# Genetic inhibition of vascular endothelial growth factor receptor-1 significantly inhibits the migration and proliferation of leukemia cells and increases their sensitivity to chemotherapy

BING XIU[1*], WENJUN ZHANG[1*], BINBIN HUANG[1], JINGDE CHEN[2], HUINA LU[1], JIANFEI FU[1], HONG XIONG[1] and AIBIN LIANG[1]

[1]Department of Hematology, Tongji Hospital of Tongji University, Putuo, Shanghai 200065; [2]Department of Oncology, Oriental Hospital of Tongji University, Pudong, Shanghai 200120, P.R. China

**Abstract.** Little is known about the role of vascular endothelial growth factor (VEGF) receptor-1 (VEGFR-1) in acute leukemia. In this study, using real-time PCR and ELISA, we found that VEGF and VEGFR-1 are highly expressed in U937 leukemia cells and primary leukemia cells (M4/M5 subtypes), which are associated with an increased migration rate and extramedullary disease. In order to elucidate the role of VEGFR-1 in acute leukemia, we used a lentivirus-mediated shRNA expression system to specifically inhibit VEGFR-1 expression in the U937 cell line. In addition, a series of *in vitro* experiments were conducted, including cell proliferation and migration assays and drug treatments. Our results showed that shRNA reduced the proliferation and migration of U937 cells. RNA interference targeting VEGFR-1 in combination with bevacizumab did not exert synergistic antitumor effects. However, shRNA enhanced the sensitivity of the U937 cells to cytarabine by decreasing the $IC_{50}$ of cytarabine, reducing the number of cells in the S phase and suppressing the expression of the survivin gene. Taken together, these results suggest that VEGFR-1 interference may serve as a novel antitumor therapeutic strategy for the treatment of leukemia.

## Introduction

In spite of continuing advances in the treatment of acute myeloid leukemia (AML), the 5-year event-free survival rate remains low, and the majority of patients succumb to the disease due to relapse and chemotherapy resistance. Therefore, the development of novel therapeutic strategies is urgently required. Experiments and clinical trials have demonstrated vascular endothelial growth factor (VEGF) and VEGF receptors (VEGFRs) are overexpressed in patients with acute or chronic leukemia, and are closely associated with the occurrence, development and prognosis of the disease (1-4). Therefore, the direct or indirect suppression of VEGF and its receptors are therefore promising novel therapeutic approaches to improve patient outcome.

VEGFR-1 is widely expressed in hematological malignancies, and the functional role of VEGFR-1 is complex and dependent on both the developmental stage and cell type (4). Although it has been reported that VEGFR-1 regulates the distribution of leukemia cells in the bone marrow of patients with acute lymphoblastic leukemia, and, in conjunction with VEGF and placental derived growth factor modulate the survival and exportation of leukemia cells (5), studies specifically defining the role of VEGFR-1 in AML are limited.

RNA interference (RNAi) is a novel strategy for the specific suppression of target gene expression and has been applied in the development of antiviral and antitumor drugs (6-9), making it possible to elucidate the role of VEGFR-1. In the present study, we used a lentivirus-mediated shRNA expression system to specifically inhibit VEGFR-1 expression in the U937 leukemia cell line. Our results showed that shRNA reduced the proliferation and migration of U937 cells. In addition, RNA interference targeting VEGFR-1 in combination with bevacizumab did not exert synergistic inhibitory effects on the U937 cells. However, the knockdown of VEGFR-1 by shRNA made the U937 cells more sensitive to cytarabine. These findings suggest that lentivirus-mediated RNA interference targeting VEGFR-1 specifically inhibits the expression of VEGFR-1, resulting in a series of changes in the biological characteristics of leukemia cells, and may be applied as a novel antitumor therapeutic strategy for the treatment of leukemia.

## Materials and methods

*Cell culture and primary leukemia cell lines.* The leukemia cell lines, HL-60, HEL, NB4, U937 and THP-1, and lentivirus

*Correspondence to:* Professor Aibin Liang, Department of Hematology, Tongji Hospital of Tongji University, 389 Xincun Road, Putuo, Shanghai 200065, P.R. China
E-mail: lab7182@tongji.edu.cn

*Contributed equally

packaging cell line (293T cells) were purchased from the Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, Shanghai, China. The 293T cells were grown in DMEM containing 10% fetal bovine serum (FBS) and the other cells were maintained in RPMI-1640 medium supplemented with 10% FBS. Cells were incubated at 37˚C in humidified 5% $CO_2$ in air and those in the logarithmic growth phase were used for further experiments. The diagnosis of primary leukemia in 100 patients was confirmed by bone marrow biopsy and flow cytometry. After obtaining informed consent, 5 ml of heparin anti-coagulated bone marrow were collected and mononuclear cells were separated with Ficoll, and were then cultured under regular conditions or stored at -80˚C for further use. In all samples, the proportion of leukemia cells was >80% and trypan blue excluding fraction was >98%. For the control group, peripheral venous blood was obtained from 15 healthy volunteer donors who also provided written informed consent before the samples were obtained.

*Reagents*. Lentiviral vector for RNA interference (Shanghai Innovation Biotechnology Co., Ltd., Shanghai, China), RPMI-1640 medium, DMEM (Gibco, Carlsbad, CA, USA), FBS (HyClone, Logan, UT, USA), TRIzol for RNA extraction (Invitrogen, Carlsbad, CA, USA), the reverse transcription kit (Promega, Madison, WI, USA), the SYBR-Green Real-time PCR kit (Takara, Shiga, Japan), the cell counting kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan), the Annexin V-PE/7-AAD apoptosis detection kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China), cell cycle staining solution (MultiSciences Biotech Co., Ltd., Shanghai, China), VEGFR-1 monoclonal antibody (Abcam, Cambridge, MA, USA), secondary antibodies (R&D Systems, Minneapolis, MN, USA), cytarabine (Pharmacia, Kalamazoo, MI, USA) and bevacizumab (Genentech, South San Francisco, CA, USA) were used in the present study.

