*The Journal of Laryngology & Otology (2007), **121**, 476–482.*

doi:10.1017/S0022215106005019
Printed in the United Kingdom
First published online 8 December 2006

Main Article

Effect of RNA interference targeting human telomerase reverse transcriptase on telomerase and its related protein expression in nasopharyngeal carcinoma cells

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Abstract

Objective: Analysis of the correlation between telomerase and the expression of its related proteins may provide insight into the molecular mechanism of nasopharyngeal carcinogenesis. We investigated the effect of short hair pin ribonucleic acid (RNA) specific for human telomerase reverse transcriptase messenger RNA on the expression of the proteins c-myc (the transcription factor c-myc is a shortlived nuclear phospho-protein involved in cell proliferation and differentiation, belongs to the myc family), proliferating cell nuclear antigen and Caspase-3 in nasopharyngeal carcinoma cells.

Methods: Short hairpin RNA expression vectors targeting the messenger RNA of human telomerase reverse transcriptase were constructed. Cells were treated with the short hairpin RNA expression vectors targeting human telomerase reverse transcriptase or vectors that included mismatched short hairpin RNA, and telomerase activity was measured by telomeric repeat amplification enzyme-linked immunosorbent assay. Cell viability was examined using the 3-(4,5-dimethyl thizol-2-yl) 2,5-diphenyl tetrazolium bromide assay. The expression of the three proteins (c-myc, proliferating cell nuclear antigen and Caspase-3) was determined by Western blotting.

Results: Short hairpin RNA specific for human telomerase reverse transcriptase messenger RNA significantly inhibited telomerase activity. In addition, the expression of and proliferating cell nuclear antigen were both inhibited, while the expression of Caspase-3 was up-regulated.

Conclusions: Our results suggest that short hairpin RNA directed against human telomerase reverse transcriptase inhibits cell viability by regulating telomerase activity and its related proteins expression in nasopharyngeal carcinoma cells. Therefore, RNA interference technology may be a promising strategy for the treatment of nasopharyngeal cancer.

Key words: Telomerase; Proliferating Cell Nuclear Antigen; Caspase-3; Nasopharyngeal Neoplasms

Introduction

Telomeres are structures composed of deoxyribonucleic acid (DNA) and proteins, which are located at the chromosome extremities. Telomeres protect the structural integrity of eukaryotic chromosomes. Telomerase, a ribonucleoprotein enzyme complex, helps to stabilise telomere length by adding TTAGGG repeats (T = thymine, A = adenine and G = guanine) to the telomeres. While known to maintain telomeres in the germline, in normal somatic cells the enzyme's activity is low or undetectable.[1] Activation of telomerase has been implicated in cell immortalisation and tumorigenesis.[2]

The two main components of telomerase are the template human telomerase ribonucleic acid (RNA) and a human telomerase reverse transcriptase catalytic subunit. Studies have shown that human telomerase reverse transcriptase expression is likely to be the rate-limiting factor in regulating telomerase activity,[3] indicating that telomerase activity is predominantly regulated by the expression of human telomerase reverse transcriptase. Telomerase activity has been demonstrated in most cancer cells and has been associated with several advanced premalignant lesions, especially stratified squamous cell epithelium.[1,4] Many studies suggest that telomerase activation is common in nasopharyngeal carcinoma and that telomerase reactivation plays a critical role in nasopharyngeal carcinogenesis.[5]

Although human telomerase reverse transcriptase itself is immortalising, it does not seem to be

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Accepted for publication: 6 September 2006.

oncogenic.[6] Multiple factors are involved in cell cycle regulation. Head and neck squamous cell carcinoma has been shown to progress through a series of dysplastic histopathologic changes before becoming an invasive cancer.[7] Normal epithelium and cancer cells from the upper aerodigestive tract differ in some of the specific genes controlling proliferation and immortalisation. The protein products of these genes include growth factor receptors, cell cycle regulators and tumour suppressors. Due to their oncogenic or tumour-suppressive capabilities, the potential effect of these factors in the regulation of both telomerase activity and carcinogenesis is of great interest and importance.

