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Cancer Biology & Therapy

ISSN: 1538-4047 (Print) 1555-8576 (Online) Journal homepage: http://www.tandfonline.com/loi/kcbt20

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**To cite this article:** Xianling Guo, Weifen Wang, Feiguo Zhou, Zhenghua Lu, Ruifang Fang, Fengqi Jia, Xinxin Bu, Rong Li, Baihe Zhang, Mengchao Wu & Lixin Wei (2008) siRNA-mediated inhibition of hTERT enhances chemosensitivity of hepatocellular carcinoma, Cancer Biology & Therapy, 7:10, 1555-1560, DOI: 10.4161/cbt.7.10.6539

**To link to this article:** http://dx.doi.org/10.4161/cbt.7.10.6539

Published online: 01 Oct 2008.

[Cancer Biology & Therapy 7:10, 1555-1560; October 2008]; ©2008 Landes Bioscience

Research Paper

# siRNA-mediated inhibition of hTERT enhances chemosensitivity of hepatocellular carcinoma

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**Abbreviations:** hTERT, human telomerase reverse transcriptase; Ad-si/hTERT, adenovirus mediated hTERT-specific siRNA; MTT, 3-(4,5-dimethylthiazol-2-y;)-2,5-diphenil tetrazolium bromide; DMSO, dimethyl sulfoxide; MOI, multiplicity of infection; TUNEL, TdT-mediated dUTP nick-end labeling; TRAP, telomeric repeat amplification protocol; $IC_{50}$, median inhibitory concentration

**Key words:** siRNA, hTERT, chemosensitivity, cisplatin, hepatocellular carcinoma, telomerase, apoptosis





**Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. However, there is no effective treatment for HCC. It has been shown that sustained activation of telomerase is essential for the growth and progression of HCC, suggesting that telomerase is a rational target for HCC therapy. Here, we investigated the effects of siRNA-mediated knockdown of hTERT, the catalytic and rate-limiting subunit of telomerase, on the sensitivity of HCC cells to cisplatin. While silencing of hTERT and the resultant inhibition of telomerase activity by infection with the recombinant adenoviruse expressing a hTERT siRNA (Ad-si/hTERT) alone did not affect the proliferation and viability of SMMC7721 and HepG2 HCC cells within five days, co-administration of Ad-si/hTERT, but not the empty adenovirus vector, with cisplatin caused much greater extent of apoptosis in vitro under the same conditions and induced significantly more robust inhibition of SMMC7721 and HepG2 tumors growth in a mouse tumor xenograft model than cisplatin monotherapy. Our results demonstrated the synergistic effect between hTERT siRNA and cisplatin in the suppression of HCC progression and indicated that the combination of hTERT-specific siRNA and cisplatin could be an effective therapy for HCC.**

## Introduction

Human telomeres are the end of chromosomes and consist of tracts of repetitive DNA (TTAGGG).[1,2] The telomere caps the end of chromosomes and protects chromosomes from being recognized as double-strand breaks. In human somatic cells, telomere typically contains 5–15 kb pairs of repeating TTAGGG sequences and shortens by 50–200 bp with each cell division because the DNA polymerase is unable to replicate the extreme end of the DNA lagging strand.[3] This telomere shortening can be corrected by the expression of telomerase, a unique ribonucleoprotein that is capable of adding the telomeric repeats onto the 3' ends of chromosomes. The expression of telomerase has been detected in ~85–90% of all human tumors but not in the adjacent normal cells.[4] Telomerase has two major components: (1) a template RNA named hTR (the human telomerase RNA) which serves as the template on which telomeric repeats are added to the chromosome, and (2) the human telomerase reverse transcriptase (hTERT) which is the catalytic subunit of telomerase. Since hTR is constitutively and ubiquitously expressed, the activity of telomerase is largely dependent on the expression of hTERT under normal conditions, the expression of hTERT is restricted to the stem cells and progenitor cells after birth

HCC is one of the most common malignant tumors in the world. Unfortunately, no significant progress in the prevention and treatment of HCC has been made. It has been reported that 80% to 90% of HCCs express telomerase, suggesting that telomerase is a potential target for the treatment of these HCCs.

In the present study, we investigated the potential synergic effect of hTERT-specific siRNA and cisplatin on human hepatocellular carcimoma SMMC-7721 and HepG2 cells. We found that hTERT-specific siRNA can significantly inhibit hTERT expression and increase sensitivity of SMMC-7721 and HepG2 cells to the chemotherapeutic agent-cisplatin.

