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Inhibitory Effects of Thioredoxin Reductase Antisense RNA on the Growth of Human Hepatocellular Carcinoma Cells

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Abstract Thioredoxin reductase (TrxR) in conjunction with thioredoxin (Trx) is a ubiquitous intracellular oxidoreductase system with antioxidant and redox regulatory roles. In some human tumors, the thioredoxin system is found overexpressed. We have used an antisense approach to investigate whether inhibition of TrxR overexpression can suppress the growth of human hepatocellular carcinoma SMMC-7721 cells. TrxR cDNA fragment was inserted in the antisense direction into pcDNA3.1/myc-His and SMMC-7721 cells were stably transfected with the plasmid construct. The results showed that TrxR antisense RNA could significantly reduce TrxR mRNA level and activity, and suppress the growth of SMMC-7721 cells. Cell-cycle analysis showed G_2/M phase arrest in SMMC-7721 cells transfected with TrxR antisense plasmid. TrxR antisense RNA could significantly increase p53 mRNA level and decrease Bcl-2 mRNA level by reverse transcriptase-polymerase chain reaction (RT-PCR). Furthermore a significant decrease in human telomerase reverse transcriptase (hTERT) mRNA level was found in SMMC-7721 cells transfected with TrxR antisense plasmid. Flow cytometry and telomere fluorescence in situ hybridization (Flow FISH) showed that TrxR antisense RNA could significantly reduce the telomere fluorescence in SMMC-7721 cells. The results suggested that TrxR antisene RNA inhibited the growth of SMMC-7721 cells through an accumulation of cell cycle at G_2/M phase, an increase in p53 mRNA level and a reduction in telomere fluorescence and Bcl-2, hTERT mRNA levels. J. Cell. Biochem. 96: 653–664, 2005.

Key words: thioredoxin reductase; antisense RNA; cell proliferation; cell cycle; human telomerase reverse transcriptase; telomere length

Thioredoxin reductase (TrxR), which catalyzes the reduction of the active site disulfide of thioredoxin (Trx), is an NADPH-dependent homodimeric oxidoreductase with one FAD molecule per subunit [Holmgren, 1997; Mustacich and Powis, 2000]. Mammalian TrxR is a selenoprotein that contains a penultimate selenocysteine (SeCys) [Tamura and Stadtman, 1996] in the sequence-Gly-Cys-Secys-Gly-, which serves as a critical redox center [Lee et al., 2000; Zhong et al., 2000]. The codon for SeCys is UGA, which encodes a stop codon in general. To translate the UGA codon to a SeCys residue, SeCys insertion (SECIS) element should be present in the 3′ untranslated region (3′UTR) [Grundner-Culemann et al., 2001].

The Trx system (NADPH, TrxR/Trx) plays several key roles in DNA synthesis and activation of transcription factors that regulate cell growth. Studies of a variety of human primary tumors have shown that TrxR and Trx are overexpressed in the tumor comparing to levels in its equivalent normal tissue [Gasdaska et al., 1994; Berggren et al., 1996; Choi et al., 2002]. It has been reported that the elevation of TrxR or/and Trx levels in many human primary cancers appears to contribute to the increase of cancer cell growth including an increase in the sensitivity of cells to other cytokines and growth factors, inhibition of the normal mechanism of programmed cell death and resistance to chemotherapy [Shi et al., 2003]. Based on the

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fact that the Trx system stimulates cancer cell growth and inhibits apoptosis, inhibiting intracellular TrxR may be an effective method to treat and prevent cancer.

Antisense technology offers the potential to block the expression of specific genes within cells. It involves the suppression of constitutive gene expression, using plasmid vectors or virus vector, or the suppression of transient gene expression using antisense oligonucleotides. However, the use of antisense oligonucleotides faces the problems of degradation since short oligonucleotides are easily degraded by DNase present in medium or in blood when they are used clinically. And high concentration of oligonucleotides is required to inhibit gene expression, which may produce toxic effects to the cells. To investigate the effects of decrease of TrxR on the growth of human hepatocellular carcinoma SMMC-7721 cells, we constructed the antisense recombinant TrxR expression vector by molecular clone technique and stably transfected it into SMMC-7721 cells. Furthermore, its related antitumor molecular mechanism was explored.

MATERIALS AND METHODS

Cell Culture

The human hepatocellular carcinoma SMMC-7721 cell line was provided by Academy of Military Medical Science (Beijing, China), grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% newborn bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in the presence of 5% CO_2 at 37°C. The medium was changed twice each weak.

Plasmid Construct

Total RNA of SMMC-7721 cells was extracted using TRIzol reagent (Gibco BRL) according to the manufacture's instruction. Complementary DNA (cDNA) was prepared by incubation of the RNA with Molony murine leukemia virus reverse transcriptase (200 U, Promega), 10 mM dNTP (Roche), and oligo$(dT)_{15}$ (200 ng, Promega) at 37°C for 60 min in 25 μl Tris-HCl buffer (50 mM, pH 8.3). Following inactivation of the enzyme by incubation at 95°C for 5 min, the cDNAs were amplified in a polymerase chain reaction (PCR) with the following primer set: 5′-tattcagcagagcggttcct-3′ and 5′-aggccacaacagccatattc-3′. After heating samples at 95°C for 5 min, 35 cycles of PCR were performed consisting of denaturation at 94°C for 45 s, annealing at 58°C for 45 s, and extension at 72°C for 1 min with a final extension at 72°C for 10 min. A 701-bp TrxR cDNA fragment was obtained from PCR amplification (Fig. 1A) and was cloned into pGEM-T Easy Vector (Promega) (Fig. 1B). Multiple clones were sequenced to ensure the accuracy of gene sequence and digested with *Eco*R I to obtain TrxR fragment. TrxR fragment, with flanking *Eco*R I ends,

