microRNA-452 exerts growth-suppressive activity against T-cell acute lymphoblastic leukemia

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Accepted 2 December 2017

ABSTRACT

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematological cancer. Although microRNA (miR)-452 serves as a tumor suppressor in multiple solid tumors, its expression and function in hematological cancers including T-ALL is largely unknown. We measured the expression of miR-452 in 38 T-ALL and 22 normal lymph node samples by real-time PCR analysis. The methylation levels in the promoter of miR-452 were determined using MethyLight assay. The effects of miR-452 overexpression on proliferation, cell cycle distribution, and tumorigenesis were explored. It was found that miR-452 expression levels were significantly lower in T-ALL specimens than in normal lymph node biopsies (P=0.0079). T-ALL specimens had a significantly higher methylation level in the promoter of miR-452 than normal lymph node tissues (P=0.0014). Consistently, miR-452 was downregulated in Jurkat and Molt-4 T-ALL cells, whose expression was restored after treatment with a demethylation agent 5-aza-2′-deoxycytidine. Ectopic expression of miR-452 inhibited the proliferation of Jurkat and Molt-4 cells and induced a G0/G1 cell cycle arrest. Overexpression of miR-452 suppressed the protein expression of BMI1 in T-ALL cells. Rescue experiments revealed that overexpression of BMI1 partially reversed the growth-suppressive effect of miR-452 on T-ALL cells. Xenograft tumor studies confirmed that overexpression of miR-452 suppressed tumor growth in nude mice and reduced the expression of BMI1. Collectively, miR-452 is epigenetically silenced and targets BMI1 to exert a growth suppressive activity in T-ALL. Restoration of miR-452 expression may represent a promising therapeutic strategy for this malignancy.

Significance of this study

What is already known about this subject?
- T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematological cancer.
- microRNA (miR)-452 serves as a tumor suppressor in multiple solid tumors.

What are the new findings?
- miR-452 expression levels were significantly lower in T-ALL specimens than in normal lymph node biopsies.
- T-ALL specimens had a significantly higher methylation level in the promoter of miR-452 than normal lymph node tissues.
- miR-452 was downregulated in Jurkat and Molt-4 T-ALL cells.
- Ectopic expression of miR-452 inhibited the proliferation of Jurkat and Molt-4 cells and induced a G0/G1 cell cycle arrest, suppressed the protein expression of BMI1 in T-ALL cells.
- Xenograft tumor studies confirmed that overexpression of miR-452 suppressed tumor growth in nude mice and reduced the expression of BMI1.

How might these results change the focus of research or clinical practice?
- miR-452 may represent a potential therapeutic target for T-ALL.

INTRODUCTION

T-cell acute lymphoblastic leukemia (T-ALL), which arises from the malignant transformation of T-cell progenitors, is an aggressive hematological cancer.[1] It is estimated that T-ALL accounts for 15 per cent of pediatric and 25 per cent of adult ALL cases.[2] High-dose, multiagent combination chemotherapy is a primary strategy to treat T-ALL.[3] Although there is an improvement in cure rates, some patients develop chemoresistant disease and have a poor outcome.[4] Understanding of the molecular mechanism governing T-ALL growth and progression is of significance to develop effective therapeutic approaches. microRNAs (miRs) are small non-coding RNAs that negatively modulate gene expression via binding to the complementary sequences in the 3′-untranslated region (3′-UTR) of target mRNAs.[5] Mounting evidence indicates the implication of miRs in the pathogenesis of many human cancers.[6 7] It has been documented that miR-590 facilitates the proliferation and invasion of T-ALL cells by suppressing the expression of RB1.[8] Another study showed that ectopic expression of miR-212 induces apoptotic death in T-ALL cells.[9] Downregulation of miR-452 occurs in several cancer types such as non-small cell lung cancer (NSCLC)[10] and osteosarcoma[11] and shows a poor prognostic impact on patient survival. In vitro studies

To cite: Wang H, Guo Q, Zhu G, *et al*. *J Investig Med* Epub ahead of print: [*please include* Day Month Year]. doi:10.1136/jim-2017-000591