*RT-PCR of VEGF, VEGFR-1 and VEGFR-2 in leukemia cell lines*. The leukemia cells were first analyzed for VEGF and VEGFR expression by RT-PCR. Total cellular RNA was extracted, and cDNA was synthesized following conventional protocols. PCR was performed using a PCR thermal cycler (GeneAmp PCR System 9700; Life Technologies, Carslbad, CA, USA). The primers used were synthesized as follows: VEGF forward, 5'-AGGGAAGAGGAGGAGATGAG-3' and reverse, 5'-GCTGGGTTTGTCGGTGTT-3' (anticipated size, 148 bp); VEGFR-1 forward, 5'-AGACTAGATAGCGTCACCAGC-3' and reverse, 5'-GAAACCGTCAGAATCCTCC-3' (anticipated size, 102 bp); VEGFR-2 forward, 5'-CCAATAATCAGAGTGGCAGTG-3' and reverse, 5'-CATAGACATAAATGACCGAGGC-3' (anticipated size, 162 bp). The cycling conditions for PCR were 38 cycles of denaturation (94˚C for 20 sec), annealing (58 or 60˚C for 20 sec) and extension (72˚C for 30 sec). A pre-heating step at 94˚C for 5 min and a final extension step consisting of 7 min at 72˚C were also carried out. The primer sequences for β-actin were: forward, 5'-CCTGTACGCCAACACAGTGC-3' and reverse, 5'-ATACTCCTGCTTGCTGATCC-3'. The temperature for annealing was 56˚C and a total of 30 cycles were conducted. The other conditions were as mentioned above. The experiment was performed 3 times.

*Real-time PCR of VEGF, VEGFR-1 and VEGFR-2*. Fluorescence quantitative PCR instrument (LightCycler 1.5, Roche, Basel, Switzerland) and the SYBR-Green Master Mix kit were used for the detection of the expression of target genes and β-actin was used as an internal reference. The primers of VEGF and VEGFRs were as mentioned above. The temperature for annealing was 58 or 60˚C and a total of 38 cycles were conducted. β-actin served as an internal reference and the $2^{-\Delta\Delta CT}$ method was employed to determine the relative expression of the target genes and the experiment was repeated 3 times.

*Western blot analysis for VEGFR-1*. Cells were harvested and incubated with lysis buffer for protein extraction followed by the determination of protein concentration. The proteins were separated by SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were incubated with primary antibodies (VEGFR-1, 1:500; β-actin/GAPDH, 1:5,000) followed by secondary antibodies conjugated to horseradish peroxidase. Color development was performed with the ECL kit. The experiment was performed 3 times.

*Detection of VEGF content by ELISA*. The leukemia cell lines ($1x10^6$) were grown in 1 ml of serum-free RPMI-1640 medium at 37˚C in humidified 5% $CO_2$ in air for 48 h. Following centrifugation, the supernatant was obtained and the VEGF content was determined by ELISA. Each experiment was performed in duplicate according to the manufacturer's instructions. The experiment was repeated 3 times.

*Construction of lentiviral vector carrying VEGFR-1 shRNA, transfection of U937 cells and determination of the efficiency of RNA interference*. According to the principles for the design of the RNA interference sequence, 3 sequences targeting the VEGFR-1 gene (NM_002019) were designed: -sh1: 704-723 bp, 5'-CACACCACGCCCAGTCAAA-3'; -sh2: 3221-3240 bp, 5'-AATCTACAGCACCAAGAGC-3'; and -sh3: 2639-2658 bp, 5'-AATCTTGACCCACATTGGC-3'. The shRNAs targeting the VEGFR-1 gene were then connected to the lentiviral vector, pRNAT-U6.2/Lenti, yielding recombinant vectors. Following PCR and sequencing, the recombinant vectors together with lentiviral packaging plasmids (at a ratio of 1:1:1) was transfected into the 293T cells using Lipofectamine 2000. The viral solution was prepared and named sh-VEGFR-1 KD. In addition, the siRNA sequence unrelated to VEGFR-1 (5'-TTCTCCGAAC GTGTCACGT-3') was synthesized and served as the negative control (shRNA-NC). Lentiviral particles were conjugated to green fluorescent protein (GFP). The serial dilution method and flow cytometry were performed to determine the lentivirus titer. The lentivirus in the sh-VEGFR-1 KD and shRNA-NC group was used to transfect the U937 cells at the appropriate titer and named sh-KD-U937 (KD group) and sh-NC-U937 (NC group), respectively. In addition, untreated U937 cells were used as the controls (CON group). The proportion of GFP-positive cells was observed under a fluorescence microscope 72 h later. When the proportion was >90%, the cells were used for the following experiments. These cells were harvested at 120 h after being transfected, real-time PCR and western blot analysis were performed to determine the efficiency of RNA interference at the mRNA level and protein level. The experiment was repeated 3 times.

Table I. Expressions of VEGF and VEGFRs in 5 AML cell lines.

| Cell line | Cell stage | VEGF | VEGFR-1 | VEGFR-2 | VEGF (ng/ml) |
|---|---|---|---|---|---|
| HL-60 | Promyelocytic leukemia | + | + | - | 0.81±0.13 |
| NB4 | Promyelocytic leukemia | + | + | - | 0.71±0.09 |
| U937 | Monocytic cells | + | + | - | 1.77±0.07[a] |
| THP-1 | Monocytic cells | + | + | - | 1.59±0.12[a] |
| HEL | Erythroleukemia | + | + | + | 0.94±0.11 |

[a]Compared with the other groups, P<0.05.

*Growth curve assay*. Twenty-four hours after transfection, 3 groups of cells ($5\times10^3$) in the logarithmic growth phase were seeded in 96-well plates (100 $\mu$l/well) and each experiment was performed in sextuplicate followed by incubation for 7 days. The medium was refreshed every 48 h. The cells were then incubated with CCK-8 solution (5 mg/ml, 10 $\mu$l/well) for 4 h followed by blending. The absorbance (A) was determined at 450 nm with a microplate reader, which represents the cell proliferation rate. The experiment was repeated 3 times.

*Transwell migration assay*. Migration assays were performed in Transwell plates (Costar, Cambridge, MA, USA) of 6.5 mm in diameter, with 5-$\mu$m pore filters, as previously described by Fragoso *et al* (5). In brief, cell lines or different groups of U937 cells ($1\times10^6$ cells/ml) were placed in serum-free medium with or without bevacizumab (1 $\mu$g/ml) for 1 h. Cell aliquots (100 $\mu$l) were subsequently added to the upper compartments, and 600-$\mu$l of serum-free medium with or without VEGF (50 ng/ml) were added to the lower compartments. The transwell plates were incubated at 37°C, 5% $CO_2$ for 4 h. Cell migration was determined after 4 h by counting the number of migrated cells in 6 high-power fields (x400 magnification) under an optical microscope.

