

# Identification of non-coding RNA regulatory networks in pediatric acute myeloid leukemia reveals circ-0004136 could promote cell proliferation by sponging miR-142

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**Abstract.** – **OBJECTIVE:** Abnormal expression of circular RNAs (circRNAs) has been observed in various biological processes and cancer pathogenesis. However, the expression of circRNAs in pediatric acute myeloid leukemia (AML) remains largely unknown so far.

**PATIENTS AND METHODS:** Twelve bone marrow samples from pediatric AML patients and healthy controls were analyzed using Agilent circRNA microarray (n = 6, respectively). The circRNAs profiles and regulatory networks were analyzed by integrated bioinformatics methods. Functional analysis (Gene Ontology and KEGG) was performed by KOBAS. The expression of circRNA in patient samples was validated *via* qRT-PCR assay (n > 30). Luciferase reporter assay was performed to validate the binding of miRNAs. CCK8 and colony formation assay were conducted to measure cell proliferation.

**RESULTS:** A total of 273 circRNAs were up-regulated in AML and 296 were downregulated (Fold change > 2, *p*-value < 0.05), the majority of these circRNAs were distributed among chr1, chr6, and chr16, while few in chr13 and chr21. Top 20 differentially expressed circRNAs were chosen to build circRNAs-miRNAs regulatory relationships. Bioinformatics algorithms indicated that circ-0004136 acts as a sponge for several pediatric AML-related miRNAs. Target genes involved in the circ0004136-miRNA-mRNA network were enriched in leukemia-related functions and signaling pathways. Circ-0004136 was found to be significantly upregulated in pediatric AML and could sponge AML-related miRNAs, including miR-29a and miR-142. Furthermore, circ-0004136 was demonstrated to promote the proliferation of AML by sponging miR-142.

**CONCLUSIONS:** Taken together, this study revealed the circRNAs expression profile and regulatory networks of circRNAs-miRNAs-mRNAs in pediatric AML for the first time. Circ-0004136

was significantly upregulated in pediatric AML and could promote cell proliferation by sponging miR-142.

*Key Words:*

CircRNAs, MiRNAs, Network, Circ-0004136, MiR-29a, MiR-142.

## Introduction

Pediatric AML is a heterogeneous and malignant disease, which accounts for about 15-20% of leukemia in children<sup>1,2</sup>. Although the clinical outcome of AML has been improved in recent years with the development of therapeutic treatment, still a portion of patients dose not achieve long-term survival and has the potential for recurrence<sup>3-5</sup>. Therefore, there is an urgent need to identify diagnostic and prognostic biomarkers for pediatric AML. CircRNAs are a class of novel non-coding RNAs which have been identified to be widely expressed in human tissues and participated in various biological processes or cancer pathogenesis<sup>6,7</sup>. Recently, accumulating evidence suggested that circRNAs could serve as competing endogenous RNAs (ceRNAs) that act as sponges of miRNAs to regulate gene expression<sup>8</sup>, indicating the important role and functional regulations of circRNAs in human diseases<sup>9,10</sup>.

Although the role of many protein-coding genes has been well established, much less is known about the regulatory influence of circRNAs in pediatric AML<sup>11,12</sup>. Here we performed the genome-wide circRNAs expression analyses of pediatric AML patient samples, and healthy control samples using microarray (n = 6, respectively). A

total of 273 circRNAs were identified to be upregulated in AML, for which circ-0004136 was the highest expressed differential circRNA, and 296 were downregulated (Fold change > 2 and  $p$ -value < 0.05). Top 20 differential circRNAs were chosen to build circRNA-miRNA regulatory relationships. Furthermore, we focused on circ-0004136 which was significantly upregulated in pediatric AML patient samples. The experiment confirmed that circ-0004136 could act as sponge for AML-related miRNAs, including miR-29a and miR-142. Moreover, circ-0004136 was demonstrated to promote the proliferation of AML by sponging miR-142, which could be considered as a promising biomarker for the diagnosis of pediatric AML.

