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Survivin-specific small interfering RNAs enhance sensitivity of glioma U-87MG cells to paclitaxel by promoting apoptosis☆

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Abstract

A survivin siRNA expression vector was transfected into glioma U-87MG cells and these cells were then treated with paclitaxel. The results showed that survivin-specific siRNA combined with paclitaxel treatment synergistically inhibited glioma U-87MG cell proliferation and promoted apoptosis. This treatment also inhibited the expression of the cell cycle regulatory proteins, survivin, cyclinD1, c-Myc and CDK4 and enhanced the sensitivity of U-87MG cells to paclitaxel.

Key Words

RNA interference; paclitaxel; survivin; glioma; neural regeneration

Abbreviations

PTX, paclitaxel; RNAi, RNA interference; siRNAs, small interfering RNAs

INTRODUCTION

Malignant glioma is characterized by an invasive nature, impracticable resection and resistance to chemotherapy and radiotherapy. This results in extraordinarily high morbidity, which has stimulated vigorous research for novel and more specific therapeutic approaches[1].

The anti-cancer agent paclitaxel (PTX) is derived from the Pacific Yew tree and it exerts its anti-tumor activity through a unique mechanism; binding to microtubules and stabilizing their structure[2]. The resulting complex of PTX and microtubule prevents the progression of the mitotic cell cycle from metaphase to anaphase, leading to nonhomologous separation of the chromosomes due to G_2/M arrest and thereby inducing cell cycle exit[3]. PTX also induces apoptosis due to reduced function of the anti-apoptotic survivin protein[4]. However, PTX has serious side effects[5], which has limited its clinical use.

One promising method to treat glioma is the inactivation of proteins that are essential for survival or progression by RNA interference (RNAi)[6]. Optimal RNAi targets for glioma include molecules that are functionally relevant in the tumor and that avoid side effects and that are not expressed at significant levels in normal tissues. Among the family of apoptosis inhibitors, survivin has received special attention because it is highly expressed in many cancer cells and tissues including glioma[7]. High level of survivin expression has been correlated with high malignancy, poor prognosis and drug resistance[8-9]. It is widely accepted that survivin exerts anti-apoptotic function through its baculoviral inhibitor of apoptosis protein repeats domain by binding to activated caspases[10]. A further anti-apoptotic function of survivin occurs through inhibition of the second mitochondria derived apoptosis pathway[11]. Therefore, it is anticipated that high survivin

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levels enhance the resistance of cancer cells to chemotherapeutic drugs or radiation[12-14]. It is feasible to increase cell apoptosis using RNAi methods. Nevertheless, synthetic small interfering RNAs (siRNAs) can only transiently decrease target gene expression in proliferating cancer cells. A sustained supply of anticancer siRNAs is important to produce a strong therapeutic benefit[15]. Recently, short hairpin RNAs have been shown to be effective both *in vitro* and *in vivo* at inhibiting target gene expression. Short hairpin RNAs on plasmids are economic to deliver and are quite stable relative to antisense DNAs[16]. However, RNAi cannot completely inhibit target gene expression, particularly abnormally high gene expression. Combination therapy is particularly important in the clinic. Survivin expression levels are significantly higher in glioma than in normal nervous tissue, so it is feasible to decrease survivin expression levels with RNAi to increase the sensitivity of glioma to PTX.

To improve the anti-tumor effect and to reduce the toxicity of PTX, PTX and survivin specific siRNAs were combined to treat glioma cells to yield a synergistic anti-tumor effect.

RESULTS

PTX combined with survivin siRNA synergistically inhibited U-87MG cell growth

U-87MG cells treated with phosphate buffered saline (PBS) for 24 hours had large and darkly stained nuclei, with fine and short processes. The morphological features of U-87MG cells treated with PTX (final concentration 1 μM) were different, with small and retracted cells bodies having elongated cytoplasmic processes. This morphology was similar to the survivin siRNA treated cells. Cells treated with PTX combined with survivin siRNA were less numerous and were darkly stained and had long processes on one side or both sides (Figure 1). The cells were counted within one × 200 magnified visual field; the cell number was 829 ± 67 after PBS treatment. The cell number was significantly reduced in the PTX or survivin siRNA groups (521 ± 74, 608 ± 72, $P < 0.05$). The cell number was significantly diminished in the PTX + survivin siRNA group (162 ± 23; $P < 0.05$).

