ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Aug; 21 (8): 742 - 746
http://www.chinaphar.com

Human hepatoma cell telomerase activity inhibition and cell cycle modulation by its RNA component antisense oligodeoxyribonucleotides

ZHANG Ru-Gang, WANG Xing-Wang, YUAN Jin-Hui, XIE Hong[1]
(*Shanghai Institute of Cell Biology, Chinese Academy of Sciences, Shanghai 200031, China*)

KEY WORDS telomerase; RNA; hepatocellular carcinoma; antisense oligodeoxyribonucleotides; cell cycle

ABSTRACT

AIM: To investigate the effects of human telomerase RNA component antisense oligodeoxyribonucleotides on telomerase activity of human hepatoma cells and their effects on cell cycle distribution. **METHODS**: Modified telomeric repeat amplification protocol was used to detect telomerase activity. Cell cycle was analyzed by flow cytometer. **RESULTS**: Telomerase activity was detected in all of four human hepatoma cell lines but absent in normal liver cells. Antisense oligomers to human telomerase RNA component (hTR) inhibited telomerase activity of BEL-7404 human hepatoma cells markedly *in vitro*. After *in vitro* treatment with antisense oligomers for 96 h, cell cycle of BEL-7404 human hepatoma cells was mainly arrested at G_2/M phase. **CONCLUSION**: Antisense oligomers to hTR inhibited telomerase activity of BEL-7404 human hepatoma cells *in vitro* and resulted in cell cycle arrest at G_2/M phase.

INTRODUCTION

Telomeres form the ends of eukaryotic chromosomes consisting of an array of tandem repeats of the hexanucleotide 5'-TTAGGG-3'. They are believed to protect the ends of chromosomes against exonucleases and ligases, to prevent the activation of DNA-damage checkpoints[1]. Telomerase, a ribonucleoprotein enzyme, utilizes its own RNA as a template to add the hexanucleotide to the ends of replicating chromosomes. Because of its expression being prevalent in almost all human malignant processes but not in benign or normal tissues, telomerase has been considered as a potential target for cancer therapy. Antisense oligodeoxyribonucleotides for specific inhibition of telomerase activity have also been suggested to have therapeutic effectiveness against various tumors[2,3].

Liver cancer is one of the most common tumor in China, and no great progress of its prevention and treatment has been made up to date. Moreover, systematic studies on the relationship between telomerase and liver cancer are also relatively absent. As the first step of our research work, the present *in vitro* investigations assessed the possibilities of telomerase inhibition and further cell cycle modulation in human hepatoma cells by using antisense oligodeoxyribonucleotides to human telomerase RNA component (hTR).

MATERIALS AND METHODS

Cells and culture condition BEL-7404, SMMC-7721, QGY-7903, and HCCM human hepatoma cell lines were obtained from Cell Bank of Chinese Academy of Sciences. Human normal liver cells derived from human normal liver tissues, which were a gift from Dr CHANG Yun-Chao of our Institute, were cultured in RPMI-1640 medium (Gibco) supplemented with 10 % heat-inactivated new-born calf serum, at 37 ℃ in a humidified CO_2 incubator containing 5 % CO_2 and 95 % air.

Synthesis of oligomers Fifteen-mer antisense, sense, and missense oligomers were synthesized by β-cyanoethyl-phospharamidite chemistry using a model 381 A automated DNA synthesizer (Applied Biosystems). Deprotection and purification were carried out according to the protocol on the user's manual (Applied Biosystems). Oligomers added to cell culture medium contained stabilizing modifications: there were phosphorothiodate residues in the last two linkages at each end of the oligomers. The sequences were: antisense 5'-GGTAGGGTTAGACAA-3', sense 5'-GTAGGCGCCCT-

[1] Correspondence to XIE Hong. Phn 86-21-6471-5017.
Fax 86-21-3401-0138. E-mail laiw@990.net
Received 1999-12-14 Accepted 2000-03-10

GCTT-3′, missense 5′-ACGTCGATCTGAGCA-3′[4,5].

Telomerase activity assay The telomerase assay was performed following a previously described telomeric repeat amplification protocol (TRAP) with some modifications[6,7]. Briefly, cells were washed twice in phosphate-buffered saline, and suspended in ice-cold CHAPS-lysis buffer. After 30 min on ice, the supernatant was rapidly frozen and stored at −80 ℃. The concentration of protein was measured with the use of Bradford protein assay, an aliquot of extract containing 1 μg of protein was used for each telomerase assay.