Little is known about the relation between the expression of telomerase and its related proteins in nasopharyngeal carcinoma. Our previous work showed that short hairpin RNA directed against human telomerase reverse transcriptase inhibited telomerase activity and cell viability.[8,9] For this study, we transfected nasopharyngeal carcinoma cells with plasmid that could express short hairpin RNA specific to human telomerase reverse transcriptase. We then investigated the effect of short hairpin RNA targeting human telomerase reverse transcriptase on cell proliferation and expression of c-myc (the transcription factor c-myc is a shortlived nuclear phospho-protein involved in cell proliferation and differentiation, belongs to the myc family), proliferating cell nuclear antigen and Caspase-3.

Materials and methods

Cells, chemicals, enzymes and antibodies

Human nasopharyngeal carcinoma cells were purchased from the China Center for Type Culture Collection (Wuhan, China). Dulbecco's modified Eagle's medium and fetal bovine serum were both obtained from Gibco-BRL (Carlsbad, California, USA). The transfection reagent Metafectene™ was obtained from Biontex (Munich, Germany). The telomeric repeat amplification enzyme-linked immunosorbent assay kit was obtained from Roche Diagnostics (Mannheim, Germany). Antibodies against c-myc, Caspase-3 and ß-actin were obtained from Cell Signaling Tech Inc, (Beverly, Mass) and antibody against proliferating cell nuclear antigen was obtained from Santa Cruz Biotech (Santa Cruz, California, USA). Secondary antibody and the enhanced chemiluminescence kit were obtained from Pierce Biotech Inc (Rockford, Illinois, USA).

Construction of short hairpin RNA human telomerase reverse transcriptase plasmid

The plasmid encoding human telomerase reverse transcriptase messenger RNA was constructed by Wuhan Genesil Biotechnology Co Ltd (Wuhan, China). The selected coding sequence for the short hairpin RNA was 19-nucleotide oligonucleotides. The primary structure of human telomerase reverse transcriptase comptementary DNA was obtained from GenBank (Bethesda, Maryland, USA; GenBank accession number AB085628). Structure analysis was performed according to the strategy of RNA interference (RNAi), which determines the specific base sequences to design the short hairpin RNA plasmid. Short hairpin RNA1, involved in the fluorescein gene protein, was synthesised based on the specific base sequence 5′ GTT CCT GCA CTG GCT GATG 3′ (1629–1647) (G = guanine, T = thymine, C = cytosine and A = adenine). Control short hairpin RNA2, a random sequence without additional specific sequences, was also constructed; its base sequence was 5′ GCT TCA TAA GGC GCA TAGC 3′ (definitions as above). The construction of the short hairpin RNA plasmids was as described in our previous work.[8]

Cell culture and transfection

Cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10 per cent heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were cultured at 37°C and in a 5 per cent CO_2 gas phase. Twenty-four hours before transfection, cells were seeded onto six-well plates filled with antibiotic-free growth medium, at a density of 2×10^4 cells per well, so that the confluence would reach approximately 75 per cent at the time of transfection. Cells were transfected with 2 μg per well of short hairpin RNA1 plasmid targeting human telomerase reverse transcriptase, another gene, or a control short hairpin RNA plasmid, using 4 μl of transfection reagent, according to the manufacturer's protocol. At 24 and 48 hours, fluorescence expression was detected by confocal microscopy (model TCS / SP2 / MP, Leica, Germany).

