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Original Article

MicroRNA-25 promotes T-cell acute lymphoblastic leukemia cell proliferation and invasion by directly targeting EphA8

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Abstract: MicroRNAs (miRNAs) are a class of small non-coding RNAs that have been suggested to play an essential role in tumorigenesis. In the present study, we investigated the role of miR-25 in human T-cell acute lymphoblastic leukaemia (T-ALL). In this study, using qRT-PCR, our data showed that miR-25 was significantly up-regulated in T-ALL. We further found that ectopic expression of miR-25 significantly promoted the proliferation, migration and invasion of T-ALL cells in vitro. Further integrated analysis revealed that EphA8 was as a direct and functional target of miR-25. Luciferase reporter assay demonstrated that miR-25 directly targeted 3'-UTR of EphA8. Knockdown of EphA8 promoted proliferation, migration and invasion of T-ALL cells which was similar with effects of overexpression of miR-25. This study suggested that miR-25 could act as tumor oncogene in T-ALL cell partially via targeting EphA8. Thus, miR-25 may represent a potential therapeutic target for T-ALL intervention.

Keywords: MicroRNAs, miR-25, T-cell acute lymphoblastic leukaemia, EphA8

Introduction

Acute lymphoblastic leukemia (ALL) is a hematologic malignancy arising from hematopoietic precursors of the lymphoid lineage [1]. ALL is the most common leukemia in pediatrics, nearly up to 80% of ALL cases occurs in children [2]. T-cell acute lymphoblastic leukemia (T-ALL) is the ALL transformed from developing thymocytes and resulting from cooperative genetic lesions [3]. Despite the improvements in therapeutic modalities, the prognosis of T-ALL in children is still poor [4]. Thus, a better understanding of the molecular mechanisms will be useful to develop novel therapeutic targets and improve the current therapeutics.

MicroRNA (miRNA) is a class of non-coding small RNA that comprised of about 18-23 nucleotides [5]. MiRNAs regulate the gene expression post-transcriptionally, through its base-pairing with the 3'-untranslated region (3'-UTR) of the messenger RNA (mRNA) of the target gene [6]. MiRNAs are involved in the regulation of many key biological processes including cell proliferation, differentiation and migration [7, 8]. Emerging evidence showed that numerous miRNAs participate in the regulation of T-ALL initiation and progression. For example, Lv et al found that miR-142-3p could serve as an oncogene in T-cell acute lymphoblastic leukaemia by targeting glucocorticoid receptor-α and cAMP/PKA pathways [9]. Zhu et al showed miR-664 negatively regulated PLP2 and promoted cell proliferation and invasion in T-cell acute lymphoblastic leukaemia [10]. Mets et al suggested that miR-193b-3p could act as a tumor suppressor by targeting the MYB oncogene in T-cell acute lymphoblastic leukemia [11]. However, the role of miR-25 in T-ALL cells remains unclear.

In this study, we found that miR-25 was significantly increased in T-ALL and over-expression of miR-25 substantially promoted T-ALL cells proliferation, migration and invasion in vitro. Furthermore, our data showed that EphA8 was one of direct target genes of miR-25, and con-

firmed that miR-25 exerted its effect on the promotion of cell proliferation, migration and invasion by downregulating EphA8 in T-ALL cells. Thus, our study demonstrated that miR-25 could act as a novel miRNA with a potential oncogenic role in T-ALL through targeting of EphA8.

Materials and methods

Patients

This study included 28 patients with confirmed T-ALL. Their diagnosis was based on routine morphological evaluation, immunophenotyping and cytochemical smears using the French-American-British classification. Peripheral blood was obtained from patients (T-ALL) and healthy volunteers (NC). Informed consent was obtained from all patients in accordance with the Declaration of Helsinki and with approval of the Clinical Research Ethics Board of the Zhumadian Central Hospital.

Cell culture and transfection

The human T-ALL cell lines CCRF-CEM and Jurkat were obtained from American Type Culture Collection (ATCC). The CCRF-CEM and Jurkat cell line was cultured in RPMI-1640 media and supplemented with 10% fetal bovine serum (FBS) (Life Technologies). Cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C.