PTX combined with survivin siRNA induced U-87MG cell apoptosis

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to detect the inhibitory effect of PTX combined with survivin siRNA on U-87MG cell growth. As shown in Table 1, PTX combined with survivin siRNA synergistically inhibited U-87MG cell proliferation. Significant differences were determined between the PTX + survivin siRNA group and the PTX or survivin siRNA groups ($P < 0.05$ or $P < 0.01$).

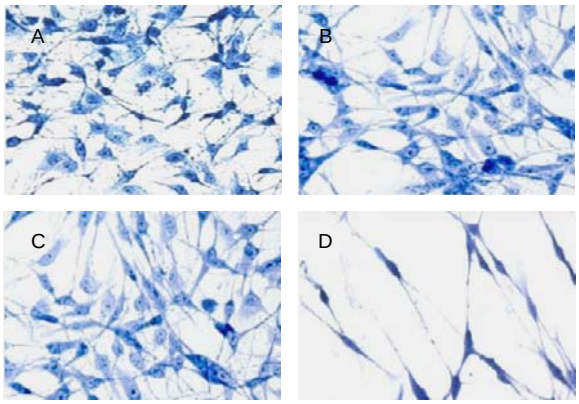

Figure 1 Treatment with survivin small interfering RNA (siRNA) combined with paclitaxel (PTX) decreased U-87MG cell proliferation (× 200).

(A) In the mock group, the U-87MG cells treated with phosphate buffered saline showed large and darkly stained nuclei with fine and short processes.

(D) In the PTX + survivin siRNA group, the number of the cells was significantly reduced compared with the mock group, and was reduced compared with the PTX (B) and survivin siRNA (C) groups.

Table 1 Inhibitory effect (%) of paclitaxel (PTX) combined with survivin small interfering RNA (siRNA) on U-87MG cell growth

Group	Time after treatment (hour)		
	24	48	72
Mock	0.0	0.0	0.0
PTX	8.72±0.93	10.23±1.07	18.14±2.20
Survivin siRNA	7.63±7.43	11.38±5.18	19.21±1.59
PTX+survivin siRNA	21.33±1.28[a]	38.69±7.19[a]	70.42±13.02[b]

Data are presented as mean ± SD. [a]$P < 0.05$, [b]$P < 0.01$, *vs.* the mock, PTX and survivin siRNA groups respectively by independent samples *t*-test analysis.

To determine whether survivin siRNA with PTX had a synergetic inhibitory effect on the U-87MG cell cycle, we determined cell apoptosis by flow cytometry. In the PTX treated cells, the apoptosis rate was significantly higher than that in the mock group. More cells were arrested in G_2 phase. The apoptosis rate was similar with survivin siRNA treated cells, where cells were arrested in G_0/G_1 phase. In the PTX + survivin siRNA group, the apoptosis rate was significantly higher than in the other groups ($P < 0.05$ or $P < 0.01$; Table 2), which indicated that PTX combined with survivin siRNA showed a synergistic effect in promoting apoptosis (supplementary Figure 1 online).

Table 2 Apoptosis induction of U-87MG cells by paclitaxel (PTX) combined with survivin small interfering RNA (siRNA)

Group	Apoptotic cells (%)	G_0–G_1 (%)
Mock	1.31±0.25	30.94±2.73
PTX	5.81±0.73	40.70±2.84
Survivin siRNA	10.16±2.38[c]	45.38±3.28[c]
PTX+survivin siRNA	26.34±5.88[b]	55.38±13.20[a]

Group	G_2 (%)	S (%)
Mock	5.35±0.18	62.76±7.28
PTX	7.92±3.24[c]	42.52±7.23[c]
Survivin siRNA	8.02±0.94	34.91±5.22[c]
PTX+survivin siRNA	5.62±4.94[b]	11.34±4.28[a]

Data are presented as mean ± SD. [a]$P < 0.05$, [b]$P < 0.01$, *vs*. the PTX and survivin siRNA groups; [c]$P < 0.05$, *vs*. the mock group (independent samples *t*-test analysis).

