Downregulation of hTERT contributes to ovarian cancer apoptosis and inhibits proliferation of ovarian cancer cells

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Background: Our study aims to study the effects of the exogenous human telomerase reverse transcriptase (hTERT) interfering gene on the ovarian cancer cell line SKOV3 through proliferation and apoptosis.

Methods: Lipofectamine TM2000 was used to transfer the hTERT interfering gene into the SKOV3 cells. After a predetermined amount of time after transfection with the hTERT interfering genes, the expression of the tumor-related genes was detected using real-time quantitative polymerase chain reaction (RT-qPCR), and relative protein level was detected using western blot analysis. Cell morphology was acquired by microscopy. Cell viability was detected by middle-time-spray (MTS), and cell cycle and apoptosis were detected by flow cytometry.

Results: Forty-eight hours after transfection, the expression of tumor-related proteins in the experimental group was increased compared with the control group, and the difference was statistically significant ($P<0.05$). The cell morphology showed a significant difference between the control group and the hTERT shRNA group. Furthermore, 48 and 72 h after transfection with the hTERT interfering gene, the cell viability inhibition rates of the hTERT shRNA group were 0.77 ± 0.02 and 0.88 ± 0.01 respectively. Compared with the control group, the cell viability inhibition rates were 11.97 ± 2.37 (%) and 18.72 ± 1.01 (%), respectively, with statistically significant differences ($P=0.009$, $P=0.004$). Flow cytometry detected the apoptosis peak of SKOV3 cells in the experimental group 48 h after transfection with the hTERT interfering gene. Propidium iodide (PI) and Annexin V-FITC revealed that the apoptotic cells accounted for 18.13% of the total number, which was significantly higher than the 3.85% demonstrated by the control group.

Conclusions: The amount and activity of SKOV3 cells in ovarian cancer was decreased after the exogenous introduction of the hTERT interfering gene.

Keywords: Ovarian cancer; hTERT gene; downregulation; proliferation; apoptosis

Introduction

Among gynecological malignant tumors, ovarian cancer ranks third in incident rate in the female reproductive system. Also, ovarian cancer has the highest mortality rate among all malignant tumors (1-3). The incidence rate of ovarian cancer has become the third highest of the female reproductive system tumors, and the mortality rate ranks the first among gynecological malignant tumors (1-3). Early diagnosis and treatment options are vital for recovery (4,5); thus, our study focused on ovarian cancer prevention and treatment.

Finding novel effective drugs with fewer side-effects is the focus of cancer research (6,7). In recent years, many researchers have been devoted to developing new anti-ovarian cancer drugs in order to find more effective treatments (8). Therefore, it has become necessary to understand the biological mechanisms of ovarian cancer. hTERT is a crucial nutrient factor in primary tumor and cancer cell lines. It has been shown to have a high expression among tumors, while there being no expression in normal tissues. Therefore, the clinical values and multiple biological effects are not understood clearly (9,10).

Recently, several genes have been reported to exert apoptosis-inducing activity for human cancers. In our study, we investigated and compared the apoptosis-inducing activity of p21, p53, cmc-y, tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) expression in SKOV3 cell lines (11). The hTERT gene was silenced in this study after electrotransfection into ovarian cancer SKOV3 cells to investigate the therapeutic effect of this method on ovarian cancer persistence.

Methods

Materials and reagents

The human ovarian cancer cell line SKOV3 was bought from the ATCC corporation (USA). Vector RNAi-Ready pSIREN-DNR-dsred-express was bought from Santa Cruz Biotechnology company (USA). Lipofectamine 2000 was obtained from Invitrogen, and McCoy's 5A (modified) cell medium was purchased from Thermo Fisher Scientific (USA). Streptomycin and potassium phosphate buffer (PBS) were also purchased from Hyclone. Meanwhile, the cell lysates were bought from Life Technologies, and Super Signal West Pico chemiluminescent substrate kit was bought from Thermo Scientific. A BCA protein concentration kit (Shanghai Biyuntian biotechnology) and a Alexa Fluor 88 annexinV cell cycle apoptosis kit were also purchased (Invitrogen, USA); all the other reagents were analytical pure (8).