*Drug treatment*. Seventy-two hours after transfection, 3 groups of U937 cells in the logarithmic growth phase were harvested and seeded in 96-well plates at a density of $1\times10^5$ cells/ml (100 $\mu$l/well). These cells were then treated with cytarabine or bevacizumab (10 $\mu$l) at various concentrations in RPMI-1640 medium containing 10% FBS. For each concentration, the experiment was performed in sextuplicate. Additionally, a normal control and blank group were also prepared. After 48 h of incubation, cells were incubated with CCK-8 solution (10 $\mu$l) for 4 h followed by blending (5 min). The absorbance (A) was determined at 450 nm with a microplate reader and the cell proliferation and inhibition rate were calculated as follows: proliferation rate/inhibition rate (%) = [($A_{control}$ - $A_{experiment}$)/$A_{control}$ - $A_{blank}$)] x100%. The experiment was repeated 3 times.

*Flow cytometry for apoptosis analysis*. After 48 h of treatment with different drugs, 3 groups of U937 cells were collected followed by washing with PBS twice, then resuspended in PBS (1X) at a density of $1\times10^6$ cells/ml. Subsequently, 1 $\mu$l of Annexin V-PE and 5 $\mu$l of 7-AAD were added followed by blending. A blank group, Annexin V-PE group and 7-AAD group were also prepared. Cells were incubated for 15 min followed by flow cytometry for the apoptotic rate.

*Flow cytometry for cell cycle analysis*. Three groups of U937 cells were seeded in 24-well plates at a density of $1\times10^6$ cells/ml and maintained in medium containing different drugs for 48 h. Cells ($5\times10^5$) were then collected and washed with PBS (4°C) twice. The cells were then resuspended in 1 ml of PBS and added to 4 ml of cold absolute ethanol, which was blended and fixed at -20°C for 15 min followed by centrifugation. The supernatant was removed and 5 ml of PBS were added to the sediment followed by incubation for 15 min. Centrifugation was then performed and the supernatant was removed. The sediment was stained with DNA staining solution [propidium iodide (PI), 50 mg/l and RNase A, 10 mg/ml] in the dark for 30 min. In addition, a blank group and PI group were also prepared. Flow cytometry was performed within 1 h.

*Statistical analysis*. Statistical analysis was performed using SPSS 14.0 statistical software, and the t-test was used for comparisons between two means. The $\chi^2$ test was conducted for comparisons between apoptotic rates. A value of P<0.05 was considered to indicate a statistically significant difference.

## Results

*Expression of VEGF, VEGFR-1 and VEGFR-2 in acute leukemia cell lines and primary leukemia samples*. The expression of VEGF, VEGFR-1 and VEGFR-2 was detected by RT-PCR in 5 AML cell lines. As shown in Table I, VEGF and VEGFR-1 were expressed in all 5 AML cell lines, whereas only HEL cells expressed VEGFR-2. We also detected the VEGF protein levels in the cell supernatants by ELISA (Table I). The U937 cell line had the highest VEGF protein level and the THP-1 cell line the second highest (P<0.05 for both). Furthermore, quantitative PCR and western blot analysis were used to detect VEGFR-1 expression in the 5 AML cell lines. As shown in Fig. 1A and B, the expression of VEGFR-1 was the highest in the U937 cell line and the lowest in the NB4 cell line.

In order to determine whether the migratory behavior of the leukemia cells is associated with the high expression of VEGF/VEGFR-1, VEGF-induced cell migration was performed in transwell migration assays with the 5 AML cell lines. As shown in Fig. 1C, the U937 cells showed the most potent chemotactic response to VEGF (with a 2.35-fold

Table II. VEGF and VEGFR mRNA expression in different groups of patients with acute myeloid leukemia.

| Group | VEGF mRNA | VEGFR-1 mRNA | VEGFR-2 mRNA |
|---|---|---|---|
| Extramedullary infiltration | | | |
| Yes | $1.325\pm0.23^a$ | $0.987\pm0.16^b$ | $0.623\pm0.22$ |
| No | $0.976\pm0.25$ | $0.625\pm0.18$ | $0.60\pm0.13$ |
| FAB subtype | | | |
| M4 + M5 | $1.225\pm0.09^c$ | $1.105\pm0.16^d$ | $0.645\pm0.27$ |
| Favorable FAB | $0.876\pm0.25$ | $0.587\pm0.13$ | $0.512\pm0.26$ |

[a,b]Compared with non-extramedullary infiltration group, P<0.05. [c,d]Compared with favorable FAB group, P<0.05. FAB, French-American-British classification system of acute leukemia.
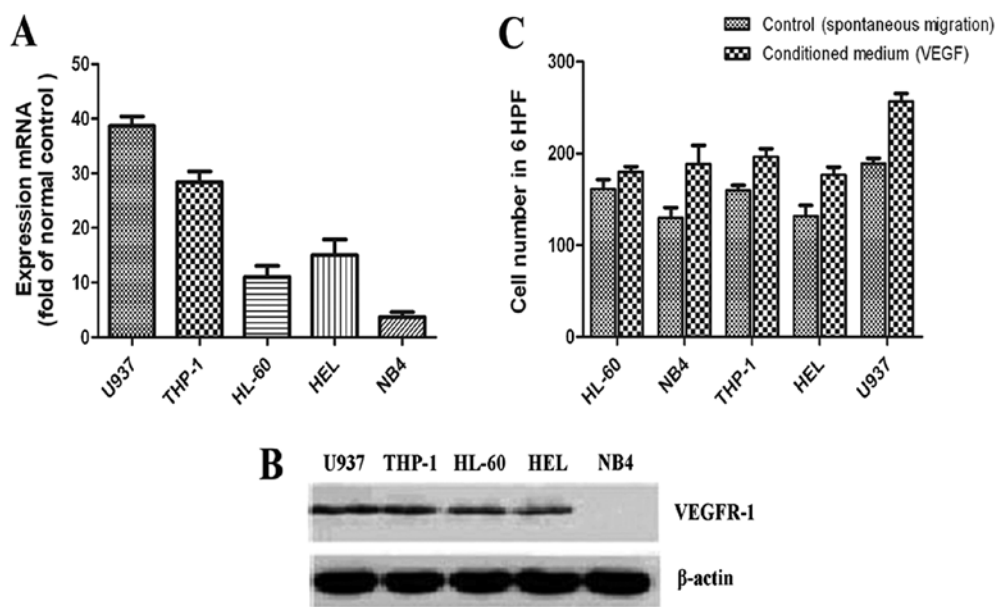


Figure 1. VEGFR-1 (FLT-1) expression level in the different acute myeloid leukemia. (AML) cell lines used in our study. (A) Relative expression of the VEGFR-1 gene in AML cell lines; (B) expression of VEGFR-1 protein in AML cell lines; (C) migration assay of the 5 AML cell lines. HPF, high power field.

ratio of VEGF-migrated cells/control; P<0.05), the THP-1 cell line showed the second most potent chemotactic response to VEGF (with a 1.75-fold ratio of VEGF-migrated cells/control; P<0.05), with the remaining 3 cell lines showing comparable migration capacity.