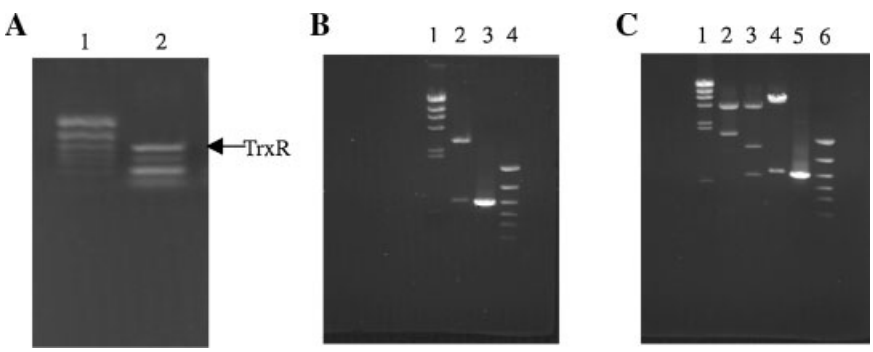

Fig. 1. Construction of TrxR antisense expression vector. **A**: Total RNA was extracted from SMMC-7721 cells and TrxR cDNA fragment was amplified by RT-PCR. **Lane1**: PCR marker; **Lane 2**: RT-PCR product of TrxR cDNA fragment. **B**: TrxR cDNA fragment was cloned into pGEM-T Easy Vector and pGEM-T/TrxR recombinant plasmid was identified by PCR and *Eco*R I digestion. Lane 1: λDNA/*Hin*d I marker; Lane 2: pGEM-T/TrxR recombinant plasmid digested with *Eco*R I (showing 5.5 kb and 720 bp fragments); **Lane 3**: PCR analysis of pGEM-T/TrxR recombinant plasmid (showing a specific band of 701 bp length); **Lane 4**: PCR marker. **C**: TrxR fragment with flanking *Eco*R I ends was subcloned into the mammalian expression vector pcDNA3.1/myc-His *Eco*R I site. The pcDNA3.1/TrxR construct was screened by PCR and enzyme digestion. Lane 1: λDNA/*Hin*d III marker; Lane 2: Restriction enzyme analysis of TrxR Sense RNA with *Hin*d III and *Sma* I (showing 165 bp, 1.8 kb, and 4.3 kb fragments); Lane 3: Restriction enzyme analysis of TrxR antisense RNA with *Hin*d III and *Sma* I (showing 640 bp, 1.3 kb, and 4.3 kb fragments); Lane 4: Restriction enzyme analysis of pcDNA3.1/TrxR with *Eco*R I (showing a specific fragment of 720 bp length): **Lane 5**: PCR analysis of pcDNA3.1/TrxR (showing a specific band of 701 bp length); **Lane 6**: PCR marker.

was subcloned into the mammalian expression vector pcDNA3.1/myc-His *Eco*R I site. The pcDNA3.1/TrxR construct was screened for orientation by cut with *Hin*dIII/*Sma* I. The *Hin*dIII/*Sma* I digest produced DNA fragments of 640, 1300, and 4300 bp, indicating cloning of the TrxR cDNA in the antisense direction (as opposed to 165, 1,800, and 4,300 bp in the sense direction) (Fig. 1C).

DNA Transduction

SMMC-7721 cells were transfected with TrxR antisense vector, TrxR sense vector and the control vector. Transfection was performed by LipoVec^{TM} anion liposome (Invivogen) following the protocol supplied by the manufacturer. Forty-eight hours after transfection, G418 was added into the medium at a concentration of 400 μg/ml. Twenty-five days after transfection, individual colonies were picked up and grown in culture for further analysis. SMMC-7721 cells transfected with TrxR antisense vector, TrxR sense vector and the control pcDNA3.1/myc-His vector were referred as SMMC-7721 pcDNA3.1/as-TrxR, SMMC-7721 pcDNA3.1/s-TrxR, and SMMC-7721 pcDNA3.1 cells, respectively.

PCR Analysis of Integration of TrxR into Genomics

Total genomic DNA was extracted from the parental and transfected cells and subjected to PCR analysis by using the primer sets: 5′-tgactgggcacaacagaca-3′ and 5′-agatcatcctgatcgacaa-3′. After heating samples at 95°C for 5 min, 35 cycles of PCR were performed consisting of denaturation at 94°C for 45 s, annealing at 56°C for 45 s, and extension at 72°C for 1 min with a final extension at 72°C for 5 min.

Analysis of TrxR, p53, Bcl-2, and Human Telomerase Reverse Transcriptase (hTERT) mRNA Levels

TrxR, p53, Bcl-2, and human telomerase reverse transcriptase (hTERT) mRNA levels were determined by a semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) method. Total RNA was extracted from the parental and transfected cells using TRIzol reagent and then reverse transcribed. PCR was performed with the following primer sets: 5′-ctaaaaatgaacggccctga-3′ and 5′-cttccccaaacttctccaca-3′ for TrxR (the product size 1,195 bp, 5′-aaggaaatttgcgtgtggag-3′ and 5′-ttctgacgcacacctattgc-3′ for p53 (the product size 702 bp), 5′-actggccagggtcagagtta-3′ and 5′-cttccaactccctgatccaa-3′ for Bcl-2 (the product size 998 bp), 5′-cggaagagtgtctggagcaa-3′ and 5′-ggatgaagcggagtctgga-3′ for hTERT (the product size 145 bp). To control equal template concentrations and amplification efficiency, a cDNA sequence of β-actin was amplified using the following primers: 5′-tgctatccctgtacgcctct-3′ and 5′-gtacttgcgctcaggaggag-3′ (the product size 596 bp). After heating samples at 95°C for 5 min, 35 cycles of PCR were performed consisting of denaturation at 94°C for 45 s, annealing at 58°C for 45 s, and extension at 72°C for 1 min with a final extension at 72°C for 5 min. An aliquot of PCR products was separated on a 1% agarose gel containing ethidium bromide and measured semiquantitatively using a Bio-Rad Multi-Analyst System. To compare the relative mRNA expression level from each of the samples, the value is presented as the ratio of the band intensity of RT-PCR product over the corresponding β-actin RT-PCR product. The PCR products were sequenced to confirm the specificity of the oligonucleotide primers.