## Patients and Methods

### *Patient Samples and Cell Line*

This study involved cases of 32 children diagnosed with AML at Zhongshan Hospital Affiliated to Fudan University Qingpu branch and 15 healthy children as a control group. Bone marrow samples were collected following informed consent from all participants. This study was approved by the Ethics Committee of Zhongshan Hospital Affiliated to Fudan University Qingpu branch and followed the Declaration of Helsinki. None of the participants had any chemotherapy treatment previously. The OCI-AML3 cell line was kindly provided by Liang Lab.

### *RNA Extraction and Microarray*

Total RNA was extracted from the above samples according to manufacturer's instruction by using TRIzol (Invitrogen, Carlsbad, CA, USA). Six AML samples and six control samples were used for microarray analysis. Next, 1-10  $\mu$ g of total RNA was isolated for RNase R (Qiagen, Hilden, Germany) treatment to remove linear RNAs. Subsequently, the purification of RNAs was performed by RNeasy Mini Kit (Qiagen, Hilden, Germany), and the integrity and concentration of RNAs were assessed by NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA). The circRNA microarray analysis was conducted by CapitalBio (Beijing, China) as circRNAs were labeled and hybridized onto Human 8x15 K circRNA Array (Arraystar, Rockville, MD, USA).

### *Bioinformatics and Statistical Analysis*

Raw data was extracted by Agilent Feature Extraction software (Santa Clara, CA, USA).

Subsequently, the circRNA expression data from each sample were normalized by R software. The statistical significance of differential circRNAs between AML and control group was measured by DESeq2 package<sup>13</sup> and setting threshold parameter as fold change (FC) > 2 and  $p$  < 0.05. CircRNA annotations were mapped to CircBase<sup>14</sup>. The target miRNA prediction of circRNAs was performed by miRanda<sup>15</sup> and TargetScan<sup>16</sup> algorithms. The experimentally validated target genes for miRNAs were extracted from miRTarBase<sup>17</sup>. CircRNA-miRNA-mRNA regulatory network was constructed by CytoScape<sup>18</sup> software. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of the target genes were carried out by KOBAS<sup>19</sup> software.  $p$  < 0.05 was considered as statistically significant.

### *qRT-PCR Experiment*

Total RNA was reverse transcribed into cDNA with random primers using PrimeScript RT Reagent Kit (Invitrogen, USA) following the manufacturer's protocol. The relative expression of circ-0004136 and miRNAs were validated by qRT-PCR using SYBR Premix Taq (TaKaRa, Otsu, Shiga, Japan). GAPDH and U6 were used as an internal control for circRNAs and miRNAs, respectively. Primers: miR-142 (Forward: 5'-GGGTGTAGTGTTTCTACT-3'; Reverse: 5'-CAGTGCCTCGTGGAGT-3'); U6 (Forward: 5'-TGCGGGTGCTCCGCTTCGGCAGC-3'; Reverse: 5'-CAGTGCAGGGTCCGAGGT); circ-0004136 (Forward: 5'-GGTCCACATATG-GAACT; Reverse: 5'-CACCTTGAGGACC-3'); GAPDH (Forward: 5'-TCCTCTGACTTCAA-CAGCGACAC-3'; Reverse: 5'-CACCT-GTTGCTGTAGCCAAATTC-3').

### *Cell Transfection*

The negative control vectors, miR-142 mimics, and siRNA of circ-0004136 were synthesized by Capital Biotech Company (CapitalBio, Beijing, China). The OCI-AML3 cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

### *Luciferase Reporter Assay*

The wild type (WT) and mutant (Mut) of circ-0004136 fragments were cloned and inserted into vectors (Promega, Madison, WI, USA) and co-transfected with miRNAs mimic or NC. Luciferase activities were measured using a detection kit (Promega, Madison, WI, USA) after 24 h according to the instruction.

### **Cell Counting Kit-8 (CCK8) Assay and Colony Formation**

CCK8 assay and colony formation were conducted to measure cell proliferation. The transfected OCI-AML3 cells were cultured and recorded by a spectrophotometer (450 nm) at 0, 24, 48, and 96 hours respectively. As for colony formation, OCI-AML3 cells were incubated with 4% paraformaldehyde and then stained with crystal violet (CapitalBio, Beijing, China). A high-power microscope was used to count colony numbers.