As regards the primary leukemia samples, we detected the mRNA expression of VEGF, VEGFR-1 and VEGFR-2 in 100 patients with AML. VEGF was expressed in 92% of the AML samples, and VEGFR-1 and VEGFR-2 were expressed in 87 and 37% of the samples, respectively. The patients were divided into 2 groups according to the French-American-British (FAB) classification system of acute leukemia (Table II). The quantification of VEGF and VEGFR-1 mRNA levels demonstrated significantly higher levels in the M4/M5 vs. M1, M2 and M3 FAB subtypes (P<0.05 for both). In addition, the significantly higher expression of VEGF and VEGFR-1 mRNA was observed in patients with extramedullary infiltration vs. those with no extramedullary infiltration, whereas VEGFR-2 levels did not significantly differ between the 2 groups.

Given the high expression level of VEGF and VEGFR-1 in the U937 cells and the M4 and M5 subtypes of AML samples characteristic of a higher migration rate or extramedullary infiltration, we hypothesized that VEGF-VEGFR-1 may play significant roles in the regulation of leukemia cell migration and other biological characteristics. In the following experiments, the U937 cells were used as target cells, and lentivirus-mediated RNA interference was performed to suppress the VEGFR-1 expression in the U937 cell line.

*Lentivirus-mediated RNA interference targeting VEGFR-1 effectively inhibits the VEGFR-1 expression in U937 cells.* The shRNA-NC and sh-VEGFR-1 KD lentiviral vectors were used to transfect the U937 cells at a multiplicity of infection (MOI) of 10. Seventy-two hours after transfection, the transfection efficiency of the U937 cells was >90%, as observed under an inverted fluorescence microscope. One hundred and twenty hours after transfection, we collected the cells and used quantitative PCR to detect VEGFR-1 mRNA expression.
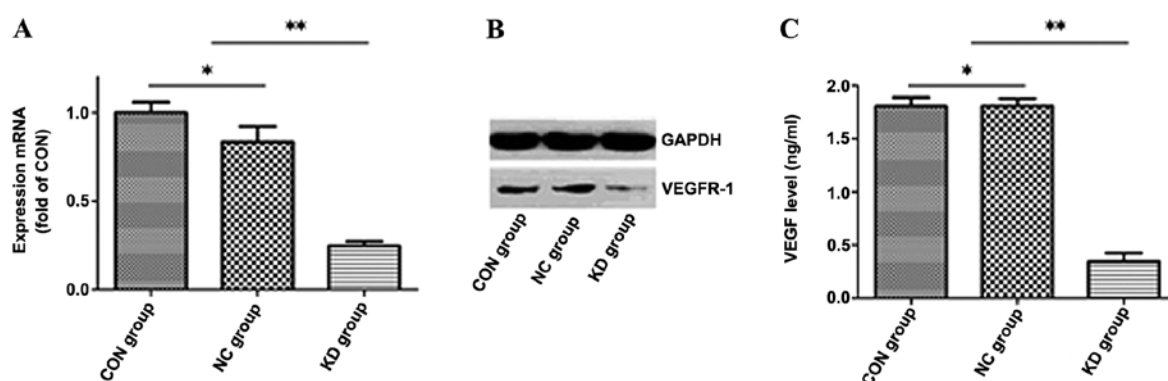
Figure 2. Construction of lentiviral vector carrying VEGFR-1 shRNA, transfection of U937 cells and determination of the efficiency of RNA interference. (A) The mRNA expression of VEGFR-1 in the KD group was suppressed by 75.98 and 73.53% when compared with CON and NC group (P<0.05). (B) The expression of VEGFR-1 protein was decreased by 94.14% (P<0.05) when compared with the CON group. No significant difference in the expression of VEGFR-1 protein was observed between the CON and NC group. (C) There was no significant difference in VEGF content in the U937 cell supernatants between the CON and NC group, which was 1.69±0.17 and 1.65±0.04 ng/ml, respectively, while that in the U937 cell supernatants in the KD group decreased dramatically by >75% (P<0.05) when compared to the U937 cell supernatants in the CON and NC group, which was 0.41±0.06 ng/ml. *P>0.05, indicates that there is no statistically significant difference between the groups. **P<0.05, indicates that there is a statistically significant difference between the groups.
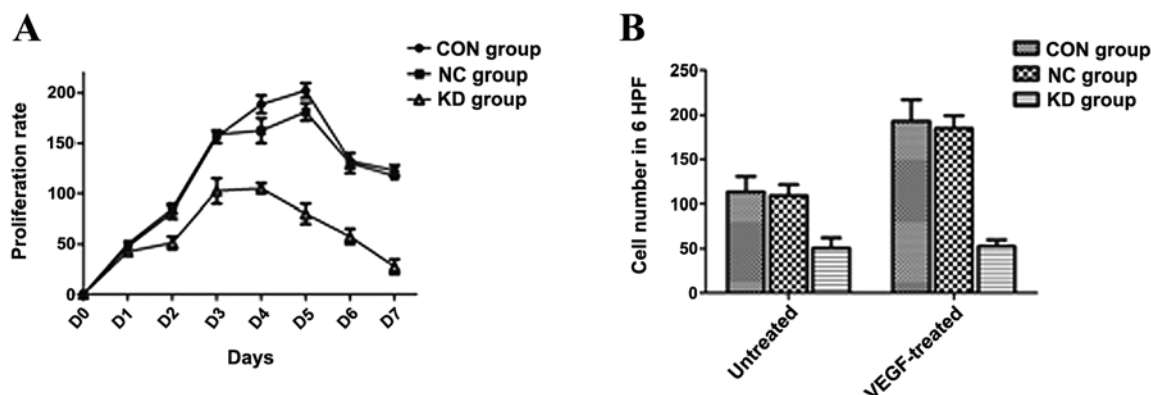


Figure 3. Changes in U937 cell characteristics before and after the suppression of VEGFR-1 with lentivirus-mediated shRNA. (A) Growth curves of U937 cells; (B) migration assay. Before treatment, no significant difference in the number of migrated cells was observed between the NC and CON group. However, the number of migrated cells in the KD group was markedly lower than that in the CON group (~49% of that in the NC group, P<0.05). Following treatment with VEGF, the number of migrated cells in the NC and CON group was significantly increased by 75 and 76%, respectively (P<0.05 vs. before treatment). No significant changes in the number of migrated cells were observed in the KD group (by 19%, P>0.05 vs. before treatment). The number of migrated cells in the NC and CON group was dramatically increased when compared with the KD group (P<0.05). HPF, high power field.

