miR-153-3p Suppresses Inhibitor of Growth Protein 2 Expression to Function as Tumor Suppressor in Acute Lymphoblastic Leukemia

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Abstract
Alterations in microRNAs expression can accelerate the development of human cancers. However, the role of miR-153-3p in acute lymphoblastic leukemia remains unknown. The expression of miR-153-3p in acute lymphoblastic leukemia cell lines was measured by quantitative real-time polymerase chain reaction. Effects of miR-153-3p expression on acute lymphoblastic leukemia cell proliferation, migration, and invasion were examined by Cell Counting Kit-8 assay, wound healing assay, and Transwell invasion assay, respectively. We then validated inhibitor of growth protein 2 as a direct target of miR-153-3p through bioinformatics analysis, luciferase activity reporter assay, and Western blot assay. The miR-153-3p expression was decreased in acute lymphoblastic leukemia cell lines. Cell proliferation, migration, and invasion of acute lymphoblastic leukemia were obviously decreased by miR-153-3p overexpression. Moreover, inhibitor of growth protein 2 was validated as a direct target of miR-153-3p and the overexpression of inhibitor of growth protein 2 reversed the suppressive effects of miR-153-3p on acute lymphoblastic leukemia cell behaviors. Based on these results, we provided evidence that miR-153-3p might be a target for the treatment of acute lymphoblastic leukemia.

Keywords
miR-153-3p, ING2, acute lymphoblastic leukemia, tumor suppressor, cell behaviors

Abbreviations
aGVHD, acute graft-versus-host disease; ALL, acute lymphoblastic leukemia; CCK-8, Cell Counting Kit-8; DMEM, Dulbecco’s Modified Eagle Medium; FBS, fetal bovine serum; ING2, inhibitor of growth protein 2; miRNAs, microRNAs; miR-153-3p, microRNA-153-3p; mt, mutant; NC-miR, negative control miRNA; PBMC, peripheral blood mononuclear cell line; snRNA, small nuclear RNA; qRT-PCR, quantitative real-time PCR; 3′-UTR, 3′-untranslated region; Wt, wild-type.

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Introduction
Acute lymphoblastic leukemia (ALL) is the most commonly diagnosed childhood cancer originated from B or T progenitor cells.1 Overall survival of patients with ALL exceeded 80% at present; however, 15% to 20% of patients will endure recurrence.2,3 The main reason is the pathogenesis of ALL was not fully understood. Therefore, exploring the mechanisms of ALL to identify novel targets for ALL treatment is necessary.

MicroRNAs (miRNAs) are conserved noncoding RNAs to suppress target gene expression by 3′-untranslated region (3′-UTR) binding.4 A body of literature has pointed out the importance of miRNAs in the regulating human cancer progression by functioning as tumor suppressor or oncogene.5,6 Micro-RNAs were also showed to have the potential to be developed as diagnosis, prognosis, and even as therapeutic targets in ALL.7

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The miR-153-3p is an miRNA that implicated to have crucial roles in disease progression.8-11 Zhao et al revealed that miR-153-3p could inhibit the progression of acute graft-versus-host disease (aGVHD) via inhibition of indoleamine-2,3-dioxygenase and correlated with the survival of aGVHD mice.8 Meanwhile, miR-153-3p was also reported to function as tumor suppressor in human cancers including melanoma, thyroid carcinoma, and glioma.9-11 MicroRNA-153-3p expression was found downregulated in melanoma tissues and cells, and the overexpression of miR-153-3p inhibited cell proliferation and invasion but promoted apoptosis by regulating snail family transcriptional repressor 1.9 Besides that, miR-153-3p was found to have reduced expression in radioresistant glioma.11 Moreover, it was found miR-153 overexpression promoted the radiosensitivity and apoptosis in glioma, suggesting miR-153-3p is a potential target to enhance radiosensitivity on glioma.11

In this study, quantitative real-time polymerase chain reaction (qRT-PCR) was used to investigate miR-153-3p expression in ALL cell lines. Moreover, miR-153-3p expression in patients with ALL was also detected to utilize the public data set in gene expression omnibus (GEO). We then investigated the biological roles of miR-153-3p in ALL cell lines using gain-of-function studies. Luciferase activity reporter assay and Western blot assay were employed to investigate the connection between miR-153-3p and inhibitor of growth protein 2 (ING2). Furthermore, we overexpressed ING2 in the miR-153-3p-upregulated ALL cell lines to assess the role of ING2 in ALL.

