Down-regulation of *survivin* expression reversed multidrug resistance in adriamycin-resistant HL-60/ADR cell line[1]

WANG Lei, ZHANG Gui-Mei, FENG Zuo-Hua[2]

Department of Biochemistry and Molecular Biology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China

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ABSTRACT

AIM: To investigate the ability of an antisense RNA eukaryotic expression plasmid pcDNA3.1/*survivin* in down-regulating the expression level of *survivin* mRNA and survivin protein and reversed multidrug resistance (MDR) in adriamycin-resistant HL-60/ADR cell line. **METHODS:** The expression of *survivin* mRNA was measured by RT-PCR and the expression of survivin protein was measured by Western blot. Caspase-3 activity was determined by Phar Mingen colorimetric assay kit. Apoptosis was assessed by flow cytometry. The chemosensitivity of HL-60/ADR cells to adriamycin (ADR) was measured by MTT assay. **RESULTS:** pcDNA3.1/*survivin* down-regulated the expression level of *survivin* mRNA and survivin protein obviously, and induced apoptosis of HL-60/ADR cells in a time-dependent manner during 12-48 h. After transient transfection with pcDNA3.1/*survivin* for 48 h, *survivin* mRNA decreased by 67 %, survivin protein decreased by 57 %, caspase-3 activity increased 4.37 times, and the apoptosis rate increased by 4.41 % compared with control. Compared with ADR alone, pcDNA3.1/*survivin* significantly reversed MDR in HL-60/ADR cells, the chemosensitivity of HL-60/ADR cells to ADR was increased to 5.36 folds. **CONCLUSION:** pcDNA3.1/*survivin* down-regulated the expression level of *survivin* mRNA and survivin protein obviously, the threshold of apoptosis was decreased and MDR was reversed.

INTRODUCTION

Diminished apoptosis plays a critical role in tumor initiation, progression, and drug resistance. Several proteins that inhibit apoptosis have been identified, including bcl-2 family members bcl-2 and bcl-xl and the IAPs. Certain members of the latter family directly inhibit terminal effector caspases engaged in the execution of cell death[1]. The gene encoding the IAP survivin was cloned recently, and the protein was characterized[2]. Survivin is expressed during embryonal development but lacks expression in terminally differentiated adult tissues. Interestingly, it becomes reexpressed in transformed cell lines and a variety of human tumors. Survivin is expressed in the G_2-M phase in a cell cycle-regulated manner, and its interaction with the mitotic spindle apparatus is essential for antiapoptotic function[3]. This could imply that the IAP survivin counteracts a default induction of apoptosis in the G_2-M phase of the cell cycle. Overexpression of survivin has oncogenic po-

[1] Project supported by the National Natural Science Foundation of China (No 39970322).
[2] Correspondence to Prof FENG Zuo-Hua.
Phn/Fax 86-27-8365-7574. E-mail fengzhg@public.wh.hb.cn
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tential because it may overcome the G_2-M-phase checkpoint to enforce progression of cells through mitosis. Because survivin inhibits the processing of downstream effector caspase-3 and -7 in cells receiving an apoptotic stimulus, its overexpression in tumors is implicated in the resistance to a variety of apoptotic stimuli, including chemotherapy.

Leukemia is a primary cause of cancer death, and its incidence continues to rise. The main reason for the unfavorable prognosis of leukemia is their propensity to develop resistance to a wide range of functionally unrelated anticancer agents. Although *survivin* has been widely recognized as an attractive target for cancer therapy, the use of antisense cDNA to inhibit its expression has only recently been described[4-8]. Whereas these studies were designed to unravel the biological function of survivin, the promise of *survivin* antisense to facilitate apoptosis of tumor cells and overcome chemoresistance in cancer therapy remains to be determined.

In the present study, we transfected an antisense RNA eukaryotic expression plasmid pcDNA3.1/*survivin* into the survivin-overexpressing human leukemia multidrug resistant HL-60/ADR cells transiently, it down-regulated *survivin* mRNA and protein levels efficiently and directly induced apoptosis. Moreover, in a combination experiment with the chemotherapeutic agent ADR, the evidence was provided that antisense-mediated down-regulation of survivin has the potential to reverse MDR.

MATERIALS AND METHODS

Drugs and reagents Adriamycin (ADR) and 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co, USA. Fetal calf serum and RPMI-1640 were purchased from Gibco, USA. The antisense RNA eukaryotic expression plasmid of *survivin* pcDNA3.1/*survivin* was kindly provided by Dr ZHANG Wan-Guang in Wuhan Tongji Hospital.

