

# Telomerase inhibition and telomere loss in BEL-7404 human hepatoma cells treated with doxorubicin

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## Abstract

**AIM:** To study the effects of doxorubicin on telomerase activity and telomere length in hepatocellular carcinoma.

**METHODS:** Telomerase activity was assayed with a non-radioisotopic quantitative telomerase repeat amplification protocol-based method. The effect of doxorubicin (DOX) on the growth of BEL-7404 human hepatoma cells was determined by microculture tetrazolium assay. Mean telomere length (terminal restriction fragment) was detected by Southern blot method. The expression of telomerase subunits genes was investigated by RT-PCR. Cell apoptosis and cell cycle distribution were evaluated by flow cytometry.

**RESULTS:** Telomerase activity was inhibited in a dose and time-dependent manner in BEL-7404 human hepatoma cells treated with DOX for 24, 48 or 72 h in concentrations from 0.156 to 2.5  $\mu\text{M}$  which was correlated with the inhibition of cell growth. No changes were found in the mRNA expression of three telomerase subunits (hTERT, hTR and TP1) after drug exposure for 72 h with indicated concentrations. The cells treated with DOX showed shortened mean telomere length and accumulated at the G<sub>2</sub>/M phase. However, there was almost no effects on cell apoptosis by DOX.

**CONCLUSION:** The telomerase inhibition and the telomere shortening by DOX may contribute to its efficiency in the treatment in hepatocellular carcinoma.

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## INTRODUCTION

Telomeres form the ends of eukaryotic chromosomes consisting of an array of tandem repeats of hexanucleotide 5'-TTAGGG-3'. Telomeres protect the chromosomes from DNA degradation, end-to-end fusions, rearrangements and maintain nuclear structure<sup>[1]</sup>. Human telomerase is a ribonucleoprotein complex, composed of a catalytic reverse transcriptase subunit (hTERT), an RNA component (hTR) that serves as a template for the synthesis of telomeric repeats, and an associated protein

subunit (TP1)<sup>[2-4]</sup>. It adds telomeric repeats to the 3' end of telomeric DNA. This telomere stabilization by telomerase can lead to unlimited cell proliferation.

Hepatocellular carcinoma (HCC), one of the most common malignancies in the world especially in Asia and Africa, is an aggressive cancer. It causes approximately 250 000 deaths annually<sup>[5]</sup>. It was reported that HCC exhibited a high incidence of telomerase activity and that the activity increased in accordance with the HCC degree of histological undifferentiation which was absent in normal liver tissue<sup>[6,7]</sup>. Other reports revealed that hTERT expression was the rate-limiting determinant of HCC telomerase activity<sup>[8-10]</sup>.

Doxorubicin (DOX), an antitumor antibiotic, can intercalate into base pairs of DNA and generate toxic oxygen free radicals, which not only causes single- or double-strand DNA breaks but also damages a variety of necessary macromolecules such as proteins, lipids and RNA<sup>[11]</sup>. DOX is one of the most efficient chemotherapy agents in the treatment of HCC, and its total efficiency rate can be up to 44 %<sup>[12]</sup>. However, the relationship between the efficiency of DOX and telomerase activity in HCC has not yet been elucidated. In the present study, we investigated the effects of DOX on the telomerase activity and telomere length in BEL-7404 human hepatoma cells.

## MATERIALS AND METHODS

### Cell and culture condition

BEL-7404 human hepatoma cell line from Cell Bank of Chinese Academy of Sciences<sup>[13]</sup>, was cultured in RPMI-1640 medium (Gibco) supplemented with 10 % heat-inactivated newborn calf serum, at 37 °C in a humidified CO<sub>2</sub> incubator containing 5 % CO<sub>2</sub> and 95 % air.

### Drug

DOX (Sigma) was dissolved in RPMI-1640 to the final concentration of 5mM and stored at 4 °C.

### Assessment of cell proliferation

An MTT assay was conducted to determine the cell proliferation. Cells were seeded at  $1 \times 10^4$  cells/well in a 96-well plate and incubated overnight. The drug was added to the cultured cells with the final concentrations from 0.156  $\mu\text{M}$  to 2.5  $\mu\text{M}$  and culturing further for another 24, 48 or 72 h respectively. Following culture, the cells were incubated with 800 mg/L 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma), which was used to assay the activity of mitochondrial dehydrogenases. Four hours later, 10 % sodium dodecyl sulphate - 5 % isobutanol-0.12 % hydrochloric acid solution was added to solubilize the formazan product. The plate was then incubated at 37 °C for another 12 h. The absorbance at 570 nm was measured with a model 550 microplate reader (Bio-Rad). The percent of cell growth inhibition was expressed as:  $(A-B)/A \times 100\%$ , where A was the absorbance value from the controls and B was that from the experimental cells.

