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MiR-152-3p promotes the development of chronic myeloid leukemia by inhibiting p27

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Abstract. – OBJECTIVE: The aim of this study was to investigate the relationship between micro ribonucleic acid (miR)-152-3p and chronic myeloid leukemia (CML), and to explore the underlying mechanism.

PATIENTS AND METHODS: The expression level of miR-152-3p in the bone marrow of 40 CML patients and 30 normal controls was detected by fluorescence quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). MiR-152-3p was up-regulated or down-regulated in the CML cell line (K562) by transfection with overexpressed lentivirus LV5-miR-152-3p or interfered lentivirus LV3-miR-152-3p, respectively. Transfection efficiency of miR-152-3p was detected by qRT-PCR. Cell counting kit-8 (CCK-8) assay was applied to measure the proliferation ability of K562 cells. Flow cytometry was used to assess the cell cycle and apoptosis rate of K562 cells after transfection. P27Kip1 (p27) was confirmed as the potential target of miR-152-3p by on-line target gene prediction software. Moreover, the interaction between miR-152-3p and p27 was analyzed by luciferase reporter gene assay and Western blot.

RESULTS: MiR-152-3p was highly expressed in the bone marrow of CML patients and cell lines. In vitro experiments indicated that the apoptosis rate of K562 cells in the lentivirus LV3-miR-152-3p interference group was significantly increased, and the cell cycle was arrested in G0G1 phase. Meanwhile, the proliferation of K562 cells was markedly inhibited. However, LV5-miR-152-3p transfection remarkably promoted the proliferation and cell cycle progression of K562 cells. We searched three online public databases to predict the potential target of miR-152-3p and found that p27 was a direct target of miR-152-3p. Luciferase reporter gene assay and Western blot confirmed our hypothesis. In addition, subsequent experiments showed that up-regulation of p27 attenuated the effect of miR-152-3p on the ability of CML cells.

CONCLUSIONS: MiR-152-3p was highly expressed in the bone marrow of CML patients and cells. Meanwhile, miR-152-3p inhibited the expression of its downstream protein p27, thus promoting the proliferation of K562 cells and the entry of cells into the cell cycle. In addition, it inhibited cell apoptosis, which might be a potential target for the incidence and development of CML.

Key Words:
Chronic myeloid leukemia (CML); MiRNA-152-3p (MiR-152-3p); $P27^{Kip1}$ (p27).

Introduction

Chronic myeloid leukemia (CML), known as a malignant colonel disease, is caused by abnormalities of hematopoietic stem cells. It's reported that CML accounts for about 20% of adult leukemia, with a strong malignant and invasive ability[1]. Ph chromosome-specific cytogenetic changes may occur in most CML patients with CML. This means the translocation of ABL proto-oncogene on the long arm of chromosome 9 to the breakpoint cluster region (BCR) of the long arm of chromosome 22, thereby forming a BCR-ABL fusion gene[2]. The protein exerts a sustained activation of tyrosine kinase activity. Meanwhile, BCR-ABL tyrosine kinase activates downstream signaling pathway to promote cell proliferation, enhance cell migration, affect normal cell apoptosis, block cell differentiation, and ultimately trigger CML[3,4]. So far, the reasons for the development of CML, as well as the mutation of targeted sites or the activation of by-pass signals leading to drug resistance remain unclear. This greatly hinders

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the diagnosis and treatment of CML. Therefore, more in-depth researches are needed to elucidate the mechanism of the incidence and development of CML and to explore more effective treatments.

Micro-ribonucleic acids (miRNAs) are endogenous non-coding RNAs with 20-24 nucleotides in length. MiRNAs are expressed in eukaryotic cells and may degrade target messenger RNA (mRNA) or inhibit its translation by interacting with the three prime untranslated regions (3'-UTRs) of target genes. Moreover, they may participate in post-transcriptional gene regulation[5]. It's reported that miRNAs can be expressed in a variety of malignant tumors[6]. The pathogenesis of miRNAs can be achieved through proto-oncogenes[7-9]. In addition, miRNAs can be used as tumor suppressor genes affecting the incidence and development of tumor cells[10-12].

