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The influence of HOXA5-specific siRNA on the expression of Livin and Smac proteins

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Abstract. – OBJECTIVE: To Knockdown Homeobox A5 (HOXA5) expression by HOXA5-specific siRNA and evaluate the effects on Livin and Smac proteins expression in acute T cell leukemia Jurkat cells.

MATERIALS AND METHODS: We designed and constructed HOXA5-specific siRNA, and using liposomes we transfected Jurkat cells with this siRNA. The experiment was designed for three groups: (i) experimental group with Jurkat cells transfected with HOXA5-specific siRNA (siRNA transfection group), (ii) negative control group (irrelevant siRNA transfection) with Jurkat cells transfected with pRNAT-U6.1-siD and (iii) normal control group (untransfected Jurkat cells, only with equivalent amounts of cells and medium). We used FQ-PCR and Western blot to detect the relative expression levels of HOXA5 mRNA and protein in each group separately. The Western blot was also used to detect Livin and Smac protein levels in Jurkat cells.

RESULTS: Expression levels of HOXA5 mRNA and protein were significantly reduced in the group with Jurkat cells transfected with HOXA5 siRNA ($p<0.05$). The expression of Livin protein was significantly down-regulated ($p<0.05$) while the expression of Smac protein was significantly up-regulated ($p<0.05$).

CONCLUSIONS: HOXA5-specific siRNA effectively silenced the HOXA5 gene expression and down-regulation of HOXA5 induced the down-regulation of Livin protein expression and up-regulation of Smac protein. We suggest the HOXA5 gene to be considered as the new target for acute leukemia gene therapy.

Key Words:
HOXA5, RNA interference, Jurkat cells, Livin, Smac.

Introduction

Acute leukemia (AL) is one of children's most common malignant tumors, accounting for about 25% of all malignant tumors in children, with an incidence rate about 3 to 4 in hundred thousand. In recent years, it has shown a rising trend, with an annual increase of 6000 to 8000 cases. AL is a disease that seriously threaten children's health in China[1,2]. Presently, the etiology and pathogenesis of leukemia have not been fully elucidated. Studies have shown that apoptosis suppression was an important regulatory mechanism in leukemia. AL resulted from an imbalance between cell proliferation and apoptosis[3]. Livin is a new member of the inhibition apoptosis protein (IAP) family, showing high expression levels in most malignant tumors. Studies found abnormal expression levels of Livin and anti-Livin antibody in blood serum and solid tumor tissues, such as colorectal cancer, bladder cancer and lung cancer. Over-expression of Livin has been closely linked to the occurrence of acute leukemia[4,5]. The abnormal expression of Second Mitochondria-derived Activator of Caspases (Smac) protein may result in tumor development by influencing the cell apoptosis. Smac is a mitochondrial intermembrane protein that can be released into the cytoplasm to promote apoptosis[6].

The occurrence of cancer is a very complex pathological process, which involves the induction of several factors, the participation of multiple genes, and the development of numerous stages. Activation of oncogenes and inactivation of anti-oncogenes were the material bases for the formation and development of human tumors. Development of certain tumors has been closely related to the abnormal expression of homeobox genes (Hox) that are usually involved in cell growth regulation[7-10]. Homeobox genes are transcriptional regulators that modulate embryonic morphogenesis and pathological tissue remodeling in adults via regulation of genes associated with cell-cell or cell-ECM (extracellular matrix) interactions. The abnormal expression of homeotic gene HOXA5 showed to play an important role in breast tumorigenesis and leukemia[11-13]. Abnormal expression of HOXA5 might be involved through adjusting cell cycle,

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inhibition, or promotion of the occurrence of tumor through influencing the cell apoptosis. HOXA5 is a master regulator of the morphogenesis and cell differentiation. HOXA5 is also involved in the adjustment of the proliferation and the apoptosis. The HOXA5 over-expression has been linked to the occurrence and development of leukemia. In children with acute leukemia, the bone marrow cells show an irregular expression of Livin and Smac proteins. We examined the possibility of adjusting the Livin and Smac protein expression levels by interfering with the expression of HOXA5 gene using RNA interference (RNAi) technology.

RNA interference (RNAi) technology is a highly efficient gene blocking technique that could silence some functional genes at the post-transcription level[14]. It has been widely used in recent leukemia studies. To further investigate the relationship between HOXA5 gene and apoptosis of leukemia cell, HOXA5-specific siRNA was designed and synthesized, and human acute T leukemia cells (Jurkat cells) were transfected using cationic liposomes. We planned to silence HOXA5 gene and study the effects of this silencing on HOXA5 mRNA expression as well as changes in expression levels of HOXA5, Livin, and Smac proteins. This study attempts to offer a new idea for the pathogenesis and targeted therapy for leukemia.

