Original Article

Upregulation of hsa-miR-625-5p Inhibits Invasion of Acute Myeloid Leukemia Cancer Cells through ILK/AKT Pathway

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Abstract
Objective: Acute myeloid leukemia (AML) is characterized by abnormalities of differentiation and growth of primary hematopoietic stem cells (HSCs) in the blood and bone marrow. In many studies, miR-625-5p has been shown to inhibit downstream pathways from affecting the metastasis and invasion of the integrin-linked kinase (ILK) signaling pathway. It has been proved that the expression of miR-625-5p decreases in AML cell lines. This study aimed to investigate the effect of miR-625-5p upregulation on the invasion of KG1 ell line *in vitro*.

Materials and Methods: In this experimental study, we investigated the impact of upregulation of miR-625-5p on invasion via the ILK/AKT pathway in the KG1 cell line. After transfection using the viral method, the cellular invasion was assessed by invasion assay and the levels of miR-625-5p genes and protein were evaluated by quantitative polymerase chain reaction (qPCR) and western blotting. Moreover, CXCR4 level was assessed by flow cytometry.

Results: The invasion significantly reduced in MiR-625-5p-transfected KG1 cells (P<0.01) that was concomitant with remarkably decreasing in the expression levels of *ILK, NF-κB*, and *COX2* genes compare with the control group (P<0.01). Incontrast, *MMP9, AP1*, and *AKT* significantly increased (P<0.01, P<0.001 and P<0.01, respectively) and GSK3β did not change significantly in MiR-625-5p-transfected KG1 cells. The protein level of NF-κB decreased (P<0.01) and MMP9 increased, however it was not significant. Moreoever, the expression of CXCR4 was significantly lower (P<0.01) in comparison with the control group.

Conclusion: miR-625-5p leads to a reduction in cell invasion in the AML cell line through ILK pathway. Therefore, it could be a breakthrough in future AML-related research. However, further studies are needed to support this argument.

Keywords: Acute Myeloid Leukemia, COX2, Integrin-Linked Kinase, Invasion, MMP9

Introduction

Acute myeloid leukemia (AML) is a heterogeneous class of aggressive hematopoietic malignancies with abnormal hematopoietic stem cells (HSCs) in the blood and bone marrow. AML leads to dysregulation and activation of cellular cascades such as invasion and migration. Invasiveness and resistance to therapy are two main issues that challenge research and treatment of refractory cancer (1). Some mechanisms of invasion and migration are cell migration and motility, extracellular matrix (ECM) destruction, and interaction with stromal and other cells (2).

The main problem is that the tumor cells, unlike normal cells, do not stop the signaling pathways to end this process, leading to the emergence of invasion (3). The majority of patients are not treated fully, and therefore identification of the mechanisms involved in AML invasion may culminate in innovative therapeutic methods, improve the treatment rate and reduce the rate of recurrence. Many studies have been conducted on AML, but the complex molecular mechanisms of the disease invasion and progression have not yet been adequately identified and further studies are needed to investigate this subject (4).

Much evidence has revealed dysregulation of microRNAs expression in cancer cells, so that miRNAs serve as tumor suppressors or oncogenes in cancer (5).

MiR-625-5p dysfunction has been identified in many diseases, often with reduced expression of the microRNA. This molecule can suppress various tumors, such as hepatocellular carcinoma, breast cancer, gastric cancer, and acute lymphocytic lukemia (ALL) (6). The increased expression of this miR can inhibit the proliferation and invasion of cancer cells. It has been confirmed that miR-625-5p expression decreases in AML cell lines (7).

Upregulation of miR-625-5p in gastric cancer significantly suppresses cell invasion and metastasis. ILK is a target gene of miR-625-5p that regulates cell invasion and metastasis. The integrin signaling pathway plays a crucial role in mediating the interaction between cells and the ECM. The ligand of ILK binds to the integrin and initiates out-to-inside signals by modulating the changes in various intracellular pathways including the expression of the genes MMP9 and NF-κB contributed in cell migration and invasion (8, 9).

ILK contributes substantially to regulating anchorage cell survival and growth, cell cycle progression, the epithelial-mesenchymal transition, invasion, and migration as well ascell movement. The invasion of the ILK signaling pathway occurs through two pathways: the GSK3β-Ap1-MMP9 signaling pathway and the AKT- NF-κB-COX2 pathway (10).

ILK activity increases in many cancers, and therefore ILK inhibitors have been identified that contribute to cancer treatment by inhibiting cell invasion, proliferation and survival so far (11, 12). It has also been demonstrated that this miR y, can control the downstream pathways by influencing the ILK signaling pathway and thus involved in the invasion and metastasis of cancers (8, 13).

Increased expression of miR-625-5p leads to induction of apoptosis and reduction in migration and invasion in AML by decreasing oncogenes. Studies on ILK gene expression throughout miR-625-5p's exerting effect on AML cell and changes in the expression of proteins involved in invasion and metastasis have increased our understanding of how this miR and its target genes in various processes lead to leukemia in the bone marrow.

This study investigated the effect of miR-625-5p upregulation on the invasion of AML cell *in vitro*. Finally, the mechanism of effect of miR-625-5p of KG1 cell line invasion is evaluated, and mRNA and protein levels of factors involved in the invasion, are measured through the ILK signaling pathway, including COX2, NF-ƙB, GSK3β, MMP9, AP1, and ILK.

Materials and Methods

Cell lines and cell cultures

In this experimental study, mycoplasma-negative KG1 human cells were purchased from the Pasteur Institute (Tehran, Iran). The KG1 cell line was taken from a patient with erythroleukemia in the myeloblastic phase and has the phenotype and function of myeloblasts. Human embryonic kidney 293 T cells were obtained from the Royan Institute (Tehran, Iran).

KG1 cell lines were cultured in RPMI1640 medium (Gibco-BRL, Eggenstein, Germany) containing10% fetal bovine serum (FBS, Gibco BRL, USA) and 1% penicillin/ streptomycin and 2mm L-glutamine (Gibco, Germany) and in the presence of 5% CO_2 at 37°C.

HEK cells were cultured in Dulbecco's Modified Eagle Medium (DMEM ,Gibco-BRL, Eggenstein, Germany) with 15% FBS at 37°C and 5% CO_2. Every two days, the cell lines medium was changed.