As a member of the miRNA family, miR-152-3p is involved in the development of multiple diseases such as human glioma[13], type 2 diabetes[14], prostate cancer[15], and heart failure[16]. However, no relevant literature has reported the exact role of miR-152-3p in the progression of CML. Therefore, the aim of this study was to explore the role of miR-152-3p in the incidence and development of CML as well as the possible underlying mechanism.

Patients and Methods

Collection of Samples

From July 2014 to July 2017, CML patients admitted and treated in the Outpatient Clinic and the Department of Hematology, the Affiliated Yantai Yuhuangding Hospital of Qingdao University were selected as research subjects. Bone marrow tissue samples were collected from all patients. Meanwhile, all patients were diagnosed in accordance with the diagnostic criteria of *Hematology* (Version 2). During the same period, 25 patients with non-proliferative bone marrow diseases were enrolled as normal controls. This study was approved by the Ethics Committee of the Affiliated Yantai Yuhuangding Hosptial of Qingdao University. Signed informed consent was obtained from each patient before the study.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Human peripheral blood lymphocyte separation fluid (Tianjin Haoyang Biology Co., Ltd. Tianjin, China) was applied to isolate mononuclear cells in bone marrow cells. Total RNA was extracted according to the instructions of the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Subsequently, extracted total RNA was reversely transcribed into complementary deoxyribonucleic acid (cDNA). QRT-PCR was employed to detect the expression level of miR-152-3p normalized to miRNA U6. MiR-XTM miRNA Reverse Transcription Kit and MiR-XTM miRNA qRT-PCR Kit were purchased from TaKaRa (Otsu, Shiga, Japan). Three replicates were set for each sample, and the qRT-PCR reaction was achieved by a Real Time-PCR-iQ5 detector (Bio-Rad, Hercules, CA, USA). Relative gene expression changes were calculated by the $2^{-\Delta\Delta CT}$ ΔCT method. Primers used in this study were as follows: miR-152-3p, F: 5'-ACACTCCAGCTGGGTCAGTGCATGACAG-3', R: 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCCAAGTT-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'.

Cell Culture and Transfection

The CML cell line (K562) was purchased from Shanghai Drug Institute Cell Bank of the Chinese Academy of Sciences. All cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Corning, Corning, NY, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) in a 37°C, 5% CO_2 incubator.

K562 cells in the logarithmic growth phase were collected and inoculated into 24-well plates at a concentration of 1×10^5/mL. When the confluence reached 80%, cells were transfected with overexpressed lentivirus LV5-miR-152-3p, interfered lentivirus LV3-miR-152-3p and corresponding negative controls (LV5 and LV3) according to the instructions of Polybrene. After transfection for 48 h, total RNA was extracted, and qRT-PCR was employed to detect miR-152-3p expression in each group.

Cell Counting Kit-8 (CCK-8) Assay

Transfected K562 cells were collected and seeded into 96-well plates at a density of 2×10^4 cells/mL, with 100 μL cell solution in each well. A total of 5 replicates were set for each group. After culture for 24, 48, 72, and 96 h, 10 μL CCK-8 solution (Dojindo, Kumamoto, Japan) was added in each well, followed by culture at 37°C for 3 h in the dark. The absorbance value (A) at the wavelength of 450 nm was measured by a microplate reader (Bio-Rad, Hercules, CA, USA). The blank controls were zeroed.

Cell Cycle

After transfection, 1×10^6 cells in the logarithmic growth phase were collected from each group. The cells were washed twice with phosphate-buffered saline (PBS), and the supernatant was discarded. For cell cycle detection, each sample was first fixed with pre-cooled 70% ethanol (-20°C), and placed at -20°C overnight. Then, the cells were washed twice with pre-cooled PBS, followed by 15 min of incubation with ribonucleases (RNases) (50 μg/mL) (Shanghai Li Rui Biological Technology Co., Ltd. Shanghai, China). Subsequently, 50 μg/mL propidium iodide (PI) was added in each sample for 30 min of incubation. Finally, stained cells were detected *via* flow cytometry (Arial, BD, Franklin Lakes, NJ, USA).

