

# Survivin deficiency induces apoptosis and cell cycle arrest in HepG2 hepatocellular carcinoma cells

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**Abstract.** The postulated dual roles of survivin as an anti-apoptotic factor and a mitotic inducer have placed this factor in the spotlight of cancer research. The purpose of this study was to investigate whether survivin might connect the cell cycle with apoptosis. Here, by simultaneously monitoring survivin deficiency-induced morphological changes of HepG2 cells using time-lapse imaging as well as determining apoptosis progression, we observed synchronized defective mitosis characterized by multinucleated and polyploid cells and cell cycle arrest at S phase or G2/M phase followed by apoptosis, the processes of which depended on the simultaneous destruction of specialized subcellular compartments of survivin and activation of caspase-3-like protease. These findings showed that the survivin protein acted as mitotic regulator and apoptosis inhibitor, but may also possess the role of a bridge in integrating apoptosis and cell division. An essential prerequisite of this pathway was the specialized subcellular localization of survivin. The overexpression of survivin was required to maintain cell viability and proper cell cycle transitions, and to preserve genetic fidelity during cell division in HepG2 cells.

## Introduction

Survivin, a member of the inhibitor of apoptosis (IAP) gene family (1), is overexpressed in almost all human tumors and cancer cell lines including HepG2, Huh7, sk-Hep1, and are frequently associated with resistance to cancer therapy with unfavorable outcome (2-4). Experimental work carried out

*in vitro* (5) and in transgenic animals (6,7) has assigned a dual function to survivin, protection from apoptosis and regulation of cell division. In recent years, several important mitotic functions have been associated with survivin, leaving little doubt about the importance of survivin as a regulator of cell cycle. As a part of the large chromosomal passenger complex (CPC), survivin might interact directly with other chromosomal passengers, including INCENP, Aurora B and Borealin (7-10). Evidence shows that survivin has four-dimensional regulation of mitotic events concentrated near centromeres in G2, diffuse at chromosome arms and concentrated at inner centromeres in prophase and (pro) metaphase. In anaphase, survivin is no longer associated with the centromeres but relocates to the central spindle and is positioned at the midzone in anaphase B. During cytokinesis, survivin accumulates at the midbody and is eventually expelled from the cell after abscission of the two daughter cells (11-13). Interestingly, the cell cycle-dependent expression of survivin and its antiapoptotic function has led to the hypothesis that survivin connects the cell cycle with apoptosis, thus providing termination of defective mitosis (14,15). However, recent experiments show that, although division in the absence of survivin results in defects in chromosome alignment, failure of cytokinesis and eventually cell death (9,10,12,16,17), the ensuing cell death appears to be a late event (18), does not occur in mitosis, and coincides with a decrease in the number of polyploid cells. Thus, it is suggested the cell death induced after survivin depletion is more likely to be an indirect consequence of aberrant cell division rather than of a failure to directly inhibit caspases, indicating that survivin might not be a survival factor or that its role of cytoprotective function is independent of caspase inhibition. Furthermore, although survivin has been widely recognized as an attractive target for cancer therapy and the survivin pathway is beginning to be explored in liver cancer (19,20), it is still unclear whether the control of apoptosis is an obligatory requirement for hepatocellular cell cycle transitions (14,21). In this study, by simultaneously time-lapse imaging of HepG2 cells after antisense depletion of the survivin antiapoptotic effect, we observed synchronized defective mitosis characterized by multinucleated and polyploidy cells and cell cycle arrest at S phase or G2/M phase followed by apoptosis, the processes of which depended on the simultaneous destruction of specialized subcellular compartment of survivin and activation of caspase-3-like protease.

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**Abbreviations:** IAP, inhibitor of apoptosis; CPC, chromosomal passenger complex; INCENP, inner centromere protein

**Key words:** apoptosis, cell cycle, survivin, hepatocellular carcinoma

Table I. Sequences of oligonucleotides for survivin.

Antisense	5'-CCCAGCCTTCCAGCTCCTTG-3'
Sense	5'-CAAGGAGCTGGAAGGCTGGG-3'

## Materials and methods

**Antisense oligonucleotides for survivin.** According to the method of Olie *et al.* (22), antisense and sense oligonucleotides for survivin were designed. The antisense oligonucleotide which targets nucleotides 232-251 revealed the strongest effect and was used in the following experiments. The sequences are shown in Table I. Phosphorothioate oligonucleotide ODNs were purchased from Sangon (Shanghai China), and were delivered in the form of complex with Lipofectamine 2000 (Lip) (Invitrogen, USA).

