Effect of silencing *HOXA5* gene expression using RNA interference on cell cycle and apoptosis in Jurkat cells

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Abstract. Acute lymphocytic leukemia (ALL) is a common malignant tumor with a high morbidity rate among children, accounting for approximately 80% of leukemia cases. Although there have been improvements in the treatment of patients frequent relapse lead to a poor prognosis. The aim of the present study was to determine whether HOXA5 may be used as a target for gene therapy in leukemia in order to provide a new treatment. Mononuclear cells were extracted from the bone marrow according to the clinical research aims. After testing for ALL in the acute stage, the relative mRNA and protein expression of HOXA5 was detected in the ALL remission groups (n=25 cases per group) and the control group [n=20 cases, immune thrombocytopenia (ITP)]. Gene silencing by RNA interference (RNAi) was used to investigate the effect of silencing *HOXA5* after small interfering RNA (siRNA) transfection to Jurkat cells. The HOXA5-specific siRNA was transfected to Jurkat cells using lipofectamine. The experiment was divided into the experimental group (liposomal transfection of HOXA5 targeting siRNA), the negative control group (liposomal transfection of cells with negative control siRNA) and the control group (plus an equal amount of cells and culture media only). Western blotting and quantitative fluorescent polymerase chain reaction (QF-PCR) were used to detect the relative HOXA5 mRNA expression and protein distribution in each cell group. Cell distribution in the cell cycle and the rate of cells undergoing apoptosis were determined using flow cytometry. The expression of HOXA5 at the mRNA and protein levels in the acute phase of ALL was significantly higher than that in ALL in the remission and control groups. In cells transfected with HOXA5-specific siRNA, the expression of HOXA5 at the mRNA and protein levels decreased significantly ($P<0.05$). The distribution of cells in the cell cycle was also altered. Specifically, more cells were present in the G0/G1 phase compared to the S phase ($P<0.05$). In addition, the apoptotic rate was significantly higher in cells transfected with HOXA5-specific siRNA ($P<0.05$). In conclusion, high expression levels of HOXA5 mRNA and protein in children with ALL indicate that HOXA5 is closely associated with childhood ALL. In addition, HOXA5-specific siRNA effectively silences HOXA5 gene expression and induces apoptosis and cell-cycle arrest in Jurkat cells, thus inhibiting cell proliferation.

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Introduction

Acute lymphocytic leukemia (ALL) is one of the most common malignant tumors and has the highest morbidity rates among children, accounting for ~80% of leukemia cases. The incidence rate of ALL is 5-fold higher than that of acute myeloid leukemia (AML). The development of medical technology, has led to improvement in the treatment of ALL. However, 20-30% of children with leukemia suffer ALL relapse and subsequently have a poor prognosis (1-3).

Clinical studies have shown that the relapse of AML after treatment is strongly associated with the expression of homeobox (*HOX*) genes, whose main role is to control the proliferation and differentiation of hematopoietic stem and progenitor cells (4). It has also been shown that even the development of various types of acute leukemia such as acute myeloid leukemia, is associated with HOX gene expression (5,6). HOX genes are divided into four clusters according to the similarity and chromosomal location of the human HOX gene sequence. These clusters are HOXA, HOXB, HOXC and HOXD, which are located on chromosome number VII, XVII, XII and II, respectively. Each of the HOX contains 9-11 genes. HOXA5 belongs to type 1 of the *HOXA* gene and is located on chromosome VII (7p15.2). HOXA encodes a DNA-binding transcription factor that regulates the expression of genes which control cell differentiation (6). The abnormal expression of HOX may affect cell differentiation and maturation in hematopoietic disorders (6). It may also decrease hematopoietic ability and result in the occurrence and development of leukemia (6). Findings by Delval *et al* (7) have shown that HOXA1 interacts with B-cell leukemia transcription factor through a HOX polypeptide. The mutation of the conserved tryptophan and methionine residues led to loss of its ability to stimulate cell proliferation, anchorage-independent cell growth and loss of contact inhibition (7). A study by Okada *et al* (8) showed

that HOXA5 methylation plays an important role in leukemic transformation, which is induced by the CALM-AF10 fusion protein (8). Bach *et al* (9) found that the high expression of HOXA5 may contribute to the occurrence and phenotype of leukemia.

RNA interference (RNAi) is a type of simple and effective genetic tool that has been developed in recent years and is used instead of gene knockout (10,11). RNA interference (RNAi) is the process of sequence-specific, post-transcriptional gene silencing in the same direction, initiated by double-stranded RNA (10). RNAi technology is a type of small-interfering RNA (siRNA) with 21-23 bp that is derived from double-stranded DNA (dsRNA) by effect of RNase III endonuclease Dicer (11). It is a highly efficient gene-blocking technology that blocks the expression of target genes by mediating specific degradation of complementary homologous mRNA (12). In the present study, *HOXA5* gene expression in ALL was detected by clinical tests, and the expression levels of HOXA5 mRNA and protein were detected by quantitative fluorescent-polymerase chain reaction (QF-PCR) and western blot analysis. Subsequently, through the synthesis of HOXA5 targeting-specific siRNA, cationic liposome was used to transfect Jurkat cells, a human acute T-cell leukemia cell line. HOXA5-specific siRNA may inhibit the expression of *HOXA5* gene. We detected the expression of HOXA5 mRNA and protein in Jurkat cells and investigated the effect of *HOXA5* gene in cell cycle and apoptosis. In the present study, the results showed that HOXA5 can be used as a target for gene therapy in leukemia and provide a new treatment for acute lymphoblastic leukemia.

Materials and methods

Cell lines and reagents. Human lymphocyte separation medium were obtained from Tianjin TBD Co. (Tianjin, China), and Jurkat leukemic T cells from human peripheral blood (Shanghai Institute of Cell Library, Shanghai, China). RPMI-1640 and fetal bovine serum (FBS) were purchased from HyClone (Logan, UT, USA) and Lipofectamine™ 2000 from Invitrogen-Life Technologies (Carlsbad, CA, USA). G418 and CCK-8 were obtained from Beyotime Institute of Biotechnology (Shanghai, China) and DMSO from Sigma (St. Louis, MO, USA). Annexin V-PE/7AAD and the cell apoptosis detection kit were obtained from Nanjing KeyGen Biotech (Nanjing, China) and the TRIzol reagent from Invitrogen-Life Technologies. The RNA extraction reagent was purchased from BioFlux, Hangzhou, China. The QF-PCR kit, HOXA5, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primer, restriction endonuclease *Bam*HI, T4 DNA ligase, and gel purification kit were purchased from Takara (Shiga, Japan). siRNA sequence targeting HOXA5, and the negative control siRNA sequence were purchased from Adicon Co. (Shanghai, China). Rabbit anti-human HOXA5 polyclonal antibodies were purchased from Abcam (Cambridge, UK) and horseradish peroxidase-labeled goat anti-rabbit secondary antibodies were purchased from the Beyotime Institute of Biotechnology. Other reagents were developed and purified in China.

