

## Original Article

# RNA interfering of CXCR4 inhibits the adhesion, invasion, and tumorigenicity of acute monocytic leukemic cells *in vivo*

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**Abstract:** To investigate the effect of CXCR4 gene expressing on the proliferation, adhesion, and invasion of human monocytic leukemic cell line SHI-1 and its tumorigenic capacity in nude mouse. The SHI-1 cells were firstly infected with the rescued recombinant lentivirus to establish SHI-1/CXCR4i cell line. The expression of CXCR4, MMP-2 and MMP-9 in the SHI-1/CXCR4i cell was determined by real time quantitative PCR and flow cytometry. MTT assay was performed to detect the SHI-1/CXCR4i cell proliferative rate. The co-culture system of the leukemia cells with bone marrow stromal cells was utilized to detect the adhesive and migratory ability of SHI-1/CXCR4i cell. The nude mouse was subcutaneously inoculated with both cell lines, and then used to evaluate the growth ability of leukemia cells without CXCR4 expressing. The expression of CXCR4 mRNA in established SHI-1/CXCR4i cells decreased by 76% comparing with that in SHI-1/NC cells, which were infected with negative control virus. The proliferative rate of the SHI-1/CXCR4i cells *in vitro* did not show obvious difference with the SHI-1/NC cells, however, the adhesive and trans-Matrigel invasive ability were significantly decreased. Notably, the neoplasm was not observed in mice subcutaneously inoculated with SHI-1/CXCR4i cells, but presented in the SHI-1/WT and SHI-1/NC cells-inoculated mice in neoplastic volume of both groups. The present data showed that the CXCR4 silencing reduced the adhesive and migratory ability of SHI-1 cells *in vitro*, and suppressed the formation of subcutaneous neoplasm *in vivo*, demonstrating that the CXCR4 can be served as a novel target for leukemia gene therapy.

**Keywords:** Acute monocytic leukemia cells, CXCR4, RNA interference, mouse model, tumorigenesis

## Introduction

Acute monocytic leukemia possesses a considerable extra-medullary infiltrative ability into hepar, spleen, lymphonodus, and gingiva, in a multi-factor regulating manner. CXCR4 is specific chemokine receptor which was highly-expressed in multiple types of leukemia cells, especially during the period of pathogenesis process of acute monocytic leukemia. Leukemia cells with highly-expressed CXCR4 possessed a stronger extra-medullary infiltrative ability which interfere the chemotherapeutic effect [1] and indicates faulty prognosis [2-4]. In the previous study, we found that anti-CXCR4 antibody blocking its receptor function prevented the marrow stromal cells-induced trans-Matrigel migratory capacity of SHI-1 cells *in vitro* [5]. To further confirm the effect of CX-

CR4 in the infiltrative process of acute monocytic leukemia, the present study utilized a highly-aggressive human monocytic leukemia cell SHI-1 [6] and the RNA interference of CXCR4 by recombinant lentivirus infection to reveal its effect on proliferation, adhesion, invasion, and tumorigenicity ability of SHI-1 cell.

## Material and methods

### *Reagents and apparatus*

SHI-1 cell line was kindly provided by Prof. Yongquan Xue from Institute of Hematology of Jiangsu Province. Fetal bovine serum and IMDM culture medium were purchased from Gibco inc. (United States). Trizol and Lipofectamine 2000 were obtained from Invitrogen Company (United States). Total RNA reverse

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**Table 1.** Sequences of the primers

Gene	Primer
$\beta$ -actin	F: 5'-AGCGAGCATCCCCAAAGTT-3'
	R: 5'-GGGACGAGGGCTCATCATT-3'
MMP-2	F: 5'-GCTATGGACCTTGGGAGAA-3'
	R: 5'-TGGAAGCGGAATGGAAAC-3'
MMP-9	F: 5'-TCCCTGGAGACCTGAGAACC-3'
	R: 5'-CGGCAAGTCTCCGAGTAGTTT-3'
CXCR4	F: 5'-TTCTACCCCAATGACTTGTG3'
	R: 3'-ATGTAGTAAGGCAGCCAACA-3'

transcription kit and quantitative PCR kit were purchased from Takara company (Japan). Fluorescence ration PCR instrument 7300 was purchased from ABI Company (United States). PCR primers and shRNA oligonucleotides were synthesized in Shanghai Sangon Company. Anti-CXCR4, anti-GAPDH antibody, and Matrigel were purchased from R&D Company (United States). Millicell was obtained from Millipore Company (United States).