Study methods

siRNA design

shRNA was designed to select RNAi target sites (hTERT mRNA sites 1812-1832 nucleotide, long 19 nt, target sequence: AGCATTGGAATCAGACAGC). In combination with the specification of RNAi ready

Psc-36641-SH-hTERT-expressing plasmid from BD Biosciences Clontech, an shRNA online design tool was used to design oligonucleotide chains that could transcriptionally target hTERT shRNA, including BamH 1 and EcoR 1 restriction sites at both ends and reverse complementary target sequences, which were separated by loop intervals of 9 bp non-homologous sequence (TTCAAGAGA). 3' end with a TTTTTT termination sequence, chemical synthesis Invitrogen company Shanghai branch is from the United States. The two oligonucleotides chain are Sense: 5'-GAT CCAGCATTGGAATCAGACAGCTTCAAGAGAGCT; GTCTGATTCCAATGCTTTTTTTGGAAG -3' and Antisense: 5'- AATTCCTTCCAAAAAAAGCATTGGAA TCAGACAGCTCTCTTGAAGCTGTCTGATTCCA ATGCT -3'. Meanwhile, negative control sequences were designed by the same method.

Cells transfection

We divided ovarian cancer SKOV3 cells into 4 test groups: the non-transfection group as the control group, the pIRSC2-blank as the blank control group, the transfection (blank group) as the control, the non-specific siRNA vector (pIRSC2-NC) transfection as the negative control (NC group), and the pIRSC2-shTERT transfection as the observation group (shTERT group). According to the procedure provided in the transfection kit, transfection was carried out instantaneously. In the transfection process, 3 g recombinant plasmid and 6 mL liposomes were dissolved in 500 mL serum-free medium to make the transfer solution, and the transfection was suspended after 6 h with the addition of 2 mL per well to the final volume of serum-free medium. It was observed that the red fluorescence could be seen in SKOV3 cells 12 h after the staining, and the fluorescence expression was strongest at 48–72 h, with the fluorescence still being until day 7. In this study, the 24–72 h timeframe after transfection was selected as the experimental time. The Psc-36641-SH-hTERT transfection group (SKOV3/p-shRNA) and the blank control group were then established (12,13).

Real-time quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR detected the expression of hTERT, C-MYC, P21, P53, TRAIL mRNA. GAPDH was used as the control. Total RNA was isolated from SKOV3 cells and was quantified by nucleic acid electrophoresis. When the ratio of 28 s rRNA to 18 s rRNA was greater than or equal to 1, the quality of total RNA extracted was

qualified. When the expression of all samples all went through GAPDH level correction, according to the $2^{-\Delta\Delta Ct}$ formula, the relative expression level of samples was calculated. hTERT primer sequence in upstream was 5'-GGCCTTCACCACCAGCGTGC-3', and in downstream was 5'-AGGACCCCTGCCCAACGGGC-3', with amplification fragment 427 bp; within the referring to the sequence of GAPDH primers, upstream was 5'-AGGTAGGACGAGGTCGGAGTC-3', and downstream was 5'-GAATTAGGTACTAGGATTTC-3' (14,15).

Western blotting

SKOV3 cell lines were transfected with a plasmid for 48 h. After that, the cells of each group were collected, and proteins were extracted for later analysis. Next, RIPA cell lysate was added to each well of the 6-well plate (PMSF, Protease inhibitor) to extract protein, using the BCA protein concentration determination kit to determine protein concentration. Then, 20 mg protein was transferred to sds-page gel, followed by transfer to PVDF. They were incubated by primary antibody and secondary antibody, and ECL exposure was used to acquire the image and analysis.

Cell proliferation assay

MTS cell proliferation confirmed SKOV3 cell lines at 36 h after transfection. After inoculation, the cells were cultured in a 96-well culture plate, and the number of cells per well was the same. The proliferation of MTS cells was detected after the incubators were set for 12 h. The proliferation of SKOV3 cells in each well was detected, continuously for 3 days by MTS assay kit. MTS was added to each well and mixed with diluent 100 µL (10 µL MTS reagent and 90 mL 10% FBS+1640 medium) in a cell culture plate at 37 ℃ and incubated for 2 h in the dark. Finally, the absorbance value at 490 nm was detected by a microplate reader. The conditions of each test were consistent (12).

