

Boosting effects of MiR-4262 on acute myeloid leukemia advancement via governing KLF6

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Abstract

Background: Purpose of this study was to explore the influence of *miR-4262* on the progression of acute myeloid leukemia (AML) and its molecular mechanism.

Methods: Quantitative real-time polymerase chain reaction (qRT-PCR) was carried out to assess the expression of *miR-4262* in AML serum and cell lines. MTT, Transwell assays and flow cytometry were adopted to investigate the effect of *miR-4262* on cell proliferation, invasion, migration and apoptosis abilities of HL-60 cells respectively. Luciferase reporter assay was conducted to reveal the target relationship of *miR-4262* and *KLF6*. Western blot analysis was utilized to evaluate the expression level of proteins.

Results: Relative expression of *miR-4262* was up-regulated in AML serum and cell lines ($P<0.05$). *miR-4262* expression was closely related to FAB classification ($P=0.002$) of AML patients. *miR-4262* mimics could promote the proliferation, invasion and migration of HL-60 cells, while *miR-4262* inhibitor obviously weakened these biological behaviors. Luciferase assay illustrated that *miR-4262* can directly interact with *KLF6* 3'UTR. Up-regulation of *miR-4262* could decrease *KLF6* level, and increase EGFR level, while the down-regulation of *miR-4262* showed the opposite results. Moreover, *KLF6* gene knockdown reversed the results caused by *miR-4262* inhibitor ($P<0.05$). Inhibition of *KLF6* could significantly promote the proliferation, invasion and migration of HL-60 cells which is caused by *miR-4262* inhibitor.

Conclusions: *miR-4262* was obviously increased in AML serum and cells, it promotes the progression of AML by regulating *KLF6*.

Background

Acute myeloid leukemia (AML) is a heterogeneous hematopoietic stem cell neoplasm, and is the most common malignant myeloid disease in adults, accounting for 80% of adult leukemia [1, 2]. Leukemic cells accumulate in peripheral blood, bone marrow and other tissues, leading to a large reduction of normal blood cells [3]. With the development of science and technology, AML biomarkers have been found continuously, which is very helpful for AML screening, diagnosis, prognosis and monitoring [4]. Although great progress has been made in medicine and chemotherapy, the recurrence rate of AML patients is high, and the overall 5-year survival rate is about 30% - 40%, which is still very low [5]. Heterogeneity of disease and resistance to treatment are two major problems of AML [6]. MiRNAs and miRNAs regulatory networks are widely involved in the pathogenesis, treatment and prognosis of AML [7]. Therefore, it is very important to identify and develop effective novel molecular approaches for the diagnosis and treatment of AML.

MicroRNAs (miRNAs) are a kind of non-coding RNAs that target the 3'untranslated region (3'UTR) of mRNAs and cause translation inhibition or mRNA degradation. They play a key role in regulating cell migration, proliferation, apoptosis and invasion [8-10]. Recently, many miRNAs have been proved to play an important role in AML, such as miR-15/16, miR-34c-5p and miR-182-5p, etc [11-13]. A previous study

showed that *miR-4262* is a cancer gene, which promotes the proliferation of skin malignant melanoma cells through *KLF6* mediated EGFR inactivation and p21 up regulation [14]. *miR-4262* is up-regulated in the bone marrow and serum of AML patients, which is a useful tool for predicting the development and prognosis of AML [15]. This suggested that *miR-4262* plays an important role in AML. Unfortunately, *miR-4262* in AML is still poorly elucidated. Therefore, it is necessary to verify the mechanism of *miR-4262* in the development of AML.

In our research, we explored the expression pattern of *miR-4262* in AML serum and cells, as well as investigated the target gene of *miR-4262*. In addition, the possible mechanism of *miR-4262* in AML progression was also revealed.

Methods

Patients and specimens

This study collected 128 AML patients who visited PanYu Central Hospital. Meanwhile, 109 healthy volunteers were enrolled into the study as controls. The controls were frequently matched with cases both in age and gender. No patients had received any treatment including radiotherapy or chemotherapy before diagnosis. Individuals younger than 18 years old, had other tumors, systemic diseases were excluded from this study. This study was approved by the Ethic committee of PanYu Central Hospital, and written informed consents were also signed by all patients or their family before sampling.

Blood specimens were taken from 128 AML patients and 109 healthy volunteers. Then the blood samples were put into blood collection tube of EDTA and centrifuged to collect leukocytes. Leukocytes were stored at -80°C until used. Clinicopathological features of the patients were listed in **Table 1**, including age, gender, WBC, FAB classification, immunophenotype and lymphadenopathy.

