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LncRNA UCA1 inhibits proliferation and promotes apoptosis of cervical cancer cells by regulating β-catenin/TCF-4

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Abstract. – OBJECTIVE: The aim of this study was to explore the regulatory effect of long non-coding ribonucleic acid (lncRNA) urothelial carcinoma antigen 1 (UCA1) on the proliferation and apoptosis of HeLa cells and to elucidate its potential regulatory mechanism.

MATERIALS AND METHODS: HeLa cells were cultured in vitro and randomly divided into three groups, including blank control group (Control group), lncRNA UCA1 negative control (NC) group, and lncRNA UCA1 interference group [lncRNA UCA1 small interfering RNA (siRNA) group]. The expression level of lncRNA UCA1 in HeLa cells was detected by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Methyl thiazolyl tetrazolium (MTT) assay was performed to determine the effect of lncRNA UCA1 on the proliferation of HeLa cells. The effect of lncRNA UCA1 on the apoptosis of HeLa cells was determined via Hoechst 33258 staining assay and flow cytometry. In addition, qRT-PCR and Western blotting were employed to measure the messenger RNA (mRNA) and protein expression levels of β-catenin and TCF-4 in HeLa cells, respectively.

RESULTS: There were no statistically significant differences in the proliferation and apoptosis of HeLa cells as well as the mRNA and protein levels of β-catenin and TCF-4 in HeLa cells between Control group and lncRNA UCA1 NC group ($p>0.05$). In comparison with lncRNA UCA1 NC group, lncRNA UCA1 siRNA group exhibited overtly reduced proliferation, enhanced apoptosis rate of HeLa cells and down-regulated mRNA and protein levels of β-catenin and TCF-4 in HeLa cells ($p<0.05$).

CONCLUSIONS: LncRNA UCA1 inhibits proliferation and promotes the apoptosis of HeLa cells. Furthermore, its mechanism of action may be related to the inhibition on the β-catenin/TCF-4 signaling pathway.

Key Words:
LncRNA UCA1, Cervical cancer (CC), Cell proliferation, Cell apoptosis, β-catenin/TCF-4 signaling pathway.

Introduction

Cervical cancer (CC) is one of the most common malignant tumors in women worldwide at present. It ranks second in cancer-related deaths in women, severely affecting the physical and mental health of females[1]. Based on statistics, 500,000 females are newly diagnosed with CC every year, and they tend to be younger. With the continuous progress in technologies and the popularization of CC screening, increasingly more CC patients can be effectively treated. However, the best timing for CC treatment is missed since patients have already been at the middle or advanced stage when first diagnosed due to unobvious clinical manifestations in the early stage[2,3]. Therefore, searching for efficient and stable biomarkers is conducive to the early diagnosis of CC. Meanwhile, exploring the pathogenesis of CC is of great significance for its prevention and treatment[4].

Long non-coding ribonucleic acids (lncRNAs) have been found closely correlated with the development, progression and metastasis of tumors[5]. As a class of non-coding RNAs with about 200 nt in length in the transcriptome, lncRNAs account for about 98% of human gene transcription products. They are able to participate in many processes, such as physiological and pathological processes through epigenetic regulation[6]. Currently, lncRNAs have been proven to be abnormally expressed in non-small cell lung cancer, liver cancer, prostate cancer, and CC[7-9]. Wang et al[10] have shown that urothelial carcinoma antigen 1 (UCA1), located at chromosome 19p13.12, is significantly up-regulated in CC. UCA1 is positively associated with the size, clinical stage, and lymphatic metastasis of many tumors, while negatively related to the prognosis of patients[11].

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Therefore, it is suggested that inhibiting the expression of lncRNA UCA1 may be a new way to treat cancer of the uterus.

Wnt signaling pathway is activated in various malignancies. β-catenin is the most important regulatory factor in the Wnt signaling pathway, which plays a vital role in the development and progression of tumors. The activation or degradation of β-catenin will lead to changes in the Wnt signaling pathway[12]. When the Wnt signaling pathway is activated, β-catenin in the cytoplasm begins to recruit and transfer to the nucleus. This may produce the transcription complexes of β-catenin and the T-cell factor 4 (TCF-4)/lymphoid enhancer factor family of transcription factors in the nucleus. Such complexes regulate the expression of downstream targets, eventually affecting the development, progression and metastasis of tumors[13,14]. However, whether lncRNA UCA1 affects the proliferation and apoptosis of CC HeLa cells by regulating the β-catenin/TCF-4 signaling pathway in CC needs to be further studied.