### Telomerase assay

Telomerase activity was assayed with PCR-based telomeric repeat amplification protocol (TRAP) as previously described<sup>[14, 15]</sup>. Cells were collected and washed with PBS, lysed in 1×3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid (CHAPS, Sigma) buffer, incubated on ice for 30 min, and centrifuged at 12 000 g for 30 min. The protein concentration was determined by Coomassie Protein Assay. Each of TRAP reactions contained 1 µg of total protein. The reaction mixture [20 mM Tris-HCl (pH 8.3), 1.5 mM MgCl<sub>2</sub>, 63 mM KCl, 0.005 % Tween-20, 1mM EGTA, 50 µM of each dNTPs and 0.1 µg TS (5' - AATCCGTCGAGC AGAGTT-3' )] was incubated at 30 °C for 30 min, heated at 94 °C for 5 min. Then 0.1 µg of return primer ACX(5' - GCGCGG[CTTACC]<sub>3</sub>CTAACC-3' ), 0.1 µg of internal control primer NT(5' -ATCGTCTCTCGCCTTTT-3' ), 0.01 aM of internal control template TSNT(5' -AATCCGTCGAGC AGAGTTAAAGGCCGAGAAGCGAT-3' ) and 2 units Taq DNA polymerase ( Promega) were added. The reaction mixture was then subjected to 28 PCR cycles: 94 °C for 30s and 60 °C for 30 s. PCR products were separated by electrophoresis on 12 % nondenaturing polyacrylamide gels and stained with SYBR Green I ( FMC ) for 15 min, visualized and analyzed by UVP system. In every experiment, a negative control (1 µl CHAPS lysis buffer) was included. All of experiments were repeated at least twice. The relative telomerase activity was quantified by the formula: TP=[(A/B)/(A cell control/ B cell control)]×100 Where TP=total product, A=total intensity of telomerase product (50 bp,56 bp,62 bp.), and B=intensity of internal control (36 bp).

### RT-PCR

Total cellular RNA was extracted from cells using Trizol (Life Technologies, Inc.) according to the instructions of the manufacturer. In each reaction, 1 µg of total RNA was reverse transcribed into cDNA using M-Mlv reverse transcriptase (Promega). Primer sets used to amplify specific sequences were 5' -CGGAAGAGTGCTCTGGAGCAA-3' and 5' -GGATGAAGCGGAGTCGGA-3' for hTERT (146 bp); 5' -TCTAACCTAACTGAGAAGGGCGTAG-3' and 5' -GTTTGCTCTAGAATGAACGGTGGAAAG-3' for hTR (126 bp); 5' -TCAAGCCAAACCTGAATCTGAG-3' for TP1 (264 bp); 5' -GTGGGGCGCCCCAGGCACCA-3' and 5' -GTCCTTAATGTCACGCACGATTTTC-3' for β-actin (539 bp). The PCR conditions of hTERT and TP1 were 94 °C, 45s; 60 °C, 45 s; 72 °C, 90 s for 31 and 29 cycles, respectively. And the PCR conditions of hTR and β-actin were 94 °C, 45 s; 55 °C, 45 s; 72 °C, 90 s for 28 and 22 cycles, respectively<sup>[2]</sup>.

### Telomere length assay

Genomic DNA samples were prepared as described<sup>[16]</sup>. Cells were lysed and proteins were digested in 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 25 mM EDTA, 0.5 % SDS, 0.1 mg/ml proteinase K at 48 °C overnight. Following two extractions with phenol and one with chloroform, DNA was precipitated with ethanol and dissolved in 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (TE). Telomere length was detected using TeloTAGGG telomere length assay (Roche) according to the manufacturer's protocol. For each sample, 1 µg of genomic DNA was digested with Rsa I/Hinf I (Sigma), separated on a 0.8 % agarose gel, transferred to a nylon membrane (Amersham Hybond-N<sup>+</sup>), and hybridized with a telomere specific digoxigenin(DIG)-labeled probe, incubated with anti-DIG-alkaline phosphatase and detected by chemiluminescence. The blotted signal was divided into 30 equidistant intervals from 1.9 to 21.2 kilobases to calculate mean telomere length

(terminal restriction fragment, TRF) using the formula  $TRF = \frac{\sum (OD_i)}{\sum (OD_i/L_i)}$ , where OD<sub>i</sub> was the chemiluminescent signal and L<sub>i</sub> was the length of the TRF fragment at position i<sup>[17]</sup>.

