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Reversal of drug resistance by silencing Survivin gene expression in acute myeloid leukemia cells

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The role of Survivin in the pathogenesis of leukemia was explored in order to discover the effective avenues for gene therapy. Most primary leukemia cells isolated from patients as well as three leukemia cell lines (HL-60, K562, and U937) all expressed Survivin gene. To investigate the relationship between Survivin and chemotherapeutic resistance, HL-60 cells were treated with daunorubicin (DNR), mitoxantrone (MIT) or arsenious oxide (As_2O_3), and it was found that after 24 h the level of Survivin mRNA was decreased by 9.7%, 41.0% and 27.5%, respectively. At 72 h, the level of Survivin mRNA was increased by 21.2% and 65.2% in HL-60 cells treated with DNR or MIT, but decreased by 33.2% in those treated with As_2O_3 as compared with that in the cells treated for 24 h. These results showed that DNR and MIT could initally decrease the expression of Survivin and then increase it, but As_2O_3 could decrease the Survivin expression continually. Furthermore, shRNA plasmids targeting the Survivin gene (pEGFP-Survivin), which can silence the expression of Survivin with a high specificity, were constructed. pEGFP-Survivin and pEGFP-H1 were transfected into HL-60 cells *via* electroporation and selected by G418, and HL-60/Survivin and HL-60/EGFP cells were obtained. After treatment with DNR, the cell survival rate and IC_{50} of DNR in HL-60/Survivin cells were decreased substantially as compared with those of HL-60/EGFP and HL-60 cells (IC_{50} of DNR: 18.3±2.45 *vs* 40.8±6.37 and 39.2±5.91 ng/ml, respectively), and the apoptosis rate was elevated ((84.3±19.7)% *vs* (45.8±13.8)% and (50.9±12.4)%, respectively). These results suggest that shRNA can down-regulate the expression of Survivin in HL-60 cells substantially and improve their sensitivity to DNR. They also further explain the pathogenesis of leukemia drug resistance and provide new theory in the design of clinical therapies.

Keywords: Survivin, chemotherapeutic resistance, shRNA, acute myeloid leukemia, apoptosis

INTRODUCTION

The balance between cell death and cell viability is important in tissue homeostasis. Abnormalities in the control of programmed cell death (apoptosis) play an important role in tumorigenesis (Tazzari *et al.*, 2008). The evolutionarily conserved multi-step apoptosis cascade is regulated by proteins that promote or inhibit apoptotic cell death (Jakubowska *et al.*, 2007). The inhibitor of apoptosis proteins (IAPs) were originally identified in baculoviruses and are highly conserved across species. The expression of at least some IAPs is up-regulated by various growth factors (Carter *et al.*, 2001), supporting their roles in survival. Among them, Survivin is considered important because it links cell death and cell proliferation (Ai *et al.*, 2006). Survivin is not expressed in adult terminally differentiated tissues, but is expressed in most human cancers and cancer cell lines. Disruption of Survivin expression causes

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Abbreviation: AML, acute myeloid leukemia; ALL, acute lymphocyte leukemia; DNR, daunorubi; dsRNAs, double-stranded RNAs; IAPs, inhibitor of apoptosis proteins; MIT, mitoxantrone; PBS, phosphate-buffered saline; RNAi, RNA interference; SDS/PAGE, SDS/polyacrylamide gel electrophoresis; shRNA, short hairpin RNAs ; siRNAs, small interfering RNAs.

cell death and cell division defects, which results in polyploidy and multinucleated cells (Bhardwaj *et al.*, 2007). Studies from different groups have demonstrated that elevated expression of Survivin is associated with poor prognosis and increased tumor recurrence in many cancers (Adida *et al.*, 2000; Sarela *et al.*, 2000) including acute myeloid leukemia (AML) (Adida *et al.*, 2000).

During the last years it has become evident that apoptosis-related molecules may be useful as prognostic markers because several antiapoptotic mechanisms are operational in acute leukemia. Suppression of apoptosis contributes to leukemogenesis by different mechanisms, including prolonging cell life span, thus facilitating the accumulation of gene mutations, permitting growth factor-independent cell survival, promoting resistance to immune-based cytotoxicity, and allowing disobeyance of cell cycle checkpoints which would normally induce apoptosis (Altieri, 2003). In addition, Survivin appears to be involved in tumor cell resistance to some anticancer agents and ionizing radiation (Zaffaroni *et al.*, 2005).

To assess the relationship between Survivin and chemotherapeutic resistance in AML, the expression of Survivin in myeloid leukemia cells treated with daunorubicin (DNR), mitoxantrone (MIT) or arsenious oxide (As_2O_3) was studied. An shRNA vector targeting Survivin (pEGFP-Survivin) was constructed and transfected into HL-60 cells. It was found that pEGFP-Survivin could decrease the expression of Survivin substantially and improve the sensitivity of HL-60 cells to chemotherapeutic drugs. It is suggested that Survivin can be a potential target for leukemia therapy against drug resistance.