Materials and Methods

Reagents

LncRNA UCA1 negative control (NC) and small interfering RNA (siRNA) (Shanghai GenePharma Co., Ltd., Shanghai, China), penicillin/streptomycin (P/S), fetal bovine serum (FBS) and Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA), methyl thiazolyl tetrazolium (MTT) kits (Beyotime Biotechnology, Shanghai, China), 96-well culture plates and culture dishes (Corning, Corning, NY, USA), β-catenin, TCF-4 and β-actin primers (Thermo Fisher Scientific, Waltham, MA, USA), Hoechst 33258 staining kits (Nanjing Genechem Co., Ltd., Nanjing, China), β-catenin, TCF-4 and β-actin primary antibodies and horseradish peroxidase (HRP)-labeled secondary antibodies (CST, Danvers, MA, USA), enhanced chemiluminescence (ECL) kits (Solarbio, Beijing, China), and LightCycler 480 SYBR Green I Master reagents (Roche, Basel, Switzerland).

Instruments

A micro-plate reader (Biotek, Biotek Winooski, VT, USA), a high speed centrifuge (Eppendorf, USA), a CO_2 incubator (Shanghai Hasuc Instrument Manufacture Co., Ltd., Shanghai, China), a quantitative polymerase chain reaction (qPCR) instrument and a electrophoresis apparatus (Bio-Rad, Hercules, CA, USA), a super clean bench (Suzhou Antai Equipment Co., Ltd., Suzhou, China), and a flow cytometer (Jiyuan Biotechnology Co., Ltd., Jiyuan, China).

Cells

Human HeLa cells (Catalog number: TCHu187) used in experiments were bought from the cell bank of the Chinese Academy of Sciences (Beijing, China). All cells were cultured in DMEM containing 1% P/S and 10% FBS. Culture medium was replaced at regular time, and the cells were sub-cultured. HeLa cells were randomly divided into three groups, namely, blank control group (Control group), lncRNA UCA1 NC group and lncRNA UCA1 siRNA intervention (lncRNA UCA1 siRNA) group.

Culture and Transfection of HeLa Cells

HeLa cells were removed from liquid nitrogen and quickly thawed *via* water bath at 37°C. The resulting cell suspension was transferred into a centrifuge tube and centrifuged. Next, the cell cryopreservation solution was aspirated, and the cell pellet was re-suspended in medium and cultured in a culture dish. When cell fusion rate reached about 80%, the cells were digested and sub-cultured. For transfection, HeLa cells in the logarithmic growth phase were collected and cultured in serum-free medium. After that, the cells were transfected with Lipofectamine 2000 transfection reagents NC as lncRNA UCA1 NC group and siRNA as lncRNA UCA1 siRNA group. UCA1 level in transfected cells was measured through quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The sequences of forward and reverse primers of UCA1 were 5'-ACGCTAACTGGCACCTTGTT-3', and 5'-CTCCGGACTGCTTCAAGTGT-3'. β-actin was used as an internal reference. The sequences of forward and reverse primers of β-actin were 5'-GGCTGTATTCCCCTCCATCG-3', and 5'-CCAGTTGGTAACAATGCCATGT-3'. SYBR reaction was carried out using a 10 μL system for 40 cycles according to relevant instructions. The relative expression level of UCA1 was calculated based on the $2^{-\Delta\Delta Ct}$ method.

HeLa Cell Proliferation Through MTT Assay

Transfected cells were first seeded into 96-well plates at a density of 3×10^4 cells/well, with six replicates for each group. After the cells were adhered for 24 h, 48 h, and 72 h in a 5% CO_2

incubator, 10 μL of MTT solution was added to each well for 2 h of incubation in the dark. 4 h later, the medium in wells was discarded, and 100 μL of DMSO solvent was added to each well and shaken for 10 min. Next, the crystals were fully dissolved. Absorbance at 450 nm was finally determined using 1 micro-plate reader.

HeLa Cell Apoptosis Via Hoechst 33258 Staining Assay

Hoechst 33258 is a blue fluorescent dye that can penetrate cell membranes, which is extremely low toxic to cells and is used for the detection of apoptosis. Transfected cells in each group were spread in 96-well plates, with 3 replicates for each group. After overnight culture, 150 μL of Hoechst 33258 staining solution was added to each well to fully cover the samples and incubated for 30 min. Subsequently, the staining solution was discarded and the cells were washed with phosphate-buffered saline (PBS). An inverted fluorescent microscope was employed to observe the staining.

