

# Tumor-specific expression detected with the use of an expression vector driven by human telomerase reverse transcriptase gene promoter

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To construct the tumor-specific expression vector driven by human telomerase reverse transcriptase gene promoter, we amplified a fragment of the enhanced green fluorescent protein (EGFP) gene from the pEGFP-N1 plasmid and cloned it into the multiple cloning site of the pLNCX vector, then named the recombinant as pLNCX-EGFP. The fragment of human telomerase reverse transcriptase gene promoter (hTERT) was amplified from the human genome with the use of human telomerase reverse transcriptase gene-specific primers and cloned into the pLNCX-EGFP vector, from which the cytomegalovirus promoter had previously been removed through the use of restriction enzymes, in sense orientation relative to the green fluorescent protein coding sequence. Then the expression vector pLNT-EGFP—under the control of the human telomerase reverse transcriptase gene promoter, which contains green fluorescent protein reporter gene—was successfully constructed. To detect the transcriptional activity of the human telomerase reverse transcriptase gene promoter, we conducted transient transfection of this specific expression vector into human lung fibroblast (HLF) cell lines with high telomerase activity and normal human fetal lung fibroblast (WI38) cell lines without telomerase activity. The results of transient transfection showed that the pLNT-EGFP vector strongly expressed the green fluorescent protein reporter gene in telomerase-positive cells but not in telomerase-negative cells. (*J Lab Clin Med* 2004;144:302-6)

**Abbreviations:** bp = base pair; cDNA = complementary DNA; CMV = cytomegalovirus; EGFP = enhanced green fluorescent protein; ELISA = enzyme-linked immunosorbent assay; GFP = green fluorescent protein; HLF = human lung fibroblast; hTERT = human telomerase reverse transcriptase; hTR = RNA component of human telomerase; kb = kilobase; mRNA = messenger RNA; PCR = polymerase chain reaction; RT = reverse transcription; WI38 = normal human fetal lung fibroblast

**G**ene therapy is a promising approach to the treatment of malignant tumors, but problems are associated with its use. One of these problems is nonspecificity: The delivery system may nonspecifically infect adjacent cells, many of which are normal. This indiscriminate transfection can prevent the application of this technique in the treatment of

malignant tumors. There are several approaches to overcoming this problem. One is to manipulate the surface proteins on the virus so that the virus can specifically infect certain types of cells. Another avenue is to use a tumor-specific promoter to regulate the

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expression of the therapeutic gene delivered into tumor cells.

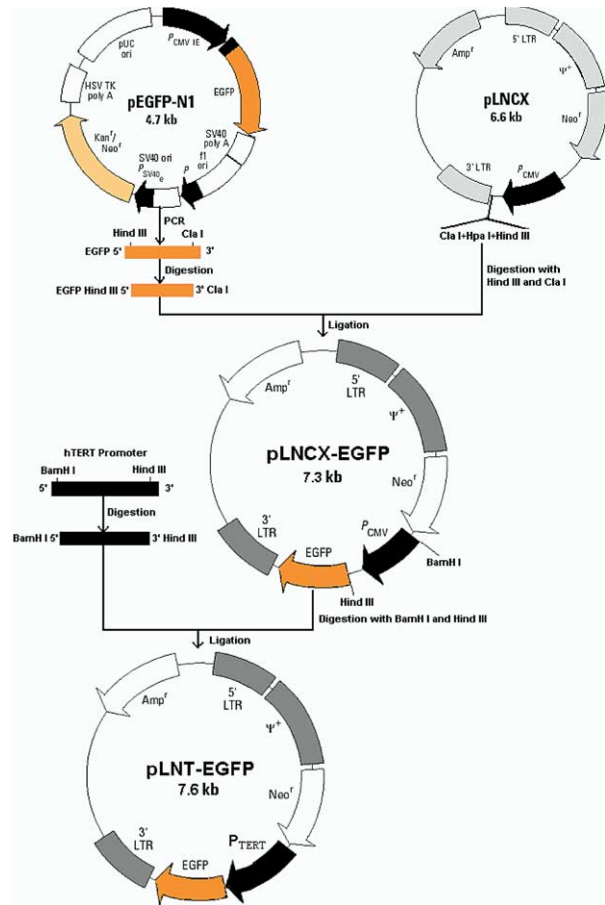
Telomerase is an RNA-dependent DNA polymerase that synthesizes telomeric DNA. Telomerase consists of several components, but the most important component responsible for the enzymatic activity of telomerase is hTERT, the catalytic subunit of telomerase, which is highly active in immortalized cells and in more than 85% of human cancers but quiescent in most normal somatic cells.<sup>1,2</sup> Because hTERT expression is regulated at the transcriptional level, the hTERT promoter can be used to regulate the targeted expression of the therapeutic genes. We therefore constructed a retroviral vector driven by the hTERT promoter specifically to express a GFP reporter gene in cells with high telomerase activity and evaluated its activity in vitro.