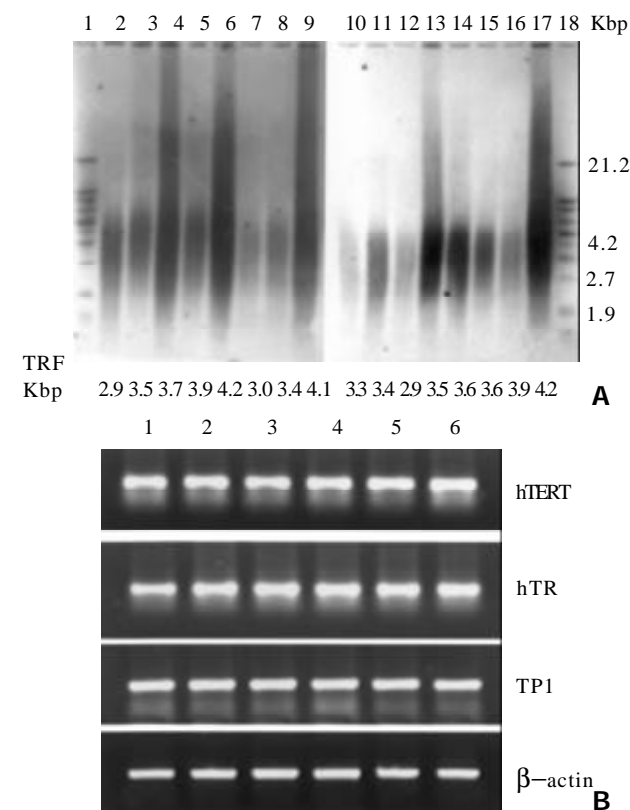
### Flow cytometry analysis of cell cycle and apoptosis

The cells were harvested and resuspended in the solution containing 40mM sodium citrate, 250mM sucrose and 5 % DMSO. The suspension was stored at -20°C for 20 min, then thawed rapidly at room temperature and centrifuged to collect the cells. The cells were resuspended in a solution containing RNase A (5×10<sup>4</sup> unit/g, 50 mg/L) and 20 mg/L propidium iodide (PI). The cell cycle distribution and apoptosis were determined by the fluorescence of individual cells measured with flow cytometry<sup>[18]</sup>.

## RESULTS AND DISCUSSION

### Inhibition of telomerase activity

Telomerase activity was inhibited in a dose and time-dependent manner in BEL-7404 human hepatoma cells treated with DOX (Figure 1A). To analyze the telomerase inhibition on the gene expression level, cells treated with DOX for 72 h were employed to study the telomerase mRNA expressions of its three major gene components, hTERT, hTR and TP1, using RT-PCR. Results showed that no changes were observed in the mRNA expression pattern of these subunits after the DOX exposure for 72 h with indicated concentrations (Figure 1B).



**Figure 1** Telomerase inhibition by DOX in BEL-7404 human hepatoma cells. **(A)** Inhibition of telomerase activity by DOX in a dose and time-dependent manner in BEL-7404 human hepatoma cells. Lane 1, negative control; Lane 2, cell control; Lane 3-7, telomerase activity in presence of DOX for 24 h (2.5, 1.25, 0.625, 0.313 and 0.156 µM); Lane 8-12, telomerase activity in presence of DOX for 48 h (2.5, 1.25, 0.625, 0.313 and 0.156 µM); Lane 13-17, telomerase activity in presence of DOX for 72 h (2.5, 1.25, 0.625, 0.313 and 0.156 µM). All experiments were repeated at least twice and representative results were shown here. **(B)** RT-PCR analysis of hTERT, hTR and TP1 mRNA expression with DOX treatment for 72 h in a concentration range from 0.156

