miR-141-3p and TRAF5 Network Contributes to the Progression of T-Cell Acute Lymphoblastic Leukemia

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Abstract
Numerous lines of evidence have shown that microRNAs (miRNAs) play a vital role in regulating the progression in many types of cancers, including T cell acute lymphoblastic leukemia (T-ALL). In this study, the potential underlying mechanism and functional role of miR-141-3p in T-ALL cells were determined. We found that the expression level of miR-141-3p was significantly downregulated, while that of tumor necrosis factor receptor-associated factor 5 (TRAF5) was strongly upregulated in tissues from patients with T-ALL compared with healthy controls. Subsequently, upregulation of miR-141-3p significantly repressed T-ALL cell proliferation and promoted cell apoptosis. Conversely, downregulation of miR-141-3p significantly inhibited cell apoptosis and enhanced T-ALL cell proliferation. We also verified that TRAF5 was the direct target of miR-141-3p in T-ALL cells. Additionally, TRAF5 overexpression significantly repressed cell apoptosis and increased T-ALL cell proliferation. In summary, miR-141-3p regulates T-ALL cell progression by directly targeting TRAF5, and may serve as a potential therapeutic target for T-ALL.

Keywords
T cells, T-ALL, miR-141-3p, TRAF5, apoptosis, proliferation

Introduction
T cell acute lymphoblastic leukemia (T-ALL) is an aggressive malignancy originating from T-cell precursors. It is characterized by high genetic, immunophenotypic, and clinical heterogeneity. The genetic landscape of T-ALL has been largely characterized by next-generation sequencing. However, the miRNAs of T-ALL have been less extensively studied¹. Growing evidence has shown that dysregulated expression and function of microRNAs (miRNAs) can contribute to T-ALL development and progression².

miRNAs are a class of highly conserved small non-coding RNAs that play critical roles in regulating diverse cellular processes by interacting with the 3'-untranslated region (3'-UTR) of target mRNAs to suppress expression³. Some miRNAs, such as miR-141-3p, have shown significantly decreased expression in several cancers, including prostate cancer, gastric cancer, non-small cell lung cancer, and colorectal cancer⁴⁻⁷. Yet, the role and underlying molecular mechanism of miR-141-3p in T-ALL still need further investigation.

TRAFs, including TRAF5, have been revealed as molecular activators for the nuclear factor NF-kB. Accumulated evidence has suggested that TRAF5 plays a pivotal role in the progression of many types of cancers. TRAF5 has been reported as the target of miR-26b and regulates cancer proliferation in esophageal squamous cell carcinoma⁸. TRAF5 has also been shown to be involved in the regulation of cell proliferation with miR-141-3p in colorectal cancer⁹. However, the role of TRAF5 in the miR-141-3p network remains unclear in T-ALL.

Here, we studied the effects of miR-141-3p on T-ALL cells and identified its direct target gene, TRAF5, in order to decipher the molecular mechanisms of miR-141-3p in the progression of T-ALL.

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Submitted: August 29, 2019. Revised: September 30, 2019. Accepted: October 11, 2019.

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Material and Methods

Cell Lines and Tissue Collection

The MOLT-4 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in RPMI medium (GIBCO, Grand Island, NY, USA) supplemented with 10% FBS at 37°C with 5% CO₂ and split every 2 or 3 days.

A total of 17 patients aged 1–17 years old at diagnosis with T-ALL, and 17 healthy controls of similar ages, were included at the Department of Hematology of Guangzhou First People’s Hospital, between March 2017 and November 2018. The diagnosis of T-ALL was based on a combination of morphology, cytogenetic analysis, and immunophenotyping, as previously described⁶. Clinical samples were obtained between March 2017 and November 2018, frozen in liquid nitrogen, and further stored at −80°C for subsequent analysis. None of the patients had received preoperative chemotherapy or radiation. Age, histological type, stage, grade, additional disease (i.e., diabetes, obesity, and high blood pressure) and treatment information were recorded in all cases. Tissue collection was approved by the Ethics Committee of Guangzhou First People’s Hospital, Guangzhou, China, and informed consent was obtained from each patient.