## Results

**siRNAs are efficiently internalized and inhibit telomerase activity in HCC cells.** We assessed the efficiency of chemically synthesized siRNAs in interfering with hTERT and telomerase function in hepatocarcinoma cancer cell lines SMMC7721. GFP labeling was instrumental to detect the uptake and intracellular distribution of siRNAs by fluorescence microscopy. Two days after viral infection,

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Submitted: 01/09/08; Revised: 05/14/08; Accepted: 07/02/08

Previously published online as a *Cancer Biology & Therapy* E-publication: http://www.landesbioscience.com/journals/cbt/article/6539

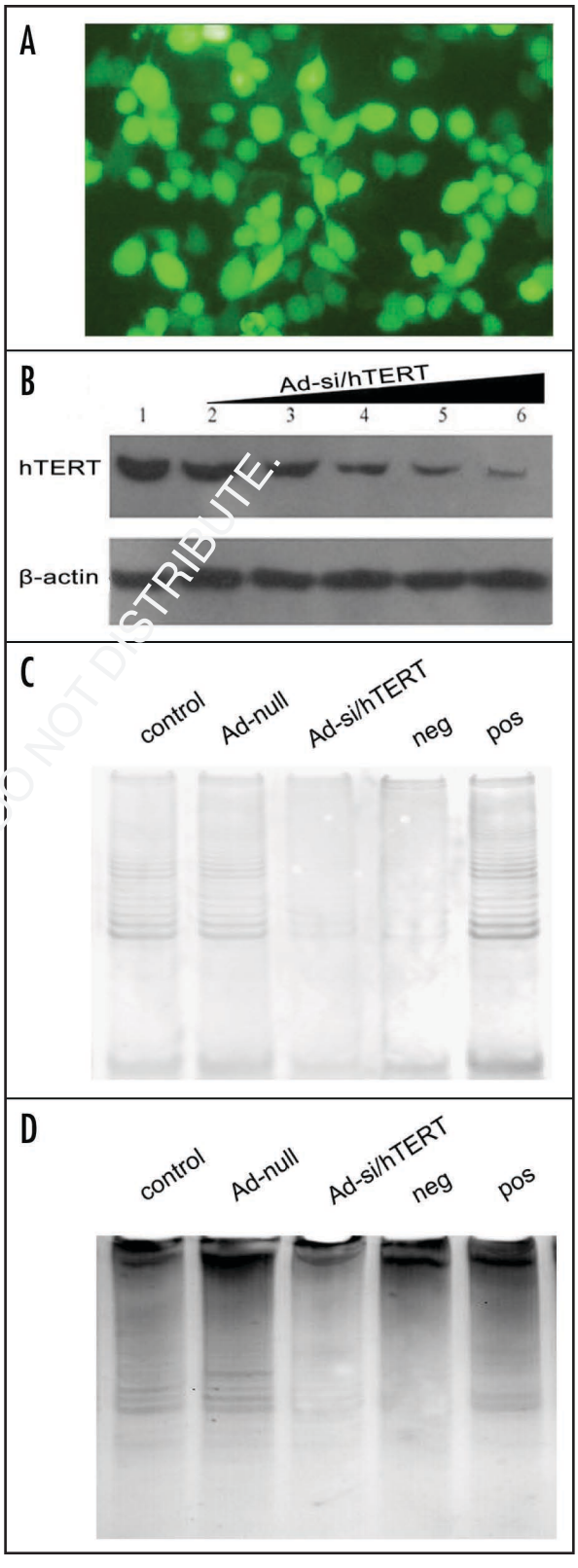



Figure 1. Effects of Ad-si/hTERT on hTERT expression and telomerase activity in SMMC7721 and HepG2 cells. (A) Representative photograph showing the uptake and cellular distribution pattern of GFP-conjugated siRNAs in HCC SMMC-7721 cells, as detected by fluorescence microscopy. (B) HCC SMMC7721 cells infected with different MOI were harvested to prepare cell lysates. The lysates were subjected to SDS-PAGE and blotted with anti-TERT antibody. A representative example of an experiment that was repeated three times is shown. Lane 1: un-infected cells. Lane 2–6: corresponding to the different MOI 1, 5, 10, 20, 50 respectively (C and D) HCC SMMC7721 (C) and HepG2 (D) cells were treated with Ad-si/hTERT and Ad-null (50 MOI) for three days, and subjected to TRAP assays to assess telomerase activity. A representative example of an experiment that was repeated three times is shown. neg, negative control. pos, positive control.