Flow cytometry detection

Apoptotic cells were harvested and transfected by flow cytometry, centrifuged at 1,000 r/min, precooled, fixed with 75% ethanol, and stained with propidium iodide (PI). Meanwhile, apoptotic cells were labeled with fitc-annexin v, and the apoptotic effect of SKOV3 cells was detected by flow cytometry. The specific steps were conducted in strict accordance with the instructions of the kit, and the optical density value of the test strip was measured by image analysis technology. The test operation of the SKOV3 group was repeated 3 times (16).

Statistical processing analysis

SPSS version 19.0 was used as the statistical analysis software while analysis and measurement data were conducted by t-test. Data are expressed as the mean ± SD (standard deviation) of at least 3 separate experiments performed in triplicate. P values <0.05 were considered statistically significant.

Results

The effect of hTERT on the expression of cell cycle- and apoptosis-related proteins

To determine whether hTERT contributes to the proliferation of SKOV3 cells, we checked the hTERT expression level in SKOV3 cell lines and found that hTERT siRNA knockdown was performed in SKOV3 cell lines. The RT-PCR results showed that p21, p53, and TRAIL expression was significantly increased in the SKOV3 cells 36 h after transfection with 2 mmol/L hTERT shRNA sequence. In contrast, the cell cycle gene (c-myc) expression was significantly decreased 36 h after transfection with 2 mmol/L hTERT shRNA sequence. This indicates that knockdown hTERT could upregulate tumor related genes (p21, p53, and TRAIL) and inhibit c-myc. The western blot analysis showed the same trend with RT-PCR (*Figure 1*).

The effect of inhibition of hTERT on the proliferation of SKOV3 cells

SKOV3 cells transfected with hTERT shRNA showed significantly slower growth and reduced proliferation compared with SKOV3 cells transfected with empty plasmid on the second and third days after transfection (*Figure 2*).

The effect of hTERT on the morphology of SKOV3 cells and the apoptosis-inducing effect of HTERT on SKOV3 cells