Cases. Fifty children newly diagnosed with ALL were enrolled in the study between October 2013 and June 2015.

The patients were divided into three groups: i) the acute phase group included 25 newly diagnosed cases of ALL. The ALL cases were confirmed by morphological analysis of bone marrow cells and MICM typing, had not previously received any treatment and excluded other neoplastic diseases, such as multiple myeloma tumor and malignant lymphoma, according to the basic criteria for the diagnosis of ALL. ii) The ALL remission group comprised 25 cases. In this group, ALL remission induction therapy administered achieved complete remission as per CR standards with efficacy standards of ALL. iii) The control group comprised 20 cases. Selected bone marrow samples were obtained from children with immune thrombocytopenia (ITP) (13). The samples were collected with the consent of the children's parents.

The ALL acute phase group comprised 13 male and 12 female children with a median age of 6.6 years (10 months to 14 years). In the ALL remission group, there were 15 male and 10 female children with a median age of 6.3 years (10 months to 14 years). The control group included 9 male and 11 female children with a median age of 6.7 years (10 months to 14 years).

Isolation of mononuclear cells from bone marrow. Prior to diagnosis with ALL, routine biopsy was performed to obtain bone marrow samples of ~2 ml from each child. Subsequently, lymphocyte bone marrow mononuclear cell samples were isolated. Bone marrow cells (2 ml) were diluted by adding an equal volume of saline solution. Human lymphocyte separation medium (4 ml) was added to the centrifuge tube. Diluted bone marrow fluid was gently and gradually layered along the wall until it adhered to the lymphocyte separation medium, and then centrifuged at 599.4 x g for 25 min. The intermediate buffy coat layer was then collected and placed into a new tube. Four volumes of saline were added, followed by centrifugation at 599.4 x g for 20 min. The cells were washed twice with RPMI-1640, which was purchased from Hyclone (Logan, UT, USA), prior to discarding the supernatant. The cell pellet was then washed with 10% FBS RPMI-1640, after the dispersion count. The cells were seeded in a culture flask (Hyclone) at a concentration of $3x10^7$/ml, and placed in a cell incubator at a temperature of 37˚C, carbon dioxide (CO_2) concentrations of 5 and 30% moisture saturation. The medium was changed after 2-3 days and passaged once.

Detection of HOXA5 mRNA expression levels in mononuclear cells using QF-PCR. RNA was extracted from the mononuclear cells of the bone marrow. The absorbance of the samples was determined by the UV spectrophotometer A ratio (A260/A280), at a range of 1.8-2.2, by identification of 1% agarose gel electrophoresis. Amplification of *HOXA5* and *GAPDH* genes was performed by QF-PCR. *HOXA5* gene was amplified using the primers: upstream, 5'-TTTTGCGGTCGCTATCC-3', and downstream, 5'-CTGAGATCCATGCCATTGTAG-3' and the amplified fragment length was 140 bp. For the *GAPDH* gene, the primers used were: upstream, 5'-ATGCTG GCGCTGAGTACGTC-3' and downstream, 5'-GGTCATGAG TCCTTCCACGATA-3' and the amplified fragment length was 262 bp. The conditions used to set up the PCR reaction were: 95˚C denaturation 30 sec, followed by 95˚C denaturation for 5 sec, 58˚C annealing for 34 sec, for 40 cycles. The condition

Table I. Sequences of the siRNA targeting *HOXA5* gene.

Group	*Hin*dIII	Sense	Loop	Antisense	Termination signal	*Hin*dIII
siRNA insert A 1:75 bp	GGATCCCG	TTATGGAGATCATAGT TCCGT	TTCAAGAGA	ACGGAACTATGAT CTCCATAA	TTTTTT	CCAAAAGCTT
siRNA insert B 1:75 bp	GGATCCCG	TACGGCTACAATGGC ATGGAT	TTCAAGAGA	ATCCATGCCATTGT AGCCGTA	TTTTTT	CCAAAAGCTT
siRNA insert C 1:75 bp	GGATCCCG	TTGCGGTCGCTATCCA AATGG	TTCAAGAGA	CCATTTGGATAGC GACCGCAA	TTTTTT	CCAAAAGCTT

siRNA, small interfering RNA; HOXA5, homeobox gene.

for drawing the dissolution curve was 95˚C denaturation for 15 min, 60˚C annealing for 60 sec, and 95˚C denaturation for 15 sec. Data were analyzed using the formula RQ = $2^{-\Delta\Delta Ct}$ while $2^{-\Delta\Delta Ct}$ was used to represent the relative expression levels of mRNA HOXA5. The gray-level ratio with HOXA5 and internal reference gene *GAPDH* expressed was used to indicate the relative expression of HOXA5 mRNA. The experiment was repeated three times.

Detection of HOXA5 protein expression in bone marrow mononuclear cells using western blot analysis. Bone marrow mononuclear cells were washed with cold phosphate-buffered saline (PBS) twice, cell lysis was performed and the cell lysate was collected and stored at -80˚C. The protein concentration in the lysate was determined using the BCA method to ensure that the same amount of protein was added in each reaction. Subsequently, 5X SDS [sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)] polyacrylamide gel electrophoresis sample buffer (Abcam, Cambridge, UK) was added to this cell lysate and boiled for 5 min. The proteins were then transferred to PVDF membranes following SDS-PAGE. Tris-HCl buffer solution (TBS) sealing liquid with 5% skim milk powder as well as 1 g/l Tween-20 were used to block the solution for 2 h on the table concentrator. Subsequently, 1X TBST was used to fully rinse the solutions three times for 5 min. HOXA5 polyclonal antibody and anti-HOXA5 antibody were used at a dilution of 1:1,000 and incubated overnight at 4˚C. After fully rinsing the primary antibody the following day, goat anti-rabbit secondary antibody was added at a dilution of 1:1,000. Following incubation at room temperature for 1 h, the blot was developed with ECL light developing film in the dark using the Gel-Pro Analyzer software (Media Cybernetics, Rockville, MD, USA). The relative ratio of target protein was determined using HOXA5 protein bands of gray value and GAPDH protein bands of gray value. The gray-level ratio with HOXA5 and the internal reference gene *GAPDH*, the relative expression quantity of HOXA5 protein expressed in the experimental group was carried out. The experiment was repeated three times.

Short hairpin RNA (shRNA) design, screening and synthesis. For the Jurkat cell experiments, three specific sequences of HOXA5 siRNA were chemically designed and synthesized. To overcome the influence of siRNA, we designed a negative control sequence (siRNA-NC), which had no homology with any of the human genes. The three siRNA sequences targeting the *HOXA5* gene are shown in Table I. The sequences contained restriction sites for the *Bam*HI and *Hin*dIII enzymes. siRNA was synthesized by Adicon Co. (Jiangsu, China).

