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Original Article

Effect of newly identified hTERT-interacting proteins on telomerase activity

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There is a close relationship between telomeres–telomerase and age-related disease. Human telomerase reverse transcriptase (hTERT) is both the catalytic component of human telomerase and the rate-limiting determinant of telomerase activity. Its transcriptional regulation is the primary mode of control of telomerase activity. It is critical to find the proteins interacting with hTERT for exploring the regulatory mechanisms of the hTERT expression and the telomerase activity. In this study, the yeast two-hybrid system was used to screen the potential interactive proteins of hTERT. Six proteins were obtained, among which T-STAR, LOXL3, HKR3, and Par-4 were further confirmed as the interacting proteins of hTERT by co-immunoprecipitation. Then the sense and antisense gene eukaryotic expression vectors containing these four genes were constructed and transfected into tumor cell lines. The correlations among the expression levels of these four proteins, the expression level of hTERT, and the telomerase activity were analyzed. Results showed that the up-regulation of T-STAR expression and down-regulation of HKR3 expression led to the increase of hTERT expression and telomerase activity, while the up- and down-regulation of LOXL3 and Par-4 expressions had no obvious effect. Our results suggested that T-STAR has a positive correlation with the telomerase activity while HKR3 may be a negative regulator. This conclusion is important to further explore the influencing factors or regulation pathways of human telomerase activity, which may be of great importance for the potential clinical application.

Keywords telomerase; hTERT; yeast two-hybrid screen; co-immunoprecipitation

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Introduction

Telomeres, the complex protein–DNA structures at the end of linear chromosomes, which are proportionally shortened with the cell division and proliferation in normal human cells [1], play an important role in maintaining genomic stability and integrity. Telomerase, known as a unique cellular ribonucleoprotein (RNP) enzyme in tissue progenitor cells and cancer cells, synthesizes and elongates the telomeric DNA sequences [2]. The telomerase activity decides the maintenance of telomeres' length and thereby controls the replicative capacity of cells. At present, studies in telomerase have shed light on a broad spectrum of research fields including normal aging, various forms of cancer, stem cells, and age-related disease pathology such as heart disease and diabetes [3]. These crucial roles of telomerase highlight the need for a better understanding of the mechanisms of human telomerase structure and function and how this enzyme contributes to human disease.

To this end, a series of studies have been launched, which tremendously improved our understanding of telomerase architecture and its regulation. Given the complexity of other polymerases and RNPs, telomerase is a unique reverse transcriptase that contains a catalytic protein subunit [4]. It is clear that the two basic cores of telomerase are the RNA subunit (called hTR or hTERC in human) that serves as the template for telomeric DNA synthesis [5] and the catalytic protein subunit called telomerase reverse transcriptase [called human telomerase reverse transcriptase (hTERT) in human] [6]. Other protein components of active telomerase include RNA-binding protein dyskerin, nucleolar protein 10 (hNOP10), non-histone protein 2 (hNHP2), pontin/reptin, and TCAB1 [7–10]. However, even the total molecular weight of these known proteins is much less than that of active human telomerase, which is estimated to be ranging from 650 to 2000 kDa, which suggests the existence of additional structural proteins [11].

The regulation of telomerase activity is fairly complicated. Besides the telomere-related proteins including TRF1 and TRF2, Tankyrase, TIN2, PinX1, and recently found processivity factor POT1-TPP1 complex, there are some membrane proteins such as β1-integrin and epidermal growth factor. Most importantly, only the catalytic component hTERT seems to be the rate-limiting determinant of telomerase activity among the core components of human telomerase. The

transcriptional regulation of hTERT is the primary mode of control of telomerase activity in initiation and progression of human cells and cancer [12]. It has been a major focus in the field of telomerase regulation. The regulation of telomerase activity occurs at various levels, including transcription, mRNA splicing, maturation and modifications of hTERT, assembly of the functional RNP telomerase holoenzyme, transportation to functionally relevant nuclear compartments, and access of the enzyme to its substrates. All of these regulations need the protein–protein interactions between hTERT and its interactive proteins. The precise actions of hTERT and its partners in the multistep processes of telomerase assembly and their potential functions in regulating telomerase activity and in the accessibility of telomerase to telomeres remain to be determined.

To address these issues, we have used the yeast two-hybrid system to screen and identify a group of proteins interacting with hTERT using hTERT as the bait. In the present study, we further studied the effects of up- and down-regulations of these factors on the active telomerase *in vitro* and *in vivo*.

Materials and Methods

Construction of bait plasmid

The recombinant bait plasmid pGBKT7-hTERT was constructed by inserting the full length of the coding region of hTERT that is from the plasmid pGRN145 (a kind gift from Geron, Menlo Park, USA) into the multiple cloning sites *Eco*RI and *Not*I of the DNA-BD vector, PGBKT7 (Clontech, Mountain View, USA). The primers were as follows: forward, 5′-TATGCCGTGGTCCAGAAGG-3′, reverse, 5′-CAAGAAATCATCCACCAAACG-3′. Then the recombinant bait plasmid pGBKT7-hTERT was transformed into host yeast strain AH109 to identify self-activation and toxicity according to Yeast Transformation System 2 manual book (Clontech).

