

# MiR-410 regulates malignant biological behavior of pediatric acute lymphoblastic leukemia through targeting FKBP5 and Akt signaling pathway

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**Abstract.** – **OBJECTIVE:** The aim of this study was to investigate the role of miR-410 in regulating the proliferation and apoptosis of pediatric acute lymphoblastic leukemia (ALL) cells and to explore the possible underlying mechanism.

**PATIENTS AND METHODS:** The expression level of miR-410 in ALL cases and cells was detected by real-time fluorescence quantitative polymerase chain reaction (qRT-PCR). Luciferase reporter gene assay was performed to evaluate the interaction between miR-410 and FKBP5. MTT and colony formation assay were used to determine the effect of miR-410 on the proliferation and colony formation ability of ALL cells. The effect of miR-410 on cell apoptosis was measured by Annexin V-fluorescein isothiocyanate 1 (FITC) and propidium iodide (PI). Western blot was used to analyze the effect of miR-410 on the protein expression levels of phosphorylated Akt (p-Akt) and cleaved caspase-3.

**RESULTS:** In our investigation, miR-410 was significantly up-regulated in ALL cases and cells. We searched three public databases to predict the potential target of miR-410, and found that FKBP5 was a direct target of miR-410. Meanwhile, Luciferase reporter gene assay confirmed our hypothesis. The overexpression of miR-410 accelerated the proliferation and colony formation ability of ALL cells, whereas remarkably decreased cell apoptosis rate. Western blotting showed that miR-410 inhibited the activation of Akt signaling pathway. However, FKBP5 could reverse the effects of miR-410.

**CONCLUSIONS:** MiR-410 regulated the proliferation, colony formation and apoptosis of ALL cells through targeting FKBP5 and Akt signaling pathway, indicating that miR-410 might be a potential therapeutic target for the treatment of ALL.

*Key Words:*

MiR-410, Pediatric acute lymphoblastic leukemia (ALL), FKBP5, Akt signaling pathway.

## Introduction

Acute lymphocytic leukemia (ALL), a heterogeneous blood system disease, is caused by abnormal cloning and hyperplasia of primitive and immature lymphocytes in bone marrow and peripheral blood. ALL infiltrates all tissues and organs. Meanwhile, it is the most common childhood leukemia, accounting for 75%-80% of children with acute leukemia<sup>1,2</sup>. With the increasing understanding of molecular genetics and pathogenesis of ALL, as well as the combination of risk grading treatment and new targeted drugs, the cure rate and survival outcome of ALL have been greatly improved in recent decades. However, around 10%-20% childhood ALL cannot achieve long-term remission<sup>3</sup>. Therefore, an in-depth study on its pathological mechanism is of great significance to find a more effective diagnosis and treatment therapy. Recent researches have shown that abnormal and complex biological processes of multiple genes are involved in the occurrence and development of ALL. Moreover, its specific molecular and regulatory mechanisms are still under investigation. It has also been demonstrated that abnormal regulation of micro ribonucleic acid (miRNA)-related pathways is an important factor in the occurrence and development of ALL.

MiRNAs are a type of endogenous, non-coding RNAs with about 18-22 nucleotides in

length. Acting as the expressions of regulatory factors and controlling genes after transcription<sup>4</sup>, miRNAs play an extremely important role in the development of multiple diseases. Studies have shown that abnormal expression of miRNAs is closely related to biological behaviors in children with ALL, such as diagnosis, drug resistance and prognosis. For example, increased level of miR-19b is positively correlated with poor prognosis of pediatric ALL<sup>5</sup>. MiR-410 can act as a potential predictive biomarker for evaluating the therapeutic effect of pediatric ALL<sup>6</sup>. Meanwhile, low expression of miR-18a may be a characteristic diagnostic marker for pediatric ALL<sup>7</sup>. In addition, low expression of miR-210 eventually predicts drug resistance of chemotherapy<sup>8</sup>.