MATERALS AND METHODS

Patients. Bone marrow aspiration samples were obtained from a group of 37 untreated leukemia patients, consisting of 23 males and 14 females, aged 14–69 years (median age: 42.2). All patients were diagnosed by cell morphology, marrow histology, and phenotype. According to the French-American-British (FAB) standard, there were 22 acute myeloid leukemia (AML) cases with the following distribution of subtype: M_1, three cases; M_2, nine cases; M_3, five cases; M_4, two cases; and M_5, three cases. Furthermore, 15 patients with acute lymphocyte leukemia (ALL) comprised two L1 cases, ten L2 cases, and three L3 cases. The control group included 10 healthy volunteers — six males and four females — aged 18–44 years (median age: 33.6). Bone marrow samples (2–3 ml each) were separated with a Ficoll-based discontinuous density gradient, resulting in mononuclear cells. These cells were washed twice with phosphate buffered saline (PBS), mixed with 1 ml TRIzol (Gibco BRL, USA), and stored at –80°C.

Cell culture and drug treatment. Three leukemia cell lines, HL-60, K562 and U937 were used in this study. All cells were cultured in RPMI-1640 medium containing 10% new born calf serum (Gibco BRL, USA) at 37ºC in a humid atmosphere of 5% CO_2 and 95% O_2. HL-60 cells were cultivated in 6-well plates at a density of 2×10^5/ml in about 5 ml, then exposed to DNR (20 ng/ml), MIT (5 ng/ml) or As_2O_3 (1 μM), respectively. Cells not treated with drug served as controls. The cells in each group were collected after treatment for 24 or 72 h.

Design and construction of shRNA. The pEGFP-H1 vector was specifically designed for the cloning of small synthetic oligonucleotides encoding two complementary sequences of 19 to 21 nt, homologous to a segment of the gene of interest, separated by a short spacer region of 5–7 nt. The insert was cloned downstream of the human H1 promoter, then the recombinant was transcribed into a short dsRNA with a hairpin structure consisting of a 19–21 nt bp double stranded region corresponding to the target sequence and a small loop formed by the spacer region. The pEGFP-H1 plasmid was digested with *Bpi*I (MBI, USA), an unusual restriction enzyme that generates asymmetric cohesive overhangs that are not mutably compatible to eliminate the risk of self-ligation of the vector. (1) The design of shRNA: according to the rule, 19–21 nt shRNA targeting Survivin gene was designed by siRNA software which was provided by the Invitrogen Company, and then blasted against the NCBI gene bank to ensure its specificity. This sequence should start with an A which corresponds to the transcription initiation point of the human H1 promoter. Two complementary oligonucleotides were designed, in which the first four bases created 5′ overhangs compatible with *Bpi*I (TCCC for the sense strand and AAAC for the antisense strand). The forward and reverse oligonucleotides were 5′ TCCCAGGAAACTGCGAAGAAAGTTCAAGAGACTTTCTTCGCAGTTTCCTTT 3′ and 5′ CAAAAAAGGAAACTGCGAAGAAAGTCTCTTGAACTTTCTTCGCAGTTTCCT 3′, respectively. (2) Annealing of siRNA insert: the siRNA insert was dissolved and diluted to obtain forward and reverse oligonucleotides at a concentration of 25 μM. The annealing solution was prepared by mixing the following components (forward oligonucleotide 2 μl, reverse oligonucleotide 2 μl, 0.5 μM NaCl 6 μl, H_2O 20 μl), incubated for 2 min at 80ºC, put in a water bath until the temperature was reduced to 35ºC, then stored at –20ºC for further use. (3) The construction of pEGFP-Survivin: pEGFP-H1 was digested with *Bpi*I, and its large fragment was eluted by using a 0.7% low-melting agarose gel and the purified DNA fragment was diluted to obtain a

solution at 0.1 μg/μl. The ligation solution was prepared by mixing the following components (*Bpi*I-digested pEGFP 1 μl (100 ng), annealed siRNA insert 1 μl, T4 DNA ligase 1 μl, 10×ligation buffer 2 μl, H_2O 15 μl). The mixture was incubated at 16°C overnight. The constructed vector, pEGFP-Survivin, was transformed into DH5α *Escherichia coli* bacteria, and confirmed by restriction digestion and DNA sequence analysis.

Cell transfection and expression of survivin. HL-60 cells, $1\times10^{6,}$ in exponential growth phase were washed once with pre-chilled PBS, re-suspended in 400 μl hypoosmolar electroporation buffer (Eppendorf, Germany), and transferred into electroporation cuvette. pEGFP-Survivin plasmid (10 μg) dissolved in 100 μl ddH_2O was added the cuvette. The cuvette was incubated at 0°C for 10 min, subjected to the electroporation (500 V, 80 μs), and keep at room temp. for 10 min. Then, the cells were transferred into a culture bottle and cultured with RPMI-1640 containing serum. After 48 h, G418 was added gradually to get a final concentration of 1 mg/ml.