Materials and methods

Materials and Reagents

In this study we used human peripheral blood leukemia T cells Jurkat cells (purchased from typical cell bank of the Chinese Academy of Sciences in Shanghai. Roswell Park Memorial Institute (RPMI)-1640, fetal calf serum (FCS: Hyclone, South Logan, UT, USA); penicillin and streptomycin with a final concentration of 100 IU/ml (self compound); HEPES (Shanghai Qianchen Biological Technology Co. Ltd.); G418 (Beyotime Institute of Biotechnology, Shanghai, China); Liposome Lipofectamine^{TM}2000 (Invitrogen, Carlsbad, CA, USA); Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA); Biozol TotM RNA Extraction Reagent (BioFlux Company, Tokyo, Japan); reverse transcription (RT-PCR) kit, HOXA5, β-actin primers, BamHI restriction endonuclease, T4DNA ligase and gel extraction kit (Takara, Otsu, Shiga, Japan); SiRNA sequence of targeted HOXA5, negative control siRNA sequence compounded by ADICON Corporation, (Northfield, IL, USA). Other reagents used in this research were provided by domestic companies.

Design and Synthesis of HOXA5siRNA Sequences

We designed and synthesized three siRNA sequences specific to HOXA5. To exclude the influence of siRNA itself on Jurkat cells, we have also designed a negative control (siRNAnc) sequence, which had no homology with any of human genes. Table I shows the sequences of HOXA5 specific siRNAs as well as the sequence of our siRNAnc used in negative control group. All siRNA sequences contained BamHI and HindIII restriction sites. The internationally accepted non-target siRNA was used as negative control. Our siRNAs were synthesized by ADICON Company (Northfield, IL, USA).

Cell Culture

We used cell culture media with 10% FCS, RPMI-1640 medium and penicillin and streptomycin with final concentration of 100 IU/ml (culture medium composition: 100 ml culture medium with antibiotic=10 ml 10% fetal bovine serum (FBS) +90 ml RPMI-1640 medium +1 ml penicillin and streptomycin with final concentration of 100 IU/ml; 100 ml culture medium without antibiotic with 10 ml 10% FBS +90 ml RPMI-1640 medium). Culture media for Jurkat cells were placed in an incubator with 5% CO_2 and 30% saturated humidity at 37°C. Cells in culture bottle were suspended and grew well in bulk (like a cluster of grape). Media were changed and cells were passaged once every 2-3 days. 1. The cell cultures were centrifuged at 1000 rpm for three minutes. 2. The supernatant was removed, and 3 to 4 ml nutrient solution with antibiotics were added to the pellets 3. The pellets were resuspended and transferred into cell culture bottles. 4. The proper amount of culture media with antibiotics were added and bottles were placed in the incubator. All cells were in their logarithmic growth phase.

Cell Transfection and Grouping

Three pairs of specific short hairpin double-stranded RNA (shRNA) of targeted HOXA5 gene were designed and synthesized. We screened one pair of those shRNAs that expressed the optimal silencing efficiency toward HOXA5 (primer sequence: sense strand

Table I. Three HOXA5-specific siRNA sequences and the nontarget siRNA (siRNAnc) sequence.

Group	Hind III	Sense	Loop	Antisense	Termination signal	Hind III
siRNA insert A 1: 75 bp	GGATCCCG	TTATGGAGATCATAGT TCCGT	TTCAAGAGA	ACGGAACTATGAT	TTTTTT CTCCATAA	CCAAAAGCTT
siRNA insert B 1: 75 bp	GGATCCCG	TACGGCTACAATGGC	TTCAAGAGA ATGGAT	ATCCATGCCATTGT	TTTTTT AGCCGTA	CCAAAAGCTT
siRNA insert C 1: 75 bp	GGATCCCG	TTGCGGTCGCTATCCA AATGG	TTCAAGAGA	CCATTTGGATAGC	TTTTTT GACCGCAA	CCAAAAGCTT
siRNA insert D 1: 75 bp	GGATCCCG	TAAGCCAACAATGCC	TTCAAGAGA ATTCAT	ATGAATGGCATTGT	TTTTTT TGGCTTA	CCAAAAGCTT

TTGCGGTCGCTATCCAAATGG, antisense strand: CCATTTGGATAGCGACCGCAA) (sequence C in Table I). A complementary double-stranded oligonucleotides that could code the corresponding short hairpin double-stranded RNA (shRNA) was synthesized. Cell concentration was adjusted with RPMI 1640 culture media up to 3×10^7 per ml before the transfection. The final concentration of siRNA was 135 ng/ul. Liposomes were mixed with siRNAnc and HOXA5 siRNA in RPMI-1640 culture media. The experiments were divided into 3 groups. (i) blank control group (Jurkat cells and Lipofectamine™ 2000), (ii) negative control group with non-silencing siRNA (Jurkat cells and Lipofectamine™ 2000), (iii) silencing siRNA; experimental group with HOXA5-specific siRNA (Jurkat cells and Lipofectamine™ 2000). According to the differences in the three siRNA sequences, we subdivided our experimental group into three subgroups: experimental group A (pRNAT-GFP-Neo-HOXA5A); experimental group B (pRNAT-GFP-Neo-HOXA5B); experimental group C (pRNAT-GFP-Neo-HOXA5C). 24 hours after transfection, we added G418 (200 μg/mL) for selection, and changed the media once every two days and added the same concentration of G418. The selection under this concentration continued for two weeks and, then, the concentration of G418 was changed to 100 μg/mL. The selection process under new concentration continued for another two weeks and, then, monoclonal cells were selected. Cultures were received G418 (100 μg/mL). We repeated the experiment three times. Follow-up experiments were divided into 3 groups. Cells from all three groups were collected for follow-up experiments.