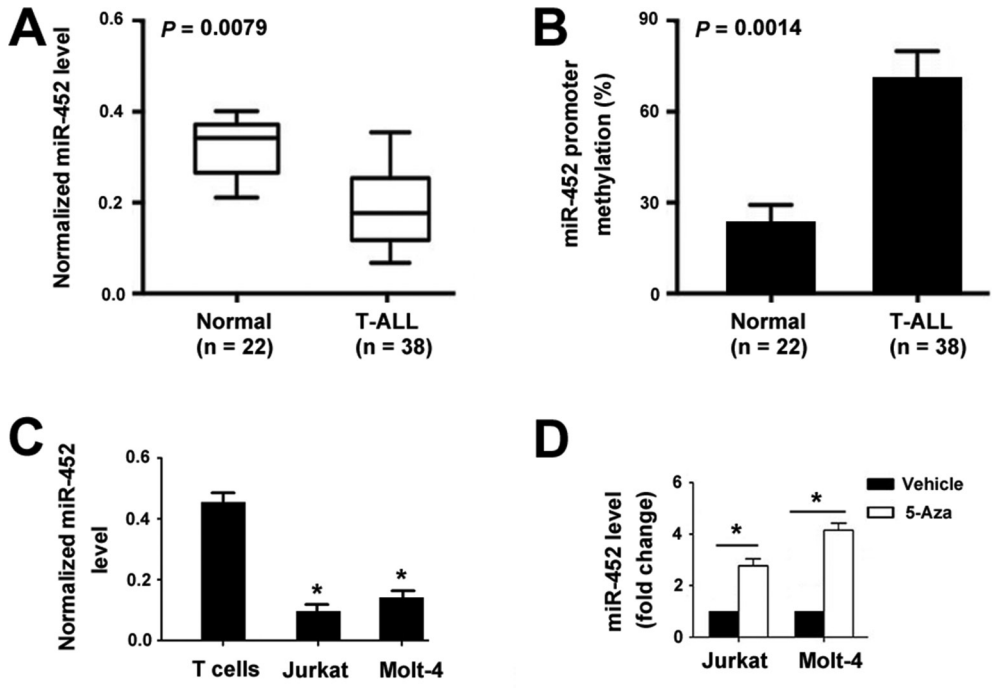

Figure 1 MicroRNA (miR)-452 is downregulated in T-cell acute lymphoblastic leukemia (T-ALL). (A) Detection of miR-452 levels in 38 T-ALL and 22 normal lymph node samples by real-time PCR analysis. (B) Analysis of methylation levels in the promoter of miR-452 in T-ALL specimens and normal lymph node biopsies by MethyLight assays. (C) Real-time PCR analysis of miR-452 levels in T-ALL cell lines and normal T lymphocytes. *P < 0.05 vs normal T cells. (D) Measurement of miR-452 levels in Jurkat and Molt-4 cells after treatment with 5-aza-2′-deoxycytidine (5-Aza). *P < 0.05.

demonstrated that miR-452 exhibits anti-invasive activity in NSCLC[12] and prostate cancer.[13] Despite the importance of miR-452 in the progression of solid tumors, relative little is known concerning its expression and function in hematological cancers including T-ALL.

In this work, we examined the expression of miR-452 in tumor samples from 38 patients with T-ALL and normal lymph node biopsies from 22 healthy controls. The effects of miR-452 on the growth, cell cycle progression, and tumorigenesis of T-ALL cells were further analyzed. In addition, the target gene(s) involved the action of miR-452 was identified.

MATERIALS AND METHODS

Tissue specimens

We collected tumor specimens from 38 patients with T-ALL and normal lymph node biopsies from 22 healthy controls. There were 22 males and 16 females in the series of patients with T-ALL, with a median age of 45 years (range, 24–76 years). All cases were histologically diagnosed. Each participant gave a written informed consent. Tissue samples were snap-frozen in liquid nitrogen immediately after surgery and stored at −80°C until use.

Cell culture

Jurkat and Molt-4 T-ALL cell lines were purchased from the American Type Culture Collection (Manassas, Virginia, USA). Normal T lymphocytes were obtained from peripheral blood using anti-CD4 and anti-CD8 monoclonal antibodies (Miltenyi Biotec, Auburn, California, USA). All cells were maintained in RPMI 1640 medium (Sigma-Aldrich, St. Louis, Missouri, USA) supplemented with 10 per cent fetal bovine serum (HyClone Laboratories, South Logan, Utah, USA) at 37°C in a humidified atmosphere with 5 per cent CO_2. For demethylation treatment, Jurkat and Molt-4 cells were incubated with 2 μM 5-aza-2′-deoxycytidine (5-Aza; Sigma-Aldrich) for 72 hours[14] and subjected to gene expression analysis.