PTX combined with survivin siRNA reduced mRNA levels of cell cycle regulators in U-87MG cells

CDK4, cyclinD1 and c-Myc genes regulated the cell cycle and determined cell division rate[17]. In glioma, the above genes are up-regulated[18], and the division rates of the cells are accelerated. To understand the molecular mechanism of cell cycle alteration, reverse transcription-polymerase chain reaction (RT-PCR) assay was applied to determine gene expression levels. Both PTX and survivin siRNA downregulated CDK4, cyclinD1 and c-Myc expression (Figure 2).

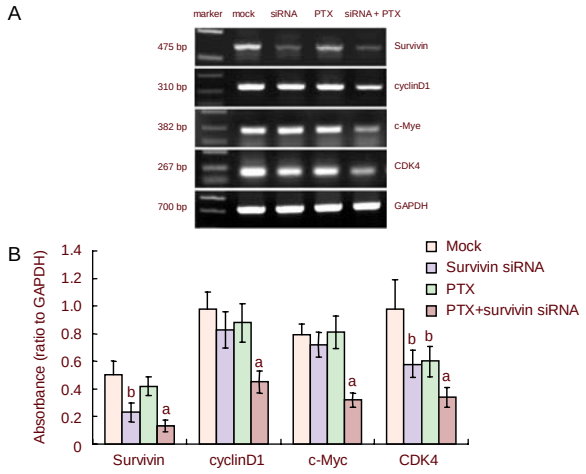

Figure 2 Reverse transcription-polymerase chain reaction analysis of mRNA expression in U-87MG cells.

(A) Expression of survivin and cell cycle genes in U-87MG cells after different kinds of treatment.

(B) Amounts of survivin and cell cycle genes (cyclinD1, c-Myc and CDK4) in U-87MG cells transfected with different treatments, and data were expressed as mean ± SD from three separate experiments.

[a]$P < 0.05$, *vs*. the paclitaxel (PTX) and survivin small interfering RNA (siRNA) groups; [b]$P < 0.05$, *vs*. the mock group by paired *t*-test. PTX combined with survivin siRNA synergistically inhibited survivin, c-Myc, cyclin-D1 and CDK4 mRNA levels.

U-87MG cells treated with PTX combined with survivin siRNA showed synergistic inhibition of CDK4, cyclinD1 and c-Myc gene expression.

Analysis of survivin expression level using immunofluorescence staining

An immunofluorescence assay was applied to determine the level of survivin protein after 48 hours of treatment. As exhibited in Figure 3, survivin expression was high in the mock group. The survivin expression level was down-regulated and cell proliferation was inhibited in both the survivin siRNA and PTX groups. Survivin immunosignal was impaired or lost in cells of the survivin siRNA + PTX group, confirming a robust synergistic role.

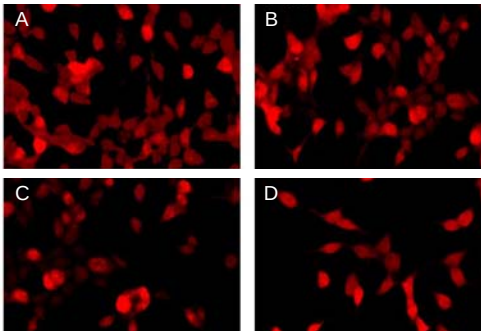

Figure 3 Treatment with survivin small interfering RNA (siRNA) combined with paclitaxel (PTX) decreased survivin expression in U-87MG cells as seen by immunofluorescence staining.