Real-Time Fluorescence Quantitative PCR Method for Measuring the Expression Level of HOA5mRNA

In the control group, Jurkat cells were harvested in logarithmic phase and were inoculated into 6-well culture plates. Each well received 3×10^5 cells. For experimental and negative control groups, transfected cells were harvested during the early period and were inoculated into 6-well culture plates (2 wells for each group). Total RNA was extracted 24 hours later. After observation on 1% agarose gel, the purity of the extracted RNA was verified, by measuring absorbance ratio of A260/A280 using spectrophotometry. We used those extracts showing A260/A280 absorbance ratio ranging from 1.8 to 2.2. Reverse transcriptase enzymes (RT) were used to convert RNA into cDNA and then quantitative PCR was applied to amplify HOXA5 and the internal reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). GAPDH primer sequence used here was: upstream, 5'- ATGCTGGCGCTGAGTACGTC- 3', downstream 5'-GGTCATGAGTCCTTCCACGATA-3'. HOXA5 primer sequence: upstream, 5'-TTTTGCGGTCGCTATCC-3', downstream 5'- CTGAGATCCATGCCATTGTAG-3'. We used the $RQ=2^{-\Delta\Delta Ct}$ equation to analyze our data. We expressed the relative expression amount of HOXA5 mRNA by the gray ratio of HOXA5 to internal reference gene GAPDH and calculated the mRNA inhibition ratio of HOXA5 in the experimental group. The mRNA inhibition ratio of HOXA5=[1-experimental group (relative expression amount of mRNA of HOXA5) / blank control group (relative expression amount of mRNA of HOXA5)]×100%. The experiment was repeated three times.

Table II. The expression level of HOXA5 mRNA in all groups and their HOXA5 inhibition ratio (x±s).

Group	Experimental group A	Experimental group B	Experimental group C	Negative control group	Blank control group
	STIR	DWI	STIR	DWI	
HOXA5mRNA relative expression amount	1.01±0.03*	0.87±0.02*	0.39±0.01*	1.34±0.16#	1.29±0.21
HOXA5 inhibition ratio (%)	24.62±2.34	35.07±3.21	70.89±6.41	-	-

*comparison with blank control group and negative control group ($p<0.05$); comparison with blank control group ($p>0.05$).

Western Blot for Detecting the HOXA5 Protein Expression

Jurkat cells in logarithmic phase were harvested and cells were inoculated into 6-well culture plates. We used the procedures explained in the previous section for the cell grouping, inoculation density and transfection. After transfection, cells were washed twice with phosphate buffered saline (PBS); then, cell lysis solution was added and kept on the ice for 30 minutes. Cell lysates were centrifuged at 10,000 rpm for 10 minutes at 4°C and supernatant were then collected and kept in -80°C. Bicinchoninic acid assay (BCA) method was applied to measure the protein concentration, and equivalent amounts of protein were used for SDS-polyacrylamide gel (SDS-PAGE). SDS-PAGE loading buffer (5X) was added to the protein samples and, after 5 minutes boiling, proteins were separated using SDS-PAGE. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes after SDS-PAGE. Membranes were blocked with Tris-HCl buffer salt solution (TBST) then rinsed completely in TBST (1X) three times each time 5 minutes. A blocking solution composed of 5% dried skimmed milk and 1 g/L, Tween-20. The HOXA5 polyclonal antibody was diluted (1:1000) and added to the membranes then incubated at 4°C overnight and then rinsed. Goat anti-rabbit secondary antibody (1:1000) was added and incubated at room temperature for one hour. ECL was applied for luminescence, and dark room films were used for imaging. Images were analyzed with Gel-Pro analyzer software and the expression levels for target proteins were measured using the ratio between the gray value of HOXA5 protein band and GAPDH protein band. pRNAT-GFP-Neo-HOXA5 plasmid vector group with the highest silence efficiency were chosen to continue the follow-on study. Protein inhibition ratio for HOXA5 in the experimental group was calculated. Protein inhibition ratio was calculated using the following equation: HOXA5= [1-experimental group (relative expression amount of protein of HOXA5) / blank control group (relative expression amount of protein of HOXA5)] x 100%

The experiments were repeated for three times.

