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ORIGINAL ARTICLE

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shRNA-targeted hTERT suppress cell proliferation of bladder cancer by inhibiting telomerase activity

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Abstract RNA interference (RNAi) has demonstrated profound prospect in human gene research. hTERT, the rate-limiting component of telomerase activity, is highly expressed in bladder cancer cells. Here, we investigated the anti-proliferation effects of small hairpin interfering RNA (shRNA)-targeted hTERT gene on bladder cancer in vitro and in vivo. The results showed that ph2-shRNA, the most-effective vector carrying shRNA-targeted hTERT, could significantly inhibit the cell proliferation by down-regulating hTERT expression, decreasing telomerase activity, decreasing cell number of S phase, increasing the cell number of G0/G1 phase in T24 cells and xenograft tumor tissues, and attenuate the tumor growth of xenograft mice model compared with controls. Our results demonstrate that hTERT-directed shRNAs are potent inhibitors of bladder cancer.

Keywords RNAi · Bladder cancer · hTERT · Telomerase

Introduction

The abnormal expression of telomerase is closely related to the occurrence and development of malignant neoplasms. hTERT, one of telomerase's three components, is the rate-limiting factor of telomerase activity. It has been reported that the positive expression of hTERT in bladder cancer was about 92% [1], while there was non-expression in normal bladder mucosae. Meanwhile, Akiyama's research demonstrated that high expression of hTERT could suppress cell apoptosis through multi-differentiation stimuli, resulting in the immortalization of cells [2]. In contrast, blocking or down-regulating hTERT expression would lead to the shortness of telomere, induce the instability of chromosome, inhibit cell growth, accelerate cell apoptosis and alter the maglinant phenotype of cancer cells [3]. hTERT is also used as a target of tumor therapy in the late literature [4–5].

Twenty-one base double-stranded RNA molecules, termed small interfering RNA (siRNA), are potent mediators of gene silencing in mammalian cells by RNA interference (RNAi) [6]. It has been reported that in mammalian cells, 21- or 22-nt siRNAs with 2-nt 3' overhangs exhibit RNAi effect [7, 8]. Some studies showed that siRNA targeting different genes could inhibit cell growth in some tumors [9]. In previous studies we have successfully applied hTERT-targeted small hairpin interfering RNAs (shRNA) to inhibit hepatocarcinoma cell growth in vitro and in vivo [10]. Here, we explored hTERT-directed shRNA to investigate the anti-proliferation effects on bladder cancer cell and its xenograft tumor model.

Materials and methods

Cells

Bladder cancer T24 cells were cultured in RPMI1640 medium (Gibco-BRL, USA) containing 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C with 5% CO_2humidified incubator.

Construction of shRNA-hTERT-pTZU6 + 1 vectors

hTERT-targeted siRNA vectors were constructed with RNA–DNA vector technique [11]. Briefly, the short

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hairpin (sh) structure of siRNA transcript template was designed as 56 bp length of transcript template, *XbaI* and *SalI* restriction endonuclease sites at each end, 4 bp loop sequence partition of inverse repetition target sequence in the middle, 5T as transcript terminator of RNA polymerase III, then ligated to the empty vector pTZU6 + 1. Different siRNA-hTERT short fragments targeted the corresponding hTERT sequence, screened and confirmed by redigesting and sequencing (ABI 7700 DNA Sequencer System, Applied Biosystems, Foster, CA), named as shRNA-hTERT-pTZU6 + 1, abbreviated as ph1-shRNA, ph2-shRNA and ph3-shRNA. The site-mutated sequence of fragment II was mutated from **T** to ***A*** and from **A** to ***T*** nucleotide at the ten site of sense and antisense sequences respectively, abbreviated as ph2'-shRNA.

The used sequences were as follows (synthesized by Bioasia, China):

Fragment I (NM 003219.1 nucleotides 1520–1540)
Sense: 5′- AGGAACACCAAGAAGTTCATC-3′,
Antisense: 5′-GATGAACTTCTTGGTGTTCCT-3′
Fragment II (NM 003219.1 nucleotides 2770–2790)
Sense: 5′-GAACTTCCCTGTAGAAGACGA-3′,
Antisense: 5′-TCGTCTTCTACAGGGAAGTTC-3′
Fragment II (NM 003219.1 nucleotides 2770–2790)**site-mutated**:
Sense: 5′-GAACTTCCC***A***GTAGAAGACGA-3′,
Antisense: 5′-TCGTCTTCT***T***CAGGGAAGTTC-3′
Fragment III (NM 003219.1 nucleotides 3040–3060)
Sense: 5′-GACGGTGTGCACCAACATCTA-3′,
Antisense: 5′-TAGATGTTGGTGCACACCGTC-3′

