

Effects of hTERT antisense oligodeoxynucleotide on cell apoptosis and expression of hTERT and bcl-2 mRNA in keloid fibroblasts

Y. HUANG, L.-X. LIN, Q.-X. BI, P. WANG, X.-M. WANG, J. LIU, Y.-T. WANG

Department of Burn and Plastic Surgery, The Affiliated Yantai Yuhuangding Hospital of Qingdao University, Yantai, China

Yong Huang and Lixin Lin contributed equally to this work

Abstract. – **OBJECTIVE:** This study was purposed to investigate the effects of hTERT antisense oligodeoxynucleotide (ASODN) on cell apoptosis and expression of hTERT and bcl-2 mRNA in keloid fibroblasts and to explore its anti-keloid effect.

MATERIALS AND METHODS: Primary cultures of dermal fibroblasts derived from 12 keloid samples were established, strains of fibroblasts at passages 3 to 4 were used in this study. After treated by hTERT ASODN the proliferation of the fibroblasts was measured by cell count and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay method, the apoptosis was analyzed by flow cytometry (FCM), and the expression of hTERT and bcl-2 mRNA were observed by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). The data was analyzed by statistical software (SPSS11.5).

RESULTS: The results showed that after sealing hTERT gene with ASODN for 72 h, the fibroblasts growth was repressed and the ability of proliferation decreased as the fibroblasts were treated with 1.0 mol/L ASODN for 72 h, the fibroblasts apoptosis was induced and the expression of hTERT and bcl-2 mRNA was lower than that of controlled group. The result was significantly different between control group and treatment group and was related to the treatment time of ASODN ($p < 0.01$), but the difference was no significant when compared 1.0 μ mol/L SODN group with untreated group ($p > 0.05$).

CONCLUSIONS: As a negative modulatory factor, hTERT-ASODN can suppress growth and proliferation of keloid fibroblasts. Decreasing the telomerase activity of keloid fibroblasts may be one of the most important mechanisms. That hTERT-ASODN inhibited telomerase activity in keloid fibroblasts is an important pathway that may play a key role in the anti-keloid therapy.

Key Words:

Keloid, hTERT, Antisense oligodeoxynucleotide, bcl-2, Apoptosis.

Introduction

Keloid is a common pathological scar with usually unobvious inducements, and sometimes it may occur without medical history of injuries. Different from normal scars, keloid often exceeds the original injury scope with an invasive growth. Its growth character is very similar to the immortal growth of tumors. Telomerase is a nucleoprotein reverse transcriptase for maintaining the length of telomere. With its RNA as a template, it synthesizes DNA sequence at the tail end of chromosome to immortalize cells. In this process, the most important component to maintaining telomerase activity is human telomerase reverse transcriptase, (hTERT)^{1,2}. Researches^{3,4} showed that the occurrence of many malignant tumors is closely related to the increase of telomerase activity and the overexpression of telomerase also exists in keloid fibroblasts. To verify the imagination of inducing keloid fibroblast apoptosis and inhibiting its proliferation by inhibiting telomerase activity, we worked hTERT antisense oligodeoxynucleotide (ASODN) on keloid fibroblasts, observed its effect on telomerase activity, fibroblast apoptosis, growth and proliferation to provide theoretical basis for clinic treatment of keloid.

Materials and Methods

Materials

12 keloid specimens are sourced from surgical patients from Yuhuangding Hospital.

Requirements on specimen selection: patients have no systemic disease; do not use hormone drugs. There is no history of medicine injection, use of drugs for external use or radiotherapy; the-

re is no inflammation or anabiosis on scars. The patient's age is 18-38 and the median age is 26; the course of the disease is half a year to one year and the average course of the disease is 8 months. After cutting for keloid specimens, the swelling and aglow marginal parts are taken for experimental study.