HeLa Cell Apoptosis by Flow Cytometry

Cell suspension in each group was collected, washed with buffer once and centrifuged. Then, the cell pellet was re-suspended with 100 μL of labeling solution, followed by incubation in the dark at room temperature for 15 min. After centrifugation, the cells were rinsed with buffer once, and added with fluorescent solution for incubation in dark at 4°C for 20 min. Flow cytometry was finally applied to detect the apoptosis rate.

Expression of Ð-Catenin and TCF-4 Messenger RNA (mRNA) in HeLa Cells Through qRT-PCR

Total RNAs were extracted from cells using TRIzol lysis buffer. Subsequently, extracted RNA was reversely transcribed into cDNAs according to the instructions of the reverse transcription kit. Specific reaction conditions were as follows: pre-denaturation at 95°C for 5 min, denaturation at 95°C for 10 s, annealing at 55°C for 10 s and extension at 72°C for 60 s for 25 cycles in total, followed by complete extension at 72°C for 5 min. Primer sequences used in this study were shown in Table I below.

Protein Expression of Ð-Catenin and TCF-4 in HeLa Cells Via Western Blotting Analysis

Transfected cells in each group were collected, washed with phosphate-buffered saline (PBS) for three times, and lysed with cell lysis solution. The resulting solution was collected and centrifuged to collect the cell lysate. Protein concentration was determined in accordance with the bicinchoninic acid assay (BCA) kit. Subsequently, protein samples were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). After blocking with 5% skimmed milk powder for 2 h, the membranes were incubated with β-catenin, TCF-4, and β-actin primary antibodies (1:1000) overnight. On the next day, the membranes were incubated with horseradish peroxidase (HRP)-labeled secondary antibodies for 1 h. Immuno-reactive bands were exposed by enhanced electrochemiluminescence (ECL) method. Optical density was calculated using ImageJ software, with β-actin as a semi-quantitative internal reference.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. All experiments were repeated for at least 3 times. Experimental results were expressed as mean ± standard deviation. Analysis of variance (ANOVA) was adopted to analyze the significance of the difference, followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ was considered

Table I. Primer information.

List	Primer
β-catenin	F: CACTCTCGAGATGGCTACTCAAGCTG R: CTGCGGATCCTACAGGTCAGTATCAAAC
TCF-4	F: CGAGGGTGATGAGAACCTGC R: CCCATGTGATTCGATGCGT
β-actin	F: GGCTGTATTCCCCTCCATCG R: CCAGTTGGTAACAATGCCATGT

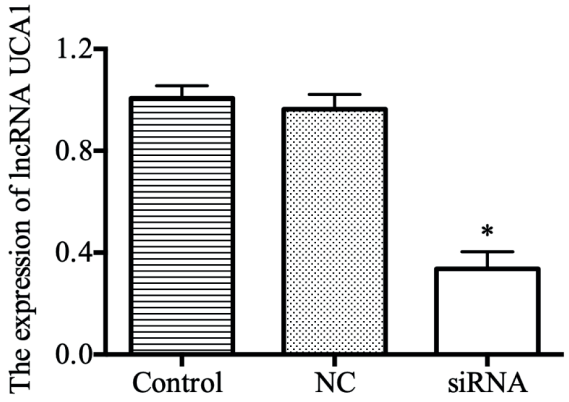

Figure 1. Expression level of lncRNA UCA1 in HeLa cells of each group ($^*p$<0.05: lncRNA UCA1 siRNA group *vs.* Control group and lncRNA UCA1 NC group).

statistically significant.

Results

LncRNA UCA1 Was Lowly Expressed in LncRNA UCA1 SiRNA Group

The results of qRT-PCR (Figure 1) showed that the expression of lncRNA UCA1 was lowly expressed in lncRNA UCA1 siRNA group compared with Control group and lncRNA UCA1 NC group ($^*p$<0.05, $^*p$<0.05).

LncRNA UCA1 SiRNA Repressed the Proliferation of HeLa Cells

MTT assay results indicated that the proliferation rate of HeLa cells was significantly lower in lncRNA UCA1 siRNA group than that in Control group and lncRNA UCA1 NC group at 24, 48 and 72 h ($^*p$<0.05, $^*p$<0.05, $^*p$<0.05) (Figure 2), suggesting that lncRNA UCA1 siRNA inhibited the proliferation of HeLa cells.