Materials and Methods

Data Source

The miRNA profiling GSE109868 submitted by Qian Jiang was extracted from GEO database (http://www.ncbi.nlm.nih.gov/geo/), which was detected on the platform of TaqMan human miRNA A/B Plates. MicroRNA-153-3p expression level in plasma samples from 3 healthy children and 3 newly diagnosed ALL patients was analyzed.

Cell Lines and Cell Transfection

Acute lymphoblastic leukemia cell lines (Molt-3 and Molt-4) and human primary peripheral blood mononuclear cell line (PBMC) purchased from American Type Culture Collection (Manassas, Virginia) were incubated in Dulbecco’s Modified Eagle Medium (DMEM; Invitrogen, Thermo Fisher Scientific, Inc, Waltham, Virginia) supplemented with fetal bovine serum (FBS; Invitrogen, Thermo Fisher Scientific, Inc) at 37°C humidified incubator containing 5% CO2. Meanwhile, miR-153-3p was also reported to function as tumor suppressor in human cancers including melanoma, thyroid carcinoma, and glioma.9-11 MicroRNA-153-3p expression was found downregulated in melanoma tissues and cells, and the overexpression of miR-153-3p inhibited cell proliferation and invasion but promoted apoptosis by regulating snail family transcriptional repressor 1.9 Besides that, miR-153-3p was found to have reduced expression in radioresistant glioma.11 Moreover, it was found miR-153 overexpression promoted the radiosensitivity and apoptosis in glioma, suggesting miR-153-3p is a potential target to enhance radiosensitivity on glioma.11

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MicroRNA-153-3p mimic (5'-UUGCUAUAGUCACAAA-GUGAUC-3'), miR-153-3p inhibitor (5'-GAUCACUUUUGU-GACUAUGCAAA-3'), and negative control (NC-mimic, 5'-ACUGUAUAAGUAGUCGUAAAC-3'); NC-inhibitor, 5'-AUCGUGCGAUAAUCUAGAUAUAC-3') were purchased from RiboBio (Guangzhou, China). The open reading frame of ING2 was cloned into pcDNA3.1 vector by GenScript (Nanjing, China) to generate pcDNA-ING2 plasmid. Cells were incubated until about 70% confluence and then transfected with miRNAs, pcDNA-ING2, or empty vector at a final concentration of 100 nM using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific, Inc) according to the provided instructions. After transfection for 48 hours, cells were collected for further analyses.

RNA Extraction and qRT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Inc) according to the manufacturer’s protocols. Complementary DNA was synthesized using Quantscript RT Kit (Tiangen, Beijing, China). MicroRNA-153-3p expression was quantified at 7500 Fast Real-time PCR System (Applied Biosystems, Foster City, California) using SYBR Premix Ex Taq II kit (Takara, Dalian, China) with U6 small nuclear RNA (snRNA) as internal control. Relative expression levels were calculated using the ΔΔCt method. The primers used were as follows: miR-153-3p, 5’-ACACTCCAGCTGGGTGATGAGTCATAAAA-3’ (forward) and 5’-CAGTGCTGCAGTGGACTGATG3’ (reverse); U6 snRNA, 5’-CCCTTGGGAGATCCCGAATA-3’ (forward) and 5’-TTTGACGTGCATCTTTG-3’ (reverse). The following thermocycling conditions were used: 10 minutes at 95°C, 40 cycles of 1 minute at 95°C, 2 minutes at 63°C, and 1 minute at 72°C.

Protein Isolation and Western Blot

Total proteins were extracted using Radio Immunoprecipitation Assay (RIPA) lysis buffer supplemented with protease inhibitor (Beyotime, Haimen, Jiangsu, China). Protein samples were separated at 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and then transferred to polyvinylidene fluoride (PVDF) membrane (Beyotime). After blocked with fat-free milk, membranes were incubated with primary antibodies (rabbit anti-ING2: ab109504; rabbit anti-MMP-9: ab76003; rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH): ab181602; Abcam, Cambridge, Massachusetts) for overnight incubation. Cells were incubated until about 70% confluence and then transfected with miRNAs, pcDNA-ING2, or empty vector at a final concentration of 100 nM using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific, Inc) according to the provided instructions. After transfection for 48 hours, cells were collected for further analyses.