Cell culture

Human leukemia cell lines HL-60 and human embryonic kidney cell lines 293T were purchased from the American Type Culture Collection (ATCC; Rockefeller, MD, USA). Cells were cultured in RPMI 1640 media with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (10,000 U/mL penicillin and 10 mg/mL streptomycin). Then they were incubated at 37°C humidified atmosphere containing 5% CO₂ for cell passage. The media was replaced every two days.

Cell transfection

When the cell density reached approximately 70% confluency, the cells were transfected. *KLF6* siRNA, *KLF6* siRNA-NC, *miR-4262* mimics, *miR-4262* mimics NC (negative control), *miR-4262* inhibitor, *miR-4262* inhibitor NC were purchased from GenePharma (Shanghai, China). They were transfected into HL-60 cells by LipofectamineTM2000 (Invitrogen, CA, USA) according to the manufacturer's instruction, then incubated at 37°C humidified atmosphere with 5% CO₂ for 6h. Medium was replaced and continuing

cultivated for 48h. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to verify whether the transfection was successful.

qRT-PCR

miRNA from leukocytes was performed with the miRNeasy™ RNA isolation kit (Qiagen, Valencia, CA, USA). RNAs of cells were extracted using Trizol method (TaKaRa, Japan). RNA was reverse-transcribed into cDNA by PrimerScript RT reagent kit (Takara, Dalian, China) according to the instruction. PCR amplification was conducted by SYBR Premix Ex Taq™ II kit in ViiATM 7 real-time fluorescent quantitative PCR system. Expression of *KLF6* were normalized to GAPDH, and *miR-4262* was normalized to U6. Relative expression of *miR-4262* was analyzed by $2^{-\Delta\Delta CT}$ method. Every sample was measured at least three times.

MMT assay

MTT method was used to detect the proliferation of HL-60 cells. Cells transfected with *miR-4262* mimics, *miR-4262* mimics NC, *miR-4262* inhibitor, *miR-4262* inhibitor NC were adjusted to 2×10^4 /mL and inoculated on 96-well plates. They were cultured at incubator containing 5% CO₂ at 37°C for 0h, 24h, 48h and 72h respectively. 50µl MTT solution (5mg/mL) were added to cells, then continuously incubated for 4h. Next step, 150µl 20% SDS was added to every well, incubation for overnight at room temperature. Cell proliferation was determined by MTT cell proliferation kit (Cayman Chemical) following the manufacturer's instruction. MTT enzyme-linked immunometric meter was used to measure OD value (490nm).

Transwell assays

Transwell assays were used for the detection of the migration and invasion abilities of HL-60 cells. Transwell chambers were pre-coated by matrigel (BD Biosciences) and 50µl serum-free medium with BSA were added to the upper compartment, 37°C dehydration for 2h. Then upper chamber was added 200µl cell suspension and the lower chamber was 500µl DMEM media with 10% FBS. Then cells were incubated at 37°C incubator with 5% CO₂ for 48h. The invasive cells were stained with 0.1% crystal violet for 30 min. Under the microscope the cells were counted in 7 random sights.

Meanwhile, without matrigel transwell assay was conducted to measure the migrative ability of HL-60 cells.

Apoptosis Analysis

Forty-eight hours after transfection, cells were harvested, stained with propidium iodide and anti-annexin-V antibody (Annexin V-FITC Apoptosis Detection kit, BD Biosciences, San Jose, CA, USA) following the manufacturer's protocol, and stained cells were detected by flow cytometry. The experiments for the apoptosis assay were performed at least three times.

Luciferase reporter assay

In order to verify that KFL-6 is the target gene of *miR-4262*, KFL-6-3'UTR-WT or KFL-6-3'UTR-MUT (100 ng) were co-transfected with 100 nM *miR-4262* mimics or *miR-4262* mimics NC into HL-60 cells using Lipofectamine 2000 (Invitrogen, CA, USA) following the instruction of manufacture. After 48h transfection, HL-60 cells were harvested and measured with a Dual-Luciferase Reporter Assay System Kit (Promega, Madison, WI, USA) according to the manufacturer's instruction.

Western blot

Transfected cells were lysed using RIPA buffer (Thermo Scientific, Belmont, MA, USA) at 4°C for 30min. Cells proteins were extracted and separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a PVDF membrane (Roche) by electroblotting. Nonfat milk was used to block PVDF membrane at 4°C for overnight. Then it was incubated by primary antibodies at 4°C for overnight, including anti-*KLF6* (1:300), anti-EGFR (1:400) (Abcam, Cambridge, MA, USA), and anti-GAPDH (1:800; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), which was used as the internal reference. Then they were incubated 1.5h at room temperature using second antibody (1:2000, Abcam, China). Target band of protein was showed using ECL Western blotting kit (Millipore, Boston, MA, USA).