### Cell culture

SHI-1 cells were cultured in IMDM medium ( $5 \times 10^5$  cells/ml,  $37^\circ\text{C}$ , 5%  $\text{CO}_2$  and under saturated humidity). The medium was changed every another day. Cells in the logarithmic growth phase were used for following experiments.

Bone marrow stromal cells (BMSCs) were extracted and isolated from the bone marrow of acute non-lymphocytic leukemia patients with Ficoll lymphocyte separation medium. BMSCs were then cultured in IMDM medium ( $1 \times 10^6$  cells/ml,  $37^\circ\text{C}$ , 5%  $\text{CO}_2$  and under saturated humidity). The medium was changed every third day. Cells in the logarithmic growth phase were used for following experiments.

### Establishment of SHI-1/CXCR4i cell line

The shRNA oligonucleotides primers were designed. Positive-sense strand: 5'-GCGGGC-TGCCTTACTACATTGGGATCTCGAGATCCCAATGT-AGTAAGGCAGCTTTTT-3' to target the interfering sequence 5'-GCTGCCTTACTACATTGGGAT-3' of CXCR4 gene. Amplified DNA fragments were cloned into GV248-EGFP vector, which was subsequently co-transfected with pHelper 1.0 and pHelper 2.0 into 293T cells. Cell culture supernatant containing the rescued viruses was collected and titrated by dilution methods.

SHI-1 cells were infected with rescued viruses of high titer, and then flow cytometry was utilized to determine infectious efficiency through examining the GFP expression in cell population. Same method was used to construct the SHI-1/NC cell lines.

### CXCR4, MMP-2, and MMP-9 expression by RT-qPCR

Total RNA was isolated from SHI-1/CXCR4i and NC cells using TRIzol. Total RNA (500 ng) from each group was reverse transcribed with random hexamers followed by RT-qPCR using two sets of primers, and the primers sequences were displayed in the **Table 1**. The PCR cycling conditions were  $95^\circ\text{C}$  for 3 sec,  $58^\circ\text{C}$  for 30 sec, and  $72^\circ\text{C}$  for 30 sec for 30 cycles. Ct value of each gene was obtained from RT-qPCR,  $2^{-\Delta\Delta\text{Ct}}$  calculating method was used to compare the gene expression difference.  $\beta$ -actin was served as an internal control.

### Detection of CXCR4 by flow cytometry

The SHI-1 cells growing in log phase under identical conditions were harvested and washed in ice-cold PBS. Cells were incubated with anti-CXCR4 (12G5) antibody and isotype control antibody for 15 min, and subsequently incubated with PE-coupled secondary antibody. Then cells were analyzed immediately with FACSORT flow cytometry for detecting membrane CXCR4 expressing. All data were analyzed using Cell Quest software.

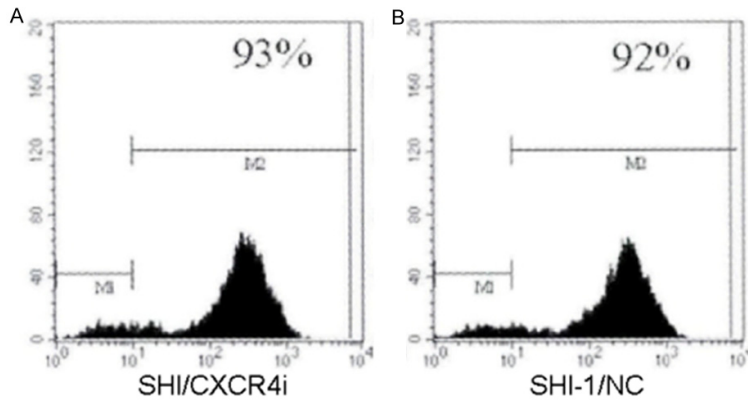
### Proliferation of SHI-1 cells assessed by MTT experiments

SHI-1 cells at logarithmic phase in each group were seeded into 96-well plate at the concentration of  $1 \times 10^6$  cells/ml. At 24, 48, 72 hrs post-seeding, 15  $\mu\text{l}$  of 5 mg/ml MTT solution was added to each well and incubated at  $37^\circ\text{C}$  for 4 h. Then 150  $\mu\text{l}$  of DMSO was added after the MTT solution in each well was deserted. Optical density values were examined on a plate reader at 490 nm and SHI-1 cells multiplication curves were graphed. The mean values of experiments were obtained by repeating the assay three times.