Treatment of cells with different shRNA-hTERT-pTZU6 + 1 vectors

T24 cells were cultured in six-well plates except for cell counting in 24-well plates. Cationic liposome transfection method was used according to the manufacture's protocol. In brief, the fresh antibiotics-free cell culture medium was changed, inoculated 1.0×10^6 cells into each well the day before transfection. Cells were treated with shRNA-hTERT-pTZU6 + 1 in the presence of Lipofectamine^{2000} (Invitrogen) and the ratio of vector/Lipofectamine2000 was 1:3, incubated at serum-free and non-antibiotics media for 6–8 h, then changed to the full media with 10% FCS till 48 h. The site-mutated vector ph2'-shRNA was simultaneously transfected as negative control for the cell proliferation and gene expression. The optimal vector and its best concentration were determined by testing hTERT expression with real-time RT-PCR. Each experiment was performed in triplicate and the standard deviation was obtained.

Analysis of cell proliferation, cell cycle and apoptosis

To evaluate cell proliferation, cells were first adjusted to the concentration, inoculated 2.5×10^5 cells, the same cell amount, into each 24-well plate, transfected with different plasmids 12 h later, and then trypsinized at 24 h after transfection, stained with trypan-blue, counted by using a hemocytometer, and continued for 5 days in succession. For cell cycle analysis, cells were harvested, washed by PBS, fixed by cold 70% ethanol, stained by propidium iodide for 30 min at room temperature and DNA content was detected by flow cytometer (Coulter, Beckman). Meanwhile, the apoptosis was quantified by the same flow cytometry for the fraction of cells containing sub-G1 DNA content. All the experiments were performed in triplicate and the standard deviation was determined.

Real-time RT-PCR

T24 cells with different treatment were lysed with TRIZOL (Invitrogen) after 48 h transfection and reverse transcription was performed according to the standard protocol of AMV reverse transcriptase (TaKaRa, Japan) at 42°Cfor 30 min. The 50 μl reaction volume of PCR amplification was the mixture of 1 μg cDNA, 300 nM primers, 5 mM $MgCl_2$, 200 μM dNTP, 5 μl 10× buffer and Taq polymerase 2.5 U. The amplification condition of hTERT was 35 cycles each including 94°C for 60 s, 60°C for 30 s and 72°C for 30 s. The 25 μl real-time PCR reaction mixture included 5× buffer 5 μl, 300 μM dNTP, 5 mM $MgCl_2$, 200 nM of each primer, TaqMan probe 120 nM, Taq polymerase 1.25 U and cDNA 2 μl. After incubating 95°C for 3 min, 40 cycles were performed with 95°C for 15 s and 65°C for 25 s in each. Each sample was analyzed in duplicate and each treatment was performed at least three times. *PO* gene (also known as 36B4) encoding human acidic ribosomal phosphoprotein was as inner control [12]. The primer sequences and probes of hTERT, c-myc and PO were referred to literature [12] and synthesized by TaKaRa.

For analyzing real-time RT-PCR results, the starting quantity of a specific mRNA in an unknown sample which was determined by preparing a standard curve using known dilutions of the standard cDNA. The standard curve was generated on the basis of the linear relationship between the Ct value (corresponding to the cycle number at which significant increase in the fluorescence signal was first detected) and logarithm of the starting quantity. The unknown samples were quantified with the software of the ABI PRISM 7700 sequence detector system, which calculated the Ct value for each sample and then determined the initial quantity of the target gene using the standard curve. To normalize the hTERT mRNA expression for sample-to-sample differences in RNA input, RNA quality and reverse transcriptase efficiency, we amplified the housekeeping gene PO. According to each standard curve, we got the copy numbers of PO, hTERT and c-myc. The ratio between copy numbers of hTERT or c-myc and PO represented the normalized hTERT (NhTERT) or Nc-myc for each sample and could be compared with that of the other samples [13]. hTERT or c-myc values were normalized to those of PO and expressed as the ratio of hTERT or

c-myc mRNA copy numbers to PO mRNA copy numbers. NhTERT = (hTERT mRNA copies $_{sample}$/PO mRNA copies $_{sample}$) ×100, Nc-myc = (c-myc mRNA copies $_{sample}$/PO mRNA copies $_{sample}$) ×100. All the experiments were performed in triplicate and the standard deviation was obtained.

Telomerase activity assay

Telomerase activity was measured by TRAP-ELISA method (Roche). Briefly, 1×10^6 cells were centrifuged 10 min at 4,000 r/min and the sediments were suspended with lysis buffer (10 mM EGTA, 10 mM EDTA, 62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate) on ice for 30 min. The supernatant was used as TRAP template after centrifuging at 14,000 r/min for 20 min. The reaction mixture was incubated at 25°C for 30 min, then performed PCR amplification for 35 cycles of 94°C for 30 s, 59°C for 60 s in each. PCR product was first mixed with hybridization solution, incubated at 37°C for 1 h, followed by washing, incubating another 30 min, chromogenized and detected the absorbance according to manufacturer's instruction. Each experiment was performed in triplicate and the standard deviation obtained.