VEGFR-1 mRNA expression decreased by 73.53±0.026% in the KD group when compared with the NC group (Fig. 2A). Western blot analysis showed that the VEGFR-1 protein expression in the KD group decreased by 94.14% (P<0.05) when compared with the NC group (Fig. 2B). No significant difference in the mRNA and protein expression of VEGFR-1 was observed between the CON and NC group.

Furthermore, we performed ELISA to demonstrate that there was no significant difference in VEGF production in the U937 cell supernatants in the CON (1.69±0.17 ng/ml) and NC group (1.65±0.04 ng/ml), while VEGF production in the U937 cell supernatants in the KD group (0.41±0.06 ng/ml) decreased dramatically by >75% (P<0.05) when compared to the CON and NC group (Fig. 2C). These results indicated that lentivirus-mediated RNA interference suppressed the auto-crine loop of VEGF/VEGFR-1 in the transfected U937 cells.

These results demonstrate the feasibility of suppressing VEGFR-1 expression by lentivirus-mediated RNA interference, which inhibits the mRNA and protein expression of VEGFR-1 and blocks the VEGF/VEGFR-1 autocrine loop in U937 cells.

*Lentivirus-mediated RNA interference targeting VEGFR-1 suppresses the proliferation and migration of U937 cells.* To determine the effects of VEGFR-1 gene silencing on the biological characteristics of U937 cells, a series of *in vitro* experiments were conducted, including cell proliferation and migration assays, cell cycle analysis and determination of the apoptotic rate.

Cells from 3 different groups were grown for 7 consecutive days and the growth curve was delineated according to the proliferation inhibition rate. As shown in Fig. 3A, the proliferation rate of the U937 cells in the KD group was markedly slower than that of the cells in the NC and CON group after 3 days of culture (P<0.05), and this tendency continued for at least 7 days.

Seventy two hours after transfection, 3 groups of U937 cells were seeded in transwell plates for cell migration assay. As shown in Fig. 3B, before drug treatment, the number of migrated cells in the KD group was markedly lower than that in the NC and CON group (with a 2.5-fold ratio of VEGF-migrated cells/ control; P<0.05). Following treatment with VEGF, the number
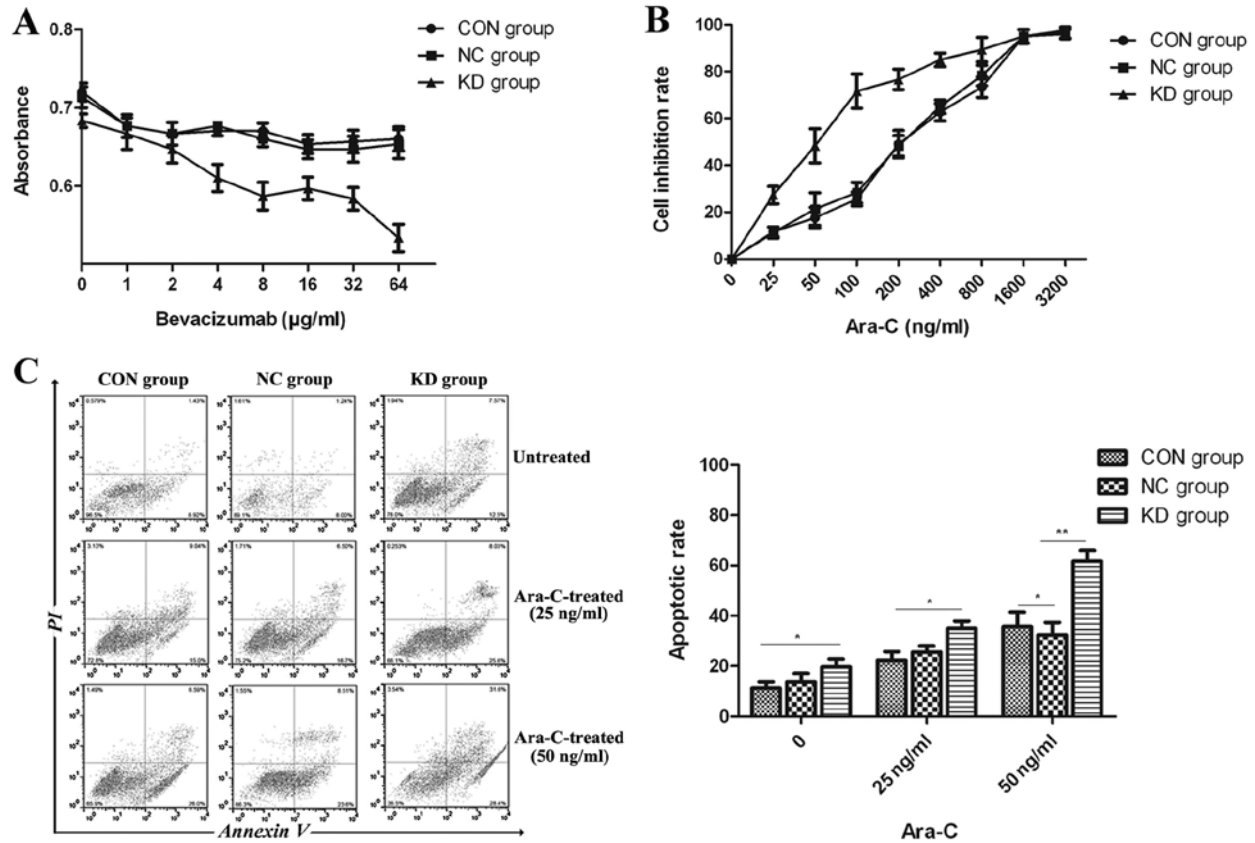
Figure 4. VEGFR-1 gene silencing in combination with drugs. (A) No difference in the cell proliferation rate was observed among the 3 groups of cells. (P>0.05). (B) Effects of cytarabine treatment on the growth of U937 cells. (C) Apoptotic rate after 48 h of treatment with cytarabine. When the cytarabine concentration was 25 ng/ml, the apoptotic rate in the KD, CON and NC group was 28.20, 16.80 and 19.90%, respectively.*P>0.05, indicates that there is no statistically significant difference between the groups. **P<0.05, indicates that there is a statistically significant difference between the groups. The apoptotic rate increased with the increase in the cytarabine concentration. Ara-C, cytarabine.

of migrated cells in the NC and CON group was markedly increased, while this phemomenon was not observed in the KD group.