Cell proliferation after treatment with different short hairpin RNAs

The 3-(4,5-dimethyl thizol-2-yl) 2,5-diphenyl tetrazolium bromide assay was performed to assess the effect of the different short hairpin RNAs on cell proliferation. Cells (5.0×10^3 cells per well) were incubated in 96-well plates and maintained in Dulbecco's modified Eagle's medium supplemented with 10 per cent fetal bovine serum. Twenty-four hours after seeding, the culture medium was replaced by fresh medium. Cells were then transfected with 45 μl of fetal bovine serum-free medium per well, containing 0.1 μg of plasmid RNA and 0.4 μl of transfection reagent (Metafectene). After 20, 44 and 68 hours, 10 μl of sterile 3-(4,5-dimethyl thizol-2-yl) 2,5-diphenyl tetrazolium bromide dye (5 mg/ml; Sigma, St Louis, Missouri, USA) was added to the cells and incubated for another 4 hours at 37°C, 200 μl of dimethyl sulphoxide was then added and thoroughly mixed for 30 minutes. Spectrometric absorbance at a wavelength of 492 nm was measured on an enzyme immunoassay analyser (model 550; Bio-Rad, Hercules, California, USA). The absorbance without the treatment was

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designated as 100 per cent. Each experiment was repeated three times.

Telomerase activity measurement

Telomerase activity was measured by the telomeric repeat amplification enzyme-linked immunosorbent assay method. Briefly, 1×10^6 cells were centrifuged for 10 minutes at 3000 *g* and the sediments suspended with lysis buffer (10 mM ethyleneglycol bix(2-aminoethyl ether)tetraacetic acid, 10 mM ethylenediamine tetraacetic acid, 62.5 mM Tris-HCl, pH 6.8, 2 per cent sodium dodecyl sulphate) on ice for 30 minutes. The supernatant was used as the telomeric repeat amplification template after centrifuging at 12 000 *g* for 20 minutes. The reaction mixture was incubated at 25°C for 30 minutes, and polymerase chain reaction amplification was then performed for 35 cycles of 94°C (30 seconds) and 59°C (60 seconds) in each cycle. First, the polymerase chain reaction product was mixed with hybridisation solution, incubated at 37°C for 1 hour, washed, incubated for another 30 minutes and chromogenised. The absorbance values of wavelengths 450 to 655 nm were then determined to calculate the overall absorbance (overall $A = A_{450} - A_{655}$). The overall absorbance value was presented as telomerase activity. Lysis buffer was used as the negative control, and each experiment was performed three times.

Western blotting

After 24 and 48 hours of administration, expression of c-myc, proliferating cell nuclear antigen and Caspase-3 was determined by Western blotting analysis. Briefly, cells were harvested at the indicated time after transfection and washed with cold phosphate-buffered saline solution. The total proteins were then extracted in the extraction buffer cells and lysed in Nonidet P-40 isotonic lysis buffer. Protein was quantitated by the Bradford standardisation assay. Equal amounts of protein (30 μg per lane) from the treated cells were loaded and electrophoresed on a 10 per cent sodium dodecyl sulphate–polyacrylamide gel and then transferred onto Hyband-enhanced chemiluminescence nitrocellulose membranes. The blotted membrane was incubated with antibodies to c-myc, proliferating cell nuclear antigen, Caspase-3 and ß-actin (1:1000), followed by treatment with secondary antibody conjugated to horseradish peroxidase (1:5000). The proteins were detected by enhanced chemiluminescence and exposed to X-ray film. Protein expressions were semi-quantitatively analysed using the Villber Lourmat scanning system (Marne-la-Vallée, France).

Statistical analysis

All values are expressed as the mean ± standard deviation. Statistical analyses were evaluated by Student's *t*-test, using the Statistical Package for the Social Sciences software (version 11.0; SPSS Inc, Chicago, Illinois, USA). Probability values of $p < 0.05$ were considered statistically significant.

Results

Recombinant plasmids successfully transfected into nasopharyngeal carcinoma cells

Green fluorescent protein was used as a plasmid marker to detect whether the short hairpin RNA expression plasmids could be successfully transfected into nasopharyngeal carcinoma cells. The efficiency of the transfection was illustrated by the green fluorescent protein fluorescence, as seen in Figure 1. Treatment with short hairpin RNA1 induced many cells to die, whereas treatment with short hairpin RNA2 did not.

