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In vitro application of RNA interference to silence *livin* gene expression to induce apoptosis in leukemia cells

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Abstract. – OBJECTIVE: To search for new targets and novel methods of anti-leukemia treatment and to discuss the mechanism of silencing the livin gene using small RNA interference technology to induce apoptosis in the K562 leukemia cell line.

METHODS: We designed and synthesized livin-specific small interference RNA (siRNA). Transfected K562 cells were cultured. Reverse-transcription polymer chain reaction (RT-PCR) was used to detect livin mRNA expression. Protein expression for livin was detected using Western blotting. A non-transfected group was used as a control. Meanwhile, vectors carrying enhanced green fluorescent protein (EGFP) were transfected as a positive control and flow cytometry was used to determine the transfection efficiency by detecting green fluorescence. The rate of apoptosis was determined using the annexin V and propidium iodide double-staining method. ELISA was used to determine the activity of Caspase-3.

RESULTS: The transfection efficiency of electroporation was as high as 50%. The siRNA sequences could knockdown livin gene expression at both the mRNA and protein levels. Apoptosis rate of the cells was 27.41 ± 2.30% 48 h after transfection with specific siRNA. This was significantly higher than that of the control group (9.63 ± 0.89%, $p < 0.05$). The 48-h apoptosis rate of the combined effect group VP-16 (5 μmol/L) and transfection rate was 45.1 ± 4.40%, which was significantly elevated ($p < 0.05$) compared with the groups treated with only VP-16 or by transfection.

CONCLUSIONS: Caspase-3 activity in cells transfected with siRNA was significantly elevated compared to the cells in the non-transfected groups ($p < 0.05$).

Key Words:
Livin, siRNA, RNA interference, Anti-apoptosis protein, Caspase-3.

Introduction

Several studies[1,2] have shown that during cell proliferation, abnormities in both pro-apoptotic and anti-apoptotic pathways could lead to the occurrence, development, drug resistance, and relapse of leukemia. Most current leukemia treatment methods function through the induction of cell apoptosis. All kinds to drugs induce apoptosis through the death receptor pathway or mitochondrial pathway, which further trigger caspase activation in the body, and eventually lead to cell apoptosis. Apoptosis-related regulators are currently a huge topic in leukemia studies. Among various *in vivo* and *in vitro* apoptosis-related regulators being studied, inhibitor of apoptosis protein (IAP)[3,4] has become one the most promising new anti-leukemia targets since it directly bind to caspase and promotes the binding of other IAP family members with caspase, thus, inhibiting apoptosis. The IAP family consists of a group of IAPs sharing the same structural domain. A newly discovered member – *livin*[5] – has been proven to have anti-apoptosis effects[6,7] and is also specifically expressed in tumour tissues[8-13]. It has, therefore, become a favoured topic in anti-tumour research work. To look for new gene targets and methods that reduce leukemia cell burden, we performed an *in vitro* study on the *livin* gene. By inhibiting the expression of the *livin* gene using siRNA interference technology[14], we observed changes in apoptosis rate in the leukemia cell line – K562 – in which the *livin* gene was suppressed, and primarily discussed the anti-apoptosis mechanism of this gene.

Materials and Methods

Reagents

RMPI-1640 was purchased from Gibco (Great Island, NY, USA). Fetal bovine serum (FBS) was a product of Hyclone (Logan, VT, USA), siRNA sequences of *livin* were chemically synthesized

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by. Ambion (Foster City, CA, USA). The transfection reagent was the product of Amaxa, Cologne, Germany. Trizol was purchased from Invitrogen (Carlsbad, CA, USA). Taq polymerase and M-MLV reverse transcriptase were both products of Promega (Madison, WI, USA). The *livin* monoclonal antibody was purchased from Active Motif (Carlsbad, CA, USA).