## **Results**

### **CircRNA Expression Profile in Pediatric AML**

To characterize the profile of circRNAs in pediatric AML and control samples ( $n = 6$ , respectively), microarray analysis was performed with Agilent circRNA chips. The identified circRNAs with  $FC > 2$  and  $p < 0.05$  were considered to be differentially expressed. Among them, 273 circRNAs were up-regulated, and 296 circRNAs were downregulated in pediatric AML patients compared to control samples. To identify the heterogeneity in circRNAs expression across twelve samples, hierarchical clustering analysis was performed by using circRNAs expression data. As shown in Figure 1A, the differentially expressed circRNAs were characterized with distinct expression patterns. Chromosomal distribution results showed that the majority of differentially expressed circRNAs were distributed among chr1, chr6, and chr16, while a small number of circRNAs were distributed in chr13 and chr21 (Figure 1B). In addition, we sorted out top 20 differentially expressed circRNAs that were upregulated or downregulated in pediatric AML (Figure 1C), including circ\_0004136, circ\_0005273, circ\_0005774, circ\_0003256, circ\_0035487, circ\_0034646, circ\_0126662, circ\_0130221, circ\_0078693, circ\_0043532, circ\_0113705, circ\_0116913, circ\_0118530, circ\_0014756, circ\_0038634, circ\_0098519, circ\_0032023, circ\_0043533, circ\_0004277, and circ\_0007609. Among them, the expression level of circ-0004136 was the highest with  $\sim 4 \log_2 FC$  in AML as compared to control.

### **Target Prediction and CircRNA-miRNA Interactions**

In view of circRNA exerts its potential regulatory function by acting as miRNA sponges<sup>8</sup>, we predicted the target miRNAs of top 20 differential circRNAs by miRanda and TargetScan algorithms.

Target miRNAs predicted by two methods were intersected and then used for the construction of the circRNA-miRNA regulatory network. As shown in **Supplementary Figure 1**, circ-0004277 exhibited the most interactions, followed by circ-0004136, circ-0018530, and circ-0007069. We then focused on circ-0004136, as microarray results indicated its highest expression in AML. The interactions between circ-0004136 and its target miRNAs were demonstrated in Figure 1D; there were several miRNAs, for example, hsa-miR-29a, hsa-miR-196a and hsa-miR-142-3p have been found to be associated with pediatric AML, suggesting that circ-0004136 may regulate functional genes by acting as a sponge for these miRNAs.