CCRF-CEM and Jurkat cell lines were seeded in 24-well plates at 1×10^5 cells/wells and incubated overnight. Transfection of the miR-25 mimic or miR-NC was taken using Lipofectamine 2000 transfection reagent (Invitrogen). Transfection efficiency was monitored by qRT-PCR.

Cell proliferation

Cell proliferations were measured using a Cell Counting Kit-8 (Dojindo). CCRF-CEM and Jurkat cells were plated in 24-well plates at 1×10^5 cells/wells. Then cells were incubated in 10% CCK-8 which was diluted in normal culture medium at 37°C for color conversion. Proliferation rates were determined at 24, 48 and 72 h after transfection.

Cell migration and invasion assays

Migration and invasion assays were performed using Transwell chambers with a pore size of 8 mm. Cells were transfected with miR-25 mimic or miR-NC and incubated for 24 h. For migration assays, 5×10^4 transfected cells were placed in the upper chamber. RPMI 1640 medium containing 10% FBS was added to the lower chamber as a chemoattractant. Chambers were incubated at 37°C in 5% CO_2 for 24 h, and then cells on the upper surface were removed. Cells that had migrated to the bottom surface were washed twice with cold phosphate-buffered saline, fixed in methanol and stained with 0.1% crystal violet. Stained cells were counted under a microscope. For invasion assays, the upper chamber was precoated with Matrigel. Both assays were performed in three independent experiments.

Luciferase reporter assay

The 3'-UTR untranslated region of EphA8 was amplified by PCR and cloned downstream of the firefly luciferase gene in the pGL3 vector (Promega). The vector was named wild-type (Wt) 3'-UTR. Site-directed mutagenesis of the miR-25 binding site in EphA8 3'-UTR was performed using the Quick change site-directed mutagenesis kit (Stratagene) and named mutant (Mut) 3'-UTR. For reporter assays, Wt or Mut 3'-UTR vector and the control vector pRL-CMV (Promega) were cotransfected. The luciferase assay was performed by using the dual Luciferase reporter assay system (Promega) 48 h after transfection.

RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cells using TRIzol reagent (Invitrogen) following the instructions of the manufacturer. The quantity and quality of RNA were checked with BioPhotometer plus. For the detection of EphA8, cDNA was synthesized by using a ReverTra Ace qPCR RT Kit (TOYOBO). For miR-25, RNA was reverse transcribed using M-MLV reverse transcriptase (Invitrogen) and specific reverse transcription primer (RiboBio). The expression levels of miR-25 and EphA8 were quantified by way of qRT-PCR using Real-time PCR Master mix kit (TOYOBO) and ABI 7500 real-time PCR system (Applied Biosystems) with snRNA U6 as their endogenous reference gene. The PCR reaction consisted of an initial denaturation step (95°C for 60 s), 48 cycles (95°C for 15 s, 60°C for 30 s, 72°C for 45 s) and melting curve analysis. The $2^{-\Delta\Delta CT}$ method was performed to calculate the relative expression and expression levels of negative controls were used as calibrator.

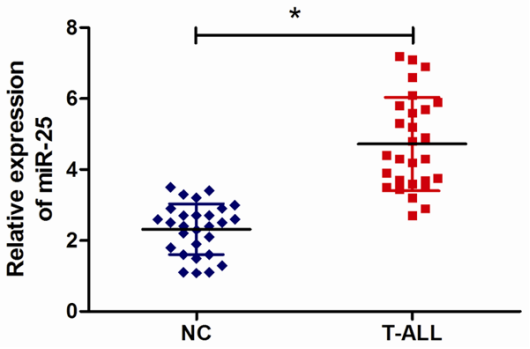

Figure 1. miR-25 was up-regulated in T-ALL. The expression level of miR-25 in T-ALL patients (T-ALL) (n=28) and healthy volunteers (NC) (n=28) were measured by quantitative real-time PCR (qRT-PCR). snRNA U6 was used as an internal control. *P < 0.05.