Methods

1. Fibroblast culture: cultured according to the tissue block method, cells of the 3rd or 4th generation are used in the experiments⁵.
2. Design and synthesis of sense and antisense sequences of telomerase oligonucleotides: refer to telomerase catalytic subunit sequence (Gene ID7015) of NCBI/GenBank, select 20 basic groups upstream of 5' end including the start codons ATG to synthesize segments of antisense oligodeoxynucleotides (ASODN) and take segments of sense oligodeoxynucleotides (SODN) as control. The synthetic sequences are as follows: its sense sequence (shTERT): 5'-CC-CGCGATGCCGCGCTCC-3'; anti-sense sequence (ashTERT): 5'-GGAGCGCGCG-GCATCGCGGG-3'. It is proved by computer retrieval that the anti-sense segments have no homology with other known human genes. All the oligonucleotides are synthesized, purified, and subpackaged by Sangon Biotech (Co., Ltd., Shanghai, China) and can be stored at -20°C for use.
3. Experiment grouping: the experiment shall be done in three groups including hTERT-ASODN treatment group (ASODN treatment), hTERT-SODN group (SODN) and blank control group. Each group of cells is all sourced from 12 patients.
4. Oligonucleotide transfection: carried out according to the specification. First, gently blend lipotap lipofection transfection reagent, then mix a defined amount of Dulbecco's Modified Eagle Medium (DMEM, Shanghai Srkbio Co., Ltd., Shanghai, China) nutrient solution not containing antibiotics and fetal calf serum (FCS), 15 µL lipidosome (Shanghai Bioleaf Biotech Co., Ltd, Shanghai, China) and a defined amount of purified ASODN and SODN with the final volume to be 75 µL. Use a gun to gently blow, beat and gently blend the diluted oligonucleotides and lipidosome. Quietly place it at the indoor temperature for 20 min to form oligonucleotide-lipidosome compound. Add the oligonucleotide-lipidosome compound into the corresponding culture plate holes. Place the culture plate into the 37°C incubator (flexcell-lint FX-4000T, Bio Excellence International Tech Co., Ltd, Beijing, China) with saturated humidity and 5% CO₂ for further culture.
5. Effects of ASODN in different concentrations on keloid fibroblast proliferation: get fibroblasts of the 3rd and 4th generation sourced from keloid, count them after they are digested with 2.5 g/L trypsin respectively, dilute them with DMEM including 10% fetal calf serum until the final density becomes 2×10⁵/ml, inoculate them successively to the 96-hole plate based on 100 µL/hole, and start experimental treatment after cell adhesion after cultivating for 24 h under the conditions of 37°C, 5% CO₂ and 95% moisture. Respectively add ASODN with the final concentration of 0.5, 1.0 And 1.5 µmol/L in the experimental groups, add SODN with the same concentration in the control group and only add DMEM culture solution in the blank control group. 3 parallel repeated holes are set up for each treatment. Culture for another 72 h under the conditions of 37°C, 5% CO₂ and 95% moisture. Measure the effect of ASODN on keloid fibroblast growth and proliferation with MTT method: after the above treatment for 72 h, get the 96-hole cell culture plate. Add 10 µl of MTT (the product of Zhenxing No. 1 Chemical Plant) solution in each hole (including MTT 5 mg/ml phosphate buffer saline (PBS)). Suck supernate after culturing for another 4h in the 37°C, 5% CO₂ and 95% moisture incubator. Add 100 µl of DMSO (analytically pure, the product of Zhenxing No.1 Chemical Plant) solution in each hole with reaction for 10 min, and shock without stop to sufficiently solve the crystal substance. Measure the optical density value (D) at 570 nm wave length with an automatic enzyme-linked detector and record results.
6. Apoptosis analysis of flow cytometry: prepare fibroblast single cell suspension and adjust the number of cells to 10⁵-10⁶ in different time. 1000 r/min centrifugation for 5 min for twice, keep 70% of cold ethanol at 4°C for 24 h. After washing with PBS for once, add 200 µg/ml RNA *enzyme* without DNA *enzyme*, add 0.5 ml of propidium iodide (PI) fluorescent staining solution and conduct analysis with flow cytometry after putting into the refrigerator at 4°C for staining for 30 min. Cells in lower than G phase are apoptosis cells.
7. Test expression of human telomerase reverse transcriptase (hTERT) and bcl-2mRNA