Real-time PCR analysis

Total RNA from tissues and cell samples were extracted using TRizol reagent (Invitrogen, Carlsbad, California, USA). Reverse transcription was achieved using the RevertAid First Strand cDNA Synthesis kit (Fisher/Thermo Scientific, Pittsburgh, Pennsylvania, USA). Mature miR-452 was amplified by PCR and quantified using the TaqMan microRNA Assay kit following the manufacturer's instructions (Applied Biosystems, Foster City, California, USA). The relative expression of miR-452 was calculated after normalization to RNU6B by the $2^{-\Delta\Delta Ct}$ method. For quantification of BMI1 transcript, real-time PCR was performed using the SYBR-Green PCR Master Mix (Applied Biosystems) with the following primers[15]: *BMI1* forward, 5′-AATTAGTTCCAGGGC TTTTCAA-3′ and *BMI1* reverse, 5′-CTTCATCTGCA ACCTCTCCTCTAT-3′. *GAPDH* was amplified using the following PCR primers: forward, 5′-CGACCACTTTGT-CAAGCTCA-3′ and reverse, 5′-AGGGGTCTACATGG-CAACTG-3′. The mRNA level of BMI1 was normalized against that of GAPDH.

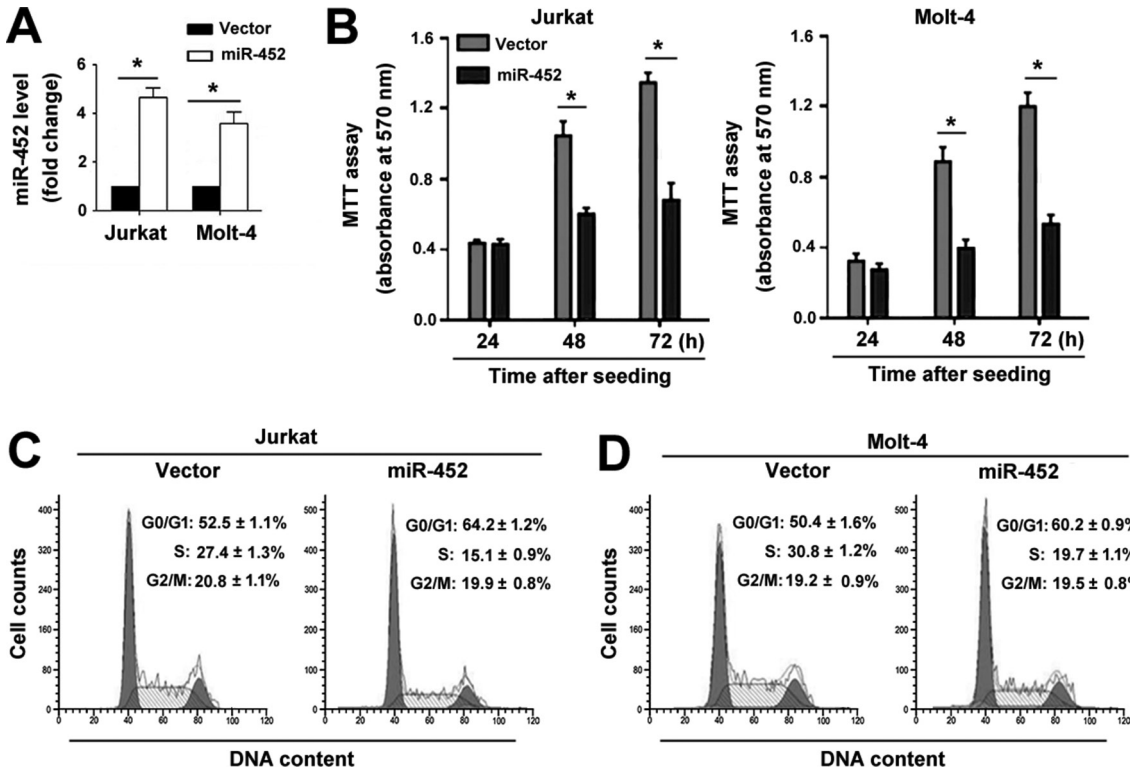

Figure 2 Ectopic expression of microRNA (miR)-452 induces G0/G1 arrest in T-cell acute lymphoblastic leukemia cells. (A) Real-time PCR analysis of miR-452 levels in Jurkat and Molt-4 cells transfected with the miR-452-expressing plasmid or empty vector. (B) 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay was done to assess the proliferation of transfected Jurkat and Molt-4 cells after culturing for 24–72 hours. (C, D) Flow cytometric analysis of cell cycle distribution in (C) Jurkat and (D) Molt-4 cells after propidium iodide staining. Data represent the results from three independent experiments. $^{*}P < 0.05$.

MethyLight assay

DNA methylation status was determined by MethyLight assay, as described previously.