Inhibition of nasopharyngeal carcinoma cell proliferation by short hairpin RNA targeting human telomerase reverse transcriptase

Cells were treated with short hairpin RNA1, short hairpin RNA2 or Metafectene alone. The cell viability of each group is shown in Table I. We found that short hairpin RNA1 exhibited potent cytotoxic activity against nasopharyngeal carcinoma cells. Following incubation with short hairpin RNA1 for 24, 48, and 72 hours, cell viability progressively decreased to 80, 42.86 and 24.66 per cent, respectively. Cell proliferation remained high in the other three groups.

Suppression of telomerase activity of nasopharyngeal carcinoma cells by short hairpin RNA targeting human telomerase reverse transcriptase

After observing the anti-proliferative effect of short hairpin RNA1, we examined the effect of short hairpin RNAs on telomerase activity. Following exposure to short hairpin RNA1 for 48 hours (Table II), the telomerase activity of the nasopharyngeal carcinoma cells was significantly decreased, declining to 8.51 per cent compared with the control group ($p < 0.05$), whereas no significant effects were observed in the other three groups.

Effect of short hairpin RNA targeting human telomerase reverse transcriptase on proteins

After treating with short hairpin RNA1 at 24 and 48 hours, the expression of proliferating cell nuclear antigen protein declined to 64.41 and 58.67 per cent ($p < 0.05$), respectively, compared with the control group. The expression of c-myc protein decreased to 68.75 and 67.68 per cent ($p < 0.05$) at 24 and 48 hours, respectively, whereas expression of Caspase-3 was up-regulated by 1.55 and 1.60 times at 24 and 48 hours, respectively ($p < 0.05$). There were no significant differences in the other three groups, as seen in Figure 2.

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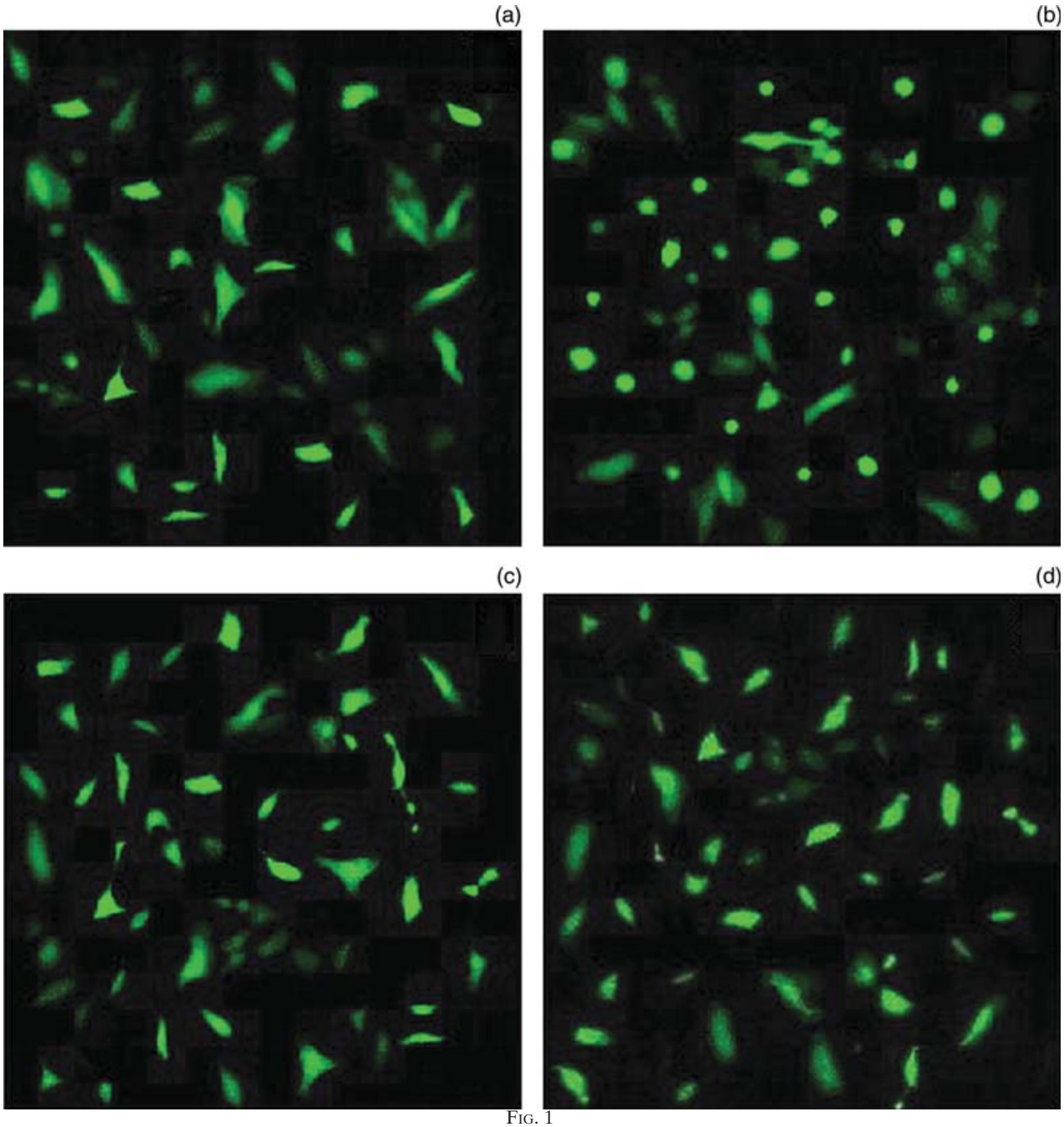