Construction of *survivin* antisense RNA eukaryotic expression plasmid *Survivin* mRNA structure region was amplified by RT-PCR from total RNA isolated from the HepG2 hepatic cancer cell line. The primers used for PCR were 5' GGGGAATTCATGGGTGCCCCACGTTGCC 3' (underline denotes *Eco*R I site), and 5' CTCTCGAGTCAATCCATGGCAGCCAGCT 3' (underline denotes *Xho* I site). The PCR product (434 bp) was inserted inversely into the eukaryotic expression plasmid pcDNA3.1 at *Eco*R I and *Xho* I sites. The construction was verified by both restriction enzymes and PCR analysis. The sequence of *survivin* gene cloned was confirmed by DNA sequencing.

Cell culture Human leukemia multidrug resistant cell line HL-60/ADR was maintained in RPMI-1640 medium supplemented with 10 % fetal calf serum, glutamine 2 mmol/L, benzylpenicillin 100 kU/L, streptomycin 100 mg/L, and ADR 0.08 μmol/L at 37 ºC in a 5 % CO_2 atmosphere. Prior to use in experiments, HL-60/ADR cells were cultured in drug-free medium for two weeks.

Transfection of plasmids into HL-60/ADR cells The plasmids were transfected transiently into the HL-60/ADR cells using lipofectAMINE (Gibco). Briefly, for each transfection, 2 μg of DNA was incubated with 12 μL lipofectAMINE in RPMI-1640 medium without serum to form complexes according to the manufacturer's recommendations. Subsequently, 1×10^6 cells seeded in 6-well plates were exposed to a DNA/ lipofectAMINE mixture for 5 h. After 5 h, the medium was adjusted to the normal serum concentration, and the incubation of cells continued under regular growth conditions until the cells were harvested at various time.

Semiquantitative RT-PCR analysis of *survivin* gene expression

Reverse transcription reaction Total cellular RNA was isolated from cells using TRIzol reagent (Sigma) in accordance with the manufacturer's instructions. The reverse transcription reaction was performed using Promega products. The cDNA was stored at -20 ºC until required for analysis.

PCR Two sets of primer were used in all reaction to result in amplication of endogenous control (306 bp of GAPDH, primer sequences: 5' CGA AGT CAA CGG ATT TGG TCG TAT 3',5' AGC CTT CTC GGT GGT GAA GAC 3') and the target gene of interest (431 bp of *survivin*, primer sequence: 5' GCA TGG GTG CCC CGA CGT TG 3',5' GCT CCG GCC AGA GGC CTC AA 3'). PCR was performed as follows: 95 ºC for 4 min followed by 30 cycles at 94 ºC for 1 min, 55 ºC for 1 min, and 72 ºC for 1 min, followed by final step at 72 ºC for 10 min. PCR products were resolved on a 2 % agarose gel and the bands were visualized by ethidium bromide staining. Each band was analyzed on Image Analysis System GDS8000. The gene expression level was determined semiquantitatively by calculating the ratio of density metric value from the target gene in relation to

internal standard.

Western blot analysis of survivin protein expression Cells were lysed in lysis buffer at 4 ºC with sonication. The lysates were centrifuged at 15 000×*g* for 15 min and the concentration of the protein in each lysate was determined with Coomassie brilliant blue G-250. Loading buffer was then added to each lysate, which was subsequently boiled for 3 min and then resolved by 12 % SDS/PAGE and transferred to a nitrocellulose membrane. The membrane was blocked for 30 min in blocking buffer, and incubated with rabbit antihuman survivin polyclonal antibody (1:500) overnight at 4 ºC. The membrane was then washed in Tris-buffered saline containing 0.2 % Tween 20, incubated with alkaline phosphatase-conjugated goat antirabbit antibody (1:1500) for 60 min, and developed with tetrazolium salts. As a control, tubulin protein was blotted concurrently. All antibodies were from Santa Cruz Biotechnology. Each band was analyzed on Image Analysis System GDS8000. The gene expression level was determined by calculating the ratio of density metric value from the target gene in relation to internal standard.

Measurement of caspase-3 activity Cells were lysed for 1 h as reported in the manufacture's instructions and centrifuged at 15 000×*g* for 15 min at 4 ºC. Cytosolic protein (40 μg) was mixed with caspase-3-specific substrate DEVD-para-nitroanilide 80 μmol/L (PharMingen) and incubated at 37 ºC. Substrate cleavage was monitored at 400 nm using a microELISA reader (Sigma). To confirm that substrate cleavage was due to caspase activity, extracts were incubated in the presence of caspase-3-specific inhibitor DEVD-CHO 10 μmol/L (PharMingen) for 30 min at 37 ºC, before the addition of substrate. The value (in arbitrary absorbance units) of the absorbance signal of the inhibited sample was subtracted from that of the noninhibited sample.