The apoptotic rate of the U937 cells in the KD group was slightly higher than that in the CON and NC group (Fig. 4C). No significant difference in cell cycle distribution was observed among the 3 groups of U937 cells by cell cycle analysis prior to drug treatment (Fig. 5).

Following treatment with VEGF (Fig. 5), the proportion of cells in the S + $G_2$ phase and $G_1$ phase increased and decreased, respectively, in the CON and NC group. However, in the KD group, no marked changes in the proportion of cells in the S + $G_2$ phase or $G_1$ phase were observed following treatment with VEGF. These results demonstrate that VEGF enhances the colony-forming ability of the U937 cells by increasing the proportion of cells in the S + $G_2$ phase. However, after VEGFR-1 gene silencing, these effects conferred by VEGF were blocked, accompanied by a suppressed colony-forming ability. These findings suggest that VEGFR-1 plays a pivotal role in the proliferation of leukemia cells.

*VEGFR-1 gene silencing in combination with cytarabine results in increased inhibitory effects on proliferation and a higher apoptotic rate of U937 cells*. As demonstrated above, VEGFR-1 gene silencing profoundly inhibits the *in vitro*

proliferation and migration of the U937 cells, but does not directly increase apoptotic rate of the U937 cells. In the following experiments, VEGFR-1 gene silencing in combination with bevacizumab or cytarabine were used to explore their synergistic effects on the inhibition of cell proliferation and apoptotic effects. As shown in Fig. 4A, bevacizumab at various concentrations did not profoundly affect the proliferation of U937 cells in the CON, NC and KD group. Flow cytometry also showed that the apoptotic rate in the 3 groups of U937 cells was not affected by bevacizumab (data not shown). These findings showed that *in vitro* VEGFR-1 gene silencing in combination with bevacizumab did not confer suppressive effects on the proliferation and did not increase the apoptosis of U937 cells. Following treatment with various concentrations of cytarabine, a higher number of shrunken and ruptured cells was observed in the KD group. As indicated by the growth curve (Fig. 4B), the inhibition rate increased with the increase in the cytarabine concentration in each group. When the cytarabine concentration reached ≥25 ng/ml, the inhibition rate in the KD group dramatically increased compared to the CON and NC group (P<0.05). The $IC_{50}$ of cytarabine in the U937 cells in the KD, CON and NC group was 50, 200 and 200 ng/ml, respectively. These results revealed that the sensitivity of U937 cells to cytarabine increased by VEGFR-1 gene silencing.
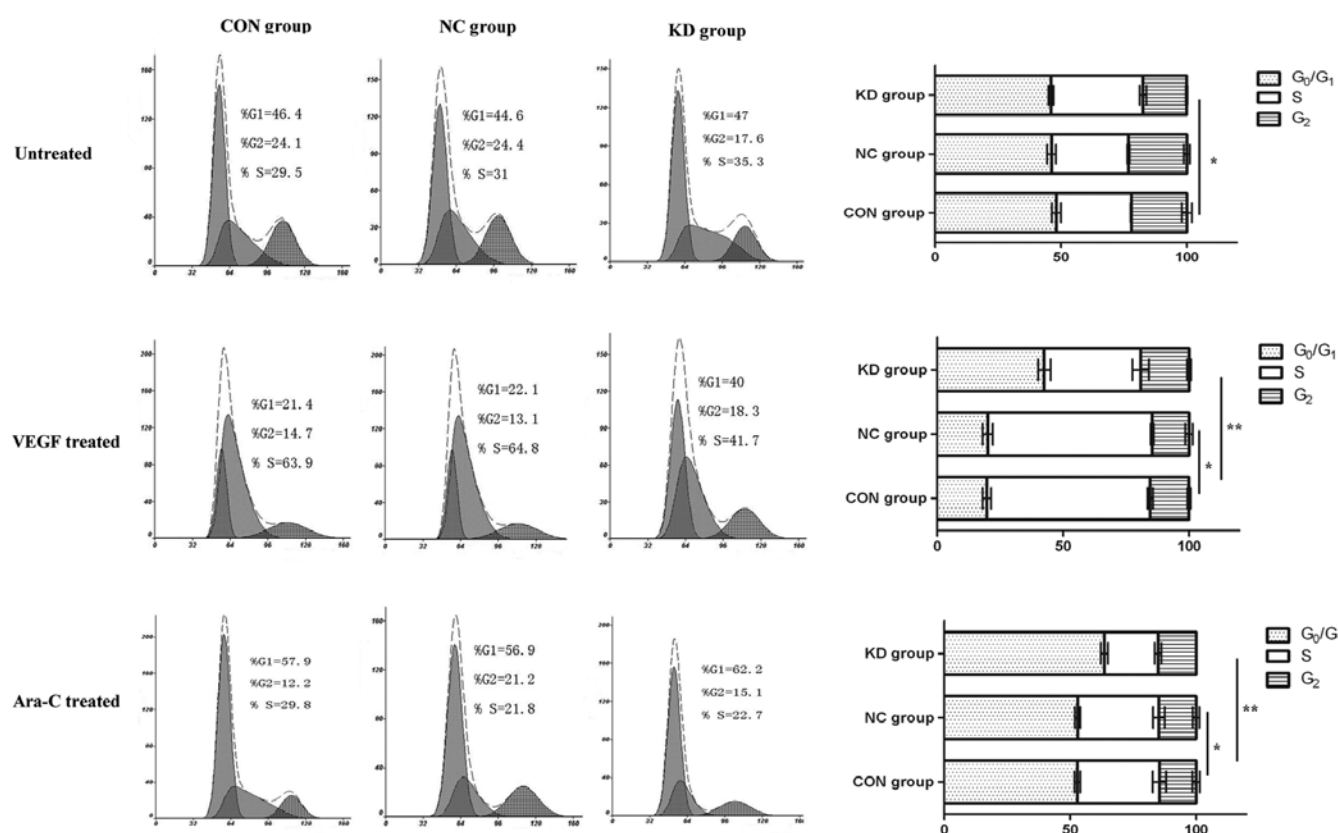
Figure 5. Changes in the cell cycle distribution of U937 cells in the different groups. Following treatment with VEGF, the proportion of cells in the S + G$_2$ phase in the CON and NC group, increased from 53.6 to 78.6% and from 55.4 to 77.9%, respectively, and that in the G$_1$ phase decreased from 46.4 to 21.4% and from 44.6 to 22.1%, respectively. However, in the KD group, the proportion of cells in the S + G$_2$ phase increased from 52.9 to 60% and that in the G$_1$ phase decreased from 47 to 40%. Flow cytometry analysis showed that in the KD group, there were not marked changes in the number of cells in the S + G$_2$ phase, and the G$_1$ phase following treatment with VEGF. Ara-C, cytarabine.$^*$P>0.05, indicates that there is no statistically significant difference between the groups. $^{**}$P<0.05, indicates that there is a statistically significant difference between the groups.
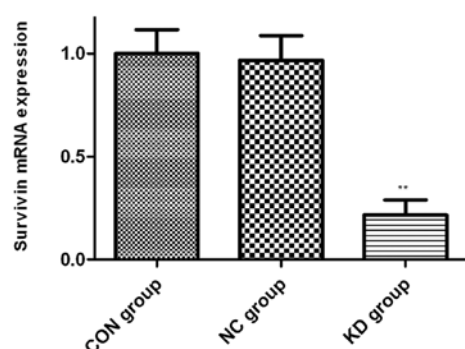


