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• *BASIC RESEARCH* •

Effect of vector-expressed shRNAs on hTERT expression

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Abstract

AIM: To study the effect of short hairpin RNAs (shRNAs) expressed from DNA vector on hTERT expression.

METHODS: Oligonucleotides coding for four shRNAs against hTERT were cloned into a mammalian shRNA expression vector pUC18U6 to form pUC18U6ht1-4, which were then introduced into HepG2 cells by using liposome-mediated transfection. HepG2 cells transfected by pUC18U6 and pUC18U6GFPsir, which expressed shRNA against green fluorescent protein (GFP), were used as controls. hTERT mRNA in the transfected cells were quantified by using real-time fluorescent RT-PCR.

RESULTS: Among the four shRNAs against hTERT, two decreased the hTERT mRNA level. Compared with the controls, pUC18U6ht which expressed the two shRNAs reduced hTERT mRNA by 39% and 49% ($P<0.05$).

CONCLUSION: hTERT expression is inhibited by the shRNAs expressed from the DNA vector.

Key words: RNA interference; Short hairpin RNA; Telomerase

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INTRODUCTION

Telomeres are the ends of eukaryotic linear chromosomes. Consisting of tandem repeats of G-rich nucleotides and associated proteins, telomeres protect chromosomes from degradation, fusion, and recombination and are therefore important for maintaining genomic stability[1,2]. In most normal somatic cells, telomeres shorten with each cycle of cell division due to a so-called "end-replication problem"[3]. When telomeres reach a critical short length, cell arrest/apoptosis will ensue, which is called cell senescence. However, for immortal cells, such as germ cells and cancer cells, telomeres maintain their length stable generally through the action of a cellular reverse transcriptase-telomerase[2,4-6]. Telomerase is a ribonucleoprotein and, using RNA subunit as a template, the catalytic subunit of telomerase (telomerase reverse transcriptase, TERT) adds telomeric hexanucleotide repeats onto chromosome ends, thus compensating for telomeric loss accompanying each cell division. Eighty-five percent of all cancers are telomerase positive, while for most normal somatic cells, telomerase activity is absent or very low[7,8]. Furthermore, recent studies have demonstrated that reactivation of telomerase is a key step in tumorigenesis[9]. Therefore, telomerase is considered to be an ideal target for cancer therapy, and many strategies have been developed to inhibit telomerase, such as antisense nucleotides, ribozymes, dominant-negative proteins and small synthetic molecules[10-13].

RNA interference (RNAi) is a potent gene silencing mechanism conserved in all eukaryotes, in which double-stranded RNAs suppress the expression of cognate genes by inducing degradation of mRNAs or blocking mRNAs translation[14-16]. In mammalian cells, double-stranded RNAs inducing RNAi must be short in length (<30 bp) so that they will not activate nonspecific interferon reaction. These short interfering RNAs (siRNAs) can be produced by four different ways: chemical synthesis, *in vitro* transcription, enzymatic digestion of dsRNAs and transfection of DNA vectors encoding siRNAs or short hairpin RNAs (shRNAs), which are converted to siRNAs in cells. Among the four ways, transfection of DNA vectors has some advantages such as low cost, lasting expression of siRNA, easiness of preparation, making it the preferential method when using siRNAs in the treatment of diseases.

As a first step to explore the possibility of using RNAi in cancer therapy, we transfected HepG2 cells, a tumor cell line, with DNA vectors which can express shRNAs against human telomerase reverse transcriptase (hTERT), and then by quantifying mRNA of hTERT with real-time fluorescent RT-PCR, we selected two shRNAs which could inhibit the expression of hTERT.

MATERIALS AND METHODS

Cell culture

Human hepatoblastoma HepG2 cell line was cultured in

Dulbecco's modified Eagle's medium (Gibco Life Technologies, Grand Island, NY, USA) supplemented with 100 mL/L fetal calf serum (Sijiqing Biotech Company, Hangzhou, China).