**Cell line and cultures.** Liver cancer cell line HepG2, which is reported to express high levels of survivin, was obtained from Shanghai Institute of Cell Biology. Cells were grown in DMEM medium (Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (Shi Ji Qing, Hangzhou, China). Cells were maintained in monolayer cultures at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air.

**Treatment of HepG2 cells with antisense survivin oligonucleotides.** One day before transfection, HepG2 cells were plated in, 96-, 24-, or 6-well tissue culture plates. Oligonucleotides were delivered in the form of survivin with Lip as described above. After a 24-h transfection, the transfection medium was replaced by medium without transfection reagent. HepG2 cells were harvested 24, 48 or 72 h after the start of transfection.

**RNA extraction and RT-PCR.** Total cellular RNA was extracted using RNeasy Plant Mini kit reagent according to the manufacturer's recommendations (Qiagen, Germany). The quantity and quality of RNA was assessed spectrophotometrically at 260 and 280 nm (the A260/ = A280 ratio of pure RNA is ~2). RT-PCR amplification product was synthesized with 0.1 µg of total RNA using OneStep RT-PCR kit (Qiagen, Germany), and was employed according to the manufacturer's protocol. Primers used for amplification were human survivin sense primer corresponding to nucleotides 47-66: 5'-GGCATGGGTGCCCCGACGTT-3', and antisense primer complementary to nucleotides 466-485: 5'-AGAGGCCTCAATCCATGGCA-3'. Amplification of human β-actin served as a endogenous control, β-actin sense primer corresponding to nucleotides 578-609: 5'-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3', and antisense primer complementary to nucleotides 1415-1384: 5'-CGTCATACTCCTGCTTCCTGATCCACATCTGC-3'. Following optimization of conditions: reverse transcription 30 min at 50°C, initial PCR activation step 15 min at 95°C; 3-step cycling, denaturation 1 min at 94°C, annealing 1 min at 62°C, extension 1 min at 72°C, number of cycle 27, final extension 10 min at 72°C. PCR products were resolved on a 2% agarose gel and the bands were visualized by ethidium bromide staining.

**Western blot analysis.** Cells were collected on ice and centrifuged at 1,500 rpm for 5 min at 4°C. The cells were resuspended and washed with ice-cold PBS buffer and centrifuged again at 1,500 rpm for 5 min at 4°C. Supernatants were discarded and cell pellets were lysed in 2X cell lysis buffer [2% Triton X-100, 300 mmol/l NaCl, 20 mmol/l Tris-HCl pH 7.2, 10% glycerol and 4% SDS] and heating at 95°C for 10 min. The lysates were centrifuged at 14,000 rpm for 30 min at 4°C and the protein supernatants were collected and stored at -20°C. The protein concentrations were determined by BCA assay according to the manufacturer's instructions (Pierce Co.). Equal amounts of proteins (20 µg/well) were separated on 7.5-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis for the detection of survivin. Following electrophoretic transfer of protein onto nitrocellulose membranes, immunoblots were sequentially incubated in 5% skimmed milk blocking solution at room temperature for 2 h. The membranes were incubated in a solution containing anti-human survivin antibody (1:800; Oncogene, USA) overnight at 4°C. After washing with three changes of 1% PBS-Tween solution at room temperature for 1 h, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000; Oncogene, USA) at room temperature for 2 h. Finally, after rewashing the membranes for 1 h, protein bands were detected by using the ECL system according to the manufacturer's instructions (Amersham Life Sciences). β-actin was used as a protein loading control.

**Measurement of caspase activation.** Caspase-3-like protease activity in cells was analyzed by flow cytometry (FACScalibur, Becton-Dickinson, USA) using of a PE-conjugated Polyclonal Rabbit Anti-Active Caspase-3 Antibody kit (Becton-Dickinson). At 0, 24, 48 and 72 h after antisense survivin induction, respectively, cells were harvested and washed twice with PBS, fixed in ice-cold 70% ethanol and stored at 4°C, prior to analysis, cells were again washed with PBS and was employed according to the manufacturer's recommendations. Cytometric analyses were performed using a flow cytometer and CellQuest software. Approximately 20,000 cells were counted for each determination.