Figure 6. mRNA expression of survivin of U937 cells following treatment with cytarabine (50 ng/ml). $^{**}$Compared with CON and NC group, P<0.05.

The apoptotic rate in the different groups was detected with a flow cytometer following treatment with cytarabine. As shown in Fig. 4C, the apoptotic rate in the KD group was higher than that in the CON and NC group. When the cytarabine concentration reached 25 or 50 ng/ml, a significant difference in the apoptotic rate was observed between the KD group and the other 2 groups (CON and NC group) (P<0.05 for both). Furthermore, the apoptotic rate increased with the increase in the cytarabine concentration.

*Changes in cell cycle distribution and anti-apoptotic gene expression are associated with the synergistic effects of VEGFR-1 gene silencing in combination with cytarabine on U937 cells.* To elucidate the mechanisms underlying the inhibitory effects of VEGFR-1 gene silencing in combination with cytarabine on the proliferation and increased apoptosis of U937 cells, we further examined the cell cycle distribution in the 3 groups of U937 cells following treatment with cytarabine (50 ng/m). As shown in Fig. 5, the proportion of G$_1$ phase cells in the KD group increased from 47 to 62.2%, and that in the S + G$_2$ phase decreased from 53 to 37.8%. However, the proportion of cells in the G$_1$ phase increased from 46.4 to 51.7% and from 44.6 to 51.9% in the CON and NC group, respectively, and that in the S + G$_2$ phase decreased from 53.6 to 48.3% and from 55.4 to 48.1%, respectively. These results suggested that, after VEGFR-1 gene silencing, cytarabine treatment markedly decreased the number of cells in the S + G$_2$ phase and that these cells were more sensitive to cytarabine when compared with those in the CON and NC group.

We further analyzed the expression of anti-apoptotic genes in the 3 groups of U937 cells following treatment with cytarabine. As shown in Fig. 6, following treatment with cytarabine (50 ng/ml), the expression of survivin in the KD group was dramatically lower than that in the CON and NC group. These findings suggest that VEGFR-1 gene silencing downregulates the expression of anti-apoptotic genes.

## Discussion

VEGF and VEGFRs not only promote angiogenesis under physiological and pathophysiological conditions, but also enhance the proliferation, survival and migration of leukemia and myeloma cells, thus resulting in resistance to chemotherapy-induced apoptosis (4). Previous studies have demonstrated that the expression of VEGF and its receptors can be used to evaluate the progression of leukemia, as well as to predict the outcome of leukemia and monitor minimal residual leukemia cells (1-3).

Since its discovery, RNAi has been widely used as a rapid reverse-genetic approach for gene function analysis, as well as for the ablation of specific genes for therapeutic purposes (10). Due to their important roles in tumor neovascularization, VEGF and its receptors have been the ideal target genes of RNAi. It has been shown that the RNAi targeting of VEGF in combination with chemotherapy inhibits the proliferation of cancer cells and promotes their apoptosis (11). Shen *et al* (12) used a vector-based siRNA expression system to specifically inhibit VEGF165 expression in leukemia cells, leading to decreased tumor vascularity and growth *in vivo*.

To date, little is known concerning the role of VEGFR-1 in AML. In the present study, we used real-time PCR to detect the mRNA expression of VEGF, VEGR-1, VEGFR-2 in leukemia cell lines and patients diagnosed with AML. We demonstrated that the U937 cell line, with the highest migration rate under both normal and VEGF-induced physiological conditions, presents the highest expression of VEGF and VEGFR-1. In addition, we demonstrated that patients with extramedullary disease express significantly higher levels of VEGF and VEGFR-1 mRNA comapred to their non-extramedullary migration counterparts. This phenomenon was also observed in leukemia cells derived from AML M4/M5 compared to non-M4/M5 subtypes. Therefore, we hypothesized that VEGF and VEGFR-1 may modulate leukemia survival, proliferation and migration. Therefore, we used a lentiviral vector-based shRNA expression system, which induced stable and long-term gene silencing (13), to elucidate the role of VEGFR-1 in leukemia biology.

We came to the conclusion that the mRNA and protein expression of VEGFR-1 was inhibited by pRNAT/sh-VEGFR-1 transfection. In addition, we concluded that transfection with pRNAT/sh-VEGFR-1 *in vitro* suppressed the proliferation of U937 cells and blocked the promotive effects conferred by VEGF. The suppressed proliferation occurred at 72 h after transfection and continued for at least 1 week. We hypothesized that there may be a positive feedback between VEGF and VEGFR-1. Following VEGFR-1 gene silencing, the expression of VEGFR-1 was suppressed and the positive stimulatory effect of VEGF/VEGFR-1 was blocked. The amount of secreted VEGF decreased and the autocrine loop of VEGF was interrupted, resulting in suppressed proliferation. These results are in agreement with those from a previous study on myelodysplastic syndrome (14).

In our study, we observed that *in vitro* transfection with pRNAT/sh-VEGFR-1 suppressed the migration of U937 cells and blocked the VEGF-induced cell migration, while Fragoso *et al* (5) demonstrated *in vitro* and *in vivo* that VEGFR-1 (FLT-1) activation results mainly in leukemia cell migration. These results suggest that VEGFR-1 is associated with the migration of leukemia cells and that the specific suppression of VEGFR-1 may delay extramedullary infiltration in leukemia patients.

