

RESEARCH ARTICLE

siRNA-mediated Silencing of Survivin Inhibits Proliferation and Enhances Etoposide Chemosensitivity in Acute Myeloid Leukemia Cells

Hadi Karami^{1,2}, Behzad Baradaran^{1*}, Ali Esfahani³, Mehrdad Asghari Estiar⁴, Mohammad Naghavi-Behzad⁵, Masoud Sakhinia⁶, Ebrahim Sakhinia^{7*}

Abstract

Background: Overexpression of survivin, a known inhibitor of apoptosis, is associated with tumor progression and drug resistance in numerous malignancies, including leukemias. The aim of this study was to investigate the effect of a specific survivin small interference RNA (siRNA) on proliferation and the sensitivity of HL-60 acute myeloid leukemia (AML) cells to the chemotherapeutic drug etoposide. **Materials and Methods:** The cells were transfected with siRNAs using Lipofectamine™ 2000 transfection reagent. Relative survivin mRNA and protein levels were measured by quantitative real-time PCR and Western blotting, respectively. Trypan blue exclusion assays were performed to monitor tumor cell proliferation after siRNA transfection. The cytotoxic effects of etoposide and survivin siRNA, alone and in combination, on leukemic cells were determined using MTT assay. Apoptosis was assessed by ELISA cell death assay. **Results:** Survivin siRNA markedly reduced both mRNA and protein expression levels in a time-dependent manner, leading to distinct inhibition of cell proliferation and increased spontaneous apoptosis. Surprisingly, survivin siRNA synergistically increased the cell toxic effects of etoposide. Moreover, survivin down-regulation significantly enhanced its induction of apoptosis. **Conclusions:** Our study suggests that down-regulation of survivin by siRNA can trigger apoptosis and overcome drug resistance of leukemia cells. Therefore, survivin siRNA may be an effective adjuvant in AML chemotherapy

Keywords: Survivin - siRNA - etoposide - HL-60 - apoptosis

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Introduction

Acute myeloid leukemia (AML) is a lethal hematologic malignancy, with high a frequency of relapse and poor outcome even with intensive chemotherapy (Robak and Wierzbowska, 2009). The development of multi-drug resistance in leukemic cells is one of the main obstacles for successful chemotherapy in leukemia (Baran et al., 2007; Ho et al., 2008). Therefore, the innovation of new treatment modalities to overcome chemoresistance of tumor cells may be a potential source of improved therapies.

Apoptosis, or programmed cell death, is a biological process essential for the regular development and maintenance of tissue homeostasis (Michels et al., 2005; Gautrey and Tyson-Capper, 2012). Disturbance in the regulation of apoptosis machinery contributes to the development of tumor and subsequent multi-drug resistance (Dai and Grant, 2007; Kang and Reynolds, 2009). As the majority of cytotoxic drugs mainly kill malignant cells by the activation of apoptosis, recent anti-

cancer approaches are focusing their efforts on specifically targeting the mediators involved within the respective apoptotic pathways. (Wacheck et al., 2006; High et al., 2010).

Survivin (BIRC5) is a unique bifunctional member of the inhibitor of apoptosis (IAP) family of proteins that is involved in the regulation of apoptosis and cell division. Unlike other IAP members, survivin is largely expressed in embryonic and fetal tissues as well as in many malignant cells, but not in normal adult tissues (Zaffaroni and Daidone, 2002; Coumar et al., 2013). Previous reports have demonstrated that the overexpression of survivin is correlated with tumor progression, poor prognosis and drug resistance in many cancers including AML (Adida et al., 2000; Fukuda and Pelus, 2006). Because of this overexpression in cancer, its important role in apoptosis as well as cell division, and its association with drug resistance, survivin has been considered as an attractive therapeutic target in malignancies (Ryan et al., 2009; Feng et al., 2013). Various studies have shown that suppression of survivin expression with dominant negative mutants,