Western Blot Method in Detecting the Expression of Livin Protein and Smac Protein

Western blot was used to evaluate the expression levels of Livin and Smac (Second Mitochondrian-derived Activator of Caspases) proteins in all groups. Experimental procedures were as explained in the previous section. Gray ratio of Livin, Smac to internal reference gene GAPDH was used to express the relative expression amount of Livin and Smac proteins.

Statistical Analysis

The SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA) was used for data analyses. Measurement data were presented by means±standard deviation; single factor variance analysis was applied among groups; q-test was applied in pairwise comparisons among multiple means. Inspection levels of $\alpha=0.05$ and $p<0.05$ were considered statistically significant.

Results

pRNAT-GFP-Neo-siHOXA5 Recombinant Vector Transfected Jurkat Cells

Jurkat cells were transfected with pRNAT-GFP-Neo-siHOXA5C recombinant vector and green fluorescent protein (GFP) expression was observed under fluorescence microscope. We obtained a transfection efficiency of about 50% (Figure 1).

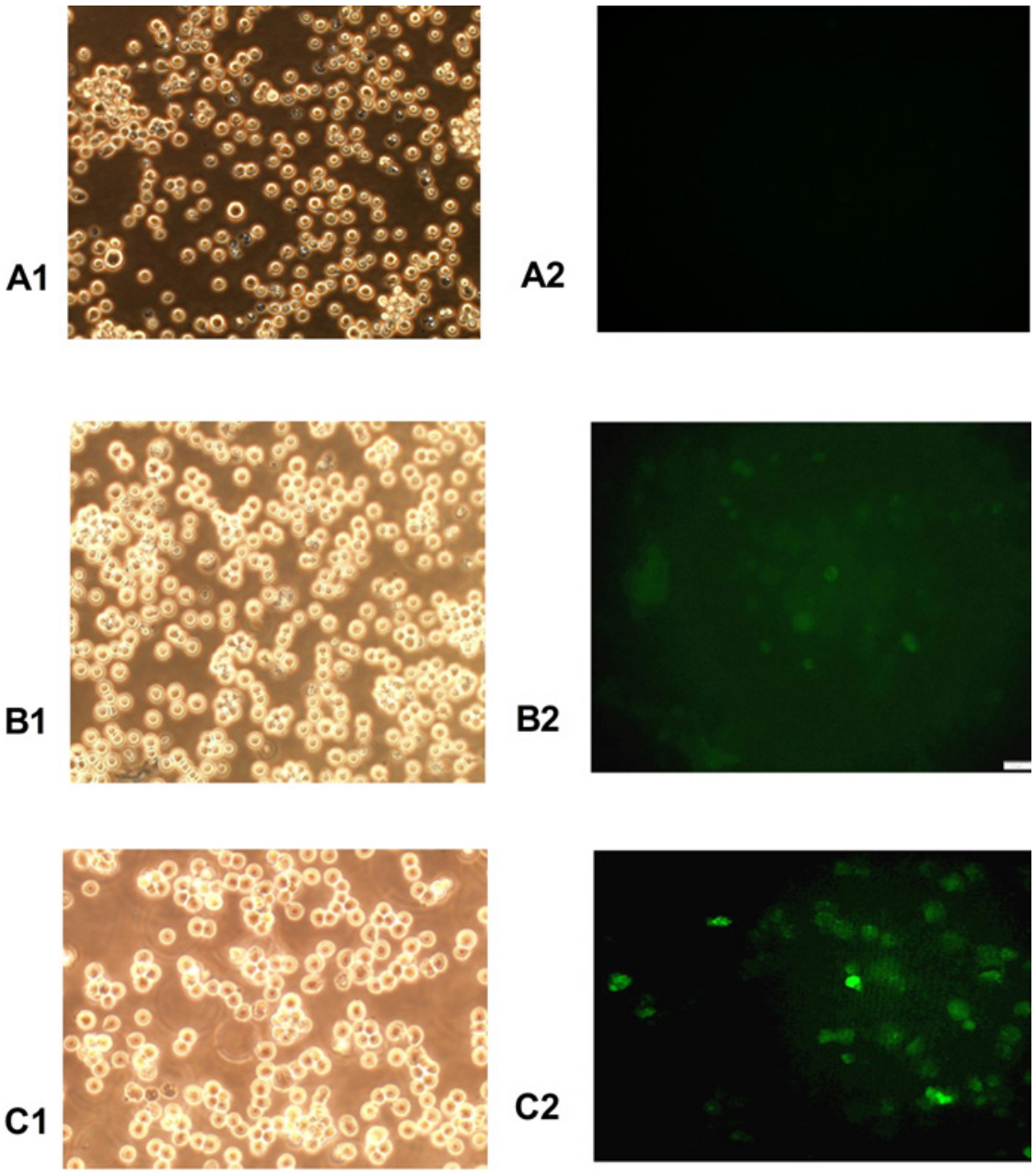

Figure 1. pRNAT-GFP-Neo-HOXA5C recombinant plasmid vector transfected Jurkat cell (×400). A1, B1, C1: ordinary microscope; A2, B2, C2: fluorescence microscope, A1, A2: blank control group; B1, B2: negative control group (pRNAT-GFP-Neo-siRNAnc); C1, C2: experimental group (pRNAT-GFP-Neo-HOXA5C).