(A) In the mock group, the cells treated with phosphate buffered saline showed the highest number of stained cells and strong survivin immunofluorescence.

In PTX (B) and survivin siRNA (C) groups, the number of cells was reduced compared with the mock group ($P < 0.05$), with relatively weak survivin immunofluorescence signal.

The cell number was significantly lower in the PTX + survivin siRNA group (D) compared with the PTX or survivin siRNA groups separately ($P < 0.05$).

Survivin expression was greatly inhibited. The statistical analysis results are displayed in supplementary Table 1 online (× 200).

Survivin specific siRNA combined with PTX reduced protein levels of cell cycle regulators in U-87MG cells

The above results showed that U-87MG cells transfected with survivin siRNA had a significant decrease in survivin protein levels compared to mock-treated cells (Figure 4). However, not all survivin protein expression was inhibited. To increase treatment effects, we combined PTX with survivin siRNA to treat glioma cells.

The synergistic effect was obvious compared with both survivin siRNA and PTX groups ($P < 0.05$). Western blot analysis showed that cyclinD1, c-Myc and cdk4 levels were significantly decreased in U-87MG cells transfected with survivin siRNA and treated with PTX compared with controls.

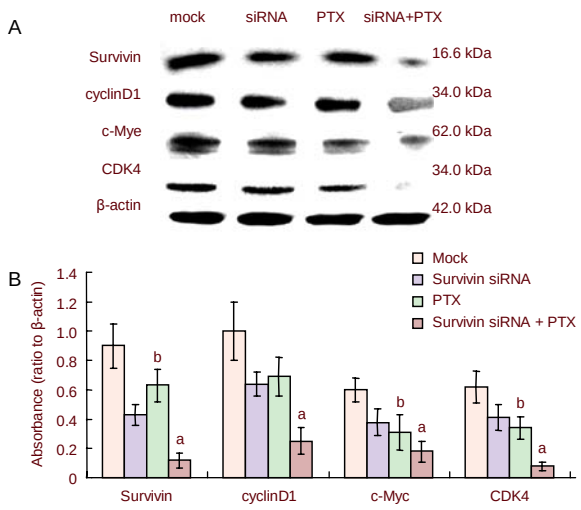

Figure 4 Western blot analysis of proteins in U-87MG cells.

(A) Survivin, c-Myc, cyclin-D1, CDK4 protein levels after different treatments.

(B) Amounts of survivin and cell cycle regulatory proteins (cyclinD1, c-Myc and CDK4) in U-87MG cells transfected with different treatments, and data are expressed as mean ± SEM from three separate experiments.

[a]*P* < 0.05, *vs*. the paclitaxel (PTX) and survivin small interfering RNA (siRNA) groups; [b]*P* < 0.05, *vs*. the mock group by paired *t*-test. PTX combined with survivin siRNA synergistically inhibited survivin, c-Myc, cyclin-D1 and CDK4 protein level.

DISCUSSION

Survivin is uniquely expressed in tumor cells and developmental cells, which can undergo either inappropriate or programmed cell growth, respectively. It participates in cell cycle entry and progression by binding with mitotic spindle fibers and by facilitating the entry of cells into S phase and by controlling apoptosis by inhibiting caspase activation[19]. Moreover, survivin has also been shown to regulate CDK4, cyclinD1 and c-myc activity in different types of cancer[18, 20-21]. PTX can inhibit cell growth and decrease survivin expression. Survivin is overexpressed in glioma and reduced survivin levels inhibit glioma proliferation and induce apoptosis of glioma cells[22-23]. It may, therefore, be feasible to treat glioma with combined PTX and survivin siRNA.