This study was ethically coded and approved by Tehran University of Medical Sciences (IR.TUMS.SPH. REC.1395.837) and Royan Institute (IR.ACECR.ROYAN. REC.1397.41).

Plasmid construct and extraction

The pLentiIII-premiR-625-5p-GFP expression vector constructs and pLentiIII-Backbone-GFP were purchased from the Bonyakhte Institute (Tehran, Iran). Vector-harboring E. coliDH5α strain was grown in an incubator for 24 hours to produce a single colony on an LB agar medium (Thermo Fisher Scientific, USA) containing ampicillin at a concentration of 50 mg/ml of culture medium. One hundred μl of the culture containing bacteria purchased in Falcon tube was cultured in 300 ml of LB Broth (Thermo Fisher Scientific, USA) containing antibiotics and ampicillin, and placed inside a shaker incubator at 37°C for 12-16 hours. The plasmid was extracted by using Gene All Exprep™ Plasmid Kit (Gene All, Dongnam, Songpa-gu, Macherey-Nagel, Korea).

Confirmation of plasmid structure of pLentiIII-miR-625-5p-GFP and pLenti-backbone-GFP

To confirm the presence of miR-625-5p, we first retrieved the sequence file for miR-625-5p and the backbone using the Snap Gene program and the gene bank. miR-625-5p restriction enzymes, called BamH1 and EcoR1(Bonyakhte, Iran). Solutions contain of vector and enzymes that were first placed in a 37°C incubator for 4-6 hours run On the 1% electrophoresis gel (Merck, Germany). Then the plasmids were electrophoresed with a 1 kb marker size (Gibco BRL, USA) on 1% agar gel. After 45 minutes, plasmids were digested and identified by a gel document (Syngen, England).

Transient transfection

The concentration of fresh KG1 cells was maintained at 0.5-1.5×10⁶ cells/ml. 5.0×10⁶ from the cell subcultures were transfected with 5 μg miR-625-5p and Backbone vector using the viral transfection according to the respective protocol. First, we added gelatin 1% into the plate and placed it in the incubator at 37°C for 1 hours. After the removal of gelatin, the complete medium was added. 293T cells were counted and added drop by drop on the culture medium inside the plate,the supernatant was removed after a night and the new medium was added. At this step, we produced a transfection solution such as DMEM low glucose media, packaging vector PD and PS (Bonyakhte, Iran), miR-625-5p or backbone plasmid, PEI

(Bonyakhte, Iran) and incubated at room temperature. We added the transfection solution to the cells, and after 6-12 hours, removed the supernatant and added preheated media. Twenty four hours later, the first virus was extracted and the complete medium was added to the cells again. 48 hours later, the second step of the virus production was performed. At this step, we centrifuged the viruses at 37565 g and 4°C for 2 hours and dissolved them in RPMI1640 media. The virus was finally added to KG1 cell line and 48 hours after transfection, the Survival rate efficiency was investigated by flow cytometry. Transfection was performed on two cell groups (Backbone and Mir-625-5p) with three replications. After 48 hours of transfection, the transfected cells were collected for further assay.

Sorting green fluorescent protein expressing cells

First, the supernatant was isolated from the cells using centrifugation at 250 g for 5 minutes. Then, the cells were rinsed with dulbecco's phosphate-buffered saline (D-PBS)- solution and finally centrifuged at 250 g for 5 minutes. Then PBS with 1% bovine serum albumin (BSA) was added, and the cells expressing GFP were isolated using Ariya FACS sorting (Becton Dickinson, Belgium),

Functional assay

Invasion assay *in vitro*

We applied transwell inserts (24 well inserts, 8 μm pore size Millipore, USA) to gain the effect of miR-625-5p on the invasion of AML cells *in vitro*. the inserts were coated with ECM gel (BD Biosciences, Bedford, MA) for one night. Briefly, 2×10^5 cells were resuspended in serum-free medium and were dumped in the upper chambers as duplicates. The bottom chamber was incubated via RPMI1640 containing 20% FBS as absorbent (chemotactic) overnight to perform an invasion test.

Upon completion of the experiment, the cells that have remained on the upper surface of the membranes were removed and finally, the invasion cells to the bottom chamber were centrifuged at 250 g for 5 minutes. Then, the supernatant was removed and were counted on the neo-bar slide that were approximately 7×10^3.

The underlying cells of the insert were also fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Randomly five visual fields were counted from each insert using an optical microscope (14).

Flow cytometry

A total of $2\text{-}3 \times 10^5$ cells were rinsed with D-PBS solution and then incubated with CXCR4-PE (Santa Cruz Biotechnology. Inc, USA). Identical iso-type antibodies were used as a control (IgG2ακ, PE-conjugated, Santa Cruz Biotechnology. Inc, USA) for 30 minutes at 4°C.

Cells were analyzed using a FACS flow cytometer (Becton-Dickinson). Analysis of CXCR-4 expression in GFP-positive KG1 gametes, GFP-positive KG1 cells were gated in a SSC/FL-3 dot plot.

A FL-1/FL-2 dot plot was applied for further analysis of GFP-positive KG1 cells. Mean fluorescence intensity was measured with reference to the fluorescence histogram and presented as corresponding units (15).

RNA extraction and cDNA synthesis

Total RNA was extracted from the KG1 cell line using Trizol (Invitrogen, Carlsbad, CA, USA) reagent 48 hours after transfection based on the manufacturer's protocol. RNA quality was then determined by electrophoresis and DNA extraction was performed using a fermentase kit (Fermentas, Lithuania) to remove any remaining DNA according to the manufacturer's protocol. cDNA of the whole RNA was synthesized by cDNA synthesis kit (Royan Biotech, Tehran, Iran) and miR-625-5p by another CDNA synthesis kit (Bonyakhte Tehran, Iran) (16).

Quantitative polymerase chain reaction for miR-625-5p expression

The reverse and forward primers with the stem-loop primers were designed for cDNA synthesis and miR-625-5p qPCR according to the procedure of Chen et al. (17).

The expression level of miRNA was assessed by miRNA diagnostic kit (Bonyakhte, Iran) using the qRT-PCR stem-loop method. U6 RNA (snord47) was used for normalization. Finally, the relative expression ratio of miR-625-5p was determined by $2^{-\Delta\Delta CT}$ in triplicate (13).