FIG. 1

Short hairpin ribonucleic acid (shRNA) expression vectors, which contained the green fluorescent protein marker gene, expressed fluorescence in nasopharyngeal carcinoma cells after 24 and 48 h of exposure. (a) Cells treated with shRNA1 after 24 h; (b) cells treated with shRNA1 after 48 h; (c) cells treated with shRNA2 after 24 h; and (d) cells treated with shRNA2 after 48 h. After treatment with shRNA1, many cells showed green fluorescence and died. The dead cells appear small and round (arrow). Cells treated with shRNA2 grew well. See text for explanation of shRNA1 and 2.

Discussion

Nasopharyngeal carcinoma is an epithelial malignancy of the head and neck region which occurs in various populations. It is endemic to certain geographic regions, such as south-east China. The overall five-year survival rate for nasopharyngeal carcinoma has not improved over the past 20 years,[10] remaining at 45–50 per cent, despite advances in surgical management and radiation oncology.[11,12]

Telomerase activity has been demonstrated in most cancer cells, including nasopharyngeal carcinoma, and has been associated with several advanced premalignant lesions, especially of stratified squamous cell epithelium.[4] Increasing telomerase activity will not necessarily make cells oncogenic; however, telomerase activation may be a necessary component and a critical step in cell immortalisation and oncogenesis. Studies have shown that inhibition of

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TABLE I

NPC CELL VIABILITY AFTER EXPOSURE TO DIFFERENT SHRNAS (BY ABSORBANCE)

shRNA	Cell viability (mean ± SD)*		
	24 h	48 h	72 h
shRNA1	0.16 ± 0.05†	0.27 ± 0.03†	0.19 ± 0.04†
shRNA2	0.20 ± 0.01	0.57 ± 0.08	0.614 ± 0.04
Metafectene	0.30 ± 0.02	0.60 ± 0.03	0.74 ± 0.06
Control	0.20 ± 0.03	0.63 ± 0.10	0.77 ± 0.12