Construction of hTERT-shRNAs expression vectors

pUC18U6 is a vector which can express siRNA in mammalian cells (Figure 1A). Eight oligodeoxyribonucleotides encoding four shRNAs against hTERT were designed according to the principles proposed in Ref. 19 and synthesized by Bioasia Company (Shanghai). The sequences of these eight oligodeoxyribonucleotides and their target sites on hTERT mRNA are shown in Table 1. To construct hTERT-shRNAs expression vectors, 400 nmol/L for each of the two corresponding oligodeoxyribonucleotides encoding a shRNA were mixed together, heated at 100 ℃ for 5 min, and cooled gradually to room temperature to anneal. pUC18U6 was digested with *Kpn*I, blunt-ended with T4 DNA polymerase, then digested with *Eco*RI, purified and ligated with the annealed oligodeoxyribonucleotides. The ligation mixtures were transformed into competent *E. coli*. The recombinant plasmids-pUC18U6ht1, pUC18U6ht2, pUC18U6ht3, and pUC18U6ht4-were then purified from transformed *E. coli*, and verified by *Hin*dIII/*Xho*I digestion analysis and automated DNA sequencing. The construction of a control vector, pUC18U-6GFPsir, which expressed a siRNA against green fluorescent protein (GFP), was reported previously.

Transfection

Twenty-four hours before transfection, HepG2 cells were seeded onto the culture plate at a density of 2×10^8/L. Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA, USA) was used for the transfection of HepG2 cells by pUC18U6ht1, pUC18U6ht2, pUC18U6ht3, pUC18U6ht4 and control vector pUC18U6 and pUC18U6GFPsir according to the manufacturer's protocol. All transfections were performed in triplicates. pEGFP-N3 (Clontech, Palo Alto, CA, USA) which can express GFP was transfected in the same way as transfection efficiency control.

Quantification of hTERT mRNA by real-time fluorescent RT-PCR

The establishment of the real-time fluorescent RT-PCR method for quantification of hTERT mRNA has been reported elsewhere[18]. The method has a good sensitivity, specificity, and reproducibility. For example, the correlation coefficient of the calibration curve was 1.00, and the mean coefficient of variation of the assay was 7.1%. After the validity of the method was confirmed, it was used in this study to quantify hTERT mRNA. Briefly, 48 h after the transfection, total RNA was isolated from transfected HepG2 cells by using Trizol® reagent (Invitrogen) according to the manufacturer's protocol. Isolated total RNA was first reverse transcribed into cDNA using random primers and SuperScript™ II reverse transcriptase (Invitrogen). Then cDNA was used as a template in real-time fluorescent PCR. The sequences of the primers were as follows: P1: 5'TCACCTCACC-CACGCGA3'; P2: 5'CAGCCATACTCAGGGACACCTC3'. The sequence of Taqman-MGB probe was 5'CTTCCTC-AGGACCCTGGT3'. P1 and P2 were designed in different exons of hTERT gene, exon 10 and exon 11, respectively, so that only mRNA of functional hTERT but not genomic DNA would be amplified in PCR. The primers and the probe were synthesized by Shanghai Genecore BioTechnologies (Shanghai). All PCR reactions were performed and analyzed using an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). For each sample, mRNA for human β-actin (hBA) was also quantified as the endogenous RNA control to which hTERT mRNA level was normalized. The primers and probe for hBA were provided by Shanghai Genecore BioTechnologies (Shanghai). Experiments were performed with triplicates for each cDNA sample.

Statistical analysis

One-way ANOVA was used for data analysis. Differences were considered significant when $P<0.05$.

RESULTS

Construction of hTERT-shRNAs expression vectors

Since there was no *Xho* I site in pUC18U6, a parental vector which was suitable to express siRNA in mammalian cells, we designed the spacer in shRNA to be *Xho* I site (CTCGAG). Therefore, the recombinant vectors pUC18U6ht1-4 which contained the shRNAs coding sequences should yield new 310-bp fragments compared with pUC18U6 after being digested with *Xho*I and *Hin*dIII (Figure 1B), and this was verified by agarose gel analysis (Figure 2). These recombinant vectors were then automatically sequenced, but only pUC18U6ht4 could be successfully sequenced, showing that shRNA encoding cassette was correctly cloned into the vector. For pUC18U6ht1-3, the sequences of the inserts could