**Flow cytometry analysis.** HepG2 cells treated with control or antisense survivin (100, 300 and 600 nM) were collected at 24-72 h after transfection. The cells were again washed twice with PBS, fixed in cold methanol, and rewashed with PBS to remove methanol. They were suspended in 500 ml of PBS and then digested with 20 mg/ml RNase at 37°C for 30 min and chilled on ice for 10 min. The cellular DNA was then stained with 50 mg/ml propidium iodide and incubated for 1 h at room temperature in the dark. The cell cycle distribution was analyzed by flow cytometry using a Becton-Dickinson FACScan.

**Electron microscopic examination.** HepG2 cells treated with control or survivin antisense were collected at 24-48 h after transfection. Cells were seeded into 30-mM dishes, collected on ice with medium by gently scraping, and washed three times in ice-cold PBS. The cell pellets were fixed with a solution of 2% formaldehyde and 3% glutaraldehyde in 0.1 mol/l sodium cacodylate buffer (pH 7.4) for 1 h at 48°C. The fixed

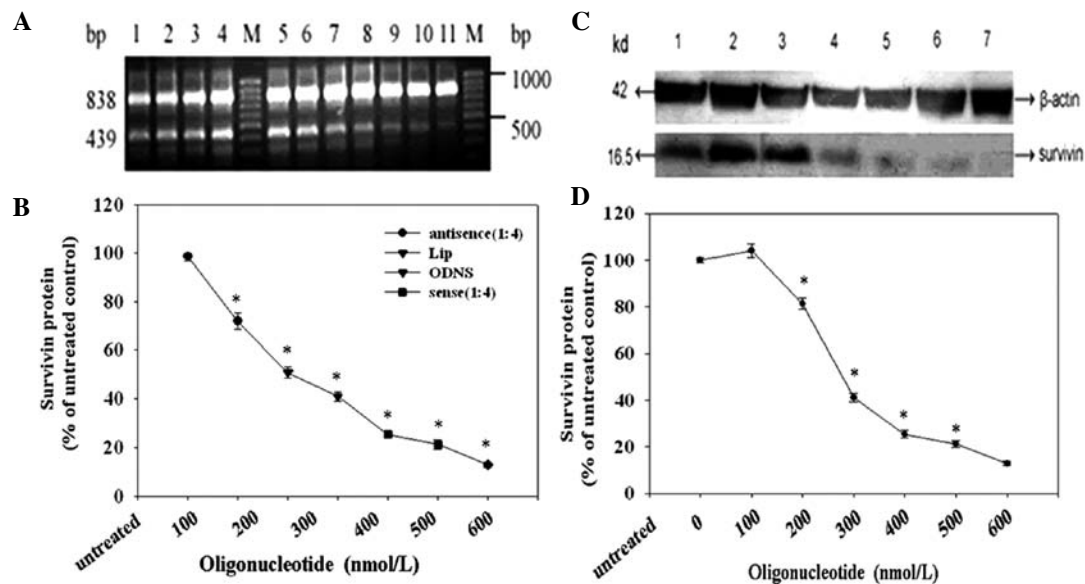


Figure 1. Survivin mRNA and protein expression in HepG2 cells was down-regulated in dose-dependent manner on treatment with antisense survivin. (A) RT-PCR assay detected the expression of survivin mRNA in HepG2 cells when treated with sense compound (lane 1), Lip (lane 2), ODNs (lane 3), untreated cells (lanes 4 and 5), 100 nmol/l antisense survivin (lane 6), 200 nmol/l (lane 7), 300 nmol/l (lane 8), 400 nmol/l (lane 9), 500 nmol/l (lane 10), and 600 nmol/l (lane 11), respectively. M, marker. (B) RT-PCR quantification of the relative expression of survivin mRNA in HepG2 cells when treated by antisense survivin, Lip, ODNs, and the sense, respectively, using  $\beta$ -actin as internal standard; the level of survivin mRNA relative to  $\beta$ -actin in untreated cell maintained under identical experimental conditions was taken as 100%. (C) Survivin protein expression was determined by Western blotting in HepG2 cells when treated with control (lane 1), 100 nmol/l antisense compound (lane 2), 200 nmol/l (lane 3), 300 nmol/l (lane 4), 400 nmol/l (lane 5), 500 nmol/l (lane 6), 600 nmol/l (lane 7). (D) Western blot quantification of the relative expression of survivin protein in HepG2 cells, using  $\beta$ -actin as internal standard.