Cell culture and transfection. Jurkat cells were cultured in RPMI-1640 medium at 37˚C, with a 5% volume fraction of CO_2 and 30% saturated humidity. The medium was supplemented with 10% fetal calf serum, penicillin and streptomycin at a concentration of 100 IU/ml. The cells were grown in suspension, the medium was changed after 2-3 days, and the cells were passaged once. Experiments were conducted with cells in the logarithmic growth phase. To perform transfection, the cell concentration was adjusted to $3x10^7$/ml in the RPMI-1640 medium with no serum and no antibiotics. The cells were divided into group A, blank control group (plus an equal amount of cells and culture media only); group B, the negative control group (liposomal transfection with negative control siRNA); and group C, the experimental group (liposomal transfection with HOXA5 targeting siRNA). The siRNA concentration for each transfection was 135 ng/μl according to the Lipofectamine™ 2000 specification, mixed with serum-free medium without antibiotics. The mixed liquid was transfected into Jurkat cells. G418 (200 g/ml) was added to the screened cells after 24 h of transfection. Monoclonal cells were selected after screening for 4 weeks and G418 (200 g/ml) medium was used to expand the culture. The experiment was repeated three times.

Detection of HOXA5 mRNA expression levels in Jurkat cells using QF-PCR. Jurkat cells in the logarithmic phase at $3x10^5$ cells/well were transfected in the 6-well culture plate as the control group. The experimental and negative control groups were established using the early stably transfected cells seeded in a 6-well plate. The cells in each group were seeded in 2 wells and total RNA was extracted after 24 h. The conditions used to set up the PCR reaction and the calculation of the relative quantity of gene expression were described earlier. The gray-level ratio with HOXA5 and the internal reference gene *GAPDH* was used to indicate the relative expression of HOXA5 mRNA, prior to calculation of the experimental group HOXA5 mRNA inhibition rate. The HOXA5 mRNA inhibition rate was calculated as: [1- experimental group (HOXA5 mRNA relative

Table II. Expression of HOXA5 mRNA in bone marrow of each group.

Group	n	HOXA5 mRNA positive rate [cases (%)]	HOXA5 mRNA (mean ± SD)
Control group	20	7 (28)	0.47±0.08
ALL acute phase	25	16 (64)[a]	0.76±0.05[a]
ALL remission stage	25	10 (40)[b]	0.48±0.07[b]

[a]Compared with the control group, P<0.05; [b]compared with the control group, P>0.05. Expression of HOXA5 mRNA in the acute phase of ALL was significantly higher than that in the ALL remission stage and the control group. HOXA5, homeobox gene; ALL, acute lymphocytic leukemia.

expression level)/blank control group (HOXA5 mRNA relative expression level)] x100%. The experiment was repeated three times.

Detection of HOXA5 protein expression levels using western blot analysis. Jurkat cells in the logarithmic phase were vaccinated in a 6-well culture plate. The cell groups, the density of inoculation and the transfection steps were similar to those described earlier. After 24 h of transfection, the cells were washed with cold PBS twice, cell lysis was performed and the cell lysate was collected and stored at -80˚C. The conditions for the PCR reaction were performed as described earlier. The gray-level ratio with HOXA5 and the internal reference gene *GAPDH* were determined, the relative expression quantity of HOXA5 protein was expressed in the experimental group, and the calculation of HOXA5 protein inhibition rate was performed. The HOXA5 protein inhibition rate was calculated as: [1- experimental group (HOXA5 protein relative expression)/blank control group (HOXA5 protein relative expression)] x100%. The experiment was repeated three times.

Detection of cell cycle. The cells in each group containing 0.5% serum in RPMI-1640 medium were cultured for 48 h after cell synchronization. The cells were cultured in complete medium for 48 h and then seeded at 5×10^5/well in a 6-well culture plate in a final volume of 1 ml. The cells were washed twice with ice-cold PBS solution, followed with ice-cold 70% ethanol and then fixed at 4˚C for 2 h overnight. Subsequently, the cells were washed with PBS to remove the ethanol. Conventional PI staining was measured using flow cytometry and FACScan DNA analysis was performed to determine the content of DNA. The results were analyzed using MultiCycle software. Experiments were repeated three times.

Determination of the apoptotic rate using Annexin V-PE/7AAD. Jurkat cells in the logarithmic phase were seeded at 5×10^5 cells/well in a 6-well culture plate in a final volume of 1 ml. The cells were transfected with HOXA5-specific siRNA or the control siRNA. The cells were collected and washed with cold PBS twice. The cells were then resuspended in 50 μl of binding buffer, followed by 5 μl of 7-AAD. Staining was performed at a room temperature of 25˚C for 5-15 min in the dark. This was followed by the addition of 450 μl of binding buffer, 1 μl of Annexin V-PE, at room temperature for 5-15 min in the dark. The apoptotic rate was determined using flow cytometry within 1 h. Experiments were repeated three times.

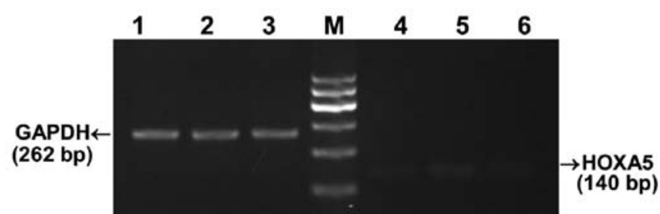

Figure 1. Detection of the relative expression of HOXA5 mRNA in bone marrow mononuclear cells using QF-PCR. Agarose gel electrophoresis images of HOXA5: GAPDH, 1-3; Lanes 1, control group; 2, acute lymphocytic leukemia (ALL) acute phase; 3, ALL remission stage; M, marker; 4, control group; 5, ALL acute phase; 6, ALL remission stage.

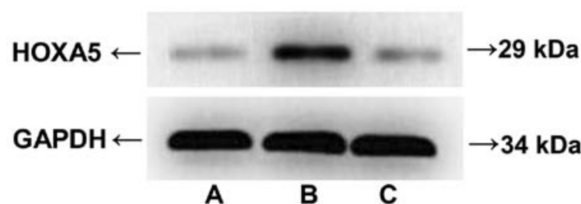

Figure 2. HOXA5 expression levels of bone marrow mononuclear cells. A, control group (ITP); B, acute lymphocytic leukemia (ALL) remission; C, control group. ALL acute phase compared with ALL remission and control group differences were statistically significant (P<0.05), while there was no significant difference in the ALL remission and control groups (P>0.05).

Statistical analysis. Data were analyzed using SPSS 13.0 statistical software (SPSS, Inc., Chicago, IL, USA). Measurement data were presented as means ± SD. ANOVA was used to compare groups, and multiple pairwise comparisons were made by the Dixon's q-test. P<0.05 was considered statistically significant.