Flow cytometry analysis of apoptosis Cells were harvested at various time by centrifugation, washed twice with PBS, and resuspended in a final volume of PBS 300 μL. One milliliter of ethanol was added to the resuspended cells with vigorous mixing. Fixed cells were stained for 5 min with propidium iodide, incubated in the dark for 30 min. then the fluorescent intensity was analysed by flow cytometer (FACScan, Beton Dickinson, USA). Data were analysed with CellQuest™ software (Beton Dickinson, USA).

Measurement of cell growth and viability Growth inhibition of HL-60/ADR cells was determined by the colorimetric MTT viability/proliferation assay. Cells were transfected for 5 h at 37 ºC and incubated for another 64 h at 37 ºC. Subsequently, 10 μL of MTT reagent (5 g/L) was added to each well. The reaction was stopped 4 h after incubation and then the absorbance values at 570 nm were measured by a microELISA reader (Sigma). The viability of HL-60/ADR cells was assessed by morphology analysis using an inverted phase-contrast microscope (Leize, Wetzlar, Germany).

Statistical analysis All data were expressed as mean±SD and statistically analyzed by *t*-test. $P<0.05$ was considered significant.

RESULTS

Down-regulating effects of the pcDNA3.1/*survivin* on *survivin* mRNA By RT-PCR, the antisense RNA eukaryotic expression plasmid was examined for its effect on *survivin* mRNA in HL-60/ADR cells, which overexpress the survivin protein. The antisense RNA eukaryotic expression plasmid pcDNA3.1/*survivin* reduced the *survivin* mRNA level significantly (Fig 1). To further characterize the potency of pcDNA3.1/*survivin*, the time course of its effect on *survivin* mRNA in HL-60/ADR cells was examined by RT-PCR. pcDNA3.1/*survivin* down-regulated the *survivin* mRNA level in a time-dependent manner. A maximum down-regulation to 33 % of the initial mRNA level was achieved after 48 h (Fig 2). After this, pcDNA3.1/*survivin* did not result in increased antisense efficacy. The mismatch control pcDNA3.1 did not down-regulate the

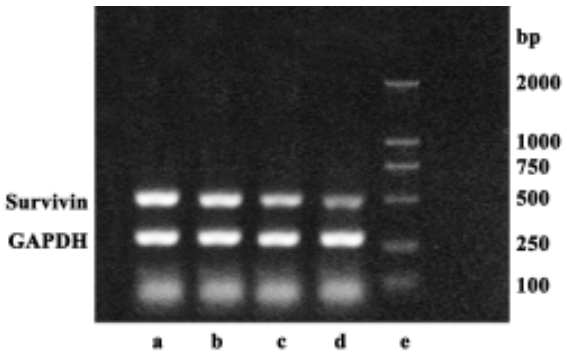

Fig 1. RT-PCR analysis of *survivin* mRNA in HL-60/ADR cells after transfection with pcDNA3.1/*survivin*. a) control; b) 12 h; c) 24 h; d) 48 h; e) DNA Marker.

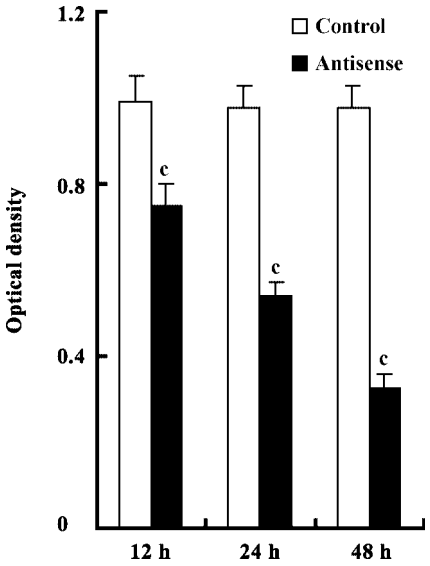

Fig 2. Time course of *survivin* mRNA expression after transfection with pcDNA3.1/*survivin*. *n*=3. Mean±SD. $^cP<0.01$ *vs* control group.