Studies in vivo

For establishing bladder cancer xenograft tumor model, T24 cells (2.0×10^6 cells in 0.5 ml of serum-free RPMI1640 medium) were injected s.c. into the right back of 8–10-week-old male BALB/c nude mice (ten mice for each group), and the tumor growth was monitored using electronic calipers every other day as described previously [14]. When the tumors reached a mean tumor volume of 50–70 mm^3, the treatment was initiated. ph2-shRNA and pTZU6 + 1 empty vector (10 mg/kg/time separately) were directly injected into the tumor in situ every 48 h for 20 times with Lipofectamine^{2000}. The tumor volume was determined dynamically, calculated and recorded after injection in each of the five instances. Mice were sacrificed by cervical dislocation the day after the final treatment. The tumors were removed and frozen rapidly, total RNA and protein were isolated from frozen tumors, then detected the expression alteration of hTERT and c-myc expression, telomerase activities as described earlier. The results represented the mean value of three independent experiments. All the animal experiments were approved by the institutional and governmental review boards.

Statistical analysis

Statistical analysis was performed by Student's *t*-test (two-tailed). The criterion for statistical significance was taken as $P < 0.05$.

Results

Identification of recombinant plasmids and screen ph2-shRNA as the optimal vector and its best concentration

ph1-shRNA, ph2-shRNA, ph2'-shRNA and ph3-shRNA vectors were redigested by *Hind* III and *EcoR* I, empty vector pTZU6 + 1 as control. The redigestion product was identified with 2% agarose gel electrophoresis. After redigestion the two fragment lengths of four recombinant vectors were 2.8 kb and 352 bp separately while it was 2.8 kb and 408 bp of pTZU6 + 1. All the vector sequences were confirmed by sequencing and coincided with designed fragments (data not shown).

To screen the optimal RNAi vectors, 1.0 μg/well of ph1-shRNA, ph2-shRNA, ph2'-shRNA and ph3-shRNA were first transfected into T24 cells, and 48 h later hTERT mRNA expression was detected with real-time RT-PCR. The results demonstrated that ph1-shRNA, ph2-shRNA and ph3-shRNA could inhibit hTERT expression at different degrees and ph2-shRNA was the most effective one, which could obviously decrease hTERT expression of T24 cells (Fig. 1b). To

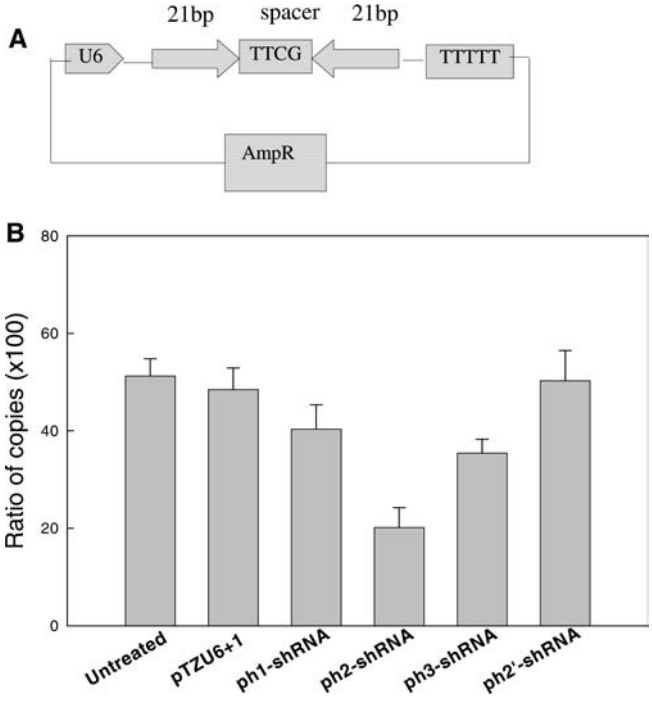

Fig. 1 shRNA vector construction scheme and screen optimal vector. **a** RNAi vector construction scheme. The short hairpin (sh) structure of siRNA transcript template was designed as 56 bp length of transcript template, *XbaI* and *SalI* restriction endonuclease sites at each end, 21 bp target sequence separated by 4 bp loop sequence partition of inverse repetition target sequence in the middle, 5T as transcript terminator of RNA polymerase III. **b** hTERT mRNA expression after different shRNA-hTERT-pTZU6 + 1 vector treatments. Real-time RT-PCR results showed that hTERT expression of T24 cells was obviously decreased after treated with ph2-shRNA but not pTZU6 + 1, ph1-shRNA, ph3-shRNA or ph2'-shRNA

further screen its best concentration, we also transfected ph2-shRNA at the gradient concentration from 0.5 μg/well to 2.5 μg/well, and then detected hTERT mRNA expression, found that the inhibition effect of hTERT expression was concentration-dependent of ph2-shRNA transfection, that is, the more the transfection vector, the less the hTERT expression (data not shown). However, when the concentration of ph2-shRNA increases to 1.5 μg/well or above, the cells were subjected to mass necrosis after transfection 24 h later. Therefore, comprehensively judging from the cell growth status, 1.0 μg ph2-shRNA was adopted for subsequent experiments.

