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Original Research Article

MiR-598-3p functions as a tumor suppressor in pediatric T-cell acute lymphoblastic leukemia

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Abstract

Purpose: *To investigate the role of miR-598-3p in pediatric T-cell acute lymphoblastic leukemia (T-ALL).*
Methods: *The expression of miR-598-3p in mononuclear cells isolated from the peripheral blood samples of children with or without T-ALL was assessed using real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Cell viability or proliferation of T-ALL cell lines was evaluated using cell counting kit-8 assay or bromodeoxyuridine staining, respectively. The target gene of miR-598-3p was predicted and validated using luciferase reporter assay, while the underlying mechanism involved in miR-598-3p-mediated T-ALL was determined by western blot analysis.*
Results: *MiR-598-3p was reduced in the peripheral blood mononuclear cells of T-ALL patients. Ectopic miR-598-3p expression decreased T-ALL cell viability and suppressed proliferation, while miR-598-3p interference showed reversed effects. Additionally, the target gene of miR-598-3p, Dishevelled, EGL-10, and Pleckstrin domain-containing mTOR-interacting protein (DEPTOR), was down-regulated by miR-598-3p in T-ALL. MiR-598-3p decreased phospho (p)-AKT protein expression, while AKT inhibition counteracted the suppressive effects of miR-598-3p silencing on T-ALL cell viability and proliferation.*
Conclusion: *MiR-598-3p/DEPTOR is involved in the proliferation of T-ALL through AKT pathway, thus providing a potential novel application in pediatric T-ALL.*

Keywords: *MiR-598-3p, DEPTOR, Progression, AKT pathway, Pediatric T-ALL*

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INTRODUCTION

Acute lymphoblastic leukemia (ALL), the most common hematologic tumor in children, accounts for approximately 25 % of pediatric cancers [1]. ALL is characterized by the abnormal malignant proliferation of cells involved in the hematopoietic system, resulting in the infiltration of various organs such as lymph nodes, liver, bone marrow, and spleen [1]. T-cell ALL (T-ALL) is an aggressive malignancy of thymocytes and contributes to approximately 15 % of pediatric ALL cases [2]. Advances in therapies have improved the outcomes for T-ALL treatment, and the 5-year event-free survival of T-ALL patients is more than 80 % [2]. However, the overall survival

of relapsed T-ALL is less than 25 % [2]. Therefore, further investigation of the pathogenesis of pediatric T-ALL might provide more effective therapeutic strategies for the disease.

MicroRNAs (miRNAs) function as post-transcriptional regulators of target gene expression within functionally linked pathways and participates in biological processes [3]. Deregulated miRNAs in pediatric ALL suggest important regulatory roles [4]. Dysregulated miRNAs are prognostic biomarkers in T-ALL [5] and act as either oncogenic miRNAs [6] or tumor suppressors [7] in pediatric T-ALL. MiR-598-3p, a promising diagnostic biomarker for breast cancer [8], showed anticancer features in glioblastoma cells [9]. However, the effect of miR-598-3p in pediatric T-ALL remains elusive.

Dishevelled, EGL-10, and Pleckstrin domain-containing mTOR-interacting protein (DEPTOR) functions as an endogenous mTOR inhibitor [10] and participates in vascular endothelial activation, glucose homeostasis, and tumor progression [11]. Over-expression of DEPTOR is associated with a poor prognosis in thyroid carcinoma [12] and promotes cell survival in esophageal squamous cell carcinoma [13]. Interference of DEPTOR suppresses multiple myeloma progression [14]. Recently, ectopic DEPTOR expression was shown to promote T-ALL cell growth [15]. However, the role of DEPTOR in miR-598-3p-mediated T-ALL has not been investigated until now.

In the present study, the miR-598-3p expression levels in the peripheral blood mononuclear cells of pediatric T-ALL patients were examined, and the role and mechanism of miR-598-3p in T-ALL were investigated.

EXPERIMENTAL

Patient samples

The study was approved by the Ethics Committee of Northwest Women and Children's Hospital (approval no. 20-066) and followed the guidelines of 1964 Helsinki Declaration and its later amendments for ethical research involving human subjects [16]. Thirty-seven pediatric T-ALL patients and 35 healthy children with written informed consent were recruited from Changchun University of Traditional Chinese Medicine Affiliated Hospital. Peripheral blood samples were collected from the children, and mononuclear cells were isolated by density gradient centrifugation using Lymphoprep (Nycomed Pharma, Oslo, Norway).