Western blot

Protein extracts were electrophoresed on 10% SDS polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat dried milk and incubated for over-night with an appropriate primary antibody, followed by the horseradish peroxidase-conjugated secondary antibody. The immunocomplexes were visualized using the chemiluminescence phototope-horseradish-peroxidase kit. GAPDH was used to ensure equivalent protein loading. The integrated density of the band was quantified by ImageJ software.

Statistical analysis

Experiments were repeated at least three times. Statistical analyses were performed using SPSS version 18.0. Values were expressed as means ± SD. Differences between groups were estimated with Student's t test or one-way ANOVA analysis. P < 0.05 was considered significant throughout the study.

Results

miR-25 expression is up-regulated in T-ALL

To explore the potential roles of miR-25 in T-ALL progression, in this study, we detected the expression level of miR-25 in normal T cells and T-leukemic cells from healthy volunteers (NC) and T-ALL patients (T-ALL) by qRT-PCR. Our results revealed that the average expression level of miR-25 was significantly up-regulated in T-leukemic cells compared to the normal T cells samples (**Figure 1**, P < 0.05). These results indicated that miR-25 was significantly increased and play an important role in T-cell acute lymphoblastic leukemia progression.

miR-25 overexpression promotes T-ALL cell proliferation, migration and invasion in vitro

In an attempt to determine the impact of miR-25 on T-ALL progression, CCRF-CEM and Jurkat cells were transfected with miR-25 mimic or miR-NC. As shown in **Figure 2A**, miR-25 mimic significantly increased the expression level of miR-25 in CCRF-CEM and Jurkat cells, respectively. MTT assays were performed to detect the effects of miR-25 on cell growth in vitro. We found that miR-25 overexpression significantly promoted CCRF-CEM and Jurkat cells proliferation compared with their corresponding control (**Figure 2B**). Furthermore, we examined the effect of miR-25 on cell migration and invasion in these cells. Transwell migration and invasion assays showed that the migration (**Figure 2C**) and invasion (**Figure 2D**) capacities of CCRF-CEM and Jurkat cells transfected with miR-25 mimic was significantly increased. Taken together, these findings suggested that miR-25 could function as a tumor oncogene in human T-ALL progression.

miR-25 directly targets EphA8 3'-UTR in T-ALL cells

miRNAs influence cell behavior by regulating the expression of target genes. To explore the molecular mechanism through which miR-25 exerts its function in T-ALL cells, we predicted and identified the candidate target genes of miR-25 by using TargetScan. Our findings revealed that EphA8 was a potential target of miR-25. The 3'-UTR of EphA8 mRNA contains a complementary site for the seed region of miR-25 (**Figure 3A**). Luciferase activity assay showed that miR-25 significantly suppressed the luciferase activity of the wild type (Wt) but not the mutant (Mut) 3'-UTR of EphA8 (**Figure 3B**). Moreover, overexpression of miR-25 significantly inhibited EphA8 expression (**Figure 3C**). These findings suggested that EphA8 was a target of miR-25 in T-ALL cells.

Knockdown of EphA8 promotes T-ALL cell proliferation, migration and invasion in vitro

To investigate the function of EphA8 in T-ALL progression, EphA8 siRNA was transfected into