Proliferation inhibition of T24 cells after transfection of ph2-shRNA in vitro

Growth of T24 cells was obviously decreased after transfection of ph2-shRNA compared with transfected empty vector pTZU6 + 1 and site-mutated vector ph2'-shRNA (Fig. 2a). There was statistical difference between ph2-shRNA treatment and pTZU6 + 1 treatment after 5 days ($P < 0.05$). The cell cycle analysis demonstrated that T24 cells treated with ph2-shRNA decreased cell number of S phase (from 65.2 to 38.6%), mainly increased cell number of G1/G0 phase (from 32 to 57.9%) and increased cell number of G2/M phase a little (from 2.8 to 3.2%). T24 cells treated with pTZU6 + 1 or ph2'-shRNA was similar to untreated cells displaying the predominant cell number of S phase and cell number of G2/M and G1/G0 phase. From the flow cytometry results, we could not observe the obvious alteration of sub-G1 DNA content in different treatments of either shRNA-hTERT-pTZU6 + 1s or pTZU6 + 1 (Fig. 2b1–b2 and Table 1). These results demonstrated that cells treated with ph2-shRNA mainly resulted in inhibiting cell proliferation other than inducing cell apoptosis. Meanwhile, the expressions of hTERT and c-myc were decreased in ph2-shRNA compared with pTZU6 + 1 treated cells ($P < 0.05$), while no obvious alteration was seen after treatment with ph2'-shRNA (Fig. 3a and Table 2). We also found that telomerase activities in T24 cells treated with pTZU6 + 1 were about 97.5% compared with untreated T24 cells, while T24 cells treated with ph2-shRNA decreased to about 65.2% ($P < 0.05$) and about 89.8% with ph2'-shRNA (Fig. 3b).

Proliferation inhibition of bladder cancer BALB/c xenograft tumor with ph2-shRNA in vivo

To determine the effect of shRNA-hTERT-pTZU6 + 1 on tumor growth in vivo, T24 cells were injected at the