Cell culture, treatment and transfection

T-ALL cell lines (6T-CEM and JURKAT) were acquired from DSMZ (Braunschweig, Germany) and cultured in RPMI-1640 medium (Transgene, Beijing, China) containing 10 % fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA). For PI3K inhibition, T-ALL cells were seeded and treated with 50 μM LY294002 (Sigma-Aldrich) 24 h before cell transfection. T-ALL cells were transfected with miR-598-3p mimic, inhibitor and the corresponding negative controls (NC mimic, NC inhibitor) (GenePharma, Shanghai, China) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Cell counting kit-8 assay

T-ALL cells (2×10^3 cells/well) were seeded and cultured for 48 h before the cell viability assay (KeyGEN, Nanjing, China). The cells were incubated with 20 μL of cell counting kit-8 solution for 4 h before measuring the absorbance at 450 nm using the Epoch microplate Reader (BioTek, Winooski, VT, USA) according to manufacturer's instructions.

Bromodeoxyuridine incorporation

T-ALL cells were seeded and cultured for 48 h before the cell proliferation assay (Bromodeoxyuridine ELISA kit; Roche Diagnostics, Basel, Switzerland). The cells were incubated with 10 μL of 10 mM bromodeoxyuridine for 2 h before measuring the absorbance at 450 nm according to the manufacturer's instructions.

Luciferase reporter assay

Wild-type or mutant 3'UTR DEPTOR sequences were constructed into the pGL3-basic vector (Promega, Madison, WI, USA). JURKAT cells were cotransfected with pRL-TK, miR-598-3p mimic/inhibitor, or NC mimic/inhibitor, and DEPTOR-WT or DEPTOR-MUT. The luciferase activities were measured 24 h later using the Luciferase Assay System (Promega) according to the manufacturer's instructions.

Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted using TRIzol (TIANGEN, Beijing, China) and reverse transcribed into cDNA. qRT-PCR was performed using the FAST SYBR Green Master Mix (Solarbio, Beijing, China) and the ViiA 7 system

(Applied Biosystems, Austin, TX, USA). The PCR conditions were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The primers used are listed Table 1.

Table 1: Primers used in PCR study

Gene	Sense	Antisense
miR-598-3p	5'-AGCTACGTCA TCGTTGTCATC-3'	5'-GTGTCGTCGAG TCGGCAATTC-3'
DEPTOR	5'-TTTGTGGTGC GAGGAAGTAA-3'	5'-CATTGCTTTGTG TCATTCTGG-3'
GAPDH	5'-GAGTCAACGG ATTTGGTCGT-3'	5'-GACAAGCTTCC CGTTCTCAG-3'
U6	5'-CTCGCTTTCG GCAGCACA-3'	5'-AACGCTTCACG AATTTGCGT-3'

Western blotting

T-ALL cell lysates (30 μg) in RIPA (radioimmunoprecipitation assay) buffer were subjected to 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then were electro-transferred onto polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA, USA). After blocking in 5 % non-fat milk, the membranes were incubated with primary antibodies: DEPTOR (1:2000; Abcam, Cambridge, UK), p-AKT and AKT (1:2500; Abcam) and β-actin (1:3000; Abcam). The membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:5000; Abcam) before incubation with Pierce ECL Western Blotting Substrate (Pierce Biotechnology, Waltham, MA, USA) according to the manufacturer's instructions.

Statistical analysis

The data were reported as means ± standard deviation and were analyzed by analysis of variance followed by the post hoc test using SPSS 17.0. $p < 0.05$ was considered statistically significant.

RESULTS

Expression of miR-598-3p in pediatric T-ALL

A significant decrease in miR-598-3p was observed in samples from pediatric T-ALL compared with those from healthy children ($p < 0.01$) (Figure 1), suggesting that miR-598-3p might be involved in T-ALL progression. Analysis of the clinical and immunophenotypic features between pediatric T-ALL patients and healthy children suggested that the hemoglobin level, white blood cell count, and platelet count showed significant differences (Table 2).