Yeast two-hybrid screening

hTERT was used as the bait to screen a ‘Matchmaker’ human testis cDNA library according to Matchmaker two-hybrid system 3 protocol (Clontech), because in the normal tissues hTERT is only expressed in germinal tissues such as testis and ovary at a high level, while the telomerase activity is repressed in other tissues due to the transcriptional repression of hTERT. The yeast strain AH109 was transformed with pGBKT7-hTERT. A human testis cDNA library in the pACT2 vector (Clontech) was pre-transformed into the yeast strain Y187 by the lithium acetate method. Transformants expressing both the bait and the interacting prey proteins were selected on nutrient-deficient culture and detected by β-galactosidase activity assay. Then the positive pACT2-cDNA constructs were transformed into AH109, and the plasmid pGBKT7-hTERT was transformed into Y187 to exchange the host yeast stain. AH109 and Y187 were mated to reciprocal-hybridization, and the true positives were obtained. Finally, the sequences from the candidate positive clones were blasted in GenBank and analyzed by bioinformatics methods.

Construction of sense and antisense gene eukaryotic expressing vectors

The constructions were made by DNA recombinant technique. The sense gene vector pEGFP-C1/T-STAR was purchased from the University of Leicester (Leicester, UK) and the antisense gene vector pneo-STAR was constructed by inserting T-STAR cDNA from the plasmid pEGFP-C1/T-STAR into the *Eco*RI and *Not*I sites of the vector pcl-neo (Third Military Medical University, Chongqing, China). The sense gene vector pCMV-SPORT6-HKR3 was purchased from Biosystems (Barcelona, Spain) and the antisense gene vector pcl-neo-HKR3 was constructed by inserting HKR3 cDNA from the plasmid pCMV-SPORT6-HKR3 (Biosystems) into the *Sal*I and *Xba*I site of the vector pcl-neo. The sense gene vector pcDNA3.1 (+)-LOXL3 was constructed by inserting LOXL3 cDNA from the plasmid pOTB7-LOXL3 (Biosystems) into the *Eco*RI and *Xho*I sites of the vector pcDNA3.1 (+) (Fourth Military Medical University, Xi’an, China) and the antisense gene vector pcDNA3.1 (−)-LOXL3 was constructed by inserting LOXL3 cDNA into the *Eco*RI and *Xho*I sites of the vector pcDNA3.1 (−) (Fourth Military Medical University). The sense gene vector pcl-neo-Par-4 was constructed by inserting Par-4 cDNA from the plasmid pDNR-LIB/Par-4 (ATCC, Manassas, USA) into the *Eco*RI and *Sal*I sites of the vector pcl-neo and the antisense gene vector pcDNA3.1 (−)-Par-4 was constructed by inserting Par-4 cDNA into the *Eco*RI and *Sal*I sites of the vector pcDNA3.1(−).

Cell transfection

Human gastric cancer cell line SGC 7901 (Department of General Surgery, Southwest Hospital, Chongqing, China) was grown in Dulbecco’s modified Eagle’s medium (Hyclone, Logan, USA) with 10% heat-inactivated fetal calf serum (Hyclone). For co-immunoprecipitation, SGC7901 cells were co-transfected with pGBKT7-hTERT and the sense eukaryotic expression vectors of *T-STAR*, *LOXL3*, *HKR3*, and *Par-4* genes, respectively, using 8 μl of Lipofectamine reagent (Invitrogen, Carlsbad, USA) per 1 μg of DNA. Four groups of transfected SGC7901 cells were selected by G418 at 48 h post-transfection.

Immunoprecipitation and immunoblot analysis

The transfected SGC7901 cells were washed with ice-cold phosphate-buffered saline and lysed in RIPA lysis buffer containing 1% protease inhibitor cocktail, 1%

phenymethylsuphonyl fluoride, and 1% sodium othovanadate (Santa Cruz, Santa Cruz, USA). Lysates were allowed to continue for 30 min on ice and then spun down at 13,000 *g* for 20 min. The cell lysates were divided into two parts: one part was incubated with 2 μg rabbit polyclonal antibody against human hTERT (Santa Cruz) and the other was incubated with anti-T-STAR (Santa Cruz), anti-HKR3 (tebu-bio, Paris, France), anti-LOXL3 (Abcam, Cambridge, UK), and anti-Par-4 (Santa Cruz), respectively, overnight at 4°C. Then Protein A/G plus-agarose beads (Santa Cruz) were added for incubation at 4°C for 3 h. The immunoprecipitates were washed five times with RIPA lysis buffer and boiled with sodium dodecyl sulfate (SDS) loading buffer for 5 min. The immunoprecipitates were separated on 12% SDS–polyacrylamide gel electrophoresis (PAGE) and blotted onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, USA). After being incubated with blocking buffer containing 5% non-fat powdered milk at room temperature for 2 h, the membranes were incubated with first antibodies (1 : 1000) at 4°C overnight. Then the membranes were probed with the goat anti-rabbit immunoglobulin G horseradish peroxidase-conjugated secondary antibodies (Zhongshan Biotechnology, Beijing, China) (1 : 2000) and detected by SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, USA).