The TRAP reaction contained 1 × TRAP buffer, 50 $\mu mol \cdot L^{-1}$ of each dNTP and 0.1 μg of a primer 5′-AATCCGTCGAGCAGAGTT-3′. After a 30-min incubation at 30 ℃, 0.1 μg of return primer 5′-GCGCGG [CTTACC]$_3$CTAACC-3′, 0.1 μg internal control primer 5′-ATCGCTTCTCGGCCTTTT-3′, 0.01 amol of the internal control template 5′-AATCCGTCGAGCAGAGTT-AAAAGGCCGAGAAGCGAT-3′ and 2 units Taq DNA polymerase (Promega) were added. The reaction mixture was then subjected to polymerase chain reaction (PCR) amplication in a thermal cycler (Perkin-Elmer 2400) with 30 cycles at 94 ℃ for 30 s, 60 ℃ for 30 s. The PCR products were resolved by electrophoresis in a 10 % polyacrylamide gel under nondenaturing conditions. The gel was then stained with SYBR Green I (FMC Bioproducts) for 15 min, visualized by UVP system and analyzed by Gelworks 1 D advanced software.

For RNase treatment, the extracts were incubated with 5 $g \cdot L^{-1}$ RNase A for 20 min at 37 ℃. Heat inactivation was performed by heating the extracts at 75 ℃ for 15 min. In every experiment, a negative control (1 μL CHAPS lysis buffer) and 0.1 amol of the quantification standard oligodeoxyribonucleotide R_8 [5′-AATCCGTCGAGCAGAGTTAG (GGTTAG)$_7$-3′] were included. The telomerase quantification was done as described previously[7]. Each of the oligomers was added to the reaction solution before the extracts. All of experiments were repeated at least twice.

Cell cycle analysis by flow cytometry BEL-7404 human hepatoma cells were seeded at 10^6 $cells \cdot L^{-1}$ in 6-well microplate. Following a 12-h incubation at 37 ℃, 5 % CO_2, antisense oligomers were added at a final concentration of 5 $\mu mol \cdot L^{-1}$, once every 24 h. Additional controls consisted of culture medium alone. The cells were harvested 96 h later and resuspended in the solution containing sodium citrate 40 $mmol \cdot L^{-1}$, sucrose 250 $mmol \cdot L^{-1}$ and 5 % dimethyl sulfoxide. The suspension was stored at −20 ℃ for 20 min, then thawed rapidly at room temperature and centrifuged to collect the cells. The cells were resuspended in a solution containing RNase A (5×10^4 $unit \cdot g^{-1}$, 50 $mg \cdot L^{-1}$) and propidium iodide (PI) 20 $mg \cdot L^{-1}$. The PI-stained cells were analyzed by flow cytometry[8].

RESULTS

Telomerase activity of human normal liver cells and different human hepatoma cell lines Several control experiments were done by using cell extracts of BEL-7404 human hepatoma cells. The specificity of telomerase activity was confirmed by the disappearance of specific TRAP products upon treatment with RNase and heat. Substitution of the extracts with lysis buffer also did not result in the synthesis of specific TPAP products. The R_8 quantification standard oligodeoxyribonucleotide exhibited a characteristic pattern of the first through seventh TRAP products (Fig 1). Further, all of four human hepatoma cell lines showed telomerase activity, whereas normal liver cells were apparently telomerase negative (Fig 2).

Inhibition of telomerase activity of BEL-7404 human hepatoma cells by hTR antisense oligodeoxyribonucleotides *in vitro* An addition of hTR antisense oligodeoxyribonucleotides inhibited telomerase activity of BEL-7404 cells *in vitro*, whereas sense and missense oligomers did not induce any noticeable change in telomerase activity in comparison with control (Fig 3). The telomerase activities of antisense, sense, missense and control were 22.5, 71.0, 70.2, and 77.1 TPG, respectively.

Effect of hTR antisense oligomers on cell cycle of BEL-7404 human hepatoma cells Cell cycle distribution of BEL-7404 cells was analyzed by using flow cytometry. After a 96-h treatment with 5 $\mu mol \cdot L^{-1}$ antisense oligomers, cell cycle was mainly arrested at G_2/M phase with a slight increase in S-phase. The antisense oligomers caused about 48 % of the cell arrest at G_2/M phase compared with about 16 % at baseline, while sense and missense oligomers did not have such effects (Fig 4).