MTT assay results indicated that PTX combined with survivin inhibited cell proliferation synergistically. According to flow cytometry results, the apoptotic rate was clearly elevated and the cell cycle stage was changed when cells were treated with PTX combined with survivin siRNA. To elucidate the mechanisms involved, the expression of the cell cycle genes and proteins, cyclinD1, CDK4 and c-Myc, was detected. It is widely accepted that in the normal cell cycle, cyclin D1 and CDK4 form cyclin D1-CKD4 and the complex phosphorylates the Rb protein. The anti-oncogene Rb cannot then inhibit the E2F gene and DNA synthesis is strengthened. The cell cycle progresses from G_1 to S phase and cell proliferation continues. In cancer cells, cyclinD1 expression is greatly elevated. The *c-Myc* gene is also expressed in G_1 phase and participates in cell phase modulation. If the expression of *c-Myc* is decreased, G_2/M phase will arrive ahead of schedule. Survivin mRNA is undetectable in freshly isolated G_0 cells, but is present in G_1 cells, and after cytokine treatment, the expression of survivin was detected in G_0 and elevated in G_1 glioma cells and remains high in S and G_2/M phases[24]. High survivin expression induces cyclinD1 and c-Myc and CKD4 expression and the cell proliferation phase is shortened. Thus, disturbance between cell proliferation and cell apoptosis plays a crucial role in tumorigenesis. For this reason, a survivin siRNA vector was used to inhibit survivin expression in glioma cells. RT-PCR, immunofluorescence and western blot assays were used to detect the inhibitory effects of survivin. All the results confirmed that the survivin level was downregulated and that the downstream genes were also modulated accordingly.

In conclusion, the blockade of survivin signaling using the RNAi approach combined with PTX significantly suppressed survivin and the expression of downstream genes *in vitro*, suggesting that induction of glioma differentiation resulted in sustained downregulation of the cell proliferation markers leading to cell cycle arrest. The prolongation of cell cycle arrest promoted PTX-mediated activation of apoptosis pathways. Plasmid based siRNA combined with chemotherapy for tumor suppression may offer an effective and inexpensive approach for the treatment of glioma.

MATERIALS AND METHODS

Design

A cytology, paired observation study.

Time and setting

Experiments were performed in the Department of Neurology Research of the First Clinical Hospital, Jilin University, China, and at the Molecular Medicine Research Center of Basic Medical College, Beihua University, China, between June 2009 and October 2010.

Materials

Glioma cell line U-87MG[25-26] was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA).

Methods

Construction of pSilenceTMneo3.1-H1-Survivin siRNA

DNA templates for the synthesis of siRNAs were

constructed under the control of the H1 promoter in the plasmid pSilence^{TM}neo3.1-H1 (Ambion Inc Austin, TX, USA). In brief, the double stranded DNA template encoding survivin siRNA oligonucleotides (GenBank: accession number for human survivin: NM-001168[27]) that contained a sense strand of 19 bp followed by a short spacer sequence (TTC AAG AGA), the reverse complement of the sense strand, and five thymidines as a RNA polymerase III transcriptional stop signal were synthesized. The forward sequence was 5'-GAT CCC GGA CCA CCG CAT CTC TAC Att caa gag aTG TAG AGA TGC GGT GGT CCT TTT TGG AT-3' and the reverse sequence was 5'-AGC TAT CCA AAA AGG ACC ACC GCA TCT CTA C Atc tct tga aTG TAG AGA TGC GGT GGT CCG G-3'. The oligonucleotides were annealed in a buffer (potassium acetate 100 mM, 30 mM HEPES-KOH pH 7.4, and magnesium acetate 2 mM) and the mixture was incubated at 90°C for 3 minutes and then at 37°C for 1 hour. The double stranded oligonucleotides were cloned into the *Bam*HI and *Hin*dIII sites of pSilence^{TM}neo3.1-H1, to enable short hairpin RNA to be expressed under the control of the H1 promoter[28].

Cell treatment and morphological analysis

The human glioma cancer cell line, U-87MG, was cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, penicillin (North China Pharmaceutical Co, China; 100 kU/L) and streptomycin (North China Pharmaceutical; 100 mg/L) at 37°C in a humidified incubator with 5% CO_2[29]. First, the cells were treated with 0.25, 0.50, 1.0, 1.5 and 2.0 μM PTX; after that 1 μM PTX + siRNA at 0.25, 0.5, 1.0 and 1.5 μM were used (supplementary data online).