cells were washed three times in cacodylate buffer (pH 7.2) containing 0.2 mol/l sucrose, postfixed with 1% osmic acid in 0.3 mol/l cacodylate buffer, dehydrated in a graded series of acetone, and embedded in epoxy resin. Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and assessed by using a TECNA10 electron microscope (Philips, The Netherlands).

**Immunofluorescence and confocal laser scanning microscopy.** Cells were cultured on microscope slides. Then, treated or not treated with antisense survivin (1:4) for 48 h. Cells were washed three times with PBS for 15 min and fixed in ice-cold paraformaldehyde for 30 min. Then fixed in ice-cold CAM (chloroform:acetone:methanol = 1:1:2) at  $-20^{\circ}\text{C}$  for 30 min, and washed three times with PBS for 15 min. Cells were incubated for overnight at  $4^{\circ}\text{C}$  with polyclonal anti-survivin (1:400 diluted; rabbit polyclonal IgG; Oncogene, USA), then washed three times with PBS for 30 min, and incubated for 2 h at room temperature with fluorescein (FITC)-conjugated anti-rabbit-IgG (1:1000; Oncogene). After a further washing step, cell membranes were stained for 2 min with a  $5 \times 10^{-3}$  % (w/v) solution of Alexa-labelled concanavalin A (MoBiTec, Gottingen, Germany) in BPS. Finally, the cells were embedded in a gel containing 22.73% (w/w) glycerine, 9.1% (w/w) Mowiol 4-88 (a present from Ningbo Institute of Microcirculation and Henbane), 22.73% (w/w) double-distilled water, 45.45% (w/w) 0.2 mol/l Tris-buffer, pH 8.5, and 2.5% (w/w) DABCO (a present from Ningbo Institute of Microcirculation and Henbane) as an anti-fading agent. After 12 h, confocal microscopy was performed with a microscope (TCS-SP2, Leica, Germany) equipped with an argon/krypton laser. Two channel image recording at 488 and 633 nm

laser excitation was used. Optical filters were chosen for the FITC and TRLTC range. All optical sections were recorded with the same laser and detector settings using software (Leica Confocal, Germany). Further image processing was performed with software (Leica Confocal) on a computer system. Confocal stacks of green and red fluorescence were visualized in section view mode.

**Statistical methods.** All statistical analyses were performed by the SPSS 11.0 software package for Windows (SPSS Inc., Chicago, IL). The one-ANOVA test was used to compare the distribution of individual variables. A two-tailed P-value  $<0.05$  was considered statistically significant.

## Results

**Down-regulation of survivin mRNA and protein by antisense survivin.** To determine the knock-down effect of antisense survivin in HepG2 cells, we transfected cells, where survivin was highly expressed, with different concentrations of survivin antisense-oligonucleotides (100–600 nM). Cells transfected with sense oligonucleotides (600 nM), Lip (600 nM), as well as untreated cells were used as controls. Forty-eight hours after transfection or treatment, HepG2 cells were examined by RT-PCR and Western blot analyses. As shown in Fig. 1, antisense-survivin down-regulated survivin expression at both mRNA (Fig. 1A and B) and protein levels (Fig. 1C and D) in a dose-dependent manner, with an  $\text{IC}_{50}$  of  $\sim 250$  nM. At a concentration of 600 nM, maximum down-regulation to 20% of the initial mRNA level was achieved. A further increase in oligonucleotide concentration did not result in a further increase in the knock-down efficacy of antisense survivin.