As a member of the miRNA family, miR-410 has shown its unique advantages in the diagnosis and treatment of a variety of diseases, such as systemic lupus erythematosus (SLE)<sup>9</sup>, cardiac hypertrophy<sup>10</sup>, anxiety-related behavior<sup>11</sup>, osteosarcoma<sup>12</sup>, and non-small cell lung cancer (NSCLC)<sup>13</sup>. However, few reports have investigated the role of miR-410 in the development of pediatric ALL and its possible underlying molecular mechanism. In this work, we detected the expression of miR-410 in ALL cases and cells, and further studied the biological role of miR-410 in the development of ALL.

## Patients and Methods

### *Clinical Cases and Cell Lines*

From July 2012 to April 2017, 38 newly diagnosed pediatric ALL cases treated in the Department of Hematology were enrolled in this investigation and bone marrow specimens were collected. Newly diagnosed ALL cases included 22 males and 16 females, with a median age of 4 (1-14) years old. All of the cases were definitely diagnosed and confirmed ALL by clinical medicine, morphology, immunology, cytogenetics and molecular biology. The diagnosis and treatment of ALL cases enrolled were conducted in strict accordance with the standard of the CCLG-ALL 2008 Program. Meanwhile, 15 children (8 males and 7 females) with idiopathic thrombocytopenic purpura (ITP) were included as normal controls, with a median age of 4.5 (2-12) years old. This study was approved by the Ethics Committee of our hospital. The informed consent was obtained from each patient before the study.

Human ALL cell line CCRF-CEM and human embryonic kidney cell line HEK-293T were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Invitrogen, Carlsbad, CA, USA) complemented with 10% fetal bovine serum, 100 µg/mL streptomycin and 100 IU/mL penicillin (Invitrogen, Carlsbad, CA, USA) in a 37°C, 5% CO<sub>2</sub> incubator.

### *Luciferase Reporter Gene Assays*

TargetScan, miRDB, and microRNA websites predicted that FKBP5 was a target gene of miR-410. The binding sequence of miR-410 at the 3'-UTR of FKBP5 was mutated by using a point mutation kit (Agilent Technologies, Santa Clara, CA, USA). Subsequently, mutant (Mut-type) and non-mutant FKBP5 (WT-type) were connected with the pGL3-Basic Luciferase reporter vector (Promega, Madison, WI, USA). PGL3-Basic vector with mutant FKBP5 was transfected into CCRF-CEM cells after lentivirus intervention on 24-well plates. Meanwhile, the same treatment was performed for the pGL3-Basic vector connected with non-mutant FKBP5 according to the instructions of the Luciferase Reporter Gene Assay Kit. Finally, Luciferase activity was detected by a multi-function microplate reader.

### *Cell Transfection*

MiR-410 mimics and si-FKBP5 were synthesized and transfected into ALL cells (CCRF-CEM) to analyze the biological function of miR-410. Three groups were established to study the potential relevance between miR-410 and CCRF-CEM cells, including the NC group (negative control), the miR-410 mimics group (CCRF-CEM cells transfected with miR-410 mimics) and the mimics + FKBP5 group (CCRF-CEM cells transfected with miR-410 mimics and si-FKBP5). All the stuff was purchased from RiboBio (Guangzhou, China). Cell transfection was performed according to the manufacturer's instructions of Lipofectamine RNAiMAX (Life Technologies, Gaithersburg, MD, USA).

### *Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)*

Total RNA was extracted from CCRF-CEM cells in accordance with the manufacturer's instructions of TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). SYBR green qPCR assay was used to measure the expression level of FKBP5,

and glyceraldehyde 3-phosphate dehydrogenase (GADPH) was used as an internal reference. TaqMan miRNA assay (Applied Biosystems, Foster City, CA, USA) was performed to detect the expression level of miR-410 normalized to miRNA U6. QRT-PCR reaction conditions were as follows: 94°C for 30 s, 55°C for 30 s and 72°C for 90 s, for a total of 40 cycles. The relative expression level of the target gene was calculated by the  $2^{-\Delta\Delta Ct}$  method. Primers used in this study were as follows: FKBP5, F: 5'-GCCGAACCCCTCCT-TACTC-3', R: 5'-CTTGGGATGTGTCCGAAG-GA-3'; MiR-410, F: 5'-CCAGAAGCTTAT GTC-CCATCCGTCCTCA-3', R: 5'-ACTTGGATC-CGCACAACCCTAGCATCTTC-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTGCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