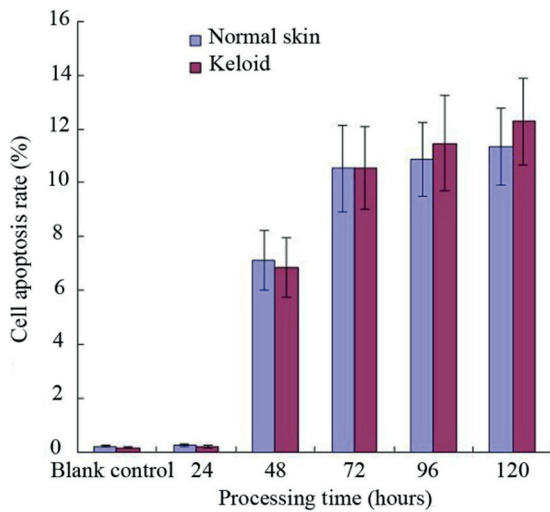


Figure 1. Apoptosis ratio of keloid fibroblasts treated by ASODN in different time.

of Keloid fibroblast with RT-PCR: (1) Primer synthesis: synthesized by Sangon Biotech (Shanghai, China) Co., Ltd. Sense primer of hTERT is 5'-CGGAAGAGTGTCTGGA-GCAA-3', Reverse primer is 5'-GGATGAA-GCGG AGTCTGGA-3', amplified fragment is 145 bp. Sense primer of bcl-2 is 5'-GACTTC-GCCGAGA TGTCCAG-3', its reverse primer is 5'-GTGCAGGTGCCGTTTCAGG-3' and amplified fragment is 228 bp. Sense primer of internal reference β actin is 5'-GTCAGA-AGGATTCCTATGTG-3', its reverse primer is 5'-AGGTCTCAAACATGATCTGG-3' and amplified fragment is 237 bp. (2) The total

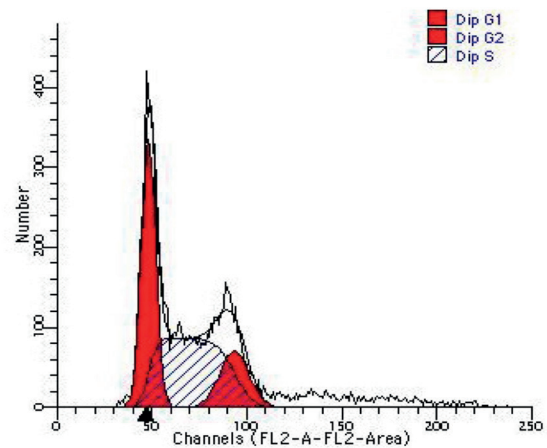


Figure 3. After 24 h ASODN treatment in keloid fibroblasts.

RNA extraction from cells: Extract total RNA of cells with TRIzol. (3) Reverse transcription and PCR Reaction: According to the kit instructions, Taq DNA Polymerase and PCR reverse transcriptase are the products of Gibeo, amplification kit was bought from TaKara. Reaction conditions of PCR are 94°C 30s, 62°C 30s, 72°C 30 s, 36 circulations amplification, 2-min extension at 72°C. (4) Result analysis: separate the amplicon with 1.5% sepharose gel electrophoretic separation, conduct densitometric scan on electrophoretic band with gelatin scanning imaging analyzer processing system, do statistical analysis according to Htert/ β actin and bcl-2/ β actin and evaluate the expression quantities of target genes in tissues.