Statistical analysis

SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) was used for data statistics analysis and GraphPad Prism (GraphPad, San Diego, CA, USA) software was applied for plotting. All experiments were repeated at least in triplicate and the data were expressed using mean±SD (standard deviation). Difference between groups was compared by Student's t-test (Mann-Whitney U test) or χ^2 test. Two-tailed $P<0.05$ indicated statistically significant difference.

Results

miR-4262 expression was up-regulated in AML patients and HL-60 cells

QRT-PCR results showed that relative *miR-4262* level in AML patients serum was significantly increased, compared with healthy controls ($P<0.001$, **Figure 1A**). Similarly, *miR-4262* expression level in HL-60 cells was also significantly higher than that in 293T cells ($P<0.05$, **Figure 1B**).

Relationship between *miR-4262* expression and AML clinical features

128 AML patients were divided into two groups according to the median expression level of *miR-4262*: low expression (n=63) and high expression (n=65) groups. *miR-4262* expression was closely associated with FAB (French–American–British) classification (**Table 1**, $P=0.002$) of AML patients, but it's not related to age, gender, white blood cell count (WBC), immunophenotype and lymphadenopathy ($P>0.05$).

miR-4262 transfection efficiency in HL-60 cells

Relative expression level of *miR-4262* was measured by qRT-PCR in HL-60 cells transfected by *miR-4262* mimics, *miR-4262* inhibitor and corresponding negative controls to detect the transfection efficiency. The results showed that the relative expression level of *miR-4262* in *miR-4262* mimics group was significantly higher than that in *miR-4262* mimics NC group. *miR-4262* expression level in *miR-4262* inhibitor group was significantly decreased, compared with the *miR-4262* inhibitor NC group ($P < 0.05$, **Figure 2**).

***miR-4262* regulated the proliferation, migration, invasion and apoptosis of HL-60 cells**

MTT assay showed that the proliferation ability of HL-60 cells was significantly increased in *miR-4262* mimics group, while that in *miR-4262* inhibitor group was significantly reduced, respectively compared with the corresponding controls ($P < 0.05$, **Figure 3A**). Transwell assays indicated that the migration and invasion number of HL-60 cells in *miR-4262* mimics group were significantly higher than that in *miR-4262* mimics NC group. Moreover, *miR-4262* inhibitor significantly reduced the migration ($P < 0.05$, **Figure 3B**) and invasion (*, $P < 0.05$, **, $P < 0.01$, **Figure 3C**) number of HL-60 cells. In addition, flow cytometry assays suggested that *miR-4262* mimics group significantly reduced the apoptosis ability of HL-60 cells, while that of *miR-4262* inhibitor group was significantly increased, respectively compared with the corresponding controls (*, $P < 0.05$, **, $P < 0.01$, **Figure 3D**). Therefore, *miR-4262* could influence the biological behaviors of AML cells.

KLF6* is identified as a target gene of *miR-4262

Targetscan 7.2 software found that *miR-4262* specifically targeted 3'UTR region of *KLF6*. In this study, the cells transfected with *KLF6*-3'UTR-WT, *miR-4262* mimics had significantly lower luciferase activity; when the binding site was mutated, the above inhibition was lost (**Figure 4**). So, *miR-4262* could play a role in AML by combining with *KLF6* promoter, and *KLF6* is identified as a target gene of *miR-4262*.

***miR-4262* regulated EGFR expression in HL-60 cells**

miR-4262 mimics could significantly inhibit the protein level of *KLF6*, while *miR-4262* inhibitor could significantly enhance the protein level of *KLF6* (**Figure 5**). Meanwhile, EGFR protein expression levels were significantly up-regulated in HL-60 cells with *miR-4262* mimics transfection, but significantly decreased in HL-60 cells transfected by *miR-4262* inhibitor (**Figure 5**), respectively compared with the controls.

***miR-4262* promotes HL-60 cells proliferation, invasion and migration through *KLF6* mediated EGFR**

When *miR-4262* inhibitor + *KLF6* siRNA was transfected, the expression of *KLF6* protein in HL-60 cells decreased significantly, while the expression of EGFR protein increased significantly (**Figure 6**). Moreover, we found that the reduced proliferation, migration and invasion ability of HL-60 cells caused by *miR-4262* inhibitor was reversed by *KLF6* knockdown (**Figure 7 A-C**). The inhibition of *KLF6* expression was also significantly reversed the enhanced the apoptosis ability of HL-60 cells caused by *miR-4262* downexpression (**Figure 7 D**).