DISCUSSION

In the present study, telomerase activity was detected in all of four human hepatoma cell lines but not in

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Aug; 21 (8)
E-mail aps@mail.shcnc.ac.cn Phn/Fax 86-21-6474-2629

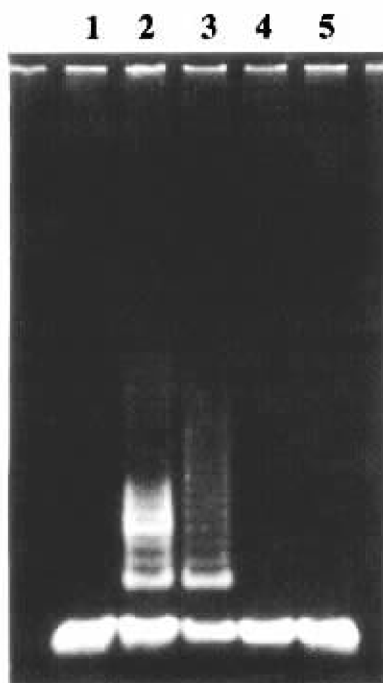

Fig 1. Control experiments confirming the specificity of telomerase activity assay in cell extracts of BEL-7404 human hepatoma cell line. Lane 1, negative lysis buffer control; Lane 2, R_8 quantification standard; Lane 3, telomerase activity in the extracts of BEL-7404 cells; Lane 4, telomerase activity in the extracts treated with RNase A; Lane 5, telomerase activity in heat inactivated extracts.

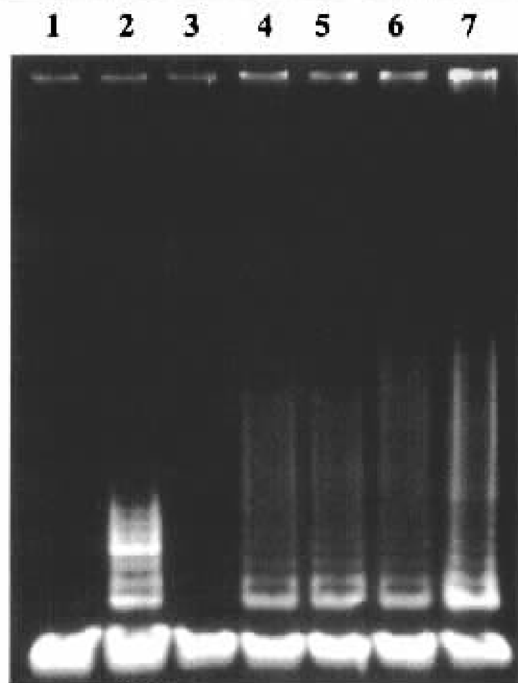

Fig 2. Telomerase activity of human normal liver cells and different human hepatoma cell lines. Lane 1, negative lysis buffer control; Lane 2, R_8 quantification standard; Lane 3, telomerase activity of human normal liver cells; Lane 4, telomerase activity of BEL-7404 human hepatoma cells; Lane 5, telomerase activity of SMMC-7721 human hepatoma cells; Lane 6, telomerase activity of QGY-7903 human hepatoma cells; Lane 7, telomerase activity of HCCM human hepatoma cells.

normal liver cells. These results indicated that telomerase activity was specific for human hepatoma cells, thus providing a potential approach for selective liver therapy by targeting telomerase[9].

The development of oligodeoxyribonucleotide-based telomerase inhibitors is especially attractive compared to other approaches for rational designing of inhibitors[2,3]. However, a main drawback in the use of antisense oligomers, possessing naturally occurring phosphodiester linkages, is their rapid degradation by nucleolytic enzymes present inside and outside cells. To overcome this limitation the present studies designed the oligomers with phosphorothioate residues, and they were more resistant to degradation by nucleases than their unmodified counterparts. The disruption of the function of telomerase RNA in *Tetrahymena* through the overexpression of an inactive form of telomerase RNA has been shown to lead to progressive shortening at telomeres[10]. Tumor cells transfected with antisense hTR also lose telomeric DNA[11]. These findings suggest that hTR function is absolutely required for telomerase activity. Hence, the antisense