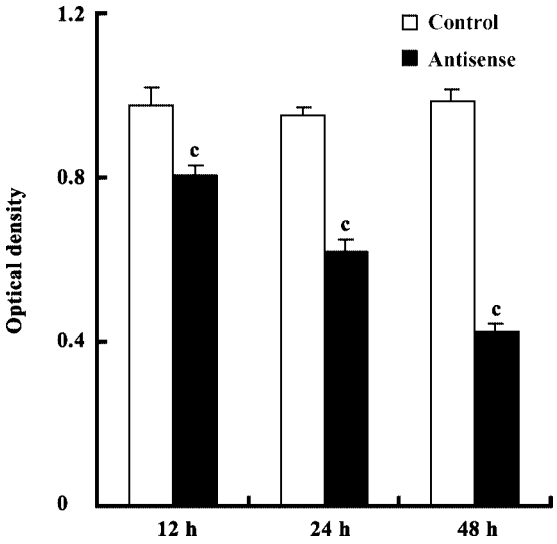

Fig 4. Time course of survivin protein expression after transfection with pcDNA3.1/*survivin*. *n*=3. Mean±SD. $^cP<0.01$ *vs* control group.

survivin mRNA level.

Down-regulating effects of the pcDNA3.1/*survivin* on survivin protein By Western blot, the antisense RNA eukaryotic expression plasmid was examined for its effect on survivin protein in HL-60/ADR cells, which overexpressed the survivin protein. The antisense RNA eukaryotic expression plasmid pcDNA 3.1/*survivin* reduced the survivin protein level significantly (Fig 3). To further characterize the potency of pcDNA3.1/*survivin*, the time course of its effect on survivin protein in HL-60/ADR cells was examined by Western blot. pcDNA3.1/*survivin* down-regulated the survivin protein level in a time-dependent manner. A maximum down-regulation to 43 % of the initial protein level was achieved after 48 h (Fig 4). After this, pcDNA3.1/*survivin* did not result in increased antisense efficacy. The mismatch control pcDNA3.1 did not down-regulate the survivin protein level.

Inhibition of cell growth by pcDNA3.1/*survivin* Sixty-four hours after the transfection, pcDNA3.1/*survivin* reduced HL-60/ADR cell gowth significantly. The unspecific growth-inhibitory effect of the mismatch control pcDNA3.1 was comparatively low (Tab 1).

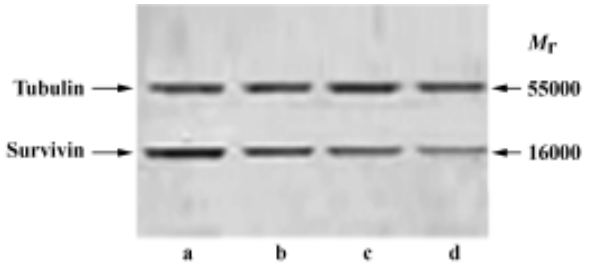

Fig 3. Western blot analysis of survivin protein in HL-60/ADR cells after transfection with pcDNA3.1/*survivin*. a) control; b) 12 h; c) 24 h; d) 48 h.

Tab 1. Inhibitory effect of pcDNA3.1/*survivin* on the growth of HL-60/ADR cells. *n*=4. Mean±SD. $^cP<0.01$ *vs* control group.

Group	A_{570}	Inhibitory rate/%
Untreated	0.65±0.07	0.0
Control	0.62±0.05	4.6
Antisense	0.49±0.05^c	24.6

Induction of apoptosis by pcDNA3.1/*survivin* Lysates from pcDNA3.1/*survivin*-treated cells revealed increased caspase-3 activity compared with control (Tab 2). Moreover, after treatment with pcDNA3.1/*survivin*, cells-contained nuclei with condensed and fragmented chromatin, provided further evidence for the induction of apoptosis as a consequence of *survivin* antisense treatment. In cultures treated with the mismatch control pcDNA3.1, very few cells showed signs

Tab 2. Time course of the activity of caspase-3 in HL-60/ADR cells after transfection with pcDNA3.1/*survivin*. *n*=3. Mean±SD. $^cP<0.01$ *vs* control group.

Group	A_{400}		
	12 h	24 h	48 h
Untreated	0.041±0.005	0.045±0.005	0.039±0.005
control	0.044±0.006	0.055±0.006	0.046±0.004
Antisense	0.046±0.005	0.124±0.006^c	0.247±0.025^c

of apoptosis. Flow cytometry analysis also confirmed that the induction of apoptosis in HL-60/ADR cells was due to the down-regulation of *survivin* mRNA (Tab 3).

Tab 3. Percentage of apoptotic cells after transfection with pcDNA3.1/*survivin*. *n*=3. Mean±SD. $^cP<0.01$ *vs* control group.