Cell Apoptosis

Cell apoptosis was conducted according to the instructions of the Apoptosis Test Kit (BD, Franklin Lakes, NJ, USA). Briefly, 200 μL binding buffer was added to each group for cell re-suspension. 5 μL Annexin and 5 μL PI were added and mixed gently. Subsequently, the cells were placed at room temperature for 15 min in the dark. After that, the cells were washed with 1 mL binding buffer once, followed by centrifugation at 2000 r/min for 5 min. 200 μL binding buffer was added after washing. Finally, the average fluorescence intensity was measured by flow cytometry.

Western Blot

K562 cells from each group were collected and washed three times with PBS, and then the supernatant was discarded. Afterwards, 100 μL lysate was added in cells, followed by incubation on ice for 5 min and centrifugation at 10000 g for 10 min. Then, the supernatant was collected, and the total protein concentration was determined by Coomassie brilliant blue (Shanghai Majorbio Co., Ltd. Shanghai, China). 50 μg protein was separated by polyacrylamide gel electrophoresis and transferred to the polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% skim milk at room temperature for 2 h, the membranes were incubated with primary antibodies (dilution at 1:1000) at 4°C overnight. After washing three times with Tris-buffered Saline with Tween 20 (TBST) (Beyotime, Shanghai, China), the membranes were incubated with horseradish peroxidase-labeled secondary antibody at 37°C for 1.5 h. Immuno-reactive bands were exposed by enhanced chemiluminescence (ECL) method (Thermo Fisher Scientific, Waltham, MA, USA). Results were analyzed *via* gray-scale analysis, and the ratio of $p27^{Kip1}$ (p27) to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was regarded as a parameter for protein expression level.

Luciferase Reporter Gene Assay

K562 cells were co-transfected with pMIR-30UTR-WT-p27/pMIR-30UTR-Mut-p27, miR-152-3p mimics/negative control (NC), and the pMIR-Renilla plasmid (Promega, Madison, WI, USA). Transfected cells were seeded into 24-well plates, and lysed for subsequent procedures. Luciferase activity was assessed by the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). The results were normalized to Renilla luciferase activity.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 16.0 Software (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. Measurement data were expressed as mean ± standard deviation (x±s). The *t*-test was used to compare the difference between the two groups. $p<0.05$ was considered statistically significant.

Results

MiR-152-3p Was Significantly Up-Regulated in CML Patients and Cell Lines

The expression level of miR-152-3p in CML patients and normal controls was determined by qRT-PCR. Results showed that the mRNA expression of miR-152-3p in CML patients was remarkably higher than that of normal controls (Figure 1A). Besides, the same results were obtained at the cellular level (Figure 1B).

Expression of MiR-152-3p After Cell Transfection in K562 Cells

After transfection with LV3-miR-152-3p, LV5-miR-152-3p, as well as LV3 and LV5 vector lentiviruses for 48 h, the cells were collected and the expression of miR-152-3p was detected. QRT-PCR revealed that the expression level of miR-152-3p in K562 cells was significantly up-regulated after LV5-miR-152-3p transfection. However, miR-152-3p expression was remarkably suppressed by LV3-miR-152-3p transfection. There was no statistical difference in LV5 and LV3 empty vector NC groups (Figure 2).

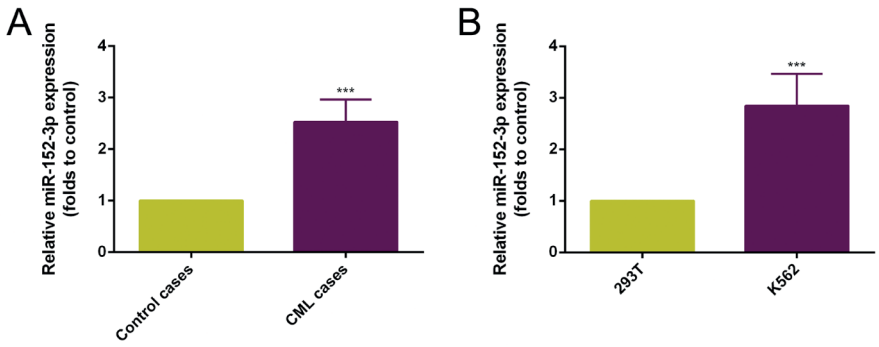

Figure 1. The expression level of miR-152-3p in patients and cell lines. ***A,*** Difference in the expression of miR-152-3p in clinical samples (***p<0.001). ***B,*** Difference in the expression of miR-152-3p in CML cells (***p<0.001).