[16] In brief, genomic DNA from T-ALL and normal lymph node tissues was extracted using the AllPrep Mini DNA/RNA kit (Qiagen, Hilden, Germany). DNA was then bisulfite converted using the EpiTect Fast DNA Bisulfite Kit (Qiagen). Quantitative methylation-specific PCR (MethyLight) was performed using the following primers[16]: forward, 5′-GATGTTT AGGAGGATTGGAAGA-3′ and reverse, 5′-CTCCGCG-CAAATAATCG-3′. A CpG-free region of *MYOD1*, which was used as an internal control, was amplified in parallel using the primers: forward, 5′-CCAACTCCAAATCCC CTCTCTAT-3′ and reverse, 5′-TGGTTTTTTTAGGGA GTAAGTTTGTT-3′. The relative methylation level on the promoter region of miR-452 was determined after normalization to MYOD1 expression levels.

Plasmids and transfections

A fragment containing miR-452 precursor was amplified by PCR from human genomic DNA and cloned into pcDNA3.1(+) expression vector. Human *BMI1* cDNA lacking the entire 3′-UTR was obtained from Origene Technologies (Rockville, Maryland, USA) and inserted into pcDNA3.1(+). Cell transfections were performed using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's instructions. For generation of stable cell lines, Jurkat cells transfected with the pcDNA3.1/miR-452 plasmid or empty vector were selected in the presence of G418 (800 µg/mL; Sigma-Aldrich) for 2 weeks.

Cell proliferation assay

Cells were seeded onto 96-well culture plates (4×10^3 cells/well) and cultured for 24–72 hours. Afterwards, cells were incubated with the 3-(4,5-dimethyl-2-thiazolyl)−2,5-diphenyl-2-H-tetrazolium bromide (MTT) solution (0.5 mg/mL; Sigma-Aldrich) for 4 hours at 37°C. Insoluble formazan crystals were dissolved by dimethyl sulfoxide, and absorbance was measured at 570 nm.

Cell cycle analysis

For analysis of cell cycle distribution, cells were stained with 50 µg/mL propidium iodide (PI; Sigma-Aldrich) in the presence of RNase A (20 µg/mL; Sigma-Aldrich) for 1 hour. Stained cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, New Jersey, USA).

Animal studies

Jurkat cells stably transfected with the pcDNA3.1/miR-452 plasmid or empty vector were subcutaneously injected into the flanks of male BALB/c nude mice (4×10^6 cells/mouse; n=5). Tumor volumes were measured weekly from 1 week after cell injection. Animals were sacrificed 5 weeks later, and xenograft tumors were weighed.

