MiR-10a-5p regulates proliferation, apoptosis and cell cycle of NPM1-mutated acute myeloid leukemia cells by targeting SHANK3

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**Research**

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Abstract

Objective: Although NMP1 mutation plays a crucial role in regulating the occurrence and development of acute myeloid leukemia (AML), there is still a lack of effective means to improve the prognosis of AML. Studies showed that miR-10a-5p is significantly highly expressed in leukemia and other cancers. However, the function and mechanism of miR-10a-5p in NPM1-mutated AML remain unclear.

Methods: The differential expression of miRNAs and mRNAs related to AML from GEO database were analyzed by bioinformatics. NPM1-mutated AML cell lines were constructed, while miR-10a-5p and SHANK3 were overexpressed to analyze cell proliferation, apoptosis and cell cycle. The targeting relationship between SHANK3 and miR-10a-5p was predicted by bioinformatics and further validated by dual luciferase assay.

Results: Bioinformatics analysis on NPM1-mutated AML samples revealed that miR-10a-5p was highly expressed while SHANK3 was poorly expressed, and miR-10a-5p might target to regulate SHANK3 expression. Overexpression of miR-10a-5p promoted the proliferation, inhibited the apoptosis and regulated the cell cycle of NPM1-mutated AML cells, while opposite results were observed when SHANK3 was overexpressed. Collectively, miR-10a-5p regulated the proliferation, apoptosis and cell cycle of NPM1-mutated AML cells partially by inhibiting SHANK3 expression.

Conclusion: These results demonstrate the role of miR-10a-5p/SHANK3 in NPM1-mutated AML, which provides a potential method for prognosis prediction of AML patients.

Introduction

Leukemia is a malignant clonal disease originating from hematopoietic stem cells, and it is regarded as an acute and chronic disease according to the maturity of leukemia cells and the natural course of the disease. The differentiation of acute leukemia cells ceases at an early stage. Acute myeloid leukemia (AML), accounting for 60% of all leukemia cases, is the predominant type of leukemia in adults and a hematopoietic malignancy that severely threatens human life and health[1–3]. However, the etiology and pathogenesis of AML remain unclear.

NPM1 (nucleophosmin, NPM1), which codes 12-exon-contained nucleophosmin protein, is the gene highly mutated in AML at present[4]. Up to date, NPM1 mutations have been identified to be present not only in AML patients with normal karyotype[5], but also in patients with myelodysplastic syndromes[6], or in patients with chronic myeloid leukemia in blast crisis[7]. Research has shown that NPM1 mutations can be used as a proto-oncogene synergistically working with E1A to induce cell malignant transformation[8]. Meanwhile, NPM1 mutations can also promote the malignant proliferation of leukemia cells by regulating the activity of p53 and p53-dependent pathways[9], enhancing the stability of c-Myc and its cytoplasmic accumulation, and cooperating with FLT3-ITD and other genes[10]. These findings suggest that NPM1 mutations are beneficial for the occurrence and development of leukemia.
Nevertheless, studies on the specific prognosis and pathogenesis of patients with NPM1-mutated AML remain scarce.

MicroRNAs (miRNAs), known as small RNA molecules with a length of approximately 22 nucleotides, are widely present in eukaryotes and highly conserved in evolution, with molecular functions of regulating cell differentiation, proliferation and apoptosis [11, 12]. Research indicated that the expression level of miRNAs in tumor tissues is significantly different from that in normal tissues, and its tissue-specific characteristic might help to identify the origin of tumor[13]. Therefore, miRNAs play an essential role in the processes of tumor differentiation and metastasis. MiR-10a-5p, an important miRNA belonging to the miR-10 family, has drawn extensive attention from people and been used in the study of the prognosis and pathogenesis of AML patients[14, 15][16, 17]. Bryant et al. discovered that miR-10a-5p is over-expressed in NMP1-mutated AML, which provides a new evidence for the high expression of the miR-10 family in AML patients. Zhi et al. reported that miR-10a-5p expression in AML patients is higher than that in normal individuals and the finding illustrated that miR-10a-5p could act as a prognostic biomarker for AML patients[18]. However, the specific functional role of miR-10a-5p in patients with NMP1-mutated AML remains unknown.

This study focused on miR-10a-5p and its predicted target gene SHANK3 to identify the regulatory role of their targeting relationship in the proliferation, apoptosis and cell cycle of NPM1-mutated AML, so as to search for a new targeted therapy against AML.