Table 1 Sequences of oligonucleotides used in the research

shRNA	Sequences of oligonucleotides	Target site on hTERT mRNA (GenBank accession no. NM_003219)
1	F: 5′ttcctgcactggctgatgctcgagcatcagccagtgcaggaacttttttg3′	1 682-1 702nt
	R: 5′aattcaaaaaaagttcctgcactggctgatgctcgagcatcagccagtgcaggaa3′	
2	F: 5′agtgtctggagcaagttgctcgagcaacttgctccagacactcttttttg3′	1 787-1 807nt
	R: 5′aattcaaaaaagagtgtctggagcaagttgctcgagcaacttgctccagacact3′	
3	F: 5′catggactacgtcgtgggactcgagtcccacgacgtagtccatgcttttttg3′	1 958-1 978nt
	R: 5′aattcaaaaaagcatggactacgtcgtgggactcgagtcccacgacgtagtccatg3′	
4	F: 5′agccacgtctctaccttgctcgagcaaggtagagacgtggctcttttttg3′	2 333-2 353nt
	R: 5′aattcaaaaaagagccacgtctctaccttgctcgagcaaggtagagacgtggct3′	

shRNA encoding cassettes 1-4 were formed by annealing two corresponding oligonucleotides, respectively. F stands for forward and R for reverse. The underlined bases are spacer region and recognition site for *Xho*I. nt: nucleotide.

not be reliably obtained, possibly due to strong secondary structure formed by the hairpin sequences (see Discussion).

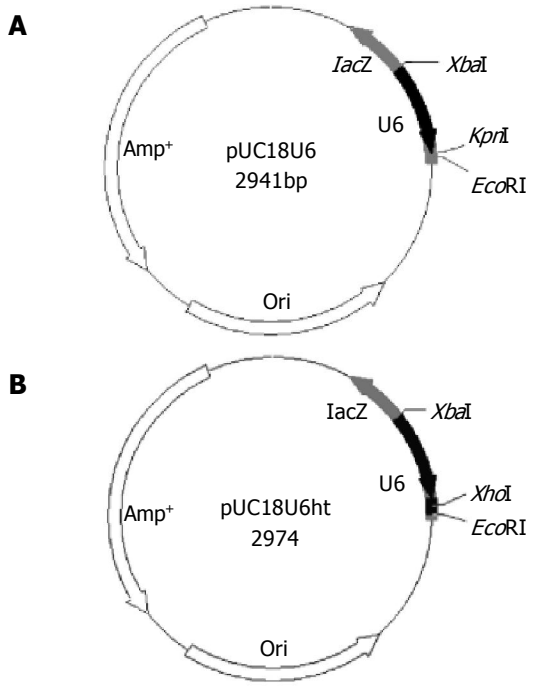

Figure 1 Maps of pUC18U6 and pUC18U6ht. **A:** pUC18U6. **B:** pUC18U6ht.

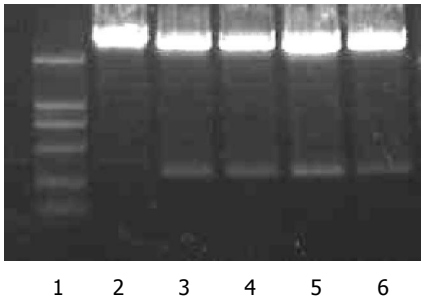

Figure 2 Restriction digestion analysis of recombinant vector pUC18U6ht. 1: DNA marker (2 000, 1 000, 750, 500, 250, 100 bp from top to bottom); 2: pUC18U6; 3-6: pUC18U6ht1-4, respectively.

Effect of shRNA expressed by pUC18U6ht on hTERT mRNA

pUC18U6ht1-4 were transfected into HepG2 cells by using liposome. As controls, parental vector pUC18U6 and pUC18U6GFPsir expressing shRNA against GFP were also introduced into HepG2 cells by liposome transfection. Forty-eight hours after the transfection, total cellular RNA was isolated from transfected cells and hTERT mRNA was quantified by real-time fluorescent RT-PCR as described in Materials and methods. As shown in Figure 3, transfection of all pUC18U6ht1-4 reduced hTERT mRNA level, but only the reduction caused by pUC18U6ht1 and pUC18U6ht2 was statistically significant as compared with the controls ($P<0.05$). Normalized hTERT mRNA levels of HepG2 cells transfected by pUC18U6ht1 and pUC18U6ht2 were reduced by 39% and 49%, respectively, as compared with that of HepG2 cells transfected by pUC18U6. This suggested that shRNA expressed by pUC18U6ht1 and pUC18U6ht2 suppressed the expression of hTERT, and this suppression was specific because transfection of pUC18U6GFPsir, which expressed siRNA against GFP, had no effect on hTERT mRNA.