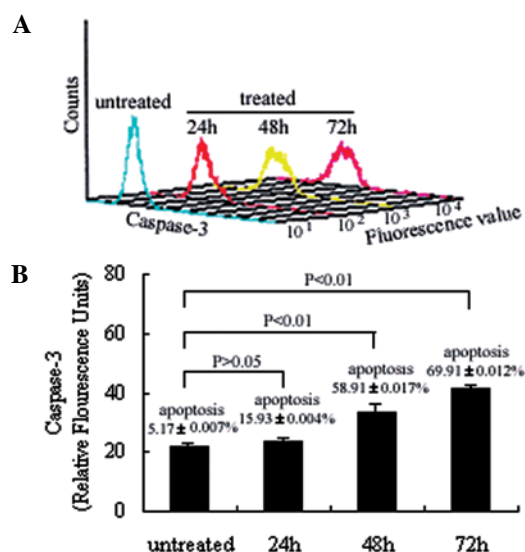


Figure 2. Flow cytometric analysis of caspase-3-like protease activity (A) and the rate of apoptosis (B). HepG2 cells were treated with antisense survivin at 24, 48 and 72 h after the start of transfection. At 24 h, treated cells had detectable active caspase-3 activity corresponding to measurable value 23, whereas about 16% of the cell population were induced to undergo apoptosis; at 48 h, measurable value 33 corresponding to 60%; at 72 h, measurable value 42 corresponding to 70%; untreated cells as control primarily negative for the presence of active-caspase-3, measurable value 16 corresponding to 5% apoptotic cells.

The sense oligonucleotide (600 nM), Lip (600 nM), and anti-sense without Lip (600 nM), did not down-regulate survivin expression.

**Induction of apoptosis by antisense survivin.** Having demonstrated that down-regulation of survivin expression reduced the viability of HepG2 cells, we analyzed whether cell death was due to the induction of apoptosis. As shown in Fig. 2, HepG2 cells were treated with antisense survivin at 24, 48 and 72 h after the start of transfection. Then, the cells were analyzed by flow cytometry using PE-conjugated polyclonal rabbit anti-active caspase-3 antibody. Antisense survivin-treated cells revealed increased caspase-3-like protease activity compared with untreated cells. Treated cells for 24 h had detectable active caspase-3 corresponding to measurable value 23, whereas about 16% of the cell population were induced to undergo apoptosis; at 48 h, measurable value 33 corresponding to 60%; at 72 h, measurable value 42 corresponding to 70%; untreated cells as control primarily negative for the presence of active-caspase-3, measurable value 16 corresponding to 5% apoptosis cells.

**Antisense survivin produces cell cycle arrest and apoptosis.** To determine whether survivin is required for the control of apoptosis during the cell cycle, the cell cycle distribution