almost all SMMC-7721 cells were found to be infected by Ad-si/hTERT at 20 MOI (Fig. 1A). To select an optimum concentration of adenovirus, HCC SMMC-7721 cells were infected by increasing MOI of Ad-si/hTERT. The hTERT expression level was examined by Western-blot three days later. As shown in Figure 1B, the increasing RNA interference efficiency was tightly correlated with the increasing MOI of Ad-si/hTERT, and the hTERT expression was silenced almost completely at 50 MOI which we applied in the following experiments. Then, we evaluated the ability of Ad-si/hTERT to inhibit telomerase activity with TRAP assay. As shown in Figure 1C and D, significant inhibition of telomerase activity was observed with respect to control and Ad-null (negative control) in hepatocarcinoma cell line SMMC-7721 and HepG2.

**Ad-si/hTERT has no effects on the proliferation of HCC cells in a short period.** To evaluate the effect of Ad-si/hTERT on SMMC-7721 and HepG2 cells proliferation, we counted cell numbers for five days at 24 h intervals. SMMC-7721 (2 x $10^5$) and HepG2 cells (2 x $10^5$) were seeded in a six-well plate one day after being infected with Ad-si/hTERT. Cells infected by Ad-null of the same MOI were used as the negative control while cells under normal cultured conditions were used as the blank control. Cells were digested by trypsin at fixed time points and counted under microscope. As shown in Figure 2A and B, compared with control groups, the proliferation of the Ad-si/hTERT group was not significantly inhibited. These results indicated that certain MOI of Ad-si/hTERT has no effect on SMMC-7721 and HepG2 cells proliferation within a short period (five days).

**Ad-si/hTERT enhances the chemosensitivity of SMMC-7721 cells to cisplatin in vitro.** To investigate the combination effect of cisplatin plus Ad-si/hTERT, we first identified the effect of different concentrations of cisplatin on cell proliferation by MTT assay, and select a concentration of about 12.5 μM to evaluate the effect of low dose of cisplatin on SMMC-7721 cells (Fig. 3A). Subsequently, we examined DNA content by cytometry in SMMC-7721 cells after exposure to indicated concentration cisplatin for different time points up to 72 h. The cell cycle analysis demonstrated that SMMC7721 cells treated with cisplatin decreased the number of cells in the $G_1$ phase, and mainly increased the number of cells in the S phase in a time dependent manner. Untreated cells displaying the predominant cell number of $G_1$ phase. From the cytometry results, we could not observe the obvious alteration of sub-$G_1$ DNA content in different time point treatments. These results demonstrated that cells treated with cisplatin mainly resulted in cell cycle arrest rather than inducing cell apoptosis (Fig. 3B).

To evaluate the effect of combination of Ad-si/hTERT and low dose cisplatin on SMMC-7721 cells, the SMMC-7721 cells were treated with cisplatin two days after Ad-si/hTERT infection for 72 hours, the cells then were performed to TUNEL assay. As shown in Figure 4, the combined treatment group (Ad-si/hTERT plus