### Adhesion assay

BMSCs were seeded into 12-well plate at the concentration of  $1 \times 10^4$  cells/ml. When BMSCs

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**Figure 1.** Expression of GFP in SHI-1 cells. A. 93% of SHI-1/CXCR4i cells were GFP-positive; B. 92% of SHI-1/NC cells were GFP-positive.

confluence reached 90%,  $1 \times 10^6$  cells/ml SHI-1 cells were added to each well for co-culturing. At 24 hrs post co-culturing, medium supernatant was deserted and cells were slightly washed. Quantity of leukemia cells adhering on the BMSCs were then counted in three views to obtain the mean value.

### Cell migration assay

For migration assays,  $2 \times 10^5$  SHI-1 cells and  $2 \times 10^4$  BMSCs were plated into upper chamber of a 24-well Millicell chamber (Millipore) coated with Matrigel (diluted with IMDM with a ratio of 1:1, BD Biosciences) as described in the manufacturer's protocol. Sole SHI-1 cells and BMSCs were served as control group. After incubation for 24 hrs, the cells that invaded through the membrane were stained with crystal violet and then stained and counted under microscope.

### Tumorigenicity of SHI-1 cells in nude mice

The SHI-1/CXCR4i cells and controls growing in log phase under identical conditions were trypsinized and harvested. Approximately  $1 \times 10^7$  cells in 0.2 ml PBS were injected subcutaneously into each flank of BALB/c-nu/nu athymic mice ( $n=5$ ). For each mouse, the SHI-1/CXCR4i cells were injected on the left flank and the SHI-1/WT and NC cells were injected on the right flank. After 4 weeks, these mice were killed and the tumors were harvested and observed for its tumorigenesis condition. The maximum diameter (A) and minimum diameter (B) were measured to calculate the neoplasm volume (V) [ $V=(A \times B^2)/2$ ].

All animal research was approved by The Chinese Academy of Medical Sciences Institute of Experimental Animals and complied with the Beijing Laboratory Animal Welfare and Ethical Guidelines of the Beijing Administration Committee of Laboratory Animals.

### Statistical analysis

Data were analyzed using SPSS 22.0 software and were summarized as mean  $\pm$  SD. T test was used for independent-samples. \* $P$  value  $< 0.05$  was considered to indicate a statistically significant difference between the groups.

## Results

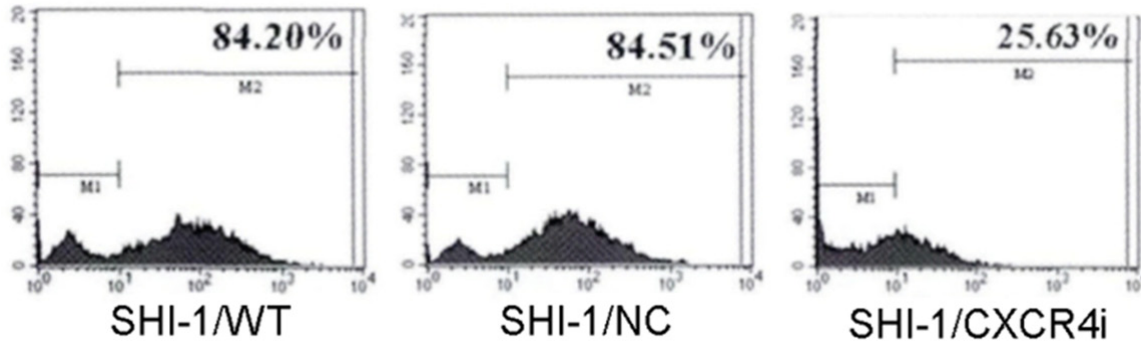
### Establishment of cell lines

CXCR4 interfering segment has been constructed into lentivirus vector GV248-EGFP, and co-transfected in 293T cells with virus rescue vectors. The rescued recombinant lentivirus have been titrated as  $8 \times 10^8$  TU/ml by dilution method. Then the SHI-1 cells were infected with the rescued viruses (SHI-1/CXCR4i cells), while negative control viruses were also used to infect SHI-1 cells to construct the negative control cells (SHI-1/NC cells). Flow cytometry was performed to examine the GFP fluorescence rate among SHI-1/CXCR4i cells and SHI-1/NC cells, the ratio of GFP-positive cells in each group were 93% and 92%, respectively (**Figure 1**).