#### Western Blot Analysis

Total protein in CCRF-CEM cells was extracted by radio-immunoprecipitation assay (RIPA) lysate (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The concentration of extracted protein was detected by the bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Extracted proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Sigma-Aldrich, St. Louis, MO, USA) and transferred with polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). After blocking with 5% skimmed milk, the membranes were incubated with primary antibodies of FKBP5, phosphorylated-Akt (p-Akt), cleaved caspase-3 and GADPH [diluted at 1:1000, Cell Signaling Technology (CST) Inc. Danvers, MA, USA] at 4°C overnight. After washing with Tris-Buffered Saline-Tween 20 (TBST), the membranes were incubated with corresponding secondary antibodies (CST, Inc. Danvers, MA, USA) at room temperature for 1-2 h. Immunoreactive bands were exposed by the enhanced chemiluminescence method (ECL) (Thermo Fisher Scientific, Waltham, MA, USA).  $\beta$ -actin was used as an internal reference. Relative changes in protein expression were finally calculated.

#### Cell Proliferation

When cells grew to the logarithmic growth phase, they were collected, diluted into  $1 \times 10^6$  cell suspension, and seeded into 96-well plates at a density of  $5 \times 10^3/100 \mu\text{L}$  per well. The wells only

added with culture medium were used as blank controls. Cell viability was determined *via* MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) colorimetric assay. Briefly, 15  $\mu\text{L}$  MTT reagents (500  $\mu\text{g}/\text{mL}$ ) was added into each well, followed by incubation for 2 h. The absorbance was measured by using an enzyme-labeled spectrophotometer, and zero setting was performed by using blank controls.

#### Colony Formation Assay

CCRF-CEM cells were digested with trypsin to obtain a single cell suspension. A total of  $6 \times 10^3$  cells were seeded into culture dishes with a diameter of 60 mm and incubated for 14 d. Subsequently, bacterial colonies were fixed, stained with 0.5% crystal violet for 15 min, and then washed with PBS 3 times. 10 randomly selected fields were observed under a light microscope. The number of colonies was counted (cell group consisting of more than 50 cells was taken as one cell colony), and the differences in the number of colonies in each group were compared. The experiment was repeated three times.

#### Cell Apoptosis

48 h after transfection, CCRF-CEM cells were collected, washed with pre-cooled polybutylene succinate (PBS), and re-suspended in 300  $\mu\text{L}$  binding buffer. The cell concentration was adjusted to  $1 \times 10^6$  cells/mL. Next, 100  $\mu\text{L}$  cell suspension was added to the flow tube, followed by 5  $\mu\text{L}$  Annexin V-fluorescein isothiocyanate 1 (FITC) and 5  $\mu\text{L}$  propidium iodide (PI). Then the mixture was incubated at room temperature for 15 min in the dark. 400  $\mu\text{L}$  PBS was added to the reaction tube. Finally, cell apoptosis was detected by a flow cytometer within 1 h.

#### Statistical Analysis

Prism 6.02 software (La Jolla, CA, USA) was used for all statistical analysis. Student's *t*-test or *F*-test was performed to compare the differences among groups. All *p*-values were two-sided, and  $p < 0.05$  were considered statistically significant.

## Results

### MiR-410 Was Down-Regulated in ALL Cases and Cells

To examine the role of miR-410 in ALL development, we first detected its expression level in bone marrow specimens of pediatric ALL and

ITP cases by qRT-PCR. Results showed that the expression level of miR-410 in ALL cases was significantly higher than that of ITP cases (Figure 1A). The same results were obtained *in vitro* (Figure 1B). Taken together, we thought that miR-410 might have a certain regulating effect during the development of ALL.