Cell Culture

RPMI-1640 containing 10% FBS was used for culture in an incubator containing 5% CO_2 at 37°C. Cell density was adjusted to 1×10^5 mL^{-1}. The media was changed 1 day before transfection by centrifugation at 1,200 rpm (centrifugal radius: 13 cm; duration: 5 min).

siRNA Sequences

Based on known *livin* gene sequences NM_022161 (*livin*-β) and NM_139317 (*livin*-α) from GenBank and according to the design principle of siRNA, we started from the initiation codon AUG of the transcript (mRNA), searched for amino acid ("AA") sequences and marked down the 19-bp sequences at its 3' end as potential siRNA targets. We performed homologous sequence search against those potential sequences using BLAST to exclude sequences that were homologous to the other coding sequences. Three pairs of siRNA sequences that each contained 21 bases were chemically synthesized by Ambion. Sequences of the sense strands and anti-sense strands were as follows: siRNA-1: 5'-GGUGAGGUGCUUCUUCUGCtt-3' and 5'-GCAGAAGAAGCACCUCACCtt-3'; siRNA-2: 5'-GGUGCUUCUUCUGCUAUGGtt-3' and 5'-CCAUAGCAGAAGAAGCACCtc-3'; siRNA-3: 5'-GGAAGAGACUUUGUCCACAtt-3' and 5'-UGUGGACAAAGUCUCUUCCtt-3'. Both siRNA-1 and siRNA-2 were from the second exon, siRNA-3 was from the third exon.

Determination of Transfection Rate

For the determination of the transfection rate nucleofection was carried out using program T16 of an Amaxa nucleofector. Nucleofection of 2×10^6 K562 cells with 2 μg green fluorescent protein (GFP) plasmid DNA was performed in 100 μL of nucleofaction solution. Transfected cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 containing 10% FBS. A flow cytometer was used to determine transfection rate after 48 h of culture.

Cell Transfection

Nucleofection of cells was performed using program T16 of an Amaxa nucleofector. The transfection of 4×10^6 K562 cells with 2 μg *livin* siRNA was performed in 100 μL of nucleofection solution. Transfected cells were cultured in RPMI-1640 containing 10% FBS. Total RNA was extracted after 12 h and 24 h for RT-PCR analysis. Total protein was extracted after 24 h and 48 h for Western blot analysis.

RT-PCR

Total RNA was extracted using the Trizol method. The A_{260}/A_{280} absorbance ratio within the range of 1.8-2.0 was used to determine extraction quality. RNA concentration was calculated based on absorbance. Approximately 5 μg total RNA and 1 μL of Oligo $(dT)_{15}$ were used and diethylpyrocarbonate (DEPC)-treated water was used to fill up to a volume of 12 μL. After preheating for 5 min at 70°C, 5 × buffer 4 μL, 1 μL of an RNAase inhibitor, and 2 μL of 10 mmol/L dNTP were added and incubated for 5 min at 37°C. Then, 1 μL of reverse transcriptase was added and incubated for 60 min at 42°C. Touchdown polymerase chain reaction (touchdown PCR) was used to amplify the target gene. Primers of the *livin* sequence were as follows: sense strand 5'-TGGGACCCGTGGGAAGAACCG-3' and anti-sense strand, 5'CCGCACGGCACAAAGACGATG-3'. The product of *livin* α was 275 bp, whereas that of *livin* β was 221 bp. Primers of the β-actin sequence were as follows: sense strand 5'-GACAGGATGCAGAAGGAGATTACT-3', anti-sense strand 5'-TGATCCACATCTGCTGGAAGGT-3. The amplification product was 141 bp in size.

Western Blot Analysis

Cells of each group were washed twice with 4°C precooled phosphate buffered saline (PBS), then lysed with tri-detergent lysis buffer 50 mmol/L tris-HCl pH 8.0, 150 mmol/L NaCl, 0.2 g/L sodium azide, 1 g/L sodium dodecyl sulphate (SDS), 100 mg/L aprotin, 10 g/L NP-40, 5 g/L deoxysodium cholate, 100 mmol/L phenylimethylsulfonyl fluoride (PMSF). After a 30-min incubation in an ice bath that broke down cell membranes, the solution was centrifuged at 4°C, 12,000 r/min for 20 min with a centrifugal radius of 13 cm. The supernatant was collected and denatured in boiling water. Approximately 5 μL of the solution was taken before denaturation and diluted with Coomassie brilliant blue to perform colorimetry at a wavelength of 595 nm. Protein concentration was cal-

culated according to the standard curve and record. After SDS-PAGE electrophoresis, the denatured proteins were transferred to a nitrocellulose membrane. Rat anti-human *livin* monoclonal antibody and β-actin antibody were diluted at 1:1,000 and incubated with the proteins. After rinsing the membrane, chemiluminescence, X film exposure, and development were carried out.