Results

HOXA5 mRNA expression levels in bone marrow mononuclear cells. HOXA5 expression was observed in 7 of 20 control group patients with ITP (positive rate of 28%). HOXA5 was expressed in 16 of the 25 cases of children with ALL in the acute stage (positive rate of 64%). In the ALL remission group, HOXA5 was expressed in 10 of 25 cases (positive rate of 40%). The results of QF-PCR for the HOXA5 mRNA relative expression analysis in each group were: ALL acute phase $2^{-\Delta\Delta Ct}$ 0.76±0.05% (F=16.31, P<0.05); ALL remission $2^{-\Delta\Delta Ct}$ 0.48±0.07%; and control group (ITP) $2^{-\Delta\Delta Ct}$ 0.47±0.08% (Fig. 1 and Table II).

HOXA5 protein expression levels in bone marrow mononuclear cells. The results of the western blot analysis of HOXA5 protein expression levels in bone marrow mononuclear cells were: ALL acute phase (0.70±0.02), ALL remission (0.39±0.03), control group ITP (0.42±0.02) (Fig. 2).

Jurkat cells transfected with recombinant vector. Green fluorescent protein was expressed in Jurkat cells transfected with

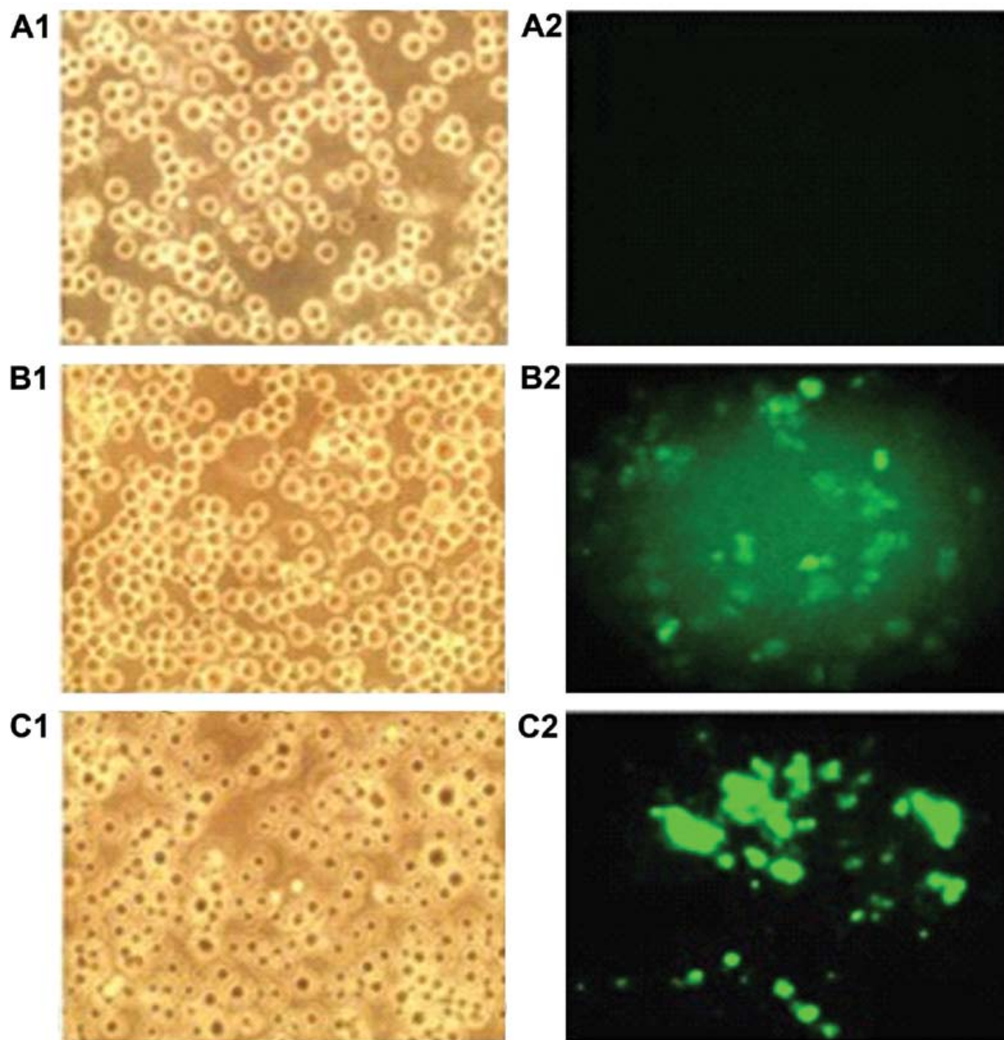

Figure 3. Jurkat cells were transfected into pRNAT-GFP-Neo-HOXA5C recombinant vector (magnification, x400). (A1, B1 and C1) Ordinary microscope; (A2, B2 and C2) fluorescent microscope; (A1 and A2) normal control group; (B1 and B2) negative control group (pRNAT-GFP-Neo-siRNA-NC); and (C1 and C2) experimental group (pRNAT-GFP-Neo-HOXA5C).

pRNAT-GFP-Neo-siHOXA5C recombinant vector (Fig. 3). The transfection efficiency was ~60%.

QF-PCR amplification and melting curve. The experimental amplification curve shown in Fig. 4A and B is an s-shaped curve that demonstrates line dynamics. Following QF-PCR reaction at a temperature of 65-65˚C, melting curve analysis was conducted and the results are shown in Fig. 4C and D. The homogeneous melting point of the *HOXA5* gene and GAPDH was 84-85˚C. The graphs have a single sharp absorption peak (Fig. 4C and D). No other product was observed and primer-dimer formation did not occur, indicating that the design of the primer had a good specificity (Fig. 4).

Effects of the recombinant vector on the expression of HOXA5 mRNA in Jurkat cells. QF-PCR results for the relative expression quantity of HOXA5 mRNA were: PRNAT-GFP-Neo-HOXA5A (1.01±0.03%), PRNAT-GFP-Neo-HOXA5B (0.87±0.02%), PRNAT-GFP-Neo-HOXA5C (0.39±0.01%), negative control group (1.34±0.06%), and blank control group (1.29±0.21%) (Fig. 5). The difference between the experimental, negative control and blank control groups was not statistically significant (P>0.05). The difference between the experimental, blank control and negative control groups was statistically significant (P<0.05), while there was no significant difference between the negative control and blank control groups (P>0.05). HOXA5 mRNA inhibition ratios were as follows: PRNAT-GFP-Neo-HOXA5A (24.62±2.34%), PRNAT-GFP-Neo-HOXA5B (35.07±3.21%) and PRNAT-GFP-Neo-HOXA5C (70.89±6.41%) (Fig. 5). It was evident that of the three selected siRNAs, PRNAT-GFP-Neo-HOXA5C had the best interfering interference effects (Fig. 5). Consequently, PRNAT-GFP-Neo-HOXA5C was selected to conduct the subsequent experiments.