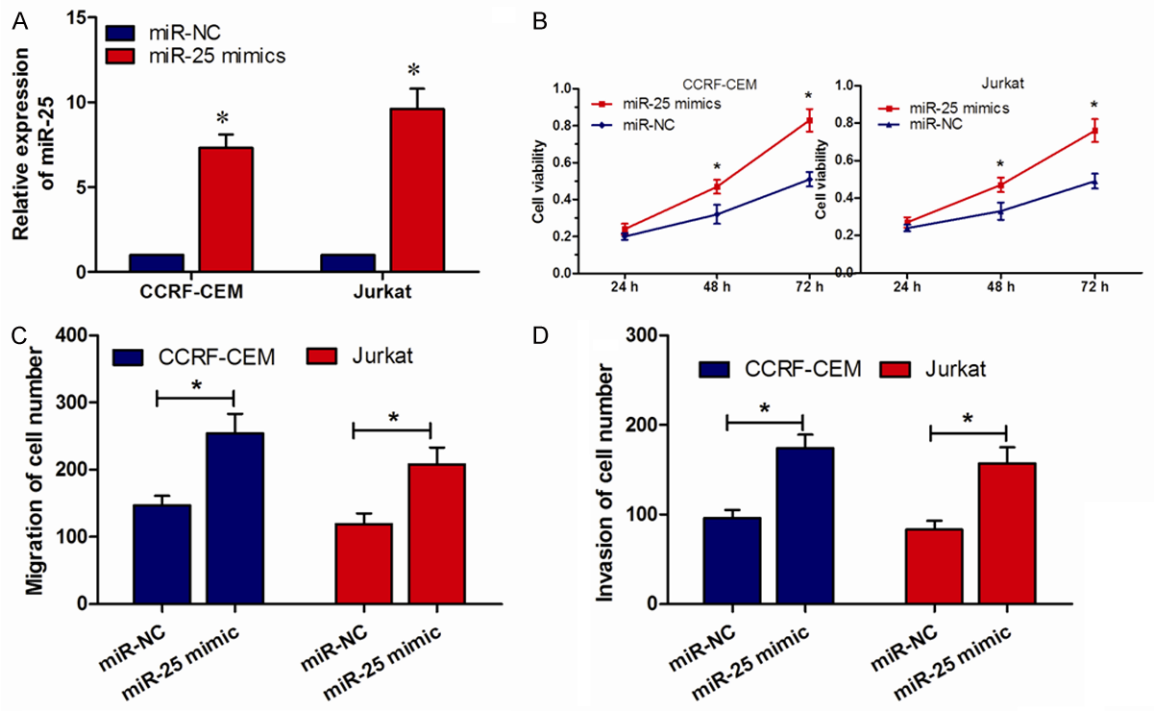

Figure 2. miR-25 promoted T-ALL cell proliferation, migration and invasion. A. Expression of miR-25 in CCRF-CEM and Jurkat cells transfected with miR-25 mimic or miR-NC. B. CCK-8 assay of CCRF-CEM and Jurkat cells transfected with miR-25 mimic or miR-NC. C. Transwell migration assay of CCRF-CEM and Jurkat cells transfected with miR-25 mimic or miR-NC. D. Transwell invasion assay of CCRF-CEM and Jurkat cells transfected with miR-25 mimic or miR-NC. Data were drawn from three independent experiments. *P < 0.05.