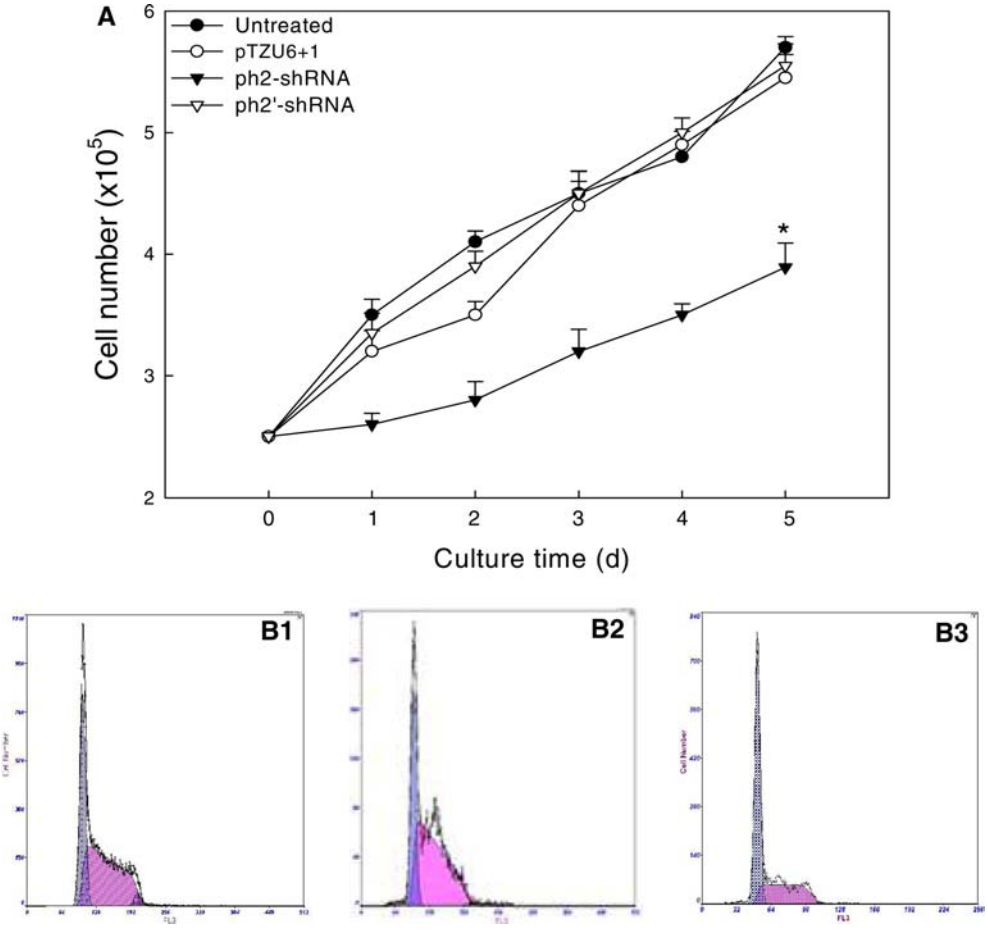

Fig. 2 Proliferation inhibition of T24 cells with different treatment. **a** cell proliferation of T24 cells with different treatment. The proliferation of T24 cells treated with ph2-shRNA was obviously suppressed. While T24 cells treated with both ph2'-shRNA and pTZU6 + 1 was still proliferating highly as untreated T24 cells. **b1**–**b3** cell cycle and apoptosis analysis of T24 cells with different treatments. **b1** T24 cells treated with pTZU6 + 1, and the cell number of S phase was predominant; **b2**, T24 cells treated with ph2'-shRNA, and the cell number of S phase was also dominant; **b3**, T24 cells treated with ph2-shRNA, cell number of S phase was decreased and the number of cells G0/G1 phase increased. All the T24 cells with different treatments had not shown obvious sub-G1 DNA content peak

Table 1 The distribution of cell cycle of T24 cells with different treatment (Mean ± SD)

Groups	Distribution of cell cycles (%)			
	G1/G0	S	G2/M	Sub-G1
Control (untreated)	32.0 ± 5.1	65.2 ± 4.2	2.8 ± 1.2	0 ± 0.3
pTZU6 + 1	31.9 ± 4.6	63.6 ± 2.9	4.3 ± 1.4	0.2 ± 0.2
ph2-shRNA	57.9 ± 3.9*	38.6 ± 3.3*	3.2 ± 0.8	0.3 ± 0.1
ph2'-shRNA	35.5 ± 3.7	58.4 ± 5.2	4.8 ± 1.0	0.3 ± 0.2

* $P < 0.05$ versus pTZU6 + 1

Table 2 Quantitative real-time RT-PCR results of hTERT and c-myc expressions in T24 cells with different treatment (Mean ± SD normalized RNA copies)

Groups	NhTERT	Nc-myc
Control (untreated)	44.3 ± 2.65	38.5 ± 2.55
PTZU6 + 1	38.6 ± 3.24	36.4 ± 2.76
ph2-shRNA	10.2 ± 3.05*	16.2 ± 2.83*
ph2'-shRNA	40.5 ± 4.23	35.4 ± 3.16

* $P < 0.05$ versus pTZU6 + 1

right back of BALB/c nude mice. After 5–14 days, the xenograft tumor started to grow and about 21–30 days the model was successfully established to initiate treatment. Either ph2-shRNA or empty vector pTZU6 + 1 was directly injected into the tumor as a different treatment every 48 h. The concentration of ph2-shRNA and pTZU6 + 1 was 10 mg/kg/times with Lipofectamine^{2000} (ratio of 1:3). The tumor volume was calculated and recorded after injection in each of the five instances. After it was injected 15 times, tumor growth significantly reduced in ph2-shRNA-treated bladder tumors while no obvious effects on pTZU6 + 1 control (Fig. 4a). After 20 times of treatment, the mice were sacrificed and the tumor tissues were isolated for detecting the alterations of hTERT and c-myc expression. The results demonstrated that the mean tumor volume of ph2-shRNA treatment decreased compared with pTZU6 + 1 treatment ($P < 0.05$). Meanwhile, the expressions of hTERT and c-myc in tumor tissues treated with ph2-shRNA declined compared with that treated with pTZU6 + 1 ($P < 0.05$) (Table 3). We also found that telomerase activities in tumor tissues treated with ph2-shRNA were decreased compared with that treated with pTZU6 + 1 ($P < 0.05$) (Fig. 4b).