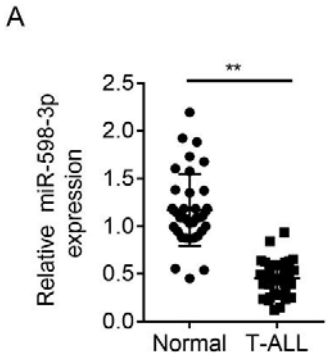

Figure 1: Expression of miR-598-3p in pediatric T-ALL. The miR-598-3p expression level was reduced in peripheral blood mononuclear cells isolated from pediatric T-ALL patients compared with that in healthy children. ** T-ALL vs. Normal $p < 0.01$

MiR-598-3p suppresses T-ALL proliferation

T-ALL cell lines (6T-CEM and JURKAT) were transfected with miR-598-3p mimic or inhibitor to assess the effect of miR-598-3p on T-ALL cell proliferation capability. Significant up-regulation of miR-598-3p was found in 6T-CEM and JURKAT cells transfected with miR-598-3p mimic compared with those transfected with NC mimic ($p < 0.01$) (Figure 2 A).

Table 2: Clinical and immunophenotypic features of T-ALL patients and normal controls

Characteristic	T-ALL patient (n=37)	Normal control (n=35)	*P*-value
Sex (M/F)	(21/16)	(19/16)	0.833
Age (years)	6.0(1-14)	6.5(1-14)	0.088
Hemoglobin level	9.58(4.21-17.5)	151.02(102.11-198.63)	<0.001***
WBC count*10^9	150.20(4.41-236)	17.78(3.1-36.89)	<0.001***
Platelet count*10^9	107.59(9.89-608)	165.33(103.13-278.92)	0.025*
Thymic cell count (%)	11/37(29.72)	15/35(42.86)	0.246
Mature T-cell count (%)	13/37(35.14)	12/37(32.43)	0.806
Prethymic cell count (%)	12/37(32.43)	6/35(17.14)	0.134

The data from the cell counting kit-8 assay (Figure 2 B) and bromodeoxyuridine incorporation (Figure 2 C) revealed that forced expression of miR-598-3p decreased the cell viability and suppressed T-ALL proliferation, suggesting the anti-proliferative role of miR-598-3p in T-ALL. However, miR-598-3p knockdown by transfection with miR-598-3p inhibitor (Figure 3 A) increased cell viability (Figure 3 B) and promoted cell proliferation (Figure 3 C) of T-ALL, confirming the suppressive role of miR-598-3p in T-ALL.

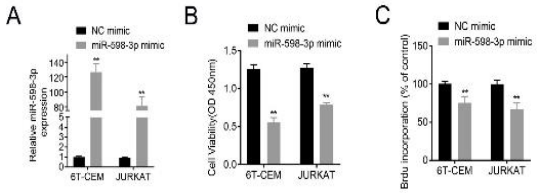

Figure 2: MiR-598-3p suppresses T-ALL proliferation. (A) Transfection of miR-598-3p mimic increased miR-598-3p expression in 6T-CEM and JURKAT cells compared with NC mimic transfection. (B) MiR-598-3p mimic transfection decreased the cell viability of 6T-CEM and JURKAT cells compared with NC mimic transfection. (C) Transfection with miR-598-3p mimic decreased the cell proliferation of 6T-CEM and JURKAT cells compared with NC mimic transfection; ***p* < 0.01, miR-598-3p mimic vs NC mimic

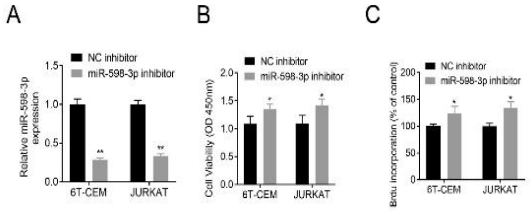

Figure 3: Knockdown of miR-598-3p promotes T-ALL proliferation. (A) Transfection of miR-598-3p inhibitor decreased miR-598-3p expression in 6T-CEM and JURKAT cells compared with NC inhibitor transfection. (B) Transfection with miR-598-3p inhibitor increased the cell viability of 6T-CEM and JURKAT cells compared with NC inhibitor transfection. (C) Transfection with miR-598-3p inhibitor increased the cell proliferation of 6T-CEM and JURKAT cells compared with NC inhibitor transfection. **p* < 0.05, miR-598-3p inhibitor vs NC inhibitor. ***p* < 0.01, miR-598-3p inhibitor vs NC inhibitor