After the optimal drug concentration was confirmed, the cells were divided into four groups. One was mock treated with PBS only, one was transfected with plasmid containing survivin specific siRNA (0.50 μM); the third group was treated with 1 μM PTX; the last group was treated with survivin siRNA (0.5 μM) combined with 1 μM PTX. Cells were cultured in monolayers in 6-well plates for 24 hours. At the appropriate time, cells were washed twice with ice-cold PBS, pH 7.4, before fixing in ice-cold 95% ethanol. The cells were stained with 0.2% (v/v) methylene blue solution (Sigma, St. Louis, MO, USA) (prepared in 50% ethanol) for 30 seconds and washed twice in ice-cold distilled water. The plates were air dried before being examined under a light microscope (Olympus, Tokyo, Japan) at 400 × magnification.

Proliferation and apoptosis of U-87MG cells

U-87MG cells were incubated in 96-well plates. Cell proliferation was determined by the MTT assay (Sigma) and cell numbers were counted using a hemocytometer (All-Time Commercial Co, Shanghai China) at 24, 48 and 72 hours after treatment. The absorbance values at 570 nm (A_{570}) were measured using a multiwell plate reader (Sunostik Medical Technology, Changchun, China). The inhibitory rate of cell growth was calculated according to the following formula: inhibitory rate (%) = [(A_{570}c– A_{570}e)/A_{570}c] × 100%. A_{570}c: A_{570} in control group; A_{570}e: A_{570} in experimental group.

For flow cytometry analysis of apoptosis, U-87MG cells were treated with Mock, PTX, survivin siRNA and PTX + survivin. After 48 hours, cells were collected and washed in cold PBS containing 4 mM acetic acid. Cells were fixed in 70% cold ethanol, collected by centrifugation, and washed once again in PBS containing acetic acid. Cells were resuspended in PBS containing 4 mM acetic acid, 0.2% Triton X-100, 20 mL/L of propidium iodide (Sigma) and 40 mg/L RNase A (Sigma). The cells were incubated for 30 minutes at 4°C and analyzed by flow cytometry (FACScan, Becton Dickinson, Franklin Lakes, NJ, USA), using Cell Quest software.

RT-PCR determination of survivin, c-Myc, Cyclin-D and CDK4 mRNA levels in U-87MG cells

Quantitation of specific mRNAs was done essentially as described previously[30]. Briefly, after treatment for 48 hours, cells were collected and total RNA was extracted from cells with Trizol (Invitrogen) following the instructions of the manufacturer. For RT-PCR analysis, 10 μg of total RNA was subjected to reverse transcription using a RT-PCR kit (Promega, Madison, WI, USA). For the amplification of mRNA, the primer pair sequences are as follows:

Primer	Sequences (5'–3')	Product size (bp)
Survivin	Sense: GAA TTC ATG GGT GCC CCG ACG TTG CC Antisense: AGA TCT TTC TTC TTA TTG TTG GTT TCC	475
c-Myc	Sense: CTG CTG CCA AGA GGG TCA Antisense: CGT TTC CGC AAC AAG TCC	310
Cyclin-D1	Sense: CCT GTG CTG CGA AGT GGA AA Antisense: GAT GGA GTT GTC GGT GTA GAT	382
CDK4	Sense: GGG ACC GTC AAG CTG GCT GA Antisense: TCG AGG CCA GTC GTC TTC TG	267
GAPDH	Sense: GGG TGA TGC TGT GCT GAG TAT GT Antisense: AAG AAT GGG AGT TGC TGT TGA AGT	700

Taq polymerase (Invitrogen, Carlsbad, CA) was added and reactions run under the following cycling conditions: 94°C for 3 minutes, followed by denaturing at 94°C for 45 seconds, annealing at 56 to 60°C for 30 seconds, and amplification at 72°C for 30 seconds, repeated for 30 cycles. RNA dilutions not subjected to the reverse transcription reaction were included as controls. The PCR products were analyzed by standard agarose gel electrophoresis with ethidium bromide staining, and the relative absorbance values of target bands were quantified with Image Quant 5.0 software (Molecular Dynamics, Sunnyvale, CA, USA) and compared with GAPDH.