Group	Apoptotic cells/%		
	12 h	24 h	48 h
Untreated	4.2±0.3	4.3±0.3	3.9±0.2
Control	4.6±0.4	4.3±0.3	4.2±0.3
Antisense	4.7±0.4	6.8±0.3^c	8.6±0.4^c

pcDNA3.1/*survivin* sensitizes HL-60/ADR cells to ADR To investigate whether down-regulation of survivin expression has the potential to sensitize HL-60/ADR cells to chemotherapy, a combination treatment with pcDNA3.1/*survivin* and ADR was performed. Treatment with ADR after transfection of pcDNA3.1/*survivin*, the chemosensitivity was increased significantly. Compared to treatment with ADR alone, the chemosensitivity increased about 4.36 times (Tab 4).

Tab 4. Reversal effect of pcDNA3.1/*survivin* on MDR in HL-60/ADR cells. IC_{50} of ADR was measured by MTT assay. *n*=4. Mean±SD. $^cP<0.01$ *vs* ADR group.

Group	IC_{50}/μmol·L^{-1}	Reversal fold
ADR	1.50±0.17	-
Antisense+ADR	0.283±0.012^c	5.36

DISCUSSION

The development of resistance of tumor cells to anti-cancer drugs is one of the critical issues for successful chemotherapy. Multi-drug resistance is an adaptable reaction of tumor cells to chemotherapeutic drugs. Various kinds of protein expression and/or the changes of enzyme activity could be seen in MDR-type tumor cells. Through the changes of these proteins, tumor cells could avoid being killed by chemical agents. Much evidence suggests that survivin may be involved in drug resistance[9,10]. Down-regulation of survivin is likely to reduce the apoptotic threshold in cells.

Antisense RNA is the RNA that is complementary with the mRNA. The antisense RNA could combine with mRNA specifically and inhibit the translation of the mRNA. In comparison of the ribozyme technique, antisense RNA technique has the advantages of simple designing, strong specificity, easy operating, and being economic and time saving.

In the present study, we describe an antisense cDNA approach to down-regulate surivin expression and demonstrate its ability to induce apoptosis and reverse MDR in human leukemia multidrug resistant cell line HL-60/ADR.

RT-PCR and Western blot showed that the expression of *survivin* was decreased as compared with their parental cells and their pcDNA3.1-transfected groups. This indicated that the antisense RNA of *survivin* had partly blocked the transcription of *survivin* mRNA and inhibited the translation of survivin protein.

The coding strand of the *survivin* gene shows extensive complementary to the *EPR*-1 gene[11], therefore it is likely to amplifies *EPR*-1 cDNA, which may lead to a false positive *survivin* signal in cases where only *EPR*-1 is expressed or to an underestimation of *survivin* down-regulation on antisense treatment if both genes are coexpressed. The expression of both genes, however, is reported to be mutually exclusive[12]. This suggests that the amplified transcript corresponds to the *survivin* mRNA and not the *EPR*-1 mRNA.

In our study, pcDNA3.1/*survivin* indeed induced a growth-inhibitory effect and apoptosis in HL-60/ADR cells in the absence of any further cytotoxic stimulus. The sensitivity of the pcDNA3.1/*survivin* transfected cells to chemotherapeutic drugs (ADR) increased more significantly than their parental cells. This means that lowered expression of *survivin* could increase sensitivity of the tumor cells to chemotherapeutic drugs.

This observation is in agreement with the finding of others describing the necessity of interaction between survivin and microtubules of the mitotic spindle apparatus to prevent a default induction of apoptosis at the G_2-M phase of the cell cycle[13]. This mode of action may constitute a safeguard mechanism against the proliferation with unwanted properties. Whether down-regulation of survivin expression sensitizes cells to apoptosis only when passing the G_2-M checkpoint remains to be investigated.

Two major apoptosis signaling pathways are known: (a) the mitochondrial pathway; and (b) the death receptor pathway. The antiapoptotic proteins bcl-2 and bcl-xL block the apoptotic event of mitochondrial cytochrome c release into the cytosol and have been shown to inhibit mainly the first of these two pathways. Because survivin directly blocks the processing and activation of effector caspase-3 and -7, which act at a common downstream part of the two major apoptosis pathways, the *survivin* antisense approach has the potential to facilitate apoptosis through both pathways. Using pcDNA3.1/*survivin* in combination with ADR, which triggers cell death via cytochrome c release[14], we provide the evidence that down-regulation of survivin sensitizes cells to death induction via mitochondrial pathway. Whether it also increases cell death induced by the death receptor pathway and whether it sensitizes other survivin-overexpressing tumor cells to chomotherapy are currently under investigation. To normal cells which lacks survivin expression, pcDNA3.1/*survivin* does not induce apoptosis[15]. Our data suggest that the use of pcDNA3.1/*survivin* deserves further investigation as a novel approach to selective cancer therapy.

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