MiR-152-3p Stimulated the Proliferation of K562 Cells

The CCK-8 assay demonstrated that the proliferation ability of K562 cells was significantly increased after overexpression of miR-152-3p. However, the proliferation of K562 cells transfected with LV3-miR-152-3p was markedly decreased (Figure 3B). To further understand the underlying mechanism, subsequent experiments were performed to confirm whether the miR-152-3p had an influence on cell cycle progression. Surprisingly, results demonstrated that after overexpression of miR-152-3p, the percentage of cells in G0/G1 phase was remarkably decreased when compared with G2SM phase. However, LV3-miR-152-3p transfection exhibited the opposite effect. Furthermore, cell cycle indicated that overexpression of miR-152-3p promoted cell transformation from G0G1 to G2SM phase, eventually resulting in enhanced cell proliferation (Figure 3D).

MiR-152-3p Blocked Apoptosis of K562 Cells

Subsequently, flow cytometry was used to detect the apoptosis of K562 cells. Results manifested that compared with the LV3-miR-152-3p group and the LV3/LV5 vector group, the number of apoptotic K562 cells in the LV5-miR-152-3p group was significantly decreased, suggesting that LV5-miR-152-3p could inhibit cell apoptosis (Figure 3C).

P27 Was Predicted as a Target of MiR-152-3p

By searching TargetScan, miRDB and microRNA websites, we found that miR-152-3p could bind to the 3'-UTR of p27 (Figure 4A). Luciferase reporter gene assay revealed that overexpression of miR-152-3p could significantly down-regulate the luciferase activity of wild-type p27 3'UTR, suggesting that miR-152-3p 5 had a targeting effect on p27. However, the luciferase activity in the report vector group transfected with mutant p27 3'UTR showed no evident decrease. Experimental results further indicated that miR-152-3p could complement and combine with p27 3'UTR (Figure 4B).

Subsequently, we detected the expression changes of p27 in K562 cells after miR-152-3p overexpression or inhibition by Western blot. Results showed that the protein expression level of p27 in the LV5-miR-152-3p group was remarkably declined, whereas the level of p27 in those transfected with LV3-miR-152-3p was notably increased (Figure 3A). These results revealed that p27 was a downstream target of miR-152-3p.

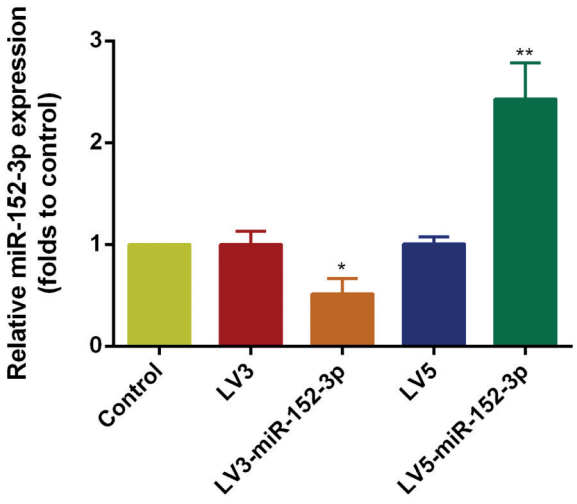

Figure 2. Transfection efficiency detected by qRT-PCR (*p<0.05, **p<0.01)