Effects of siRNA on HOXA5 protein expression levels in Jurkat cells. Western blot analysis was used to examine the expression of HOXA5 protein. The results revealed that, siRNA targeting of HOXA5 in Jurkat cells after 24 h decreased the expression of HOXA5 protein, pRNAT-GFP-Neo-HOXA5A (0.64±0.15), pRNAT-GFP-Neo-HOXA5B (0.41±0.06), pRNAT-GFP-Neo-HOXA5C (0.17±0.05), negative control group (0.73±0.12), and the blank control group (0.73±0.13) (Fig. 6). The difference between the experimental, blank control and negative control

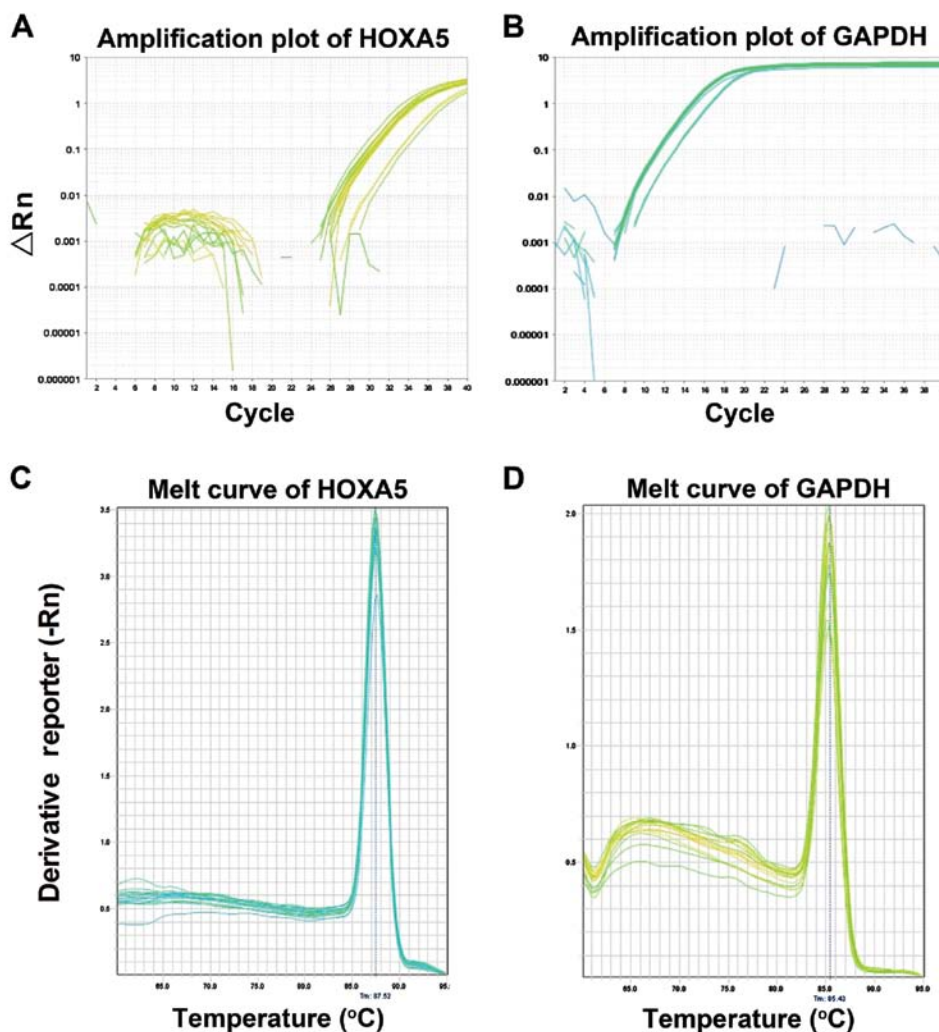

Figure 4. Amplification curve and melting curve of QF-PCR. (A) Amplification plot of homeobox (HOXA5); (B) amplification plot of GAPDH; (C) melting curve of HOXA5; and (D) melting curve of GAPDH.

groups was statistically significant ($P<0.05$), while there was no significant difference between the negative control and blank control groups ($P>0.05$). The relative HOXA5 protein expression in the experimental group was significantly lower than that in the negative control and blank control groups. The HOXA5 protein inhibitory rate was: pRNAT-GFP-Neo-HOXA5A (12.32±3.12%), pRNAT-GFP-Neo-HOXA5B (43.83±4.13%) and pRNAT-GFP-Neo-HOXA5C (76.71±5.16%) (Fig. 6). The expression levels of the pRNAT-GFP-Neo-HOXA5C vector of the HOXA5 protein had a significant inhibitory effect and were shown to be effective in the interference for subsequent experiments.

Morphological changes in each group as revealed by the Wright's stain method. When observed under light microscopy and compared with the negative control and blank control groups, the experimental nuclear mass ratio in the experimental group decreased, and rare nuclear fission and the apoptotic rate increased (Fig. 7).

Effects of siRNA on cell cycle of Jurkat cells. Following the transfection of Jurkat cells with HOXA5 siRNA for 48 h, the ratio of Jurkat cells in the G0/G1 phase significantly increased (56.70±6.4 vs. 38.55±6% and 38.69±2.2%), whereas the ratio of cells in the S phase significantly decreased (29±5.5 vs. 49.53±8.3% and 48.86±6%) (Fig. 8 and Table III). This difference was statistically significant ($P<0.05$). No statistically significant difference was identified in the distribution of cells

Table III. Distribution of the cell cycle 48 h after transfection (%, mean ± SD).

Group	G0/G1	S	G2/M
Control	38.69±2.2	48.86±6.0	11.70±2.8
Negative control	38.55±6.0	49.53±8.3	11.60±3.5
Experimental (pRNAT-GFP-Neo-HOXA5C)	56.70±6.4[a]	29.00±5.5[a]	14.29±1.5[b]

[a]Experimental group (pRNAT-GFP-Neo-HOXA5C) compared with the control and negative control groups, $P<0.05$; [b]experimental group (pRNAT-GFP-Neo-HOXA5C) compared with the empty vector and control groups, $P>0.05$.