¹Immunology Research Center, ²Department of Biochemistry, Faculty of Medicine, ³Hematology and Oncology Research Center, ⁴Medical Philosophy and History Research, ⁵Tuberculosis and Lung Diseases Research Center, Tabriz University of Medical Sciences, Tabriz, ⁶Scientific Research Center, Tehran University of Medical Sciences, Tehran, Iran, ⁷Faculty of Medicine, University of Liverpool, Liverpool, United Kingdom *For correspondence: baradaranb@tbzmed.ac.ir, esakhinia@yahoo.co.uk

ribozymes, antisense oligonucleotides (ASOs) and small interfering RNAs (siRNAs) increased spontaneous apoptosis and sensitized tumor cells to chemotherapy and ionizing radiation (Zaffaroni et al., 2005; Ryan et al., 2009). Yet, a study on whether the siRNA targeting of survivin could enhance the sensitivity of AML cells to antileukemic agent etoposide has not been investigated until now.

The objective of this study was to investigate whether the down-regulation of survivin by siRNA could sensitize AML cells to etoposide. To this end, we examined the effects of either survivin specific siRNA or etoposide treatments alone versus the combination, in HL-60 human AML cell line.

Materials and Methods

Cell culture

The HL-60 AML cell line (Pasteur Institute, Tehran, Iran) was grown in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 15% fetal bovine serum (FBS) (Sigma-Aldrich), 1% antibiotics (100 IU/ml penicillin, 100 µg/ml streptomycin) (Sigma-Aldrich), 1% sodium pyruvate and 2 mM of glutamine, at 37°C in a humidified atmosphere containing 5% CO₂. The cells were sub-cultured 24-48 h later with an initial concentration of 4×10⁴ cells/ml and used in the logarithmic phase in all experiments.

siRNA transfection

The survivin specific and negative control (NC) siGENOME siRNAs were purchased from Dharmacon (Lafayette, CO, USA). Just before transfection, the cells were cultivated in RPMI-1640 medium free of serum and antibiotics. siRNA transfection (at a final concentration of 80 nM in all experiments) was performed using Lipofectamine™ 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. Briefly, siRNAs and lipofectamine (4 µl/ml of transfection medium) were diluted in Opti-MEM I Reduced Serum Medium (Invitrogen) separately and incubated for 10 min at room temperature. The diluted solutions were then mixed and incubated for 20 min at room temperature. Subsequently, the mixtures were added to each well containing cells and medium. Moreover, the treated cells with only the transfection reagent were considered as a blank control. The cell culture plates were then incubated for 6 h at 37°C in a CO₂ incubator. Following on, RPMI-1640 medium containing FBS (final FBS concentration of 15%) was added, with cells being incubated under the above mentioned conditions. To evaluate the effects of siRNAs on gene silencing, transfections (5×10⁵ cells/well) were performed in 6-well cell culture plates for 24-48h. The suppression of survivin gene expression was then assessed by quantitative real-time PCR (qRT-PCR) and Western blotting.

Cytotoxicity assay

The effect of survivin siRNA on the chemosensitivity of leukemic cells was determined using 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium

Bromide (MTT) assay. The experiment was subdivided into eight groups: survivin siRNA, NC siRNA, etoposide, survivin siRNA and etoposide, NC siRNA and etoposide, siRNA blank control, etoposide blank control and combination blank control. Briefly, cells were cultured at a density of 15×10³ cells/well in 96-well cell culture plates and then transfected with siRNAs. After 6h of incubation, the cells were exposed to different concentrations of etoposide (0.001, 0.2, 0.5, 1, 2 and 4 µM). Cells treated with only 1% DMSO (solvent of etoposide) or a mixture of 1% DMSO plus lipofectamine were also considered as etoposide or combination blank controls, respectively. Eighteen hours later, the cytotoxicities of the treatments were assessed using the MTT cell proliferation kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol. The amount of formazan dye was determined by quantifying its absorbance (A) at 570 nm (with a reference wavelength of 650 nm) using a microplate reader (Awareness Technology, Palm City, FL, USA). The survival rate (SR) was measured from the following equation: SR (%)=(A Treatment /A Control)×100%. The concentration that produced 50% cytotoxicity (IC₅₀) was determined using GraphPad Prism 6.01 software (GraphPad Software Inc., San Diego, CA, USA).