Discussion

AML is a highly invasive malignant disease of hematopoietic system, which is characterized by proliferation enhancement, differentiation retardation and apoptosis disorder [16, 17]. Accumulated data show that *miR-4262* plays an important role in various human diseases [18, 19]. AML has the highest mortality, and abnormal expression of miRNAs is involved in the pathogenesis of AML [20]. Therefore, we analyzed the effect of *miR-4262* on the biological behavior and progression of AML in this study.

Previous studies have shown that *miR-4262* is up-regulated in non-small cell lung cancer and glioma tissues, and participates in biological processes such as cell proliferation, migration and apoptosis [21, 22]. In our study, we proved that the expression of *miR-4262* was significantly up-regulated in AML patients and cell lines. This is consistent with the results of a previous study [15]. We also found that the *miR-4262* mRNA expression was closely related to FAB classification. That is also consistent with previous study. Han et al. Indicated that increased *miR-4262* expression was obviously associated with FAB classification and cytogenetics [15]. So, *miR-4262* may be a novel potential therapeutic strategy for AML.

Expression of *miR-4262* in breast cancer tissues and cell lines is increased, which plays the role of oncogene and promotes the proliferation and invasion of breast cancer cells through directly targeting *KLF6* and *KLF15* [23]. However, Weng et al showed that the expression of *miR-4262* in colon cancer was significantly decreased, which may be due to the different expression trends of *miR-4262* in different diseases [18]. In our study, *miR-4262* mimics could promote the proliferation, migration and invasion, as well as inhibit the apoptosis of AML cells, while *miR-4262* inhibitor has the opposite results. In addition, previous studies have shown that a large number of miRNAs are up-regulated in AML, including miR-216b, miR-23a-3p and miR-210 [24-26], and they play an important role in the process of AML. Therefore, *miR-4262* might play an important role in the development and progression of AML.

Wang et al. demonstrated that *miR-4262* targets the 3'-UTR region of *KLF6* and *KLF15* mRNA [23]. In our research, we confirmed that *KLF6* is the target gene of *miR-4262* by luciferase reporter gene analysis. In addition, the overexpression of *miR-4262* can inhibit the expression of *KLF6*, while the transfection of *miR-4262* has the opposite effect. A previous study found that *KLF6* was down regulated in AML [27]. This suggests that *miR-4262* may play a role in AML through *KLF6*.

Zhang et al. Proved that *miR-4262* promotes the proliferation of human cutaneous malignant melanoma cells through *KLF6*-mediated EGFR inactivation and p21 upregulation [14]. Zhuang et al. found that low expression of GLUT3 could promote apoptosis and chemosensitivity of AML cells through EGFR signal [28]. A previous study found that in lung adenocarcinoma, whether in vivo or in vitro, *KLF6* is negatively regulated by the activated EGFR signal, and inhibition of EGFR signal can increase *KLF6* expression [29]. In our study, we found that the expression of *miR-4262* was positively correlated with the expression of EGFR, and the inhibition of *KLF6* could significantly promote the progression of HL-60 cells with *miR-4262* down-expression. This indicated that *miR-4262* promotes the AML process by targeting *KLF6* to regulate the expression of EGFR.

Several shortcomings in this study should be not avoided, although we obtained some achievements. Firstly, the sample size was relatively small, which may reduce the accuracy of our results. Secondly, only one cell line was used in this study, so this result should be verified in other cell lines. Moreover, in vivo experiment was not conducted to verify the relative results. Therefore, further studies are needed to verify our results with well-design and large sample size.

Conclusions

In conclusion, the expression of *miR-4262* is significantly increased in AML patients and cell lines. Overexpression of *miR-4262* can promote the proliferation, migration and invasion of AML cells, and decrease the apoptosis of HL-60 cells. Moreover, *KLF6* is a target of *miR-4262* in the progression of AML through affecting EGFR.

List Of Abbreviations

acute myeloid leukemia (AML)

Quantitative real-time polymerase chain reaction (qRT-PCR)

MicroRNAs (miRNAs)

3'untranslated region (3'UTR)

fetal bovine serum (FBS)

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Declarations

Ethics approval and consent to participate

This study was supported by the Ethics Committee of PanYu Central Hospital and also has been carried out in accordance with the World Medical Association Declaration of Helsinki.

The subjects had been informed the objective. Certainly, written consents were signed by every subject in this study.