LncRNA UCA1 SiRNA Facilitated Apoptosis of HeLa Cells

Hoechst 33258 staining results revealed that compared with Control group and lncRNA UCA1 NC group, the number of HeLa cells stained fluorescent blue increased evidently in lncRNA UCA1 siRNA group (Figure 3A). Based on the statistical graph (Figure 3B), the apoptosis rate of HeLa cells rose remarkably after transfection with lncRNA UCA1 siRNA ($^*p$<0.05, $^*p$<0.05).

Flow cytometry was utilized in this study to detect the effect of lncRNA UCA1 on HeLa cell apoptosis. As shown in Figure 4, the apoptosis in lncRNA UCA1 siRNA group increased significantly ($^*p$<0.05, $^*p$<0.05). The above results of Hoechst 33258 staining and flow cytometry indicate that lncRNA UCA1 siRNA promotes the apoptosis of HeLa cells.

LncRNA UCA1 SiRNA Suppressed mRNA Expressions of β-Catenin and TCF-4 in HeLa Cells

QRT-PCR results (Figure 5) demonstrated that the mRNA levels of β-catenin and TCF-4 in HeLa cells were markedly lower in lncRNA UCA1 siRNA group than those in Control group and lncRNA UCA1 NC group ($^*p$<0.05, $^*p$<0.05). These findings imply that lncRNA UCA1 siRNA is capable of inhibiting the mRNA expressions of β-catenin and TCF-4 in the β-catenin/TCF-4 signaling pathway.

LncRNA UCA1 SiRNA Inhibited Protein Expressions of β-Catenin and TCF-4 in HeLa Cells

Western blotting band graph (Figure 6) showed that the protein levels of β-catenin and TCF-4 in HeLa cells were overtly lower in lncRNA UCA1 siRNA group than those in Control group and lncRNA UCA1 NC group ($^*p$<0.05, $^*p$<0.05). These findings suggest that lncRNA UCA1 siRNA can repress the protein expressions of β-catenin and TCF-4 in the β-catenin/TCF-4 signaling pathway.

Discussion

CC is a common gynecological malignant tumor with about 190,000 new cases each year and over 30,000 deaths each year in China. The incidence rate of CC shows an increasing trend year

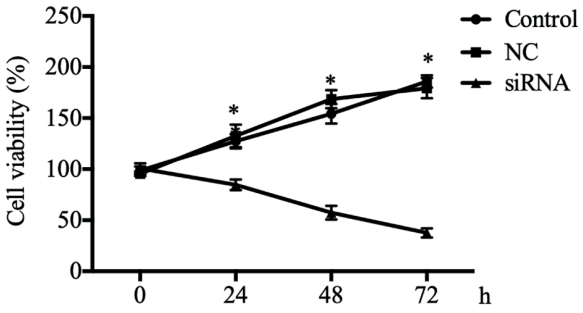

Figure 2. Effect of lncRNA UCA1 on HeLa cell proliferation ($^*p$<0.05: lncRNA UCA1 siRNA group *vs.* Control group and lncRNA UCA1 NC group).

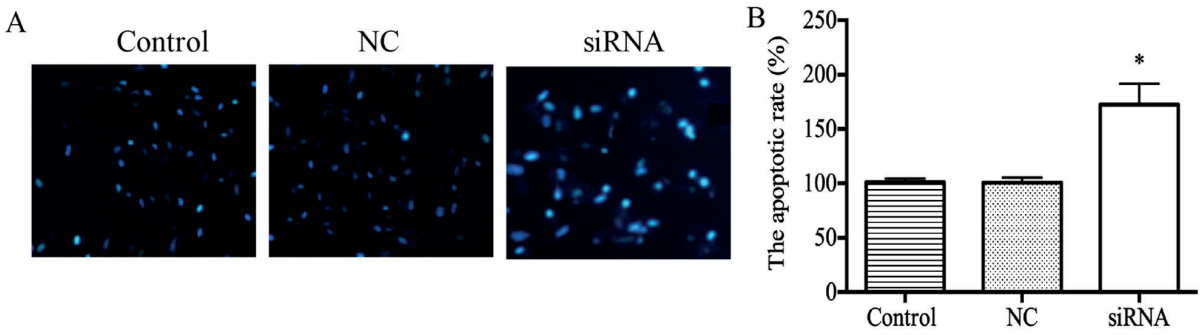

Figure 3. Effect of lncRNA UCA1 on HeLa cell apoptosis. **A,** Hoechst 33258 staining map (magnification: 200×), **B,** Apoptosis rate (**p*<0.05: lncRNA UCA1 siRNA group *vs.* Control group and lncRNA UCA1 NC group).

by year[15]. In recent years, continuous improvement has been achieved in the measures for the prevention and treatment of CC. However, the therapeutic effect on advanced CC is still far from satisfactory. In addition to conventional treatment schemes, novel treatment schemes or small molecule regulators shall be urgently found to fundamentally cure CC. This is of great significance for improving the survival rate, prognosis and quality of life of patients[16].