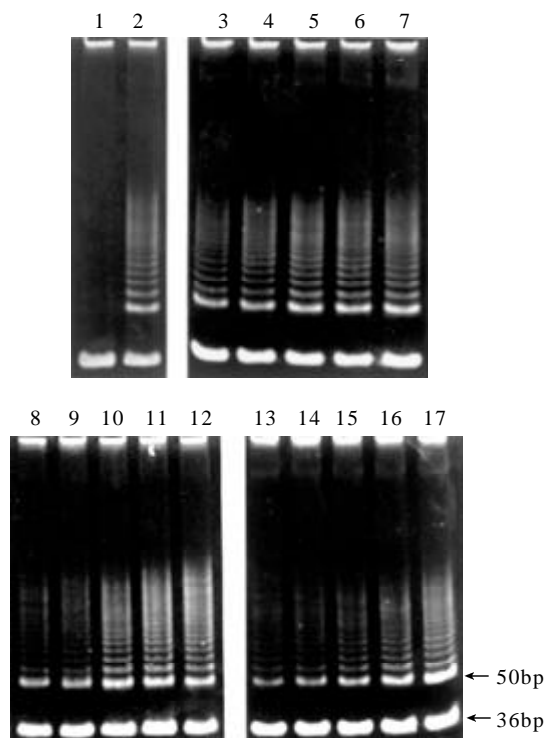
to 2.5  $\mu\text{M}$ . Lane 1, cell control; Lane 2, 2.5  $\mu\text{M}$ ; Lane 3, 1.25  $\mu\text{M}$ ; Lane 4, 0.625  $\mu\text{M}$ ; Lane 5, 0.313  $\mu\text{M}$ ; Lane 6, 0.156  $\mu\text{M}$ .  $\beta$ -actin was used as standard. Amplified sequences for hTERT, hTR, TP1 and  $\beta$ -actin are 146, 126, 264 and 539 bp, respectively.

The first report on the telomerase inhibition by DOX was presented by Zhu *et al.*, regarding the SW480 colon carcinoma cells<sup>[19]</sup>. However, the opposite observations were reported later, in which authors found no effect of DOX on telomerase regulation in nasopharyngeal cancer, testicular cancer and squamous cell carcinoma<sup>[20-22]</sup>. In this study, we found that the DOX treatment inhibited the telomerase activity in a dose and time-dependent manner in BEL-7404 human hepatoma cells. Considering the different results above, We think that this kind of inhibition might be cell-type specific and may be correlated with the clinical different efficiency of DOX in the treatment of different kinds and stages of tumors.

Abundant evidence indicated that the regulation of telomerase was multifactorial in mammalian cells and involves telomerase gene expression, post-translational protein-to-protein interactions, and protein phosphorylation<sup>[23]</sup>. However, there was no information regarding three telomerase subunits expression affected by DOX in Zhu's study<sup>[18]</sup>. In our investigation, there were no changes found in the expression of hTERT, hTR or TP1 mRNA. This indicated that the telomerase inhibition by DOX might be indirect with its antibiotic activity (eg.J damaging of necessary macromolecules).

### Shortening of mean telomere length

Mean telomere length of BEL-7404 human hepatoma cells was decreased by the DOX treatment (Figure 2). Here, we believe that this is the first report regarding the shortening of telomere length by DOX treatment in BEL-7404 human hepatoma cells.



**Figure 2** Shortening of mean telomere length ( terminal restriction fragments,TRF) in DOX treated BEL-7404 human hepatoma cells. Lane 1 and 18, molecular marker;Lane 17,cell control; Lane 2 – 6, DOX treated for 72 h (2.5, 1.25, 0.625, 0.313 and 0.156  $\mu\text{M}$ ); Lane 7– 11:DOX treated for 48 h (2.5,1.25,0.625, 0.313 and 0.156  $\mu\text{M}$ ); Lane 12-16:DOX treated for 24 h (2.5,1.25, 0.625, 0.313 and 0.156  $\mu\text{M}$ ).