Consent for publication

We obtaining permission from participants to publish their data.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

Not applicable.

Authors' contributions

Y.C. design of the work; W.Z. the acquisition, analysis, X.H. interpretation of data; S.C. the creation of new software used in the work; H.Q. have drafted the work or substantively revised it. All authors read and approved the final manuscript.

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Not applicable.

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Table 1

Table 1. The association of *miR-4262* expression with clinical characteristics of AML patients.

Characteristics	No. (n=128)	<i>miR-4262</i> level		<i>P</i>
		Low expression (n=63)	High expression (n=65)	
Age (years)				0.460
≥55	55	25	30	
<55	73	38	35	
Gender				0.350
Male	76	40	36	
Female	52	23	29	
WBC				0.545
<10	46	21	25	
≥10	82	42	40	
FAB Classification				0.002
M1-M6	92	53	39	
M7	36	10	26	
Immunophenotype				0.758
T cell type	27	14	13	
Pre-B cell type	101	49	52	
Lymphadenopathy				0.749
Yes	98	49	49	
No	30	14	16	

Figures

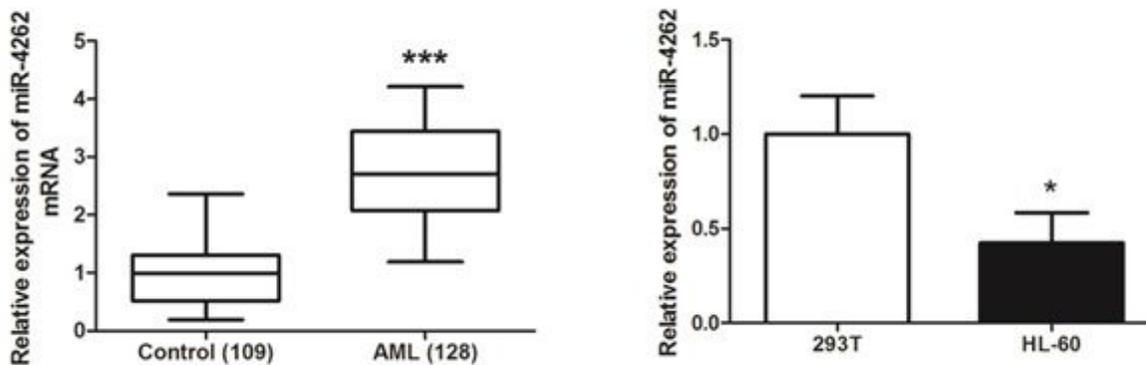


Figure 1

Relative miR-4262 expression AML patients and cell line. A, Relative expression level of miR-4262 was significantly up-regulated in AML patients and than in healthy individuals; B, miR-4262 expression level was up-regulated in human leukemia cell lines HL-60 than in human embryonic kidney cell lines 293T. *, $P < 0.05$, ***, $P < 0.001$.

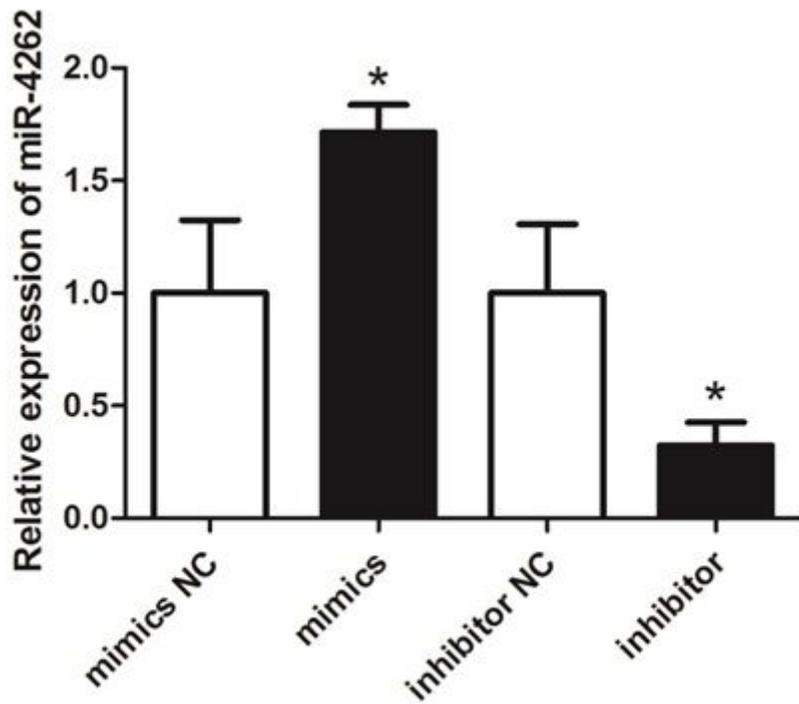


Figure 2

Relative expression level of miR-4262 in HL-60 cells transfected by miR-4262 mimics, miR-4262 inhibitor and corresponding controls. *, $P < 0.05$.