Quantitative polymerase chain reaction for genes expression

ILK and *NF-κB* as direct targets and *AKT, GSK3β, AP1 (c-FOS), MMP-9, COX2* as indirect targets of miR-625-5p, were determined using the miRNAs target prediction site (http//: miRtarbase.mbc.nctu.edu.tw) and according to the study of Wang et al. (8). Gene primers were then designed and blasted for using Primer Premier 5 software (Premier Biosoft International, USA) and Gen Runner software (ver.5.1). *GAPDH* was used a reference gene (Table 1).

To detect *ILK, AKT, GSK3β, AP1, MMP-9, NF-κB, COX2,* and *GAPDH* transcription levels, cDNA was made from the total RNA using SuperScript III First-Strand Synthesis System and then measured using Takara SYBR PCR Green Kit (Takara Bio Inc., Shiga, Japan).

The expression levels of *ILK, AKT, GSK3β, AP1, MMP-9, NF-κB and COX2* mRNA were normalized to

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GAPDH mRNA level. Target genes relative expression ratio was calculated by the $2^{-\Delta\Delta CT}$ method in triplicate.

To plot the standard curve of the primers, we prepared their 1:5 to 5 titrations in distilled water and placed them in the ABI StepOnePlus device to determine the CT. The qRT-PCR reaction (ABI StepOnePlus) was used to measure gene expression changes (8).

Western blotting analysis

After 48 hours, cell lines transfected with miR-625-5p and backbone vector were cultured and rinsed three times with cold PBS solution.

Total protein was extracted from cells by trisol (Invitrogen, Carlsbad, CA, USA) and cell lysis buffer (Biyuntian Biotechnological Co., USA). The protein concentration of the lysate was calculated from the standard line of BSA.

First 5 µl protein was boiled at 95°C for 5 minutes and then cooled on ice. Then it was run on an SDS-PAGE gel (Millipore, USA) to determine its quality and electrotransferred to PVDF membrane (Life Science, Amersham, Braunschweig, Germany). Membranes were blocked by non-fat dry milk (w/v) and then immunoblotted with anti-NF-κB-p65 (Abcam, Inc., Cambridge, MA, USA) and anti-MMP-9 (Santa Cruz Biotechnology. Inc, USA) at 4°C overnight (dilutions 1: 200 and 1: 700, respectively), followed by horseradish peroxidase-conjugated rabbit (Abcam, Inc., Cambridge, MA, USA) and goat (Santa Cruz Biotechnology. Inc, USA) secondary antibodies (dilution1:3000) incubated at room temperature for one hour. NF-κB and MMP-9 protein bands were visualized with ECL (Kodak Image Station; New Haven, CT, USA). The band densities were analyzed to use Image J software (n=3) (18).

Table 1: The list of primers used in quantitative real time polymerase chain reaction analysis

Genes	Primer sequence (5´-3´)	Length	Annealing temp.(°C)	Reference
miR-625-5p	StemloopRT: AGGGUAGAGGGAUGAGGGGGAAAGUUCUAUAGUCCUGUAAUU AGAUCUCAGGACUAUAGAACUUUCCCCCUCAUCCCUCUGCCCU	503	60	http://www.mirbase.org/
	F: CTCTGCTTGACTGTGCTG			
	R: TACCAGAACCTAACCAACTG			
SNORD 47	StemloopRT: GTCGTATGCAGAGCAGGGTCCGAGGTAT TCGCACTGCATACGACAACCTC	71	60	Designed by AlleleID and oligo sofware
	F: ATCACTGTAAAACCGTTCCA			
	R: GAGCAGGGTCCGAGGT			
GAPDH	F: AGGGTCTCTCTCTTCCTCTTGTGC TCT	224	60	Designed by AlleleID and oligo sofware
	R: CCAGGTGGTCTCCTCTGACTTCAA CAG			
ILK	F: CACCTGCTCCTCATCCTACTC	209	60	Designed by AlleleID and oligo sofware
	R: CTCATCAATCATTACACTACGGCT			
AKT	F: AACGAGTTTGAGTACCTGAAGC	204	60	Designed by AlleleID and oligo sofware
	R: GTACTTCAGGGCTGTGAGGA			
GSK3β	F: AGTGGTGAGAAGAAAGATGAGGT	207	60	Designed by AlleleID and oligo sofware
	R: GAGGTTCTGCGGTTTAATATCCC			
MMP-9	F: CAAGGATGGGAAGTACTGGC	117	60	Designed by AlleleID and oligo sofware
	R: TCCTCAAAGACCGAGTCCAG			
NF-κB	F: ACTGCCCAATTTAACAACCTG	220	60	Designed by AlleleID and oligo sofware
	R: CATCACTGGCTCTAAGGAAGG			
COX2	F: ACCAATTGTCATACGACTTGCAG	197	60	Designed by AlleleID and oligo sofware
	R: AAGGATTTGCTGTATGGCTGAG			
C-FOS (AP1)	F: TCGGGCTTCAACGCAGAC	85	60	Designed by AlleleID and oligo sofware
	R: GAGTGGTAGTAAGAGAGGCTATCC			

Statistical analysis

In this study, the GraphPad Prism software (V.7, GraphPad Software, Inc., San Diego, CA) was employed to conduct statistical analysis. The results of our tests were analyzed with t test and ANOVA. The data were expressed as mean ± standard deviation (SD). The significance level P<0.05 was considered statistically significant. In the charts, the P<0.05 shown with a star (*), P<0.01 shown with two stars (**), P<0.001 shown with three stars (***), and P<0.0001 shown with four stars (****). All the experiments were repeated three times.

Results

Transfected and overexpression of miR-625-5p in KG1 cells

To study the impact of miR-625-5p on the regulation of *ILK, AKT, GSK3β, AP1(C-FOS), MMP-9, NF*-κB*, COX2*, and finally invasion in KG1 cells, the cells were first transfected with premiR-625-5p and backbone expression vectors construct by viral transfection followed by detection of invasion.

Fluorescent microscope (Fig.1A) and flow cytometry analysis confirmed the efficacy of transfection after 48 hours (Fig.1B) where around 60% of the cells were transfected. The qRT-PCR showed a significantly increased miR-625-5p expression in the cells after 48 hours of transfection (Fig.1C). MiR-625-5p expression was approximately 27-fold higher than that of backbone vector-transfected cells (P=0.01).

miR-625-5p expression reduced cell counts after transfection

After adjoining the virus to the cells, the number o cells, and cell viability were studied after 24 and 48 hours of the transfection. After 48 hours, it was observed that the average number of cells and viability percentage reduced in miR-625-5p-transfected cells compared to the backbone group (P<0.01).