Combination effect analysis

To explore the interaction between survivin siRNA and etoposide, combination effect analysis was performed, based on the principles described by Chou and Talalay (Chou and Talalay, 1984). The value of coefficient of drug interaction (CDI) was determined using the following formula: CDI=SAB / (SA×SB), where SA and SB are the survival rate of etoposide and survivin siRNA relative to the corresponding control, SAB is the survival rate of the combination treatment relative to the control. CDI<1, CDI=1 and CDI>1 indicate synergistic, additive and antagonistic effects, respectively.

Cell proliferation assay

The antiproliferative effect of survivin siRNA was assessed by trypan blue exclusion assay. Cells (5×10⁴ cells/well) were transfected with survivin specific and NC siRNAs in 24-well cell culture plates and then incubated for 5 days. At different time points, the cells were harvested and then stained with 0.4% trypan blue (Merck KGaA, Darmstadt, Germany) for 2 min. Subsequently, the number of unstained cells (N, viable cells) was counted using an inverted microscope (Nikon Instrument Inc., Melville, NY, USA) and a hemocytometer. The percentage of viable cells was quantified from the equation as follows: Cell viability (%)=(N Treatment /N Control)×100. Moreover, the percentage of viable cells for control group was considered as 100%.

qRT-PCR

At different time points after transfections, total cellular RNA was isolated by AccuZol™ reagent (Bioneer, Daedeok-gu, Daejeon, Korea) as described by the manufacturer. Complementary DNA (cDNA) was synthesized from 1 µg of total RNA by use of MMLV

reverse transcriptase (Promega, Madison, WI, USA) and oligo-dT primer according to the manufacturer's instructions. qRT-PCR was performed in the Rotor-Gene™ 6000 system (Corbett Life Science, Mortlake, NSW, Australia) using SYBR Premix Ex Taq (Takara Bio, Otsu, Shiga, Japan). The reaction system of PCR was: 12 μ l of SYBR green reagent, 0.2 μ M of each primer, 1 μ l of cDNA template, and 6 μ l of nuclease-free distilled water. The primer sequences were as follows: forward, 5'-GGACCACCGCATCTCTACAT-3', reverse, 5'-CAACGCGAAAGGAAAGACAG-3', for survivin, and forward, 5'-TCCCTGGAGAAGAGCTACG-3', and reverse, 5'-GTAGTTTCGTGGATGCCACA-3', for β -actin. The initial denaturation step at 95°C for 10 min was followed by 45 cycles at 95°C for 20 sec and 60°C for 1 min. Relative survivin mRNA expression was measured with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), using β -actin as the reference gene.

Western blot analysis

Following treatments, the cells were washed twice with cold PBS and resuspended in lysis buffer (1% SDS, 1% Triton X-100, 1 mM EDTA, pH 8, 50 mM Tris-HCl, pH 7.4 and 150 mM NaCl) containing protease inhibitor cocktail (Roche Diagnostics GmbH) for 30 min on ice. Suspensions were centrifuged at 14,000 rpm for 10 min at 4°C and cellular debris were discarded. Protein concentrations were quantified using Bradford reagent (Sigma-Aldrich). Fifty micrograms of each protein sample was separated on 15% SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes (GE Healthcare, Amersham, Buckinghamshire, UK), and then blocked with 3% bovine serum albumin (BSA) in PBS/Tween-20 (0.05%, v/v) for 45 min at room temperature. Following on, the membranes were probed overnight at 4°C with primary mouse monoclonal antibodies against β -actin (1:1000, Abcam, Cambridge, MA, UK) and survivin (1:500, Abcam) diluted in 3% BSA in PBS. After four 5 min washes with a buffer containing PBS and 0.05% Tween-20, membranes were incubated with appropriate horseradish peroxidase-linked goat anti-mouse secondary antibody (1:3,000, Abcam) diluted in PBS for 2 h at room temperature. Subsequently, the membranes were washed and protein bands visualized using enhanced chemiluminescence detection Kit (GE Healthcare) and autoradiography films (Estman Kodak, Rochester, NY, USA). Signal intensity of each band was measured by ImageJ 1.62 software (National Institutes of Health, Bethesda, Maryland, USA) and normalized to its actin loading control.