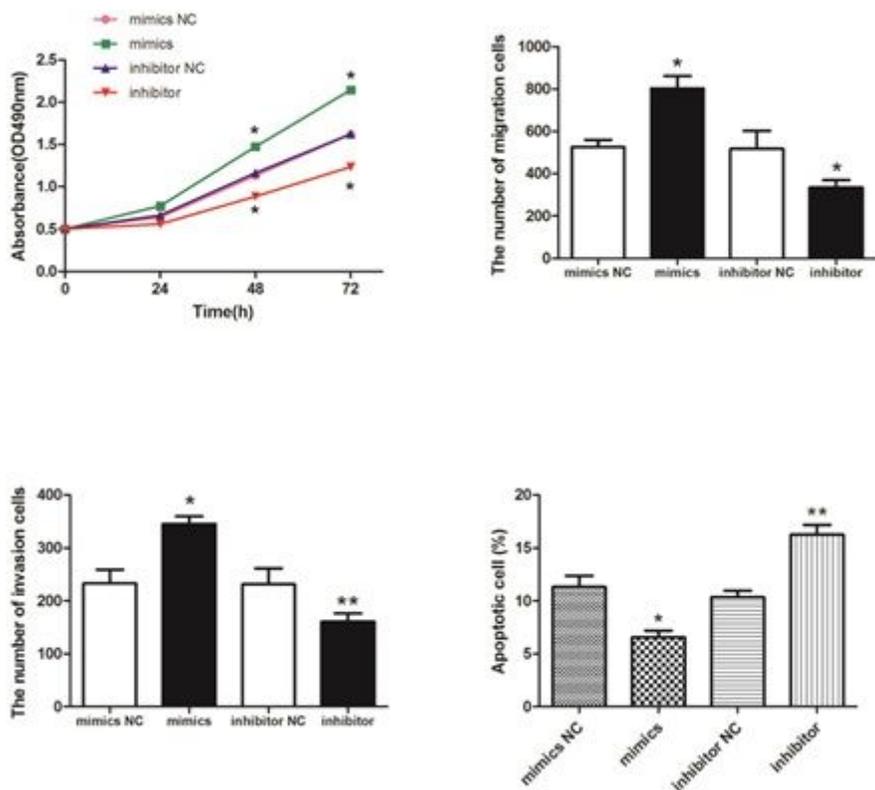


Figure 3

Influence of miR-4262 expression on biological behaviors of HL-60 cells. A, MMT assay showed that miR-4262 mimics significantly increased the proliferation of HL-60 cells and miR-4262 inhibitor reduced cell proliferation; B, Transwell analysis indicated that miR-4262 mimics obviously enhanced the migration of HL-60 cells and miR-4262 inhibitor inhibited cell migration; C, miR-4262 mimics obviously increased the invasion of HL-60 cells and miR-4262 inhibitor decreased cell invasion. D, Flow cytometry assay suggested that miR-4262 mimics obviously decreased the apoptosis of HL-60 cells and miR-4262 inhibitor enhanced cell apoptosis. * $P < 0.05$, ** $P < 0.01$.

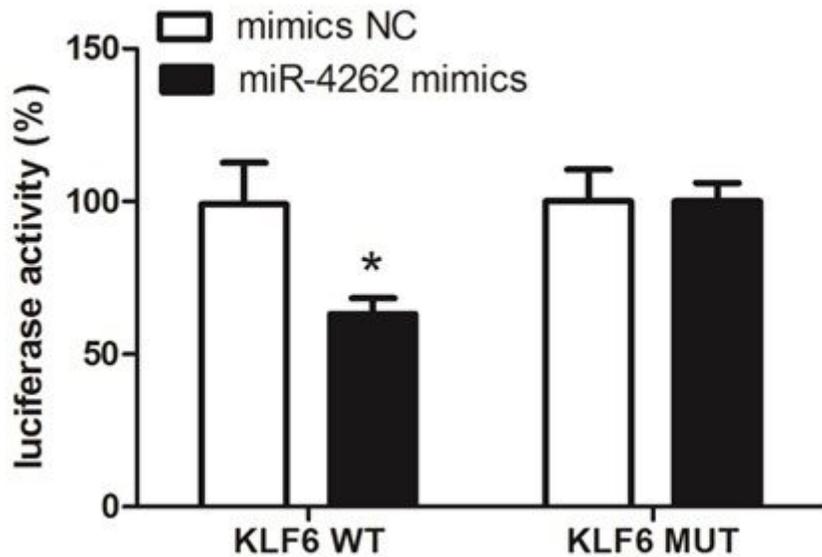


Figure 4

Luciferase activity assay. KLF6 was as the target binding site of miR-4262; *, $P < 0.05$.