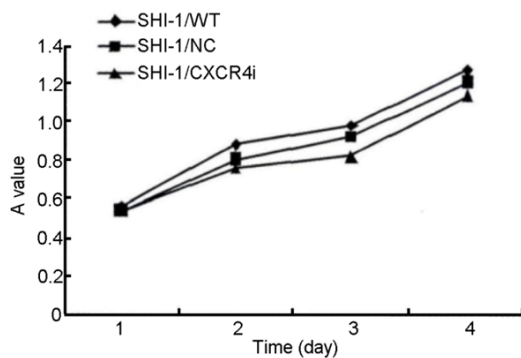
### Expression level of CXCR4 on the cellular membrane

To confirm the RNA silence efficiency in experimental group, flow cytometry was performed to detect the CXCR4 expressing on the cellular membrane of SHI-1/CXCR4i cells, while SHI-1/wild type (WT) cells and SHI-1/NC cells were served as negative control. The expressing rate of CXCR4 in SHI-1/WT and SHI-1/NC cells were 84.20% and 84.51%, respectively, in contrast, the expressing rate of CXCR4 in SHI-1/CXCR4i cells was 25.63%, demonstrating that CXCR4 gene in experimental group was knocked-down obviously (**Figure 2**).

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**Figure 2.** CXCR4 expressing in different lines of SHI-1 cells. The proportion of membrane protein CXCR4-positive cells in SHI-1/WT, SHI-1/NC, and SHI-1/CXCR4i cells were 84.20%, 84.51%, and 25.63%, respectively. The decreased CXCR4 expressing in experimental group indicated that CXCR4 gene was effectively knocked-down by CXCR4 RNAi.



**Figure 3.** Cell proliferative ability. The replicative rate of cells in each group were determined by MTT assay. There was no significant difference in proliferative ability among SHI-1/WT, SHI-1/NC, and SHI-1/CXCR4i cells ( $P>0.05$ ).

### Expression of CXCR4, MMP-2, and MMP-9 mRNA in each cell line

Highly-expressed MMPs in acute leukemia cells possesses higher extra-medullary infiltrative capacity than other leukemia cells, and the previous study demonstrated that MMP-2 and MMP-9 were able to be secreted by SHI-1 cells, therefore, real-time quantitative PCR was performed to detect the CXCR4, MMP-2, and MMP-9 mRNA expressing in each cell line. The expressing level of CXCR4, MMP-2, and MMP-9 mRNA in SHI-1/CXCR4i cells were reduced by 76%, 63%, and 62%, respectively, when comparing with SHI-1/NC cells. These genes expressing between SHI-1/NC and SHI-1/WT group did not show significant difference. These data suggested that the expressing level of MMP-2 and MMP-9 might be associated with or mediated by CXCR4 through a sealed mechanism.

### Effect of CXCR4 on proliferative ability of SHI-1 cells

MTT assays were used to evaluate the effect of decreased CXCR4 on the replicative capability of SHI-1 cells. As result showed, the in vitro proliferative rate of SHI-1/CXCR4i cells did not display any statistical difference with SHI-1/WT and SHI-1/NC cells at day 1, 2, 3, and 4 after culturing ( $P>0.05$ ) (Figure 3).

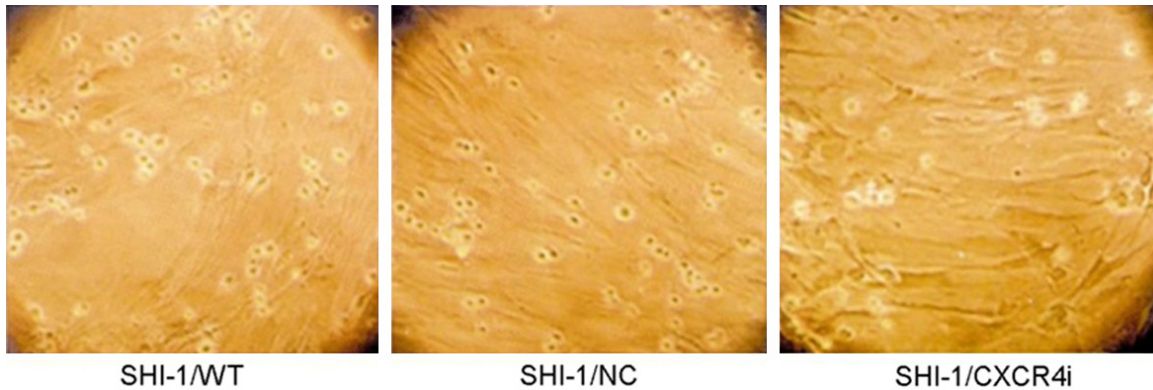
### Effect of CXCR4 on adhesive ability of SHI-1 cells in vitro