1 Materials And Methods

1.1 Bioinformatics analysis

MiRNA expression matrix data of GSE68467 (109 samples, including 11 mutant samples and 98 wild-type samples) and mRNA expression matrix data of GSE68466 (109 samples, including 11 mutant samples and 98 wild-type samples) were download from GEO database (https://www.ncbi.nlm.nih.gov/geo/). Limma package was used for differential analysis with the wild-type samples as control. MiRNAs with |logFC|>1.5 and padj < 0.05 were considered differentially expressed miRNAs and mRNAs with |logFC|>1.0, padj < 0.05 were considered differentially expressed mRNAs. The database mirDIP (http://ophid.utoronto.ca/mirDIP/index.jsp) was used to predict potential target genes of miR-10a-5p, the miRNA differentially up-regulated in mutant samples. The target gene of interest was obtained from intersection of the differentially down-regulated mRNAs in GSE68466 and the predicted potential target genes of miR-10a-5p.

1.2 Cell lines

MOLM-14 leukemia cell line (BNCC341023) was purchased from BeNa Culture Collection. NPM1-mutated plasmids pEGFP-NPM1 and blank control plasmids were respectively transfected into the MOLM-14 cell line to construct stable NPM1-mutated MOLM-14 cell line (NPM1-mut) or control MOLM-14 cell line (NPM1-wt), using the xfect™ transfection reagent (631318, Takara, Japan). Cells were placed in an...
incubator at 37 °C with 5% CO₂ and cultured with 90% Iscove's Modified Dulbecco's Medium (IMDM; sigma, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 100 U/ml penicillin/streptomycin (Corning, NY, USA).

1.3 Cell transfection
100 nmol/L miR-10a-5p mimic, 100 nmol/L oe-SHANK3 and their corresponding negative controls (NC-mimic, oe-NC) were purchased from GenePharma (Shanghai, China). Cells (1 × 10⁵) were seeded into a 12-well plate before cell transfection. Oe-SHANK3, miR-10a-5p mimic and negative controls were transfected into cells using the Lipofect reagent kit (Hanbio, Shanghai, China) in accordance with the manufacturer’s instructions. Total RNA and proteins were extracted 48 h after transfection. Primer sequences were listed in Supplementary Table 1.

1.4 Real-time fluorescence quantitative PCR (qRT-PCR)
Total RNA was extracted from treated cells using Trizol (Invitrogen, Carlsbad, USA) according to the manufacturer’s instructions. Complementary DNA (cDNA) was synthesized by the reverse transcription reagent kit (Invitrogen, Carlsbad, USA). qRT-PCR was performed using the ABI 7900HT device (Applied Biosystems, USA) with the miScript SYBR Green PCR Kit (Qiagen, Germany) under the following thermal cycling conditions: 95 ℃ 10 min, followed by 40 cycles of 95 ℃ 5 s and 60 ℃ 30 s, finally 72 ℃ 2 min. SHANK3 and miR-10a-5p expression levels were normalized to GAPDH and U6, respectively. All the primers used were shown in Supplementary Table 1. The differences in the relative expression of target genes in the control and experimental groups were analyzed by 2⁻ΔΔCt method. The experiment was performed in triplicate and repeated three times.

1.5 Western blot
After transfection for 48 h, cells in different treatment groups were washed with cold PBS for 3 times (Thermo fisher, USA). Total proteins were extracted using whole cell lysate on ice for 10 min, and quantitation was performed using the BCA protein assay kit (Thermo fisher, USA). Cell extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V after boiled for 10 min at 95 ℃ with 10 µl of loading buffer. Thereafter, the proteins were transferred onto nitrocellulose membrane at 100 mA within 120 min, and the membrane was incubated with primary antibodies overnight at 4 ℃ after blocked with 5% bovine serum albumin or Tris-Buffered Saline Tween-20 for 60 min. Next, the membrane was washed with 1 × TBST (Solarbio, Beijing, China) on a shaker for three times with 5 min per time, and sequentially incubated with horseradish peroxidase (HRP) - conjugated secondary antibody goat anti-mouse IgG for 120 min at room temperature. The membrane was washed by TBST 3 times, 20 min each time. Protein bands were visualized using the electrochemiluminescence (ECL) reagent kit (Solarbio, Beijing, China), and images were captured for observation. The experiment was performed in triplicate. All antibodies were shown in Supplementary Table 2.