RT-PCR analysis. The levels of Survivin and β-actin mRNA were detected by using RT-PCR. Primers for human Survivin were as follows: sense 5′ CTCAAGGACCACCGCATCTC 3′ and antisense 5′ CCTCAATCCATGGCAGCCAG 3′ (synthesized by Shanghai Biology Engineering Company, China). The product of Survivin was 392 bp in size. Primers for human β-actin were as follows: sense 5′ TGTACGTTGCTATCCAGGCT 3′ and 5′ CTCCTTAATGTCACGCACGA 3′. The product of β-actin was 247 bp in size. Total RNA was extracted from cells by Trizol reagents and cDNA was synthesized by M-MLV reverse transcription-polymerase (Promega, USA). PCR was performed in pre-aliquoted tubes at a final volume of 50 μl, containing cDNA 3 μl, 10×PCR buffer 5 μl, 25 mM $MgCl_2$ 10 μl, 10 mM dNTP 1.5 μl, Taqase 2 U, Survivin primers 25 pmol, and β-actin primers 25 pmol. The product was amplified over 30 cycles according to the following procedure: denaturing at 94°C for 30 s, annealing at 58°C for 45 s and extension at 72°C for 1 min. There was an additional pre-denaturing at 94°C for 5 min and final extension at 72°C for 10 min. PCR products were visualized under ultraviolet transillumination after separation in 1.5% agarose gels containing ethidium bromide. Results were expressed as Survivin/β-actin ratios. Each result was averaged over three tests.

Western blot analysis. Cells were washed twice with phosphate-buffered saline (PBS) and lysed at 4×10^4 cells/μl in cell lysis buffer (20 mM Hepes, pH 7.4, 0.25% NP-40, containing protease inhibitor cocktail; Boehringer Mannheim, USA) for 10 min on ice. Equal amounts of lysate (equivalent to 5×10^5 cells) were subjected to SDS/PAGE 12% polyacrylamide gels. Proteins were transferred to Hybond-P (Amersham Pharmacia Biotech, UK) membranes and reacted with polyclonal antibodies against Survivin and β-actin (Santa Cruz, USA) for 2 h at room temp. After they were washed, membranes were probed with a horseradish peroxidase-conjugated secondary antibody and reacted with ECL reagent (Amersham Pharmacia Biotech, UK). Signals were detected by a PhosphorImager (Storm 860, version 4.0; Molecular Dynamics, Sunnyvale, CA, USA) and quantified *via* Scion Image software (Scion, Frederick, USA).

The effect of shRNA on sensitivity of HL-60 cells to DNR. Three leukemia cell lines, HL-60 (no transfection), HL-60/EGFP (transfected with pEGFP-H1 vector) and HL-60/Survivin (transfected with pEGFP-survivin) were assayed. (1) Cell survival rate: 1×10^5/ml of cells in exponential growth phase was cultivated in 24-well plates and treated with 40 ng/ml DNR. Then the cells were collected at 0, 12, 24, 36, 48 and 60 h, and the cell survival graph was drawn. (2) Sensitivity of cells to DNR: 1×10^5/ml of cells in exponential growth phase was cultivated in 96-well plates and treated with DNR at concentrations of 0, 10, 20, 30, 40, 50 and 60 ng/ml. The cell viability was detected by MTT after 48 h and IC_{50} was calculated. (3) Apoptosis rate detected by FACS: 1×10^5/ml of cells in exponential growth phase was cultivated in 24-well plates and treated with 40 ng/ml DNR for about 48 h. Cells were collected and washed with PBS, followed by resuspension in binding buffer (Hepes/NaOH 10 mM, pH 7.4, NaCl 140 mM, $CaCl_2$ 2.5 mM). The samples were then incubated with 5 μl/l of Annexin-V in the dark for 15 min, washed with binding buffer and resuspended in 1% formaldehyde in the binding buffer at 4°C for 30 min. After being washed with binding buffer again, the cells were stained with 500 μl/l PI (1 μg /ml, Sigma, USA) for 15 min, and then were analyzed in a flow cytometer (Becton Dickinson, Stanford, USA).

Statistical analysis. All data are expressed as mean ± standard deviation (S.D.), and differences are considered significant when $P<0.05$. Significant-difference values were determined using SPSS, version 10.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

The expression of Survivin in leukemia cells

The amplified product of Survivin and β-actin was 392 bp and 247 bp, respectively. All values were normalized by Survivin/β-actin ratios. The expression of Survivin was checked in about 67.6% (25/37) of leukemia patients and only in 20.0% (2/10) of control healthy volunteers, and it was slightly