### **Circ0004136-miRNA-mRNA Regulatory Network and Functional Analysis**

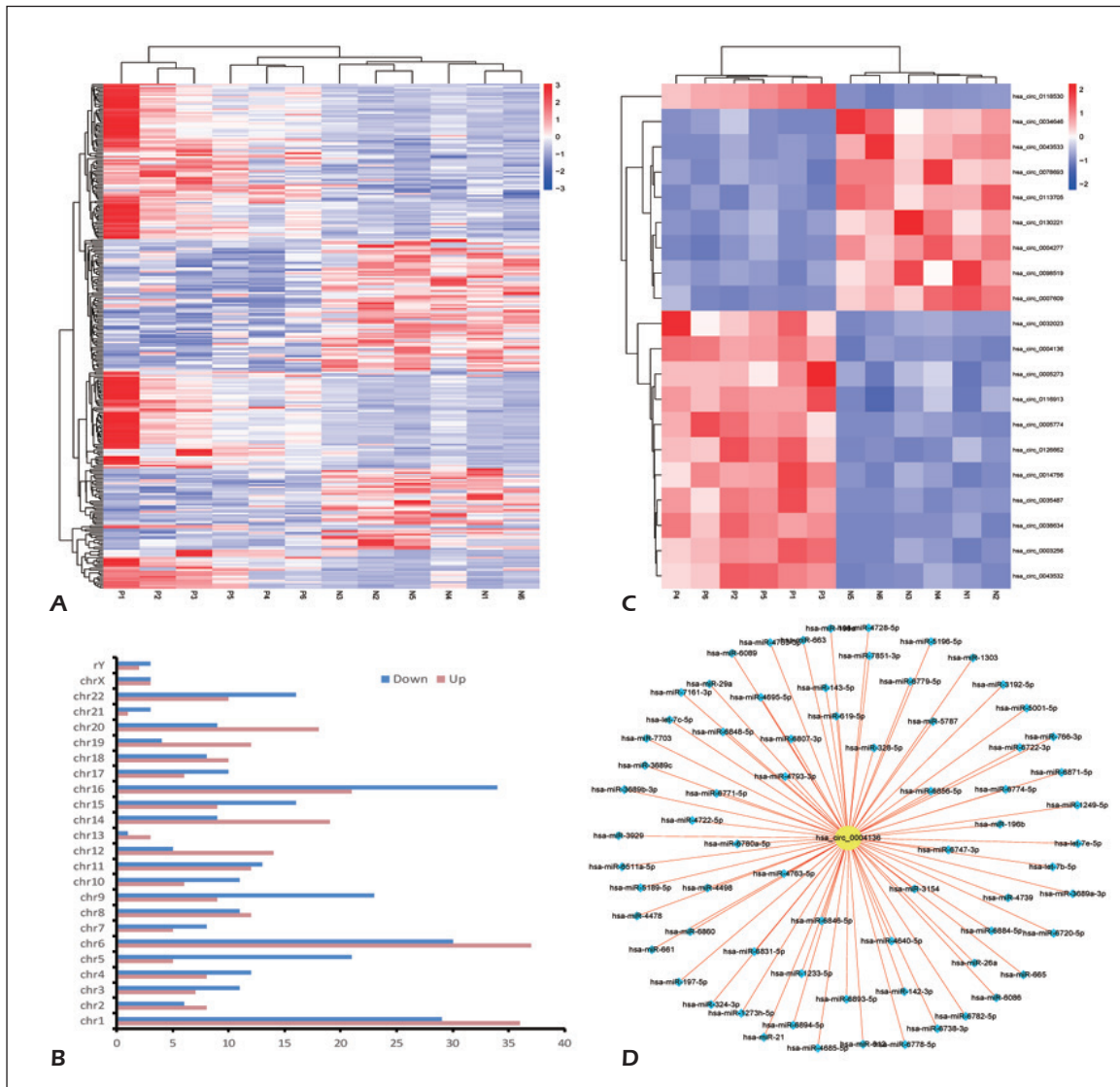
To further investigate the biological function of circ-0004136, we constructed the circRNA0004136-miRNA-mRNA regulatory network by searching the miRTarBase to filter out validated target genes for miRNAs. In the discovery cohort, over one hundred genes were involved in the network (**Supplementary Figure 2**). We analyzed biological functions and pathways of target genes by KOBAS algorithm; in brief, several biological functions including leukocyte chemotaxis, myeloid leukocyte migration and cell adhesion are associated with these targets (Figure 2A). Pathway analysis has generated several pathways including cytokine-cytokine receptor interaction, hematopoietic cell lineage, and cell adhesion molecules (Figure 2B).

### **Upregulation of Circ-0004136 Was Validated in AML by qRT-PCR**

To examine the upregulation of circ-0004136, qRT-PCR was conducted in AML patient samples and control samples as described in Patients and Methods section ( $n > 30$ ). The result showed that circ-0004136 was significantly upregulated in patient samples (Figure 3A). Validation of circ-0004136 expression in OCI-AML3 cell line showed a consistent result (Figure 3A).

### **Knockdown of Circ-0004136 Inhibited Proliferation of AML Cells**

To explore the biological function of circ-0004136 in AML, we performed a knockdown experiment in OCI-AML3 cells. As shown in Figure 3B, there was a notable decrease in circ-0004136 expression after transfection. CCK-8 and colony



**Figure 1.** **A**, Characterization of circRNAs expression profile in pediatric AML and control samples by hierarchical cluster analysis. **B**, Chromosomal distribution of differentially expressed circRNAs. **C**, Top 20 differential circRNAs. **D**, circ-0004136 regulated network. red: upregulated circRNAs, blue: downregulated circRNAs.

formation experiments showed that knockdown of circ-0004136 suppressed the proliferation of AML cells *in vitro* (Figures 3C and 3D).