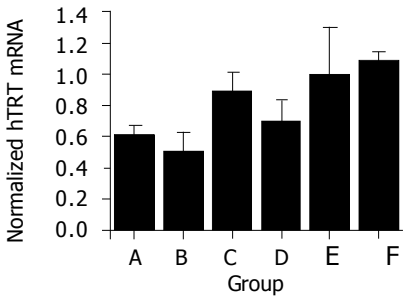

Figure 3 Normalized hTERT mRNA of transfected HepG2 cells. Groups A-F represent HepG2 cells transfected by pUC18U6ht1, pUC18U6ht2, pUC18U6ht3, pUC18U6ht4, pUC18U6, and pUC18U6GFPsir, respectively ($n = 3$ for each group). Normalized hTERT mRNA levels of HepG2 cells transfected by pUC18U6ht1 and pUC18U6ht2 were reduced by 39% and 49%, respectively, as compared with that of HepG2 cells transfected by pUC18U6.

DISCUSSION

Due to its high efficiency and specificity, RNAi is now being widely used as a method to knockdown target gene, to study gene function or to be used in experimental treatment of some diseases[19-23]. As the first step to explore RNAi in the treatment of tumor, in present study we constructed four recombinant DNA vectors which could express shRNAs against hTERT. The parental vector used in this study, pUC18U6, was constructed by us and could drive the expression of shRNA in mammalian cells under the action of U6 promoter. shRNAs transcribed in the cells could be processed by Dicer, a key enzyme in RNAi, to yield siRNAs, which would guide degradation of cognate mRNA[14-16,24,25]. A diagnostic *Xho*I/*Hin*dIII restriction enzyme analysis indicated that the four recombinant DNA vectors were constructed successfully. However, automated DNA sequencing could only verify the insert sequence in one vector, pUC18U6ht4, but failed in the other three. Devroe and Silver reported that they had encountered the same difficulty, which may be caused by the strong secondary structure formed by hairpin sequence[26]. This problem might be overcome by inclusion of a longer non-palindromic loop sequence instead of the *Xho*I sequence, or by placing one or two mismatched nucleotides in the sense strand of the hairpin[26,27].

We then tested whether the expressed shRNAs could inhibit hTERT expression by quantifying hTERT mRNA using quantitative real-time fluorescent RT-PCR. Real-time fluorescent PCR is a simple, sensitive, specific and precise method to quantitate nucleic acids over a vast dynamic range[28]. Due to these advantages, real-time fluorescent PCR has been widely used in both basic research and clinical diagnosis to measure the quantity of nucleic acids. Real-time fluorescent RT-PCR has been reported in quantifying hTERT mRNA level in human tumors, and has shown to have several advantages over conventional assay to measure telomerase activity-telomeric repeat amplification protocol, such as sensitivity,

reproducibility, precision, and quantifiability[29-31]. The results of quantitative real-time fluorescent RT-PCR showed that two out of four shRNAs used in the present study reduced hTERT mRNA level by 39% and 49%, respectively. Considering the transfection efficiency for transient transfection (about 50% in our study), the results of our research indicated that the two shRNAs expressed from DNA vector efficiently degraded hTERT mRNA. Further studies are needed to verify if these two shRNAs can also reduce hTERT protein level efficiently and lead to growth arrest and/or apoptosis of tumor cells *in vitro* and *in vivo*.

One problem in using siRNA to knockdown gene expression is target sequence selection. siRNAs target at different sites of the same gene can vary from strong to no inhibition of the gene expression. The mechanism of this selection is not well known. Therefore, it is still an empirical matter to design the most effective siRNAs, although some principles have been put forward and some softwares were invented to facilitate the selection process[17,32,33]. In present study, four target sites were chosen according to these criteria, but only half of them turned out to be effective. Interestingly, two recent reports showed that siRNAs against other two sites respectively could also suppress hTERT expression[8]. Further studies are needed to systemically evaluate the efficacy of siRNAs against different target sites of hTERT to pick out the most effective siRNAs in knocking down hTERT expression.

In summary, we demonstrated that two shRNAs expressed from DNA vectors could suppress hTERT expression. The results of our study provide basis for future research to utilize RNAi in tumor treatment.

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