Quantitative Real-Time PCR

We used the mirVanaTM miRNA isolation kit (Ambion, Austin, TX, USA) to extract total RNA from tissue samples and cells according to the manufacturer’s instructions. The 260/280 and 260/230 ratios of absorbance values were used to assess the purity of RNA using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNAs were reverse transcribed into cDNA using the high capacity RNA-to-cDNA kit (Thermo Fisher Scientific, Waltham, MA, USA) after removing the residual DNA by DNase I (Invitrogen, Carlsbad, CA, USA). Quantitative real-time PCR (qRT-PCR) was performed in triplicate on an ABI 7500 system (Applied Biosystems, Foster City, CA, USA) using SYBR Green I detection and gene-specific primers (Applied Biosystems, Foster City, CA, USA) after removing the residual DNA by DNase I (Invitrogen, Carlsbad, CA, USA). RT-qPCR was performed using the RQI MAX Reagent (Invitrogen, Carlsbad, CA, USA) based on the manufacturer’s protocol. TRAF5 overexpression plasmid (pcDNA3.1-TRAF5, TRAF5-OE) and its negative control (pcDNA3.1, Ctrl-OE) were purchased from RiboBio.

Overexpression and Silencing

MiR-141-3p mimic, miR-141-3p inhibitor, and miRNA control (miR-NC) were commercially available from RiboBio Co., Ltd. (Guangzhou, China). Transfection of miR-141-3p mimic, inhibitor and miR-NC were performed using the RNAiMAX Reagent (Invitrogen, Carlsbad, CA, USA) based on the manufacturer’s protocol. TRAF5 overexpression plasmid (pcDNA3.1-TRAF5, TRAF5-OE) and its negative control (pcDNA3.1, Ctrl-OE) were purchased from RiboBio.

3’ UTR Luciferase Assay

For miRNA target validation, the 3’ UTR luciferase reporter assay was performed. MOLT-4 cells were seeded in a six-well plate (BD Biosciences, Bedford, MA, USA) at a density of 0.25 M cells per well with complete medium. The cells were co-transfected with 1 µg TRAF5 3’-UTR luciferase reporter construct or mutant with 20 nM miR-141-3p mimic or control (miR-NC) using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA)) with Opti-MEN (GIBCO) for 48 h. The firefly luciferase and Renilla signals were measured using the dual-luciferase reporter assay reagent from GeneCopeia (Rockville, MD, USA). Data were normalized by dividing firefly luciferase activity by Renilla luciferase activity.

The wildtype and mutant of TRAF5 3’ UTR luciferase reporter constructs were generated as previously described⁶.

Western Blotting

Total protein was extracted from cells using RIPA lysis buffer (Beyotime, Shanghai, China) according to the manufacturer’s protocol. Western blotting was performed according to a standard method¹¹. All the experiments were repeated in triplicate. All the results are from separate blots to avoid possible problems related to incomplete stripping.

The primary antibodies: TRAF5 (1:1000 dilution, Abcam, Cambridge, MA, USA), CDK2, 1:1000, (Abcam), cleaved caspase-3 (1:500, Abcam) and GAPDH (1:500, Abcam). The secondary antibodies were anti-rabbit IgG conjugated with HRP (1:3000, Abcam).

Flow Cytometry

Apoptosis was detected by flow cytometry. Cell viability of cell lines was compared against their treated (MiR-141-3p mimic, miR-141-3p inhibitor or TRAF5 overexpression) and their negative controls (miR-NC or Ctrl-OE) after treatment with TNF-α (20 ng/ml) (Sigma-Aldrich, St Louis, MO, USA) for 16 h. Apoptotic or dead cells were detected by staining with Annexin V–FITC and propidium iodide during flow cytometry according to the manufacturer’s protocol (BD Biosciences, Bedford, MA, USA). The percentage of apoptotic cells by flow cytometry at different time points was quantitated.

Report

The statistics were calculated with the comparative threshold cycle (2−ΔΔCt) method¹⁰.
Cell Proliferation Assay
The effect of miR-141-3p on the growth of T-ALL cells was carried out by Cyquant assay (Thermo Fisher Scientific, Waltham, MA, USA). Cells were seeded in a 96-well plate (BD Biosciences, Bedford, MA, USA) at a density of 5000 cells per well. Plates were frozen at certain indicated times following incubation; 100 µl of fresh prepared Cyquant solution was added to the wells and incubated in the dark for 45 min at room temperature. Plates were read at excitation at 497 nm and emission at 520 nm.

Two-Dimensional Colony Formation Assay
The effect of miR-141-3p on the ability of T-ALL cells to form colonies was tested in two-dimensional (2D) culture. For 2D culture, T-ALL cells were seeded into individual wells of a six-well plate at a density of 500 cells per well and cultured for 14 days. The cells were then stained with crystal violet solution (0.5%, v/v) (Millipore, Billerica, MA, USA) for 30 min.