**Circ-0004136 Could Sponge MiR-29a and MiR-142**

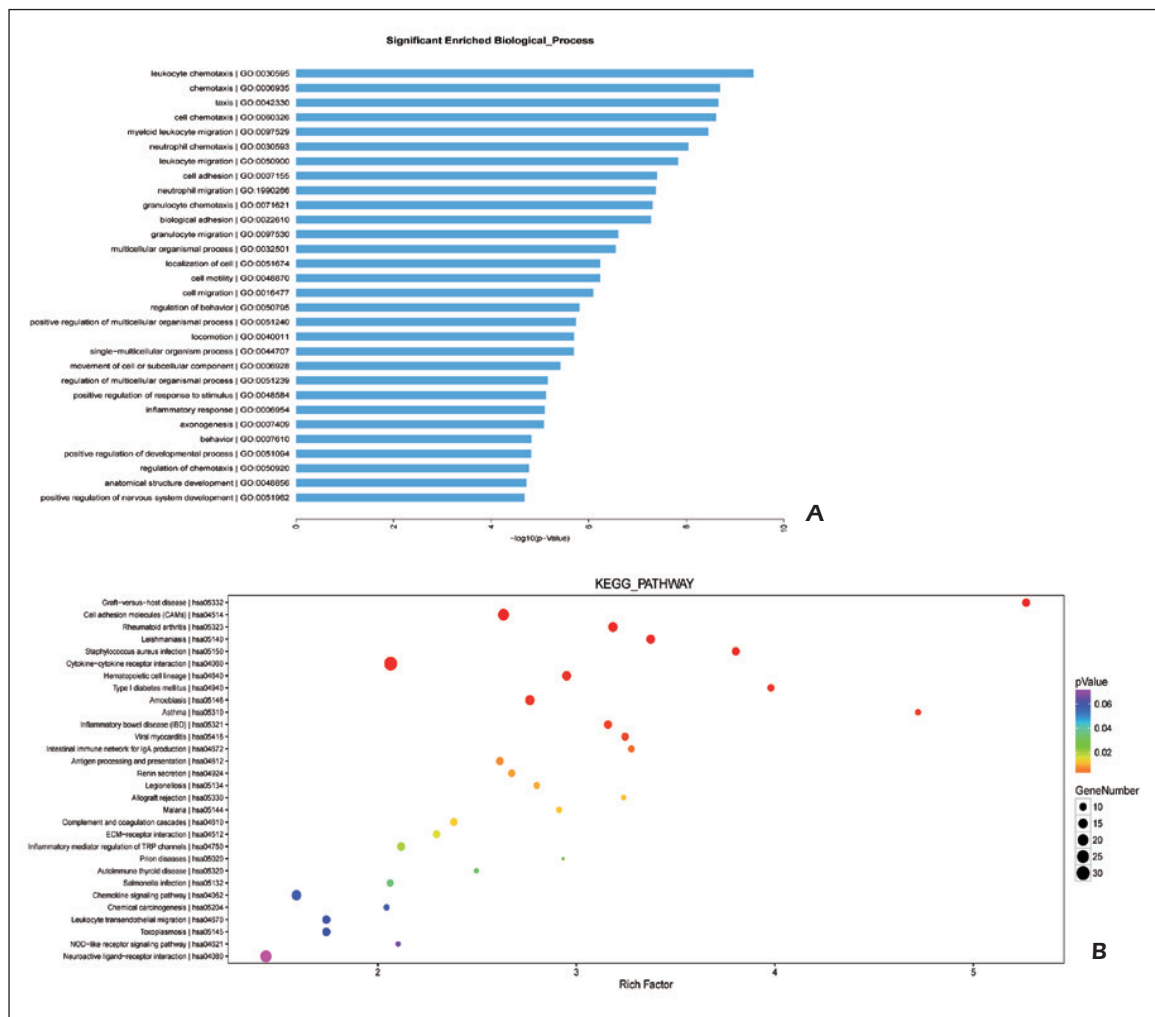
As bioinformatics analysis indicated that circ-0004136 may interact with several miRNAs in pediatric AML, we examined the expression of these miRNAs by qRT-PCR. The results showed that miR-29a and miR-142 were downregulated in the AML cell line (Figure 4A). Luciferase reporter assay was performed to verify the direct binding sites (Figure 4B), miR-29a and miR-142

mimics that co-transfected with circ-0004136 WT significantly inhibited the Luciferase activity while had no effect on the reporter vector containing the mutant binding sites (Figure 4C). Taken together, the results indicated that circ-0004136 could sponge miR-29a and miR-142.