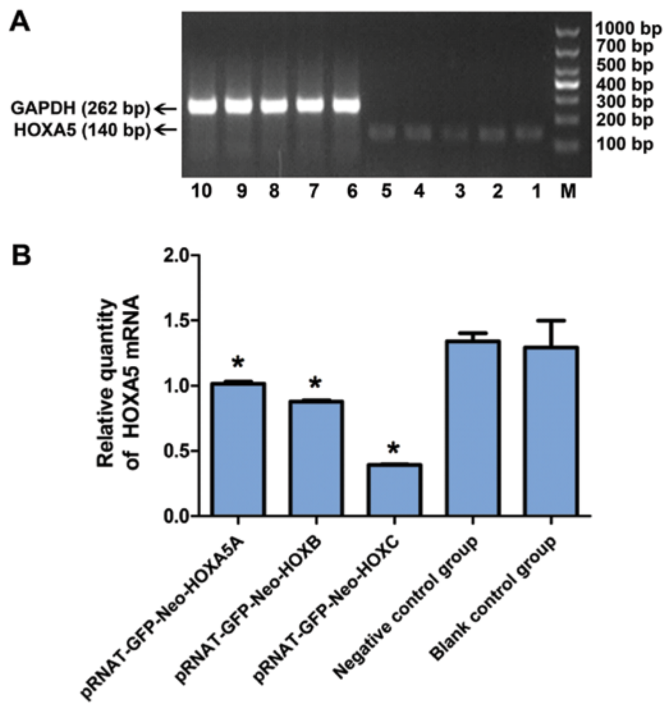

Figure 5. Inhibitory effect of siRNA on the expression of Jurkat in HOXA5 mRNA cells. (A) Agarose electrophoresis images of HOXA5; M, marker; Lanes 1, pRNAT-GFP-Neo-HOXA5A; 2, pRNAT-GFP-Neo-HOXA5B; 3, pRNAT-GFP-Neo -HOXA5C; 4, negative control group: pRNAT-GFP-Neo-siRNAnc; 5, blank control group; GAPDH; 6-8, experimental group; 9, negative control group; and 10, blank control group. (B) Statistical results of the relative quantity of HOXA5 mRNA in (A). Experimental group compared with the control and negative control groups, *P<0.05.

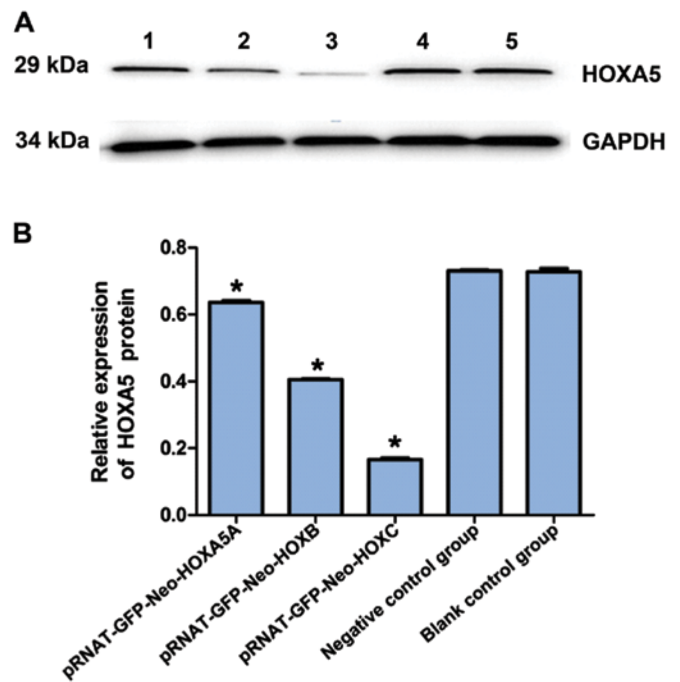

Figure 6. Detection of protein expression levels of HOXA5 using western blot analysis. (A) HOXA5 protein expression. Lanes 1, experimental group (pRNAT-GFP-Neo-Neo-HOXA5A); 2, experimental group (pRNAT-GFP-Neo-HOXA5B); 3, experimental group (pRNAT-GFP-Neo-HOXA5C); 4, negative control group: (pRNAT-GFP-Neo-siRNAnc); and 5, blank control group. (B) Statistical results of the protein relative expression of HOXA5 protein blot in (A). Experimental group compared with the control and negative control groups, *P<0.05.

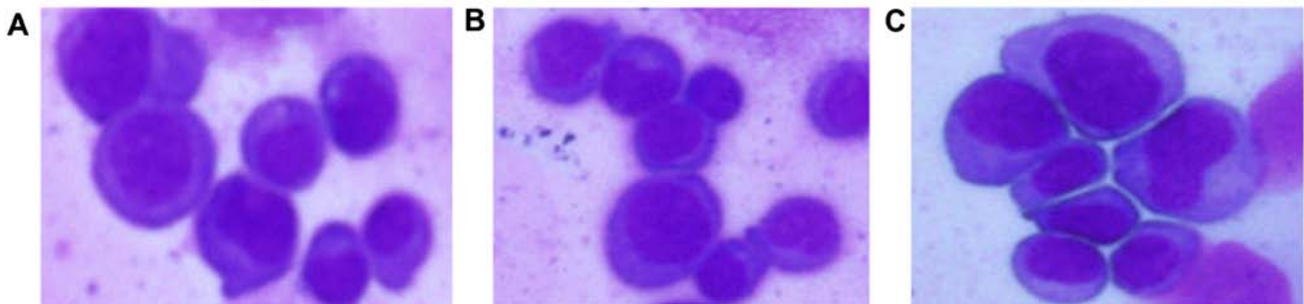

Figure 7. Morphological changes assessed by Wright's staining in each group (magnification, x400). (A) Control group; (B) negative control group: pRNAT-GFP-Neo-siRNAnc siRNA-NC; (C) experimental group: pRNAT-GFP-Neo-siHOXA5C. Blank control and negative control groups: evident nuclear atypia, large and deeply stained nuclei, less cytoplasm; nuclear cytoplasm ratio is reduced in the experimental group, the nucleus becomes smaller, lighter cytoplasmic ratio is decreased, nuclear cytoplasm becomes smaller and lighter in color.

in the control and negative control groups (Fig. 8A and B, respectively, and Table III).

Effects of recombinant vector on apoptosis in Jurkat cells. After staining with Annexin V-PE and 7-AAD, double labeling flow cytometry showed that the recombinant vector was transfected in Jurkat cells after 48 h. The apoptotic cell rate in the control, negative control and experimental groups was 13.98±1.05, 13.94±0.98 and 24.99±5.16%, respectively. The difference in the apoptotic rate between the experimental, control and negative control groups was statistically significant (P<0.05), whereas the difference between the negative control and control groups, was not statistically significant (P>0.05) (Fig. 9 and Table IV).