The expression level of lncRNAs in cells changes with variations in the body. The abnormal expression of specific lncRNAs may also reflect the development of certain cancers, including CC. The Wnt signaling pathway is also considered as a key participant in the development and progression of CC. As a housekeeping gene in the Wnt signaling pathway, β-catenin is closely involved in the proliferation, migration and invasion of cells[17]. Liu et al[18] have discovered that HCFU can inhibit the growth and metabolism of CC cells by repressing the Wnt/β-catenin signaling pathway. This indicates that inhibition of β-catenin expression may be a new therapeutic target of CC. Furthermore, Hua et al[19] have observed that lncRNA Nicotinamide Nucleotide Transhydrogenase-antisense RNA1 (NNT-AS1) is an important player in the proliferation and invasion of CC cells. High

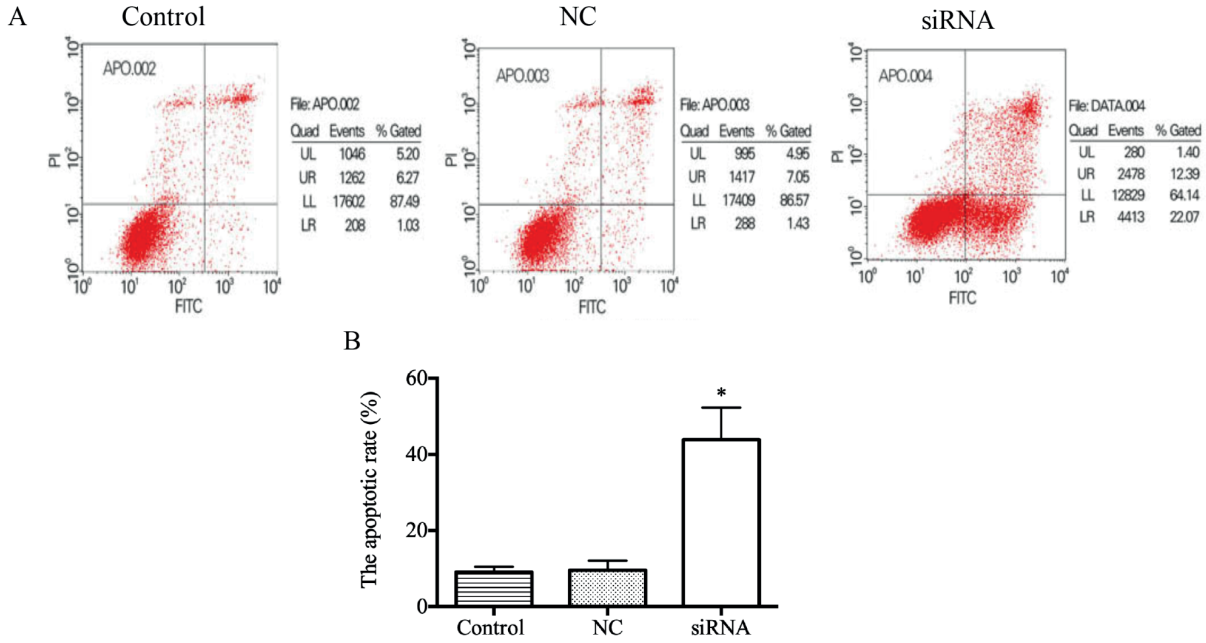

Figure 4. Effect of lncRNA UCA1 on HeLa cell apoptosis. **A,** Flow cytometry graph, and **B,** Apoptosis rate (**p*<0.05: lncRNA UCA1 siRNA group *vs.* Control group and lncRNA UCA1 NC group).