Survivin immunofluorescence staining

U-87MG cells grown on poly-L-lysine-mounted glass slides after treatment for 48 hours were fixed for 20 minutes with 4% paraformaldehyde in PBS. The samples were treated with ice cold permeabilization solution, containing 0.1% sodium citrate in PBS, and 0.1% Triton X-100, for 5 minutes. Samples were incubated for 1 hour with a rabbit anti-human survivin polyclonal antibody (1:200; Santa Cruz Biotech, Santa Cruz, CA, USA). Samples were washed three times with PBS plus 0.1% bovine serum albumin and were subsequently incubated with a secondary rabbit anti-avidin-Cy3 conjugated antibody (dilution, 1:200; Jackson IR Florida, USA) for 1 hour. Finally, the stained cells were washed three times in PBS/0.1% bovine serum albumin and once in double-distilled water, prior to examination by standard immunofluorescence microscopy (Olympus).

Western blot analysis of proteins

Total protein was extracted from harvested sample cells by freeze-thawing in protein lysis buffer (5 M acetic acid, 300 mM NaCl, 0.5 mM NaF, 0.1% Igepal, 0.5 mM Na_3VO_4, 0.5 mM phenylmethylsulphonyl fluoride and antiprotease mixture). The lysates were centrifuged at 15 000 × *g* for 30 minutes. Determination of protein concentrations of the supernatants was performed by the Bradford procedure (Bio-Rad Hercules, CA, USA). For survivin, cyclinD1, c-Myc and CDK4 analysis, supernatant containing 50 μg total protein was separated by electrophoresis on 10% sodium dodecyl sulphate-polyacrylamide gels and transferred onto polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA) and blocked with 5% nonfat dried milk in PBS containing 0.1% Tween-20. Blots were incubated with specific rabbit anti-human antibodies against the above antigens (1:200 or 1:250; Santa Cruz Biotech) or with a rabbit anti human-β-actin antibody (1:300; Santa Cruz Biotech) and then washed with Tris-buffered saline containing 0.1% Tween-20. Blots were then incubated with corresponding mouse anti-rabbit horseradish peroxidase-conjugated secondary antibodies (1:2 000; Bio-Rad, CA, USA) as indicated. Blots were washed again with Tris-buffered saline containing 0.1% Tween-20 and visualized by enhanced chemiluminescence detection (Amersham International, Buckinghamshire, UK). The western blot products were analyzed by relative absorbance of target bands compared with β-actin and were quantified with Image Quant 5.0 software (Molecular Dynamics, Sunnyvale, CA, USA).

Statistical analysis

Data are presented as mean ± SD. All statistical calculations were performed using the statistical software package SPSS V14.0 (SPSS, Chicago, IL, USA). The paired *t*-test was performed to evaluate the significance of differences between the experimental groups for RNA levels (RT-PCR) and protein levels (western blot analysis). For a single comparison of two groups, independent samples *t*-test was used for cell number analysis, cell cycle distribution and apoptosis induction. A value of $P < 0.05$ was considered statistically significant.

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Author contributions: This study was designed by Weigao Shen and Qun Liu. Yunliang Xie and Yanbo Liu carried out the majority of the experiments and performed the data analysis. Bo Zhang helped with cell culture and data analysis. Qun Liu provided data analysis and participated in the study design and revision of the manuscript. All authors read and approved the final manuscript.

Conflicts of interest: None declared.

Ethical approval: All the experimental protocols were approved by the ethics committee for animal research, Beihua University, China, and were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of the People's Republic of China.

Supplementary information: Supplementary data associated with this article can be found, in the online version, by visiting www.nrronline.org, and entering Vol. 7 No. 13, 2012 after selecting the "NRR Current Issue" button on the page.

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