Apoptosis assay

The HL-60 leukemic cells were cultivated at a density of 5×10^4 cells/well in 24-well plates and then exposed to survivin specific or NC siRNAs, etoposide (IC_{50} dose) and their combination, as described in the MTT assay section. After 24h of incubation, cells were collected and apoptosis was detected using an ELISA cell death detection kit (Roche Diagnostics GmbH) according to the supplier's recommendations. This assay determines the amount of cytosolic mono- and oligonucleosomes

produced during apoptosis. Briefly, the cells were lysed and centrifuged at 200 g for 10 min. Following the addition of 20 μ l of the cell supernatant and 80 μ l of a mixture of anti-DNA-peroxidase and anti-histone-biotin to each well, streptavidin-coated plate was incubated for 2h at room temperature. After washing with incubation buffer, 100 μ l of 2, 2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) solution was transferred to each well. Finally, the reaction was stopped and absorbance at 405 nm was determined immediately with an ELISA plate reader (with a reference wavelength of 490 nm). The fold increase in apoptosis was calculated by dividing the absorbance of the treatment group by the absorbance of the blank control group.

Statistical analysis

All data in this study were presented as mean \pm standard deviation (SD). Statistical significance of differences between groups was explored by using analysis of variance (ANOVA) and Bonferroni's test using GraphPad Prism software. Value of P less than 0.05 was considered significant.

Results

siRNA suppressed survivin mRNA and protein levels in leukemic cells

First, we explored the effect of siRNA on survivin gene expression in HL-60 cells by qRT-PCR and Western blot analysis. Relative survivin gene expression was calculated in relation to the blank control group, which was considered as 100%. As shown in Figure 1 and 2, survivin siRNA led to a marked time-dependent reduction of both survivin mRNA and protein levels ($p < 0.05$; relative to the blank control). At 24, 48 and 72h after the transfection, the relative survivin mRNA expression levels were 26.10%, 18.44% and 12.02%, respectively (Figure 1), while the relative survivin protein expression levels were 40.09%, 21.53% and 11.86%, respectively (Figure 2B) ($p < 0.05$). Meanwhile, NC siRNA had an insignificant effect on survivin gene expression compared to the blank control group.

Suppression of survivin expression inhibited the proliferation of HL-60 cells

As up-regulation of survivin is associated with

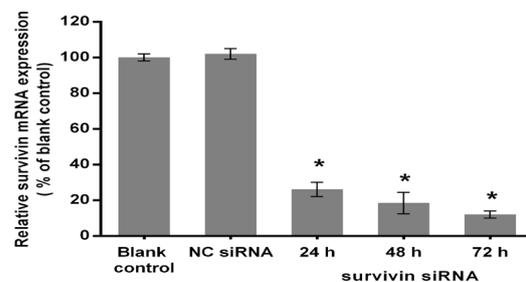


Figure 1. Suppression of Survivin mRNA Expression by siRNA in Leukemic Cells. HL-60 cells were transfected with negative control (NC) siRNA or survivin siRNA for 24, 48 and 72h. Relative survivin mRNA expression was measured by qRT-PCR using $2^{-\Delta\Delta Ct}$ method. The results are expressed as mean \pm SD (n=3); * $p < 0.05$ versus blank control