Detection of Cell Apoptosis

Cell density was adjusted to 1×10^5 mL^{-1}. A 1-ml cell solution was centrifuged at 4°C, 1,200 r/min for 5 min with a centrifugal radius of 13 cm. The supernatant was discarded; 1 ml cold PBS was added and gently shaken to resuspend the cells. It was, then, centrifuged at 4°C, 1,200 r/min for 5 min and, then, the supernatant was discarded. These steps were repeated twice. The cells were resuspended in 100 μL of a binding buffer; 5 μL of Annexin V_{FITC} and 10 μL of propidium iodide (PI) were added and mixed gently. The mixture was allowed to react in the dark for 20 min at room temperature. Then, 400 μL of a binding buffer was added and a flow cytometer was used for examination. Non-transfected cells were used as control to exclude the effects of electroporation on apoptosis.

Determination of Caspase-3 Protease Activity

The concentrations of each K562 cell group were adjusted to a density of 1×10^5/ml 48h after siRNA transfection. Approximately 180 μL of the cell suspension was added to each well of a 96-well plate. A control group that was not-induced for apoptosis (negative control) was set up at the same time, together with another control group with VP-16 (5 μmol/L) inducing apoptosis + Caspase-3 inhibitor (DEVD-fmk). After 4 h of culture, the complete cell lysis was performed in accordance with the manufacturer's instructions of the Caspase-3 activity determination kit. The supernatant was collected and the substrate (DEVE-PNA) and reaction buffer were added. The mixture was placed in an ice bath for 30 min and the OD value of each group was measured at a wavelength of 405 nm using a spectrophotometer.

Statistical Analysis

Data were analysed using the SPSS 19.0 statistical software (SPSS Inc., Chicago, IL, USA). Rate comparisons were performed using the chi-square test, with $p < 0.05$ considered as being statistically significant.

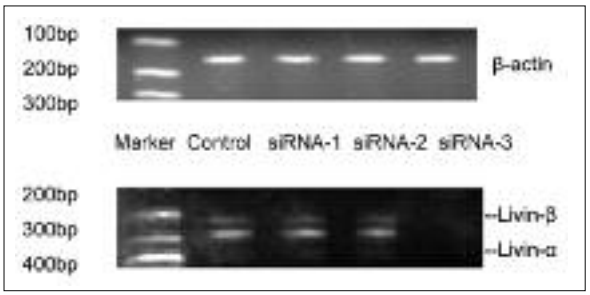

Figure 1. *Livin* mRNA expression 24 h after siRNA treatment.

Results

Determination of Transfection Rate

Expression of GFP was examined by a flow cytometry analysis 48 h after transfection, which had a transfection rate > 50%.

Knockdown of Livin mRNA

All three siRNA showed knockdown of *livin*, especially the 3rd one, which almost achieved complete knockdown. The inhibiting effect after 24 h was more evident than that after 12h. Electrophoresis images of PCR products at 24 h are shown in Figure 1.

Knockdown of Livin Protein

All three siRNAs showed knockdown of *livin*, especially the 3rd sequence, whose inhibiting effect was the most evident. The inhibiting effect after 48 h was more evident than that after 24h. Result of the Western blot analysis after 48 h are shown in Figure 2.

Apoptosis Rate Determination by Flow Cytometry

As shown in the results of flow cytometry, the apoptosis rate of the group transfected with specific siRNA-3 was 12.07 ± 1.39% at 24 h, and 12.07 ± 1.39% at 48 h, which were both signifi-

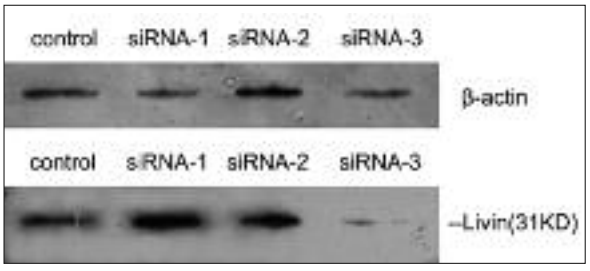

Figure 2. Reduced *livin* protein expression 48 h after siRNA treatment.

cantly elevated ($p < 0.05$) compared to the non-transfection group (9.63 ± 0.89%). The results are presented in Figure 3.