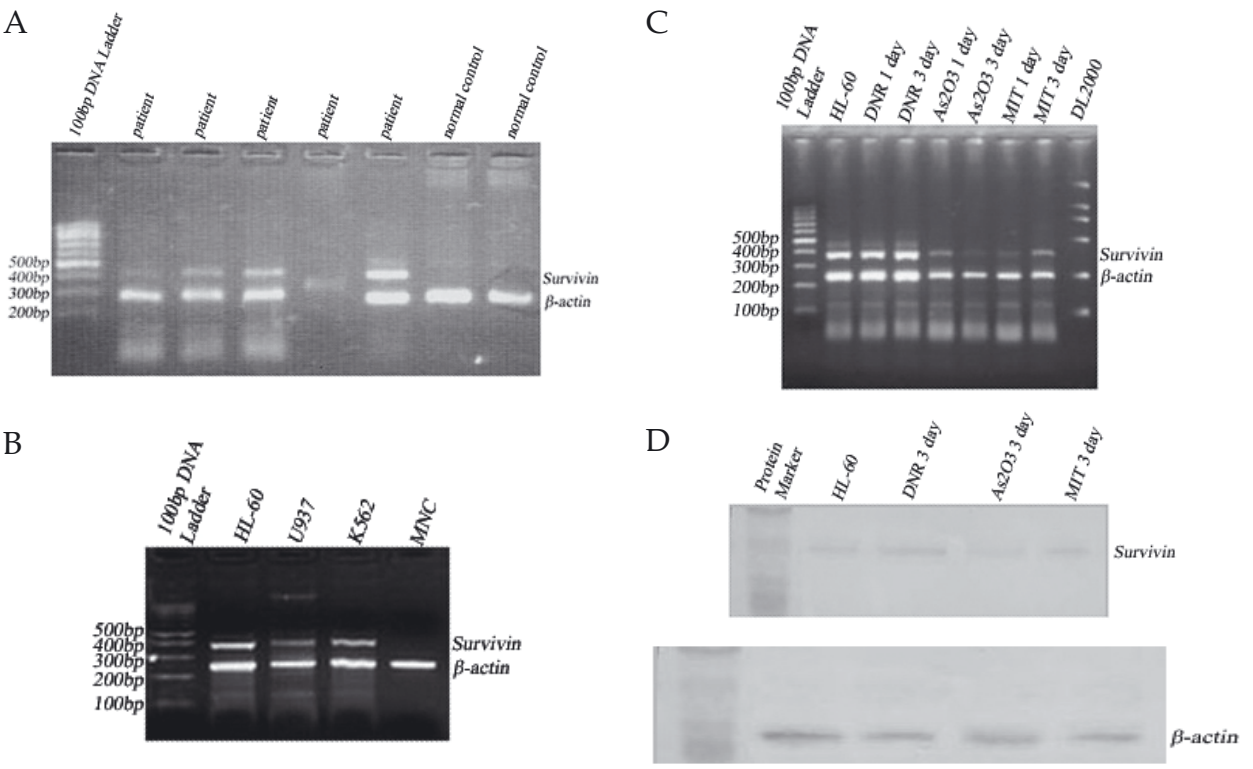

Figure 1. Expression of Survivin in leukemia cells treated with DNR, MIT or As_2O_3.
A. Survivin is expressed in marrow samples from leukemia patients (lanes 2 through 6), but not in normal control subjects (lanes 7 and 8); **B**. Survivin is expressed in three different leukemia cell lines; **C**. RT-PCR was used to analyze changes of Survivin expression in HL-6 cells treated with DNR, MIT or As_2O_3; after 24 h the expression of Survivin mRNA was decreased by 9.7%, 41.0% and 27.5%, respectively. After 72 h, the expression of Survivin mRNA was increased by 21.2% and 65.2% in HL-60 cells treated with DNR or MIT, but decreased by 33.2% in those treated with As_2O_3 as compared with that in HL-60 cells treated for 24 h; **D**. Expression of Survivin protein was increased by 14.6% and 11.3%, respectively, in HL-60 cells treated with DNR or MIT for 72 h, but decreased by 81.3% in those treated with As_2O_3 as compared with control group.

more common in ALL patients (73.3%) than in AML patients (63.6%) (Table 1, Fig. 1A). In addition, three leukemia cell lines, HL-60, U937 and K562, all expressed the Survivin gene (Fig. 1B). It is evident that Survivin was expressed in leukemia cells.

After HL-60 cells were treated with DNR, MIT or As_2O_3 for 24 h, the expression of Survivin mRNA was decreased by 9.7%, 41.0% and 27.5%, respectively, as compared to the controls. At 72 h of treatment, the expression of Survivin mRNA was increased by 21.2% and 65.2%, respectively, in HL-60 cells treated with DNR or MIT, but decreased by 33.2% in those continually treated with As_2O_3 as compared with that in the cells treated for 24 h. (Table 2 and Fig. 1C). There was no significant difference in Survivin protein expression between control HL-60 cells and those treated with DNR, MIT or As_2O_3 for 24 h. At 72nd h, the expression of Survivin protein was increased by 14.6%, 11.3% in HL-60 cells treated with DNR or MIT, but decreased by about 81.3% in those treated with As_2O_3 as compared with controls (Fig. 1D). These results showed that the expression of Survivin in HL-60 cells treated with DNR or MIT was decreased at first, but then increased substantially. However, As_2O_3 down-regulated the Survivin expression consistently. This might contribute to the occurrence of drug resistance in chemotherapy.