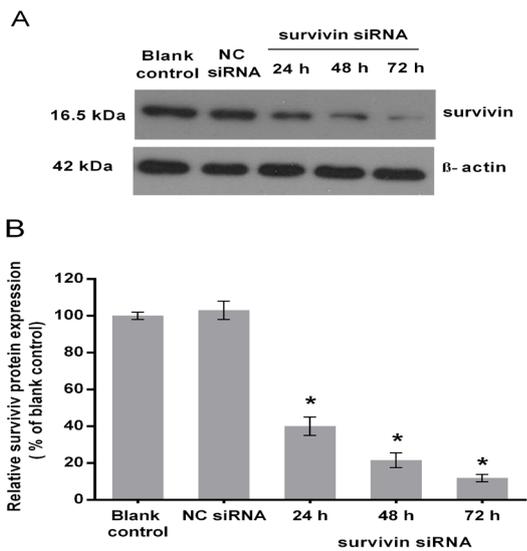


Figure 2. Survivin Protein Expression Levels in HL-60 Cells Transfected with siRNAs. (A) Representative western blot of β -actin and survivin proteins from cells transfected with NC siRNA or survivin siRNA. (B) The expression level of each band was quantified using densitometry and normalized to the respective β -actin. The results are expressed as mean \pm SD (n=3); *p<0.05 versus blank control

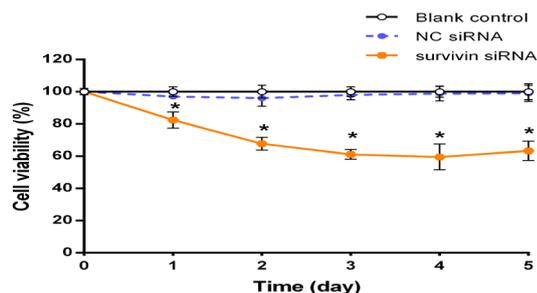


Figure 3. Proliferation Inhibition of HL-60 Cells Treated with NC siRNA or Survivin siRNA. Cell viability was determined by trypan blue assay over a period of 5 days. The results are expressed as mean \pm SD (n=3); *p<0.05 versus blank control

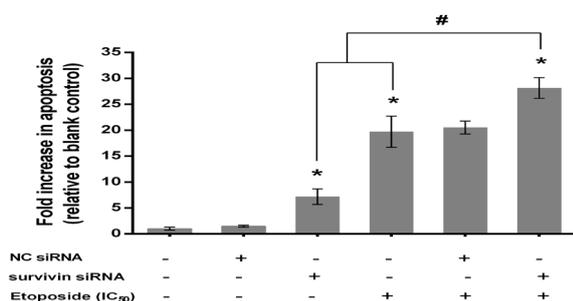


Figure 5. Effect of Survivin Suppression on Etoposide-induced Apoptosis. The cells were exposed to etoposide and siRNAs, alone or in combination, as described in methods section. At 24 h after transfection, apoptosis was measured by ELISA cell death assay. The results are expressed as mean \pm SD (n=3); *p<0.05 versus blank control; #p<0.05

tumor progression; we therefore sought to test whether suppression of this protein could arrest the proliferation of leukemic cells. The HL-60 cells were transfected with survivin specific and NC siRNAs and cell viability was measured by trypan blue assay. Compared with

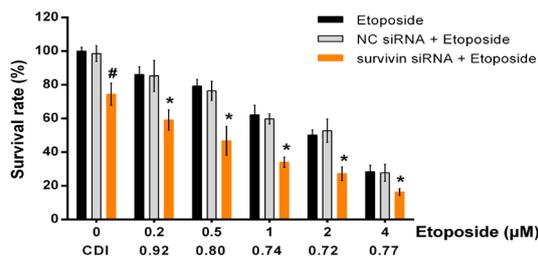


Figure 4. Effect of Survivin siRNA on the Chemosensitivity of the HL-60 Cells. The cells were treated with etoposide, NC siRNA or survivin siRNA and their combination for 24 h and then the cytotoxicities of the treatments were determined by MTT assay. The interaction effect between siRNA and etoposide was determined using combination effect analysis and coefficient of drug interaction (CDI) values. The results are expressed as mean \pm SD (n=4); *p<0.05 versus etoposide or siMcl-1 alone; #p<0.05 versus blank control