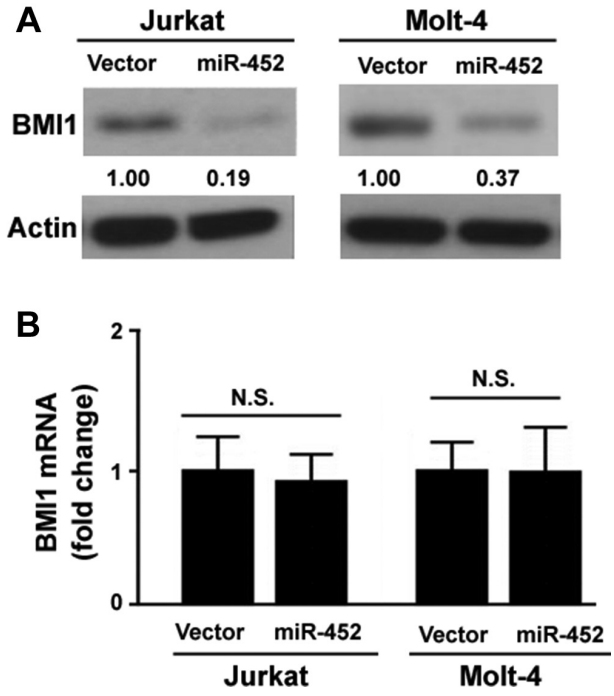

Figure 3 MicroRNA (miR)-452 targets BMI1 in T-cell acute lymphoblastic leukemia cells. (A) Western blot analysis of BMI1 protein levels in Jurkat and Molt-4 cells transfected with the miR-452-expressing plasmid or empty vector. Numbers indicate fold-change in normalized BMI1 levels. (B) Real-time PCR analysis of BMI1 mRNA levels in Jurkat and Molt-4 cells treated as in (A). N.S., no significance.

Tumor samples were paraffin-embedded and sectioned. Immunohistochemistry for BMI1 was performed with rabbit anti-Bmi1 polyclonal antibody (ab38295; Abcam, Cambridge, UK; 1:300 dilution) following standard procedures. The experiments involving animals were approved by the Animal Care and Use Committee of Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology.

Statistical analysis

Data are expressed as mean±SD. Statistical differences were analyzed using the Student's t-test or one-way analysis of variance followed by the Tukey's test. The levels of miR-452 between T-ALL specimens and normal lymph node biopsies were determined using the Mann-Whitney U test. $P<0.05$ was considered to indicate a statistically significant difference.

RESULTS

miR-452 is downregulated in T-ALL

First, we examined the expression of miR-452 in 38 T-ALL and 22 normal lymph node samples. Real-time PCR analysis demonstrated that miR-452 expression levels were significantly lower in T-ALL specimens than in normal lymph node biopsies ($P=0.0079$; figure 1A). MethyLight assay further revealed that methylation levels in the promoter of miR-452 were significantly increased in T-ALL compared with normal lymph node tissues ($P=0.0014$; figure 1B).