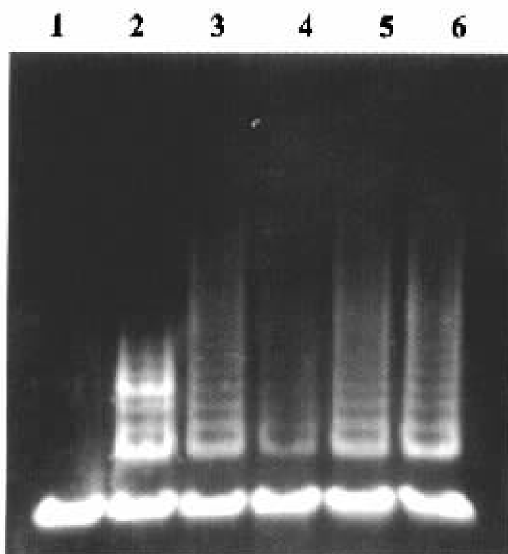

Fig 3. Effects of oligodeoxyribonucleotides on telomerase activity of BEL-7404 cell extracts. The concentration of each oligomers in reaction mixture was 1 μmol·L^{-1}. Lane 1, negative lysis buffer control; Lane 2, R_8 quantification standard; Lane 3, telomerase activity in the absence of oligomers; Lane 4, antisense treatment; Lane 5, sense treatment; Lane 6, missense treatment.

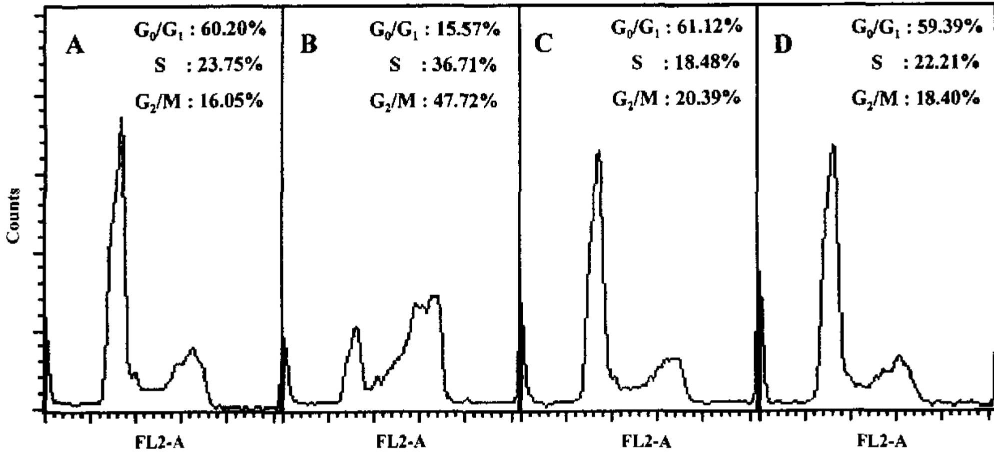

Fig 4. Cell cycle distribution of BEL-7404 human hepatoma cells after *in vitro* treatment with telomerase RNA component antisense oligodeoxyribonucleotides (5 $\mu mol \cdot L^{-1}$) for 96 h. A: Control, B: Antisense treatment, C: Sense treatment, D: Missense treatment.

oligomers to hTR were synthesized in the present studies and were found to inhibit telomerase activity of human hepatoma cells *in vitro*. The specifities of this strategy were also determined by using sense and missense oligomers as controls.

Previous several reports indicate that loss of a single telomere in *Saccharomyces cerevisiae* resulted in cell cycle arrest at G_2-phase[12]. In *cdc*13 mutants *Saccharomyces cerevisiae*, DNA lesions located in telomere regions arrested cell cycle in G_2/M phase in a checkpoint-dependent manner[13]. However, we reported for the first time that inhibition of telomerase activity of human hepatoma cells by antisense oligodeoxyribonucleotides resulted in human cell cycle arrest at G_2/M phase. Although the signaling pathways between telomerase and cell cycle machinery remain undefined, recently described homologies between telomeric protein and lipid protein kinase activities important in chromosome stability provide evidence for the existence of pathways transducing signals originating in chromosome structure to cell cycle regulatory processes[14]. Understanding exact mechanisms of cell cycle modulation by telomerase inhibition may enhance the therapeutic effects of telomerase antisense oligomers and further investigations are necessary[15]. In order to clarify the therapeutic potential of the antisense strategy, the effects of the antisense oligomers on human hepatoma cell growth and apoptosis will also be carefully studied in the near future.