Discussion

Leukemia is a malignant hyperplastic disease of the hematopoietic system, which ranks first among tumor diseases in children (14). Homeobox genes encode transcription factors that are members of the Hox gene family and participate in hematopoietic stem/progenitor cell (HSPC) proliferation, differentiation and maturation (15). They are a type of regulatory gene that controls embryonic and cell differentiation and

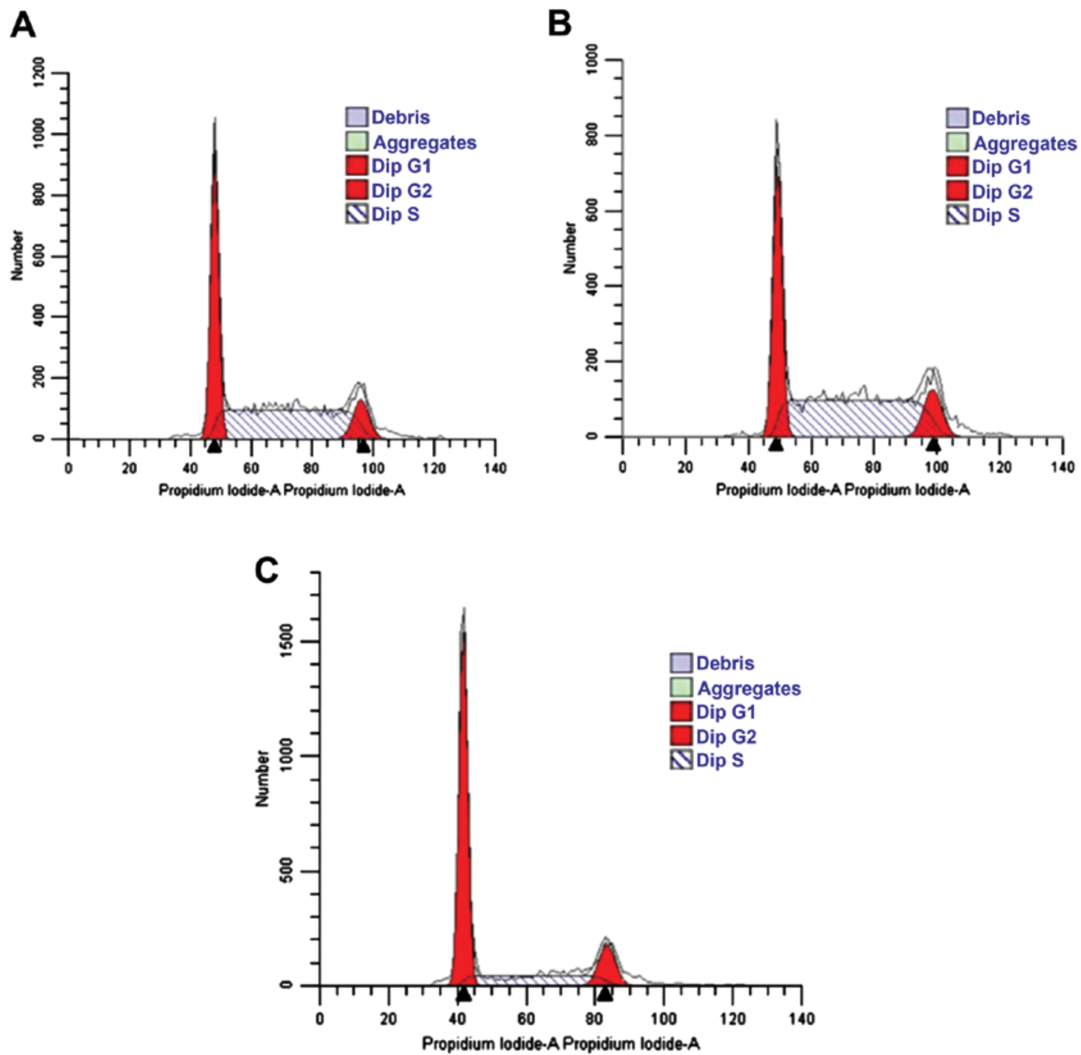

Figure 8. Effect of plasmid-mediated siRNA on Jurkat cell cycle. (A) Control group; (B) negative control group: pRNAT-GFP-Neo-siRNA-NC; (C) experimental group: pRNAT-GFP-Neo-HOXA5C. Distribution of cells in cell cycle after transfection for 48 h detected by flow cytometry (%, mean ± SD). Experimental group (pRNAT-GFP-Neo-siHOXA5C) compared with the negative control and blank control groups. G0/G1 phase cell proportion increased and their proportion in S-phase cells decreased. Thus, HOXA5 siRNA retarded the G0/G1 phase in cell proliferation, while S phase cycle arrest decreased significantly in the experimental group.

is closely associated with the incidence of leukemia (15-18). Normal mature tissues express *HOX* genes, which are silent, or expressed in the embryonic state during organization, leading to tumor development (19). HOX genes are important in the regulation of the hematopoietic proliferation and differentiation, as well as the abnormal expression of *HOX* genes, leading to the occurrence of leukemia (20). The head end (HOXA1-HOXA5) *HOX* gene, a positive marker of AML of mixed leukemia genes [mixed lineage leukemia (MLL)] is often characterized by abnormal protein expression (21). It has been suggested that MLL protein fusion is achieved by disordering the transcription of *HOX* genes (21). As a member of the family of *HOX* genes, HOXA5 is expressed in many organs and regulates gene expression, cell differentiation and the morphogenesis of body function (22). HOXA5 is a key regulator of the haematopoietic stem cell (HSC) cycle, and the inappropriate expression of HOXA5 in lineage-committed progenitor cells leads to aberrant erythropoiesis (22). Its structure and dysfunction is closely associated with the occurrence of leukemia. Kim *et al* (23) performed pyrosequencing to quantify the methylation level of the *HOXA5* gene in the bone marrow samples obtained from 50 patients with AML and 19 normal controls. The results showed that the survival rate of AML patients with stage 3A cancer correlated with HOXA5 methylation (23).

Table IV. Restructuring carrier effects on Jurkat cell apoptosis (%, mean ± SD).

Group	Flow rate of apoptosis (%)
Control	13.98±1.05
Negative control	13.94±0.98
Experimental (pRNAT-GFP-Neo-HOXA5C)	24.99±5.16[a]

[a]Experimental group (pRNAT-GFP-Neo-HOXA5) compared with the cells in the control and negative control groups, $P<0.05$.