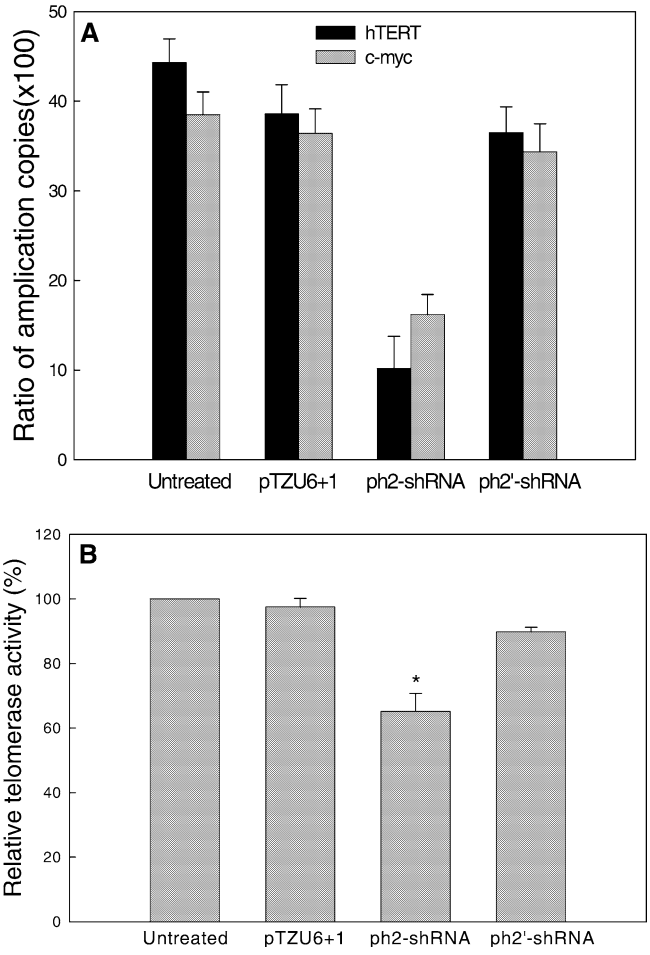

Fig. 3 The expression of hTERT and c-myc, telomerase activity of T24 cells with different treatment. **a** the bar graph of hTERT and c-myc expressions in T24 cells with different treatment. hTERT and c-myc expressions in T24 cells were decreased in ph2-shRNA-treated compared with pTZU6 + 1- or ph2'-shRNA-treated. There is a statistical significance between T24 cells treated with pTZU6 + 1 and ph2-shRNA ($P < 0.05$). **b** telomerase activity of T24 cells with different treatment. The results of TRAP-ELISA assay showed that telomerase activities in T24 cells treated with ph2-shRNA were decreased compared with cells treated with pTZU6 + 1 ($P < 0.05$) while it remained high for cells treated with ph2'-shRNA

Discussion

Bladder cancer is the most common malignant neoplasm in urinary system, in which morbidity and mortality have shown increasing tendency in recent years. The latest research showed that the abnormal expression of telomerase was closely related to its occurrence, development and prognosis of bladder cancer [15]. The holoenzyme of telomerase [16] is composed of telomerase RNA (hTR), telomerase-associated protein (TP1/TLP1) and telomerase reverse transcriptase (hTERT). Among them, hTERT is the key regulator of telomerase activity, which is more closely correlated with cancer than telomerase [17]. It is reported that more than 85% malignant tumors expressed hTERT positively [3]. Our previous study [18] showed that the expression of hTERT in bladder cancer tissue was 100%, also correlated to the occurrence, development and prognosis of bladder cancer. Therefore, it is reasonable to select hTERT as the target gene to suppress bladder cancer cell proliferation.

RNAi was developed rapidly in recent years, which had become one of the main techniques in molecular