**Circ-0004136 Promotes Proliferation of AML by Sponging MiR-142**

As a direct target of circ-0004136, we further examined the expression of miR-142 in circ-0004136 knockdown AML cells; the result showed that miR-142 was significantly upregu-





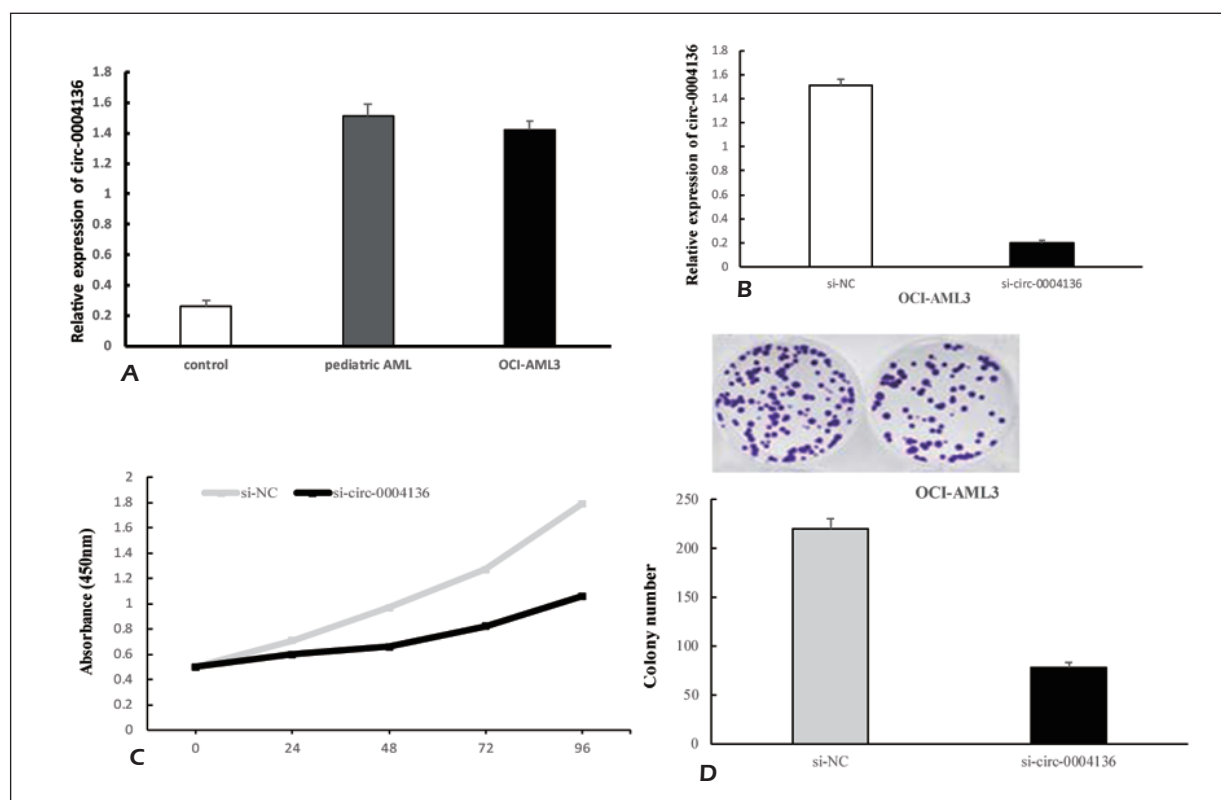
**Figure 2. A-B,** Biological functions and pathways enriched for genes involved in circ0004136-miRNA-mRNA regulatory network.

lated (Figure 4D). Moreover, miR-142 inhibitor treatment in circ-0004136-silenced OCI-AML3 cells prominently rescued cell proliferation (Figure 4E). The above results suggested that circ-0004136 could act as a sponge of miR-142 to promote AML cell proliferation.