BMSCs were co-cultured with SHI-1/WT, SHI-1/NC, and SHI-1/CXCR4i cells for 24 hrs, and then the culture supernatants were removed for low-magnification optical microscope examination. Apparently, more leukemia cells in SHI-1/WT and SHI-1/NC groups were found adhering to the BMSCs than in SHI-1/CXCR4i group. The quantity of cells adhering to BMSCs in each group was also counted with microscope observation (40 $\times$ ), there were  $56.1\pm 5.1$  in SHI-1/WT group,  $57.6\pm 5.4$  in SHI-1/NC group, and  $25.1\pm 5.5$  in SHI-1/CXCR4i group. The adherence cell amount in SHI-1/CXCR4i group was significantly reduced comparing with other two groups ( $P<0.01$ ), while there was no obvious difference between SHI-1/WT and SHI-1/NC groups ( $P>0.01$ ), demonstrating that decreased CXCR4 also suppressed the adhesion of SHI-1 cells to BMSCs (Figure 4).

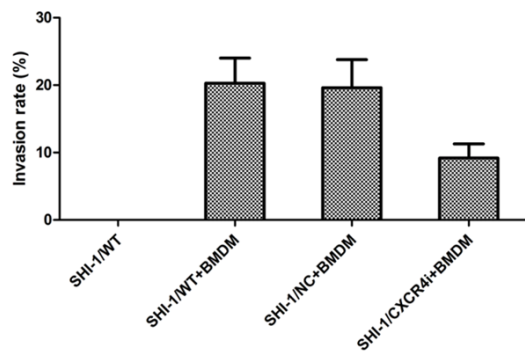
### Trans-Matrigel migratory capacity of SHI-1 cell lines

To evaluate the effect of CXCR4 on migratory capacity of SHI-1 cells, the trans-Matrigel

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**Figure 4.** Adhesive capability of each type of SHI-1 cells to BMSCs. The quantity of SHI-1/WT, SHI-1/NC, and SHI-1/CXCR4i cells adhered to BMSCs were  $56.1 \pm 5.1$ ,  $57.6 \pm 5.4$ , and  $25.1 \pm 5.5$ , respectively, indicating that adhesion of SHI-1 cells were inhibited by the reduced CXCR4 expressing ( $P < 0.05$ ).



**Figure 5.** Trans-Matrigel migratory ability of SHI-1 cells. When co-cultured with BMSCs, the migratory rate of SHI-1/WT and SHI-1/NC cells were statistically higher than that of SHI-1/CXCR4i cells ( $P < 0.01$ ).

migratory experiment in Millicell chambers was performed, however, there were only few cells observed in the lower layer of Millicell chamber at 24 hrs after SHI-1 cells in these three groups were culture in the upper layer, suggesting that sole SHI-1 cell did not possess the trans-Matrigel migratory ability. Nevertheless, while SHI-1 cells were co-cultured with BMSCs spreading on the Matrigel, these adhered SHI-1 cells were able to be found in the lower layer of Millicell. The proportions of migrated SHI-1 cell amount in the total cultured cell amount were  $20.3 \pm 3.7\%$  (17.0%-23.0%) in SHI-1/WT+BMSC group,  $19.6 \pm 4.2\%$  (15.5%-22.0%) in SHI-1/NC+BMSC group, and  $9.2 \pm 2.1\%$  (6.5%-11.0%) in SHI-1/CXCR4i group. The ratio in experimental group displayed statistical difference with other two groups ( $P < 0.01$ ), while there was no significant difference between these other gr-

oup ( $P > 0.01$ ), suggesting that decreased CXCR4 in SHI-1 cells impaired its migratory ability when co-culturing with BMSCs (**Figure 5**).