Measurement of TrxR Activity

TrxR activity was measured by the method of Wu et al. [2003]. Briefly, the cells were homogenized in 10 volumes of 50 mM HEPES buffer (pH 7.6), 5 mM EDTA at 0°C, then the homogenates were centrifuged at 110,000*g* for 1 h to provide a supernatant fraction. To remove endogenous Trx and other small-molecular-weight reductants, 0.2 ml aliquots of supernatant were mixed with 0.2 ml of fresh adenosine 2′,5′-diphosphate coupled-agarose beads (ADP-agarose) (Sigma) for 1 h at 4°C. The beads were washed twice by centrifugation at 1,000*g* with 1.0 ml of 0.1 mM NaCl, and then TrxR was eluted with 0.5 ml of 0.1 mM KCl. TrxR activity was performed spectrophotometrically which involved the NADPH-dependent reduction of the disulfide bond in 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) by TrxR [Oblong et al., 1993]. One enzyme unit of TrxR activity is that amount of enzyme that reduces 1 μM Trx per min. Protein concentrations were determined by the Brandford assay system, with bovine serum albumin (BSA) as standard [Bradford, 1976].

Cell Viability Assay

Cell viability was assessed using 3-(4,5-diethyl-2-thiazolyl)-2,5-diphenyl tetrazolium

bromide (MTT) method as previously reported [Mosman, 1983]. Cells were cultured at a density of 7.5×10^3 cells/well in a 96-well plate. After 20, 32, 44, 56 h, 10 μl MTT (5 mg/ml) was added to each well and incubated for another 4 h. The medium was carefully discarded and the formazan crystals were dissolved in 150 μl dimethyl sulfoxide. The absorbance was read at 570 nm on the ELX800 Microplate Reader (Bio-TEK).

Flow Cytometric Analysis

Cell-cycle analysis was detected according to the method of Hotz et al. [1994]. After 48 h culture, cells were harvested and washed with phosphate buffered saline (PBS). Samples of cells (1×10^6) were fixed in 70% ethanol at 4°C overnight. Subsequently, cells were rinsed with PBS, incubated with RNase A. A 50 μg/ml at 37°C for 1 h and stained with propidium iodide (PI) 50 μg/ml at 4°C for 1 h in the dark. Cell-cycle analysis was performed by using a flow cytometry (FACScan, Becton Dickinson).

Flow Cytometry and Telomere Fluorescence in situ Hybridization (Flow FISH)

The telomere length in individual cells was indirectly measured using the Flow FISH protocol as previously described [Rufer et al., 1998]. Briefly, the cells were harvested and resuspended in a hybridization mixture containing 70% deionized formamide, 20 mM Tris pH 7.0, 1% BSA, and either 0.3 μg/ml FITC labeled $(C_3TA_2)_3$ telomere specific peptide nucleic acid (PNA) probe or an equivalent amount of distilled water for the negative control cells. The volume of hybridization was 100 μl/10^5 cells. Samples were subjected to heat denaturation at 80°C for 10 min, followed by hybridization at room temperature for 2 h in the dark. Cells were washed twice with 1 ml wash buffer containing 70% deionized formamide, 10 mM Tris, 0.1% BSA, and 0.1% Tween-20 for 7 min and once with PBS, 0.1% BSA and 0.1% Tween-20 for 5 min. After the last wash, the cells were resuspended in 300 μl of PBS, 0.1% BSA containing RNase A. A 5 μg/ml and PI 5 μg/ml and incubated overnight at 4°C prior to flow cytometric analysis. For data analysis, cells were gated on single diploid cells (Fig. 7A; gate R1). The mean tolemere fluorescence was calculated as the difference between the mean fluorescence of cells hybridized with the FITC PNA $(C_3TA_2)_3$ probe and the background control cells hybridized in the absence of the probe.

Statistical Analysis

All data were expressed as the mean ± SD. Statistical analysis was examined using an unpaired two-tailed Student's *t*-test. P values <0.05 were considered significant.

RESULTS

PCR Identification of Plasmid DNA in SMMC-7721 Transfectants

To ensure that these expression vectors were stably integrated, PCR was used to amplify the neo resistance gene contained in plasmids. Using specific primers we amplified a 409 bp DNA fragment from SMMC-7721 cells transfected with pcDNA3.1/myc-His, TrxR antisense plasmid, and TrxR sense plasmid. Amplified product was undetectable in the nontransfected SMMC-7721 cells (Fig. 2).

Effects of TrxR Antisense RNA on TrxR mRNA Level and Its Activity

To determine if the transfection of TrxR antisense constructs had any effect on TrxR expression, RT-PCR and activity assay were used to investigate TrxR mRNA levels and their activities in SMMC-7721 transfectants. The results showed that there was a significant

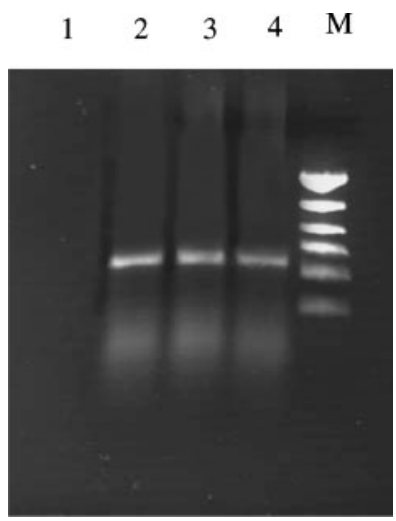

Fig. 2. PCR analysis of SMMC-7721 transfectants. Total DNA was extracted from SMMC-7721 cells (**Lane 1**) and SMMC-7721 cells transfected with pcDNA3.1/myc-His, TrxR sense plasmid, and TrxR antisense plasmid (**Lane 2–4**, respectively). PCR was performed using specific primers to detect the neo-resistance gene. **Lane M**: PCR marker.

reduction in TrxR mRNA level and activity in SMMC-7721 cells transfected with TrxR antisense plasmid compared with that in SMMC-7721 cells, but no significant difference in TrxR mRNA levels and activities was found in SMMC-7721 cells transfected with TrxR sense plasmid and pcDNA3.1/myc-His (Fig. 3). This suggested that the expression of TrxR was specially inhibited by antisense RNA.