SKOV3 cells were transfected with hTERT (2 mmol/L) for 48 h, and the morphological changes induced by the hTERT treatments were examined under an inverted microscope. The NC group cell structure was clear, and the cell nucleus was full. Compared with the NC group, the number of the SKOV3 cells was markedly reduced after treatment with 2 mmol/L hTERT for 48 h. The
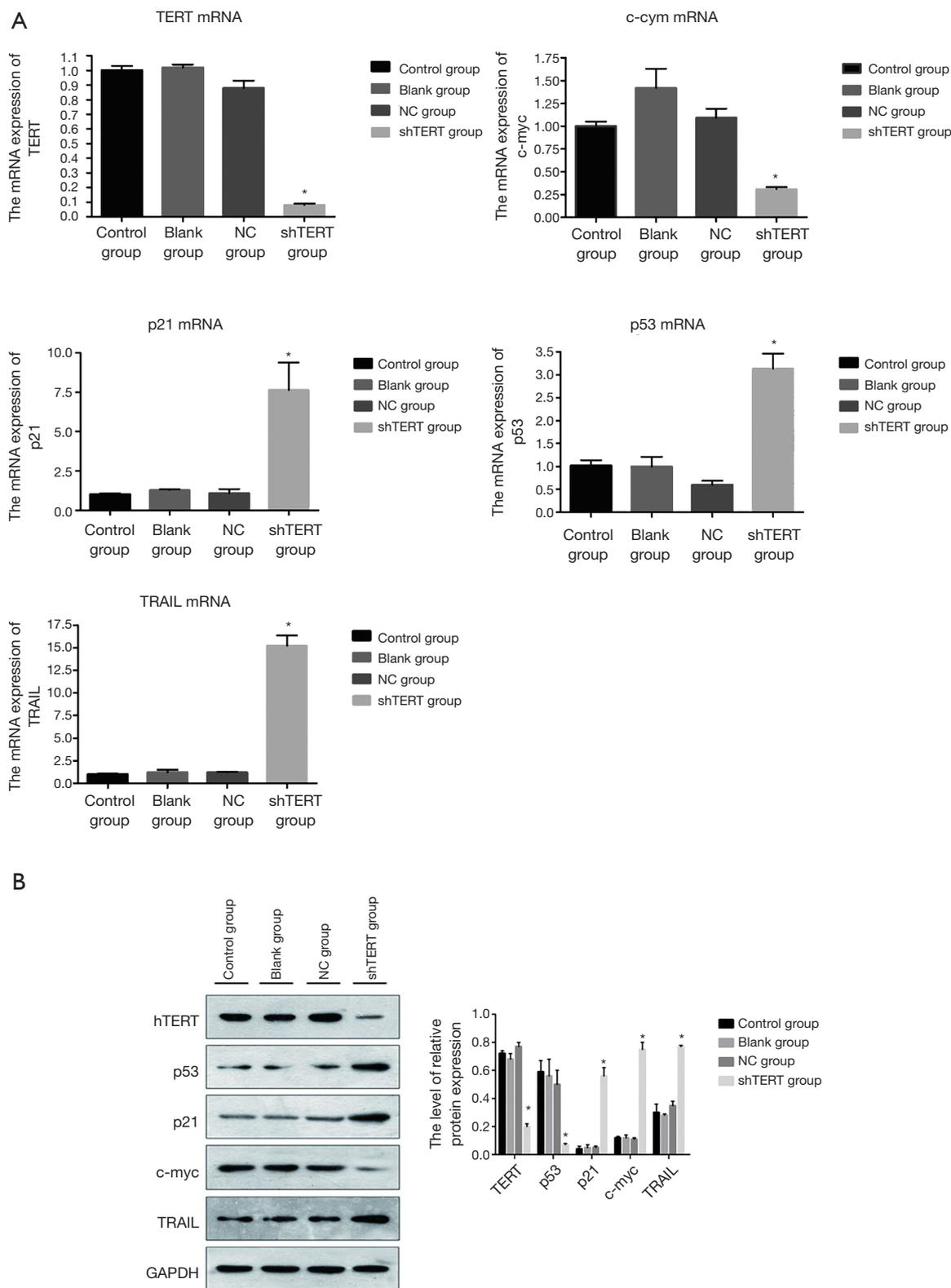
Figure 1 The expression of hTERT mRNA, p53, p21, c-myc, and TRAIL was detected by RT-PCR (A) and western blot (B). *P<0.05 *vs*. NC group.

1452

Wang et al. Effect of hTERT down-regulation on ovarian cancer cells
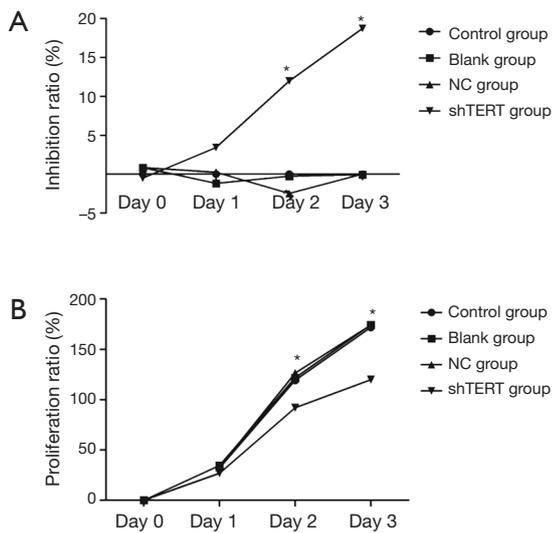
Figure 2 Proliferation and inhibitory effect of hTERT on SKOV3 cells detected by MTS assay. SKOV3 cells were transfected with hTERT for 1 day, 2 days, and 3 days. (A) Inhibition rate = (1– od value mean/control group OD value mean) ×100% (at the same time), (B) proliferation rate = (OD value mean at other time points/0 h OD value mean –1) ×100% (at the same time). *P<0.05 compared with the corresponding NC group.

hTERT-treated cells became long and thin and displayed an elongated spindle-shaped morphology. Some hTERT-treated cells even appeared star-shaped (*Figure 3*).