#### **FKBP5 Was a Direct Target of miR-410**

To elucidate the putative and possible target of miR-410, we checked three publicly available algorithms, including TargetScan, miRDB, and microRNA. FKBP5 was found the potential target of miR-410 (Figure 2A). Thus, FKBP5 caught our attention and was implemented to further studies. Firstly, we detected the expression level of FKBP5 in ALL cells by qRT-PCR, and found that FKBP5 was down-regulated in ALL cells when compared with that of 239T cells (Figure 1C). Then, Luciferase reporter vectors containing wild-type or mutant-type miR-410 seed sequences of the FKBP5 3'UTR were constructed. Increased expression of miR-410 with mimics transfection resulted in the decrease in the Luciferase activity of wild-type FKBP5 3'UTR reporter gene, whereas had no effect on mutant-type (Figure 2B). These results suggested that the expression of FKBP5 could be regulated by miR-410.

#### **MiR-410 Decreased the Expression Level of FKBP5**

Three groups were established in CCRF-CEM cells to conduct similar experiments, including the miR-NC group, the miR-410 mimics group and the mimics + FKBP5 group. Western blot results indicated that up-regulation of miR-410 remarkably decreased the expression level of

FKBP5 in CCRF-CEM cells (Figure 4A). These findings further illustrated the regulatory effect of miR-410 on FKBP5 expression.

#### **MiR-410 Promoted the Proliferation of ALL Cells**

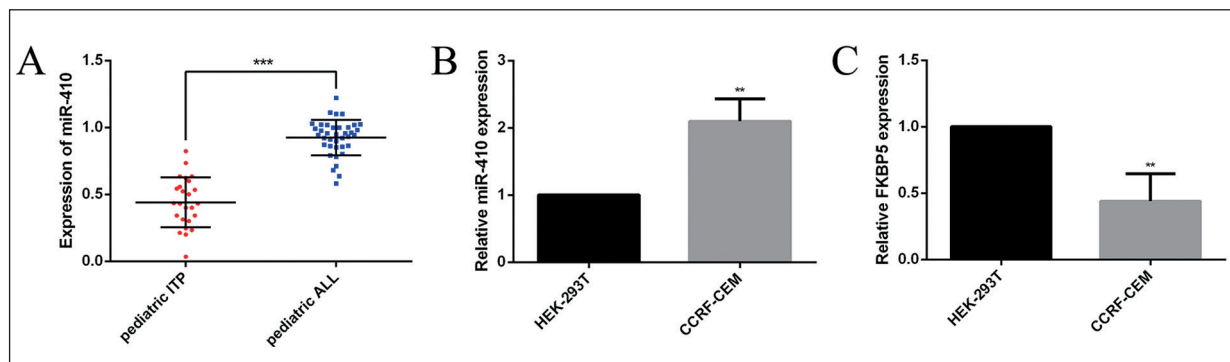
MTT assay was then performed to detect the proliferation rate of ALL cells. Results suggested that the proliferation rate of CCRF-CEM cells was significantly accelerated by miR-410 mimics transfection. In contrast, the proliferation of CCRF-CEM cells was remarkably inhibited in the mimics + FKBP5 group (Figure 3C). Moreover, colonies formed by CCRF-CEM cells after miR-410 mimics transfection were more in number and bigger in size than those formed by control cells (Figure 3A, 3B).

#### **MiR-410 Suppressed the Apoptosis of ALL Cells**

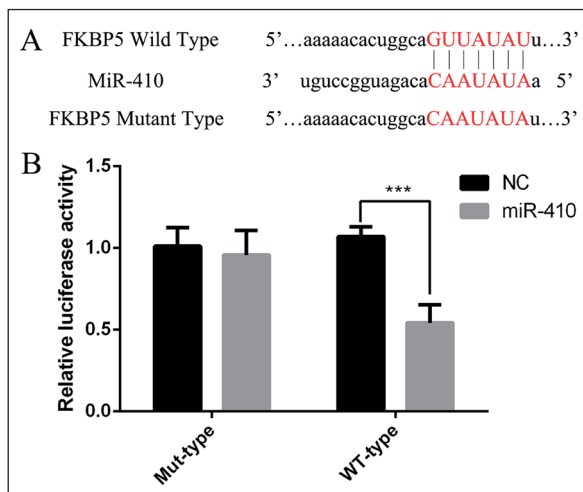
The apoptosis rate of CCRF-CEM cells was detected by flow cytometry. As shown in Figure 3D, after transfection of miR-410 mimics, the percentage of apoptotic cells was significantly decreased. However, the apoptosis rates of the miR-NC group and the mimics + FKBP5 group were both higher (35.85% and 35.41%, respectively), indicating the inhibitory effect of miR-410 on cell apoptosis.