Effects of TrxR Antisense RNA on the Growth of SMMC-7721 Cells

Based on MTT assay, SMMC-7721 cells transfected with TrxR antisense plasmid showed remarkable growth retardation compared with SMMC-7721 cells. But there was no significant difference in the rate of growth between SMMC-7721 cells, SMMC-7721 cells transfected with TrxR sense plasmid and pcDNA3.1/myc-His (Fig. 4).

Effects of TrxR Antisense RNA on Cell-Cycle Progression

To examine whether the inhibitory effects of TrxR antisense RNA on the growth of SMMC-7721 cells was associated with cell-cycle arrest, the cell-cycle distribution of SMMC-7721 transfectants was assessed by flow cytometry. As shown in Figure 5, the distribution of SMMC-7721 cells transfected with TrxR antisense plasmid in the cell cycle was significantly increased in the G_2/M phase and decreased in the G_0/G_1 phase. There was no significant change in the cell-cycle distribution of SMMC-7721 cells transfected with TrxR sense plasmid and pcDNA3.1/myc-His.

Effects of TrxR Antisense RNA on p53 and Bcl-2 mRNA Levels

p53 and Bcl-2 mRNA levels on the SMMC-7721 transfectants were determined by RT-PCR. As shown in Figure 6, TrxR antisense RNA could significantly increase p53 mRNA level and decrease Bcl-2 mRNA level. No significant difference in p53 and Bcl-2 mRNA levels was found in SMMC-7721 cells transfected with TrxR sense plasmid and pcDNA3.1/myc-His.

Effects of TrxR Antisense RNA on Telomere Fluorescence and hTERT mRNA Level

Flow Fish was carried out to determine telomere fluorescence on the SMMC-7721 transfectants. Cells were analyzed after hybridization with or without a FITC labeled $(C_3TA_2)_3$ PNA probe and gated on the basis of PI and forward scatter (FSC) (Fig. 7A; gate R1). As shown in Figure 7, the telomere fluorescence for SMMC-7721 cells transfected with TrxR antisense plasmid was significantly decreased compared with that for SMMC-7721 cells. No significant difference in telomere fluorescence was observed in SMMC-7721 cells transfected with TrxR sense plasmid and pcDNA3.1/myc-His.

RT-PCR also showed that there was a significant reduction in hTERT mRNA level in SMMC-7721 cells transfected with TrxR antisense plasmid compared with that in SMMC-7721 cells. No significant difference in hTERT mRNA level was found in SMMC-7721 cells transfected with TrxR sense plasmid and pcDNA3.1/myc-His (Fig. 8).

DISCUSSION

Oxidative stress is considered as a key factor for DNA damage. Therefore it is clear that the antioxidant Trx system is regarded as a tumor-preventing system. This applies not only to the detoxification of reactive oxygen metabolites but also to signaling processes. Recent results suggest that the selenol group of TrxR may function as a primary sensor for mutagenic H_2O_2 and initiate a signal cascade leading to the transcription of genes encoding antioxidative proteins [Sun et al., 1999]. Once a tumor has become established, the effects of Trx/TrxR system are no longer solely beneficial for the patient. Tumor proliferation is crucially dependent on a constant deoxyribonucleotide supply, which in turn depends on an active Trx/TrxR system [Spyrou and Holmgren, 1996]. Furthermore, this system provides reduced extracellular Trx as a growth factor and it protects the tumor cells from NK-lysin, tumor ncerosis factor-α, and from the respiratory burst of immune cells [Massuda et al., 1991; Andersson et al., 1996]. It is therefore not surprising that tumor cells have been observed to express several fold increased TrxR levels and it is very important to control the activity of TrxR in cancer cells, which is probably related to the pathway of cancer treatment.

This study was designed to investigate antisense RNA-mediated suppression of TrxR gene overexpression in tumor cells to determine the function of TrxR in tumor growth. We showed that the expression of TrxR antisense RNA resulted in a strong reduction in TrxR mRNA