The effect of inhibition of hTERT on the apoptosis rate of SKOV3 cells

The flow cytometric analysis results showed that the SKOV3 cells underwent apparent apoptosis after treatment with hTERT shRNA. The number of early apoptotic cells and late apoptotic cells both increased after treatment with hTERT shRNA compared with the control group (*Figure 4*). The total apoptosis rate in the hTERT shRNA group was 18.66±1.33, which was significantly higher than that in the control group (2.92±0.33), blank group (3.02±0.65), and NC group (3.66±0.34).

Discussion

In clinical practice, radiation and chemotherapy can not be tolerated by normal tissues, which in turn affects the survival rate for ovarian cancer patients. Currently, gene therapy is a hot-topic issue in the domestic and overseas fields of biological research. New genes have been discovered as one
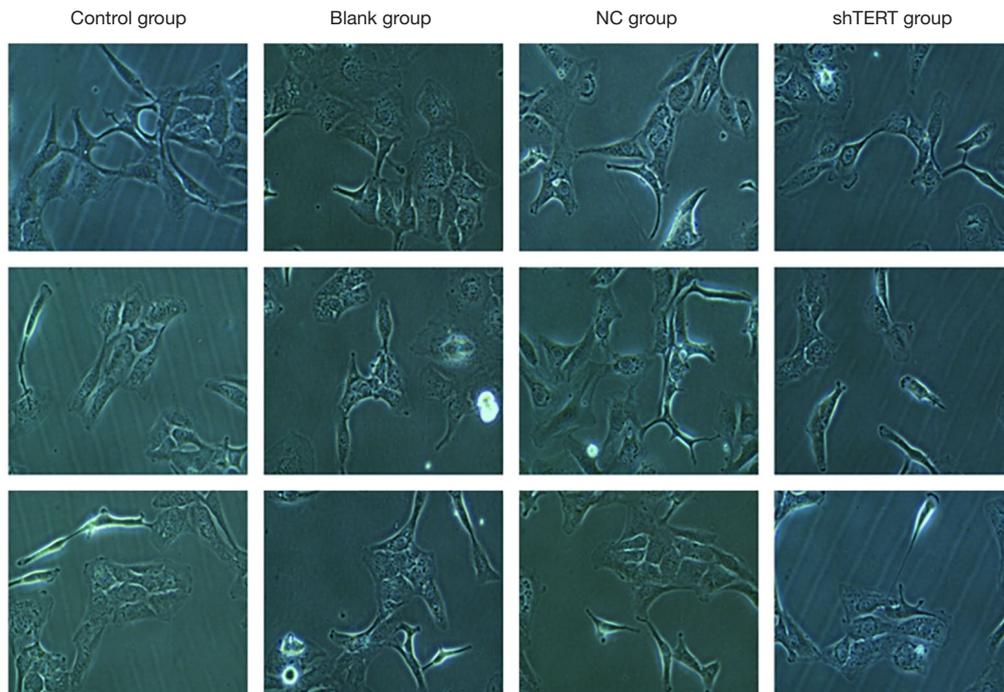
Figure 3 The morphology changes of SKOV3 cells treated with hTERT shRNA for 48 h (10×40).
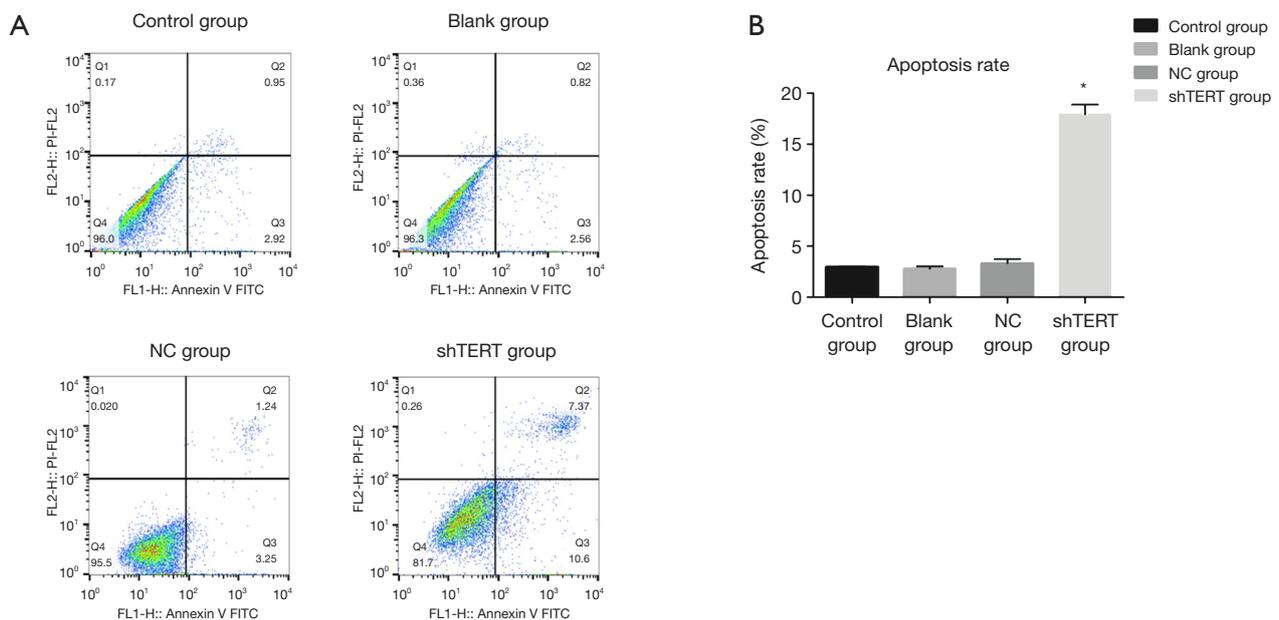
Figure 4 Apoptosis-induced effects of hTERT on SKOV3 cells by flow cytometry. (A) Apoptosis rates of SKOV3 cells treated with hTERT at 2 mmol/L for 48 h; (B) the column diagram of apoptosis rates of SKOV3 cells treated with hTERT at 2 mmol/L for 48 h. *P<0.05 *vs.* NC group.