The association of miR-625-5p expression with the invasive activity of acute myeloid leukemia cell line

To figure out the association of miR-625-5p with cell invasion, cellular invasion in the KG1 cells was evaluated by transwell insert 48 hours after transfection. As illustrated in Figure 2A and B, the count of cells attached to the filter bottom decreased in cells treated with miR-625-5p. The number of miR-625-5p transfected KG1 cell line was lower than that of Backbone transfected cells. The count of these cells was approximately 0.6% fold lower than control (Fig.2C, P<0.01).

Overexpression of miR-625-5p reduced CXCR-4 expression in the surface of the KG1 cell

The CXCR-4 surface marker was examined using flow cytometry with an antibody attached to the PE as a marker of invasion. As illustrated in Figure 3A and B, the count of miR-625-5p transfected KG1 cell line was lower than that in backbone transfected cells. The count of cells transfected with pre miR-625-5p expressing CXCR was around 13.7% lower than Backbone vector-transfected cells (Fig.3C).
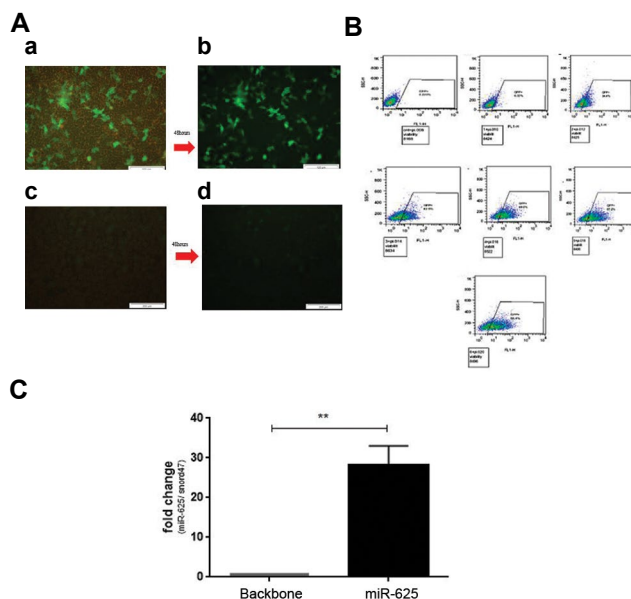

Fig.1: GFP expression in 293T cell and KG1. **A.** GFP expression in 293Tcell and KG1 after 48 hours by Fluorescent microscope. 293T cells as controls for GFP expression (a, b), KG1 cells without and with GFP expression (c), KG1 cells expressing GFP positive (d). **B.** Virus-free control cells with 82% viability (a) and cells with 10 μl of concentrated virus and 7% GFP expression (b). Cells with 20μl of concentrated virus and 34% GFP expression (c). Cells with 50 μl of concentrated virus and 43% GFP expression (d). Cells with 70 μL of concentrated virus and 66% GFP expression (e). Cell with 90 μl concentrated virus and 67% GFP expression (f) and cell with 120 μl concentrated virus and 66% GFP expression (g). Based on the percentage of expression GFP, the amount of concentrated virus was found to have a constant expression at 70, 90 and 120 μl. **C.** Confirmation of miR-625-5p expression in KG1 cells after transfection by qRT-PCR. KG1 cells were transfected with pre miR-625-5p expression vector construct or Backbone. The expression of the miR-625-5p in the KG1 cells transfected with the recombinant vector was considerably higher than Backbone after 48 hours (**P<0.01). GFP; Green fluorescent protein and qRT-PCR; Quantitative real time polymerase chain reaction.
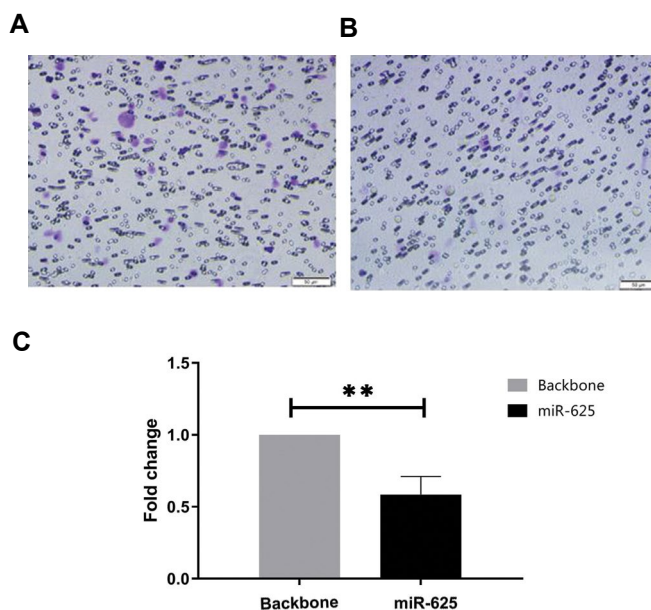