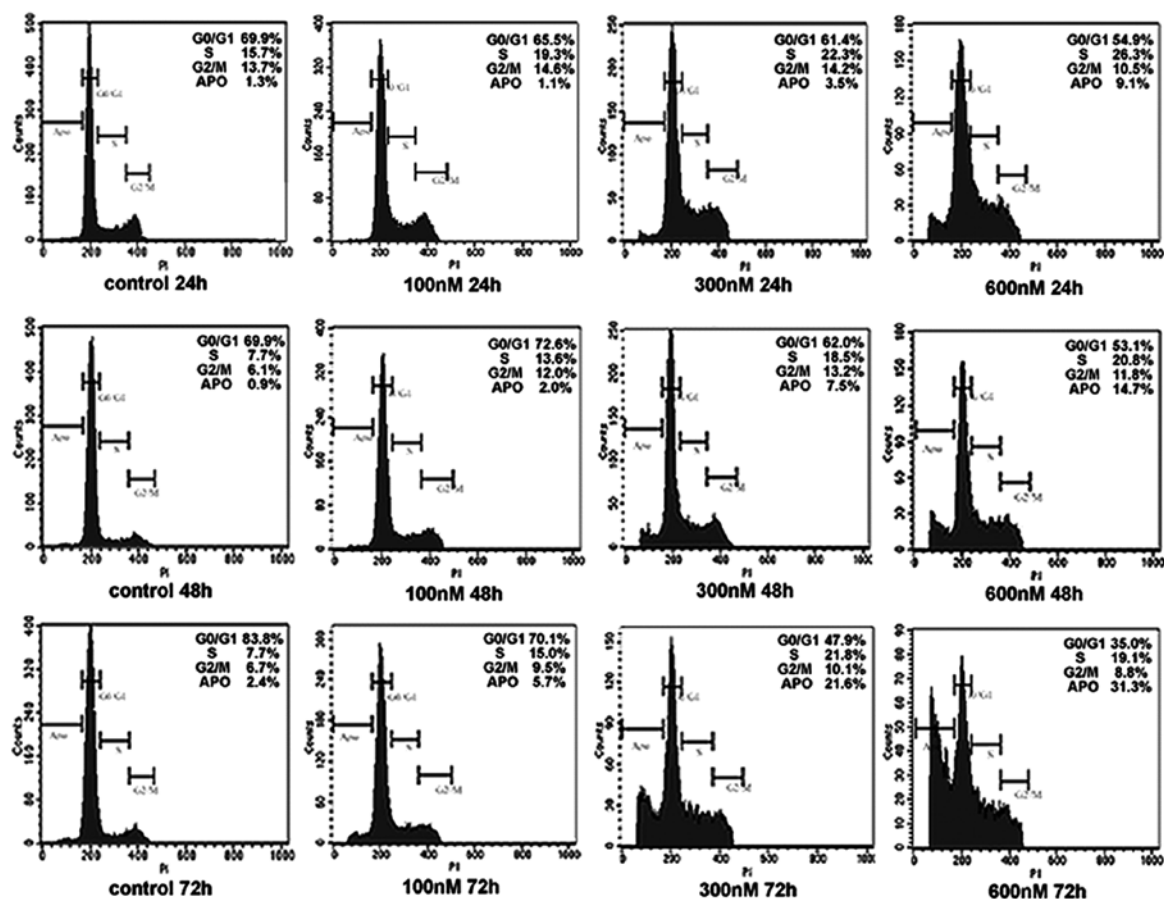


Figure 3. Cell cycle analysis of HepG2 cells treated with control or survivin antisense compound (100, 300 and 600 nmol/l) at 24-72 h after transfection. At low concentration of survivin (100 nM), the tendency of the cell of the G0/G1 phase decreased and the same time the cells of the S phase or G2/M phase increased; at high concentration (30 and 600 nM), the cell cycle arrest was strengthened in S phase and G2/M phase, the rate of apoptosis was increased in a time- and concentration-dependent manner.

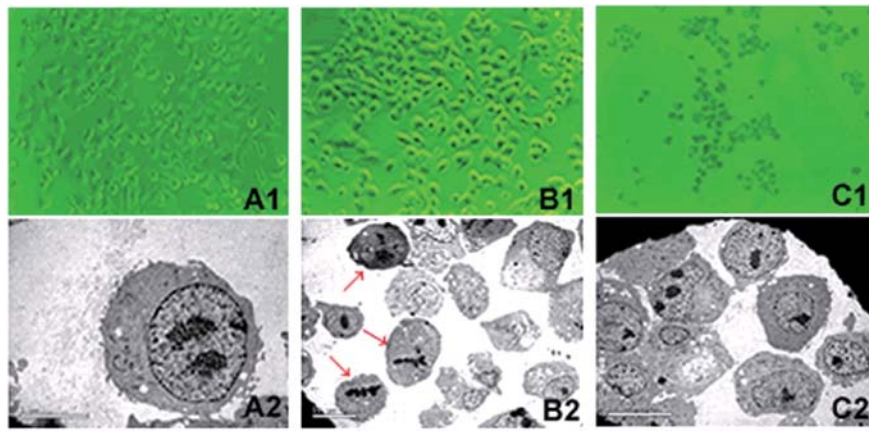


Figure 4. Effect of survivin targeting on cell nuclear morphology and ploidy. (A1 and A2) Untreated cells showed normal structure by inverted phase-contrast microscope and electron microscopy. (A1, x200; A2, x3500). (B1) Cells treated with antisense survivin for 24 h became abnormally large and flattened as judged by the inverted phase-contrast microscope (x200). (B2) Cells treated for 24 h showed accumulating multiple nuclei and polyploidy as judged by the electron microscope (x2500). (C1 and C2) Cells treated for 48 h showed morphological changes of apoptosis by the inverted phase-contrast microscope and the electron microscope (C1, x200; C2, x3500).