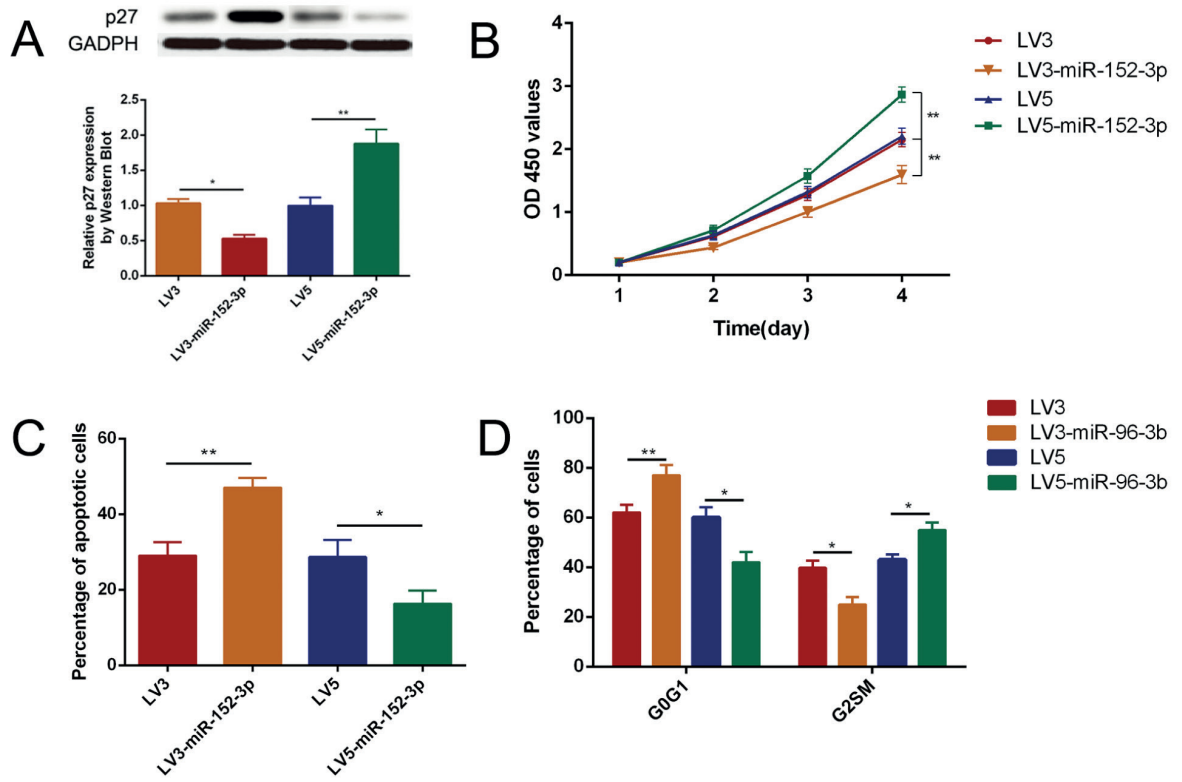

Figure 3. ***A,*** The changes of miR-152-3p expression after mimics transfection were analyzed by Western blot. ***B,*** The proliferation of CML cells detected by CCK-8 assay. ***C,*** Apoptosis level of CML cells tested by flow cytometer. ***D,*** Cell cycle phases of CML cells analyzed by flow cytometry. All data were presented as means ± standard deviations (*p<0.05, **p<0.01).

We then further analyzed the correlation between p27 and miR-152-3p in CML cells. Three groups were established in K562 cells to conduct similar experiments, including the miR-NC group, the miR-152-3p mimics group, and the mimics + p27 group. As expected, the restoration of p27 had an opposite effect on the promoting effect induced by miR-152-3p on p27 expression (Figure 5A), cell proliferation (Figure 5B), cell apoptosis (Figure 5C), and cell cycle (Figure 5D). These results indicated that up-regulation of miR-152-3p exerted a negative relationship with p27 in CML cells. MiR-152-3p stimulated cell proliferation and prevented the apoptosis of CML cells. Moreover, p27 restoration could partially weaken the promotion of miR-152-3p.

Discussion

As a member of the miRNA family, miR-152-3p exerts an effect on the incidence and development of various diseases. Recent studies[13,15] have demonstrated that miR-152-3p, acting as a tumor suppressor gene, inhibits the malignant behavior of prostate cancer cells and glioma cells through targeted regulation of TMEM97 and DNMT1, respectively. However, the role of miR-152-3p in CML has not been fully elucidated. In the present study, we found that the expression level of miR-152-3p was significantly up-regulated in CML patients and cells. Besides, the same results were obtained at the cellular level, suggesting that miR-152-3p might contribute to the development of CML.