*Mean ± standard deviation (SD) of independent experiments. †$p < 0.05$, compared with other groups. See text for explanation of shRNA1 and 2. NPC = nasopharyngeal carcinoma; shRNA = short hairpin ribonucleic acid

TABLE II

TELOMERASE ACTIVITY AFTER 48 H EXPOSURE TO DIFFERENT SHRNAS (BY ABSORBANCE)

shRNA	Telomerase activity
shRNA1	0.08 ± 0.02*
shRNA2	1.21 ± 0.20
Metafectene	1.43 ± 0.17
Control	0.94 ± 0.14

*$p < 0.05$, compared with other groups. See text for explanation of shRNA1 and 2. shRNA = short hairpin ribonucleic acid

telomerase activity could suppress cancer cell growth and induce cell apoptosis.[13] Human telomerase reverse transcriptase expression is the rate-limiting determinant of the enzymatic activity of human telomerase. Our previous study showed that inhibition of human telomerase reverse transcriptase with the RNAi technique could suppress the growth of Hep-2 cells. In the present study, nasopharyngeal carcinoma cells were transfected with

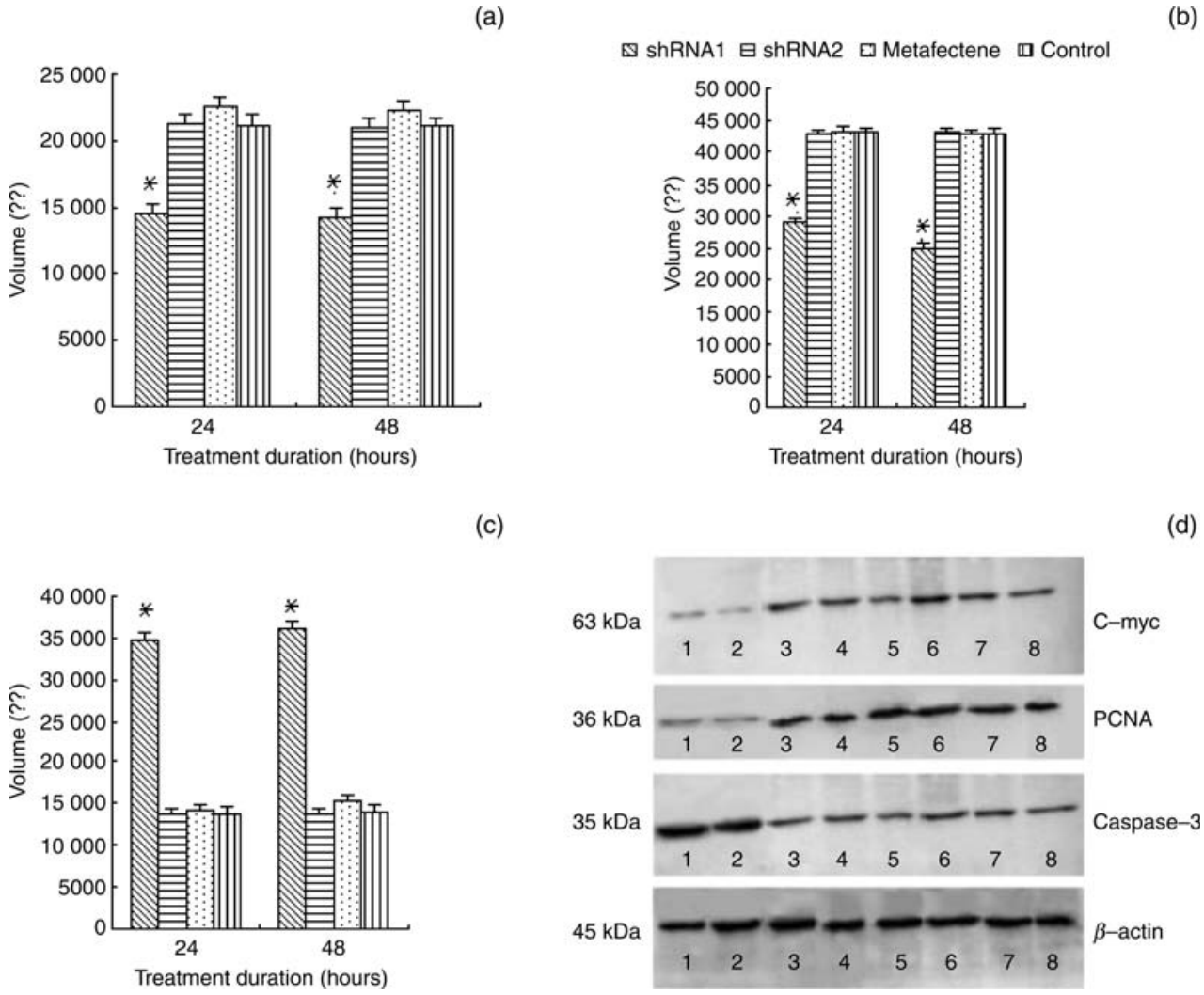

FIG. 2

Results for (a) the transcription factor c-myc is a shortlived nuclear phospho-protein involved in cell proliferation and differentiation, belongs to the myc family (c-myc), (b) proliferating cell nuclear antigen (PCNA) and (c) Caspase-3. (d) Lanes 1 and 2 = expression of protein treated with shRNA1 at 24 and 48 h; lanes 3 and 4 = expression of protein treated with shRNA2 at 24 and 48 h; lanes 5 and 6 = expression of protein treated with Metafectene at 24 and 48 h; and lanes 7 and 8 = expression of protein treated with medium only. See text for explanation of shRNA1 and 2. *$p < 0.01$, compared with control.

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plasmid that could express short hairpin RNA target at human telomerase reverse transcriptase messenger RNA. We found that such short hairpin RNA could significantly suppress the proliferation of nasopharyngeal carcinoma and telomerase activity.