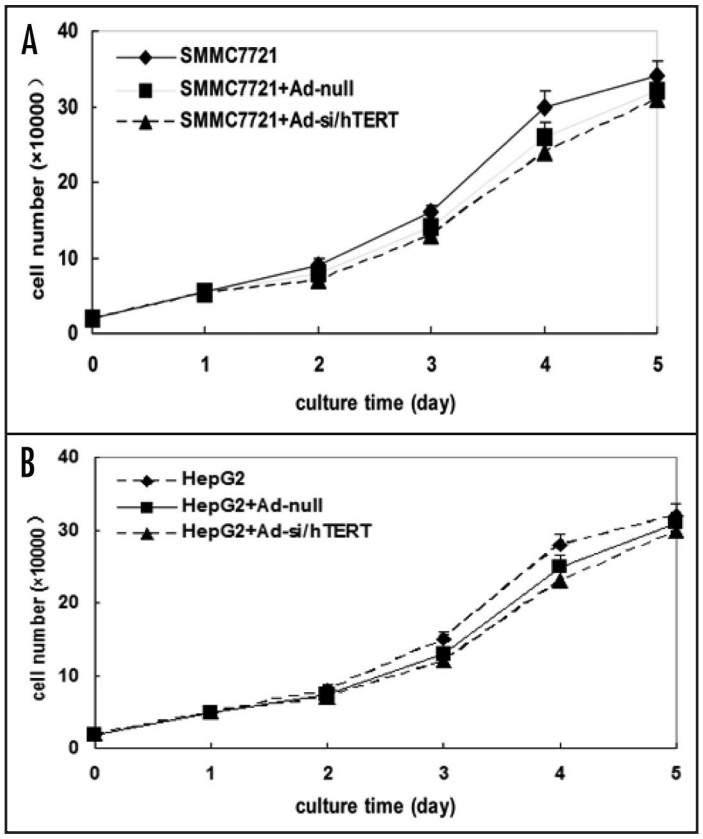
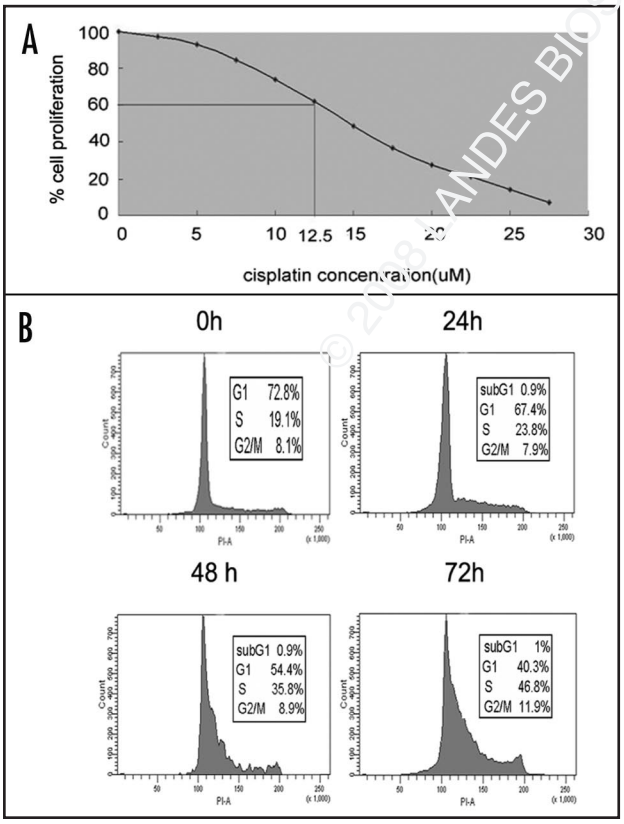



Figure 2. Growth curve of SMMC7721 and HepG2 cells. (A and B) HCC SMMC-7721 and HepG2 cells were infected with Ad-si/hTERT or Ad-null respectively, 24 h later trypsinized and continued counting for five days in a successive. The data was processed by Student's t test to determine the differences.




cisplatin) showed a significant number of apoptotic nuclei compared with the other indicated four groups. We used the Ad-null plus cisplatin group as the negative control to eliminate whether the synergic effect was caused by the adenovirus itself. The result of this group did not show any significant apoptotic nuclei, and was close to the cisplatin group. Taken together, these results suggested that Ad-si/hTERT plus cisplatin have synergistic effect on SMMC-7721 cells; inhibition of telomerase with Ad-si/hTERT can increase susceptibility of SMMC-7721 cells to cisplatin.

**Proliferation inhibition of hapatocellular cancer BALB/c xenograft tumor with Ad-si/hTERT plus cisplatin in vivo.** To determine the effect of Ad-si/hTERT plus cisplatin on tumor growth in vivo, SMMC-7721 and HepG2 cells were injected into the right back of BALB/c nude mice. After four days, the xenograft tumor started to grow and after about 15 days the model was successfully established to initiate treatment. Ad-si/hTERT and empty vector Ad-null (1 x $10^9$/30 ul/time, separately) was directly injected into the tumor, and cisplatin (3 g/Kg/100 ul/time) was intraperitoneally injected. All types of treatment were administered at the same time every five days for four administrations. Tumor sizes were subsequently measured daily. Figure 5 shows the growth curve of the SMMC7721 and HepG2 tumors, plotted as average tumor volume versus time. Ad-si/hTERT plus cisplatin treatment inhibited tumor growth significantly. However, other treatments failed to inhibit the growth of SMMC7721 and HepG2 tumors (Fig. 5). These results indicate that combination Ad-si/hTERT with cisplatin can significantly inhibit tumor growth in vivo.

## Discussion

Since telomerase is specifically activated in most malignant tumors but not in normal somatic cells, the inhibition of telomerase has been pursued as a compelling strategy for cancer therapy. Numerous approaches have been employed to target telomerase, among which blocking hTERT[8] activity or expression holds the promise. Targeting hTERT leads to the shortness or dysfunction of telomeres and impairs the stability of chromosomes, thereby blocking cell growth or triggering apoptosis.[9] In this study, we found that siRNA-mediated silencing of hTERT could sensitize HCC cells to cisplatin-induced apoptosis and enhance the efficacy of cisplatin in the suppression of HCC growth in vivo.