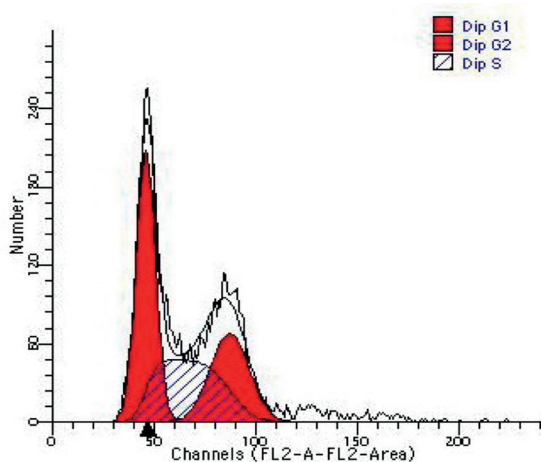


Figure 2. Keloid fibroblasts control group.

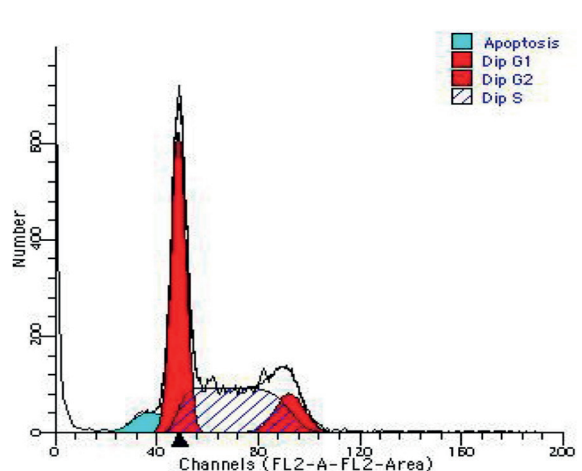


Figure 4. After 48 h ASODN treatment.

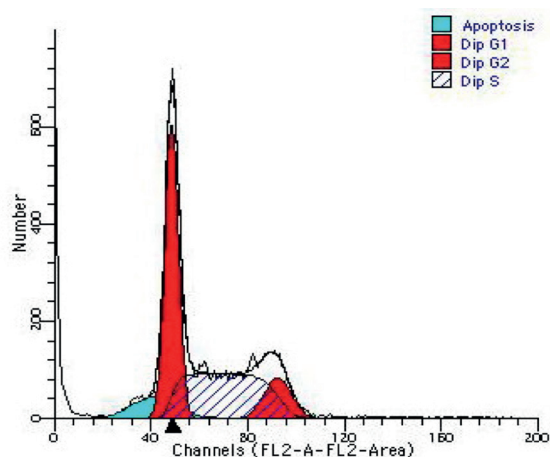


Figure 5. After 72 h ASODN treatment.

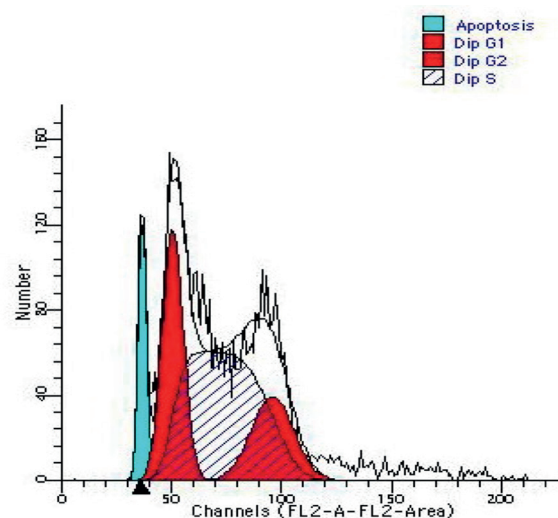


Figure 6. After 96 h ASODN treatment.