DEPTOR is a target of miR-598-3p in T-ALL

The TargetScan bioinformatics prediction algorithm (http://www.targetscan.org/vert_72/) predicted a potential binding site between miR-598-3p and DEPTOR (Figure 4 A). The luciferase activity of the DEPTOR-WT luciferase reporter vector was decreased by miR-598-3p mimic and increased by miR-598-3p inhibitor (Figure 4 B). However, NC mimic/inhibitor transfection had no significant effect on the luciferase activity of DEPTOR-MUT (Figure 4 B), suggesting that DEPTOR was a target of miR-598-3p in T-ALL. In addition, the mRNA (Figure 4 C) and protein levels (Figure 4 D) of DEPTOR were decreased in JURKAT cells transfected with miR-598-3p mimic but increased with miR-598-3p inhibitor, suggesting the functional role of miR-598-3p/DEPTOR in T-ALL. Furthermore, miR-598-3p decreased the protein expression of p-AKT in JURKAT cells, while miR-598-3p knockdown increased p-AKT expression (Figure 4 D), suggesting that the miR-598-3p/DEPTOR axis might regulate the AKT pathway in T-ALL.

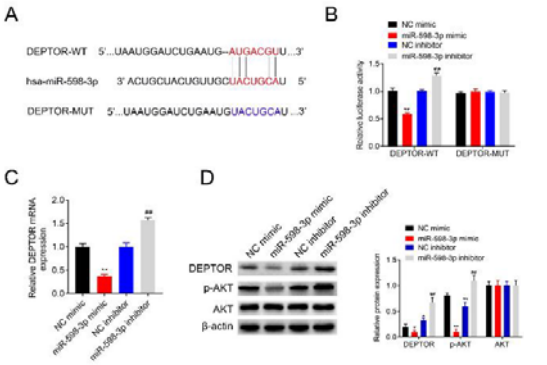

Figure 4: DEPTOR is a target of miR-598-3p in T-ALL. (A) Potential binding site, as well as the mutant site, between miR-598-3p and DEPTOR. (B) Transfection with miR-598-3p mimic decreased the luciferase activity of DEPTOR-WT, while transfection with miR-598-3p inhibitor increased the luciferase activity of DEPTOR-WT in JURKAT cells. (C)Transfection with miR-598-3p mimic decreased DEPTOR mRNA expression, while transfection with miR-598-3p inhibitor increased DEPTOR mRNA expression in JURKAT cells. (D) Transfection with miR-598-3p mimic decreased DEPTOR protein expression, while transfection with miR-598-3p inhibitor increased DEPTOR protein expression in JURKAT cells. *p < 0.05, miR-598-3p mimic vs NC mimic. **p < 0.01, miR-598-3p mimic vs NC mimic. ##p < 0.01, miR-598-3p inhibitor vs NC inhibitor

Interference of AKT counteracts the suppressive effect of miR-598-3p on T-ALL cell proliferation

To determine whether the AKT pathway was involved in miR-598-3p/DEPTOR-mediated T-ALL cell proliferation, 6T-CEM and JURKAT cells were pretreated with the PI3K inhibitor LY294002 and then transfected with miR-598-3p inhibitor. Pretreatment with LY294002 decreased T-ALL cell viability (Figure 5 A) and suppressed T-ALL proliferation (Figure 5 B). Moreover, LY294002 treatment attenuated miR-598-3p inhibitor-induced increased cell viability (Figure 5 A) and proliferation (Figure 5 B) of T-ALL. Thus,

DEPTOR is a direct miR-598-3p target gene that promotes cell proliferation in T-ALL by inactivating the AKT pathway.