Fig.2: Effect of miR-625-5p overexpression on invasion in kG1 cells 48 hours post-transfection. The transfected KG1 cells were treated with invasion assay (transwell insert) and followed by count. Invasive cells and connected to bottom the filter. **A.** Cells transfected with backbone (scale bar: 100 μm). **B.** Cells transfected with miR-625-5p (scale bar: 100 μm). Overexpression of miR-625-5p in KG1 cells significantly decreased the invasive cell compared to Backbone. **C.** There was a significant 0/6% reduction in the invasive cells transfected with miR-625-5p construct (**P<0.01).
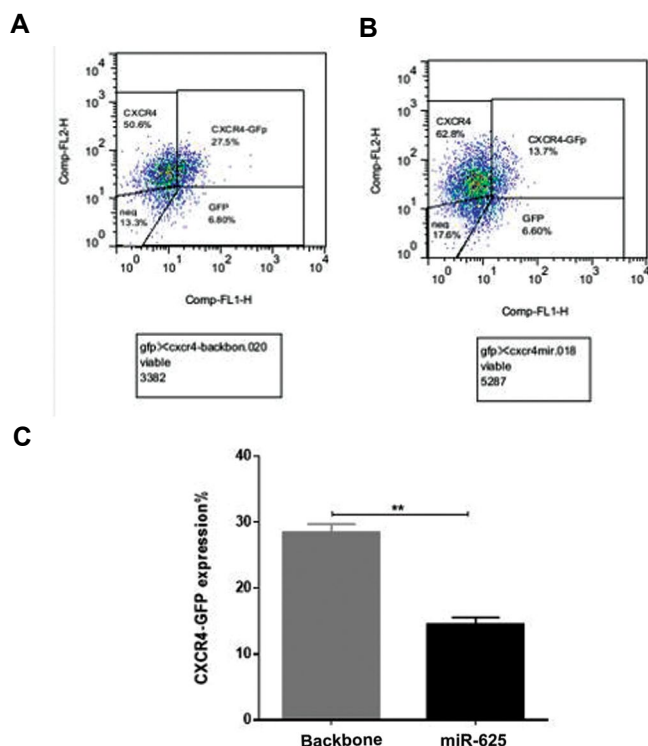
A

B

C

Fig.3: Effect of miR-625 overexpression on cells expressing CXCR-4 in KG1 cells 48 hours post-transfection. The transfected KG1 cells were treated with Antibody CXCR-4-PE and followed by flow cytometry analysis. **A.** In the KG1 cells transfected with pre miR-625-5p-GFP construct, approximately 13.7% of cells became CXCR-4-PE positive. **B.** About 27.5% of KG1 transfected cells with Backbone-GFP showed CXCR-4-PE positive. **C.** Overexpression of miR-625-5p in KG1 cells significantly decreased cells expressing CXCR-4compared to Backbone (**P<0.01).

ILK and NF-κB are potential downstream targets of miR-625-5p

To figure out the miR-625-5p-mediated invasion molecular mechanism in KG1 cells, we identified the targets of miR-625-5p. The sequence analysis of ILK demonstrated that ILK harbored potential miR-625-5p target sites at 136-143nt, which are the ILK 3′UTR, and the sequence analysis of NF-κB indicated that NF-κB harbored potential miR-625-5p target site 3′UTR of the microRNA.org site. Regarding the correlation of miR-625-5p to ILK, the ILK and downstream oncogenes mRNA levels were measured in the KG1 cell line. Cell mRNA was used 48 hours after transfection to evaluate changes in the expression of *ILK, AKT, GSK3β, C-FOS (AP1), MMP-9, NF*-κB genes using qRT-PCR (Fig.4). Our results from qRT-PCR demonstrated that the expression of ILK, as the main target at the mRNA level, was dramatically reduced in KG1 compared with the Backbone-transfected KG1 cell line. The expression of *ILK* in these cells caused a significant decrease (0.53 times lower than Backbone group) (P<0.01). The expression of the *NF*-κB and *COX2* genes decreased [0.55 (P<0.01), 0.32 (P<0.001), respectively] and the expression of genes *MMP-9, C-FOS (AP1)* and *AKT* increased [1.36 (P<0.01), 3 (P<0/001) and 1.43 (P<0.01), respectively]; however *GSK3β* did not show a significant change (0.85 with P>0.05).
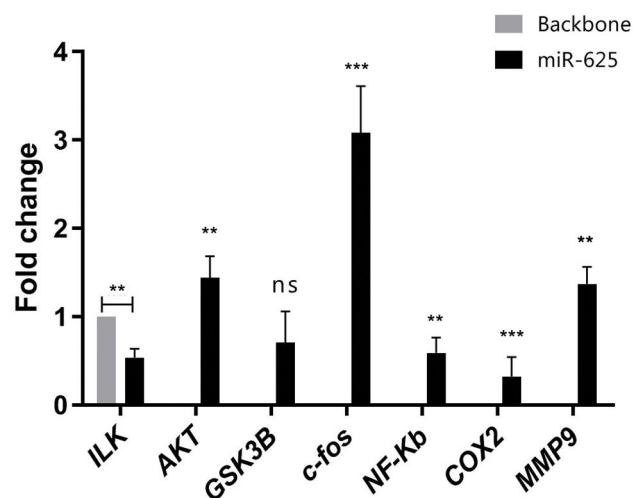

Fig.4: Expression of *ILK, AKT, GSK3β, AP1, MMP-9, NF-*κB genes by qPCR. KG1 cells transfected either with the premiR-625-5p construct or Backbone vector followed by expression evaluation *ILK, AKT, GSK3β, AP1, MMP-9, NF-*κB genes 48 hours after transfection. Overexpressed miR-625-5p resulted in downregulation of *ILK, NF-*κB and *COX2* expression and upregulation of *AKT, MMP-9* and *C-FOS* (*AP1*) but caused no alteration in *GSK3β* expression (P<0.01, P<0.001). In the diagrams, P<0.01 and P<0.001 are shown with ** and ***, respectively. qPCR; Quantitative real time polymerase chain reaction and ns; Not significant.

Overexpression MiR-625-5p reduction of NF-κB expression of protein

The western blotting was performed to evaluate the expression of NF-κB, MMP-9 and β-Actin proteins in invasion KG1 cells compared with the Backbone cells 48 hours after transfection. As illustrated in Figure 5A, the NF-kB protein showed a significant reduction of 0.6% fold (P<0.01) when it transfected with miR-625-5p versus the backbone group and MMP9 protein expression that did not show a significant change (Fig.5B). The results showed that miR-625-5p could inhibit cell invasion by inhibiting ILK and NF-κB as well as the COX-2 signaling pathway.
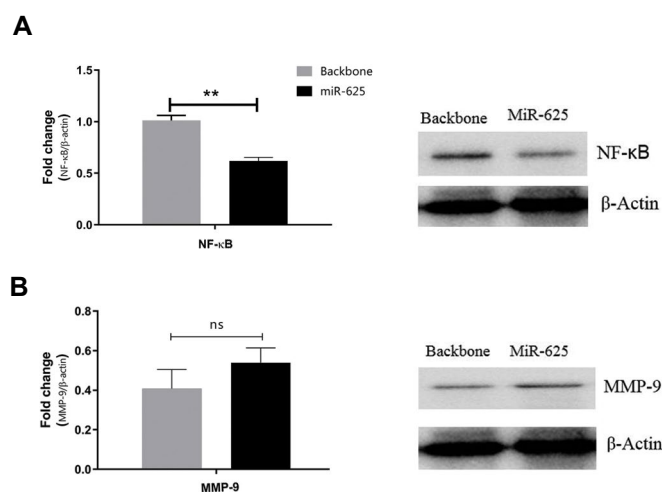
A

B

Fig.5: Western blot and Densitometry analysis of NF-κB and MMP-9 protein expression in KG1 cells transfected by either premiR625-5p construct or Backbone vector 48 hours post-transfection. **A.** miR-625-5p downregulated (0.6 fold lower) NF-κB protein. **B.** miR-625-5p caused no alteration in MMP-9 expression. Densitometry analysis of bands by ImageJ software. β-actin was used as loading control (P<0.01). In the diagrams, P<0.01 is shown with (**).

