The Effects of siRNA SIRT1 on the Proliferation of Human Cervical Cancer Cells

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Abstract: To investigate the effects of small interfering RNA (siRNA) SIRT1 on the proliferation of human cervical cancer cells with the hopes of finding new diagnostic and therapeutic modalities. Chemical synthesis of siRNA targeted against SIRT1 were transfected into human cervical cancer C33A cells by Lipofectamine RNAi-MAX liposomes, on harvesting at 72h after transfection, the total RNA were extracted by the TRIZOL reagent and reverse transcribed into cDNA with the PrimeScript RT-PCR kit, and the expression level of SIRT1 mRNA was detected by RT-PCR. The cell proliferation was performed by CCK-8 method. RT-PCR results indicated that the mRNA expression of siRNA SIRT1 group was significantly down-regulated (P<0.05) compared to the control groups. CCK-8 proliferation assay showed that the inhibition rate of siRNA SIRT1 group was 52.1%, while the negative control group was 35.4%, the inhibition rate of transfection reagent control group was 11.5%. The inhibition rate of siRNA-SIRT1 group was significantly higher than other groups. Conclusion SIRT1 siRNA could down-regulate the expression of SIRT1 mRNA, inhibit the proliferation of C33A cells, suggesting that SIRT1 has promoting abilities in human cervical cancer.

Keywords: SIRT1, RNAi, Cervical cancer cell

Introduction
Cervical cancer is one of the most common cancer and the second leading cause of cancer-related death among women worldwide, with approximately 470,000 new cases and over 200,000 deaths per year[1,2,3]. Although chemotherapy and radiotherapy are still the major option for the treatment of invasive cervical cancer, its efficacy is limited because of high toxicity and drug resistance [1,2,3]. Therefore, it is necessary to develop more effective and less toxic anticancer agents.

SIRT1, a NAD(+) dependent histone deacetylase, is known to play an important role in epigenetic silencing, suppressing recombination of rDNA, progression of the cell cycle, and longevity [3,4]. The role of SIRT1 in mammals is complex and less well characterized, however contradictory reports have suggested SIRT1 act as either tumor suppressing or promoting abilities, at present, little is known regarding the function of SIRT1 in human cervical cancer.

RNA interference (RNAi) referred to a process characterized by sequence specific, post-transcriptional gene silencing(PTGS) directed by short interfering 21-23nt double-stranded RNAs. Several studies conducted in vivo and in vitro showed that RNAi application for targeting functional carcinogenic molecules, tumor resistance to chemotherapy and radiotherapy is required in today's cancer treatment[5,6]. Therefore, the present study was designed to investigate the effects of siRNA SIRT1 on the proliferation of human cervical cancer cells C33A. The purpose of this study was to use siRNA to characterize the effect of SIRT1 on the proliferation of human cervical cancer cells with the hopes of finding new diagnostic and therapeutic modalities.

Materials and Methods
Reagents
siRNA specific to SIRT1 (5'-ACUUUGCUGUAACCCUGUAdTdT-3') and the control siRNA were synthetized by Life technology Co., Lipofectamine RNAiMAX was pursased from Life technology Co., hGAPDH and SIRT1gene primers were synthetized by Life technology Co.. Cell Counting Kit-8 kit was from Wuhan boster biological engineering co., Ltd, China.

Cell culture
The human cervical cancer cells C33A were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum. All cells were incubated at 37 °C in an incubator containing 5% CO₂.

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siRNA Transfection
siRNA SIRT1 and negative siRNA control were transfected by using Lipofectamine RNAi-MAX according to the manufacturer’s instructions. Briefly, C33A cells( 3x10^4/well) were inoculated in the 24-well plate and incubated to reach 30%-50% confluency 24 h after plating. Dilute 6 pmol RNAi duplex in 100 μL of DMEM in each well of the culture plate to be used for transfection. Mix the contents gently. Mix Lipofectamine RNAiMAX Reagent gently before use. Add 1 μL of Lipofectamine® RNAiMAX Reagent to each well containing the diluted RNAi molecules. Mix gently and incubate for 10–20 min at room temperature. Prepare 500 μL of cells for each transfection to be performed. Dilute trypsinized adherent cells in complete growth medium so that 5 00 μL contain enough cells to reach 50–70% confluency 24 h after plating. Dilute suspension cells at a concentration of 40–100 cells/μL. Add 500 μL of the diluted cells to each well containing RNAi duplex- Lipofectamine® RNAiMAX Reagent. Mix gently by rocking the plate back and forth. The final RNA concentration is 10 nM in a total volume of 60 μL. Incubate the cells 24–72 h at 37°C in a CO2 incubator until you are ready to assay for gene knockdown.