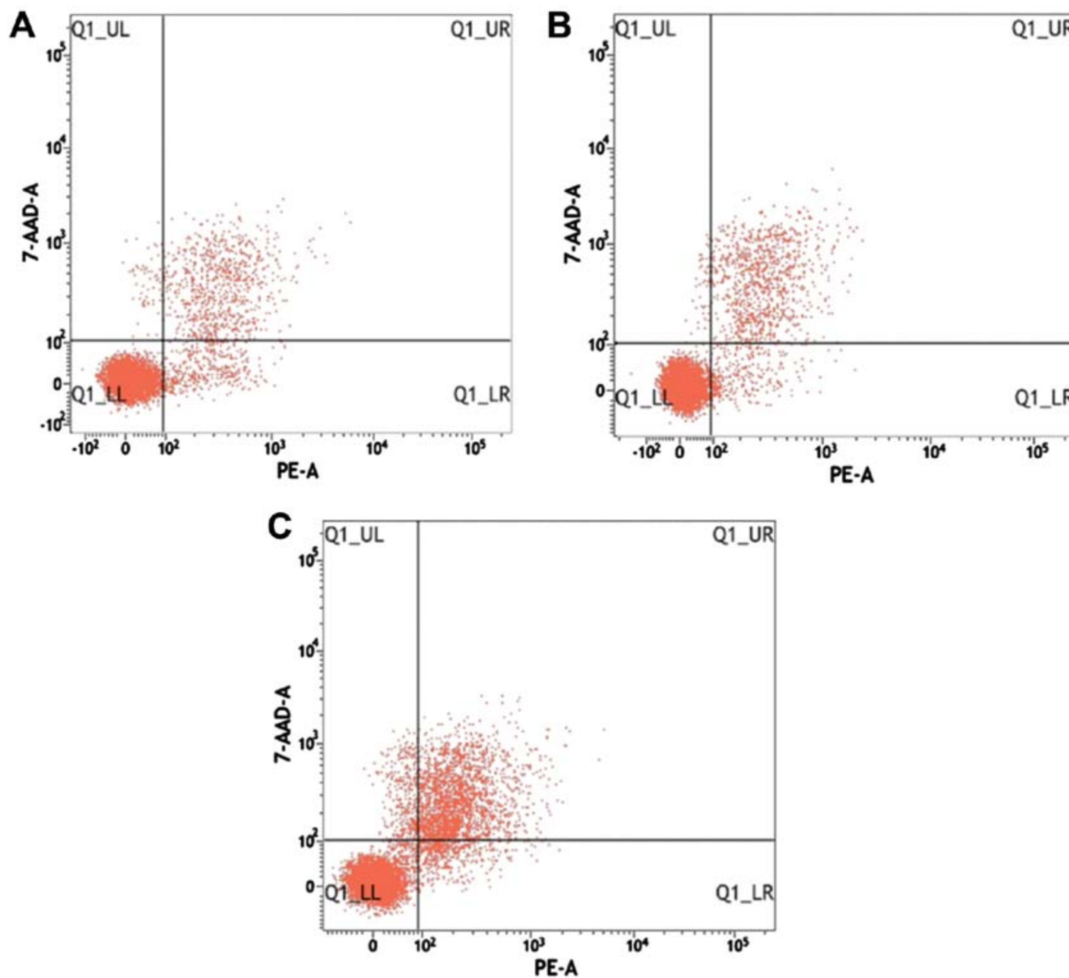

Figure 9. Effect of plasmid-mediated siRNA on Jurkat cells apoptosis. (A) Control group; (B) negative control group (pRNAT-GFP-Neo siRNAnc); (C) experimental group (pRNAT-GFP-Neo-HOXA5C). Apoptotic rate was significantly increased in the experimental group compared with the blank control and negative control groups.

Under certain conditions, changing the expression level of the *HOXA* gene may promote or inhibit the occurrence and development of a tumor (24). Although there are many methods of inhibiting gene expression, RNAi is the most commonly used. RNAi technology is a simple and effective alternative knockout genetic tool that has been developed in recent years (25,26). Moore *et al* (27) found that a significant expression of HOXA5 mediated by retrovirus causes myeloid differentiation but not erythroid differentiation of hematopoietic stem cells and progenitor cells. The findings of Liu *et al* (28) indicated that miR-196a is significantly upregulated in non-small cell lung cancer (NSCLC) tissues and regulates NSCLC cell proliferation, migration and invasion, partially via the downregulation of HOXA5. Thus, miR-196a is a potential therapeutic target for NSCLC intervention. The study of Wang *et al* (29) suggested that specific siRNA of CXCR4 effectively downregulates the expression of the *CXCR4* gene and induces cell cycle arrest and apoptosis of Jurkat cells, while inhibiting cell proliferation. Other studies have shown that Jurkat cells of human leukemia cell line is an ideal RNAi experimental cell model (30,31). Therefore, the application of RNAi technology to downregulate HOXA5 expression may inhibit the proliferation and apoptosis of leukemia cells. A study by Zhang *et al* (32) showed that shRNA targeting of silent *HOXA10* gene mediated by lentiviral vector, can effectively inhibit the proliferation and promote the apoptosis of U937 cells. Fan *et al* (33) study showed that RNAi technology combined with a small dose of Ara-C effectively inhibits the proliferation and induces the apoptosis of K562 cells.

The specific detection of QF-PCR and western blot analysis in the present study indicated that *HOXA5* gene was expressed at high levels in ALL patients. The expression of ALL mRNA (0.76±0.05%) and protein (0.70±0.020%) in the acute phase was significantly higher than that in the remission stage and control groups. The experimental group (pRNAT-GFP-Neo-siHOXA5C) affected by siRNA showed lower mRNA (0.39±0.01%) and protein (0.17±0.05%) levels compared to the negative control and blank control groups. The results showed that pRNAT-GFP-Neo-siRNAHOXA5C HOXA5 carrier effectively silences gene expression and inhibits Jurkat cell proliferation. The cell cycle detected through flow cytometry showed that, compared with the negative control and blank control groups, the proportion of G0/G1 cells increased and the proportion of S phase cells decreased. Annexin V-PE/7-AAD double staining is an ideal method for detection of the apoptotic rate (34). In the present study, the experimental group (pRNAT-GFP-Neo-siHOXA5C) under the influence of siRNA showed a flow apoptotic rate of 24.99±5.16, which was higher

as compared to that of the negative control group (13.94±0.98) and the blank control group (13.98±1.05). Following transfection, mRNA in the pRNAT-GFP-Neo siHOXA5C group was effectively reduced, and the apoptotic rate was significantly increased compared with the other groups. Another study has shown that the overexpression of HOXA5 inhibits apoptosis (34) by inhibiting its target genes. Flow cytometry showed that, in this group, siRNA carrier inhibited the ability of HOXA5 to promote Jurkat cell apoptotic rate, which was 24.99±5.16%. Compared with the negative control and blank control groups, the mRNA and protein expression of HOXA5 in Jurkat cells in the experimental group (pRNAT-GFP-Neo-siHOXA5C) was significantly reduced, the cell cycle was suppressed, and the apoptotic rate increased. The evidence showed that the construction of pRNAT-GFP-Neo-siHOXA5 in this experiment was successful.

The aforementioned results show that *HOXA5* gene is highly expressed in ALL and closely associated with the occurrence of ALL in children. Eukaryotic expression carrier targeting HOXA5 constructed in the present study can effectively reduce the expression of HOXA5 in Jurkat leukemia cells and inhibit its proliferative ability by silencing the *HOXA5* gene. Therefore, this eukaryotic expression carrier has the potential to become an effective gene therapy to treat leukemia.

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