Effect of siRNA Treatment on K562 Cell Apoptosis

The 48-h apoptosis rates of the non-transfected group, transfected group, sole VP-16 (5 μmol/L) group, combined treatment of VP-16 (5 μmol/L), and transfection group were 9.63 ± 0.89%,27.41 ± 2.30%, 31.4 ± 3.09%, and 45.1 ± 4.40%, respectively. The apoptosis rate of combined treatment group significantly increased compared to the sole VP-16 treatment group ($p < 0.05$) (Figure 4).

Changes in Caspase-3 Activity

The cells in all treatment groups showed caspase-3 activity, which could be inhibited by caspase-3 inhibitor. The caspase-3 activity of the non-transfected group, where apoptosis was induced by treatment with VP-16 (5 μmol/L), was considered 100%. Caspase-3 activity of the siRNA transfection group, where apoptosis was induced by treatment with VP-16 (5 μmol/L), was 145 ± 12.9%. Caspase-3 activity of cells transfected with siRNA significantly increased compared to the non-transfected group ($p < 0.05$) (Figure 5).

Discussion

There are two main cell apoptosis pathways[1,2]: (1) Death signal receptor pathway. Death signal receptors are a group of transmembrane proteins of the TNF family. Currently, six types of human death receptors have been characterized, of which the most thoroughly studied is the Fas/Fasl system. Polymerization occurs immediately after the death receptor Fas protein on the cell membrane binds to its ligand, Fasl, which complements and, thus, activates the two-part molecules of the death structural domain FADD in cytoplasm. FADD binds to Fas, forming the death signal transduction complex, which hydrolyses and activates the cytoplasmic protease caspase-8, and eventually activates the apoptosis executor caspase-3 through a series of protease cascade reactions. Caspase-3 initiates DNA excision by endonucleases to trigger apoptosis; (2) Mitochondrial pathway, which is activated by the precursor components of Bcl-2 family. The Bcl-2 family includes Bid, Bim, Harikari, Noxa, and Bax. Bax (which is comprised of Bax, Bak, and Bok) exists in the outer mitochondrial membrane and the cytoplasm. These proteins enter the mitochondrial membrane through interactions with each other, trigger the release of cytochrome C (Cytc), as well as other proteins. Cytc binds to