#### **MiR-410 Inhibited the Activation of Akt Signaling Pathway**

As an important factor in life activities, Akt exerts an anti-apoptotic effect *via* the caspase pathway<sup>14</sup>. Therefore, Western blot was employed to detect the effect of miR-410 on the expression levels of the Akt signaling pathway in ALL cells.



**Figure 1.** The expression levels of miR-410 and FKBP5 in ALL bone marrow specimens and cells. **A**, Difference in the expression of miR-410 between ALL and TPI bone marrow specimens. (\*\*\*)  $p < 0.001$ . **B**, and **C**, The expression of miR-410 and FKBP5 in ALL cells (CCRF-CEM) and human embryonic kidney cells (HEK-293T). (\*\*  $p < 0.01$  compared with HEK-293T).



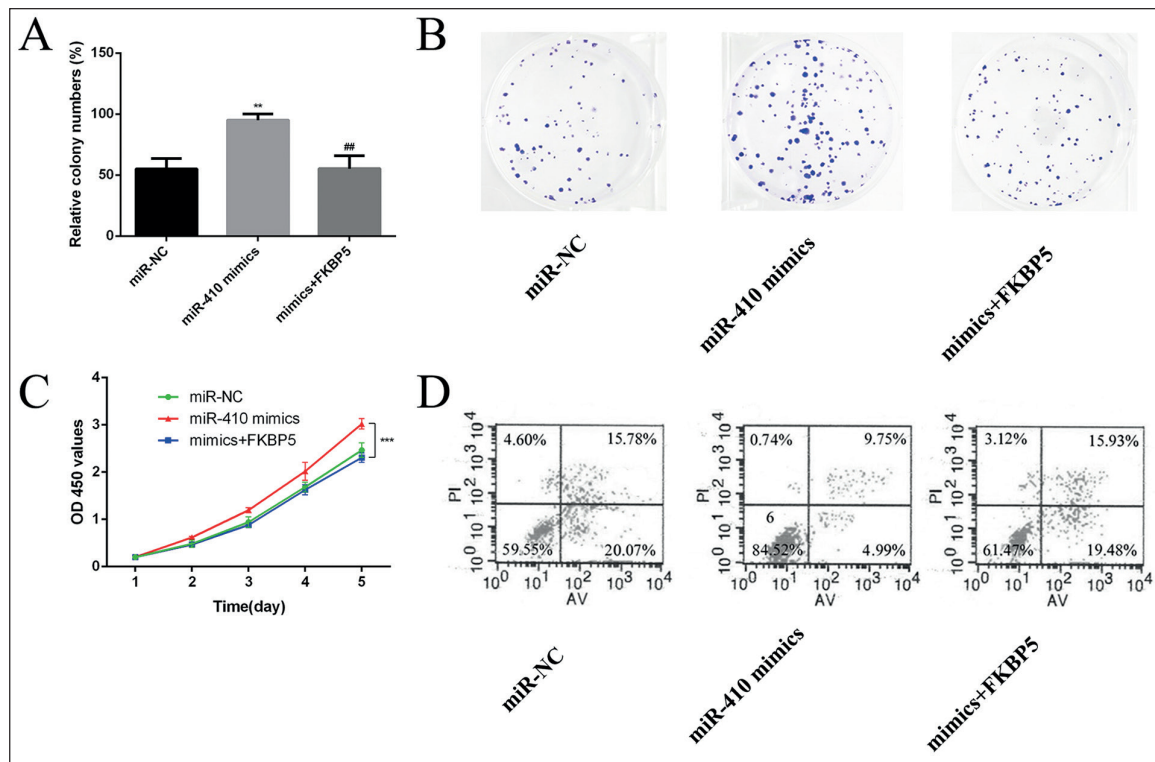
**Figure 2.** FKBP5 was a direct and functional target of miR-410. CCRF-CEM cells were transfected with miR-410 mimics and inhibitor. **A**, Diagram of putative miR-410 binding sites of FKBP5. **B**, Relative activities of Luciferase reporters (\*\* $p < 0.001$ ).