Statistical Analysis
All the statistical analyses were performed using the SPSS software (version 20.0, SPSS Inc, Chicago, IL, USA). Each experiment was carried out in triplicate in parallel. The data were subjected to the two-tailed, unpaired Student’s t test between two conditions. All data were expressed as mean ± standard error (SE). p < 0.05 was defined as statistical significance.

Results
miR-141-3p Is Downregulated in T-ALL Tissues
MiR-141-3p is one of the downregulated miRNAs in T-ALL tissues in our preliminary miRNA microarray data (data not shown) for differentially expressed miRNAs in the tissues from 17 patients with T-ALL and 17 healthy controls. To verify the preliminary analysis results, we detected the expression of miR-141-3p in T-ALL tissues and control healthy tissues. As shown in Fig. 1, miR-141-3p expression was significantly downregulated in patients with T-ALL compared with healthy controls.

Effect of miR-141-3p Alternation on T-ALL Cell Apoptosis and Proliferation
To elucidate the biological functions of miR-141-3p in T-ALL, we used miR-141-3p mimic and inhibitor to regulate the expression levels of miR-141-3p in MOLT-4 cells. The overexpression and silencing of miR-141-3p were revealed by qRT-PCR (Fig. 2A).
As shown in Fig. 2B and C, upregulation of miR-141-3p significantly enhanced MOLT-4 cell apoptosis and suppressed MOLT-4 cell proliferation, whereas downregulation of miR-141-3p significantly inhibited MOLT-4 cell apoptosis and promoted MOLT-4 cell proliferation compared with miR-NC and untransfected cells. The common caspase-mediated signaling cascade considered a hallmark of apoptosis was assessed by Western blotting of the cleaved forms of caspase-3 in MOLT-4 cells. We found the protein expression level of cleaved caspase-3 was higher in MOLT-4 cells overexpressing miR-141-3p (Fig. 2D); in contrast, the protein expression level of cleaved caspase-3 was lower in MOLT-4 cells silencing of miR-141-3p (Fig. 2D). Additionally, the number of colonies formed also decreased markedly compared with miR-NC and untransfected cells when miR-141-3p was upregulated (Fig. 2E). Conversely, the number of colonies formed increased notably compared with miR-NC and untransfected cells when miR-141-3p was downregulated (Fig. 2E).

Subsequently, we detected the protein expression level of CDK2. CDK2 is a cell proliferation-related marker. We found that the protein expression level of CDK2 was lower in MOLT-4 cells overexpressing miR-141-3p (Fig. 2F); in contrast, the protein expression level of CDK2 was higher in MOLT-4 cells silenced for miR-141-3p (Fig. 2F). These results suggest that miR-141-3p is involved in regulation of T-ALL cell proliferation.

TRA5 is Repressed by miR-141-3p
TRA5 has been identified as a direct target in colorectal cancer6. However, the expression and functions of miRNAs are cell and tissue specific. So, we next verified whether TRAF5 was also the direct target of miR-141-3p in MOLT-4 cells. The mRNA expression level of TRAF5 was found to be significantly upregulated in tissues from 17 patients with T-ALL compared with tissues from 17 healthy controls (Fig. 3A). We also found that miR-141-3p significantly suppressed the luciferase activities of wildtype
TRAF5 3' UTR but not miR-NC (Fig. 3B). In contrast, the mutation of binding site resulted in no inhibition of reporter activity by miR-141-3p (Fig. 3B).

Moreover, mRNA and protein expression levels of TRAF5 in MOLT-4 cells were determined after transfection with miR-141-3p mimic or inhibitor or miR-NC by qRT-PCR and western blotting. TRAF5 expression decreased significantly in MOLT-4 cells transfected with miR-141-3p mimics compared with those transfected with miR-NC at both mRNA and protein levels (Fig. 3C and D), whereas miR-141-3p silencing increased the expression levels of TRAF5 at both mRNA and protein levels (Fig. 3C and D).