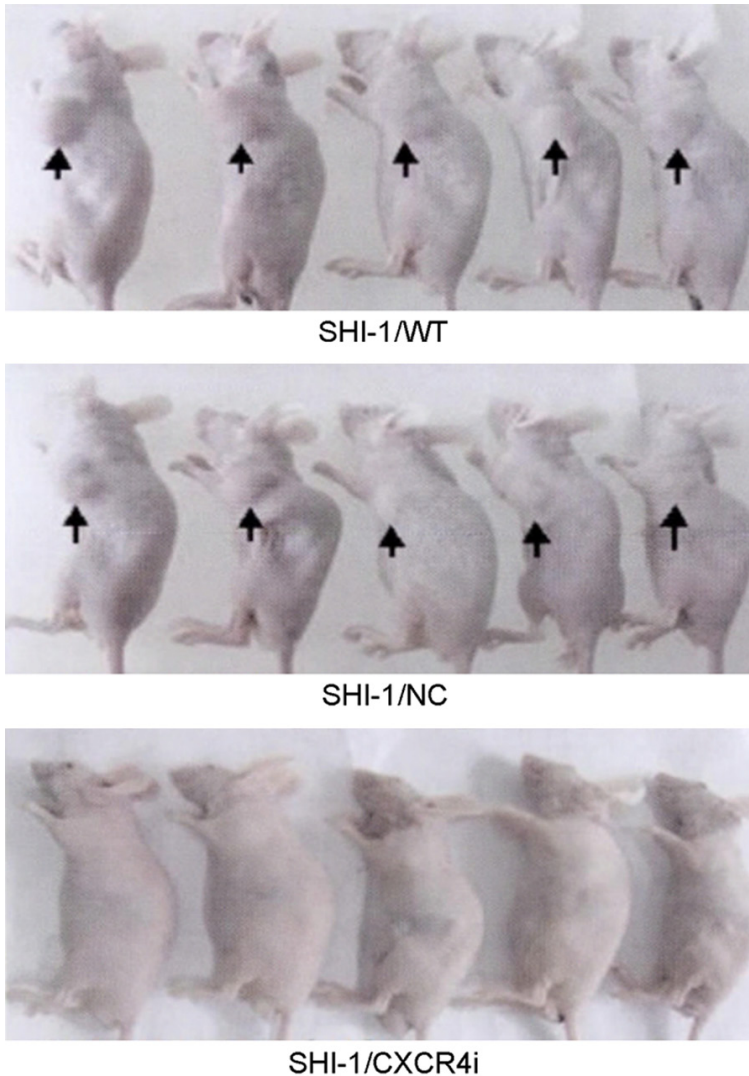
### Effect of CXCR4 on tumorigenesis in vivo

To assess the effect of CXCR4 on tumorigenesis of SHI-1 cells in nude mouse, nude mice were inoculated subcutaneously with different SHI-1 cells (WT, NC, and CXCR4i). Tumor mass appeared at day 20 post inoculation in SHI-1/WT and SHI-1/NC groups, and neoplasm volume increased gradually. In contrast, any neoplasm could not be detected and observed in SHI-1/CXCR4i group, indicating that the subcutaneous tumorigenesis ability of SHI-1 was inhibited following with the RNA silencing of CXCR4 (**Figure 6**). Inoculated nude mice were sacrificed at day 55 post inoculation, and then the maximum diameter and the vertical minimal diameter of subcutaneous were measured and calculated. The volume the tumor derived from SHI-1/WT cells were  $378.8 \pm 122.7 \text{ mm}^3$ , and did not show statistical difference with that from SHI-1/NC cells which were  $264.9 \pm 67.4 \text{ mm}^3$  ( $P > 0.05$ ) (**Figure 7**).

### Discussion

Chemokine is a kind of cytokine superfamily possessing chemotactic effect. Among them, CXCR4, a seven-span transmembrane protein, can bind with its specific ligand stromal cell-derived factor-1 (SDF-1) to activate the downstream signal pathway and play an essential role in embryogenesis, inflammation, innate immunity, angiogenesis, hematopoiesis, AIDS,

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**Figure 6.** Tumorigenesis of SHI-1 cells in nude mice. After nude mice were inoculated with SHI-1 cells, obvious neoplasm mass (black arrows) were able to be formed in SHI-1/WT and SHI-1/NC group while SHI-1/CXCR4i groups could not, demonstrated that growth capability of SHI-1/CXCR4i cells were totally inhibited after *CXCR4* gene was RNA-silenced.