Cell Counting Kit-8 Assay

Cells (4000 cells/well) were seeded in 96-well plates and incubated for 24 hours. At indicated time, Cell Counting Kit-8 (CCK-8) reagent was added to each well and further incubated for 2 hours, after that the optical density of each well was measured at 450 nm.

Wound Healing Assay

A total of 3.5 × 105 cells were seeded in 12-well plates and incubated for 24 hours. A 10-μL pipette tip was used to create a
wound in cell surface. At 0 hours and 24 hours, images were captured under light microscopy and analyzed with Image J version 1.42 software (NIH).

**Transwell Invasion Assay**

Transwell invasion assay was conducted using a Matrigel-coated chamber (8-μm, Costar, Cambridge, United Kingdom). The lower chamber was filled with DMEM supplemented with FBS, while 1 × 10^5 cells in serum-free medium were seeded in the upper chamber. After 24 hours of incubation, invasive cells were stained with crystal violet (Beyotime). Cell number in 5 random fields was counted under a microscope.

**Luciferase Activity Reporter Assay**

TargetScan algorithm revealed that ING2 3′-UTR contains a binding site for miR-153-3p. The wild-type (wt) or mutant (mt) 3′-UTR of ING2 were cloned into pGL3 vector (Promega, Madison, Wisconsin) and designated as wt-ING2 or mt-ING2. Cells were cotransfected with NC-miR or miR-153-3p mimic and wt-ING2 or mt-ING2 using Lipofectamine 2000. After 48 hours of transfection, cells were collected and analyzed using dual-luciferase reporter assay system (Promega), according to the manufacturer’s instruction.

**Statistical Analysis**

Data were presented as the mean (standard deviation) and analyzed using Student t test (2 groups) or 1-way analysis of variance with a Tukey post hoc test (3 or above groups). Data analysis was conducted using GraphPad Prism version 6.0 software (GraphPad Software, Inc, La Jolla, California). *P < .05 was considered to indicate a statistically significant.

**Results**

**Expression Level of miR-153-3p in ALL**

MicroRNA-153-3p expression in ALL cell lines (Molt-3 and Molt-4) and human primary PBMC was analyzed by qRT-PCR. As shown in Figure 1A, miR-153-3p expression was significantly reduced in ALL cell lines compared to PBMC cell line. In addition, we found miR-153-3p expression level in ALL patients was lower than in health people (Figure 1B).

**Overexpression of miR-153-3p Inhibits All Cell Proliferation, Migration, and Invasion**

To explore the role of miR-153-3p in ALL, we manipulated miR-153-3p expression in ALL cell lines using synthetic miRNAs. As shown in Figure 2A, the transfection of miR-153-3p mimic increased the levels of miR-153-3p in ALL cell lines. Cell proliferation analysis by CCK-8 assay revealed that cell vitality with miR-153-3p mimic transfection was lower than that in the NC-miR group (Figure 2B). In addition, wound healing assay showed that the presence of miR-153-3p mimic obviously decreased cell migration (Figure 2C). Furthermore, transwell invasion assay confirmed the inhibitory effect of miR-153-3p on cell invasion (Figure 2D). In addition, we examined the levels of MMP-9 in ALL cells transfected with synthetic miRNAs. It was found MMP-9 expression was downregulated by miR-153-3p mimic (Figure 2E).

**Downregulation of miR-153-3p Promotes PBMC Cell Proliferation, Migration, and Invasion**

To further confirm the biological function of miR-153-3p, the miR-153-3p expression level in PBMC cell line was reduced by miR-153-3p inhibitor. The qRT-PCR results showed that miR-153-3p expression was reduced in PBMC cell line by miR-153-3p inhibitor (Figure 3A). Cell Counting Kit-8 assay, colony
formation assay, and transwell invasion assay revealed that the introduction of miR-153-3p inhibitor increased cell proliferation, colony formation ability, and cell invasive ability compared to NC inhibitor (Figure 3B-D). In the meantime, MMP-9 expression was also shown to be upregulated by miR-153-3p inhibitor (Figure 3E).