Table 1. Expression of Survivin mRNA in leukemia cells

Groups	Cases	Survivin mRNA positive (%)	Survivin/β-actin ratio mean±S.D. (range)
Leukemia	37	67.6*	0.45±0.20 (0.22–0.69)
AML	22	63.6*	0.48±0.22 (0.26–0.63)
ALL	15	73.3*	0.55±0.18 (0.33–0.69)
Control	10	20.0	0.21±0.15 (0.11–0.27)

Note: *As compared with control group, $P<0.05$.

Construction, identification and transfection of recombinant vector

The recombinant vector, pEGFP-Survivin, was constructed on the basis of pEGFP-H1. pEGFP-H1 exploits the white-blue selection system to further facilitate the cloning. The two *Bpi*I sites flank the lacZ α-peptide allowing the discrimination between

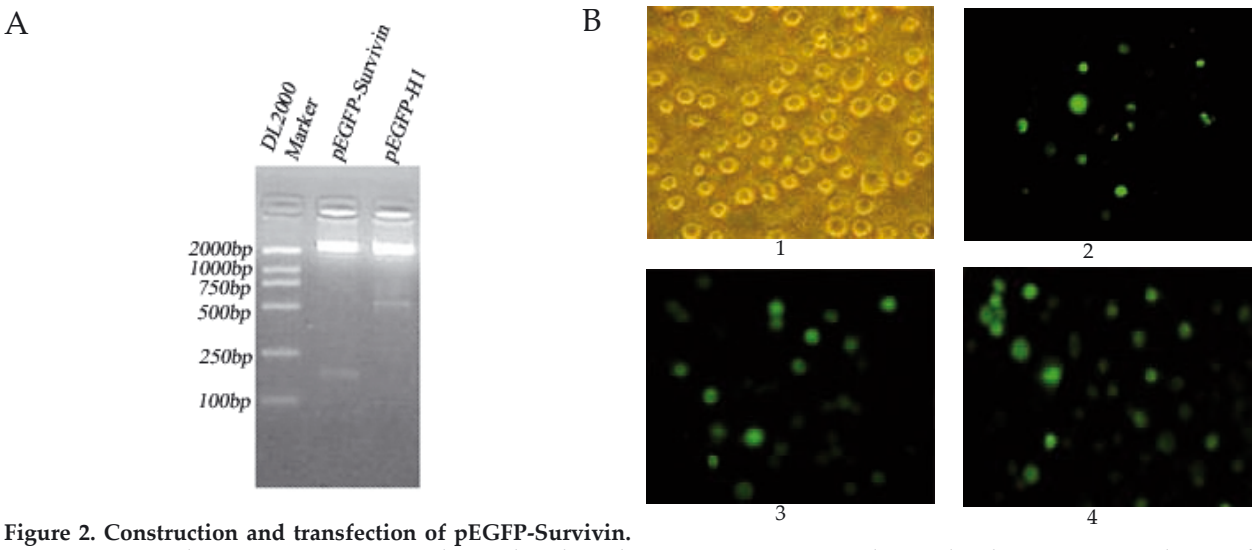

Figure 2. Construction and transfection of pEGFP-Survivin.
A. pEGFP-H1 and pEGFP-Survivin were digested with *Mul*I. Because EM7 α-peptide (339 bp) between two *Bpi*l sites of pEGFP-H1 was replaced with shRNA sequence (19 bp) in pEGFP-Survivin, the difference between two small fragments is about 300 bp; **B**. Transfection of HL-60 cells observed under fluorescence microscopy (×200) (1, HL-60, 2, HL-60/EGFP, 3, HL-60/Survivin, 4, HL-60/Survivin selected with G418).

blue parental clones and white recombinant clones. Furthermore, pEGFP-H1 has the *neo* gene. The *neo* gene from Tn5 encodes an aminoglycoside 3′-phospho-transferase that confers resistance to the antibiotics kanamycin in bacteria and G418 in mammalian cells. The recombinant plasmid was constructed and transformed into DH5α *E. coli* bacteria, and some white clones named pEGFP-Survivin were obtained. Plasmids from the white clones were extracted and confirmed by digestion with *Mul*I. As shown in Fig. 2A, pEGFP-H1 and pEGFP-Survivin were all divided into two fragments. The small fragments of pEGFP-H1 and pEGFP-Survivin were about 500 bp and 200 bp, respectively. The difference was 300 bp because the EM7 α-peptide (339 bp) between the two *Bpi*l sites of pEGFP-H1 was replaced with the shRNA sequence (19 bp) in pEGFP-Survivin. This proved the success of pEGFP-Survivin construction. In addition, the DNA sequence of pEGFP-Survivin plasmid was confirmed. pEGFP-Survivin and pEGFP-H1 were transfected into HL-60 cells *via* electroporation. After selection by G418, cells containing pEGFP-Survivin or pEGFP-H1, named HL-60/Survivin and HL-60/EGFP, were obtained (Fig. 2B).