## METHODS

**Cells.** Human normal fibroblast HLF, WI38, and human breast-cancer MCF-7 cells were purchased from the Cell Bank of the Chinese Academy of Science (Shanghai, China). Cells were cultured in RPMI-1640 medium (Gibco-BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (Gibco-BRL), 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin under an atmosphere of 95% air 5% CO<sub>2</sub> at 37°C. HLF and MCF-7 cells were positive for telomerase, whereas WI38 cells were negative.

**Clone of the hTERT gene promoter.** The 1.1-kb fragment of hTERT gene promoter was amplified from the human genome of circulating blood cells with the use of hTERT-specific primers (5'-AAAGGATCCGCTCTGGATTCTGGGAAGTC-3' as forward and 5'-AAAAAGCTTGACGCAGCGCTGCCTGAAAC-3' as reverse) in accordance with the method set forth by Wick, Zubov, and Hagen,<sup>3</sup> then analyzed on an ABI Prism 377 automated DNA sequencer (Applied Biosystems, Foster City, Calif).

**Construction of the expression vector driven by the hTERT promoter.** We amplified the 720-bp fragment of EGFP gene from the pEGFP-N1 plasmid using EGFP-specific primers (5'-CAAAGCTTATGGTGAGCAAGGGCGAGGA-3' as forward and 5'-CAATCGATTACTTGTACAGCTCGTCCA-3' as reverse). This 720-bp fragment and retroviral vector pLNCX were digested with the use of Hind III and Cla I. After they were recovered and purified, the EGFP fragment and vector were ligated. The reaction mixture was transduced into *Escherichia coli* JM109, and the resistance colonies were screened with the use of ampicillin, after which the plasmids were extracted and sequenced. The CMV promoter region was removed from the plasmid proved by means of sequencing with Hind III and BamH I, and the vector fragment was recovered. The hTERT promoter fragment was digested with the use of Hind III and BamH I, after which the 1.1-kb promoter cDNA and vector fragment were ligated. The reaction mixture was transduced into *E coli* JM109, and the resistance colonies were screened by ampicillin. Next, plasmids were extracted and sequenced. The design of the specific vector is shown in Fig 1.



**Fig 1.** The design of tumor-specific expression vector driven by human telomerase reverse transcriptase gene promoter. An EGFP gene fragment was amplified from the pEGFP-N1 plasmid and ligated with retroviral vector pLNCX after both were digested with Hind III and Cla I. The CMV promoter region was replaced with an hTERT promoter fragment digested with Hind III and BamH I.

**Analysis of telomerase activity in cells.** We assessed telomerase activity using a Telomerase PCR ELISA kit (Roche, Mannheim, Germany) in accordance with the manufacturer's instructions. We harvested  $2 \times 10^5$  cells and counted them with the use of trypan-blue staining. The extraction of telomerase and the telomerase repeat amplification protocol (TRAP) reaction were performed in accordance with the kit's protocols. Conditions for the TRAP reaction are as follows: After 30 minutes' incubation at 25°C for TRAP extension and 5 minutes at 94°C for telomerase inactivation, the reaction mixture is subjected to PCR amplification in a thermal cycler for 30 cycles, including denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, polymerization at 72°C for 90 seconds, and extension at 72°C for 10 minutes. After TRAP, we performed hybridization and ELISA in each sample in accordance with the manufacturer's protocol. Using a microtiter-plate reader, we measured the absorbance of the samples at 450 nm (reference wavelength 690 nm). The

extract of human telomerase-positive embryonic kidney cell line 293 was used as a positive control, and heat treatment of the cell extract for 10 minutes at 65°C before the TRAP reaction was used to inactivate telomerase protein to produce negative controls.

**Transient-transfection assay.** To determine whether GFP expression driven by the hTERT promoter is limited to telomerase-positive cells, we performed transient-transfection assays with the use of LipofectAMINE-mediated gene transfer (Invitrogen, Carlsbad, Calif) as described previously.<sup>4</sup> On the day before transfection, cells were seeded at a density of  $2 \times 10^5$ /mL in 6-well plates. Targeted expression vectors under the control of the hTERT promoter were transfected into the cells. Twenty-four hours after transfection, the medium was changed and the cells were incubated for an additional 48 hours. Cells were visualized with the use of an inverted fluorescence microscope to detect GFP expression 48 and 72 hours after transfection. We harvested cells to detect the expression of GFP mRNA 72 hours after transfection.