Inhibitor of Growth Protein 2 Was a Direct Target of miR-153-3p

TargetScan algorithm showed ING2 was a potential target of miR-153-3p (Figure 4A). Meanwhile, it was found miR-153-3p mimic transfection decreased the expression of ING2 in ALL cell lines (Figure 4B). To investigate whether miR-153-3p inhibits ING2 expression through 3'-UTR binding, luciferase activity reporter assay was conducted. It was found miR-153-3p mimic transfection inhibited the luciferase activity of cells transfected with wt-ING2, but not mt-ING2 (Figure 4C). We found ING2 expression was higher in ALL cell lines than in PBMC cell line (Figure 4D).

MicroRNA-153-3p Regulates ALL Cell Behaviors Through Targeting ING2

To confirm the tumor suppressive activity of miR-153-3p by downregulating ING2, we cotransfected pcDNA-ING2 and miR-153-3p mimic into ALL cell lines. As expected, pcDNA-ING2 transfection increased the levels of ING2 and reversed the inhibitory effect of miR-153-3p mimic on ING2 expression (Figure 5A). Meanwhile, MMP-9 level was found to be increased by pcDNA-ING2 (Figure 5A). Cell Counting Kit-8 assay showed that overexpression of ING2 significantly reversed the miR-153-3p-induced suppression on cell proliferation (Figure 5B). Wound healing assay showed ING2 overexpression promoted cell migration and partially reversed the effect of miR-153-3p on cell migration (Figure 5C). Transwell invasion assay confirmed ING2 expression could stimulate cell invasion and reverse the inhibitory effects of miR-153-3p on cell invasion (Figure 5D).

Discussion

Evidence has indicated that miRNAs function as critical roles in regulating various biological processes including...
miR-181a was found to promote ALL cell proliferation and cell cycle progression through targeting EGR1. Interestingly, a recent study showed miR-181a functions as tumor suppressor in pediatric ALL through interaction with Smad7-regulated TGF-β1 signaling and other signaling pathway. Moreover, miR-124 was found to target glucocorticoid receptor to regulate ALL cell proliferation and apoptosis and contribute to glucocorticoid resistance, which demonstrated miR-124 may be a therapeutic target in ALL with poor glucocorticoid response. miR-192 was recently reported as a tumor suppressive miRNA in ALL progression by targeting a handful of target genes.

In our study, we investigated miR-153-3p expression and its mechanism in ALL. We found miR-153-3p expression was decreased in ALL cell lines and patients, indicating miR-153-3p might be involved in the tumorigenesis of ALL. We further investigated the biological roles of miR-153-3p in ALL and found miR-153-3p overexpression inhibits cell proliferation, migration, and invasion. Collectively, these results suggested that miR-153-3p suppressed ALL progression by downregulating cell growth, migration, and invasion. Moreover, we showed MMP-9, an invasive marker, was decreased by miR-153-3p mimic in ALL cell lines.

To explore the mechanisms underlying miR-153-3p-induced inhibitory effects on ALL, we identified ING2 as a potential target of miR-153-3p. As expected, miR-153-3p
overexpression decreased the protein levels of ING2 in ALL cell lines. Luciferase activity reporter assay validated that miR-153-3p regulates ING2 expression through 3'UTR binding.

Inhibitor of growth protein 2, a member of the ING family, plays important roles in regulating cellular processes including cell cycle regulation, apoptosis, and so on.16 The importance of ING2 as critical modulator for tumor progression has been appreciated.16-19 Pan et al found ING2 message RNA and protein expression level was decreased in non-small cell lung cancer.17 They also observed translocation of ING2 from nucleus to cytoplasm, which might be a critical event for tumorigenesis.17 Decreased ING2 expression was also discovered in osteosarcoma, and the loss expression of ING2 was a predictor for poor overall survival of osteosarcoma patients.18 Another study reported the role of ING2 in regulating cell cycle progression through targeting p21 expression.19 In line with previous studies, our results showed that miR-153-3p negatively regulate ING2 expression by 3'UTR binding. Furthermore, ING2 overexpression reversed the miR-153-3p-induced suppression on ALL cell events, indicating that miR-153-3p functions as tumor suppressor in ALL by repressing ING2.

In summary, our study revealed miR-153-3p expression was decreased in ALL cell lines and patients. Moreover, we provided evidence that miR-153-3p regulates cell proliferation, migration,
and invasion by repressing ING2 expression. Taken together, miR-153-3p may act as biomarker for the treatment of ALL.