Statistical Analysis

Data are expressed as average value \pm standard deviation ($\bar{x} \pm s$). SPSS 11.5 statistical software (SPSS Inc., Chicago, IL, USA) is adopted. The comparison between the groups can be detected with *t*-test and $p < 0.05$ is the standard for significant differences.

Results

1. Effects of ASODN in different concentrations on keloid fibroblast proliferation. ASODN in different concentrations all have inhibiting effect on keloid fibroblasts, and inhibition ratio is positively related to the dosages. SODN group and blank control group have no obvious inhibiting effect. Differences have statistical significance compared with ASODN group ($p < 0.05$) (Table I).
2. Flow cytometry detection. The differences between keloid fibroblast apoptosis ratio of ASODN group after 24 h and that of SODN group and blank control group have no stati-

tical significance ($p > 0.05$). The cell apoptosis ratios after 48, 72, 96 and 120 h have an obvious growth. There are obvious cell apoptosis peaks. The differences have a statistical significance compared with SODN group and blank control group ($p < 0.01$, Figures 1-7).

3. Effects of ASODN treatment on the expression levels of keloid fibroblast hTERT and bcl-2 mRNA at different time. According to electrophoretic band brightness, it is discovered that expression quantities of hTERT and bcl-2 mRNA have an obvious down-regulation tendency (Figure 8-10) in keloid fibroblasts after 48 h and 96 h ASODN treatment. Through statistical treatment, the results show that the differences in the expression of hTERT and bcl-2 mRNA in keloid fibroblasts after 48 h ASODN treatment compared with blank group and SODN group have remarkable significance ($p < 0.05$); there are very remarkable differences in expressions after 96 h ASODN treatment compared with control group and SODN group ($p < 0.01$) Figures 8-12).

Table I. Effects of ASODN on keloid fibroblasts in cell proliferation [D570, $\bar{x} \pm s$].

Group	Oligonucleotide concentration		
	0.5 μmol	1.0 μmol	1.5 μmol
ASODN group	0.612 \pm 0.038*	0.535 \pm 0.027*	0.423 \pm 0.023*
SODN group	0.915 \pm 0.049	0.893 \pm 0.042	0.926 \pm 0.048
Blank control group	0.898 \pm 0.058		

Note: compared with blank control group and SODN group, * $p < 0.05$.

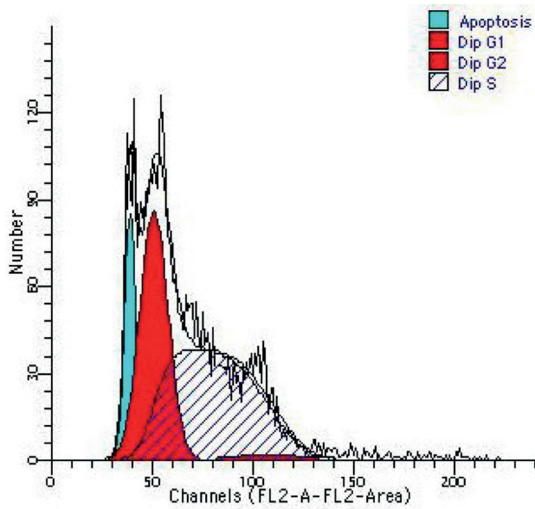


Figure 7. After 120 h ASODN treatment.