There were controversies in the literature regarding the consequences of telomerase inhibition. Some reports showed that telomerase inhibitors suppressed cancer cell growth in vitro,[12-14] whereas others revealed that acute hTERT depletion did not have any deleterious effect on cell proliferation and survival for up to 72 hours.[15] For instance, no growth-inhibitory effect could be detected in HCT116 and HeLa cells up to 5–10 days post hTERT knockdown by siRNA.[13,16] Consistent with these observations, our results showed that silencing of hTERT alone did not inhibit the proliferation of SMMC7721 and HepG2 cells within five days (Fig. 2). The

Figure 3. The effect of low dose cisplatin on SMMC7721 cells. (A) Tumor cells were seeded at 4 x $10^3$ cells per well (0.1 mL) in 96-well flat-bottomed plates and incubated overnight at 37°C. After exposure to different concentrations of cisplatin for 72 hours, cell proliferation was determined by MTT assay. The proliferation of the untreated cells was regarded as 100%. Points mean of three independent experiments. (B) Cell cycle analysis of SMMC7721 cells treated with 12.5 μM cisplatin in different time point.

differential consequesces of hTERT knockdown may be due to different lengths of telomeres or the distinct sensitivities to hTERT inhibition and telomere dysfunction between different cell lines.

Although telomerase inhibition could trigger cell death via erosion of telomeres after multiple cell divisions, telomerase inhibitors may sensitize cells to apoptosis in a telomere length-independent fashion. It has been reported that telomerase has an antiapoptotic function independently of its activity to maintain the length of telomeres. This pro-survival function of telomerase is likely related to the ability of hTERT to enhance genomic stability and to facilitate DNA damage repair, which may be especially critical for the survival of cells stressed by DNA damage.[17] Our observation that Ad-si/hTERT sensitized HCC cells to cisplatin-induced apoptosis while not killing cells by itself is consistent with this hypothesis. Therefore, potentially hTERT inhibitors could synergize with many other DNA damaging drugs for HCC treatment.

While a number of approaches have been adopted to target telomerase, the recent emergence of siRNA as a powerful tool to silence gene expression specifically offers a new modality to block telomerase and potentially a novel modality of cancer therapeutics. Our results provide a proof of concept that siRNA-mediated silencing of hTERT can synergize with cisplatin and potentially other DNA damaging agents for HCC treatment. Since adenovirus infection is toxic to the liver, adenovirus vector-mediated delivery of hTERT siRNA to HCC may not be practical or have many limitations for clinical application. However, with the development of safer delivery vehicles, hTERT siRNA could be used, in combination with other drugs, for HCC therapeutics.