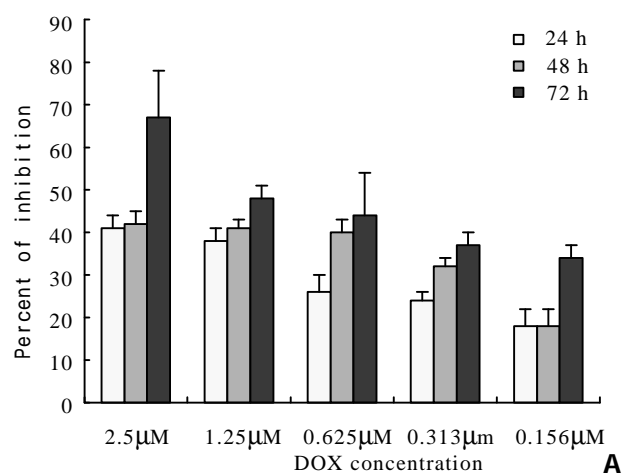
Compared with normal cells and some other carcinoma cells (such as HeLa cells), the mean telomere length of BEL-7404 human hepatoma cells is relatively short. As a result, the hepatoma cells are sensitive to telomere shortening, which may account, at least in part, for their chemosensitivity. Results from telomere shortening by cisplatin indicate that the nucleotide-excision repair system and cell division dynamics might be involved in the telomere shortening process, which make the process showing in a non- dose or time dependent manner<sup>[24]</sup>. We assume that both of the factors may likewise participate in telomere shortening by DOX, because DOX can intercalate into DNA base pairs and result in non-dose or time dependent manner of telomere shortening.

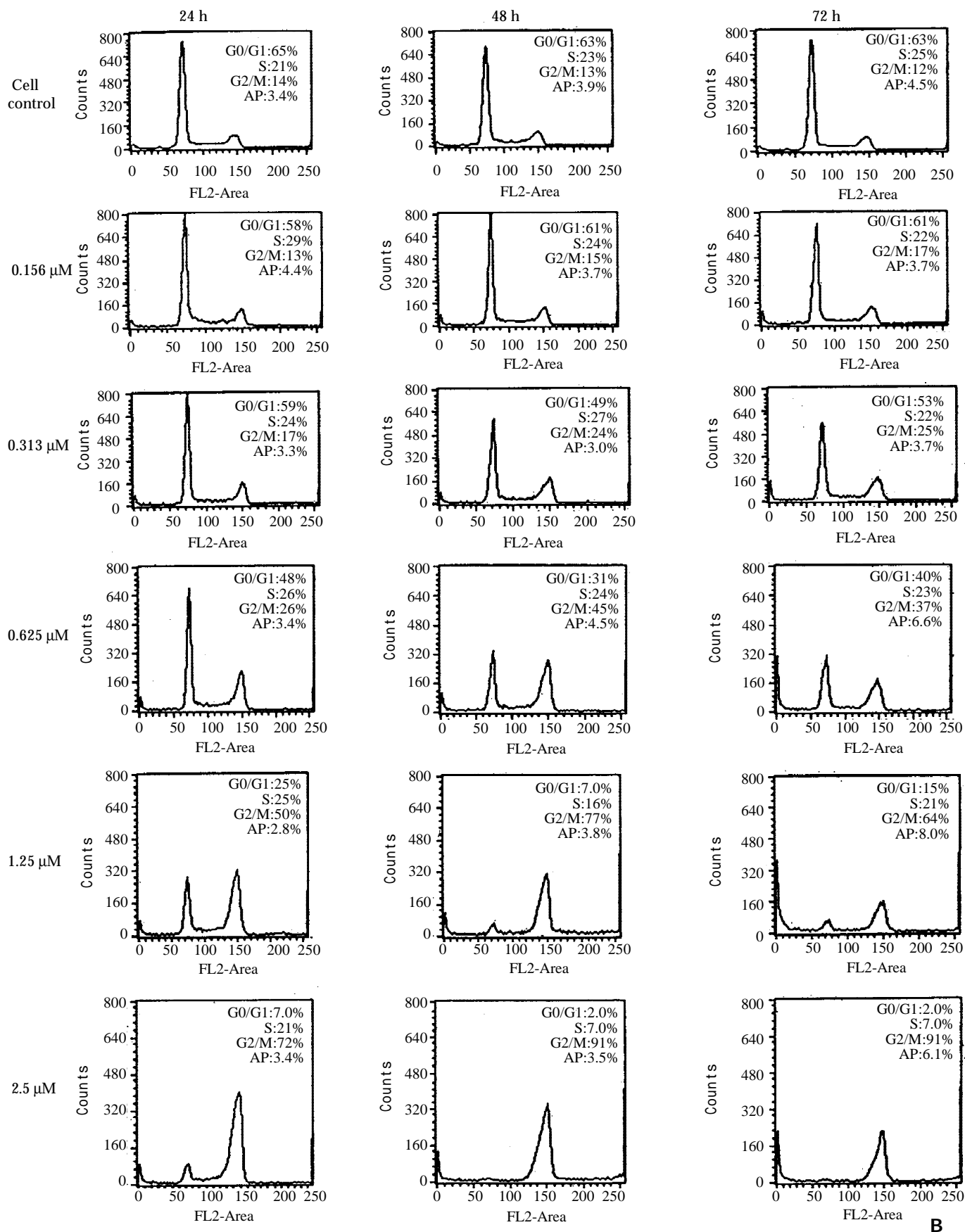
### Accumulation of cell cycle in G<sub>2</sub>/M phase