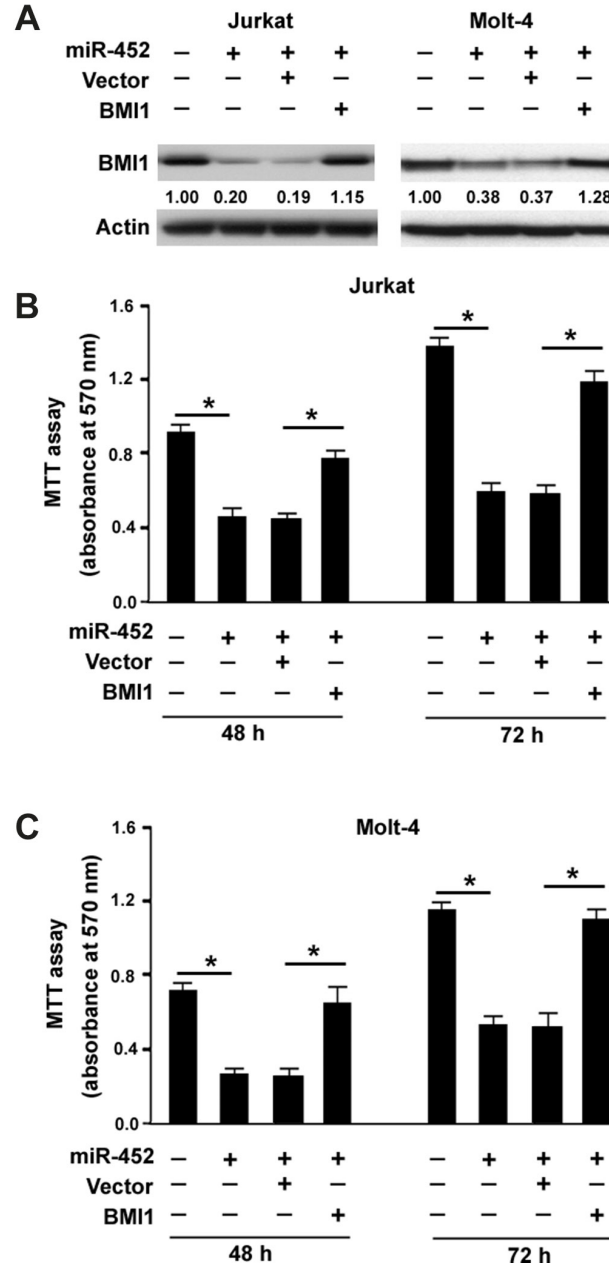

Figure 4 Enforced expression of BMI1 rescues T-cell acute lymphoblastic leukemia cells from microRNA (miR)-452-induced growth suppression. (A) Western blot analysis of BMI1 protein levels in Jurkat and Molt-4 cells transfected with indicated constructs. Numbers indicate fold-change in normalized BMI1 levels. (B) Jurkat and (C) Molt-4 cells transfected with indicated constructs were cultured for 48 and 72 hours and tested for proliferation by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assays. *$P < 0.05$.

The downregulation of miR-452 was confirmed in two T-ALL cell lines (Jurkat and Molt-4) relative to normal T lymphocytes ($P<0.05$; figure 1C). Moreover, incubation of both Jurkat and Molt-4 cells with the demethylation agent, 5-Aza led to an induction of miR-452 (figure 1D). Together, these observations indicate that DNA hypermethylation contributes to downregulation of miR-452 in T-ALL.