Subsequently, K562 cells were transfected with LV5-miR-152-3p and LV3-miR-152-3p to further explore the role of miR-152-3p in CML. Relevant results indicated that the apoptosis rate of K562 cells was significantly increased in the LV3-miR-152-3p group. Meanwhile, cell cycle was arrested in the G0G1 phase, and cell proliferation ability was markedly impeded. However, the proliferation ability of K562 cells transfected with LV5-miR-152-3p was remarkably enhanced, and the number of cells transforming from G0G1 phase to G2SM phase S was significantly increased. These results demonstrated that overexpression of miR-

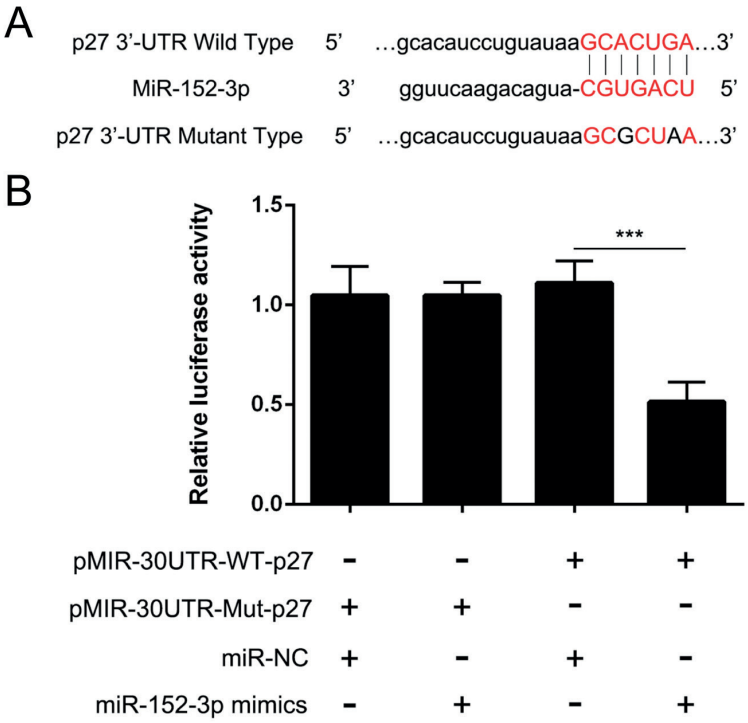

Figure 4. P27^{Kip1} (p27) was a direct and functional target of miR-152-3p. K562 cells were transfected with miR-152-3p mimics and inhibitor. ***A,*** Diagram of putative miR-152-3p binding sites of p27. ***B,*** Relative activities of luciferase reporters (***p<0.001).