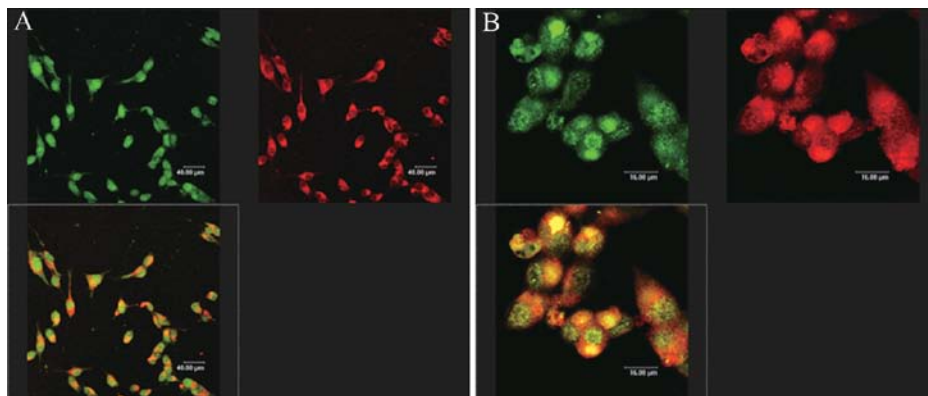


Figure 5. Subcellular localization of survivin protein in HepG2 cells detected by confocal laser scanning microscopy. (A) In cells untreated with antisense survivin, green-immunofluorescence staining of survivin clearly showed that survivin was expressed mainly in the form of a spotted distribution inside the cytoplasm, as a survivin pool. (B) In cells treated with antisense survivin, however, there was rare and weak green fluorescence inside cells of which the membranes had been counterstained with Alexa-concanavalin A.

was analyzed by flow cytometry in HepG2 cells treated with antisense survivin. As showed in Fig. 3, antisense survivin induced cell cycle arrest, followed by apoptosis. By treatment with low concentration of survivin (100 nM), the cells of the G0/G1 phase decreased and at the same time the cells of the S phase or G2/M phase increased; at high concentration (300 and 600 nM), the cell cycle arrest was strengthened in S phase and G2/M phase, but the rate of apoptosis was increased in a time- and concentration-dependent manner.

**Antisense survivin produces polyploidy and apoptosis.** To determine whether survivin is required to preserve cell viability and ploidy. We analyzed nuclear morphology and ploidy of the cells treated with antisense survivin at 24-48 h after transfection by electron microscopy, and the morphological changes were observed by inverted phase-contrast microscope. As show in Fig. 4, at 24 h, the cell of survivin antisense transfectants became abnormally large and flattened as judged by inverted phase-contrast microscope, accumulating multiple nuclei and polyploidy as judged by electron microscope; at 48 h, the cells exhibited morphological features typical of apoptosis, including

condensed chromatin and nuclear fragmentation, whereas cells transfected with control survivin had normal ploidy.

**Localization of survivin protein in HepG2 cells.** We carried out double immunofluorescence staining using FITC-labelled antibody against survivin and Alexa-labelled concanavalin A with which cells of the membranes were counterstained. The stained cells were investigated by confocal laser scanning microscopy. As shown in Fig. 5, the cells not treated with antisense survivin, green-immunofluorescence staining of survivin clearly showed that survivin was expressed mainly in the form of a spotted distribution inside the cytoplasm, which was named termed the survivin pool (Fig. 5A). In cells treated with antisense survivin, however, there was rare and weak green fluorescence inside cells of which the membranes had been counterstained with Alexa-concanavalin A (Fig. 5B).

## Discussion

In this study, we observed synchronized defective mitosis characterized by multinucleated and polyploidy cells, and cell

cycle arrest in S phase or G2/M phase followed by apoptosis in hepatocellular carcinoma cells after antisense depletion of survivin, the mechanism of which were involved with the simultaneous destruction of specialized subcellular compartment of survivin and activation of caspase-3-like protease.