Reverse-transcriptase polymerase chain reaction analysis

SGC7901 cells were transfected with the sense and antisense eukaryotic expression vectors of *T-STAR*, *LOXL3*, *HKR3*, and *Par-4* genes as described above, respectively. The mRNA expression levels of *T-STAR*, *LOXL3*, *HKR3*, *Par-4*, and *hTERT* in SGC7901 cells before and after sense and antisense gene transduction were measured. Total RNA was extracted from cultured cells with Trizol reagent (Gibco, Gaithersburg, USA) according to the manufacturer's protocol. Reverse transcription and polymerase chain reaction (PCR) were carried out according to the instructions given in the kits (Toyobo, Tokyo, Japan). In brief, cDNA was made at 50°C for 30 min using Avian Myelobastosis Virus (AMV) reverse transcriptase and oligo (dT) as a primer. The PCR optimal reaction conditions were determined. Specific primers were designed according to the sequence registered in GenBank, and synthesized by Shanghai Bioaisa Biotech (Shanghai, China) (**Table 1**). Reaction conditions were incubated at 95°C for 5 min, and then cycled at 95°C for 45 s, 48°C for 30 s, and 72°C for 1 min for 35 cycles, and finally at 72°C for 10 min for extension. PCR products were electrophoresed on 10 g/l agarose gel to detect the mRNA expression levels of *hTERT* and hTERT-interacting protein genes. Expression levels of target genes were normalized to *β-actin* mRNA level.

Western blot analysis

The protein expression levels of T-STAR, LOXL3, HKR3, Par-4, and hTERT were measured in SGC7901 cells before and after sense and antisense gene transduction. Total proteins were isolated from cells by using RIPA lysis buffer, separated on SDS–PAGE, and transferred to PVDF membranes. After being blocked, the membranes were incubated with first antibodies including T-STAR, HKR3, LOXL3, Par-4, and hTERT (1 : 1000) overnight at 4°C with gentle agitation, then incubated with secondary antibodies (1 : 2000) for 2 h at room temperature, and detected by SuperSignal West Dura Extended Duration Substrate.

Telomeric repeat amplification protocol PCR-enzyme-linked immunosorbent assay

To determine telomerase activity levels in SGC7901 cells before and after sense and antisense gene transduction, the telomeric repeat amplification protocol (TRAP) from the Telo TAGGG telomerase PCR-enzyme-linked immunosorbent assay kit (Roche, Basel, Switzerland) was used according to the manufacturer's protocol. Briefly, TRAP reaction involved the following steps: (i) telomerase extraction from cells by using lysis reagent for 30 min; (ii) primer elongation at 25°C for 30 min and telomerase inactivation at 94°C for 5 min; (iii) amplification for 30 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 90 s, and one cycle of 72°C for 10 min; (iv) depuration; (v) hybridization: amplification products were incubated with hybridization buffer in multichannel pipet well at 37°C for 2 h, then with anti-DIG-POD working solution at 20°C for 30 min, and stained with 3,3′,5,5′-tetramethylbenzidine substrate solution at 20°C for 15 min. The absorbance was determined at 450 nm using a reference wavelength at ~690 nm.

Statistical analysis

The correlations between the expression levels of these four proteins, the expression level of hTERT, and the telomerase activity in cells were analyzed. Statistical analysis was performed using SPSS 16.0 (SPSS, Chicago, USA). All values were expressed as the mean ± standard deviation (SD). Differences between experimental groups were assessed for significance with two-tailed unpaired Student's *t*-test. $P < 0.05$ was considered to be statistically significant.

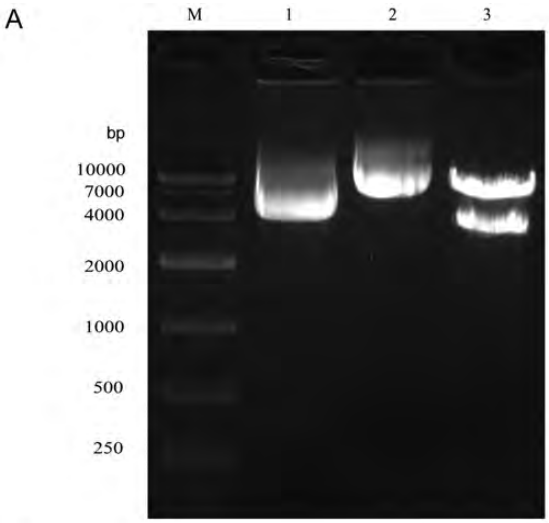
Results

Construction of bait plasmid pGBKT7-hTERT

The human telomerase catalytic subunit bait fusion protein expressing vector pGBKT7-hTERT was identified by restriction endonuclease *Eco*RI and *Not*I. Then the inserted cDNA was sequenced and verified as *hTERT* gene (GenBank ID