Discussion

The unnatural expression of miRNAs has already been investigated in different cancers, focusing on the understanding of the role and function of miRNAs in cancer progression (19). Here, we assessed miR-625-5p-mediated invasion molecular mechanism in AML cells (20). MiR-625-5p-transfected KG1 cells of invasion significantly decreased. The expression levels of ILK, NF-κB, and COX2 genes significantly decreased while MMP9, AP1, and AKT significantly increased, whereas GSK3β did not change significantly. At the protein level, NF-κB, decreased and MMP9 increased but not significantly. The expression of CXCR4 was also significantly lower.

Our results also showed that miR-625-5p inhibited cell invasion and migration in AML cells. Surprisingly, we found ILK as a possible target for miR-625-5p. We observed that miR-625-5p exhibited its tumor-suppressing function in the downregulation of cell invasion by regulating the ILK-NF-ƙB-COX2 pathways. Our study may therefore offer a new strategy for AML treatment through the upregulation miR-625-5p level.

AML is a heterozygous disease in which cell proliferation is high and apoptosis is low, and therefore its treatment is challenging due to the unknown pathogenic and intrinsic biological agents (21). The identification of AML invasion mechanisms may culminate in innovative therapeutic methods, an increase in treatment rate, and a decrease in the recurrence rate (4). Various types of miR-RNAs play part in AML and other cancers. For example, miR-625-5p is a potential biomolecule playing part in regulating cell survival and differentiation. Studies have shown miR-625-5p expression is often reduced and acts as a tumor suppressor in various tumors, including hepatocellular carcinoma, breast cancer, and malignant melanoma (22). It has also been demonstrated that miR-625-5p expression decreases in AML cell lines (23).

The expression of this miR can inhibit the proliferation and invasion of cancer cells (7). In the study Ma et al. (14) increased expression of mir-625-5p led to decreased apoptosis and cellular metastasis in patients with AML. In another study, Wang et al. (8) showed that miR-625-5p upregulation in gastric cancer resulted in a decrease in invasion through interfering with the regulation of the ILK signaling pathway. Our findings revealed that invasion was significantly decreased in KG1 cells following overexpression of miR-625-5p. In the current study, we addressed the potential anticancer effect of miR-625-5p to assist in the treatment of AML (11).

According to a previous study, the expression of ILK has constitutive activation in AML (12). Inhibition of ILK by compound-22 causes inhibition of migration, invasion, and proliferation in CML, AML, and ALL (24). ILK is a direct target of miR-625-5p, and miR-625-5p upregulation in KG1 cells results in ILK expression downregulation (8) followed by downregulation of AKT of NF-κB and COX2, resulting in invasion (25, 26). AKT1 activates the proliferation and invasion pathways in breast tumors, colorectal cancer, and leukemia (27). AKT1 seems to play an essential yet passive part in oncogenesis. AKT is activated directly via PIP3 and ILK (10). In reality, the PI3K/AKT signaling pathway contributes greatly to regulating cellular processes by which cancer is characterized, such as cell proliferation, survival, and migration (28). AKT can activate NF-κB and therefore NF-κB is an important marker in cancer cells involved in growth-independent propagation, apoptosis prevention, infiltrate replication, invasion, and tissue metastasis. ILK also leads to the activation of COX2 via the ILK-AKT-NF-κB pathway (25, 29). Previous studies have shown that the expression of COX2 increases in AML (30). Our results also showed that overexpression of miR-625-5p resulted in the downregulation of ILK, NF-κB, and COX2 because of miR-625-5p, according to microRNA.org, directly inhibited ILK and NF-κB and subsequently invasion. Overexpression of miR-625-5p led to the upregulation of AKT because AKT was separately activated via PIP3.

Ample evidence demonstrates that ILK activates the GSK3β-AP1-MMP9 signaling pathway. GSK3β contributes substantially to the cytoskeletal organization, cell polarity, and migration in organogenesis and wound healing physiological processes (2). GSK3β also contributes to cancer cell motility, migration, and invasion via a pharmacological inhibitor and, through the interference of RNA, reduces the capacity of migration and invasion of pancreatic cancer glioblastoma cells, resulting in the decrease of MMP-2 expression (31). AKT positively regulates these targets through the inhibition of GSK3 as well (32). Then, the increase in the expression of c-fos (AP1), in addition to AP1-related target genes, has been observed in many cancers. The expression of ILK induces expression of MMP-9 through the activation of AP1 transcription, causing the increase of migration and invasion. MMPs are indeed a family of endopeptidases that are functionally and structurally zinc-dependent and are responsible for the destruction of ECM components, and thus regulate metastasis and invasive tumor cells. The expression of MMPs is controlled by upstream regulation of sequences and has a connection point for AP1. MMP-9 expression increases in malignant cancers (33). In our study the miR-625-5p expression did not change the expression level of MMP-9.

Lou et al. (34) investigated osteosarcoma (OS) and its miR-625 related effects. miR-625 expression enhanced by mimic-miR-625 substantially decreased the invasion and proliferation of OS cells through the *YAP-1* gene, an important target for the treatment of OS. Wang et al. (8) studied the expression of miR-625, which contributes importantly to cancer progression. By inducing and increasing the expression of this miR in gastric cancer cells, they found that metastasis and tumor invasion were inhibited by the ILK signaling pathway and ILK was miR-625-5p's direct target.