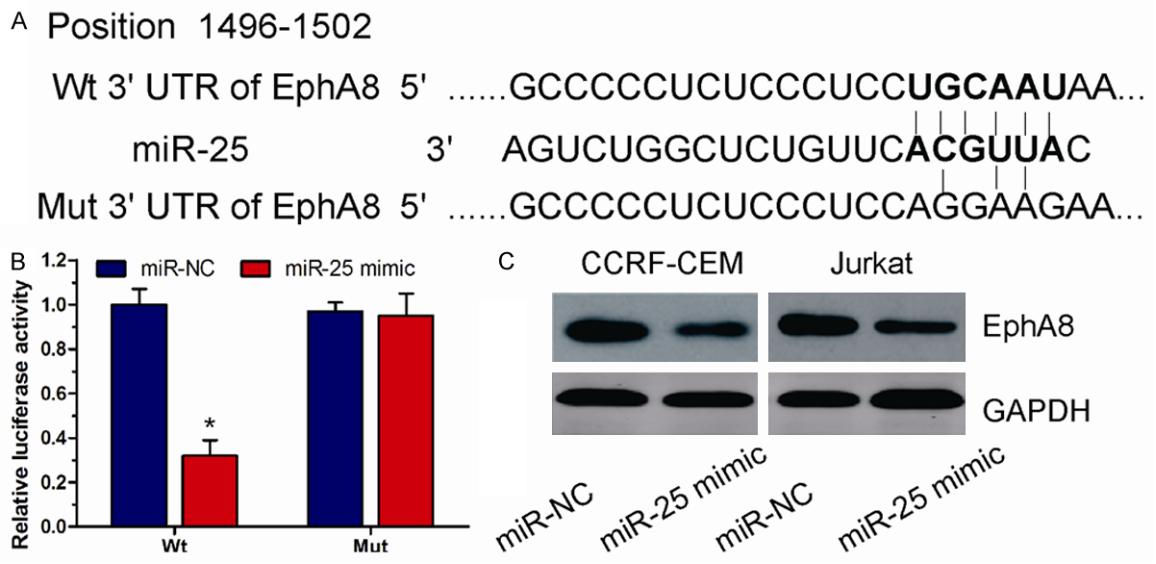

Figure 3. EphA8 was a direct target of miR-25. A. The potential miR-25 binding sites of EphA8 3'-UTR and the mutated sequences. B. Jurkat cells were co-transfected with miR-25 mimic or the vector with Wt or MutEphA8 3'-UTR. Luciferase activity was detected. C. EphA8 protein level was detected by western blot in CCRF-CEM and Jurkat cells transfected with miR-25 mimic or miR-NC. Data were drawn from three independent experiments. *P < 0.05.

CCRF-CEM and Jurkat cells. As shown in **Figure 4A**, EphA8 siRNA significantly decreased the expression of EphA8 in CCRF-CEM and Jurkat cells, respectively. MTT assay revealed that

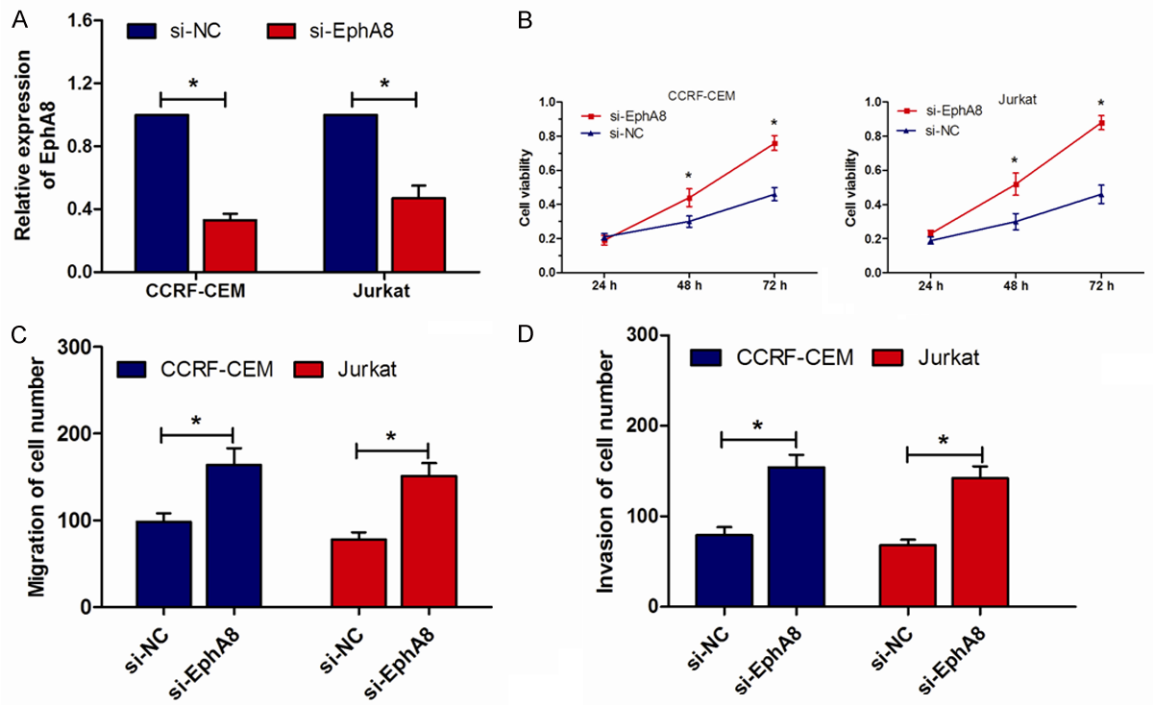

Figure 4. EphA8 inhibition resulted in similar effects as miR-25 overexpression. A. Expression of EphA8 mRNA was detected by qRT-PCR in CCRF-CEM and Jurkat cells transfected with si-EphA8 or si-NC. B. The viability of CCRF-CEM and Jurkat cells transfected with si-EphA8 or si-NC was detected using CCK-8. C. The migration ability of CCRF-CEM and Jurkat cells transfected with si-EphA8 or si-NC was detected using transwell migration assay. D. The invasion ability of CCRF-CEM and Jurkat cells transfected with si-EphA8 or si-NC was detected using transwell invasion assay. Data were drawn from three independent experiments. *P < 0.05.