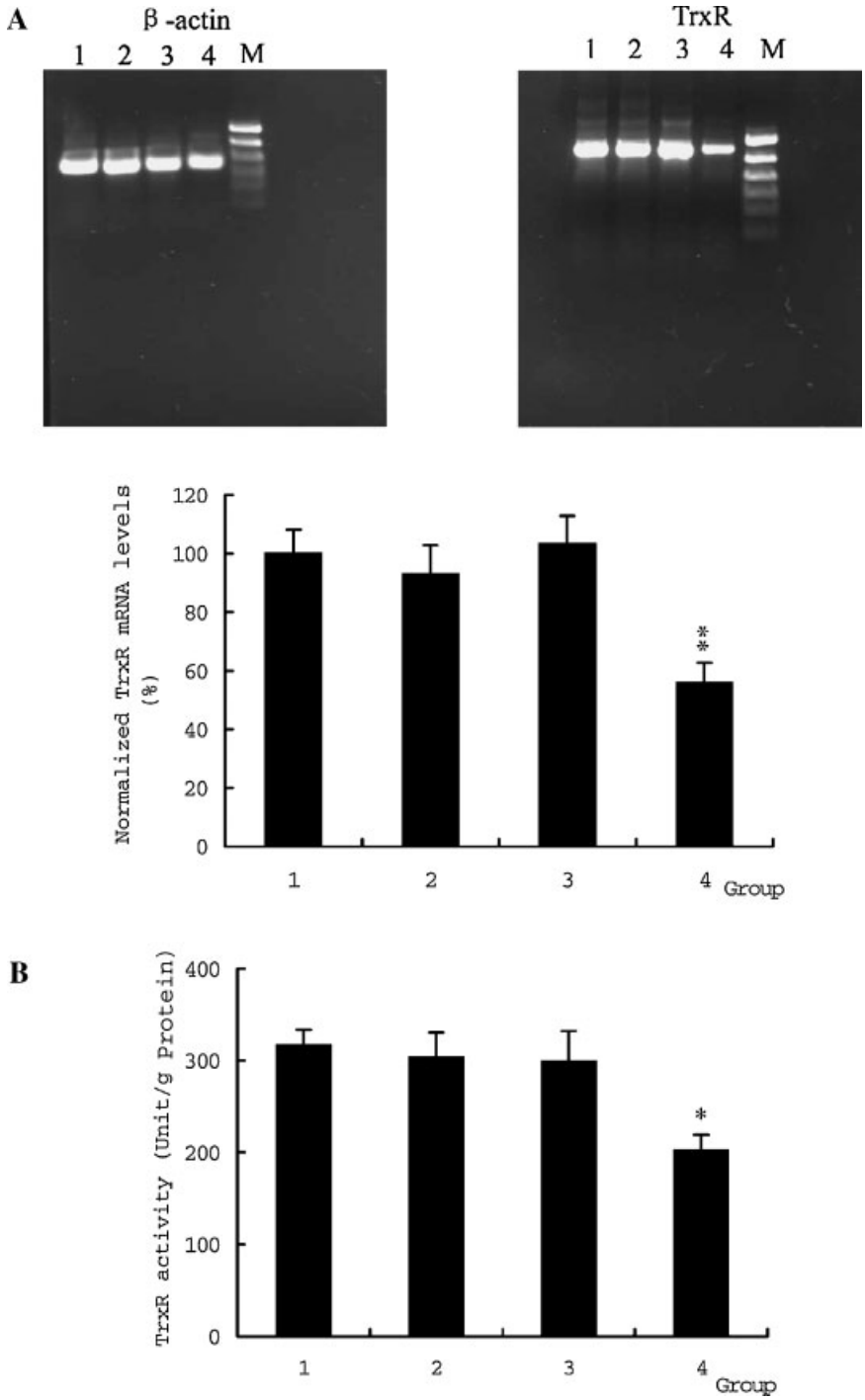

Fig. 3. Downregulation of TrxR mRNA level and activity in SMMC-7721 cells by TrxR antisense RNA. **A**: Total RNA was extracted from SMMC-7721 cells (**Lane 1**) and SMMC-7721 cells transfected with pcDNA3.1/myc-His, TrxR sense plasmid and TrxR antisense plasmid (**Lane 2–4**, respectively). TrxR mRNA levels were analyzed by RT-PCR. The value is presented as the ratio between the target mRNA and the corresponding β-actin mRNA. **Lane M**: PCR marker. **B**: TrxR activities from SMMC-7721 cells (Lane 1) and SMMC-7721 cells transfected with pcDNA3.1/myc-His, TrxR sense plasmid and TrxR antisense plasmid (Lane 2–4, respectively) were determined by enzyme analysis. Each value indicated the mean ± SD of three experiments. $^{**}P < 0.01$, $^{*}P < 0.05$ compared with SMMC-7721 cells.

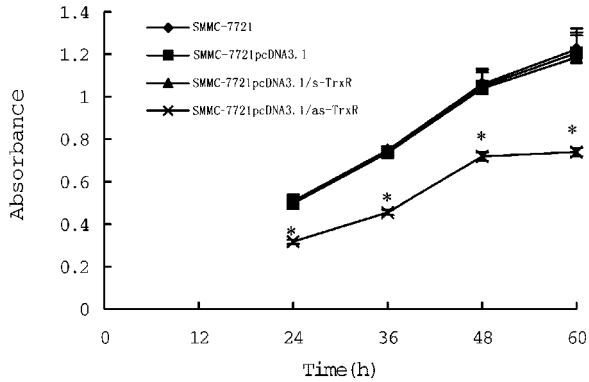

Fig. 4. Effects of TrxR antisense RNA on the viability of SMMC-7721 cells. Cells were cultured in a 96-well plate for indicated time and viability was estimated by MTT assay. Each value indicated the mean ± SD of six experiments. $^*P < 0.01$ compared with SMMC-7721 cells.

expression and activity and a significant inhibition in the proliferation of SMMC-7721 cells. Flow cytometric results revealed a reduction in the G_0/G_1 phase of the cell division cycle, but an increase of the G_2/M phase population. The results showed that the inhibition of cell growth observed in SMMC-7721 cells transfected with TrxR antisense plasmid might be due to accumulation of cells in G_2/M.

p53 and Bcl-2 protein play a central role in the regulation of cell proliferation and apoptosis, and alterations in these genes are related to oncogenesis and tumor progression [Reed, 1994; Steele et al., 1998]. The *p53* gene belongs to a class of genes known as tumor suppressor genes because of its inhibitory activity. *Bcl-2* belongs to a new class of genes that regulate cell death. TR antisense RNA resulted in a significant increase in p53 mRNA level and a decrease in Bcl-2 mRNA level, suggesting that the inhibition of cell growth might also be due to the changes of p53 and Bcl-2 mRNA levels.