The Influence of pRNAT-GFP-Neo-si-HOXA5 on the Expression Level of HOXA5mRNA in Jurkat Cells

Agarose gel electrophoresis of extracted RNA revealed the HOXA5 mRNAs (Figure 2). Real-time fluorescence quantitative PCR results (Table II) showed that the differences between the experimental group and blank control group as well as negative control group had statistical significance ($p<0.05$). On the other hand, differences between the blank control group and the negative control group had no statistical significance ($p>0.05$). Relative expression level of HOXA5 mRNA in the experimental group was significantly lower than that in the blank control group and negative control group, with inhibition ratio being pRNAT-GFP- Neo-HOXA5A (24.62±2.34)%, pRNAT-GFP-Neo-HOXA5B (35.07±3.21)%, and

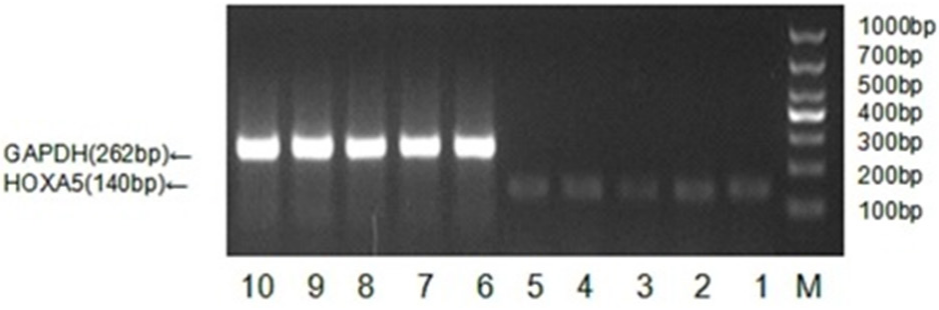

Figure 2. HOXA5mRNA agarose gel electrophoresis. ***A***, M: Marke; 1: pRNAT-GFP-Neo-HOXA5A; 2: pRNAT-GFP-Neo-HOXA5B; 3: pRNAT-GFP-Neo-HOXA5C; 4: negative control group: pRNAT-GFP-Neo-siRNAnc; 5: blank control group; GAPDH: 6 to 8: experimental group; 9: negative control group; 10: blank control group; comparison between experimental group (pRNAT-GFP-Neo-HOXA5C) and blank control group and negative control group ($p<0.05$). ***B***, The effect of siRNA on the expression of HOXA5mRNA of Jurkat cells; $^{*}p<0.05$, comparison with blank control group and negative control group had statistical significance.

pRNAT-GFP-Neo- HOXA5C (70.89±6.41)% respectively. Results demonstrated that among the three designed siRNA, the interfering effect of pRNAT-GFP-Neo-HOXA5C was the strongest. Therefore, we chose pRNAT-GFP-Neo-HOXA5C as an effective siRNA expression vector and continued the transfection process. Jurkat cells were screened in order to lay a foundation for the next step in our research on Jurkat cells proliferation and apoptosis after HOXA5 gene silenced.

The Expression Levels of HOXA5 Protein

Expression levels of HOXA5 protein in all groups were measured using Western blot. Results showed that HOXA5 expression in Jurkat cells were down-regulated after transfection with siRNAs. We obtained the following results: i) for pRNAT-GFP-Neo-HOXA5A: 0.41±0.03-, ii) for pRNAT-GFP-Neo-HOXA5B: 0.35±0.02-, iii) pRNAT-GFP-Neo-HOXA5C: 0.18±0.01, iv) negative control group: 0.85±0.04, v) blank control group: 0.84±0.05. Differences among experimental, blank control and negative control groups were statistically significant ($p<0.05$). Conversely, differences between the negative control group and the blank control group had no statistical significance ($p>0.05$). Relative expression levels of HOXA5 protein in the experimental group was significantly lower than the blank control group as well as the negative control group. Inhibition ratios observed were as follow: (1) for pRNAT-GFP-Neo-HOXA5A: 51.76±4.02%, (2) pRNAT-GFP-Neo-HOXA5B: 58.82±4.05%, (3) pRNAT-GFP-Neo-HOXA5C: 78.82±5.08%. Our results showed that pRNAT-GFP-Neo-HOXA5 vector significantly inhibited the expression of the HOXA5 protein. Therefore, it was an effective interference vector that could be applied in follow-up experiments (Table III, Figure 3).