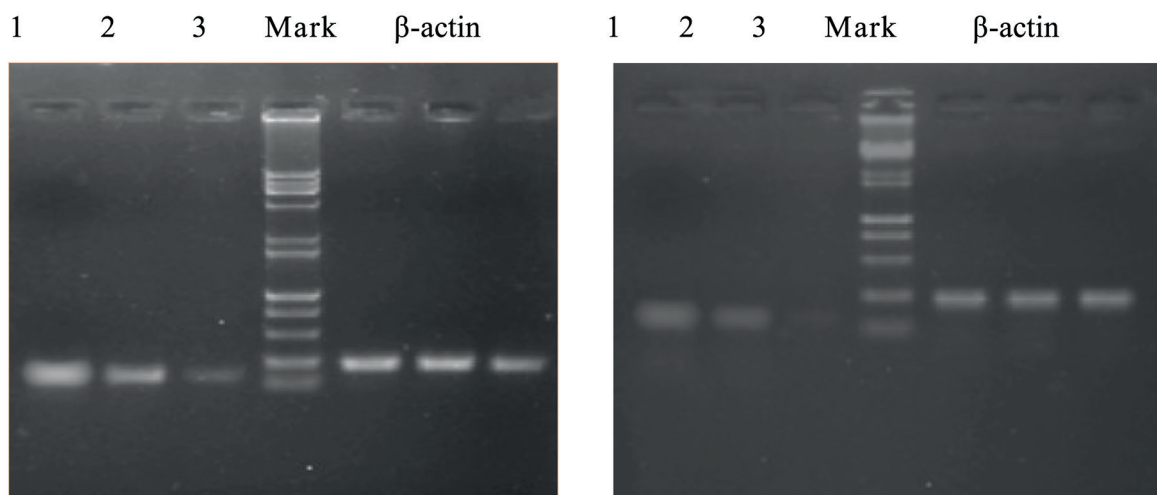
Discussion

As is known to all, with invasive growth and immortality, keloid fibroblasts have some biological characteristics different from those of normal skin fibroblasts, such as reduced demand for growth factor, enhancement of collagen synthesis ability, and insusceptible inhibition by glucocorticoid hormone, etc.⁶. Many of these characteristics are similar to those of tumor, so it is called keloid sometimes. The clinical treatment is also very

difficult, and is an extremely challenging problem for surgeons. Despite surgical excision, pressure therapy, radiation therapy, local injections of drugs (cortex steroids, IFN- γ), etc.^{7,8}, there's no way to achieve the ideal effect.

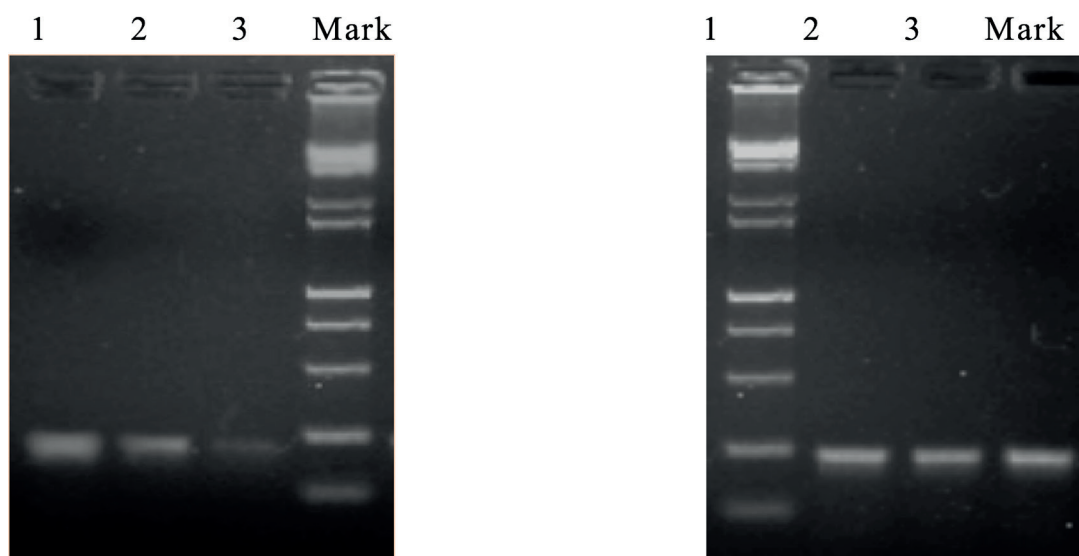
Telomerase is a special kind of nucleoprotein reverse transcriptase. It can synthesize chromosomes DNA sequence from the beginning with its RNA component as template, and perfect somatic cells chromosome DNA sequence, and maintaining telomere length stability and cell proliferation. The human telomerase reverse transcriptase (hTERT) is the most important ingredient for maintaining telomerase activity. The related research has proved that telomerase has higher expression in malignant solid tumor^{9,10}. As for whether there is telomerase mechanism in the development of keloid, the previous research results show that human telomerase reverse transcriptase (hTERT) has high expression of keloid fibroblasts, telomerase activity in human keloid fibroblasts increases. Activation of telomerase may be a key factor, and is closely associated with other high tumor gene expressions^{4,11}. Therefore, people may try inhibiting keloid fibroblast proliferation by inhibiting keloid activity with telomerase antisense nucleotide, so as to conduct gene therapy on keloid.