Telomeres are located at the ends of chromosomes and are composed of hundreds to thousands of repeats of a 6 bp sequence (5′-TTAGGG-3′) and function critically in maintaining

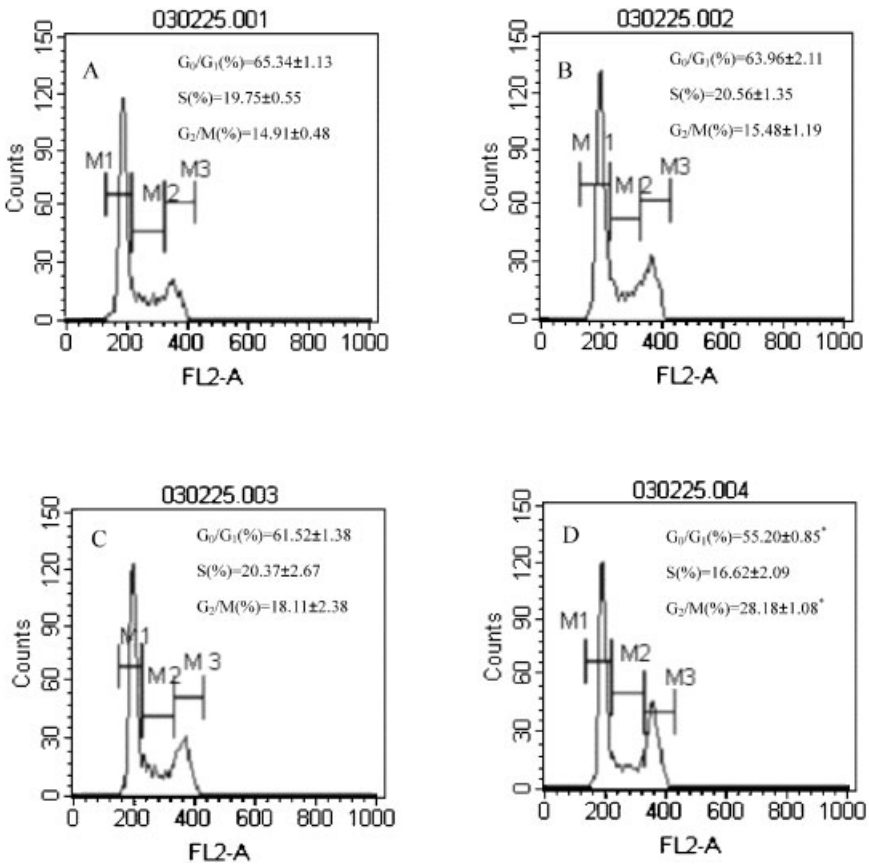

Fig. 5. TrxR antisense RNA induces G_2/M phase arrest in SMMC-7721 cells. The cell cycle phase distribution of SMMC-7721 cells (**A**), SMMC-7721 cells transfected with pcDNA3.1/myc-His (**B**), TrxR sense plasmid (**C**), and TrxR antisense plasmid (**D**) was analyzed by flow cytometry. Each value indicated the mean ± SD of three experiments. $^*P < 0.01$ compared with SMMC-7721 cells.

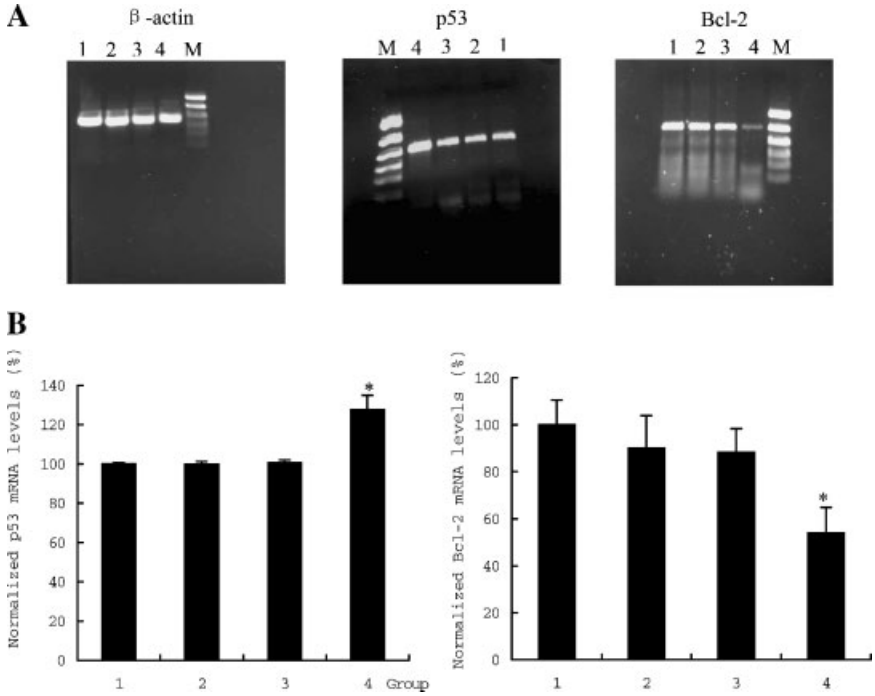

Fig. 6. (**A**) Effects of TrxR antisense RNA on p53 and Bcl-2 mRNA levels in SMMC-7721 cells. Total RNA was extracted from SMMC-7721 and SMMC-7721 cells transfected with pcDNA3.1/myc-His, TrxR sense plasmid and TrxR antisense plasmid. p53 and Bcl-2 mRNA levels were analyzed by RT-PCR. (**B**) Comparison of p53 and Bcl-2 mRNA expression levels. The value is presented as the ratio between the target mRNA and the corresponding β-actin mRNA. **Lane 1**: SMMC-7721 cells; **Lane 2–4**: SMMC-7721 cells transfected with pcDNA3.1/myc-His, TrxR sense RNA, and TrxR antisense RNA, respectively; **Lane M**: PCR marker. Each value indicated the mean ± SD of three experiments. $^*P < 0.05$ compared with SMMC-7721 cells.