Multiple factors, including oncogene and anti-oncogene dysfunction, are involved in the development of malignant tumours. Cytokine and cell cycle regulators also play key roles in the process. Research indicates that overexpression of cell cycle regulatory proteins (Epidermis Growth Factor Receptor, cyclins, c-myc and proliferating cell nuclear antigen) is correlated with advanced tumour stages in head and neck squamous cell carcinomas.[14] Data suggest that the severe chromosomal instability of telomere crisis promotes secondary genetic changes – oncogene activation and tumour suppressor loss – which facilitate carcinogenesis.[6] Poole found that multiple factors (c-myc, specific β1 glycoprotein, etc) interact with the human telomerase reverse transcriptase promoter, the p53 tumor suppress or protein is a potent transcription factor that is activated by DNA damage, its molecular weight is 53 KD leading to telomerase activation and maintenance in cervical cancer cells.[15] We can infer that molecular changes are critical for human telomerase reverse transcriptase regulation and for telomerase maintenance during the carcinogenesis of many types of cancer. Telomere dysfunction and genetic instability can accelerate carcinogenesis. Investigation of the relationship between telomerase and these factors will enhance our understanding of the complex regulation of human telomerase in cancer cells. Such investigation may also open up new strategies for anticancer therapy.

The c-myc protein is a central regulator of cell proliferation and apoptosis and is highly expressed in many tumours. The up-regulation of c-myc expression and reactivation of telomerase activity may contribute to the onset of many cancers, and c-myc expression is correlated with human telomerase reverse transcriptase messenger RNA.[16,17] Thus, the relationship between c-myc expression and human telomerase reverse transcriptase messenger RNA is receiving more attention. In this study, we successfully transfected short hairpin RNA targeting human telomerase reverse transcriptase into nasopharyngeal carcinoma cells. Telomerase activity was suppressed, and the expression of c-myc was down-regulated. Furthermore, we demonstrated that regulation between telomerase activity and c-myc may be bidirectional.