The Expression Levels of Livin Protein

Expression levels of Livin protein in all groups were measured using Western blot. Results showed that Livin protein expression in Jurkat cells was down-regulated after transfection with siRNAs.

Livin protein expression in Group C (0.20±0.02) was significantly lower compared to the negative control group (1.45±0.04) and the blank control group (1.33±0.02) ($p<0.05$). However, differences between negative control group and blank control group had no statisti-

Table III. The expression of HOXA5 protein in all groups (x±s).

Group	Experimental group A	Experimental group B	Experimental group C	Negative control group	Blank control group
The expression levels of HOXA5 protein	0.41±0.03*	0.35±0.02*	0.18±0.01*	0.85±0.04#	0.84±0.05
HOXA5 inhibition ratio (%)	51.76±4.02	58.82±4.05	78.82±5.08	-	-

*comparison with blank control group and negative control group ($p<0.05$); comparison with blank control group ($p>0.05$).

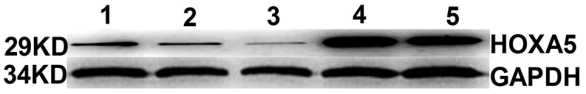

Figure 3. HOXA5 protein expression levels measured by Western blot technique.

cal significance (p>0.05), Protein bands in all groups did not show any significant differences which indicated that pRNAT-GFP-Neo-HOXA5C vector significantly down-regulated Livin protein expression (Figure 4).

The Expression Levels of Smac Protein

Expression levels of Smac protein in all groups were measured using Western blot. Results showed that Smac protein expression in Jurkat cells was upregulated after transfection with siRNAs. Smac protein expression in Group C (1.26±0.04) was significantly higher compared to negative control group (0.87±0.03) and blank control group (0.86±0.02) (p<0.05). The difference between negative control group and blank control group had no statistical significance (p>0.05). Protein bands in all groups did not show any significant differences which indicated that pRNAT-GFP-Neo-HOXA5C vector significantly up-regulated the Smac protein expression (Figure 5).

The Correlation Between HOXA5 and Livin, Smac Proteins Expression Levels

According to the results of the Western blots (Table IV) and correlation analysis of Spearman level, the expression level of HOXA5 protein was positively correlated with the expression level of Livin protein (r=0.771). Conversely, the expression level of HOXA5 was negatively correlated with the expression level of Smac protein (r=-0.714). These results suggested that the down-regulation of HOXA5 protein might be the source of the Livin protein down- regulation as well as Smac protein up-regulation.

Discussion

HOX gene has an abnormal expression in many tumors (especially in leukemia, breast cancer, and lung cancer)[11-13,15]. Under certain circumstances, altering the expression level of HOX gene can promote or inhibit the occurrence and development of the tumor. In hematopoietic cells, the abnormal expression of HOX gene could result in dysdifferentiation and dysmaturation of the cells, reduce the hematopoietic ability, and even lead to the occurrence and development of leukemia[16-19]. Based on this, some scholars took advantage of the specificity of RNAi technology to down-regulate the highly expressed HOXA gene in leukemia cells. This resulted

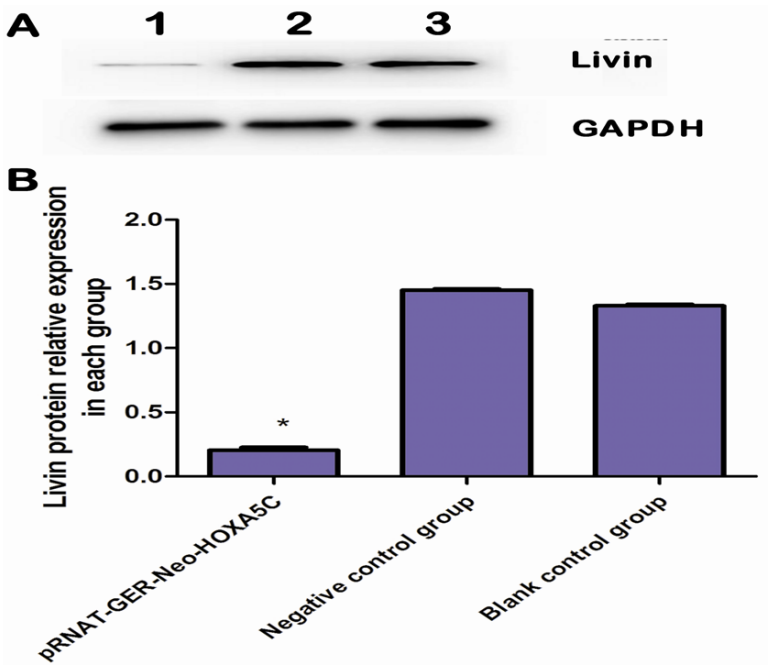

Figure 4. Western blot was used to detect the expression levels of Livin protein. ***A,*** Electrophoretogram of Western blot method in detecting the expression of Livin protein. 1: experimental group (pRNAT-GFP-Neo-HOXA5C); 2: negative control group (pRNAT-GFP-Neo-siRNAnc); 3: blank control group: the expression of Livin protein in the experimental group was significantly lower than that in the negative control group and the blank control group (p<005). ***B,*** Comparisons of the expression levels of Livin protein in all groups, p<0.05, compared with the blank control group and the negative control group.