TRAF5 Overexpression Reversed the Effects of miR-141-3p on T-ALL Cells

To assess the role of TRAF5 in the miR-141-3p regulating network in MOLT-4 cells, we upregulated TRAF5 in
MOLT-4 cells overexpressing miR-141-3p to determine whether cell apoptosis and proliferation were affected. First, we confirmed TRAF5 overexpression at the protein level by western blotting (Fig. 4A). Secondly, TRAF5 upregulation had a strong inhibitory effect on apoptosis and an enhancing effect on cell proliferation of MOLT-4 cells overexpressing miR-141-3p compared with Ctrl-OE (Fig. 4B and C). Thirdly, expression of cleaved caspase-3 protein level was also downregulated after TRAF5 overexpression (Fig. 4D). Additionally, we also observed that TRAF5 overexpression increased the number of colonies markedly compared with the Ctrl-OE (Fig. 4E). Further, CDK2 protein expression was also upregulated after TRAF5 overexpression (Fig. 4F). These results strongly indicate that the effect of miR-141-3p upregulation on MOLT-4 cells was rescued by TRAF5 overexpression.

Discussion
Mounting evidence has shown that miRNAs play widespread and diverse roles in the regulation of many processes during tumorigenesis and progression, including cell proliferation. The downregulation of miR-141-3p has been revealed to enhance cancer cell progression through different mechanisms in many types of cancers in previous studies. miR-141-3p overexpression has been reported to markedly suppress cell proliferation of HCT116 and SW480 cells. miR-141-3p suppresses tumor growth in papillary thyroid cancer via targeting Yin Yang. However, investigation of the features of expression and roles of miR-141-3p in T-ALL remains an ongoing process. In this study, we found that miR-141-3p was downregulated in T-ALL tissues compared with healthy control groups. MiR-141-3p overexpression enhanced cell apoptosis and repressed cell
proliferation and colony formation in MOLT-4 cells. Conversely, miR-141-3p inhibition repressed cell apoptosis and promoted cell proliferation and colony formation in MOLT-4 cells. These results suggest that miR-141-3p contributes to the progression of T-ALL.

TRAFs are a family of critical adaptors that are involved in many biological processes, such as cell proliferation, inflammation, and apoptosis. Among them, TRAF5 has been shown to be correlated with interleukin-5 induced cancer cell metastasis. TRAF5 has also been reported to affect colorectal cancer cell proliferation within the network of miR-873. In addition, TRAF5 was found to be directly targeted by miR-141-3p and involved in the regulation of cell proliferation, migration, and invasion in colorectal cancer. Consistent with these studies, we first found that TRAF5 expression was upregulated in T-ALL tissues. Secondly, we verified that TRAF5 was the direct target of miR-141-3p. Upregulation of TRAF5 then rescued the effect of miR-141-3p overexpression on MOLT-4 cells. The upregulation of TRAF5 had a strong inhibitory effect on apoptosis, an enhancing effect on cell proliferation, and increased the number of colonies of MOLT-4 cells overexpressing miR-141-3p compared with Ctrl-OE. Further, the CDK2 protein expression level was also upregulated after TRAF5 overexpression. Taken together, our results indicate that miR-141-3p-mediated tumor-suppressive roles in T-ALL act via the regulation of TRAF5. The present study generated the hypothesis that miR-141-3p contributes to cell progression through regulation of TRAF5. However, it is worth verifying this model in vivo in the future studies. The investigation of downstream effectors of TRAF5 in T-ALL cells is also worth future work.

Collectively, our study indicates that miR-141-3p contributes to T-ALL progression by regulating TRAF5. Our study also highlights the therapeutic potential of miR-141-3p/TRAF5 for future clinical applications.

Acknowledgments
The authors would like to thank the clinical laboratory of Guangzhou First People’s Hospital for the pathologic diagnosis.

Author contributions
RZ conceived the study. RZ and WM designed the experiments and wrote the manuscript. SW, WZ and XC collected the samples and
completed the experimental part of the study. RZ and SP analyzed the data and revised the manuscript.

**Ethical approval**
Ethical approval was obtained for all experimental procedures by the Ethics Committee of Guangzhou First People’s Hospital, Guangzhou, China.

**Statement of Human and Animal Rights**
All procedures with human subjects in this study were conducted in accordance with the human ethics committee of Guangzhou First People’s Hospital, Guangzhou, China. This article does not contain any studies with animals.

**Statement of Informed Consent**
Verbal informed consent was obtained from the patients for their anonymized information to be published in this article.

**Declaration of Conflicting Interests**
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Funding**
The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This research was supported by the Natural Science Foundation of China (No. 81600147), the Fundamental Research Funds for the Central College(No. 2175060) and the Guide Project of Guangzhou Municipal Health Bureau (No.2016A011003).

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