**RT-PCR analysis.** We used 1  $\mu$ g of RNA for first-strand cDNA synthesis with the TaKaRa RNA PCR Kit version 2.1 (TaKaRa BIOTECH, Japan). Next we amplified the cDNAs with the use of PCR involving Taq polymerase (TaKaRa BIOTECH, Japan) and a GFP-specific primer set (5'-CAAAGCTTATGGTGAGCAAGGGCGAGGA-3' as forward primer and 5'-CAATCGATTTACTTGTACAGCTCGTCCA-3' as reverse primer). PCR was carried out for 30 cycles, each consisting of 2 minutes at 94°C for predenaturation, 30 seconds at 94°C for denaturation, 30 seconds at 58°C for annealing, and 1 minute at 72°C for extension. Each PCR product was electrophoretically separated in 1.5% agarose gel. The human Glyceraldehyde-3-phosphate dehydrogenase (hGAPDH) PCR products from the same RNA samples were amplified as internal controls.

**Statistical analysis.** Data are expressed as means  $\pm$  SD. Statistical analyses were conducted with the use of Student's *t* test. We considered *P* values of less than .05 statistically significant.

## RESULTS

**Cloning of the hTERT gene promoter.** Approximately 1.1 kb of the hTERT promoter sequence was cloned, then analyzed on an ABI Prism 377 automated DNA sequencer. The nucleotide-sequence data reported here match the GeneBank nucleotide sequences with the accession number AF097365 (Fig 2).

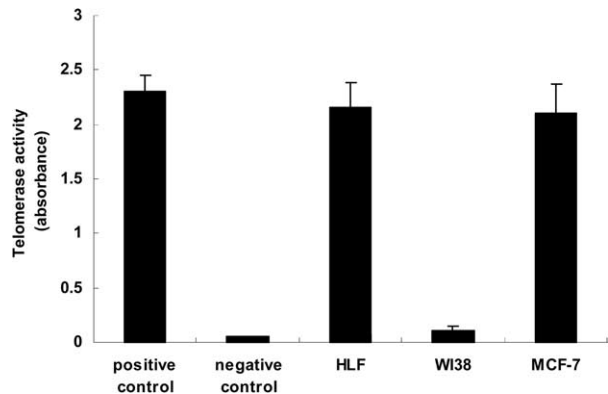
**Telomerase activity in cells.** To detect the transcriptional activity of the hTERT promoter, we selected HLF, MCF-7, and WI38 cells; HLF and MCF-7 cells express telomerase, but WI38 cells do not. Next we performed TRAP PCR ELISA assays. As shown in Fig 3, telomerase activity was demonstrated in HLF and MCF-7 cell lines, but WI38 cell lines did not express detectable activity. This finding corresponded with the results of earlier studies.<sup>5-7</sup>

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1  gggagacca gaagtttctc gcccttaga tccaaactg agcaaccgg agtctggatt
61  cctgggaagt cctcagctgt cctgcggtg tgcggggcc ccaggtctgg agggaccag
121 tggcctgtg ccttctactg ctgggctgga agtcgggccc cctagctctg cagtccgagg
181 cttggagcca ggtgcctgga ccccgagctt gccctcacc cttgctgggc gggatgtgac
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361 tcgacggag cggcccggtg ggtgattaac agatttggg tggtttgctc atggtgggga
421 cccctgcgc cctgagaacc tgcaaaaga aatgacgggc ctgtgtcaag gagccaagt
481 cgggggaag tgttcaggg aggcactcgc ggaggtccc cgtgcccgc caggagaca
541 tgcgtctgc ggttcgctc cagccgcgc taocgcctc cgtcctccc ttcactccg
601 gcaatctgg tgcgggagc cgcacgccc gcgtccgac cttgagcag cctcgggtt
661 ccgatcagg ccagcgcca aagggtgcc gcacgcact gttcccagg cctccacat
721 atggccctc cctcgggta cccacagcc taggcgatt cagcctctc ccgtggggc
781 cctcctggc gtcctgca cctgggagc cagcgggccc cggggcggg aagcgggccc
841 cagaccccc ggtccgccc gagcagctc gctgtgggg ccagggccc ctcaccatg
901 attcggggc acagacccc aggacgcgc tcccaactg cgggaggac tggggaccg
961 ggaccctgc ctgccctc accttcagc tccgcctct ccgcccggc cccggcccgt
1021 ccgaccctc cccgggtcc cggcccagc ccctccgccc cctccagcc cctcccctc
1081 ctttcgggg ccccgcctc tctcgggccc ggcagttca ggcagcctg cgtctcgtc
1141 gcacgtggg aagccctgg cccggccc cccgcatg

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**Fig. 2.** The sequence of the hTERT promoter. *Boldface letters* represent the sequence used in this experiment.



