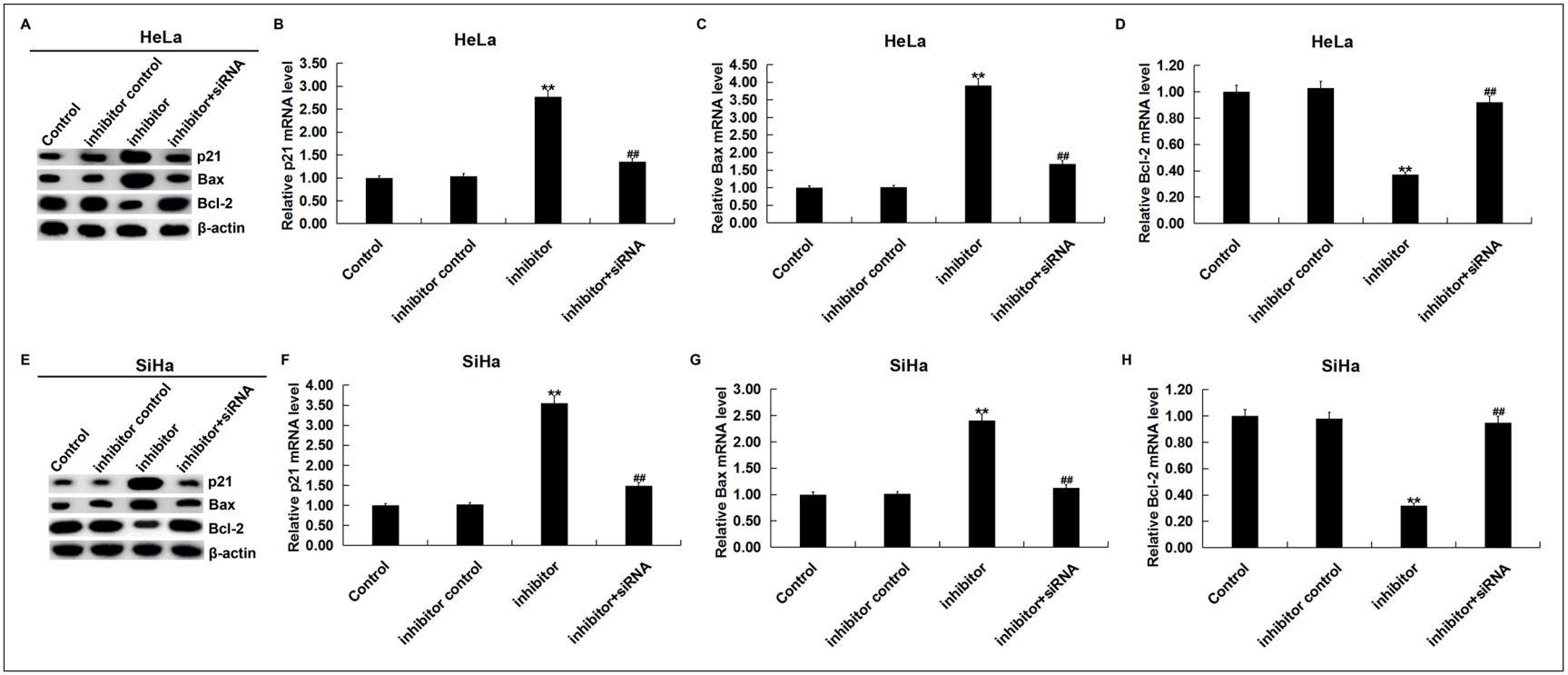

Figure 6. Effects of miR-15a-5p down-expression on cell viability and cell apoptosis of HeLa and SiHa cells. HeLa and SiHa cells were transfected with the inhibitor control, miR-15a-5p inhibitor, or miR-15a-5p inhibitor+TP53INP1-siRNA for 48 h, then the CCK-8 assay was carried out to measure the cell viability of HeLa **(A)** and SiHa **(B)** cells. Also, the Flow cytometry was performed to determine the percentages of apoptosis of HeLa **(C)** and SiHa **(D)** cells. All experiments were performed in triplicate. Each bar in the histogram represents the mean ± SD. $^{**}p<0.01$ *vs.* control; $^{\#\#}p<0.01$ *vs.* inhibitor.

miR-15a-5p level in cervical cancer tissues and cell lines through the qRT-PCR assay. The level of miR-15a-5p was significantly increased in cervical cancer tissues and cell lines. Our results were consistent with other reports, which have indicated that miR-15a-5p was also evidently up-regulated in colorectal tumor, breast cancer, glioblastoma cancer, and oral squamous cell carcinoma[30,31]. Therefore, the aberrant expression of miR-15a-5p in various types of cancer cells and tissues suggested that miR-15a-5p may play a vital role in tumorigenesis.

Many reports[32-37] have demonstrated that miR-15a-5p influenced cell growth and apoptosis in cancer cells by targeting CDK6, RBCK1, cMet, and WNT3A. Also, the putative mRNA targets of miRNAs could be predicted by bioinformatics tools. In this study, we found that miR-15a-5p directly targeted TP53INP1, thus suggesting a possible mechanism TP53INP1 with cervical oncogenesis. As we all know, TP53INP1 is a tumor-inhibitor gene, which affects cell growth and cell apoptosis by regulating the transcriptional activity of p53. Our present results suggested that TP53INP1 was a target gene of miR-15a-5p. So we inferred that miR-15a-5p may regulated cell growth by targeting TP53INP1 in cervical cancer cells. In order to confirm the hypothesis, the inhibitor control, miR-15a-5p inhibitor, or miR-15a-5p inhibitor+TP53INP1-siRNA were transfected into cervical cancer cells. We found that TP53INP1 blockage reversed the effects on cell viability and cell apoptosis, which were caused by miR-15a-5p inhibitor. As expected, we also observed that TP53INP1, Bcl-2, Bax, and p21 expression changed by miR-15a-5p inhibitor were reversed by the knockdown of TP53INP1. In summary, the biological effects of miR-15a-5p on cervical cancer cells were probably due to the suppression of TP53INP1.

Taken together, we demonstrated that miR-15a-5p was up-regulated in both cervical cancer cell lines and cervical cancer tissues for the first time. Besides, miR-15a-5p acted as a tumor promotion in cervical cancer, and that the down-regulation of miR-15a-5p reduced cervical cancer cell proliferation and induced cell apoptosis *in vitro*. We also demonstrated that TP53INP1 was a direct target of miR-15a-5p, and that it was negatively regulated by miR-15a-5p in cervical cancer. Those results may be beneficial to explore novel molecular targets for cervical cancer treatment, thus to improve treatment for patients with cervical cancer.

Conclusions

We demonstrated that miR-15a-5p was up-regulated in both cervical cancer cell lines and cervical cancer tissues, and that the down-regulation of miR-15a-5p reduced cervical cancer cell proliferation and induced cell apoptosis by targeting TP53INP1. Therefore, miR-15a-5p may be a novel therapeutic target for cervical cancer treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

The present study was supported by the National Natural Science Foundation of China (No. 81802899).

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