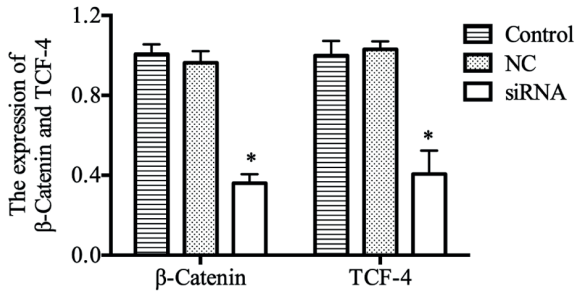

Figure 5. MRNA expression levels of β-catenin and TCF-4 in HeLa cells (*p<0.05: lncRNA UCA1 siRNA group *vs.* Control group and lncRNA UCA1 NC group).

expression of lncRNA NNT-AS1 facilitates the proliferation and migration of CC cells in a mouse model. The underlying mechanism may be correlated with the activation of the Wnt/β-catenin signaling pathway. However, there are few reports on whether lncRNA UCA1 can affect the proliferation and apoptosis of CC cells by regulating the β-catenin/TCF-4 signaling pathway.

In this study, the regulatory effect of lncRNA UCA1 on the proliferation and apoptosis of CC HeLa cells was explored. Meanwhile, the possible regulatory mechanism was analyzed and verified. First, Lipofectamine 2000 transfection reagent was adopted to transfect lncRNA UCA1 NC and siRNA into HeLa cells. QRT-PCR results (Figure 1) revealed that lncRNA UCA1 level decreased dramatically declined in HeLa cells in lncRNA UCA1 siRNA group. This indicated that HeLa cell line with lowly expressed lncRNA UCA1 was successfully constructed. Next, the regulatory effect of lncRNA UCA1 siRNA on the proliferation and apoptosis of HeLa cells was detected *via* MTT assay, Hoechst 33258 staining and flow cytometry, respectively. It was found that lncRNA UCA1 siRNA transfection significantly inhibited the proliferation and promoted the apoptosis of HeLa cells (Figures 2-4), in line with other study findings[20]. To further investigate whether lncRNA UCA1 siRNA regulated the β-catenin/TCF-4 signaling pathway to modulate the proliferation and apoptosis of HeLa cells, qRT-PCR and Western blotting were employed to measure the mRNA and protein expressions of β-catenin and TCF-4, respectively. The results (Figure 5-6) showed that expressions of β-catenin and TCF-4 were remarkably suppressed by lncRNA UCA1 siRNA transfection. All these findings suggested that lncRNA UCA1 siRNA repressed the β-catenin/TCF-4 signaling pathway to inhibit the proliferation and promote the apoptosis of HeLa cells.

Conclusions

In brief, lncRNA UCA1 siRNA effectively inhibits the proliferation and promotes the apoptosis of CC HeLa cells. Its regulatory mechanism may be associated with the inhibition on β-catenin and TCF-4 expressions in the β-catenin/TCF-4 signaling pathway. Our findings provide new ideas for the application of lncRNAs in the early diagnosis, treatment, and prognosis evaluation of CC. However, this study is performed at the *in vitro* cell level, and the *in vivo* regulatory effect of lncRNA UCA1 needs to be further investigated.

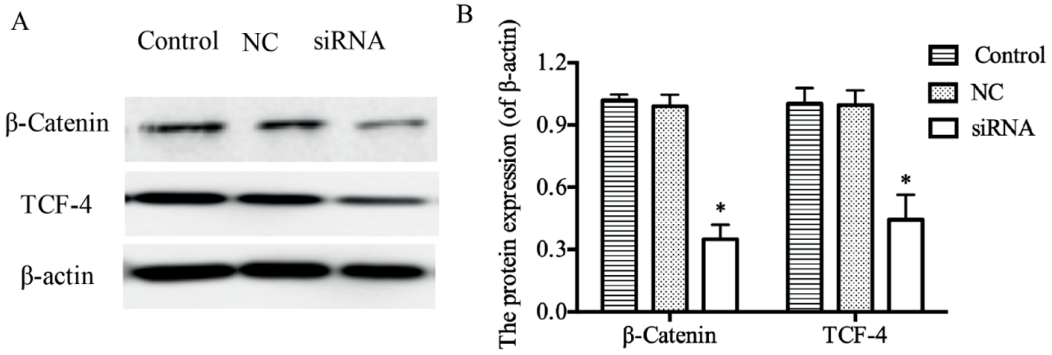

Figure 6. Protein expression levels of β-catenin and TCF-4 in HeLa cells (*p<0.05: lncRNA UCA1 siRNA group *vs.* Control group and lncRNA UCA1 NC group).

Conflict of Interest

The Authors declare that they have no conflict of interests.

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