of the treatment methods for ovarian cancer, opening new possibilities in treatment (17,18).

In recent years, the development of RNAi as a novel silencing gene technology has allowed for the determination of the genetic signature of siRNA. Our study established that the expression of hTERT siRNA, eukaryotic expression plasmid, and negative control plasmid were able to be successfully transfected into the ovarian cancer SKOV3 cell line, with a transfection efficiency of more than 50%, indicating that the transfection was successful.

As a result, the expression of hTERT mRNA was significantly decreased. DNA was cloned into a plasmid and reconstituted into hTERT mRNA-siRNA. The expression vector of hTERT mRNA was detected by RT-pcr assay, indicating target gene silencing, which is consistent with the research results of Chen *et al.* (11). Research data showed that telomerase is a reverse transcriptase, and can maintain telomere length.

hTERT is a catalytic subunit of telomerase and can be reverse transcribed using RNA as a template to synthesize telomerase. hTERT is closely related to cell growth, development, proliferation, and differentiation. hTERT can significantly inhibit the growth of carcinoma cells, which exist in multiple organisms. hTERT expression is inhibited

in normal tissues, but not in cancer cells (15,19).

However, there was high expression of hTERT that was found in tumor lines. It was also found that the hTERT gene could be transfected by electrotransfection to achieve an effective and lasting effect. Additionally, it was found that electroporation could be used for transfection because the target gene transient expression rate is much higher than the liposome transfection method so that the electro transfer can be an effective and simple method. This study showed that siRNA targeted silencing hTERT gene could be electro transferred into ovarian cancer SKOV3 cells, which can play an active role in guiding the treatment of ovarian cancer. hTERT genes may supply novel targets for gene therapy of ovarian cancer and other tumors.

Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi. org/10.21037/tcr.2020.01.39). The authors have no conflicts

of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Institutional ethical approval and informed consent were waived.

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