REFERENCES

1 Sharma HW, Maltese JY, Zhu X, Kaiser HE, Narayanan R. Telomeres, telomerase and cancer: is the magic bullet real? Anticancer Res 1996; 16: 511 – 5.

2 Zhang RG, Yuan JH, Xie H. Telomerase and tumor. Chin J Cell Biol 2000; 22: 25 – 7.

3 Zhang RG, Yuan JH, Wang XW, Xu B, Xie H. Telomerase: a novel target of antitumor agents. Chin J Oncol Res 2000; 12: in press.

4 Glukhov AI, Zimnik OV, Gordeev SA, Severin SE. Inhibition of telomerase activity of melonoma cells *in vitro* by antisense oligonucleotides. Biochem Biophy Res Comm 1998; 248: 368 – 71.

5 Norton JC, Piatyszek MA, Wright WE, Shay JW, Corey DR. Inhibition of human telomerase activity by peptide nucleic acids. Nat Biotechnol 1996; 14: 615 – 9.

6 Kim NW, Wu F. Advances in quantification and characterizaton of telomerase activity by the telomeric repeat amplication protocol (TRAP). Nucleic Acids Res 1997; 25: 2595 – 7.

7 Zhang RG, Wang XW, Yuan JH, Guo LX, Xie H. Using an non-radioisotopic, quantitative TRAP-based method detecting telomerase activities in human hepatoma cells. Cell Res 2000; 10: 71 – 7.

8 Andreassen PR, Robert L, Margolis. Microtubule dependency of $P34^{cdc2}$ inactivation and mitotic exit in mammalian cells. J Cell Biol 1994; 127: 789 – 802.

9 Boral AL, Dessain S, Chabner BA. Clinical evaluation of biologically targeted drugs: obstacles and opportunities. Cancer Chemother Pharmacol 1998; 42 (Suppl): S3 – 21.

10 Yu GL, Bradley JD, Attardi LD, Blackburn EH. *In vivo* alteration of telomere sequences and senescence cause by mutated *Tetrahymena* telomerase RNAs. Nature 1990; 344: 126 –

32.
11 Feng J, Funk WD, Wang SS, Weinrich SL, Avilion AAA, Chiu CP, *et al*. The RNA component of human telomerase. Science 1995; 269: 1236 – 41.
12 Sandell LL, Zakian V. Loss of a yeast telomere: arrest, recovery and chromosome loss. Cell 1993; 75: 729 – 39.
13 Garvik B, Carson M, Hartwell L. Single-stranded DNA arising at telomeres in cdc13 mutants may constitute a specific signal for the RAD9 checkpoint. Mol Cell Biol 1995; 15: 6128 – 38.
14 Harley CB, Sherwood SW. Telomerase, checkpoint and cancer. Cancer Surv 1997; 29: 263 – 84.
15 Lichtsteiner SP, Lebkowski JS, Vasserot AP. Telomerase. A target for cancer therapy. Ann N Y Acad Sci 1999; 886: 1 – 11.

端粒酶 RNA 组分反义寡脱氧核糖核苷酸类对人肝癌细胞端粒酶活性的抑制和细胞周期的调节

张如刚，王兴旺，袁金辉，谢　弘[1]（中国科学院上海细胞生物学研究所，上海 200031，中国）

关键词　端粒酶；RNA；肝细胞癌；反义寡脱氧核糖核苷酸类；细胞周期

目的：研究端粒酶 RNA 组分反义寡脱氧核糖核苷酸类对人肝癌细胞端粒酶活性及其细胞周期的影响. **方法**：用改良的端粒重复扩增法检测端粒酶活性. 用流式细胞仪检测细胞周期. **结果**：端粒酶在四种人肝癌细胞株中都有表达，而在正常人肝细胞中却不表达. 端粒酶 RNA 组分的反义寡脱氧核糖核苷酸类在体外明显抑制 BEL – 7404 人肝癌细胞的端粒酶活性并使细胞周期阻滞在 G_2/M 期. **结论**：端粒酶 RNA 组分的反义寡脱氧核糖核苷酸类在体外可抑制人肝癌细胞的端粒酶活性并使细胞周期阻滞在 G_2/M 期.

（责任编辑　吕　静）