Table 2. Changes of Survivin expression in HL-60 cells treated with DNR, MIT or As_2O_3

Groups	n	Survivin/β-actin	
		24 h	72 h
Control	3	0.652±0.054	0.652±0.054
DNR-treated	3	0.589±0.041	0.714±0.027$^{\#}$
As_2O_3-treated	3	0.473±0.031**	0.316±0.034$^{\#\#}$
MIT-treated	3	0.385±0.036**	0.636±0.038$^{\#\#}$

Note: **As compared with control group, $P<0.01$; $^{\#}$As compared with that at 24 h $P<0.05$; $^{\#\#}$As compared with that at 24 h, $P<0.01$.

Effect of shRNA on the expression of Survivin

The expression of Survivin in three kinds of cells, HL-60, HL-60/EGFP and HL-60/Survivin, was detected by using RT-PCR and Western blotting, and all values were normalized by Survivin/β-actin ratios. The mRNA expression of Survivin showed no significant difference between HL-60 and HL-60/EGFP cells, but it was lower in HL-60/Survivin cells (about 25.3%) than in HL-60 or HL-60/EGFP cells (Table 3, Fig. 3A). Western blotting showed similar results. The expression of Survivin protein was only about 28.6% in HL-60/Survivin cells compared to HL-60 or HL-60/EGFP cells (Table 3, Fig. 3B). These results revealed that pEGFP-Survivin could down-regulate the Survivin expression in HL-60 cells efficiently.

The effect of Survivin on the sensitivity of HL-60 cells to DNR

The HL-60/Survivin, HL-60/EGFP and HL-60 cells had different sensitivity to DNR. After treatment with 40 ng/ml DNR, the cell survival rate of HL-60/Survivin cells was decreased more quickly than that of HL-60 or HL-60/EGFP cells. After treatment with DNR for 60 h, the cell survival rate of HL-60/Survivin

Table 3. The effect of Survivin shRNA

Groups	n	Survivin/β-Actin	
		RNA level	Protein level
HL-60	3	0.489±0.143	1.12±0.34
HL-60/EGFP	3	0.477±0.152	1.05±0.28
HL-60/Survivin	3	0.124±0.035*	0.32±0.11*

Note: *As compared with HL-60 and HL-60/EGFP cells, $P<0.01$.

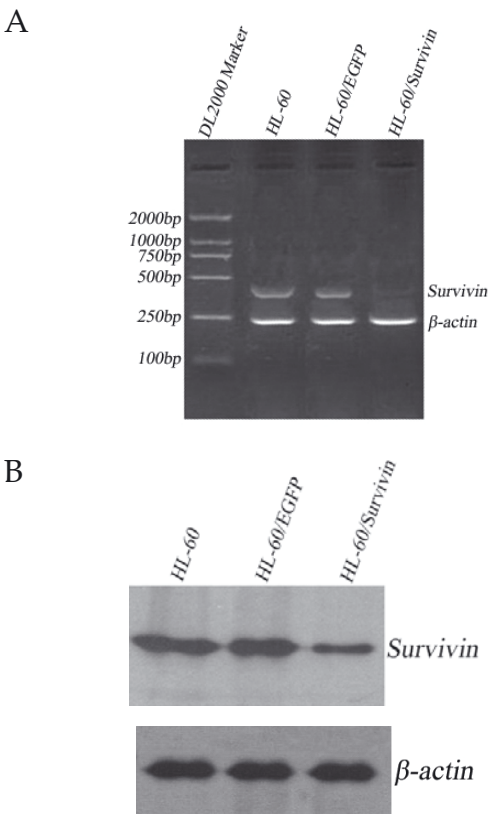

Figure 3. Expression of Survivin in HL-60, HL-60/EGFP and HL-60/Survivin cells.
A. Expression of Survivin mRNA in HL-60 and HL-60/EGFP cells showed no significant difference, but was substantially decreased in HL-60/Survivin (about 25.3% of the control level); **B**. Expression of Survivin protein was about 28.6% in HL-60/Survivin compared to HL-60/EGFP and HL-60 cells.

cells was about 9.27%, but it was 58.01% and 55.22% in HL-60 and HL-60/EGFP cells ($P<0.01$, Fig. 4A). Different concentrations of DNR were used to treat three kinds of cells and the IC_{50} was calculated. The IC_{50} of DNR was (40.8±6.37) ng/ml in HL-60, (39.2±5.91) ng/ml in HL-60/EGFP, and (18.3±2.45) ng/ml in HL-60/Survivin ($P<0.01$, Fig. 4B). The three kinds of cells were treated with 40 ng/ml for 48 h and the apoptosis rate was checked by using FACS. The apoptosis rate of HL-60/Survivin, HL-60 and HL-EGFP cells was about (84.3±19.7)%, (45.8±13.8)% and (50.9±12.4)%, respectively ($P<0.01$, Fig. 4C). It was demonstrated that HL-60 and HL-60/EGFP cells had similar sensitivity to DNR, but HL-60/Survivin cells were more sensitive, suggesting the down-regulation of Survivin expression could increase the sensitivity of HL-60 cells to DNR.