Bevacizumab is an anti-VEGF monoclonal antibody that targets VEGF-A isoform and blocks its binding to the VEGF receptors. Increasing evidence has shown that bevacizumab exhibits clinical activity against solid tumors when administered with cytotoxic chemotherapy (15). However, few studies have investigated bevacizumab treatment in hematological malignancies. Karp *et al* (16) conducted a phase II clinical trial of bevacizumab administered after chemotherapy to adults with refractory or relapsed AML, showing that cytotoxic chemotherapy followed by bevacizumab yields a favorable complete response (CR) rate and duration in adults with AML that is resistant to traditional treatment approaches. However, other studies (17,18) have revealed that bevacizumab had no effect on leukemia cells both *in vitro* and *in vivo*. In our study, we demonstrate *in vitro* that VEGFR-1 gene silencing in combination with bevacizumab does not confer synergistic effects on the suppression of cell proliferation and does not increase the apoptosis of U937 cells, which was consistent with a previous study which indicated treatment with the anti-VEGF antibody alone had little effect on cell survival (19). However, VEGFR-1 gene silencing in combination with cytarabine increased the sensitivity of U937 cells to cytarabine. As regards the mechanism by which sh-VEGFR-1 in combination with cytarabine inhibits cell prolifereation, we demonstrated that transfection with pRNAT/sh-VEGFR-1 decreased the IC$_{50}$ of cytarabine in the U937 cells in the KD group to 25% of that in the CON group, and increased the apoptotic rate in the KD group, leading to the enhanced sensitivity of U937 cells in the KD group to chemotherapy. These effects may be related to the suppressed expression of VEGFR-1 and VEGF, and the elevated oxygenation process, which facilitated the entry of the drugs to the tumors and endothelial cells, resulting in the abolishment of VEGF-induced resistance to chemotherapy (4). Additionally, cell cycle assay indicated, following treatment with cytarabine, that the proportion of cells in the S + G$_2$ phase was decreased and that the expression of survivin was suppressed by 90%. These findings suggest that the changes in the cell cycle and survivin expression also play a crucial role in the pRNAT/sh-VEGFR-1-induced apoptosis. Of note, similar effects were not observed after treatment with cytarabine in combination with bevacizumab.

The treatment of tumors with RNAi is still at the premature stage. A number of issues, such as increasing the transfection efficiency and ensuring the stability and safety of shRNA, should be resolved before this approach can be applied in clinical practice. Therefore, it is necessary to explore effective approaches for the target-specific suppression of VEGF and receptors, to construct safe and stable vectors for RNAi, and to further determine the effects of the RNAi targeting of VEGF and its receptors on the therapy of leukemia.

In conclusion, in this study, we used a lentivirus-mediated siRNA expression system to specifically inhibit VEGFR-1 expression in leukemia cells. The siRNA that we synthesized reduced the proliferation and migration of the U937 cells and enhanced their sensitivity to cytarabine. Our data suggest that the use of sh-VEGFR-1 in the treatment of leukemia, may be of therapeutic benefit.

## Acknowledgements

## References

1. Aref S, El Sherbiny M, Goda T, Fouda M, Al Askalany H and Abdalla D: Soluble VEGF/sFLt1 ratio is an independent predictor of AML patient out come. Hematology 10: 131-134, 2005.
2. de Bont ES, Fidler V, Meeuwsen T, Scherpen F, Hahlen K and Kamps WA: Vascular endothelial growth factor secretion is an independent prognostic factor for relapse-free survival in pediatric acute myeloid leukemia patients. Clin Cancer Res 8: 2856-2861, 2002.
3. Liang AB, Li L, Xie XT, et al: Preliminary study of VEGF and its receptor expression on childhood acute lymphoblastic leukemia and its relativity to clinical manifestations. Zhonghua Xue Ye Xue Za Zhi 26: 489-492, 2005 (In Chinese).
4. Podar K and Anderson KC: The pathophysiologic role of VEGF in hematologic malignancies: therapeutic implications. Blood 105: 1383-1395, 2005.
5. Fragoso R, Pereira T, Wu Y, Zhu Z, Cabeçadas J and Dias S: VEGFR-1 (FLT-1) activation modulates acute lymphoblastic leukemia localization and survival within the bone marrow, determining the onset of extramedullary disease. Blood 107: 1608-1616, 2006.
6. Elbashir SM, Harborth J, Weber K and Tuschl T: Analysis of gene function in somatic mammalian cells using small interfering RNAs. Methods 26: 199-213, 2002.
7. Hannon GJ: RNA interference. Nature 418: 244-251, 2002.
8. McManus MT and Sharp PA: Gene silencing in mammals by small interfering RNAs. Nat Rev Genet 3: 737-747, 2002.
9. Shi Y: Mammalian RNAi for the masses. Trends Genet 19: 9-12, 2003.
10. Hannon GJ and Rossi JJ: Unlocking the potential of the human genome with RNA interference. Nature 431: 371-378, 2004.
11. Filleur S, Courtin A, Ait-Si-Ali S, et al: SiRNA-mediated inhibition of vascular endothelial growth factor severely limits tumor resistance to antiangiogenic thrombospondin-1 and slows tumor vascularization and growth. Cancer Res 63: 3919-3922, 2003.
12. Shen HL, Xu W, Wu ZY, Zhou LL, Qin RJ and Tang HR: Vector-based RNAi approach to isoform-specific downregulation of vascular endothelial growth factor (VEGF)165 expression in human leukemia cells. Leuk Res 31: 515-521, 2007.
13. Manjunath N, Wu H, Subramanya S and Shankar P: Lentiviral delivery of short hairpin RNAs. Adv Drug Deliv Rev 61: 732-745, 2009.
14. Bellamy WT, Richter L, Sirjani D, et al: Vascular endothelial cell growth factor is an autocrine promoter of abnormal localized immature myeloid precursors and leukemia progenitor formation in myelodysplastic syndromes. Blood 97: 1427-1434, 2001.
15. Mukherji SK: Bevacizumab (Avastin). AJNR Am J Neuroradiol 31: 235-236, 2010.
16. Karp JE, Gojo I, Pili R, et al: Targeting vascular endothelial growth factor for relapsed and refractory adult acute myelogenous leukemias: therapy with sequential 1-β-d-arabinofuranosylcytosine, mitoxantrone, and bevacizumab. Clin Cancer Res 10: 3577-3585, 2004.
17. Kim KJ, Li B, Winer J, et al: Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. Nature 362: 841-844, 1993.
18. Zahiragic L, Schliemann C, Bieker R, et al: Bevacizumab reduces VEGF expression in patients with relapsed and refractory acute myeloid leukemia without clinical antileukemic activity. Leukemia 21: 1310-1312, 2007.
19. Santos SC and Dias S: Internal and external autocrine VEGF/KDR loops regulate survival of subsets of acute leukemia through distinct signaling pathways. Blood 103: 3883-3889, 2004.