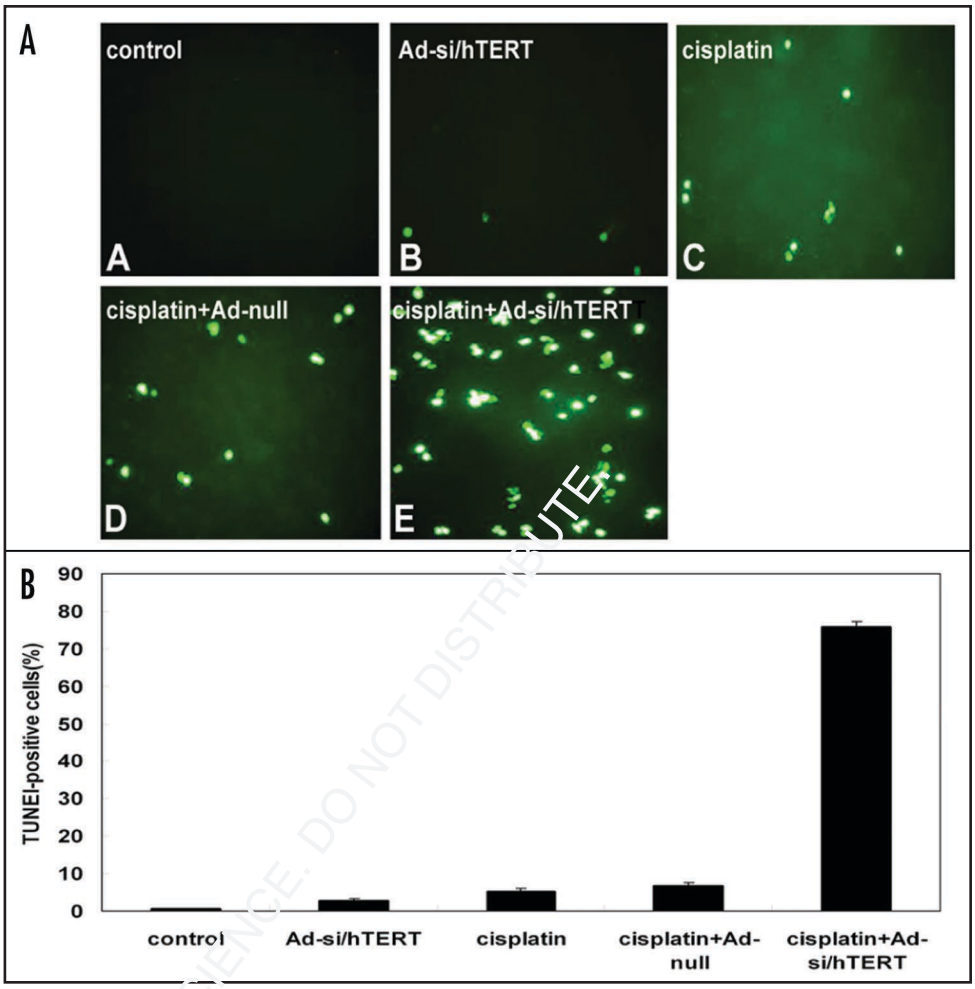



Figure 4. Ad-si/hTERT enhances chemosensitivity of SMMC7721 cells in vitro. (A) Detection of apoptotic cells in tumors. Apoptotic cells were detected by TUNEL staining. Representative picture of TUNEL-stained SMMC7721 cells treated with vehicle, Ad-si/hTERT, cisplatin, cisplatin + Ad-null and cisplatin + Ad-si/hTERT, respectively. (B) The average number of TUNEL-positive cells was scored in six randomly selected microscopic fields.

## Materials and Methods

**Construction of Ad-si/hTERT.** RNA polymerase III H1-RNA gene promoter was used in our study. Designation of siRNA was modified on the base of the published data by Masutomi. The 64 nt oligo nucleotides encoding hTERT specific siRNA were 5'-GATCCCCTTTCATCAGCAAGTTTGGATTCAAGAGATCCAAACTTGCTGATGAAATTTTTGGAA-3' and 5'-GGGAAAGTAGTCGTTCAAACCTAAGTTCTCTAGGTTTGAACGACTACTTTAAAAACCTTTTCGA-3'.These oligo nucleotides were annealed and ligated into the BglII/HindIII sites of pSUPER-EGFP (gift from Dr. Li Lin) and the targeted sequence in the recombined vector was confirmed by automatic sequence analysis. The fragment carried H-1 promoter and the target sequence was inserted into the XbaI/Hind III site of pAd-TRACK to generate TRACK-si/hTERT after adjusting the enzyme cutting site in pCDNA3.1(+). Then TRACK-si/hTERT was linearized with Pme I and transfected into BJ-5183 cells which carried the adenovirus backbone vector pAd-Easy1. Recombined viral genome was linearized with Pac I and transfected into HEK-293 cells in a six-well plate using lipofectamine2000 (Invitrogen, Carlsbad, CA, USA). Seven days after transfection, the recombinant viruses were collected and subjected to the first round of amplification in a T-25 flask, and then purified by Cscl after two further rounds of amplification. The titer was then determined by Plaque Formation Assay.

**Cell culture.** HEK-293, SMMC-7721 and HepG2 cell lines were cultured in DMEM medium (Invitrogen, Life Technologies) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified 5% $CO_2$ incubator.

**Cell growth inhibition assay.** Cells in logarithmic growth phase were seeded at 0.2 x $10^6$ in each well of six-well plates and transfected with 50 MOI siRNAs. At the end of treatment cells were washed with phosphate buffered saline and incubated at 37°C in a 5% $CO_2$ humidified atmosphere for an additional 1–5 days. Adherent cells were then trypsinized and counted. Each experimental sample was run in triplicate.