Table 1 Primer sequences of hTERT and hTERT-interacting protein genes

Gene name	Forward primer	Reverse primer
hTERT	5′-TATGCCGTGGTCCAGAAGG-3′	5′-CAAGAAATCATCCACCAAACG-3′
T-STAR	5′-CAGGATGGGACATGCTTTG-3′	5′-TCTGTAGACGCCCTTTGCT-3′
LOXL3	5′-GCTTACCAAGAAACCCAT-3′	5′-ACCATCCTGACCTCCTATT-3′
HKR3	5′-CCCTTCCAGTGCCACCTCT-3′	5′-CGCCACCACCACCATCTT-3′
Par-4	5′-GATCCAGGCAGTTCCTATC-3′	5′-TCCTGCTTTAGCTGTTCAT-3′
β-Actin	5′-GTGGGCCGCTCTAGGCACCAA-3′	5′-CTCTTTGATGTCACGCACGATTTC-3′



B

Name of the coding protein	Amount of the clones	Number of the plasmid
Testis-signal transduction and activation of RNA (T-STAR)	2	8
Lysyl oxidase-like gene 3 (LOXL3)	1	88
Human kruppel related 3 (HKR3)	1	16
Prostate apoptosis response-4 (Par-4)	1	25
SWI/SNF-related\matrix-associated, actin-dependent regulator of chromatin, subfamily b member-1 (SMARCB1)	1	66
Imidazoline receptor-1	1	38

Figure 1 Screening of the potential hTERT-interacting proteins by yeast two-hybrid system (A) Verification of the recombinant plasmid pGBKT7-hTERT by double restriction enzyme digestion. M: DNA Marker DL 10,000; line 1: pGBKT7; line 2: pGBKT7-hTERT; line 3: product of the recombinant plasmid pGBKT7-hTERT by double restriction enzyme digestion *Eco*RI and *Not*I. (B) Sequencing and database analysis of the potential interactive proteins of hTERT by yeast two-hybrid screening.

NM_198253) [**Fig. 1(A)**]. The AH109 transformed with pGBKT7-hTERT grew at the same rate as the empty vector pGBKT7 on nutrient-deficient culture, which indicated that the bait protein had no toxic effect and self-activation, and it was suitable for the yeast two-hybrid system.

Isolation of hTERT interaction proteins using yeast two-hybrid system

hTERT in the plasmid pGBKT7-hTERT was used as the bait to screen a human testis cDNA library. After four rounds of screening of diploid colony-forming units, 28 colonies were grown on SD/-Ade/-His/-Leu/-Trp (QDO) plates and showed positive phenotypes on QDO/X-α-gal plates. At last 12 plasmids were isolated from positive colonies by yeast mini-prep method. Through reciprocal-hybridization, seven prey plasmids were obtained. Their sequences were aligned with BLAST on NCBI website in the non-redundant database made up of GenBank, EMBL, and DDBJ and the results revealed that six independent gene products were reproducibly shown to be positive, including T-STAR, LOXL3, HKR3, Par-4, imidazoline receptor candidate1, and SMARCB1 [**Fig. 1(B)**].

Co-immunoprecipitation assay

According to the references [13–16], T-STAR, LOXL3, HKR3, and Par-4 were selected to further verify the interaction by co-immunoprecipitation assays. The lysates from each group of SGC 7901 cells co-transfected with pGBKT7-hTERT and the sense eukaryotic expression vectors of *T-STAR*, *LOXL3*, *HKR3*, and *Par-4* genes were immunoprecipitated with anti-hTERT and detected with partner's antibodies or immunoprecipitated with partner's antibodies and detected with anti-hTERT. hTERT could be coprecipitated with T-STAR, LOXL3, HKR3, and Par-4 in SGC 7901 cells and *vice versa*. The results confirmed that these four proteins screened by two yeast-hybrid system could interact with hTERT *in vivo* (**Fig. 2**).

Protein expression analysis after vector transfection in SGC7901 cells

The sense and antisense eukaryotic expression vectors of *T-STAR*, *LOXL3*, *HKR3*, and *Par-4* genes were successfully constructed, and then stably transfected into SGC7901 cells after G418 screening.

The effects of transduction of the above four genes, their empty control vector into SGC7901 cells, and SGC7901 cells untransfected were examined. Compared with both

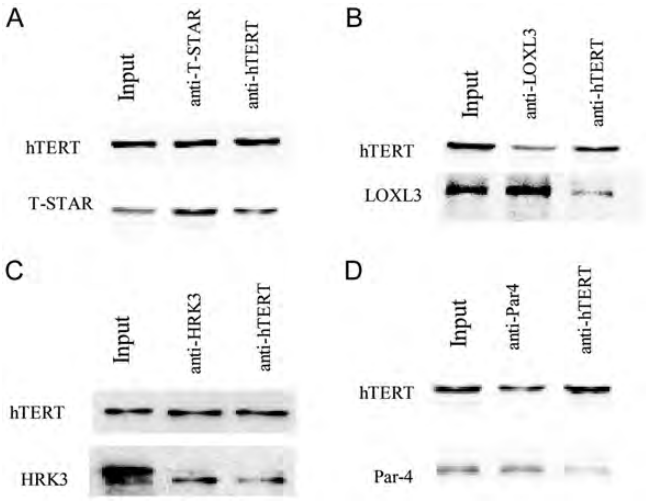


Figure 2 Binding status of hTERT and T-STAR, LOXL3, HKR3, Par-4 by co-immunoprecipitation assay hTERT was detected by anti-hTERT both in the immunoprecipitates precipitated with partner's corresponding antibodies and in those precipitated with anti-hTERT. T-STAR, LOXL3, HKR3, and Par-4 were detected by their corresponding antibodies both in the immunoprecipitates precipitated with anti-hTERT and in those precipitated with their corresponding antibodies. (A) T-STAR. (B) LOXL3. (C) HKR3. (D) Par-4. Input: total proteins from each group of SGC 7901 cells co-transfected with the sense eukaryotic expression vectors of *T-STAR*, *LOXL3*, *HKR3*, and *Par-4* genes and pGBKT7-hTERT.