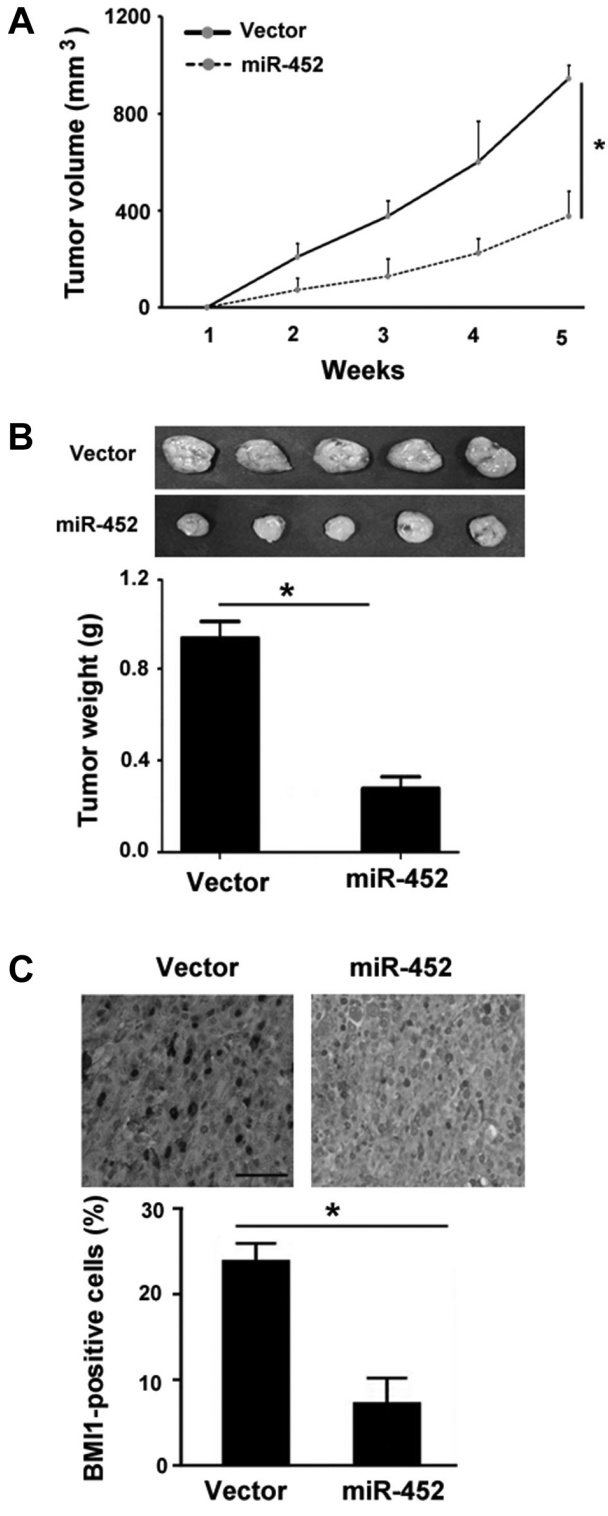

Figure 5 microRNA (miR-)452 reduces the growth of Jurkat xenograft tumors and BMI1 expression in vivo. Jurkat cells stably expressing miR-452 or vector were injected subcutaneously into nude mice (n=5), and tumor volumes were measured weekly for 5 weeks. (A) Xenograft tumor growth curves plotted based on tumor volumes. (B) Tumor weight was determined at 5 weeks after cell injection. *Upper*, representative photographs of xenograft tumors. (C) Immunohistochemistry demonstrated that BMI1 expression was significantly reduced in miR-452-overexpressing tumors relative to control tumors. Scale bar=80 µm. *P < 0.05.

Ectopic expression of miR-452 induces G0/G1 arrest in T-ALL cells

Next, we investigated the biological role of miR-452 in the proliferation of T-ALL cells. Both Jurkat and Molt-4 cells were transfected with the miR-452-expressing plasmid or empty vector and cultured for 72 hours. MTT assay showed that overexpression of miR-452 (figure 2A) led to a 49 per cent and 60 per cent decline in proliferation in Jurkat and Molt-4 cells, respectively, 72 hours after seeding (figure 2B). Flow cytometric analysis of PI-stained cells revealed that miR-452-overexpressing Jurkat cells had a higher percentage of cells in S-phase (27.4 per cent vs 15.1 per cent, P<0.05) and lower percentage of cells in G0/G1-phase (52.5 per cent vs 64.2 per cent, P<0.05; figure 2C). Likewise, miR-452-overexpressing Molt-4 cells showed a G0/G1 arrest, as evidenced by the increased percentage of G0/G1-phase cells and decreased percentage of S-phase cells (figure 2D). These results point toward the growth-suppressive activity of miR-452 in T-ALL cells.

miR-452 targets BMI1 in T-ALL cells

Bioinformatic analysis based on Targetscan software suggested that miR-452 could modulate a large number of genes (data not shown). Among them, BMI1 had attracted more attention because it is a well-defined oncogene.[17] Moreover, a previous study has reported that miR-452 can regulate BMI1 expression in NSCLC.[12] Therefore, in this study, we examined the impact of miR-452 on the expression of BMI1 in T-ALL cells. Of note, overexpression of miR-452 caused a 70 per cent and 64 per cent reduction in BMI1 protein levels in Jurkat and Molt-4 cells, respectively (figure 3A). However, the BMI1 transcript levels were not altered by miR-452 (figure 3B), suggesting a predominant repression of protein translation.

Enforced expression of BMI1 rescues T-ALL cells from miR-452-induced growth suppression

Next, we performed rescue studies to validate the role of BMI1 in mediating the growth-suppressive activity of miR-452. To this end, we constructed a miR-resistant variant of BMI1, which lacks the entire 3′-UTR. Western blot analysis showed that co-transfection with the BMI1-expressing plasmid led to a marked expression of BMI1 protein in miR-452-transfected Jurkat and Molt-4 cells (figure 4A). Overexpression of BMI1 partially restored cell proliferation in miR-452-overexpressing Jurkat (figure 4B) and Molt-4 (figure 4C) cells compared with corresponding non-transfected cells. Collectively, these data suggest that downregulation of BMI1 is responsible for miR-452-mediated growth suppression in T-ALL cells.

miR-452 reduces the growth of Jurkat xenograft tumors and BMI1 expression in vivo

Finally, we explored whether restoration of miR-452 suppresses the growth of T-ALL in vivo. To address this issue, we generated miR-452 stable expression Jurkat cells and subcutaneously injected them to nude mice. As illustrated in figure 5A, the tumors formed by miR-452-overexpressing cells grew more slowly than control tumors formed by vector-transfected Jurkat cells (P<0.05). Five weeks after cell injection, xenograft tumors were resected.