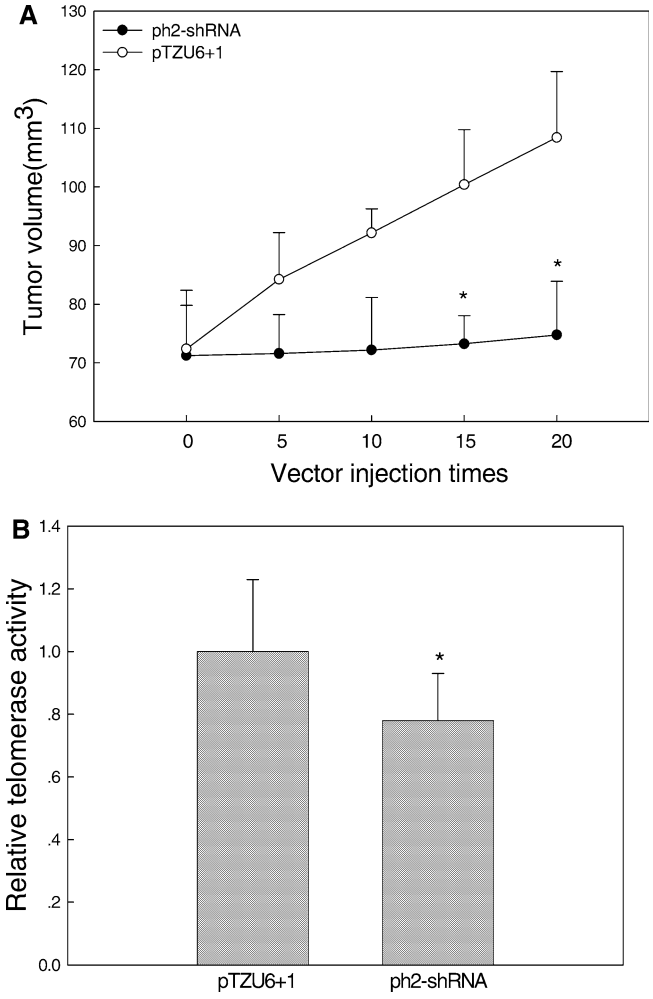

Fig. 4 Experiments in vivo. **a** the alteration of tumor size of bladder cancer BALB/c xenograft tumor tissue with different treatment. After injected with ph2-shRNA into the tumor in situ for 15 and 20 times, tumor growth was significantly reduced compared with pTZU6+1 ($P < 0.05$). **b** telomerase activity of bladder cancer xenograft tumor tissues with different treatment. Telomerase activity in tumor tissues treated with ph2-shRNA was decreased by TRAP-ELISA method compared with those treated with pTZU6+1 ($P < 0.05$)

biology and oncology. RNAi has also demonstrated great prospects in human gene function, signal transduction research and gene therapy. Nowadays, RNAi has been applied in down-regulating the expression of related genes, which has been approved in the gene therapy of leukemia [19], AIDS [20] and virus hepatitis [21, 22].

In this study, we selected three different 21 bp siRNA fragments targeted with different specific hTERT fragments of different base contents, and then designed the hairpin structure (Fig. 1a). Different 21 bp siRNA transcript templates were separately ligated to pTZU6+1 and recombined to construct shRNA-hTERT-pTZU6+1 vectors. These vectors were transfected into T24 cells to induce RNAi. The screening results demonstrated that shRNA vectors tested could

Table 3 Quantitative real-time RT-PCR results of hTERT and c-myc expressions in bladder cancer BALB/C different xenograft tumor tissues with different treatement (Mean ± SD normalized RNA copies)

Groups	NhTERT	Nc-myc
Control (untreated)	55.2 ± 4.25	43.4 ± 3.72
PTZU6+1	42.4 ± 4.06	40.2 ± 3.43
ph2-shRNA	11.5 ± 3.64*	17.8 ± 3.56*

* $P < 0.05$ versus pTZU6+1

down-regulate the expression of hTERT at various degrees, and ph2-shRNA was the most effective one. These results suggested that the design of hairpin structure was reasonable and effective. The methods that constructed shRNA-hTERT-pTZU6+1-targeted hTERT gene, then transfected into bladder cancer cells and induced RNAi directly in cells are feasible [11]. Meanwhile, the blocking efficiency of RNAi was different in different templates targeting various regions of the same gene, which was confirmed by Elbashir's report [23].

hTERT expression had been affected by many factors in cells. On one hind, hTERT expression was coordinated by c-Myc/Max/Mad network [24]. Especially c-myc could up-regulate hTERT activity by binding with its core promoter [25]. Recent studies also showed that the expression of hTERT could influence c-myc expression. There existed a mutual regulation effect between hTERT and c-myc. In our research, shRNA-hTERT-pTZU6+1 was transfected into bladder cancer T24 cells, which then inhibited the expression of c-myc in T24 cells compared to the pTZU6+1 empty vector group, which was consistent with Greenberg's report [26] that suppressing hTERT expression could lead to the inhibition of c-myc expression. This implies that shRNA-targeted hTERT could suppress bladder cancer cell proliferation by affecting hTERT expression and subsequently c-myc. We also found that after silencing hTERT expression, telomerase decreased correspondingly, which was confirmed that hTERT is the key factor of telomerase activity [17]. Meanwhile, the tumor size of T24 BALB/c nude mice was declined significantly in ph2-shRNA injected group compared with the empty vector group, and the expressions of hTERT and c-myc also decreased correspondingly. These results also suggested that the expression of hTERT and c-myc is related to the occurrence and development of bladder cancer.

Our results revealed that shRNA-hTERT could suppress the proliferation of bladder cancer by inhibiting hTERT expression and telomerase activity obviously, partly changeing the malignant phenotype, and moreover, down-regulating the expression of c-myc. Taken together, shRNA-targeted *hTERT* gene could explore a new potential for gene therapy of bladder cancer.