untransfected cells (N) and empty control cells (C), SGC7901 cells transfected with sense vectors (s) enhanced both mRNA and protein expression levels; however, SGC7901 cells transfected with antisense vectors (as) manifested the decreased expression levels. That is to say, the target genes were specifically up-regulated in s-cells, while it was opposite in as-cells [**Figs. 3(A,B)** and **4(A,B)**].

Correlation between T-STAR and telomerase activity

After transduction with *T-STAR*, both the hTERT mRNA and protein expression levels were increased [**Fig. 3(A,B)**]. Moreover, the telomerase activity was significantly increased in SGC7901-sSTAR ($P < 0.01$) [**Fig. 3(C)**], whereas transfection of SGC-7901 cells with antisense *T-STAR* gene significantly lowered the expression of hTERT [**Fig. 3(A,B)**] and decreased the telomerase activity in SGC7901-asSTAR ($P < 0.01$) [**Fig. 3(C)**]. No significant change of cellular telomerase activity was observed between groups of SGC7901 cells, SGC7901 cells transfected with blank sense vector, and SGC7901 cells transfected with blank antisense vector. The up-regulation of *T-STAR* expression increased the telomerase activity, which means that T-STAR has a positive correlation with hTERT and telomerase activity.

Correlation between HKR3 and telomerase

After transduction with HKR3, telomerase activity was decreased by 50.6% in SGC7901-sHKR3 ($P < 0.01$) [**Fig. 4(C)**] and accordingly the expression of *hTERT* mRNA was significantly decreased [**Fig. 4(A,B)**]. But the transfection of SGC-7901 cells with antisense *HKR3* gene, telomerase activity was increased by 51.8% in SGC7901-asHKR3 ($P < 0.01$) and the expression of hTERT was increased [**Fig. 4(A,B)**]. The down-regulation of *HKR3* expression could increase the telomerase activity. It suggested that HKR3 is negatively correlated with the telomerase activity, which was opposite to T-STAR.

Correlation of LOXL3 and Par-4 with telomerase activity

The sense and antisense of LOXL3 and Par-4 genes transfection in SGC-7901 cells could increase or decrease the LOXL3 and Par-4 expression, respectively. But no significant difference was found on hTERT expression between the s-cell group and as-cell group, compared with untransfected cells and empty control cells, as well as the telomerase activity. It indicated that LOXL3 and Par-4 had no effect on the telomerase activity (**Tables 2** and **3**).

Discussion

The yeast two-hybrid system is the suitable and effective method to detect protein–protein interactions. To identify the cofactors required to assemble functional telomerase, we used the catalytic component hTERT, the rate-limiting determinant of telomerase activity, as the bait to screen a human testis cDNA library. A group of proteins including T-STAR, LOXL3, HKR3, Par-4, SMARCB1, and imidazoline receptor 1 were identified as the potential molecular partners of hTERT. The reference we initially consulted indicated that: T-STAR participates in the processes of sperm generation, proliferation regulation, and immortalization of cells; HKR3 and Par-4 have an association with the occurrence and development of tumors; and LOXL3 probably has a correlation with liver fibrosis. The information of SMARCB1 and imidazoline receptor 1 that we can obtain is limited. Therefore, we selected T-STAR, LOXL3, HKR3, and Par-4 as the research targets. Our results confirmed that these four proteins are interacted with hTERT by co-immunoprecipitation and western blot analysis.

Subsequently, we investigated the effect of these newly discovered proteins on the telomerase of tumor cell lines through transduction of the sense and antisense eukaryotic expressing vectors into tumor cell lines. T-STAR is one of the three members of the SAM68 protein family [17], which is richly expressed in testis, muscle, and brain. As both a tyrosine protein and a RNA-binding protein, T-STAR participates in the processes of sperm generation, cell proliferation regulation, and immortalization. It generally acts as a growth suppressor in many cell lines [13]. Our data further showed that the expression of T-STAR is positively