We found that tumor weight was 55 per cent lower in the miR-452 group than in the control group (0.39±0.07 vs 0.86±0.09 g, P<0.05; figure 5B). In addition, immunohistochemistry demonstrated that BMI1 expression was significantly reduced in miR-452-overexpressing tumors relative to control tumors (P<0.05; figure 5C).

DISCUSSION

Aberrant expression of miR-452 has been detected in a number of cancers including NSCLC,[10] urothelial carcinoma,[18] and bladder cancer.[19] For instance, urine miR-452 levels have a high accuracy for bladder cancer diagnosis.[19] The expression of miR-452 was downregulated in glioma tissues and negatively correlated with histological grades and patient survival.[20] In this study, we extended the observations to hematological cancers and showed that miR-452 was downregulated in T-ALL. Hypermethylation of its promoter region has been suggested to be responsible for the reduced expression of miR-452 in gliomas[20] and prostate cancer.[16] Consistently, we also found that the methylation level in the promoter of miR-452 was significantly higher in T-ALL than in normal lymph node tissues. Furthermore, demethylation treatment significantly induced the expression of miR-452 in both Jurkat and Molt-4 cell lines. Therefore, our data demonstrate that miR-452 is downregulated in T-ALL largely through hypermethylation of its promoter region.

Of note, re-expression of miR-452 significantly suppressed the proliferation of both Jurkat and Molt-4 cells. Moreover, miR-452 overexpression caused a cell cycle arrest at the G0/G1 phase. These data indicate that miR-452 has a growth-suppressive activity in T-ALL cells, which provides a biological explanation for its downregulation in T-ALL specimens. In line with our findings, a previous study has reported that adenoviral delivery of miR-452 significantly inhibited the proliferation and migration of NSCLC cells.[21] In prostate cancer cells, miR-452 also exhibits the ability to suppress cell proliferation and invasion.[13] However, it should be mentioned that in some cancer types such as hepatocellular carcinoma[22] miR-452 overexpression confers a more aggressive phenotype. Therefore, the biological function of miR-452 is cancer-type dependent.

Mechanistic investigation revealed that BMI1 acts as a direct target for miR-452. Ectopic expression of miR-452 significantly reduced the BMI1 protein levels in T-ALL cells, without affecting its mRNA expression. It is suggested that miR-452 mediates translational repression of BMI1. BMI1 is a key component of the transcription suppressor complex, the polycomb repressive complex-1.[23] BMI1 serves as an oncoprotein through repression of multiple tumor suppressor genes such as p16Ink4a, p14Arf, and PTEN.[24] Growing evidence indicates the link between BMI1 and progression of lymphomas.[25 26] It has been documented that BMI1 facilitates tumorigenicity in both Ink4a/Arf-dependent and Ink4a/Arf-independent manners in mice.[27] In this study, we performed rescue experiments and found that enforced expression of BMI1 attenuated the suppression of cell proliferation by miR-452 in T-ALL cells. In vivo studies provided further evidence that miR-452 exerts a growth-suppressive activity against T-ALL, which was accompanied by reduction of BMI1 expression. Taken together, repression of BMI1 expression represents an important mechanism for miR-452-mediaated growth suppression in T-ALL. Although we showed that BMI1 acts as a functional target mediating the activity of miR-452 in T-ALL, this miR has the capacity to target multiple other genes such as E3 ubiquitin ligase-1,[13] cyclin-dependent kinase inhibitor 1B,[22] LEF1, and TCF4.[20] Ongoing studies are designed to identify novel target genes that are involved in the action of miR-452 in T-ALL cells.

In conclusion, our data demonstrate that miR-452 is epigenetically silenced in T-ALL. Re-expression of miR-452 can suppress proliferation and tumorigenesis and induce a G0/G1 cell cycle arrest in T-ALL cells through targeting of BMI1. Therefore, miR-452 may represent a potential therapeutic target for T-ALL.

Contributors SZ and PY participated in study design in vitro experiments. HW, QG, GZ, and MZ participated in study design, in vitro experiments, in vivo experiments, and drafting of the manuscript.

Competing interests None declared.

Patient consent Obtained.

Ethics approval Institutional Review Board of Huazhong University of Science and Technology (Wuhan, China).

Provenance and peer review Not commissioned; externally peer reviewed.

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