The mechanism of apoptosis is required to preserve cell viability and ploidy. Effector molecules of apoptosis may also influence cell proliferation and cell cycle transitions of all phases (12,23-25). We tested this hypothesis by antisense depletion targeting the expression of survivin, our results show that antisense survivin expression resulted in cell cycle arrest (S+G/M), followed by apoptosis. By treatment with low concentration of survivin, the cells of the G0/G1 phase decreased and at the same time the cells of the S phase or G2/M phase increased; at high concentration, the cell cycle arrest was strengthened in S phase and G2/M phase, and the rate of apoptosis was increased in a time- and concentration-dependent manner at 24, 48 and 72 h after the start of transfection of antisense survivin. This indicated that survivin might have a key role in cell cycle progression in HepG2 cells, demonstrating that anti-apoptotic survivin molecule may promote S-phase entry, and cooperate with the G2/M checkpoint to regulate proper cell cycle progression (26,27). Antisense depletion of survivin caused apoptosis of proliferating HepG2 cells, indicating that down-regulation of survivin reduced the apoptotic threshold (29,30). Accordingly, loss of survivin by antisense expression may activate a 'default' apoptotic program at mitosis and trigger caspase-dependent cell death (12,24). Overexpression of survivin in cancer may represent an exploitation of this cytoprotective mechanism, providing a uniformly increased anti-apoptotic environment in cancer cells throughout the various cell cycle phases (23,30).

To investigate whether the control apoptosis by survivin are required for hepatocellular cell mitotic progression, we carried out the time-lapse imaging of HepG2 cells treated by antisense survivin to analyzed nuclear morphology and ploidy of the cells at 24-48 h after transfection by electron microscope, and, at the same time, the morphological changes were observed by inverted phase-contrast microscope. Our findings show that the cells after transfection go on to transient morphological changes characterized by multinucleated and polyploid cells followed by apoptosis. Observed by inverted phase-contrast microscope after transfection of antisense survivin, the cells became abnormally large and flattened at 24 h, and most cells floated and eventually died at 48 h. Judged by electron microscopy, we detected accumulating multiple nuclei and polyploidy at 24 h, and the cells exhibited morphological features typical of apoptosis at 48 h, including condensed chromatin and nuclear fragmentation. However, the cells transfected with control compound had normal morphology and ploidy. The results were further evaluated by analysis of ploidy of the cells showing that cell cycle was arrested in S phase and G2/M phase, indicating these cells became progressively polyploid, with an 8N or 16N DNA content, after ablating survivin. These data demonstrated that the overexpression of survivin in HepG2 was essential to maintain polyploidy (15). During somatic cell division, faithful chromosomal segregation must follow DNA replication to prevent aneuploidy or polyploidy. After mitotic spindle damage these cells continue DNA replication in the absence of cell division and become polyploid.

The depletion of survivin can lead to damage of the mitotic spindle, a potential mechanism that interferes with chromosomal segregation. Notably, other authors reported that after removal of antisense survivin at early stage, the cells restored normal ploidy and nuclear morphology (31). In this way, whether the cells treated by ablating survivin died or become polyploidy depended on the degree of activation of caspase-3-like protease and instantaneous subcellular localization of survivin (21,32,34). To further demonstrate the bifunctional role of survivin in inhibition of apoptosis and regulation of cell division, we carried out double immunofluorescence staining to observe the spealized subcellular localization by time-lapse imaging of HepG2 cells treated by antisense survivin. The stained cells were investigated by confocal laser scanning microscopy. In the present study, double immunofluorescence analysis confirmed the presence of survivin protein pool in the cytoplasm. In untreated HepG2 cells, labeled-FITC immunofluorescence staining of survivin clearly showed that survivin was expressed mainly in the formation of a spotted distribution inside the cytoplasm. Whereas, in treated cells at 24 h after transfection, survivin was found to be rare and weak in survivin immunoreactivity. As demonstrated by Western blot analysis, in cells treated with antisense depletion, survivin expression level was decreased in a dose-dependent manner. This suggested that the survivin antisense approach was able to affect the localization of survivin. Notably, our findings agree with those reported by Fortugno *et al* (33) and Temme *et al* (32). Fortugno *et al* also identified a cytosolic pool of survivin which associated with interphase microtubules, centrosomes, spindle poles and mitotic spindle microtubules at metaphase and anaphase, and a nuclear survivin localized to kinetochores of metaphase chromosomes and to the central spindle midzone at anaphase. The findings in our study strongly indicated that anti-apoptotic survivin at the interface between cell proliferation and cell apoptosis provides a death switch for the termination of defective mitosis (21,23,32,34).

In summary, our results show that overexpression of survivin is required to maintain cell viability and proper cell cycle transitions, and preserve genetic fidelity during cell division, an essential prerequisite of this pathway is the specialized subcellular localization of survivin.

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