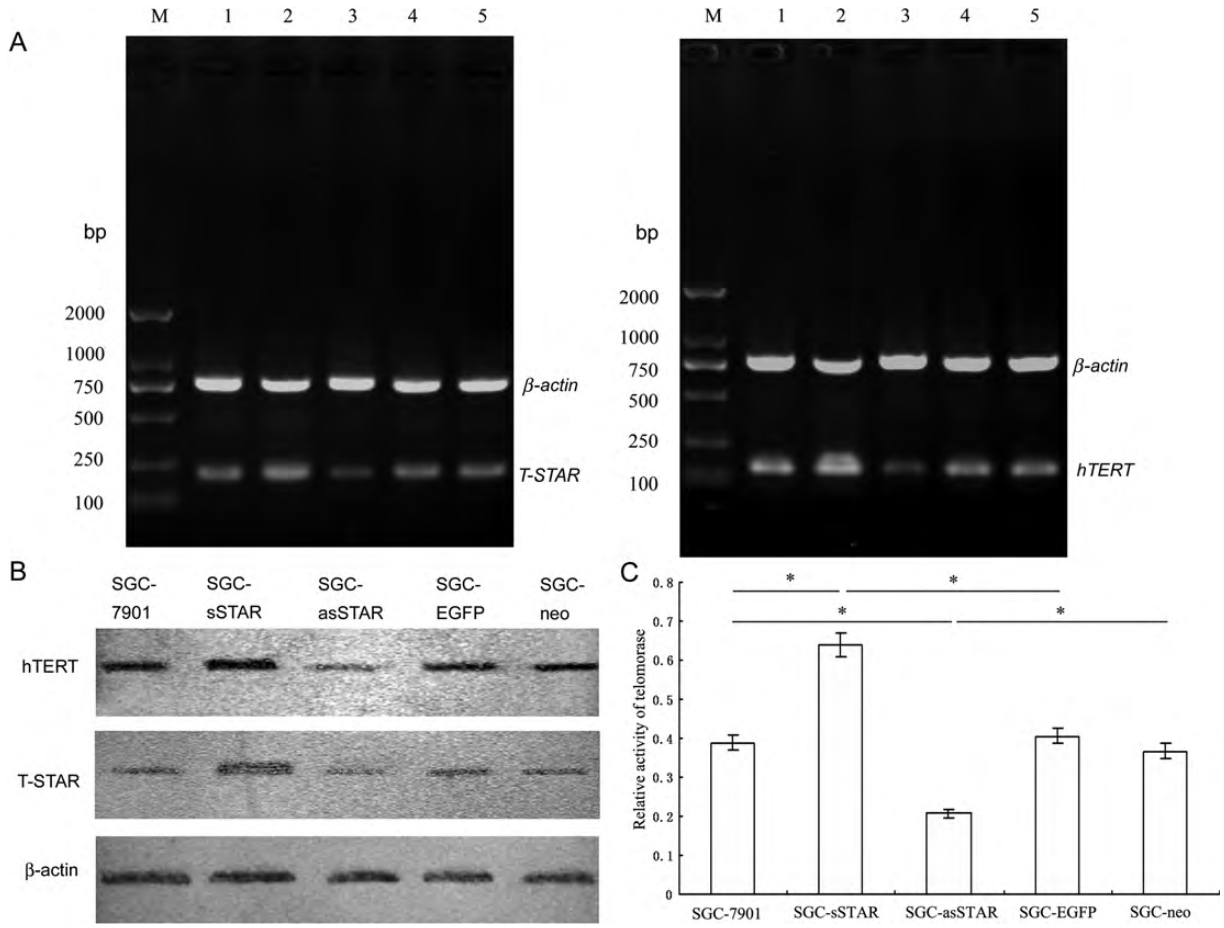


Figure 3 Effect of T-STAR on telomerase The groups were designed as SGC-7901 (untransfected), SGC-sSTAR (transfected with pEGFP-C1/T-STAR), SGC-as-STAR (transfected with pneo-STAR), SGC-EGFP (transfected with pEGFP-C1), and SGC-neo (transfected with pcl-neo). (A) The mRNA expression of *T-STAR* and *hTERT* in each group of SGC-7901 cells: total RNA was isolated from cells and RT-PCR was performed to compare *T-STAR* and *hTERT* mRNA levels in different treated groups. M, DL2000; 1, SGC-7901; 2, SGC-sSTAR; 3, SGC-as-STAR; 4, SGC-EGFP; and 5, SGC-neo. (B) The protein expression of T-STAR and hTERT in each group of SGC-7901 cells: total protein was extracted from cells and western blotting was performed to compare T-STAR and hTERT protein levels in different treated groups. (C) Quantitative analysis of telomerase activity of each group of SGC-7901 cells. $^*P < 0.01$ between the two groups. A representative analysis of three experiments is shown.

correlated with hTERT expression and telomerase activity, indicating that there is a relationship between T-STAR and oncogenesis. However, its mechanism is still unclear. One possible mechanism is that the interaction of hTERT with c-Ab1 tyrosine-kinase-binding domain SH3 may result in phosphorylation and inhibit telomerase activity [18–20]. Tyrosine phosphorylation of SAM68, the homologs of T-STAR, can interact with SH3 domain and regulate its RNA-binding proteins activity, but T-STAR can inhibit the interaction of SAM68 with its target protein. So we speculate that T-STAR might interfere with SAM68 binding with Ab1 SH3 by reverse phosphorylation that leads to impairment of Ab1 SH3's function of increasing telomerase activity. Another possible mechanism is that through the binding site of 3′-untranslated region [21], T-STAR not only participates in the processing of target pre-mRNA [22] and the regulation of specific splicing site, but also interacts with some splicing-related proteins. For example, hnRNP G-T, a new testis-specific related protein belong to the RBM gene family, is the splicing activator of pre-mRNA and related with the dependence splicing of adaptor protein Tra2β [23]. So we speculate that T-STAR may directly participate in the alternative splicing regulation of hTERT pre-mRNA, or act as the intermediate link of pre-mRNA processing through the interaction with other pre-mRNA splicing activator proteins. The exact process still needs to be further investigated.