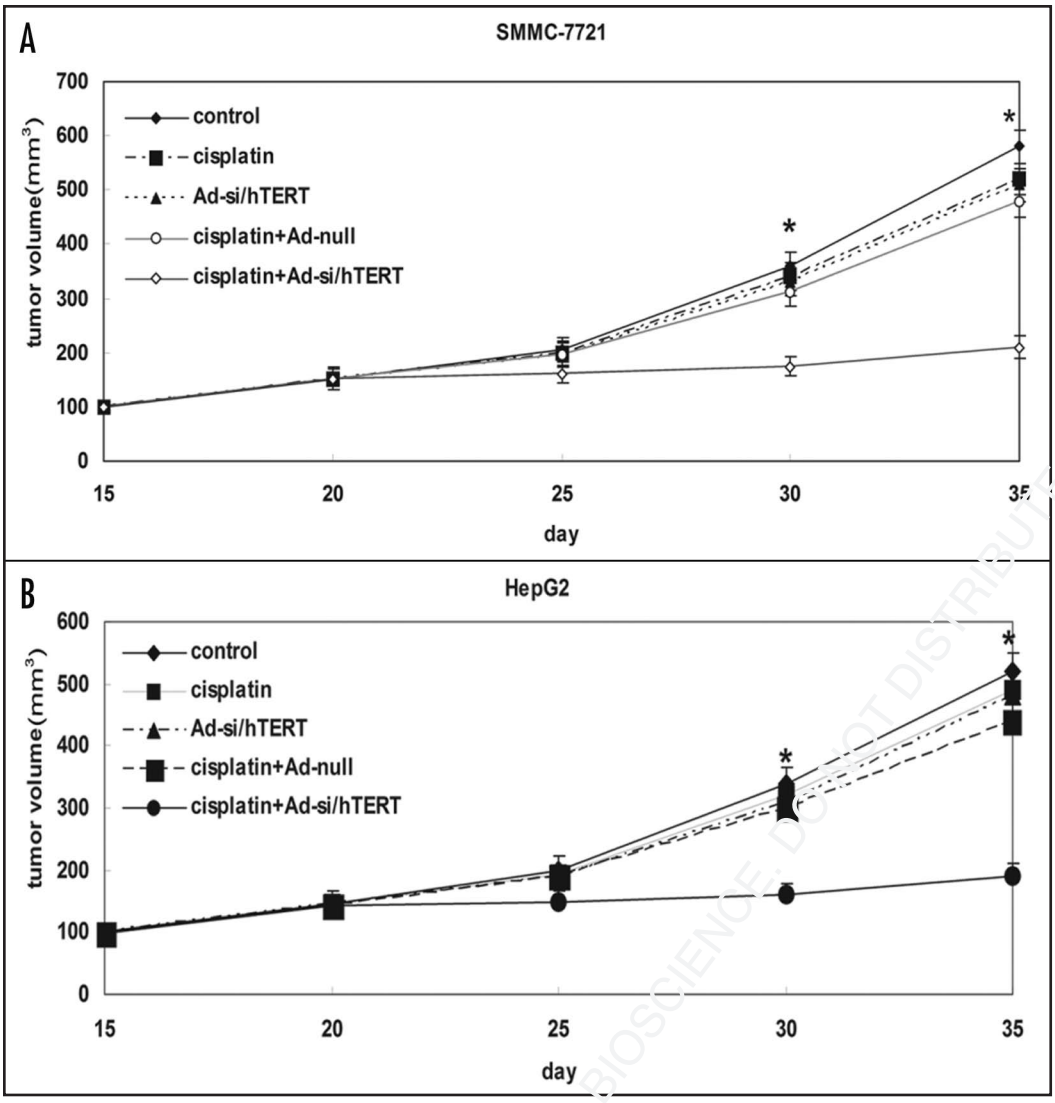



Figure 5. Ad-si/hTERT enhanced hepatocellular carcinoma to cisplatin in vivo. Mice were injected subcutaneously in the flanks with SMMC7721 and HepG2 cells. After tumors had reached 100 to 110 $mm^3$ in size, the mice were administered an intraperitoneal injection of cisplatin or vehicle, and in suit inject with Ad-null or Ad-si/hTERT. Tumor sizes were subsequently measured daily. The growth curves of SMMC7721 and HepG2 tumor are plotted. Each data point represents the mean and standard deviation. *p < 0.05 (Ad-si/hTERT plus cisplatin group vs. the other four groups separately).

**Cell cycle analysis.** SMMC-7721 cells (2 x $10^5$) were cultured in each well of six-well plates to 70% to 80% confluence. Then cells were treated with indicated concentration of cisplatin for 24, 48 and 72 hours, respectively. The cells were harvested and fixed with ice cold 70% ethanol overnight. The fixed cells were centrifuged and then incubated with propidium iodide (Sigma , St. Louis, MO) staining buffer (50 μg/mL of PI in PBS with freshly added RNase A to 10 μg/mL) for 30 minutes at room temperature; DNA analysis was performed with FACSAria cytometer (Becton Dickinson). Cells with subdiploid DNA content were considered apoptotic cells. Cell cycle distributions were analyzed by the ModFit software.