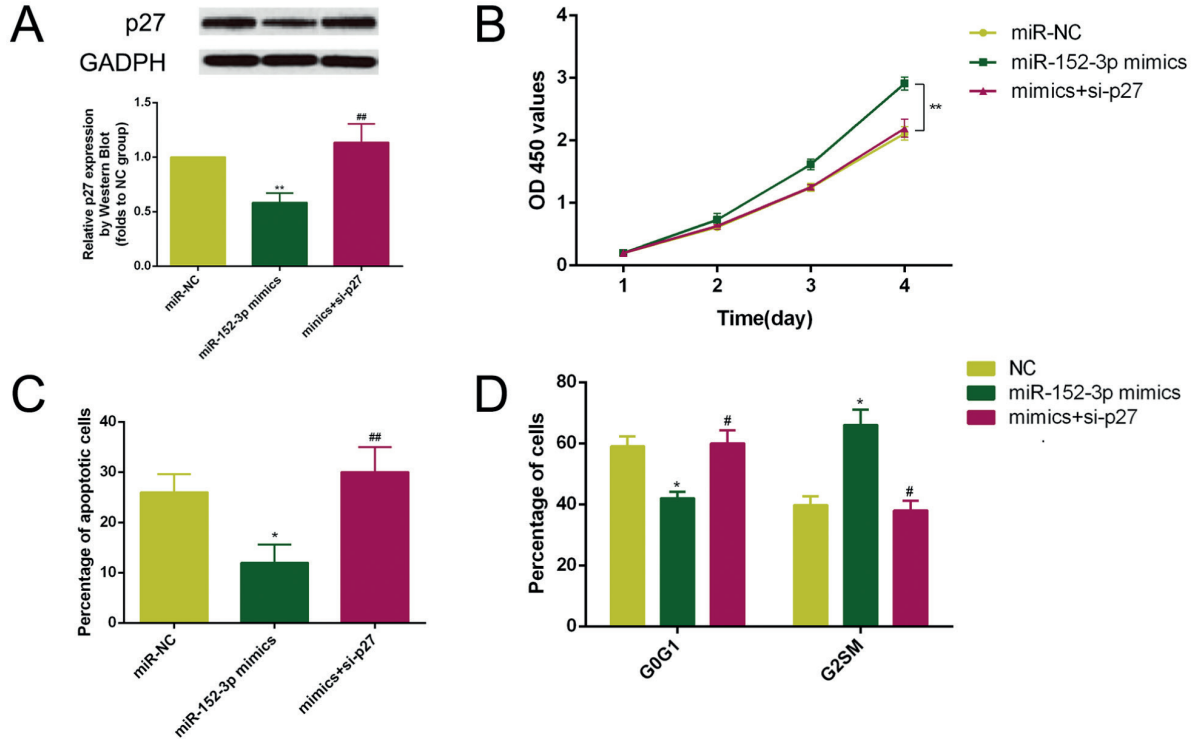

Figure 5. P27 overexpression attenuated the promotion effect of miR-152-3p on CML cells. CML cells were transfected with si-p27 and/or miR-152-3p mimics. ***A,*** The protein expression of miR-152-3p detected by Western blot. ***B,*** Cell proliferation detected by CCK-8 assay. ***C,*** Apoptosis level of CML cells detected by flow cytometer. ***D,*** Cell cycle phases detected by flow cytometry. All data were presented as means ± standard deviations (*p<0.05, **p<0.01).

152-3p could promote the malignant performance of CML cells, indicating that it might influence the progression of CML.

Since miR-152-3p has been verified highly expressed in CML tissues and cells, it is important to screen its downstream genes. By combining target gene prediction websites and previous literature reports, we found that $p27^{Kip1}$ (p27) was a potential target gene of miR-152-3p. P27, as a member of the Cip/Kip cyclin-dependent kinase inhibitors (CKI) family, can suppress cell hyperplasia by arresting cells to pass through G1/S phase based on the binding to CDK2 and cycling. Meanwhile, as a tumor suppressor in CML, p27 has been also widely investigated. Previous studies have manifested that through the phosphatidylinositol 3-kinase (PI3K) pathway, BCR-ABL fusion protein induces the expression of S-phase kinase-associated protein 2 (Skp2) in CML, promotes the degradation of ubiquitinated p27 by proteases, reduces p27 expression, and speeds up the occurrence and development of CML[17]. P27 is also a crucial target for imatinib in the treatment of CML, which can promote cell cycle arrest and reduce cell survival[18]. Some studies have also revealed that BCR-ABL1 can alter the distribution of p27 in cells, thus leading to the transfer of p27 from the nucleus to the cytoplasm. This is very likely to be a significant mechanism for drug resistance of tyrosine kinase inhibitors[19].

With the wide investigation of p27 in tumors and CML[20-25], we assumed that miR-152-3p could promote the malignant proliferation of K562 cells by regulating its downstream p27. Subsequently, the luciferase reporter gene assay indicated that miR-152-3p could complement and combine with p27 3'UTR. Western blot verified that the protein level of p27 was significantly down-regulated in K562 cells transfected with miR-152-3p overexpression plasmid. However, down-regulation of miR-152-3p significantly promoted the expression of p27, further indicating that p27 was a vital downstream regulatory protein of miR-152-3p. Moreover, p27 overexpression seriously affected the inhibitory function of miR-152-3p in the proliferation, cell cycle and cell apoptosis of CML cells. All these results indicated that the miR-152-3p/p27 axis might be an important mechanism in the development of CML.

Conclusions

We demonstrated the promotion role of miR-152-3p in CML, which could be achieved through targeted regulation of p27. Therefore, the miR-152-3p/p27 axis was expected to be one of the targets of CML targeted therapy.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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