the blank control group, survivin siRNA significantly inhibited the proliferation of tumor cells over a period of 5 days (p<0.05; Figure 3). At 24h posttransfection, the cell viability dropped to 82.40% and then to a further 63.37% at the end of the experiment (day 5). However, no significant alteration in cell proliferation was observed between the NC siRNA and the blank control groups (p>0.05; Figure 3).

Survivin siRNA synergistically enhanced the cytotoxic effect of etoposide

To assess whether down-regulation of survivin could enhance the sensitivity of the leukemic cells to etoposide, a combination treatment of etoposide and survivin siRNA on HL-60 cells was investigated. As shown in Figure 4, monotreatment with etoposide induced cytotoxicity in a dose-dependent way. The results of MTT assay showed that survivin siRNA significantly decreased the cell survival rate to 74.11%, compared with the blank control (p<0.05). Moreover, etoposide in combination with survivin siRNA further decreased the cell survival rate relative to etoposide or survivin siRNA alone (p<0.05). Surprisingly, the presence of survivin siRNA led to a clear reduction in the IC₅₀ value of etoposide from 2.03 μ M to 0.693 μ M. The CDI values were also less than 1 in all concentrations of etoposide, which indicated the synergistic effect between the two agents (Figure 4). Notably, transfection with NC siRNA had a minimal effect on the chemosensitivity of the leukemic cells relative to the etoposide treated cells (p>0.05; Figure 4).

Suppression of survivin augmented etoposide-induced apoptosis

To analyze whether the observed sensitizing effect of survivin siRNA was linked to the enhancement of apoptosis, the effects of either etoposide or survivin siRNA alone, and in their combination on apoptosis were evaluated using an ELISA cell death assay. Results demonstrated that 24 h exposure of the cells to etoposide or survivin siRNA enhanced the extent of apoptosis by 19.70 and 7.18 fold, respectively, relative to the blank control (p<0.05; Figure 5). On the other hand, combination

therapy further enhanced the extent of apoptosis to 28.14 fold ($p < 0.05$). However, no significant changes in the extent of apoptosis were detected for NC siRNA or NC siRNA plus etoposide groups compared with the blank control group or etoposide alone, respectively ($p > 0.05$). These results indicate that the chemosensitization effect of survivin suppression is partially due to the induction of apoptosis.

Discussion

Drug resistance is one of the main problems in chemotherapy of AML patients (Baran et al., 2007; Ho et al., 2008; Robak and Wierzbowska, 2009). Overexpression of survivin, a member of the IAP family of proteins, is attributed to the chemoresistance of tumor cells (Adida et al., 2000; Fukuda and Pelus, 2006; Ryan et al., 2009; Feng et al., 2013). On the contrary, different studies have shown that suppression of survivin expression can sensitize tumor cells to anti-cancer drugs (Zaffaroni et al., 2005). In the current study to investigate whether down-regulation of survivin affects chemoresensitivity in AML cells, we examined the effect of survivin specific siRNA and etoposide alone or in combination on HL-60 AML cells.

qRT-PCR and Western blot analysis revealed that transfection with survivin siRNA drastically reduced survivin mRNA and protein levels during the 3-day period. These findings suggest that survivin siRNA effectively had cleaved survivin mRNA and thereby blocked its translation to its corresponding protein. The results of the cell proliferation assay showed that the down-regulation of survivin significantly inhibited the proliferation of the HL-60 cells, demonstrating that survivin has an important role in the growth of leukemic cells. Most notably, the results of MTT assay exhibited that pretreatment with survivin siRNA distinctly lowered the IC_{50} value of etoposide and subsequently augmented its cytotoxic effect in a synergic manner. This proposes that suppression of survivin could sensitize the leukemia cells to etoposide.