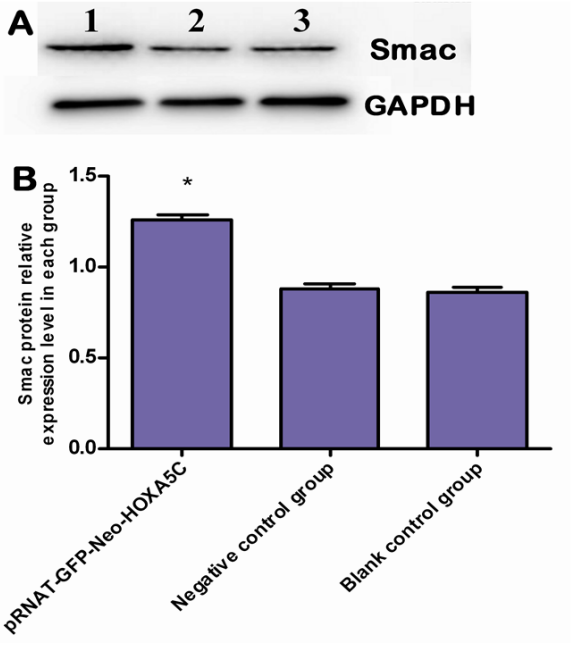

Figure 5. Western blot was used to detect the expression levels of Smac protein. ***A,*** Electrophoretogram of Western blot method in detecting the expression of Smac protein. 1: experimental group (pRNAT-GFP-Neo-HOXA5C); 2: negative control group (pRNAT-GFP-Neo-siRNAnc); 3: blank control group; ***B,*** Comparisons of the expression levels of Smac protein in all groups, $^{*}p<0.05$, compared with the blank control group and the negative control group. The difference had a statistical significance.

in an increase in the leukemia cells sensitivity to chemotherapeutic drugs. Down-regulation of HOXA gene also inhibited the proliferation of leukemia cells, promoted cell apoptosis, and improved the treatment effect of leukemia. All these evidences confirmed that this technique may become a new method of gene therapy for treating malignant blood diseases[20]. Orlovsky et al[21] used RNAi to silence HOXA9 gene in RS4;11 cells (B-cell leukemia cell line) and reported that the transition of RS4;11 cells from G0/G1 phase to S phase was obstructed. They observed that, compared to negative control cells, cells in G0/G1 phase increased, cells in S phase decreased, cell proliferation was inhibited, and cell apoptosis increased. Zhang et al[22] constructed a lentivirus that could effectively silence HOXA10 gene-shRNA vector to reduce the expression levels of HOXA10 gene. They reported that their construct effectively inhibited the cell proliferation and promoted the apoptosis in U937 cells. Wenwen et al[23] used the specific eukaryotic expression vector for HOXA10 and cationic liposomes to transfect K562 cells. The vector effectively reduced the transcription from the HOXA10 gene. The results showed that down-regulation of HOXA10 by specific eukaryotic expression vector inhibited cell proliferation, and induced apoptosis effects of vincristine and etoposide in K562 cells. RNAi of targeted HOXA10, combined with a small dose of Ara-C effectively inhibited the proliferation of K562 cells, induced apoptosis, and improved the sensitivity of K562 cells to chemotherapeutic drugs. They also held that low doses of DNR combined with HOXA10 eukaryotic expression vector could be expected to be a new effective, low toxic treatment for children with leukemia.

The homeobox gene HOXA5, located on chromosome 7 (7p15.2), encodes a DNA-binding transcription factor that has been shown to play important roles in regulation, proliferation, differentiation and apoptosis. Studies have confirmed that the transfer of hematopoietic stem/progenitor cells (HSPC) from erythroid differentiation to granulocyte differentiation is regulated by HOXA5 in pluripotent progenitor cell stage[24]. Kim et al[11] studied on 50 children with acute myeloid leukemia and 19 with healthy bone marrow to analyze the methylation levels of HOXA5. Their results showed the level of HOXA5 gene methylation had a correlation with the 3-year survival rates for patients with acute myeloid leukemia. When HOXA5 expression was inhibited (using antisense oligonucleotide technology) in hematopoietic cells in bone marrow, the myeloid progenitor cells development was also inhibited. Boucherat et al[25] reported that the down-regulation of HOXA5 might affect the expression of p53 in breast cancer and promote carcinogenic process. In contrast, the loss of HOXA5 function and specific chromosomal translocation might inhibit the occurrence of leukemia. Therefore, inappropriate expression

Table IV. The expression of HOXA5, Livin, and Smac protein in Jurkat cells (x±s).