Generally, in agreement with the results of Wang et al. (8) and Lou et al. (34). the current study indicated that

miR-625's inhibitory effect on invasion was similar to its oncogenic effects (35). In our study, similar to the findings of previous studies the overexpression of miR-625-5p altered the expression level of ILK, NF-κB, and COX2. The results of our study regarding ILK expression and invasion are consistent with the this study.

CXCR-4 is a chemokine receptor coupled to G proteins, which are expressed on the HSCs. Indeed, CXCL-12 is coupled to the CXCR-4 receptor on the surface of HSCs, which is a chemokine playing a highly important role in maintaining bone marrow, silence, implantation, survival, leading to the maintenance of the function of HSCs and gene expression, and cell migration by downstream B kinase (AKT)/(MAPK) signaling pathway activity (35). In AML patients, CXCR4 expression is significantly up-regulated and has a poor prognosis. In leukemia, CXCR4 causes the adhesion of leukemia cells to bone marrow stromal cells, resulting in resistance to chemotherapy and extramedullary infiltration into organs expressing SDF-1 (36). Metastasis of cancer cells occurs through the activation of CXCR-4 and the migration of cancer cells towards the organs expressing CXCL-12. Also, SDF-1a regulates leukemia cell trafficking through binding its cognate receptor CXCR4 on leukemia cells. In the BM, disturbance and destruction of cell anchorage by SDF1-CXCR-4 via proteolytic enzymes such as MMP-9 can lead to cellular development in the bloodstream. The CXCR-4-CXCL-12 axis is powered by AML and is a regulator of cell invasion, mobilization, implantation, and maintenance of leukemia stem cells during the onset and progression of the disease (37, 38). Panneerselvam et al. (39) reported that IL-24 disrupted the SDF-1/CXCR4 signaling axis and reduced CXCR4 expression and finally inhibited the invasion and migration of lung cancer cells. The study of Zuo et al. (40) indicated that CXCR4 overexpression enhanced cell motility and invasion by producing EGFR and MMP-9 in lung cancer (NSCLC). The results of our study regarding the expression of CXCR-4 are consistent with the studies of Panneerselvam et al. (39) and Zuo et al. (40) in which miR-625-5p overexpression caused CXCR-4 expression reduction.

Conclusion

The upregulation of the miR-625-5p expression leads to a reduction in cellular invasion in AML cell lines via the ILK pathway signaling via AKT-NF-κB-COX2 pathway. Based on the findings, that show that miR-625-5p leads to a reduction in cell invasion in the AML cell line by ILK pathway, this strategy could be a breakthrough in future AML-related research. However, further studies are needed to achieve this goal.

Acknowledgments

Authors' Contributions

S.H.M, M.E.; Contributed to conception, design, statistical analysis, interpretation of data, and were responsible for overall supervision. S.S.D; Contributed to all of the experimental works, drafted the manuscript and designed the figures which was revised by S.H.M, Sh.A., A.A.H.A. Sh.A., A.A.H.A.; Also contributed to the interpretation of the results. B.A., M.M.; Contributed to some experimental works and did transfection. All authors read and approved the final manuscript.

References

1. Ye Q, Liao X, Fu P, Dou J, Chen K, Jiang H. Portulacerebroside A inhibits adhesion, migration, and invasion of human leukemia HL60 cells and U937 cells through the regulation of p38/JNK signaling pathway. Onco Targets Ther. 2016; 9: 6953.
2. Domoto T, Pyko IV, Furuta T, Miyashita K, Uehara M, Shimasaki T, et al. Glycogen synthase kinase-3β is a pivotal mediator of cancer invasion and resistance to therapy. Cancer Sci. 2016; 107(10): 1363-1372.
3. Stuelten CH, Parent CA, Montell DJ. Cell motility in cancer invasion and metastasis: insights from simple model organisms. Nat Rev Cancer. 2018; 18(5): 296-312.
4. Ye Q, Fu P, Dou J, Wang N. Downregulation of PDIA3 inhibits proliferation and invasion of human acute myeloid leukemia cells. Onco Targets Ther. 2018; 11: 2925-2935.
5. Gupta GP, Massagué J. Cancer metastasis: building a framework. Cell. 2006; 127(4): 679-695.
6. Li JY, Liang ZA, Zheng J. MicroRNA-625 serves as a tumor suppressor in non-small cell lung cancer through targeting SOX4. Int J Clin Exp Med. 2018; 11(4): 3225-3234.
7. Lou X, Qi X, Zhang Y, Long H, Yang J. Decreased expression of microRNA-625 is associated with tumor metastasis and poor prognosis in patients with colorectal cancer. J Surg Oncol. 2013; 108(4): 230-235.
8. Wang M, Li C, Nie H, Lv X, Qu Y, Yu B, et al. Down-regulated miR-625 suppresses invasion and metastasis of gastric cancer by targeting ILK. FEBS Lett. 2012; 586(16): 2382-2388.
9. Wu C. PINCH, N(i)ck and the ILK: network wiring at cell-matrix adhesions. Trends Cell Biol. 2005; 15(9): 460-466.
10. Faes S, Dormond O. PI3K and AKT: unfaithful partners in cancer. Int J Mol Sci. 2015; 16(9): 21138-21152.
11. Krenn PW, Hofbauer SW, Pucher S, Hutterer E, Hinterseer E, Denk U, et al. ILK induction in lymphoid organs by a TNFα-NF-κB-regulated pathway promotes the development of chronic lymphocytic leukemia. Cancer Res. 2016; 76(8): 2186-2196.
12. Alasseiri M, Ahmed AU, Williams BRG. Mechanisms and consequences of constitutive activation of integrin-linked kinase in acute myeloid leukemia. Cytokine Growth Factor Rev. 2018; 43: 1-7.
13. Wang Z, Qiao Q, Chen M, Li X, Wang Z, Liu C, et al. miR-625 down-regulation promotes proliferation and invasion in esophageal cancer by targeting Sox2. FEBS Lett. 2014; 588(6): 915-921.
14. Ma L, Wang YY, Jiang P. LncRNA LINC00909 promotes cell proliferation and metastasis in pediatric acute myeloid leukemia via miR-625-mediated modulation of Wnt/β-catenin signaling. Biochem Biophys Res Commun. 2020; 527(3): 654-661.
15. Zheng J, Fu L, Li Z, Shi Q, Li J, Chen S. RNA interfering of CXCR4 inhibits the adhesion, invasion, and tumorigenicity of acute monocytic leukemic cells in vivo. Int J Clin Exp Pathol. 2018; 11(1): 68-75.
16. Khosravi MA, Abbasalipour M, Concordet JP, Berg JV, Zeinali S, Arashkia A, et al. Expression analysis data of BCL11A and γ-globin genes in KU812 and KG-1 cell lines after CRISPR/Cas9-mediated BCL11A enhancer deletion. Data Brief. 2019; 28: 104974.
17. Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, et al. Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res. 2005; 33(20): e179.
18. Pan L, Li Y, Zhang HY, Zheng Y, Liu XL, Hu Z, et al. DHX15 is associated with poor prognosis in acute myeloid leukemia (AML) and regulates cell apoptosis via the NF-kB signaling pathway. Onco-

target. 2017; 8(52): 89643-89654.