Reverse transcription polymerase chain reaction (RT-PCR)
On harvesting at 72 h after transfection, total RNA from C33A cells was extracted using the TRIZOL reagent (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions. 1μg of total RNA (in triplicate) was reverse transcribed into cDNA using the PrimeScript RT-PCR kit (Takara Bio, Inc., Dalian, China). Subsequently, cDNA product was subjected to PCR amplification with TagDNA polymerase on a thermal cycle. The RCR conditions were as follows: preliminary degeneration at 94 °C for 1 min, followed by 30 cycles of degeneration at 94 °C for 1 min, annealing at 47 °C for 1 min, extension at 72 °C for 1 min, extension at 72 °C for 10 min at last. The PCR products were loaded onto 1% agarose gels, scanning specific bandings by gel imaging system. The PCR primers used in this study are shown in Table I. Human GAPDH (hGAPDH) was used as the internal control.

| Gene  | Primer sequence                  | PCR product length (bp) |
|-------|----------------------------------|-------------------------|
| hGAPDH| Sense: 5'-GGCTTCTCCAGAACATCAT-3' | 240                     |
|       | Antisense: 5'-CACCTGGTGCTCAGTGTA-3' |                     |
| SIRT1 | Sense: 5'-TCAGTGTCTAGTCTTTTGCG-3' | 820                     |
|       | Antisense: 5'-AATCTGCTCTTTGCGACTCT-3' |                     |

Proliferation assays
Cell proliferation assays were performed using Cell Counting Kit-8 kit. Logarithmic phase of C33A cells (6x10^4/well) were inoculated in the 96-well plate and incubated for 24 h, then Chemical synthesis of siRNA and transfection after a further incubation for 24 h, 48 h and 72 h, 10 μL of CCK-8 solution was added into each well, followed by 2 h incubation at 37 °C in an incubator containing 5% CO2, and then absorption value A was measured in wavelength of 450 nm by a spectrophotometric plate reader (BioRad, Tokyo, Japan). All the experiments were carried out in triplicates. According to the formula: inhibition rate = (control group A value–experimental group A value)/ control group A value x100%.

Statistical analysis
The experimental data are expressed as the Means ± SD. The significance of the data was determined by one-way ANOVA analysis. The statistical significance of the differences between the values in each two groups was determined by q test. A P-value ≤ 0.05 was considered to indicate a statistically significant difference. All the statistical analysis were performed with SPSS13.0 software.

Results
Inhibition of siRNA SIRT1 on the expression of SIRT1
RT-PCR results showed that the mRNA expression of siRNA-SIRT1 group was significantly down-regulated (P<0.05) compared to the control groups, while the negative siRNA control group and Lipofectamine® RNAiMAX Reagent group had no significant changes (P>0.05).

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Fig1. Inhibition of siRNA SIRT1 on the expression of SIRT1 mRNA
1. Sirt1-siRNA group, 2. negative siRNA control group
3. Lipofectamine® RNAiMAX Reagent group 4. normal cell group

Effect of siRNA SIRT1 on the proliferation of C33A cells
CCK-8 proliferation assay demonstrated that the inhibition rate of siRNA-SIRT1 group was 53.1%, while the negative control group was 35.4%, the inhibition rate of transfection reagent control group was 11.5%. The inhibition rate of siRNA-SIRT1 group was significantly higher than other groups.

Discussions
SIRT1 contains at least two nuclear localization signals and two nuclear export signals, and can shuttle between the nucleus and cytoplasm under certain conditions[3,4]. It is believed that SIRT1 is involved in multiple tumors. SIRT1 expression is up-regulated in many types of cancers, such as breast cancer, lung cancer, ovarian epithelial tumours, hepatocellular carcinoma and prostate cancer et al[7-11]. SIRT1 enhances tumor angiogenesis through negatively modulating Delta-like ligand 4 (DLL4)/Notch signaling in Lewis lung carcinoma xenograft-derived vascular endothelial cells. SIRT1 also activates endothelial nitric oxide synthase by deacetylation to enhance nitric oxide production and improve vascular function. As a consequence, enhanced tumor angiogenesis induced by SIRT1 may bring more nutrition to cancer cells and facilitate their survival and growth. However, in some tumors, SIRT1 might be a tumor suppressor, and prolonged SIRT1 inhibition could have adverse effects. Several mouse studies demonstrated that over expression of SIRT1 could reduce colon and intestinal polyps in APC min/+ mice, thymic lymphoma in p53-/- mice and spontaneous carcinoma/sarcoma, showing the tumor suppressor role of SIRT1[12].

SIRT1 may exert its antitumor functions by improving genome stability through enhancing DNA damage repair.

In this study, chemical synthesis of small interfering RNA (siRNA) targeting SIRT1 gene were transfected into cervical cancer 33A cells to explore the effect of siRNA SIRT1 on the proliferation of human cervical cancer cells C33A. The results showed that SIRT1 siRNA could down-regulate the expression of SIRT1
mRNA, inhibit the proliferation of C33A cells, suggesting that SIRT1 has promoting abilities in human cervical cancer. Therefore, treatment with siRNA targeted against SIRT1 might be a future treatment option against human cervical cancer.

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