Results demonstrated that after the overexpression of miR-410 in CCRF-CEM cells, the expression of p-Akt was notably increased, whereas the

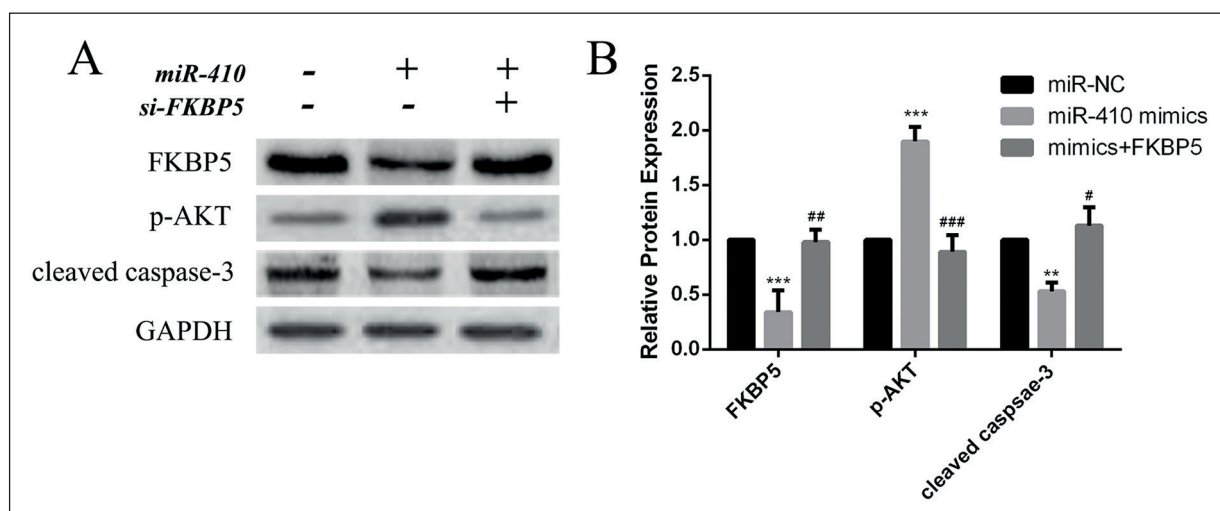
expression of cleaved caspase-3 was significantly decreased. Meanwhile, FKBP5 could reverse the effect of miR-410 (Figure 4).

## Discussion

ALL is a hematopoietic malignancy, characterized by poor differentiation and maturation of leukemia cells, clonal proliferation as well as decreased cell apoptosis. During the development of blood cells, activation of proto-oncogenes and inactivation of tumor suppressor genes can trigger leukemia by affecting each part of the development of hematopoietic cells. This process may involve the participation of multiple genes and signal pathways. Currently, risk factors related to the occurrence and prognosis of childhood ALL have been discovered. However, the causes of ALL and the different responses of ALL cases under standard chemotherapy regimen cannot be completely explained. Refractoriness and recurrence have become important factors threatening the life of pediatric ALL. Therefore, it is of great significance to further investigate



**Figure 3.** **A**, and **B**, Assessment of colony formation. Data were expressed as means  $\pm$  standard deviations (\*\* $p < 0.01$  vs. NC group, # $p < 0.01$  vs. mimics group). **C**, MiR-410 inhibited the proliferation of ALL cells (\*\* $p < 0.01$ ). **D**, MiR-410 decreased the apoptosis level of ALL cells.