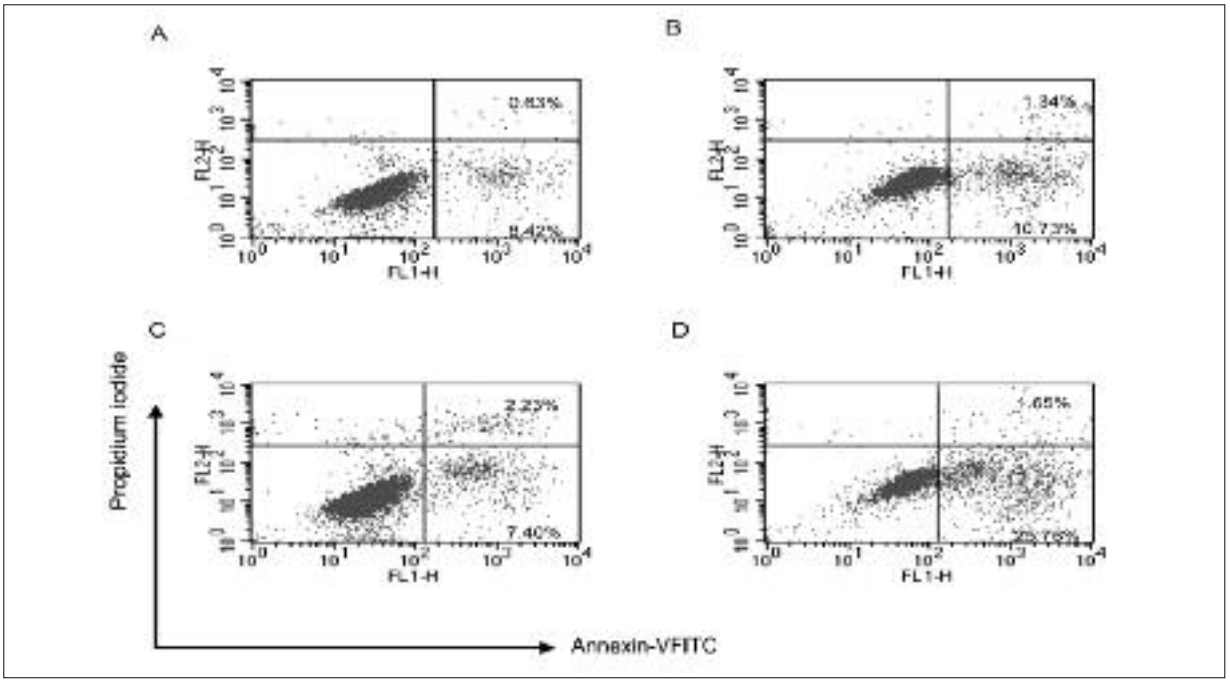

Figure 3. Increased K562 cell apoptosis rates after siRNA treatment. ***A, C,*** Are 24 h and 48 h natural apoptosis rates of K562 cells false-transfected with no siRNA. ***B, D,*** Are 24 h and 48 h apoptosis rates of K562 cells with siRNA treatment.

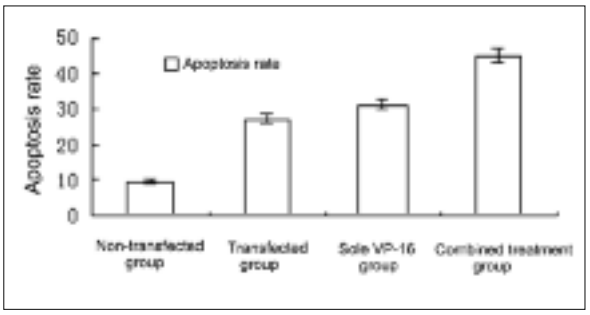

Figure 4. Effect of transfection on cell apoptosis.

Apaf-2 and Apaf-1 after being released and subsequently activates the caspase-9 precursor. Activated caspase-9 can activate the precursor of executor caspase-3, which initiates DNA excision by endonuclease to trigger apoptosis.

To summarize, cell apoptosis is progressively carried out by caspase cascade reactions of the caspase family, regardless of whether it was induced intracellularly or extracellularly. Both extracellular receptor pathway and mitochondrial pathway eventually activate caspase-3, which is why some researchers consider caspase-3 as an apoptosis executor. The function of caspase is regulated by other proteins, which are known as inhibitor of apoptosis proteins IAPs[3]. IAP members can directly bind to different types of caspase with their BIR structural domains, whereas the RING structural domain promotes the degradation of caspase that is bound to IAP. Thus, IAPs directly inhibit the occurrence of apoptosis through binding, inhibiting, and degrading caspase. Pro-apoptosis activity of caspase can be maintained through direct knockdown of IAP expression, thus hopefully to achieve anti-tumour cell proliferation, increase of chemotherapeutic drug sensitivity, reversion of drug resistance of tumour cells, and reduction of tumour recurrence.

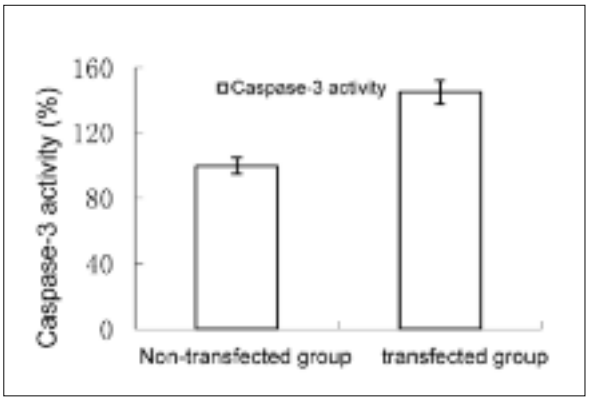

Figure 5. Caspase-3 activity changes in K562 cells before and after transfection.