HKR3 is a zinc finger gene that maps within chromosome sub-bands 1p36.2-3 [24], a region postulated to contain a tumor suppressor gene associated with advanced neuroblastomas. It is ubiquitously expressed in human tissues, especially higher expression in human fetal and adult nervous tissues, which may be related with the chromosome rearrangement and deficiency. The correlation between HKR3 and telomerase activity before and after the transfection of

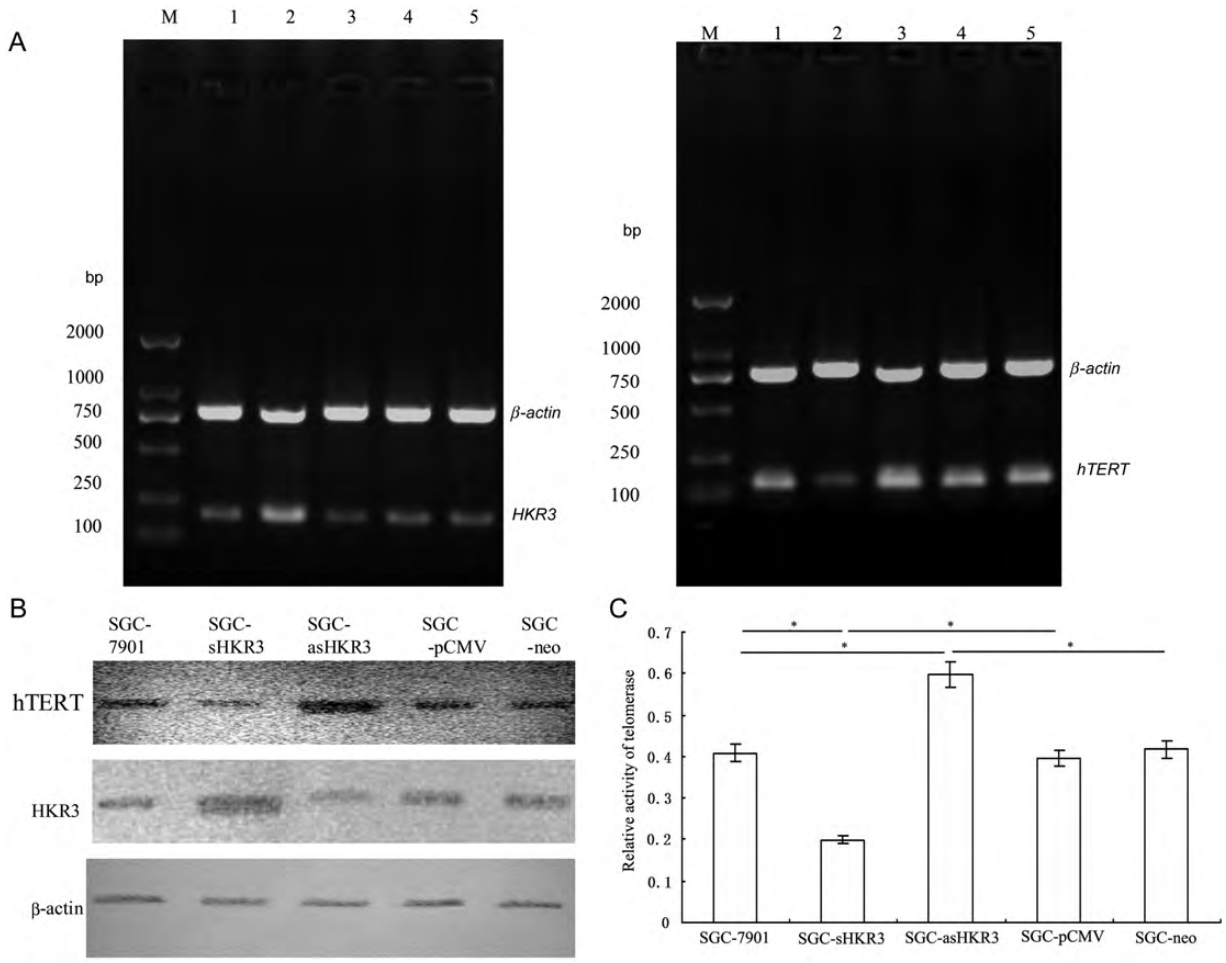


Figure 4 Effect of HKR3 on telomerase The groups were designed as SGC-7901 (untransfected), SGC-sHKR3 (transfected with pCMV-SPORT6-HKR3), SGC-as-HKR3 (transfected with pcl-neo-HKR3), SGC-pCMV (transfected with pCMV-SPORT6), and SGC-neo (transfected with pcl-neo). (A) RT-PCR results showed *HKR3* and *hTERT* mRNA expression levels of each group of SGC-7901. M, DL2000; 1, SGC-7901; 2, SGC-sHKR3; 3, SGC-as-HKR3; 4, SGC-pCMV; and 5, SGC-neo. (B) Western blot results showed HKR3 and hTERT protein expression levels of each group of SGC-7901 cells. (C) Quantitative analysis of telomerase activity of each group of SGC-7901 cells. $^{*}P < 0.01$ between the two groups. Representative analysis of three experiments is shown.