Declaration of Conflicting Interests
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References
1. Pui CH, Yang JJ, Hunger SP, et al. Childhood Acute Lymphoblastic Leukemia: Progress Through Collaboration. J Clin Oncol. 2015;33(27):2938-2948.
2. Pui CH, Pei D, Campana D, et al. A revised definition for cure of childhood acute lymphoblastic leukemia. Leukemia. 2014;28(12):2336-2343.
3. Pui CH, Pei D, Sandlund JT, et al. Risk of adverse events after completion of therapy for childhood acute lymphoblastic leukemia. J Clin Oncol. 2005;23(31):7936-7941.
4. Alvarez-Garcia I, Miska EA. MicroRNA functions in animal development and human disease. Development. 2005;132(21):4653-4662.
5. Wieser R, Scheideler M, Hackl H, et al. microRNAs in acute myeloid leukemia: expression patterns, correlations with genetic and clinical parameters, and prognostic significance. Genes Chromosomes Cancer. 2010;49(3):193-203.
6. Tutar Y. miRNA and cancer; computational and experimental approaches. Curr Pharm Biotechnol. 2014;15(5):429.
7. Trino S, Lamorte D, Caivano A, et al. MicroRNAs as new biomarkers for diagnosis and prognosis, and as potential therapeutic targets in acute myeloid leukemia. Int J Mol Sci. 2018;19(2):pii: E460.
8. Zhao XS, Wang YN, Lv M, et al. miR-153-3p, a new bio-target, is involved in the pathogenesis of acute graft-versus-host disease via inhibition of indoleamine-2,3-dioxygenase. *Oncotarget*. 2016;7(30):48321-48334.

9. Zeng HF, Yan S, Wu SF. MicroRNA-153-3p suppress cell proliferation and invasion by targeting SNAI1 in melanoma. *Biochem Biophys Res Commun*. 2017;487(1):140-145.

10. Joo LJS, Weiss J, Gill AJ, et al. A RET-related microRNA, miR-153-3p, acts as a tumor suppressor in medullary thyroid carcinoma (MTC) via S6 K signaling. *Can Res*. 2018;78(13):501.

11. Sun D, Mu Y, Piao H. MicroRNA-153-3p enhances cell radiosensitivity by targeting BCL2 in human glioma. *Biol Res*. 2018;51(1):56.

12. Verduci L, Azzalin G, Gioiosa S, et al. microRNA-181a enhances cell proliferation in acute lymphoblastic leukemia by targeting EGR1. *Leuk Res*. 2015;39(4):479-485.

13. Nabhan M, Louka ML, Khairy E, Tash F, Ali-Labib R, El-Habashy S. MicroRNA-181a and its target Smad 7 as potential biomarkers for tracking child acute lymphoblastic leukemia. *Gene*. 2017;628:255-258.

14. Liang YN, Tang YL, Ke ZY, et al. MiR-124 contributes to glucocorticoid resistance in acute lymphoblastic leukemia by promoting proliferation, inhibiting apoptosis and targeting the glucocorticoid receptor. *J Steroid Biochem Mol Biol*. 2017;172:62-68.

15. Sayadi M, Ajdary S, Nadali F, Rostami S, Edalati Fahtabad M. Tumor suppressive function of microRNA-192 in acute lymphoblastic leukemia. *Bosn J Basic Med Sci*. 2017;17(3):248-254.

16. Guérillon C, Larrieu D, Pedex R. ING1 and ING2: multifaceted tumor suppressor genes. *Cell Mol Life Sci*. 2013;70(20):3753-3772.

17. Pan YQ, Zhang X, Xu DP, et al. Decreased expression of ING2 gene and its clinicopathological significance in Chinese NSCLC patients. *Neoplasma*. 2014;61(4):468-475.

18. Han XR, Bai XZ, Sun Y, Yang Y. Nuclear ING2 expression is reduced in osteosarcoma. *Oncol Rep*. 2014;32(5):1967-1972.

19. Larrieu D, Ythier D, Brambilla C, Pedeux R. ING2 controls the G1 to S-phase transition by regulating p21 expression. *Cell Cycle*. 2010;9(19):3984-3990.