### Discussion

In our study, we employed microarray technology to identify circRNAs in pediatric AML samples and healthy controls (n = 6, respectively), and we identified a large number of differential circRNAs (273 upregulated and 296 downregulated) in AML as compared to control. Previous researches<sup>7,20</sup> indicated that circRNAs are widely expressed in human tissues with relatively high

expression level compared to their host genes. Due to their unique structures and the characteristic of resistance to exonuclease, circRNAs are more stable than other non-coding RNAs and could be considered as desirable biomarkers or therapeutic targets in disease treatment<sup>21,22</sup>. Chromosomal distribution results showed that the majority of these circRNAs were distributed among chr1, chr6, and chr16, while few in chr13 and chr21. We sorted out top 20 up regulated or downregulated differential circRNAs, including circ\_0004136, circ\_0005273, circ\_0005774, circ\_0003256, circ\_0035487, circ\_0034646, circ\_0126662, circ\_0130221, circ\_0078693, circ\_0043532, circ\_0113705, circ\_0116913, circ\_0118530, circ\_0014756, circ\_0038634, circ\_0098519, circ\_0032023, circ\_0043533, circ\_0004277, and circ\_0007609. Among them, the expression lev-



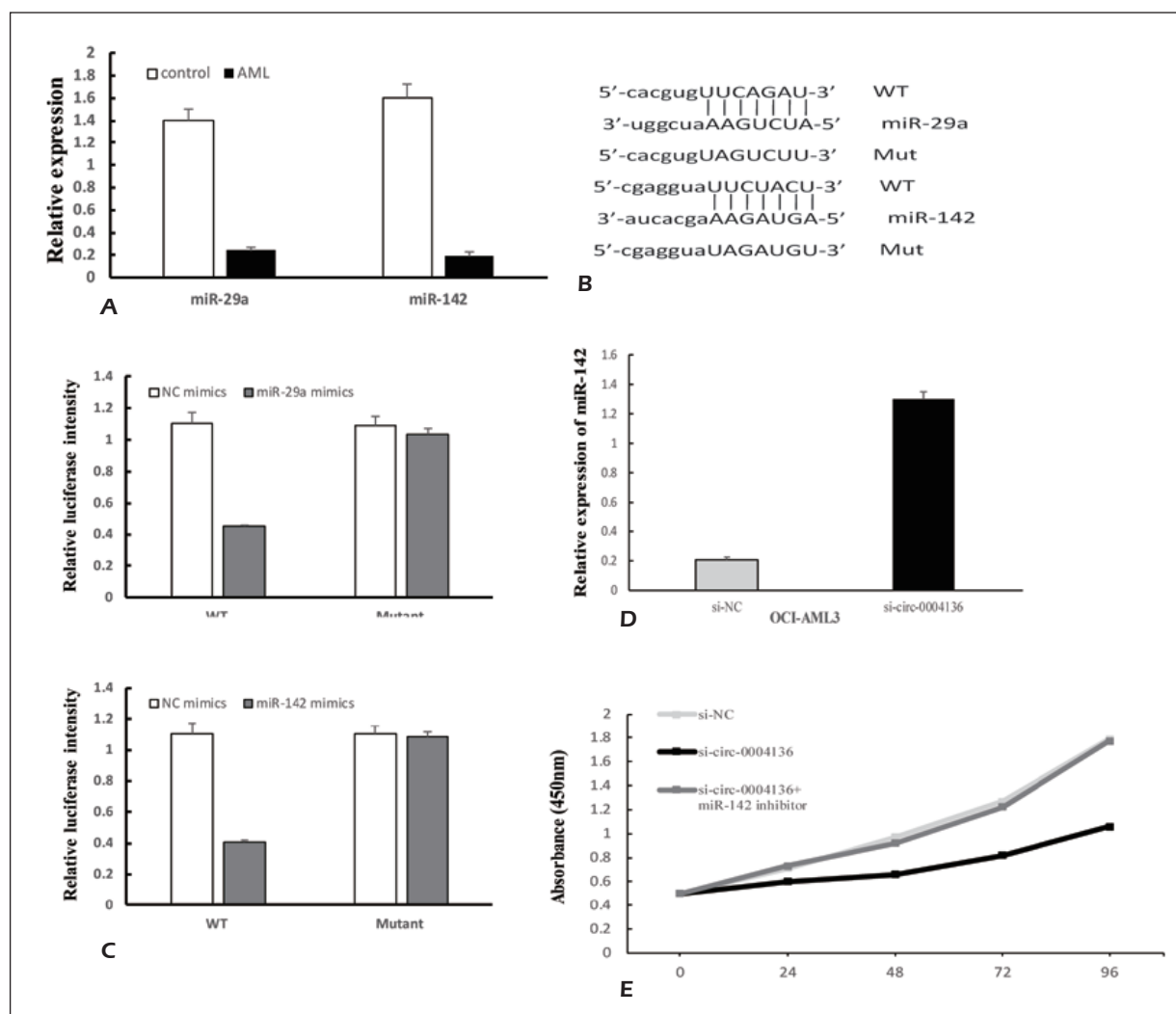
**Figure 3.** **A**, The expression level of circ-0004136 was validated by qRT-PCR in patient samples ( $n > 30$ ) and OCI-AML3 cell line. **B**, Transfection of circ-0004136 in OCI-AML3. **C-D**, CCK-8 and colony formation experiments at x50 magnification showed that knockdown of circ-0004136 suppressed proliferation of AML cells.  $p < 0.05$ .