DISCUSSION

The induction of programmed cell death is the common outcome of successful cytotoxic therapy for many different types of cancers, including AML (Greiner *et al.*, 2008). AML is a heterogeneous disease characterized by the accumulation of leukemia blasts arrested at various stages of granulocytic and monocytic differentiation. Multiple genetic alterations that result in the disruption of the physiological regulation of apoptosis are thought to account for the ability of leukemia cells to grow autonomously and for their clinical resistance to therapy (Norgaard *et al.*, 1999). Recently, a new family of downstream inhibitors of caspases, the IAPs family, has emerged as a potential key player in the regulation of apoptosis in cancer (Mita *et al.*, 2008), and some scholars have already demonstrated that Survivin, a member of the IAPs family, is expressed and has prognostic relevance in AML (Tamm *et al.*, 2000). Using immunohistochemisty, Adida and coworkers (2000) found that Survivin was expressed in 60% of patients with diffuse B cell lymphoma and leukemia. In our study, previously untreated leukemia patients including all kinds of leukemia were tested and it was found that there was a high expression rate of Survivin, and it was higher in ALL than in AML. Moreover, it was found that Survivin was expressed in leukemia cell lines. These results were consistent with the above reports.

Recently, it was reported that there was a close relationship between Survivin expression and chemotherapeutic resistance. Some studies found the expression of Survivin was increased 2–6 fold in MKN-45 cells after treatment with cisplatin (Ikeguchi *et al.*, 2002) and was involved in the tumor cell resistance to some anticancer agents or ionizing radiation (Zaffaroni *et al.*, 2005). In our experiment, the Survivin expression was detected in HL-60 cells treated with three kinds of chemotherapeutic drugs. The results showed that the expression of Survivin was decreased after HL-60 cells were treated with DNR or MIT for 24 h, but after treatment for 72 h the expression of Survivin was significantly increased as compared with that in HL-60 cells treated for 24 h. It was concluded that HL-60 cells expressed Survivin and were sensitive to the chemotherapeutic drugs (DNR or MIT), which makes leukemia cells susceptible to apoptosis through down-regulation of Survivin expression. But with prolongation of the treatment time, the expression of Survivin in surviving leukemia cells was increased gradually, leading to the resistance to chemotherapy. This could explain why leukemia cells are sensitive at first, but become insensitive gradually to the clinical chemotherapy.

Unlike DNR or MIT, As_2O_3 down-regulated the Survivin expression continually, consistently with its different mechanism. DNR and MIT mostly affect synthesis of cell nucleic acids, by conjugating with the cell DNA directly and interfering with the synthesis of DNA and RNA. As_2O_3 can induce the