relative cell growth was significantly promoted in EphA8 siRNA transfected cells (**Figure 4B**). Furthermore, our finding demonstrated that the inhibition of EphA8 by siRNA significantly promoted the migration (**Figure 4C**) and invasion (**Figure 4D**) ability of CCRF-CEM and Jurkat cells in vitro. These data showed that EphA8 knockdown had similar effects as overexpression of miR-25, which indicated miR-25 may function through down-regulating EphA8.

Discussion

Emerging role of dysregulated microRNAs in cancer has been proved by many researches [12]. Therefore, better understanding of miRNA in pathogenesis of malignancy may help find more effective cancer therapy [13]. Recently, aberrant expression of miR-25 was thought to contribute to the malignant phenotype in various types of cancer. For example, Xiang et al showed that miR-25 was increased in non-small cell lung cancer and promotes cell proliferation and motility by targeting FBXW7 [14]. Feng et al reported that miR-25 promoted ovarian cancer proliferation and motility by targeting LATS2 [15]. Li et al suggested that miR-25 could promote gastric cancer migration, invasion and proliferation by targeting ERBB2, 1 and correlated with poor survival [16]. Zoni et al found that miR-25 modulated invasiveness and dissemination of prostate cancer cells via regulation of αv- and α6-Integrin expression [17]. However, the role of miR-25 in the T-ALL progression remains unknown.

In the present study, miR-25, a new regulator of T-ALL, was shown to promote proliferation, migration and invasion of CCRF-CEM and Jurkat cells. To further explore the mechanism by which miR-25 exerted its function, we used bioinformatics analysis to predict and finally identified EphA8 as the direct target of miR-25. This conclusion is based on several experimental evidences. First, miR-25 significantly decreased the expression of EphA8 at the protein levels in T-ALL cells. Second, luciferase activity assay showed that miR-25 significantly suppressed the luciferase activity of the wild type (Wt) but not the mutant (Mut) 3'-UTR of EphA8. Third,

knockdown of EphA8 had similar effects as overexpression of miR-25. These results indicated that miR-25 targets EphA8 and down-regulates its expression in T-ALL.

Ephrin receptors comprise the largest known family of receptor tyrosine kinases (RTKs), which interact with their ligands, ephrins [18]. Accumulating evidence suggested that expression of ephrin ligands and receptors is often reduced in advanced-stage tumors and regulates tumor growth, metastasis and angiogenesis by altering cell proliferation, motility, invasion and migration [19]. Recent genetic studies suggested that EphA8 was involved in regulating cell adhesion and apoptosis [20]. Yan et al showed that miR-10a could control glioma migration and invasion through regulating epithelial mesenchymal transition via EphA8 [21]. However, reports about the relationship between EphA8 and T-ALL were still unclear. Here, our study found that miR-25 could promote T-cell acute lymphoblastic leukemia cell proliferation and invasion by directly targeting EphA8.

In conclusion, the present study reported the tumor oncogenic role of miR-25 in human T-ALL. The expression levels of miR-205 were up-regulated in T-ALL. Up-regulated expression of miR-25 could promote T-ALL cell growth and metastasis in vitro. Furthermore, EphA8 is a direct and functional target of miR-25. miR-25 mediated T-ALL cell growth and metastasis promotion is achieved through suppression of EphA8 genes. Thus, our results provided new insight into the mechanism of T-ALL, and indicated that miR-25 might serve as a potential therapeutic target of T-ALL treatment.

Disclosure of conflict of interest

None.

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