19. Landgraf P, Rusu M, Sheridan R, Sewer A, Iovino N, Aravin A, et al. A mammalian microRNA expression atlas based on small RNA library sequencing. Cell. 2007; 129(7): 1401-1414.

20. Vidigal JA, Ventura A. The biological functions of miRNAs: lessons from in vivo studies. Trends Cell Biol. 2015; 25(3): 137-147.

21. Stone RM, Mandrekar SJ, Sanford BL, Laumann K, Geyer S, Bloomfield CD, et al. Midostaurin plus chemotherapy for acute myeloid leukemia with a FLT3 mutation. N Engl J Med. 2017; 377(5): 454-464.

22. Li JY, Liang ZA, Zheng J. MicroRNA-625 serves as a tumor suppressor in non-small cell lung cancer through targeting SOX4. Int J Clin Exp Med. 2018; 11(4): 3225-32134.

23. Xiong Q, Yang Y, Wang H, Li J, Wang S, Li Y, et al. Characterization of miRNomes in acute and chronic myeloid leukemia cell lines. Genomics Proteomics Bioinformatics. 2014; 12(2): 79-91.

24. de la Puente P, Weisberg E, Muz B, Nonami A, Luderer M, Stone RM, et al. Identification of ILK as a novel therapeutic target for acute and chronic myeloid leukemia. Leuk Res. 2015; 39(11): 1299-1308.

25. Hsu HC, Fong YC, Chang CS, Hsu CJ, Hsu SF, Lin JG, et al. Ultrasound induces cyclooxygenase-2 expression through integrin, integrin-linked kinase, Akt, NF-kappaB and p300 pathway in human chondrocytes. Cell Signal. 2007; 19(11): 2317-2328.

26. Xia M, Duan ML, Tong JH, Xu JG. MiR-26b suppresses tumor cell proliferation, migration and invasion by directly targeting COX-2 in lung cancer. Eur Rev Med Pharmacol Sci. 2015; 19(24): 4728-4737.

27. Zeng Z, Sarbassov dos D, Samudio IJ, Yee KW, Munsell MF, Ellen Jackson C, et al. Rapamycin derivatives reduce mTORC2 signaling and inhibit AKT activation in AML. Blood. 2006; 109(8): 3509-3512.

28. Kumar A, Rajendran V, Sethumadhavan R, Purohit R. AKT kinase pathway: a leading target in cancer research. ScientificWorldJournal. 2013; 2013: 756134.

29. Li C, Li F, Zhao K, Yao J, Cheng Y, Zhao L, et al. LFG-500 inhibits the invasion of cancer cells via down-regulation of PI3K/AKT/NF-κB signaling pathway. PLoS One. 2014; 9(3): e91332.

30. Iachininoto MG, Nuzzolo ER, Bonanno G, Mariotti A, Procoli A, Locatelli F, et al. Cyclooxygenase-2 (COX-2) inhibition constrains indoleamine 2,3-dioxygenase 1 (IDO1) activity in acute myeloid leukaemia cells. Molecules. 2013; 18(9): 10132-10145.

31. Koul D, Shen R, Bergh S, Lu Y, de Groot JF, Liu TJ, et al. Targeting integrin-linked kinase inhibits Akt signaling pathways and decreases tumor progression of human glioblastoma. Mol Cancer Ther. 2005; 4(11): 1681-1688.

32. Manning BD, Toker A. AKT/PKB signaling: navigating the network. Cell. 2017; 169(3): 381-405.

33. Hannigan G, Troussard AA, Dedhar S. Integrin-linked kinase: a cancer therapeutic target unique among its ILK. Nat Rev Cancer. 2005; 5(1): 51-63.

34. Luo Z, Wu G, Zhang D, Liu J, Ran R. microRNA-625 targets Yes-associated protein 1 to suppress cell proliferation and invasion of osteosarcoma. Mol Med Rep. 2018; 17(1): 2005-2011.

35. Domanska UM, Kruizinga RC, Nagengast WB, Timmer-Bosscha H, Huls G, de Vries EG, et al. A review on CXCR4/CXCL12 axis in oncology: no place to hide. Eur J Cancer. 2013; 49(1): 219-130.

36. Matsuo H, Nakamura N, Tomizawa D, Saito AM, Kiyokawa N, Horibe K, et al. CXCR4 overexpression is a poor prognostic factor in pediatric acute myeloid leukemia with low risk: a report from the japanese pediatric leukemia/lymphoma study group. Pediatr Blood Cancer. 2016; 63(8): 1394-1399.

37. Rashidi A, DiPersio JF. Targeting the leukemia-stroma interaction in acute myeloid leukemia: rationale and latest evidence. Ther Adv Hematol. 2016; 7(1): 40-51.

38. Cao T, Ye Y, Liao H, Shuai X, Jin Y, Su J, et al. Relationship between CXC chemokine receptor 4 expression and prognostic significance in acute myeloid leukemia. Medicine (Baltimore). 2019; 98(23): e15948.

39. Panneerselvam J, Jin J, Shanker M, Lauderdale J, Bates J, Wang Q, et al. IL-24 inhibits lung cancer cell migration and invasion by disrupting the SDF-1/CXCR4 signaling axis. PLoS One. 2015;10(3):e0122439.

40. Zuo J, Wen M, Li S, Lv X, Wang L, Ai X, et al. Overexpression of CXCR4 promotes invasion and migration of non-small cell lung cancer via EGFR and MMP-9. Oncol Lett. 2017; 14(6): 7513-7521.