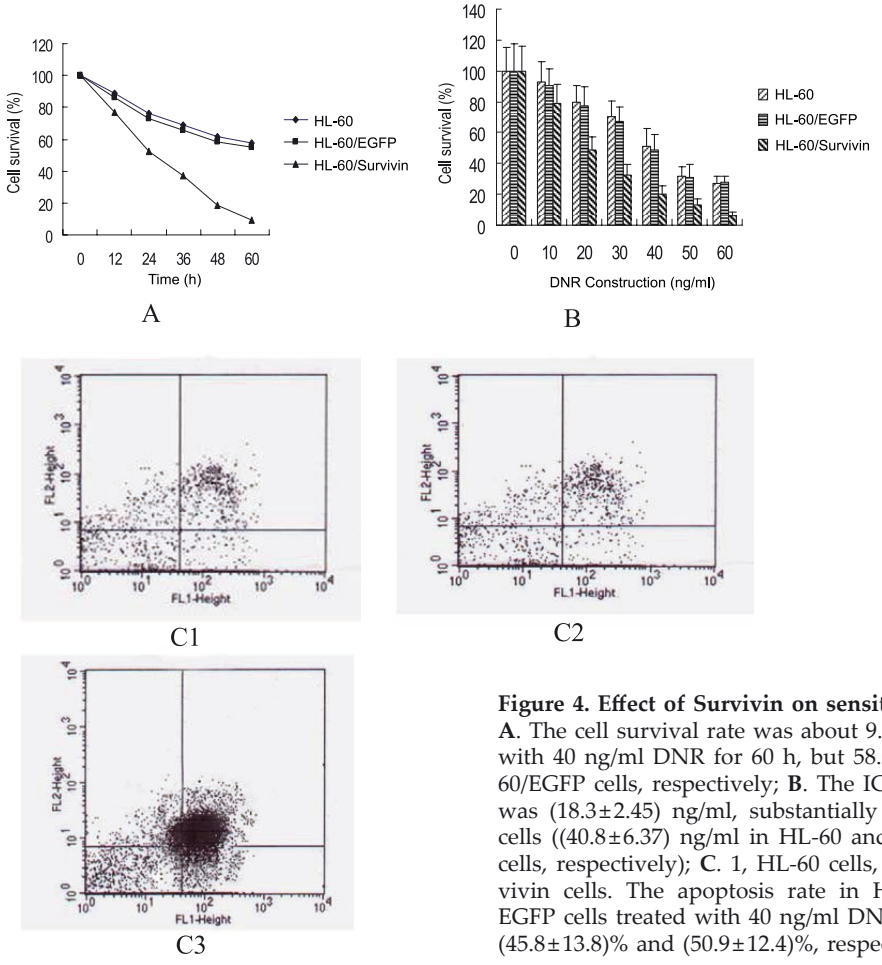

Figure 4. Effect of Survivin on sensitivity of HL-60 cells to DNR.
A. The cell survival rate was about 9.27% in HL-60/Survivin cells treated with 40 ng/ml DNR for 60 h, but 58.01% and 55.22% in HL-60 and HL-60/EGFP cells, respectively; **B**. The IC_{50} for DNR in HL-60/Survivin cells was (18.3±2.45) ng/ml, substantially lower than in other two types of cells ((40.8±6.37) ng/ml in HL-60 and (39.2±5.91) ng/ml in HL-60/EGFP cells, respectively); **C**. 1, HL-60 cells, 2, HL-60/EGFP cells, 3, HL-60/Survivin cells. The apoptosis rate in HL-60/Survivin, HL-60 and HL-60/EGFP cells treated with 40 ng/ml DNR for 48 h was about (84.3±19.7)%, (45.8±13.8)% and (50.9±12.4)%, respectively.

leukemia cell apoptosis through degrading PML-RARα, and small dosage of As_2O_3 can also induce differentiation of leukemia cells. Jing *et al.* (2001) reported that 0.1–1.0 μM As_2O_3 could induce differentiation of 20–30% of acute promyelocytic leukemia cells after co-culture for 6 days. In our experiment, the working concentration of As_2O_3 was 1 μM, which might affect HL-60 cells to differentiate and decreasing the Survivin expression. This phenomenon explained why As_2O_3 might have good therapeutic effect on some patients who respond poorly to other regular chemotherapeutic drugs. Therefore, it reminds us that As_2O_3 in combination with other regular drugs may reverse drug resistance and achieve better results.

RNAi is an evolutionarily conserved gene-silencing process triggered by dsRNAs (Hannon, 2002). The use of RNAi as a technique for analyzing loss-of-function phenotypes has revolutionized research in mammalian cells. The ways which induce RNAi in mammalian cells include the transfection of synthetic siRNAs and expression plasmids or viral vectors (Elbashir *et al.*, 2001). A common approach involves the transcription by RNA polymerase III of shRNA (Brummelkamp *et al.*, 2002). The use of shRNA offers several important advantages over siRNA such as better availability, lower cost, stability, and possibility of using both constitutive and inducible systems (Hannon *et al.*, 2004). In the experiment, the pEGFP-Survivin plasmid which could inhibit the expression of Survivin effectively and specifically was constructed successfully, transfected into HL-60 cells, and the HL-60/Survivin cells which stably expressed Survivin shRNA were obtained.

Because of the close relationship between expression of Survivin and chemotherapeutic resistance, suppressing the expression of Survivin should improve the effect of chemotherapy and have no great side-effects because of its specificity. Recently, it was found that suppressing the expression of Survivin by antisense technology could inhibit cell proliferation in a tumor and improve the sensitivity to chemotherapeutic drugs, eventually inducing the apoptosis of tumor cells (Olie *et al.*, 2000). In this experiment, it was found that the expression of Survivin was similar in HL-60 and HL-60/EGFP cells, but was about four times less in HL-60/Survivin cells. This proved that the constructed plasmid, pEGFP-Survivin, could down-regulate Survivin expression efficiently and specifically. In addition, it was found

that HL-60/Survivin cell survival rate and IC_{50} of DNR were decreased substantially, and apoptosis rate was elevated remarkably. It is proposed that the suppression of Survivin expression could improve the sensitivity of HL-60 cells to DNR and induce apoptosis. This phenomenon offers several important advantages. By increasing the sensitivity of cells to DNR, it allows lower concentration of the drug to be used in chemotherapy, thereby decreasing the likelihood of advance side-effects. This should be highly beneficial in the therapy of leukemia patients.

Above all, our study suggested that Survivin may play an important role in proliferation and progression of leukemia cells, and it level correlate with drug resistance. Inhibition of Survivin expression in leukemia cells *via* the highly efficient RNAi technique may improve their sensitivity to chemotherapy and offer a new approach to leukemia treatment.

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