Deregulation of proliferation is a malignant characteristic of cancer cells. Proliferating cell nuclear antigen is a marker of cell proliferation and is usually expressed during cell division as a proliferative index. Proliferating cell nuclear antigen is presumed to be a biological factor involved in the abnormal proliferation of malignant tumours. It is associated with head and neck tumours and is responsible for cancer growth, local invasion of host tissue and spread to distant sites. Previous research has demonstrated that telomerase activity is correlated with the cell proliferation index, and that the proliferating cell nuclear antigen index shows a significant positive correlation with telomerase activity.[18] In our study, after telomerase activity was inhibited, the growth rate of nasopharyngeal carcinoma cells was suppressed and the expression of proliferating cell nuclear antigen was down-regulated. This suggests that the activation of telomerase is a prerequisite for infinite proliferation of tumour cells, and that telomerase and proliferating cell nuclear antigen do not affect the biological behaviour of nasopharyngeal carcinoma respectively.

Suppression of apoptosis is thought to contribute to carcinogenesis via several mechanisms, including aberrant prolongation of the cellular lifespan (which facilitates the accumulation of gene mutations and permits growth factor independent cell survival).[19] Caspase-3 has been shown in numerous models to be a key executioner of apoptosis, and is partially or totally responsible for the proteolytic cleavage of key proteins. Thus, investigating the role of Caspase-3 in the development of nasopharyngeal carcinoma will help us to understand nasopharyngeal carcinogenesis. Xiang *et al.* found that introducing human telomerase reverse transcriptase into rabbit lens epithelial cells could regulate expression of the apoptosis-related genes p53 and bcl-xS, and that Caspase-3 enhanced these genes' effectiveness against apoptosis.[20] In the present study, we silenced the human telomerase reverse transcriptase messenger RNA by RNA interference, with the result that telomerase activity was down-regulated and a great number of cells died, while the expression of Protein Caspase-3 was up-regulated. This suggests that short hairpin RNA targeting human telomerase reverse transcriptase can inhibit the telomerase activity of cancer cells and disrupt the stability of their chromosomes. Caspase may also be involved in this process. The same short hairpin RNA can activate Protein Caspase-3, stimulating a series of apoptotic signals and resulting in cell death. Thus, we infer that, in nasopharyngeal carcinogenesis, regulating the activity of telomerase and the expression of the apoptosis-related gene Protein Caspase-3 can enable human telomerase reverse transcriptase to partially protect cells from induced apoptosis.

Conclusion

In this study, we found that short hairpin RNA targeting human telomerase reverse transcriptase could suppress telomerase activity, and also the proliferation of nasopharyngeal carcinoma cells by inhibiting human telomerase reverse transcriptase expression. Our research also showed that telomerase and its related proteins play a key role in nasopharyngeal carcinogenesis; there may be cross-talk behaviour between these proteins and telomerase activity. Thus, gene therapy with short hairpin RNA targeting human telomerase reverse transcriptase may represent a new approach in the treatment of nasopharyngeal carcinoma.

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- **Analysis of the correlation between telomerase and the expression of its related proteins may provide insight into the molecular mechanisms of nasopharyngeal carcinogenesis**
- **Human nasopharyngeal carcinoma cells were treated with short hair pin RNA expression vectors targeting human telomerase reverse transcriptase or vectors that included mismatched short hairpin ribonucleic acid (RNA), and telomerase activity was measured by telomeric repeat amplification enzyme-linked immunosorbent assay**
- **Results suggest that short hairpin RNA directed against human telomerase reverse transcriptase inhibits cell viability by regulating telomerase activity and its related protein expression in nasopharyngeal carcinoma cells. Therefore, use of RNA-interfering technology may be a promising strategy for the treatment of nasopharyngeal cancer**

Acknowledgement

This work was supported by grants from the National Natural Science Foundation of China (number 30471873).

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Dr Ze-Zhang Tao takes responsibility for the integrity of the content of the paper.
Competing interests: None declared