Antisense technology is a DNA or RNA fragment specifically combined with specific target gene synthesized based on the principle of complementary base pairing. As a "seal" of gene, it



Keloid (1: contrast, 2: 48 h, 3: 96 h) Normal skin (1: contrast, 2: 48 h, 3: 96 h).

Figure 8. Expression of hTERT mRNA in keloid and normal skin fibroblasts treated by IFN α -2b in different time. 123 Mark 123Mark.



Normal skin (1: contrast, 2: 48 h, 3: 96 h) β -actin.

Figure 10. Expression of bcl-2 mRNA in normal skin fibroblasts treated by IFN α -2b in different time.

can specifically block or inhibit the expression of target genes. The main mechanism of antisense nucleic acid is to form a DNA-RNA compound by combining specific base sequence of molecular antisense nucleic acid and mRNA cellular, inhibit the combination of mRNA and ribosomes, and activate the RNase to fracture mRNA and destroy its integrity, so that the specific gene cannot express and the corresponding protein synthesis is inhibited¹². The key technology of antisense therapy is to strengthen the stability of ASODN and improve the effective concentration in the cell. Research has shown that antisense nucleic acids without modification are susceptible to absorbed

by cells, but easy to be degraded by nuclease, thus having a short half-life. While AS PS-ODN has long half-life, and is not easy to be degraded by nucleic acid. So it is durable and more suitable for clinical application. Most of the antisense nucleic acids are polyanions. They are easy to interact with membrane protein through the cell membrane, but difficult to penetrate the cell membrane, and enter the cells to work. Cationic liposomes can combine with phosphate skeleton of the oligonucleotide to form compounds, and transfer liposomes into eukaryotic cells by endocytosis, fusion and other means, which greatly improves the transfection efficiency, enhances the stability

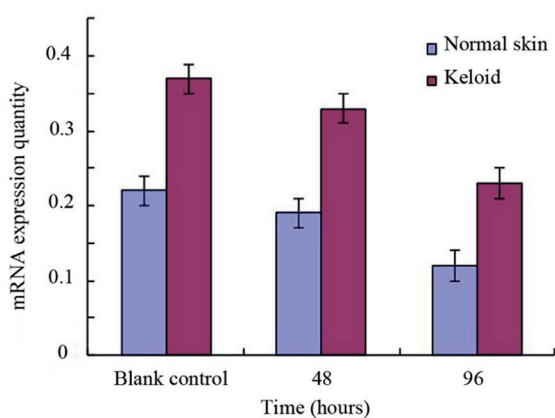


Figure 11. Expression of hTERT mRNA in keloid and normal skin fibroblasts treated by IFN α -2b in different time.