Table 2 Expression of LOXL3, hTERT, and telomerase activity in different SGC-7901 cell groups

Cell	*LOXL3* mRNA	LOXL3	*hTERT* mRNA	hTERT	Telomerase activity
SGC7901	0.411 ± 0.01	9556 ± 36	0.491 ± 0.02	6096 ± 28	2.241 ± 0.08
SGC7901-sLOXL3	0.518 ± 0.03*	12,368 ± 28	0.499 ± 0.02$^{\triangle}$	6139 ± 33$^{\triangle}$	2.405 ± 0.14$^{\triangle}$
SGC7901-asLOXL3	0.162 ± 0.03*	2492 ± 48	0.522 ± 0.01$^{\triangle}$	7378 ± 26$^{\triangle}$	2.119 ± 0.09$^{\triangle}$
SGC7901-3.1(+)	0.471 ± 0.02	9180 ± 52	0.493 ± 0.01	7005 ± 43	2.358 ± 0.11
SGC7901-3.1(−)	0.382 ± 0.02	7084 ± 32	0.503 ± 0.01	7684 ± 15	2.017 ± 0.13

$^{*}P < 0.05$ compared with SGC-7901 group.
$^{\triangle}P > 0.05$ compared with SGC-7901 group.

SGC-7901 cells through sense and antisense HKR3 showed that HKR3 may be a negative influence factor of the telomerase activity. But HKR3 is poorly understood so far, even its role in neuroblastomas is unclear yet [14]. We speculate that it may act as a transcriptional factor in the regulation of regulator of the telomerase.

There was no significant difference between the effects on telomerase activity with and without transfection of sense and antisense *LOXL3* gene in SGC7901 cells, indicating that LOXL3 has no obvious effect on the telomerase activity. The probable reasons are: one is that the *LOXL3* mRNA in three tumor cell lines is at high expression levels, but its

Table 3 Expression of Par-4, hTERT, and telomerase activity in different SGC7901 cell groups

Cell	*Par-4* mRNA	Par-4	*hTERT* mRNA	hTERT	Telomerase activity
SGC-7901	0.188 ± 0.01	2285 ± 28	0.486 ± 0.01	5996 ± 22	2.117 ± 0.02
SGC7901-spar4	0.822 ± 0.02*	5741 ± 34**	0.478 ± 0.02$^{\triangle}$	5898 ± 16$^{\triangle}$	2.088 ± 0.10$^{\triangle}$
SGC7901-aspar4	0.023 ± 0.02*	2544 ± 30**	0.492 ± 0.02$^{\triangle}$	6056 ± 24$^{\triangle}$	2.266 ± 0.08$^{\triangle}$
SGC7901-neo	0.235 ± 0.03	2062 ± 24	0.484 ± 0.01	5788 ± 28	2.024 ± 0.06
SGC7901-3.1(−)	0.304 ± 0.03	4045 ± 26	0.499 ± 0.01	6068 ± 18	2.357 ± 0.04

*$P < 0.05$ compared with SGC-7901 group.
**$P < 0.01$ compared with SGC-7901 group.
$^{\triangle}P > 0.05$ compared with SGC-7901 group.

protein expression level is low due to the lack of substrates in stomach, which belongs to lysyloxidase family [25]; and another is that LOXL3, as a secretory protein, is secreted to the extracellular [15,26], while telomerase distributes mainly in cytoplasm and nucleus. So there is a space barrier to the interaction of two proteins.

Par-4 is a pro-apoptosis protein, which plays an important role in the development and differentiation of mammalian and the occurrence and development of tumors [16,27]. hTERT is related with the cell apoptosis and proliferation, which indicates a possibility of interaction between telomerase and Par-4 [28]. In this study, we confirmed this interaction by co-immunoprecipitation. But when we increased or decreased the Par-4 expression in tumor cells, the telomerase activity was not changed. This may be explained by the following reasons: (i) the pathways and patterns of anti-apoptosis of these two proteins are different, and Par-4 may act on TERT's inducing apoptosis, which is independent of telomerase enzymatic activity; (ii) Par-4 must be transferred into nucleus to inhibit the gene transcription on condition of the activation of some factors [29], so the expression of hTERT will not be influenced; and (iii) it may be the deactivation of Par-4 that causes no influence on telomerase activity, which still needs further study on cell functions.

Although T-STAR can up-regulate the expression of hTERT, it may be only one of the numerous regulators, indicating that it cannot entirely control the telomerase activity alone. And also the regulation of HRK3 and telomerase activity needs to be further studied. An important challenge in future is the much-needed insight into the detailed structure–function understanding of hTERT-interacting proteins, which may form the basis for biochemical and molecular studies of telomerase. In a word, identification of these transcriptional repressors and tumor-specific activators of hTERT should has considerable impact on our understanding of cellular senescence and immortalization as well as on the ability to manipulate telomerase for human diseases associated with telomerase dysfunction and tissue-engineering applications.

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