chromosomal structure [Allshire et al., 1989]. The telomeric repeats at the chromosomal ends are gradually shortened during cell division [Kitada et al., 1995; Wang et al., 2002]. Telomere shortening to a critical length leads to chromosomal instability and, eventually, cell senescence and death [Harley et al., 1994]. A critical length of telomeric repeats is required for cells to sustain continuous proliferation [Hiyama et al., 1995]. Telomerase is a ribonucleoprotein enzyme that adds the TTAGGG repeats to the chromosomal ends of proliferating cells and plays a vital role in the maintenance of telomere length [Greider and Blackburn, 1989]. Telomerase activity has been detected in human germ cells and in a majority of tumor tissues and immortalized cell lines. In contrast, most mature somatic cells and tissues contain low or undetectable telomerase activity, implying that cell replication and immortality are attained through maintenance of telomere length via telomerase activity [Kim et al., 1994]. In vitro, two components are absolutely essential for the telomerase activity: hTERT, the human telomerase reverse transcriptase, and hTER, the RNA template. Recent studies have shown that hTERT expression is likely to be the rate-limiting factor in regulating telomer-

Fig. 7. Downregulation of telomere length by TrxR antisense RNA in SMMC-7721 cells. Telomere length was indirectly measured by Flow Fish in SMMC-7721 cells transfected with or without vectors. The cells were hybridized with or without a FITC labeled $(C_3TA_2)_3$ PNA probe. The cells were gated on region R1 based on the PI fluorescence and forward scatter as seen in **panel A**. The histograms obtained for telomere fluorescence intensity of SMMC-7721 cells without telomere specific probe, SMMC-7721 cells with telomere specific probe and SMMC-7721 cells transfected with pcDNA3.1/myc-His, TrxR sense plasmid and TrxR antisense plasmid with telomere specific probe are shown in **panel B–F**, respectively. **G**: Effects of TrxR antisense RNA on telomere length in SMMC-7721 cells. Q-FISH was calculated as the difference between the mean fluorescence of cells hybridized with FITC labeled $(C_3TA_2)_3$ PNA probe probe and the background control cells hybridized in the absence of the probe. **Lane 1**:SMMC-7721 cells; **Lane 2–4**: SMMC-7721 cells transfected with pcDNA3.1/myc-His, TrxR sense plasmid and TrxR antisense plasmid, respectively. Each value indicated the mean ± SD of three experiments. $^*P < 0.01$ compared with SMMC-7721 cells.

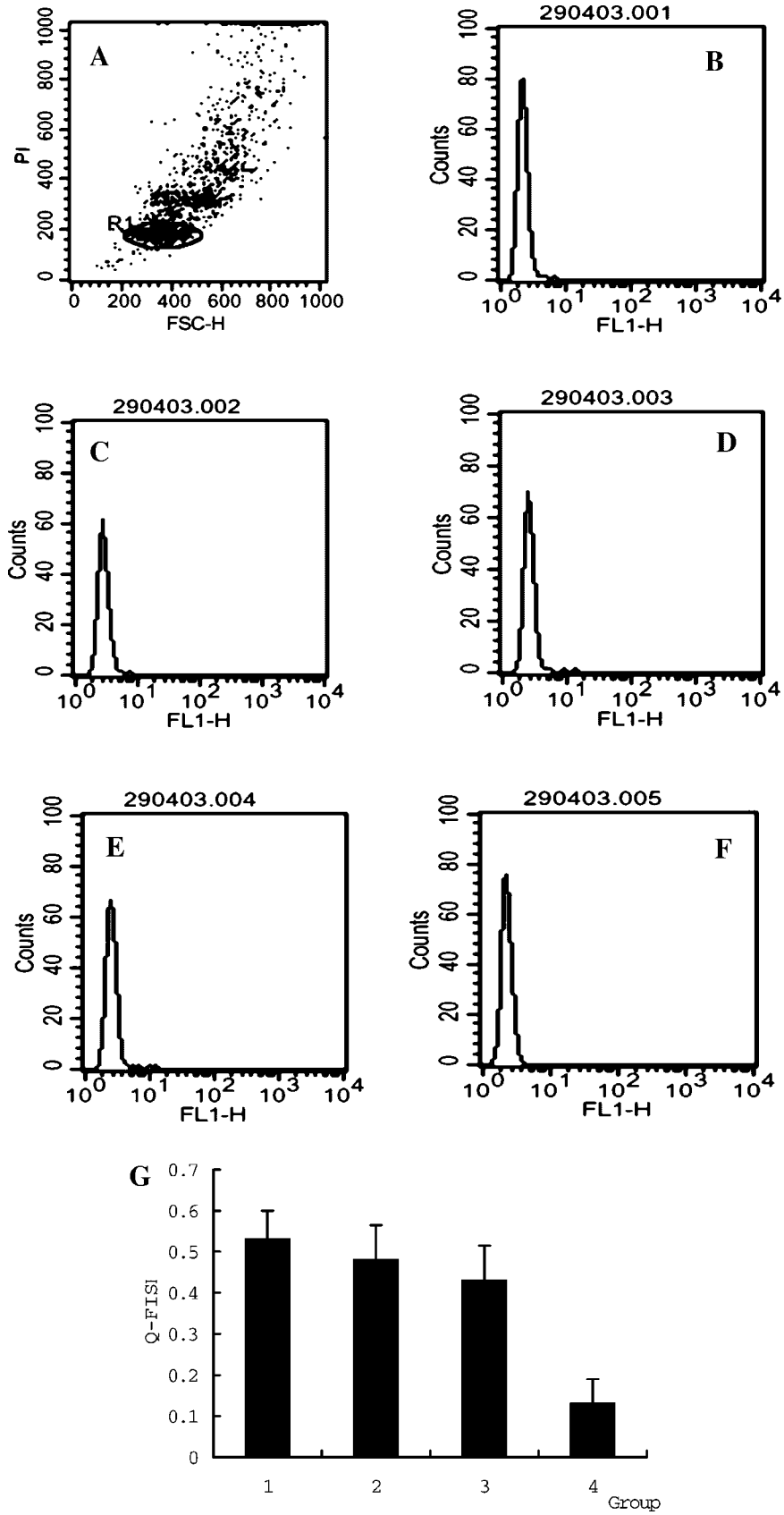
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Fig. 7.