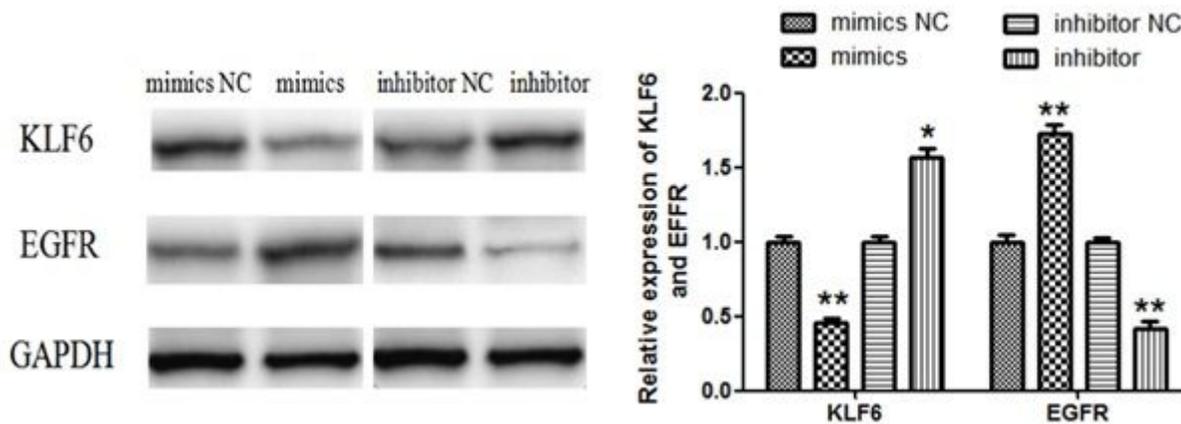


Figure 5

Western blot analysis for expression of KLF6 and EGFR in HL-60 cells. miR-4262 mimics could inhibit relative KLF6 expression and enhance EGFR expression levels than that in corresponding controls. miR-4262 inhibitor could promote relative KLF6 expression and suppress EGFR expression levels than that in corresponding controls. *, $P < 0.05$, ** $P < 0.01$.

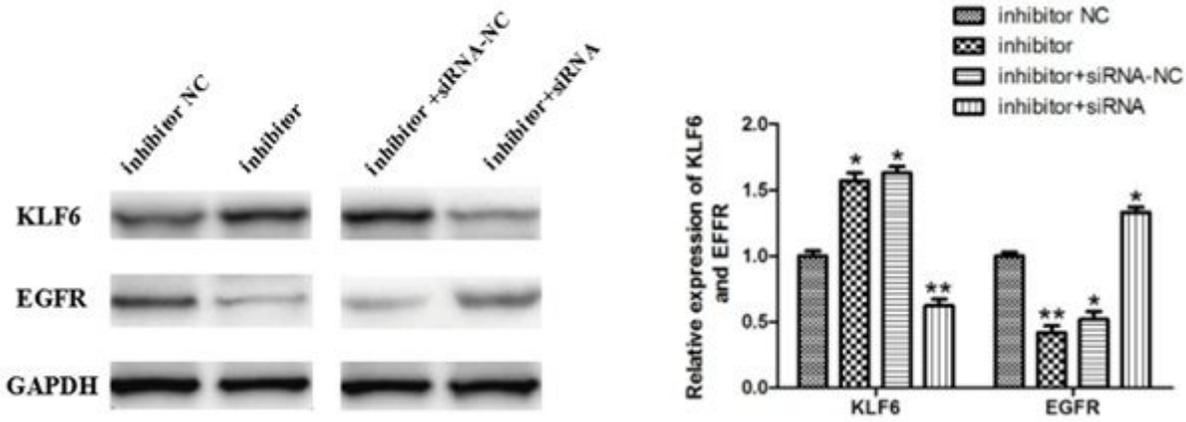


Figure 6

Expression of KLF6 and EGFR were examined by western blot analysis in co-transfected HL-60 cells. In miR-4262 inhibitor + KLF6 siRNA cells, KLF6 expression was decreased significantly, while EGFR expression was increased significantly*, $P<0.05$, ** $P<0.01$.