A new member of the IAP family, *livin*[5], possesses the characteristic structure of IAP family, which is the BIR structural domain. This structural domain mediates the direct binding of *livin* with caspase-3, 7 and 9, thus, blocking the apoptotic effect of Caspase and achieving knockdown of cell apoptosis. The *livin* gene is located on chromosome 20q13 and encodes two proteins, α and β[15]. Subcellular localization showed expression in both cytoplasm and nucleus, the RING structural domain at the carboxyl terminal mediates the subcellular localization of *livin*. Previous studies[8-15] have shown that *livin* can be expressed in tumours of various systems such as gastric cancer in the digestive system, bladder cancer in the urinary system, lung cancer in the respiratory system, lymphoblastic leukemia in the blood system, myeloid leukemia, lymphoma and myeloma, as well as in breast cancer and melanoma. It is also widely expressed in tumour cell lines. However, its expression is rarely seen in normal tissues of each system. Studies[10,16,17] have also shown significantly increased *livin* expression during drug-induced apoptosis, indicating that *livin* plays an important role in the development of tumour drug resistance. Meanwhile, recent studies[3,4] have also shown that *livin*-specific expression in tumour tissues might provide new targets for the early diagnosis, gene therapy, and cell therapy of cancer.

Hence, the *livin* gene was selected as the target in our study. Through *in vitro* experiments, we specifically inhibited *livin* gene expression in the leukemia cell line K562 and discussed new targets and new methods of anti-leukemia treatment.

RNA interference[14] (RNAi) is the specific silencing of homologous genes in cells caused by small fragment double-stranded RNAs. It is a mechanism of post-transcriptional gene regulation. Cytoplasmic nucleases, Dicer, cleave double-stranded RNA molecules into 21-23 bp fragments (siRNA). These siRNA fragments binds to certain functional proteins to form RNA-induced silencing complexes (RICS). RICS can cut and break down mRNA that is complementary to the siRNA sequence, thus leading to the silencing of certain genes. RNAi can cause systemic long-term gene silencing in plants, but currently only

temporary gene silencing in mammals. There are three main sources of siRNA: chemical synthesis, *in vitro* transcription, and intracellular transcription of plasmids expressing siRNA. Despite the high cost, chemical synthesis has good specificity and high knockdown rate, which makes it especially suitable to achieve rapid knockdown of target genes in a short period of time. Therefore, chemical synthesis was used to screen *livin*-specific sequences in our study.

The knockdown effect of siRNA is mainly related to transfection efficiency and knockdown efficiency. For K562 cells in suspension culture, our transfection efficiency of electroporation was higher than using liposomes (results not shown). Transfection efficiency could be as high as 50% by using electroporation transfection reagents from Amaxa. However, carrier molecules used as positive control were considerably larger than the 21-bp siRNA fragments. This company claimed that the siRNA transfection efficiency could reach 76%, while it maintained cell status, siRNA purity, and concentration. We speculate that the transfection efficiency should be over 50% when transfecting specific siRNA into K562 cells.

Several recent studies[5] have confirmed that *livin* is expressed in many types of tumours in the blood system. However, leukemia cell apoptosis after *livin* gene knockdown and its influence on chemotherapeutic drugs have not been extensively studied. Leukemia cell line K562 that expresses *livin* gene was selected for this study. Our study confirmed that RNA interference technology can achieve specific knockdown of the *livin* gene in both RNA and protein level. A *livin* gene-specific siRNA sequence was selected in this study. A primary functional study was carried out for this sequence and we found that after *livin* gene was silenced, the spontaneous apoptosis rate of K562 cells significantly increased compared to that in the non-silencing group. Further studies showed that RNAi silencing *livin* combined with a chemotherapeutic drug (etoposide) could cause increased apoptosis of K562 cells that showed statistically significant differences compared to the spontaneous apoptosis group, indicating that the combined application of RNAi and chemotherapy could hopefully improve leukemia treatment efficacy, and possibly reverse drug resistance of leukemia cells. Further experiments have confirmed that increased caspase-3 activity might be one of the molecular mechanisms of elevated cell apoptosis rate.

A study conducted by Bavykin et al[17] showed that after simultaneous application of two siRNA sequences to silence *cIAP2* and *livin* gene, the susceptibility of the colorectal cancer HCT-116 cell line to oxaliplatin increased, which indicated that the combined application of RNAi sequences with multiple target genes play important roles in leukemia treatment and in the reversal of drug resistance of leukemia cells. This can be further studied in future experiments.

Conclusions

Livin can be used as a new target of leukemia treatment. Interference RNA technology can effectively inhibit *livin* gene expression and, thus, provide a new method for leukemia treatment. Stable RNAi sequences are to be established in subsequent studies and are expected to achieve continuous knockdown of IAP, thus, achieving effective pro-apoptosis effects.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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