Group	HOXA5 protein	Livin protein	Smac protein
Negative control group	0.85±0.04	1.45±0.04	0.87±0.03
Blank control group	0.84±0.05	1.33±0.02	0.86±0.02
Experimental group C	0.18±0.01	0.20±0.02	1.26±0.04

of HOXA5 gene may disrupt normal growth and lead to tumorigenesis. These studies all suggested that HOXA5 was associated with the occurrence and development of leukemia.

Leukemia is a malignant disease of the hematopoietic system, topping the list of children malignant diseases, which resulted from the imbalance between proliferation and apoptosis of leukemia cells. Apoptosis blockage in leukemia cells is closely related to the over-expression of anti-apoptotic genes. Generally, two types of human genes affect cell apoptosis: (I) anti-apoptotic genes, including IAPs, Bcl-2, p53, and CrmA genes and (II) pro-apoptotic genes, including Smac/DIABLO, HtrA2, and Reaper genes. Inhibitor of Apoptosis (IAP) family plays an important role in the process of tumorigenesis. Anti-apoptotic function of IAP family is stronger than Bcl-2 family. Livin is a new member of the IAPs family with two splicing variants designated Livin α and Livin β. Through inhibiting the caspase activity, Livin exercises an anti-apoptotic effect which could promote the proliferation of malignant tumor cells[26]. Studies showed that Livin protein had low or no expression in most terminal tissues (except placenta) of normal adults and had a high expression in most malignant tumors, such as melanoma, breast cancer, colon cancer, bladder cancer, prostate cancer, leukemia, and lymphoma[27]. Ibrahim et al[28] reported that the expression of Livin protein in children with acute leukemia was significantly higher than the normal group. This over-expression suggested that Livin protein played a role in the development of acute leukemia. Over expression of Livin was reported to be the real "culprit" in the drug resistance observed in a leukemia cell. Livin could promote cancer cells against apoptosis induced by antitumor drugs through inhibiting caspase-3 and led to the drug resistance of leukemia cell[29]. Smac (Second Mitochondria-derived Activator of Caspases) is a protein present in the mitochondria which regulate the cells apoptosis. Smac, also known as DIABLO (Direct Inhibitor of Apoptosis-Binding protein with Low Isoelectric Point), is a newly discovered apoptosis regulating protein. Smac is an inhibitor of IAPs or inhibitor of apoptosis proteins. Smac promotes apoptosis through inhibiting the IAP activity. Smac expression was found to be related to the prognosis of the tumors in cancer patients. Patients with a higher expression of Smac had better disease-free progressive survival rate and overall survival rate[30]. Prior studies confirmed that Smac had a pro-apoptotic effect on many tumor cells, and increased the sensitivity of tumor cells to radiotherapy and chemotherapy. Results from a prior study suggested that Smac properties were related to the prognosis and lifetime of tumor or to the recurrence of some tumors[31]. Therefore, Livin protein and Smac proteins had inhibiting effects toward tumor cell apoptosis and promoting tumor cell apoptosis, respectively.

Through designing and synthesizing the plasmid vector pRNAT-GFP-Neo-shHOXA5 via liposome mediation, transfecting Jurkat cells and obtaining the corresponding siRNA to inhibit HOXA5 we, investigated the expression of Smac and Livin proteins after the down-regulation of HOXA5. In order to get a highly efficient gene inhibition rate, we designed three siRNA for HOXA5 gene. Using RT-PCR and Western blot, we confirmed that our vectors effectively reduced HOXA5 mRNA and protein expression levels. These findings were similar to the existing results in the literature[21-23]. The inhibition rate of HOXA5 (mRNA and protein levels) of pRNAT-GFP-Neo-shHOXA5C (Experimental group C) were as high as (70.89±6.41)%, and (78.82±5.08)%, respectively. In our study, after silencing HOXA5 gene in Jurkat cells in a human leukemia cell line, we discovered high levels of Livin protein expression in control group and negative control group and low levels of Livin protein expression in our experimental group C ($p<0.05$). We also observed low levels of Smac protein expression in the blank control group and negative control group and high expression of Smac in experimental group C ($p<0.05$). Further analyses showed that the expression level of HOXA5 gene was positively correlated with that of Livin protein and negatively correlated with that of Smac protein.

Conclusions

Results obtained from this research suggested that inhibition of the HOXA5 protein inhibited the Livin protein expression and up-regulated the Smac protein expression. It was speculated that by promoting the expression of pro-apoptotic factors and inhibiting the anti-apoptotic factors we could see an increase in the apoptosis level in tumor cells. As for how HOXA5 regulated the cell apoptosis, there is still a need for further studies.

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Conflicts of interest

The authors declare no conflicts of interest.

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