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References

1. Melissourgos N, Kastrinakis NG, Davilas I, Foukas P, Farmakis A, Lykourinas M (2003) Detection of human telomerase reverse transcriptase mRNA in urine of patients with bladder cancer: evaluation of an emerging tumor marker. Urology 62:362–367
2. Akiyama M, Yamada O, Kanda N, Akita S, Kawano T, Ohno T, Mizoguchi H, Eto Y, Anderson KC, Yamada H (2002) Telomerase overexpression in K562 leukemia cells protects against apoptosis by serum deprivation and double-stranded DNA break inducing agents, but not against DNA synthesis inhibitors. Cancer Lett 178:187–197
3. Masutomi K, Yu EY, Khurts S, Ben-Porath I, Currier JL, Metz GB, Brooks MW, Kaneko S, Murakami S, DeCaprio JA, Weinberg RA, Stewart SA, Hahn WC (2003) Telomerase maintains telomere structure in normal human cells. Cell 114:241–253
4. Li S, Rosenberg JE, Donjacour AA, Botchkina IL, Hom YK, Cunha GR, Blackburn EH (2004) Rapid inhibition of cancer cell growth induced by lentiviral delivery and expression of mutant-template telomerase RNA and anti-telomerase short-interfering RNA. Cancer Res 64:4833–4840
5. Kraemer K, Fuessel S, Schmidt U, Kotzsch M, Schwenzer B, Wirth MP, Meye A (2003) Antisense-mediated hTERT inhibition specifically reduces the growth of human bladder cancer cells. Clin Cancer Res 10:3794–3800
6. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391:806–811
7. Elbashir SM, Lendeckel W, and Tuschl T (2001) RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev 15:188–200
8. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411:494–498
9. Downward J (2004) Use of RNA interference libraries to investigate oncogenic signalling in mammalian cells. Oncogene 23:8376–8383
10. Zhang PH, Tu ZG, Yang MQ, Huang WF, Zou L, Zhou YL (2004) Experimental research of targeting hTERT gene inhibited in hepatocellular carcinoma therapy by RNA interference. Ai Zheng 23:619–625
11. Sui G, Soohoo C, Affar el B, Gay F, Shi Y, Forrester WC, Shi Y (2002) A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. Proc Natl Acad Sci USA 99:5515–5520
12. Paradis V, Bieche I, Dargere D, Bonvoust F, Ferlicot S, Olivi M, Lagha NB, Blanchet P, Benoit G, Vidaud M, Bedossa P (2001) hTERT expression in sporadic renal cell carcinomas, J Pathol 195:209–217
13. ABI Prism 7700 Sequence detection System User Bulletin#2 (2001) Relative quantification of gene expression. Available from: http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf
14. Komata T, Kondo Y, Kanzawa T, Hirohata S, Koga S, Sumiyoshi H, Srinivasula SM, Barna BP, Germano IM, Takakura M, Inoue M, Alnemri ES, Shay JW, Kyo S, Kondo S (2001) Treatment of malignant glioma cells with the transfer of constitutively active caspase-6 using the human telomerase catalytic subunit (human telomerase reverse transcriptase) gene promoter. Cancer Res 61:5796–5802
15. Longchampt E, Lebret T, Molinie V, Bieche I, Botto H, Lidereau R (2003) Detection of telomerase status by semi-quantitative and in situ assays, and by real-time reverse transcription-polymerase chain reaction (telomerase reverse transcriptase) assay in bladder carcinomas. BJU Int 91:567–572
16. Lingner J, Hughes TR, Shevchenko A, Mann M, Lundblad V, Cech TR (1997) Reverse transcriptase motifs in the catalytic subunit of telomerase. Science 276:561–567
17. Cerni C (2000) Telomeres, telomerase, and myc. An update. Mutat Res 462:31–47
18. Zou L, Luo CL, Tu ZG, Zhang PH (2002) Expression of hTERT and c-myc mRNA in transitional cell carcinoma of the bladder (TCCB), Chin Med J 15, netprints
19. Wilda M, Fuchs U, Borkhardt A (2002) Killing of leukemic cells with a BCR/ABL fusion gene by RNA interference (RNAi). Oncogene 21:5716–5724
20. Jacque JM, Triques K, Stevenson M (2002) Modulation of HIV-1 replication by RNA interference. Nature 418:435–438
21. Shlomai A, Shaul Y (2003) Inhibition of hepatitis B virus expression replication by RNA interference. Hepatology 37:764–770
22. Kapadia SB, Brideau-Andersen A, Chisari FV (2003) Interference of hepatitis C virus RNA replication by short interfering RNAs. Proc Natl Acad Sci USA 100:2014–2018
23. Elbashir SM, Lendeckel W, Tuschl T (2001) RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev 15:188–200
24. Casillas MA, Brotherton SL, Andrews LG, Ruppert JM, Tollefsbol TO (2003) Induction of endogenous telomerase (hTERT) by c-Myc in WI-38 fibroblasts transformed with specific genetic elements. Gene 316:57–65
25. Zou L, Zhang PH, Luo CL, Tu ZG (2005) Transcript regulation of human telomerase reverse transcriptase by c-myc and mad1. Acta Biochim Biophys Sin 37:32–38
26. Greenberg RA, O'Hagan RC, Deng H, Xiao Q, Hann SR, Adams RR, Lichtsteiner S, Chin L, Morin GB, DePinho RA (1999) Telomerase reverse transcriptase gene is a direct target of c-Myc but is not functionally equivalent in cellular transformation. Oncogene 18:1219–1226