**Cell viability assay.** SMMC-7721 and HepG2 cells were seeded in a 96-well plate. Twenty-four hours later cells were treated with different concentrations of cisplatin. After 72 h, MTT assay was performed. Twenty microlitters of 5 mg/ml of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT,5 mg/mL in PBS)] were added to each well followed by incubation for four hours at 37°C. The formazan crystals were dissolved in 200 μl of DMSO. Optical density values (OD) were determined at wavelength 570 nm. Each assay was performed three times and the average results were calculated.

**Western blot analysis.** Protein extracts were prepared with the Active Motif nuclear extract kit (Active Motif, Carlsbad, CA, USA). Protein concentrations were estimated using Bradford Protein Assay. Equal amounts of proteins (30 μg) were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes. After blocking with 5% nonfat dry milk in TBS containing 0.2% Tween 20, membranes were incubated at 4°C overnight with anti-TERT (Santa Cruz, CA, USA) or anti-β-actin (Sigma, St. Louis, MO, USA) primary antibodies. The immunoblots were visualized with horseradish peroxidase-coupled goat anti-rabbit or anti-mouse immunoglobulin by using a ELC chemiluminiscence substrate system (Amersham Biosciences, Piscataway, NJ, USA).

**Telomerase activity assay.** Telomerase activity was examined by silver staining telomeric repeat amplification protocol (TRAP). Briefly, five x $10^5$ cells were resuspended in 200 μl of 1 x CHAPS lysis buffer. After a 30-minute incubation on ice, the suspension was centrifuged at 12,000 xg for 20 min at 4°C. Cleared supernatant was collected, and the protein concentration was measured using Bradford Protein Assay. TRAP assays were performed on equal amounts of protein [0.5 to 2 ng protein (50 to 200 cells) per 50 μl]. A PCR-based telomerase assay and Sliver staining were done according to former protocol.[4,7] As negative control, the lysates were heat-treated at 85°C for 10 min to inactivate the telomerase prior to the TRAP assay. Cell lysates (293T) was used as a positive control.

**TUNEL assay.** The TdT-mediated dUTP Nick-End Labeling, (Promega, WI, USA) assay (TUNEL) was used to test for apoptosis. Briefly, SMMC-7721 cells with different treatments were incubated in the tested compounds and fixed in 4% paraformaldehyde (Polysciences) prepared freshly in PBS. Slides with cells were then incubated at 37°C in a humid chamber for one hour in a mixture which containing terminal deoxynucleotidyl transferase (TdT), to

promote binding of fluorescein-labeled deoxynucleotides. Then an analysis was carried out by fluorescence microscopy using a 490 nm filter. The total number of TUNEL positive cells was quantified in six randomly selected microscopic fields at x200 magnification within cell nuclei.

**Studies in vivo.** For establishing a hepatocarcinoma xenograft tumor model, SMMC7721 (2.0 x $10^7$ cells in 0.2 ml of serum-free DMEM medium) and HepG2 cells of the same number were injected subcutaneously into the right back of 8–10-week-old male BALB/c nude mice (six mice for each group), and the tumor growth was monitored using electronic calipers every day. When the tumors reached a mean tumor volume of 100–110 $mm^3$, the treatment was initiated. Ad-si/hTERT and Ad-null empty vector (1 x $10^9$/30 ul/time, separately) were directly injected into the tumor in situ. Cisplatin (3 g/Kg/100 ul/time) or vehicle was injected into the abdominal cavity. All kinds of treatments were administered at the same time every five days for four injections. Tumor sizes were subsequently measured daily; the results represented the mean value of three independent experiments. All the animal experiments were approved by the institutional and governmental review boards. Tumor volumes were measured with a caliper and calculated using the formula: Volume = a x $b^2/2$, where a was the width at the widest point of the tumor and b was the maximal width perpendicular to a.

**Statistical analysis.** Values were expressed as the mean ± standard deviation (S.D.). Differences were analyzed by Student's t-test. A p-value <0.05 was considered to be significant.

**Acknowledgements**

This project was supported by the Special Funds for National Natural Science Foundation of China (Grant No: 30700981), the Commission of Science and Technology of Shanghai Municipality (Grant No. 045407047, 08XD14003) and National High-Tech Research and Development Program of China (Grant No. 2006AA02A304)

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