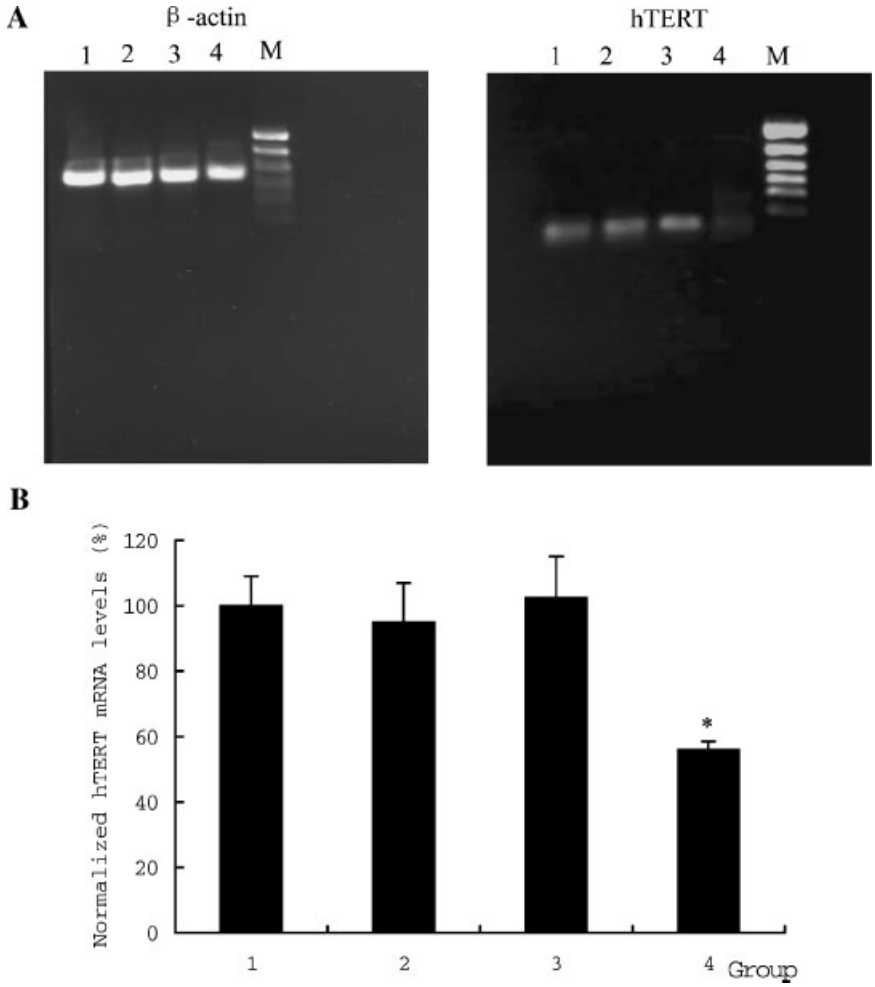

Fig. 8. Downregulation of hTERT mRNA level in SMMC-7721 cells. **A**: Total RNA was extracted from SMMC-7721 cells and SMMC-7721 cells transfected with pcDNA3.1/myc-His, TrxR sense plasmid, and TrxR antisense plasmid. hTERT mRNA levels were analyzed by RT-PCR. **B**: Comparison of hTERT mRNA expression levels. The value is presented as the ratio between the target mRNA and the corresponding β-actin mRNA. **Lane 1**: SMMC-7721 cells; **Lane 2–4**: SMMC-7721 cells transfected with pcDNA3.1/myc-His, TrxR sense RNA, and TrxR antisense RNA, respectively; **Lane M**: PCR marker. Each value indicated the mean ± SD of three experiments. $*P < 0.01$ compared with SMMC-7721 cells.

ase activity [Meyerson et al., 1997]. hTERT mRNA is undetectable in mortal, telomerase-negative cells but is induced in cells that overcome crisis by activating telomerase. More generally, hTERT is expressed at high levels in immortal cell lines and in primary human tumors. Inhibition of telomerase, telomere length and hTERT could therefore provide a new strategy for anticancer therapy. In this study, the inhibition of TrxR antisense RNA on the growth of SMMC-7721 cells might be associated with the reduced telomere length and hTERT expression.

There is abundant evidence that the regulation of telomerase is multifactorial in mammalian cells, involving telomerase gene expression, post-translational protein–protein interactions, and protein phosphorylation. Several proto-oncogenes and tumor suppressor genes have been implicated in the regulation of telomerase activity. These include c-myc, Bcl-2, Rb, p53 et al. p53 may interact directly with telomerase holoenzyme, resulting in inhibition of telomerase activity in favor of cellular senescence [Liu, 1999]. Some research have shown that p53 specific mutations were involved in telomerase activation in sun-exposed human skins [Ueda et al., 1997] and the expression of a p53 mutant in human mammary epithelial cells was occasionally associated with telomerase activation on additional genetic events [Gollahon and Shay, 1996]. In this study, we found that TrxR

antisense RNA could increase the p53 mRNA level and reduce the telomere fluorescence and Bcl-2, hTERT mRNA levels, suggesting that TrxR antisense RNA reduced the telomere fluorescence and hTERT mRNA level partly by upregulation of p53 or downregulation of Bcl-2.

In conclusion, the results provide experimental evidence that TrxR antisense RNA has an inhibitory effect on the growth of human hepatocellular carcinoma SMMC-7721 cells. Its action is probably related to accumulate the cells in G_2/M, and to increase p53 mRNA level and reduce telomere fluorescence and Bcl-2, hTERT mRNA levels in the SMMC-7721 cells. The nature of TrxR antisense RNA in mediating the growth of hepatocellular carcinoma cells could make it a potential effective therapeutic agent against liver cancer. Further studies on in vivo antitumor effect are needed.

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