**Figure 7.** Comparison of tumor volume between SHI-1/WT and SHI-1/NC groups. There was no significantly difference between the volumes of neoplasms derived from SHI-1/WT and SHI-1/NC cells ( $P>0.05$ ).

and neoplasm invasion. *CXCR4* also participate in the tumorigenesis and infiltration process during acute myeloid leukemia. The high expres-

sion of *CXCR4* was highly-related to the extra-medullary infiltrative capacity of leukemia to influence the effect of chemotherapeutic drug [7, 8]. It was also reported that highly-expressed *CXCR4* increased the drug resistance of leukemia patients to chemotherapy [9]. Our preliminary study also demonstrated that anti-*CXCR4* antibody could efficiently block the migration of leukemia SHI-1 cells. In consequence, the goal of present study was to further investigate the effect of *CXCR4* silencing on leukemia cells.

After the expression of *CXCR4* was efficiently inhibited by RNA silencing, we found that the adhesive ability and trans-Matrigel migratory capacity of SHI-1/*CXCR4i* cells were significantly decreased, but it also showed that the proliferation ability of these cells still was commensurable with SHI-1/WT cells. Matrigel is one kind of artificial basement membrane which is majorly composed of extracellular matrix, such as type IV collagen. It can be degraded *in vitro* by the metalloprotease in the culture microenvironment to induce the trans-Matrigel migration of tumor cells. Previous study showed that MMP proteins were highly expressed in the acute leukemia cases of higher extra-medullary infiltration occurrence. Our preliminary study also found that MMP-2 and MMP-9 proteins were able to be secreted by SHI-1 cells. Therefore, reduced trans-Matrigel migratory capacity of SHI-1/*CXCR4i* cells might be attribute to decreased adhesive ability to BMSCs of SHI-1/*CXCR4i* cells, and down-regulated expression of MMP-2 and -9 proteins. The following subcutaneous tumorigenesis experiments in nude

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mouse also confirmed importance of CXCR4 on growth rate of SHI-1 cells *in vivo*. A previous research has demonstrated that the interaction of CXCR4 and SDF-1 influenced the tumor microenvironment through regulating inflammatory cells and lymphocytes function which subsequently boosted abundant MMP proteins expressing. The latter introduced the vascular remodeling and regeneration of neoplasm via degrading extracellular matrix, and eventually promoted tumorigenesis [10]. The investigation on squamous cell carcinoma of the head and neck by Samara *et al.* displayed that the activated CXCR4 could recruited  $Ca^{2+}$  ion in cells and activated the extracellular signal-regulated kinase-1/2 to promote MMP-9 expression which might lead to tumor migration [11]. A recent study also indicated that Serine 339 phosphorylation at C-terminal of CXCR4 accelerated the adhesion and restorage ability of myeloid leukemia cells, and presumptively increased the drug tolerance to chemotherapy in leukemia cases [12].

Up to now, there are multiple kinds of small molecule compound designed to target and antagonize CXCR4 function, e.g., AMD3100. AMD3100 compound was found inhibited hypergrowth of acute leukemia colony forming unit [13]; in the acute promyelocytic leukemia mouse model and acute lymphocytic leukemia mouse model, AMD3100 promoted the proliferation of peripheral blood leukemia cells. The combination of AMD3100 and chemotherapy significantly decreased the tumor burden of mouse leukemia and prolonged the survival duration of leukemia mouse model [14, 15]. CXCR4 antagonist declined the adhesive and storage ability of leukemia cells in the bone marrow, and facilitated the releasing to peripheral blood of leukemia cells. It also indicated that the combination of CXCR4 antagonist and chemotherapy played an essential role in therapeutic effect.

The present study found that CXCR4 silencing restrained the adhesive, migratory, and tumorigenesis capacity *in vitro* of SHI-1 cells, and further confirmed the its inhibitory effect on hypodermic tumorigenesis in nude mouse probably through blocking SDF-1/CXCR4 signal pathway and down-regulating of MMP-2 and MMP-9 expression. In conclusion, our data suggested that CXCR4 or its antagonist might be served as a suitable candidate for the treatment targeting leukemia.

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### Disclosure of conflict of interest

None.

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