To further define the role of survivin in the drug resistance of leukemia cells, we tested the effect of survivin expression suppression on the apoptotic effect of etoposide. Apoptosis assay findings indicated that monotherapy with etoposide led to significant apoptosis in HL-60 cells. In addition, siRNA-mediated inhibition of survivin induced remarkable spontaneous apoptosis and enhanced sensitivity of the tumor cells to etoposide-mediated apoptosis. In contrast, NC siRNA or lipofectamine treatments did not alter the survivin gene expression, cell proliferation and cell toxicity of etoposide, illustrating the specific impact of survivin siRNA. However, these observations are in agreement with the findings of similar studies on a variety of tumor cells, which further confirm the critical role of survivin in the survival, proliferation and chemoresistance of tumor cells (Nakao et al., 2006; Paduano et al., 2006; Miao et al., 2007; Song et al., 2008). The above-mentioned subjects show that the presence of survivin is necessary for the survival and drug resistance of leukemic cells. Therefore,

silencing of survivin expression could trigger spontaneous apoptosis and sensitize tumor cells to antileukemic drugs.

Cellular apoptosis can be controlled by two signaling pathways. The intrinsic or mitochondrial pathway responds to toxic intracellular stimuli such as DNA damaging agents and ionizing radiation, causing release of cytochrome c from the mitochondria which lead to the activation of caspases-9. The extrinsic or death receptor pathway that is necessary for inflammation and immune selection is initiated by ligands binding to extracellular cell death receptors and results in caspase-8 activation. Both pathways converge at caspase-3 that activates other caspases leading to a proteolytic cascade and eventually the apoptotic morphology. Survivin has been demonstrated to block apoptosis via the intrinsic and extrinsic pathways by inhibition of caspases activities. Furthermore, survivin is required for stabilization of spindle microtubules and accurate chromosome segregation during mitosis. However, the exact roles of survivin in regulation of cell division and apoptosis are still unclear (Zaffaroni and Daidone, 2002; Zaffaroni et al., 2005; Kelly et al., 2011; Church and Talbot, 2012).

Etoposide is a DNA topoisomerase II inhibitor widely used for the treatment of several malignancies including leukemia. It was demonstrated that exposure of HL-60 cells to etoposide initiates both signaling pathways of apoptosis by activation of multiple caspases (Martins et al., 1997; Montecucco and Biamonti, 2007). Our study shows that down-regulation of survivin expression enhances the apoptotic effect of etoposide in HL-60 cells. Therefore, we suggest that suppression of survivin expression by siRNA may sensitize leukemic cells to etoposide through caspase-3-dependent mechanisms. Further studies are needed.

siRNA-mediated gene silencing is a powerful technology triggered by double-stranded RNA. Once it introduces to the target cells, it combines specifically with complementary mRNA and inhibits its translation to the corresponding protein. Owing to its benefits such as specificity, low cytotoxicity and high efficacy, siRNA is widely used in gene therapy investigations (Devi, 2006; Yang and Mattes, 2008; Shan, 2010). Moreover, because of the advantages of siRNA such as the greater resistance to nucleases degradation, it is preferred to the ASO and ribozyme approaches (Brantl, 2002; Aoki et al., 2003). On the contrary, transient essence of double-stranded siRNA is one of the main drawbacks of long term siRNA-based therapeutics which can be remedied by use of the constitutive vector-based systems (Devi, 2006; Yang and Mattes, 2008; Shan, 2010).

In conclusion, our data demonstrated that survivin has a critical role in the proliferation and drug resistance of HL-60 cells. Specific knockdown of survivin expression by siRNA induced apoptosis and synergistically enhanced sensitivity of leukemic cells to etoposide. Our study emphasizes the ability of survivin siRNA for chemosensitization of leukemia cells to reduce the harmful side-effects of intensive chemotherapy. We suggest that the siRNA-silencing survivin may be considered as a novel treatment strategy in the future to overcome drug resistance of AML patients.

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