**Fig 3.** Telomerase-activity analysis of 3 cell lines with the use of TRAP PCR ELISA. HLF and MCF-7 cells (which express telomerase activity) and WI38 cells (which do not) were selected for the assay of telomerase activity. Telomerase activity was demonstrated in the HLF and MCF-7 cell lines, but WI38 cell lines did not demonstrate detectable telomerase activity.

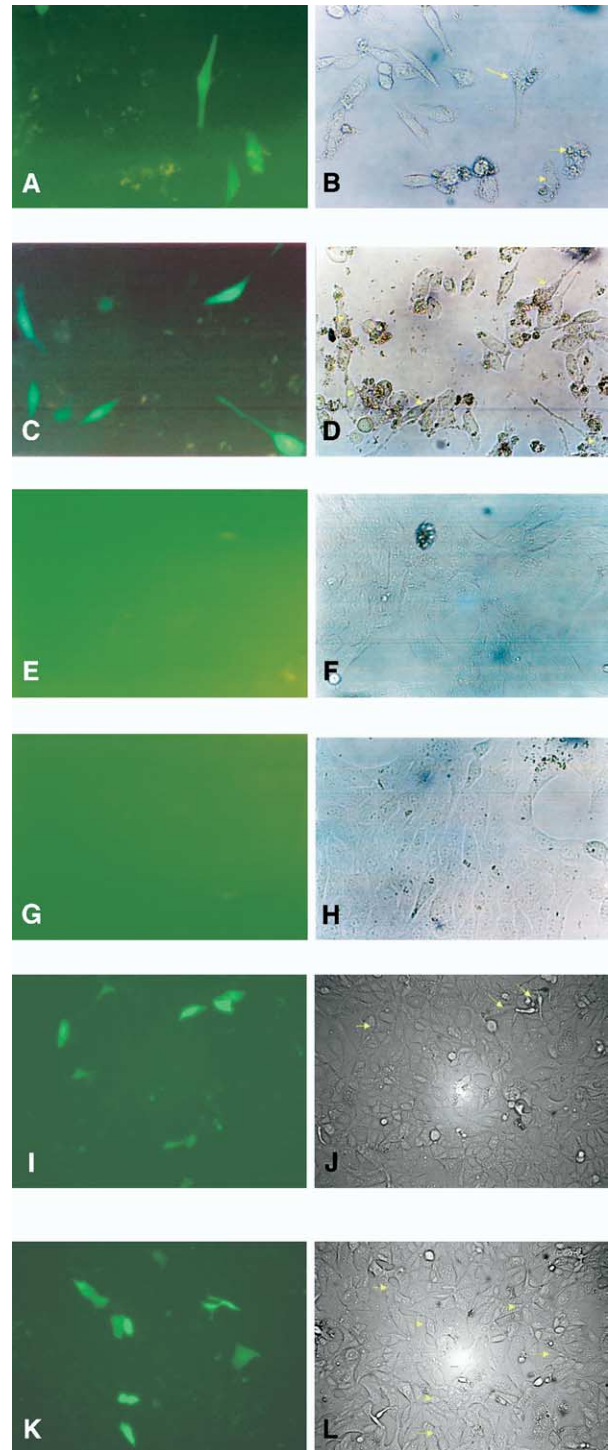
**Transcriptional activity of hTERT promoter in cells.** To confirm that the transcriptional activity of hTERT promoter is only observed in cells with telomerase activity, we conducted transient transfection of GFP reporter plasmids driven by hTERT promoter. As shown in Fig 4, the expression of GFP was observed in HLF and MCF-7 cells with telomerase activity 48 and 72 hours after transfection. In contrast, the expression of GFP was not observed in WI38 cells, which were without telomerase activity. To detect the transcriptional level of GFP mRNA, we collected cells 72 hours after transfection and analyzed them with the use of RT-PCR. The results showed transcription of GFP mRNA in HLF and MCF-7 cells but not in WI38 cells (Fig 5).