The cell growth inhibition and cell cycle progression during DOX treatment were monitored in the present study to correlate these effects with telomerase activity inhibition and telomere loss. The growth of BEL-7404 cells was inhibited by DOX, which was indicated in (Figure 3 A). Following exposure to DOX, the hepatoma cells were accumulated in G<sub>2</sub>/M phase, which revealed by flow cytometry ( Figure 3 B). There were no marked changes observed in cell apoptosis in the experimental cells exposure to DOX for 24, 48 or 72 h with the concentrations from 0.156 to 2.5  $\mu\text{M}$ , compared with the control cell.

Zhu *et al.*<sup>[19]</sup> attributed the reduction in telomerase activity by DOX to the accumulation of cell cycle in G<sub>2</sub>/M phase. But Holt *et al.*<sup>[25]</sup> found that telomerase inhibition did not correlate with the cell cycle arrested at G<sub>2</sub>/M phase but with the increasing in cell death. In present study, the cell apoptosis did not increase markedly compared with the control. Our previous research has also showed that antisense oligonucleotide to telomerase RNA component accumulated the cell cycle in G<sub>2</sub>/M phase<sup>[26]</sup>. The present observation confirmed the reports above, which indicated that telomerase inhibition was correlated with the cell cycle arrested at G<sub>2</sub>/M phase.

Previously, Ishibashi *et al.*<sup>[24]</sup> have reported that telomere loss in HeLa cells associated with the apoptosis induced by cisplatin. However, in our investigation the percent of cell apoptosis did not change markedly ( Figure 3 B), although the mean telomere length was reduced by DOX. In BEL-7404 human hepatoma cells, we previously found that the cell apoptosis occurred when the mean telomere length reached to about 1.7Kb<sup>[27]</sup>. In this study, the mean telomere did not reach to this critical length, and the apoptosis had not been induced which was consistent with the previous observation. Therefore, we deduce that telomere shortening may not be correlated with the apoptosis.





**Figure 3** Effects of DOX on cell growth, cell cycle and cell apoptosis in BEL-7404 human hepatoma cells.

**(A)** Growth inhibition of BEL-7404 human hepatoma cells treated with DOX. Each value represents mean  $\pm$  SD from triplicate wells. **(B)** Cell cycle distribution and apoptosis in DOX treated BEL-7404 human hepatoma cells, analyzed by flow cytometry. Cell cycle was arrested at G<sub>2</sub>/M with the treatment of DOX. Histograms of DNA contents of untreated cell control and treated with 0.156, 0.313, 0.625, 1.25 and 2.5  $\mu$ M were shown. Cells were maintained in the presence of DOX for 24, 48, and 72 h without a change in medium and collected at the times indicated. AP means the percent of apoptosis.

Many studies have shown that the telomerase activity was correlated with the cell growth<sup>[28]</sup>. Our finding also revealed a good correlation between the inhibition of telomerase activity and the reduction in cell growth. However, in this study, the cell growth inhibition was mainly the result of cell cycle arrest, but not the increasing of cell apoptosis.

In conclusion, we found that telomerase activity was inhibited in a dose and time - dependent manner and mean telomere length was decreased by the treatment of DOX in BEL-7404 human hepatoma cells. This process correlated with the cell growth inhibition and the cell cycle accumulation in G<sub>2</sub>/M phase. The present study indicates that the telomerase inhibition and the telomere shortening by DOX may contribute to its efficiency in the treatment of HCC.

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