**Figure 4.** MiR-410 inhibited the activation of Akt signaling pathway. Western blot showed the expression levels of phosphorylated Akt and apoptosis associated protein cleaved caspase-3. (\*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs. NC group; # $p < 0.05$ , ## $p < 0.01$ , ### $p < 0.001$  vs. mimics group).

the mechanism of the occurrence and development of ALL, as well as the risk factors associated with poor prognosis for early diagnosis and targeted treatment of ALL. This may also help to improve the disease-free survival or cure rate of pediatric ALL.

MiRNAs can lead to degradation or translation inhibition of target messenger ribonucleic acids (mRNAs) through complete or incomplete complementary binding to the 3'untranslated regions (3'UTR). Meanwhile, it can affect the proliferation, differentiation, senescence and apoptosis of cells by inhibiting the expression of target genes. Therefore, the exploration and determination of functional targeting of miRNAs are of great significance in clarifying the biological roles of the corresponding miRNAs in disease development.

Currently, studies have showed that miR-410 plays an important role in the incidence and development of many disease<sup>9-13</sup>. However, its exact role in ALL has not been clarified yet. In the present work, we found that the expression level of miR-410 was significantly increased in ALL cases and cells. Bioinformatics analysis predicted that FKBP5 was a direct target gene of miR-410. Subsequent qRT-PCR results demonstrated that FKBP5 declined remarkably in ALL cells. Also, dual Luciferase reporter gene assay indicated that miR-410 mimics impeded the Luciferase activity of WT FKBP5-3'UTR but not the Luciferase activity of Mut FKBP5-3'UTR. These results all illustrated that FKBP5 was the direct target gene of miR-410.

Immunophilins are specific receptors for immunosuppressive agents commonly existed in organisms in recent years. As a member of the immunophilin FKBP family, FKBP5 plays a certain regulatory role in steroid hormone receptors maturation, receptor-ligand binding and receptor nuclear translocation. Besides, it is closely related to the occurrence and development of hormone-dependent diseases, including autoimmune diseases, stress-related diseases and cancers<sup>15</sup>. Previous studies have shown that FKBP5 can regulate the androgen receptor signaling pathway, thus playing an important role in the progression of prostate cancer<sup>16</sup>. Furthermore, FKBP5 is involved in tumor formation, metabolism and chemotherapeutic drug resistance by modulating NF-kappaB transcription factor activity<sup>17</sup>. In addition, it has been confirmed to specifically negatively regulate the Akt signaling transduction pathway<sup>18</sup>.

Akt signaling pathway plays a crucial regulatory role in the living body<sup>19</sup>. Phosphorylated Akt acts on its downstream target proteins, thereby exerting an anti-apoptotic effect through the caspase pathway, the endothelial nitric oxide synthase (eNOS) pathway, the glycogen synthase kinase-3 (GSK-3) pathway, the nuclear factor-kB pathway, and the B-cell lymphoma 2 (Bcl-2) pathway<sup>20</sup>. Although the specific mechanism of apoptosis has not been fully understood, the key role of caspases in apoptosis has been observed. Among them, caspase-3 is an apoptotic executioner, which is also a common pathway for a variety of apoptosis protease cascades. Studies<sup>21,22</sup>

have manifested that caspase-3 plays the final key role in cell apoptosis initiated by multiple factors.

To further understand whether miR-410 intimidated the proliferation, colony formation ability, apoptosis and activation of Akt signal pathways by regulating FKBP5, MTT assay, colony formation assay, Annexin V/PI staining and Western blot were conducted *in vitro*. CCRF-CEM cells were transfected with FKBP5 siRNA and/or miR-410 mimics. Results demonstrated that FKBP5 siRNA remarkably suppressed the proliferation-promoting and apoptosis-inhibiting ability of miR-410, which also reduced the activation of Akt signaling pathway after miR-410 transfection. The above results indicated that FKBP5 was a functional target of miR-410 in ALL cells.

### Conclusions

We showed that the miR-410 expression was significantly up-regulated in ALL. Meanwhile, miR-410 acted as an oncogene and affected the biological functions of ALL cells by regulating FKBP5 expression. Our study might provide experimental evidence for searching new molecular therapeutic targets for ALL.

### Conflict of Interest

The Authors declare that they have no conflict of interests.

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