Fig 4. Transient-transfection analysis of hTERT-promoter transcriptional activities. **A**, Inverted fluorescence microscopy of GFP expression of HLF cells 48 hours after transfection (original magnification 200 $\times$ ). **B**, Light microscopy of HLF cells 48 hours after transfection (original magnification 200 $\times$ ). **C**, Inverted fluorescence microscopy of GFP expression of HLF cells 72 hours after transfection (original magnification 200 $\times$ ). **D**, Light microscopy of HLF cells 72 hours after transfection (original magnification 200 $\times$ ). **E**, Inverted fluorescence microscopy of WI38 cells 48 hours after transfection (original magnification 200 $\times$ ). **F**, Light microscopy of WI38 cells 48 hours after transfection (original magnification 200 $\times$ ). **G**, Inverted fluorescence microscopy of WI38 cells 72 hours after transfection (original magnification 200 $\times$ ). **H**, Light microscopy of WI38 cells 72 hours after transfection (original magnification 200 $\times$ ). **I**, Inverted fluorescence microscopy of GFP expression in MCF-7 cells 48 hours after transfection (original magnification 100 $\times$ ). **J**, Light microscopy of MCF-7 cells 48 hours after transfection (original magnification 100 $\times$ ). **K**, Inverted fluorescence microscopy of GFP expression by MCF-7 cells 72 hours after transfection (original magnification 100 $\times$ ). **L**, Light microscopy of MCF-7 cells 72 hours after transfection (original magnification 100 $\times$ ).

## DISCUSSION

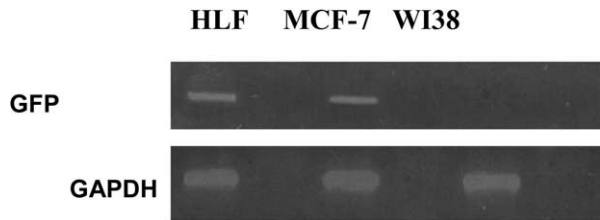
Telomerase is a ribonucleoprotein enzyme that adds telomeric repeats to the end of chromosomes.<sup>8</sup> The vast majority of malignant tumors express telomerase whereas most normal cells do not.<sup>9,10</sup> Therefore, telomerase may be a good candidate for targeted therapy. Three components of the human telomerase have been identified: hTR, the telomerase-associated protein TEP1, and hTERT. Although both hTR and hTERT are necessary for telomerase activity, their expression patterns are different. The expression of hTERT is associated with telomerase activity, whereas the expression of hTR is also detected in telomerase-negative cells.<sup>11</sup> In other words, hTERT is the major determinant of telomerase activity. The hTERT gene is highly active in tumor cells but repressed in most normal cells, and its expression is regulated at the transcription level. More recently, several groups have cloned and characterized the promoter region of the hTERT gene.<sup>12,13</sup> The transcriptional activity of the hTERT gene promoter was observed exclusively in telomerase-positive cells.<sup>14</sup> We therefore hypothesized that the hTERT promoter could be used for the expression of the tumor-specific transgene.

In the treatment of tumors through the transfer of apoptosis-inducible genes, it is essential to restrict the expression of the gene in question to tumor cells. If the apoptosis-inducible gene transduces all cells with which it comes into contact, normal cells will also be subject to apoptosis. We can solve this problem by using targeted expression vector. On the basis of the finding that the hTERT promoter is active only in telomerase-positive cells such as tumor cells, we con-



structed a tumor-specific expression vector driven by human telomerase reserve transcription gene promoter.

We observed the transcriptional activity of the hTERT promoter in 3 cell lines. The hTERT promoter construct showed significant transcriptional activity in



**Fig 5.** RT-PCR analysis of cells transfected by GFP reporter plasmids driven by hTERT promoter.

HLF and MCF-7 cells, which express telomerase activity, and the GFP gene could be transcribed and expressed, whereas transcriptional activity of the hTERT promoter was not observed in telomerase-negative WI38 cells. The telomerase-specific expression vector driven by the hTERT promoter may therefore be a novel and promising targeting approach for the treatment of tumors with telomerase activity. We can clone an apoptosis-inducible gene into this vector, and the apoptosis-inducible gene is under the control of the hTERT promoter. The specificity of apoptosis-inducible gene expression by the hTERT promoter could thus restrict the therapeutic effect to telomerase-positive tumor cells and alleviate the detrimental effect on normal cells without telomerase.

One of the major concerns with regard to the use of apoptosis-inducible gene driven by the hTERT promoter is its potential toxicity to stem cells. However, evidence suggests that hTERT is not active in the  $G_0$  stage, and stem cells are generally quiescent.<sup>9,15</sup> Of course, more experiments should be conducted to investigate the therapeutic effect of the specific expression vector on tumors and the potential negative effect on normal cells. In summary, we demonstrate here that expression of the tumor-specific expression vector driven by hTERT gene promoter is restricted to telomerase-positive cells and is not seen in normal cells without telomerase activity. We expect that this approach will increase the safety and effectiveness of gene therapy.

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