el of circ-0004136 was the highest with  $\sim 4$  log<sub>2</sub> FC in AML as compared to control. It was worth noting that circ\_0004277 has been reported to be downregulated in adult AML and increasing level of circ\_0004277 might be associated with successful treatment<sup>23</sup>.

Accumulating evidence suggested that circRNAs could serve as competing endogenous RNAs (ceRNAs) that act as miRNA sponges by binding to seed regions, and thereby regulate gene expression in an indirect way<sup>8,24</sup>. We constructed the circRNA-miRNA regulatory network in pediatric AML, involving the top 20 differentially expressed circRNAs. In the network, circ-0004277 exhibited the most interactions, followed by circ-0004136, circ-0018530 and circ-0007069. Considering the high expression of circ-0004136 in AML, we then focused on the sub-network of circ-0004136 and the results indicated that circ-0004136 interacts with several miRNAs that have been reported to play a role in pediatric AML<sup>25</sup>, including hsa-miR-29a, hsa-miR-196a, and hsa-miR-142-3p; for example, a previous study<sup>26</sup> has found that miR-29a was

downregulated in pediatric AML patients carrying MLL gene rearrangements. Furthermore, we predicted the target genes of miRNAs involved in circ-0004136 sub-network and performed functional analysis (GO and KEGG); the results indicated that circ-0004136 involved in biological functions including leukocyte chemotaxis, myeloid leukocyte migration, cell adhesion and pathways controlled by cytokine-cytokine receptor interaction, hematopoietic cell lineage and cell adhesion molecules.

We validated the aberrant expression of circ-0004136 by qRT-PCR in BM samples from pediatric AML patients ( $n > 30$ ), a much higher expression level of circ-0004136 was found in the AML compared to control. Luciferase reporter assay indicated that circ-0004136 could sponge miR-29a and miR-142. Trissal et al<sup>27</sup> indicated that miR-142 participates in myeloid progenitors/myeloblasts and ultimately contributing to leukemic transformation. Thus, we focused on miR-142 and further study found that circ-0004136 could promote AML proliferation by sponging miR-142.



**Figure 4.** **A**, The expression level of miR-29a and miR-142 in AML cells. **B**, Predicted binding sites of miR-29a/miR-142 and circ-0004136. **C**, Luciferase activities of miR-29a/miR-142 were measured. **D**, miR-142 was significantly upregulated after circ-0004136 knockdown. **E**, miR-142 inhibitor treatment in circ-0004136-silenced OCI-AML3 cells rescued cell proliferation.  $p < 0.05$ .

## Conclusions

Our results provide rich and useful resources for further investigation of circRNAs in pediatric AML; moreover, circ-0004136 may participate in the pathogenesis of pediatric AML and could be considered as a promising biomarker for diagnosis of pediatric AML.

## Conflict of Interests

The authors declare that they have no conflict of interest.

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