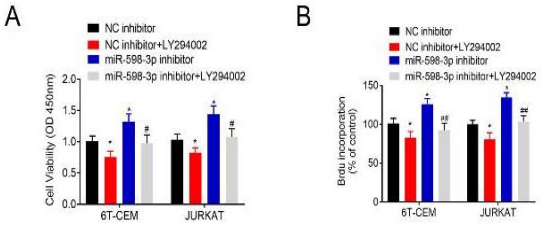

Figure 5: Interference of AKT counteracts the suppressive effect of miR-598-3p on T-ALL cell proliferation. (A) Pretreatment with the PI3K inhibitor LY294002 decreased the cell viabilities of 6T-CEM and JURKAT cells and attenuated miR-598-3p inhibitor-induced increased cell viability. (B) Pretreatment with the PI3K inhibitor LY294002 decreased the cell proliferation of 6T-CEM and JURKAT cells and attenuated miR-598-3p inhibitor-induced increased cell proliferation. *p < 0.05, NC inhibitor + LY294002 vs NC inhibitor. #p < 0.05, miR-598-3p + LY294002 vs miR-598-3p inhibitor. ##p < 0.01, miR-598-3p + LY294002 vs miR-598-3p inhibitor

DISCUSSION

Specific miRNAs have been associated with risk factors involved in T-ALL and were regarded as prognostic biomarkers in T-ALL [5]. A previous study has shown that miR-598 is down-regulated in colorectal cancer tissues and is related to the poor prognosis of colorectal cancer [17]. MiR-598 predicts a poor prognosis in osteosarcoma [18]. The role and mechanism of miR-598-3p in T-ALL were then investigated in this study. We showed a significant decrease in miR-598-3p in the peripheral blood mononuclear cells of pediatric T-ALL patients compared with those of healthy children, suggesting possible diagnostic or prognostic roles in T-ALL. Moreover, forced miR-598-3p expression decreased the cell viability and proliferation of T-ALL, while miR-598-3p interference reversed the effects. Because cell migration and invasion are important for T-ALL relapse [19], the effect of miR-598-3p on T-ALL metastasis warrants further investigation.

The luciferase activity data revealed that miR-598-3p directly binds to DEPTOR in T-ALL. In addition, qRT-PCR and western blot analysis showed the inhibitory role of miR-598-3p on DEPTOR expression. Thus, miR-598-3p may repress T-ALL by down-regulating DEPTOR. A previous study showed the controversial function of DEPTOR in tumorigenesis. On the one hand, DEPTOR binds and inhibits hyperactivated mTOR in prostate cancer and suppresses tumorigenesis [20]. On the other hand, over-expressed DEPTOR in multiple myeloma promotes cell survival [10].

Aberrant PI3K/Akt/mTOR activation has been reported to indicate a poor prognosis of T-ALL, and inhibition of the PI3K/Akt/mTOR network is a promising innovative strategy for T-ALL [21]. DEPTOR inhibits p70S6K1 phosphorylation [22] and activates AKT [23] to promote T-ALL progression. In addition, DEPTOR inhibits mTORC1 and increases mTORC2 to phosphorylate S437 and T308 residues of AKT, thus activating THE AKT pathway to promote T-ALL progression [24]. The present study showed that miR-598-3p decreased p-AKT protein expression, while miR-598-3p knockdown increased p-AKT, suggesting that miR-598-3p might suppress T-ALL proliferation by inactivating the DEPTOR/AKT pathway. Moreover, consistent with a previous study reporting that PI3K/Akt pathway inhibition by LY294002 arrests cell growth and enhances the apoptosis of T-ALL [25], T-ALL cells pretreated with LY294002 in the present study also decreased cell viability and proliferation. LY294002 treatment counteracted the suppressive effect of miR-598-3p on T-ALL cell proliferation, further confirming that the AKT pathway was involved in miR-598-3p/DEPTOR-mediated T-ALL progression.

CONCLUSION

The findings of this study demonstrate that miR-598-3p might function as a tumor suppressor in pediatric T-ALL, with miR-598-3p directly targeting DEPTOR to promote inactivation of the AKT pathway and suppression of T-ALL progression. These results may provide a means to develop a novel therapeutic miR-598-3p delivery approach for T-ALL.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Availability of data and materials

All the data generated or analyzed during this study are included in this published article.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this

article will be borne by the authors. Zhang Qiang and Jinhua Feng designed the study and supervised the data collection. Chunlian Wang analyzed and interpreted the data. Meizhu Zheng and Zhuoyu Wen prepared the manuscript for publication and reviewed the draft of the manuscript. All the authors have read and approved the manuscript.

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