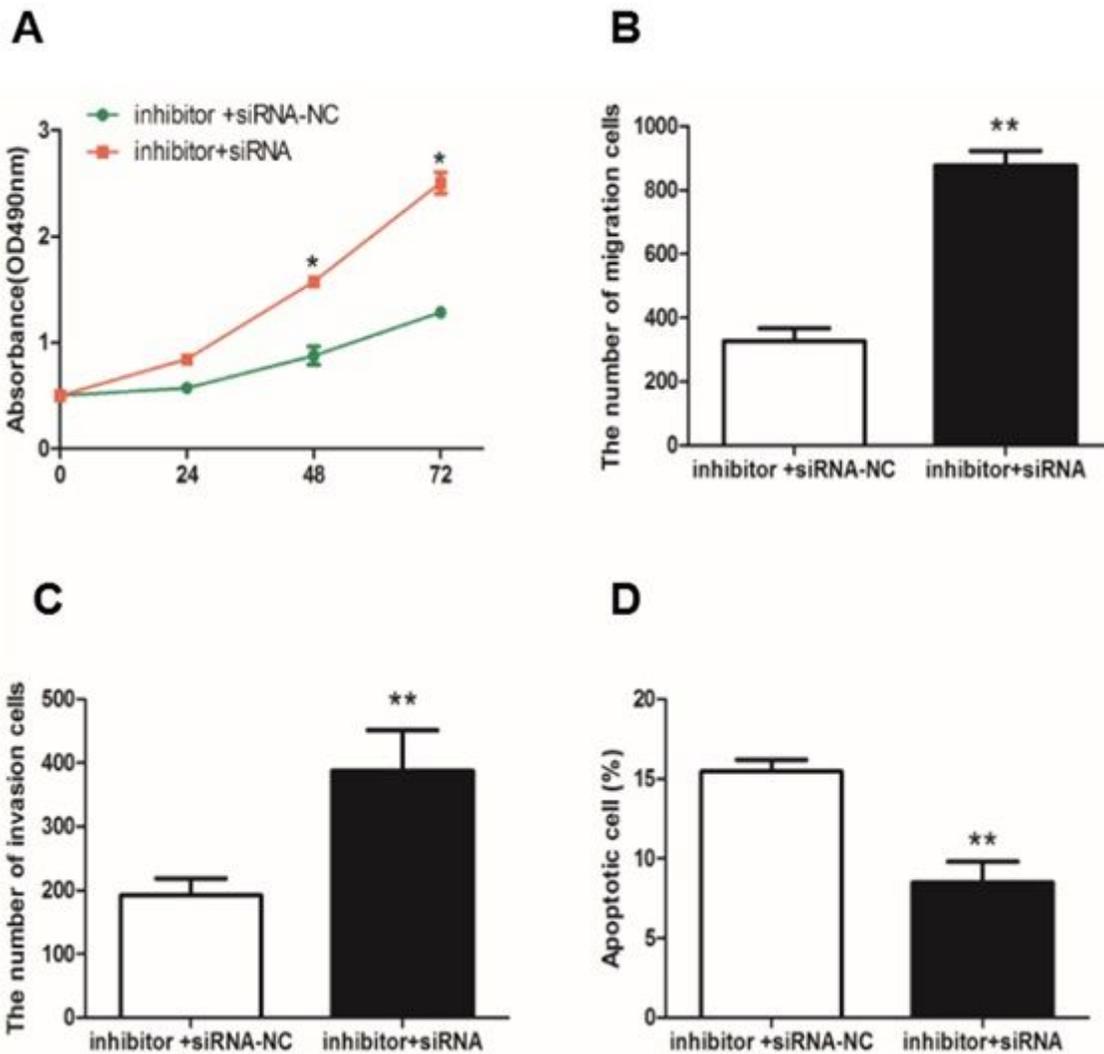


Figure 7

miR-4262 promotes the biological behaviors of HL-60 cells through KLF6 mediated EGFR. A, MMT assay demonstrated that cell proliferation was significantly enhanced in miR-4262 inhibitor+si-RNA group than in miR-4262 inhibitor+siRNA-NC group; B, Migration of HL-60 cells was obviously decreased in miR-4262 inhibitor+siRNA group; C, Invasion of HL-60 cells was obviously decreased in miR-4262 inhibitor+siRNA group; D, Apoptosis ability of HL-60 cells was obviously increased in miR-4262 inhibitor+siRNA group. **P<0.05 and **P<0.01.