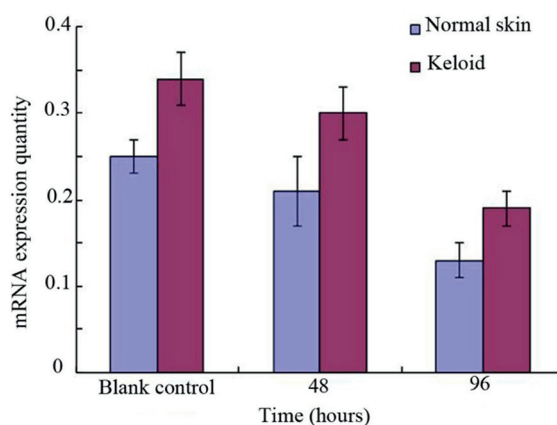


Figure 12. Expression of bcl-2 mRNA in keloid and normal skin fibroblasts treated by IFN α -2b in different time.

of antisense intracellular and prolongs the intracellular residence time of antisense nucleic acid¹³.

Antisense nucleic acid can reduce hTERT protein expression and activity of telomerase^{14,15}. By designing and synthesizing Phosphorothioate oligonucleotide containing 20 bases based on the gene of hTERT, the research confirms that the antisense oligonucleotides has inhibitory effect on keloid fibroblast proliferation *in vitro*, presenting dose dependence. Keloid fibroblasts have high proliferation and low apoptosis, which is very similar to tumor cells. This work finds that antisense oligonucleotides can inhibit the growth of keloid fibroblasts and downgrade hTERT, BCL-2 mRNA expression, and lead to cell cycle arrest and apoptosis. The regulation of telomerase activity depends on the apoptotic signal induced by IFN α -2b. Maybe telomerase controls time of the G1 phase in the process of cell cycle to make the cell cycle cross the limit point and finish the transition from G1 to S phase (begins to rise in the G1 phase, peak in S phase, and gradually degrade in the G2 phase). The overexpression of telomerase may shorten G1 phase and advance S phase. It will also cause cell cycle shortening and rapid cell proliferation. But antisense oligonucleotide can inhibit the excessive cell proliferation caused by overexpression of telomerase and induce cell apoptosis to complete the control of keloid fibroblasts. Research shows ASODN can decrease the telomerase activity of keloid fibroblasts, and presents certain time and dose effect. The results confirm that hTERT-ASODN can exactly inhibit keloid fibroblast growth, and can be used as research foundation for clinical treatment of keloid.

The relationship of the telomerase activity changes and apoptosis has not been fully elucidated. At present, most data shows that in the process of cell apoptosis regulation, various factors, such as decreased expression level of apoptosis control gene, bcl-2, increased the expression level of P53 and increased activity of Caspase 3, decrease the telomerase activity. The change of telomerase activity is just a co-event in the process of cell apoptosis. But recent data shows that in the process of cell apoptosis, telomerase may be an important regulating mechanism. Decreased activity of telomerase can promote programmed cell death. Telomerase activity inhibitor can increase the apoptosis rate of tumor cells, and increase the clinical curative effect of other chemotherapy drugs. Therefore, the relationship of telomerase activity change and cell apoptosis needs further study. As telomerase inhibitor is effective for scar treatment, "telomerase

inhibiting therapy" is potential to be a kind of widespread method for treating scars and its related diseases. Antisense technology and conventional methods may be combined to enhance comprehensive ability for the treatment of keloid. And the inhibition of telomerase activity by its antisense nucleotide to lose telomere of keloid fibroblast and promote the apoptosis is a very attractive new gene therapy target for the treatment of keloid. With the rapid development of antisense technology and the completion of human genome project, more new critical genes may appear, which will provide greater potential for antisense RNA keloid treatment.

Conclusions

As a negative modulatory factor, hTERT-ASODN can suppress growth and proliferation of keloid fibroblasts. Decreasing the telomerase activity of keloid fibroblasts may be one of the most important mechanisms. That hTERT-ASODN inhibited telomerase activity in keloid fibroblasts is an important pathway that may play a key role in the anti-keloid therapy.

Acknowledgement

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Conflict of interest

The authors declare no conflicts of interest.

Ethical Approval

The research was conducted in accordance with the Declaration of Helsinki and the United National Institutes of Health.

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