1.6 MTT and colony formation assays
Cell proliferation was detected by MTT assay. Cells were planted into 96-well plates (5 × 10^3 cells per 100 µl) and each treatment was run in triplicate. After culture for 1, 2, 3, 4 and 5 d, sterile MTT solution (Beyotime) was added into cells to evaluate cell proliferation according to the instructions. The absorbance at 490 nm was measured using the spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). For colony formation assay, cells were seeded onto 6-well plates with a density of 1 × 10^3 cells/well. After culture for 14 d, cell colonies were fixed with 30% formaldehyde for 15 min, and then stained with 0.1% crystal violet (Thermo Fisher, USA). The colonies were counted by an optical microscope (each colony was set to have over 50 cells).

1.7 Flow cytometry for cell apoptosis and cell cycle
To analyze cell apoptosis, cells were digested with trypsin and then resuspended in binding buffer containing Annexin V-FITC (BD Biosciences, San Jose, CA, USA) and propidium iodide (PI; BD Biosciences) for 15 min in the dark. The stained cells were analyzed with flow cytometry (BD Biosciences).

To analyze distribution of cell cycle, cells were fixed in 70% cold ethanol for 12 h and then incubated with PI (50 µg/ml) and RNase A (Sigma-Aldrich) for 30 min. Flow cytometry was applied for further analysis.

1.8 Dual luciferase assay
In order to determine the binding probability of miR-10a-5p to 3'UTR of SHANK3, psiCHECK luciferase reporter vectors (Sangon Co., LTD, Shanghai, China) with insertion of wild-type (wt) and mutant (mut) SHANK3 3'UTR were constructed. NPM1-mutated MOLM-14 cells were seeded into 48-well plates and cultured for 24 h. Then, miR-10a-5p-mimic/NC-mimic and SHANK3 wt/mut vectors were co-transfected into NPM1-mutated MOLM-14 cells. At last, luciferase activity was determined using the luciferase assay kit (Promega, Fitchburg, WI, USA). The experiment was conducted in triplicate.

1.9 Statistical analysis
SPSS 22.0 statistical software (SPSS, Inc, Chicago, IL, USA) was used for statistical analysis, and measurement data were presented as mean ± standard deviation. Student’s t-test was used for analyzing differences between two independent groups, and one-way ANOVA method was used for analyzing differences among multiple groups. P< 0.05 suggested a statistically significant difference, while p< 0.01 and p< 0.0001 suggested a highly statistically significant difference.

2 Results

2.1 MiR-10a-5p expression is significantly up-regulated in NPM1-mutated AML tissue and cells
Differential analysis was performed on the miRNA expression matrix data of AML patients (including NPM1-mut/wt) download from GEO database, and 10 differentially expressed miRNAs were screened (Fig. 1A and 1B). Since the expression of miR-10a-5p was highly remarkably up-regulated in NPM1-mutated patients, miR-10a-5p was in turn selected as the target miRNA for this study. We compared the expression of miR-10a-5p in NPM1-mut, NPM1-wt and common MOLM-14 cells. The results showed that,
the expression of miR-10a-5p in NPM1-mut cells was significantly higher than that in NPM1-wt cells and common MOLM-14 cells (Fig. 1C). Based on above results, we found that miR-10a-5p expression in AML with mutated NPM1 was higher than that in AML with wild NPM1.

2.2 Overexpression of miR-10a-5p modulates the proliferation, apoptosis and cell cycle of NPM1-mut MOLM-14 cells

In order to explore the regulatory effect of miR-10a-5p on biological functions of NPM1-mut cells, we transfected miR-10a-5p mimic and NC-mimic into the AML cells. Firstly, we detected miR-10a-5p expression of the two groups. The result showed that miR-10a-5p expression in the miR-10a-5p mimic group was markedly higher than that in the NC-mimic group (Fig. 2A), demonstrating that miR-10a-5p was efficiently overexpressed in the cells. Afterwards, to study the effect of miR-10a-5p on the proliferation of NPM1-mut AML cells, the proliferation of NPM1-mut MOLM-14 cells upon miR-10a-5p overexpression was measured. The results of MTT and colony formation assays revealed that overexpression of miR-10a-5p elevated the viability of NPM1-mut MOLM-14 cells (Fig. 2B and 2C). The apoptosis and cell cycle of NPM1-mut MOLM-14 cells were further analyzed under the presence of overexpressed miR-10a-5p, and it was found that miR-10a-5p overexpression significantly decreased the apoptosis of NPM1-mut MOLM-14 cells, while the cell number in G0/G1 phase was decreased and that in S phase was increased (Fig. 2D and 2E). These results demonstrated that miR-10a-5p could promote NPM1-mut MOLM-14 cell proliferation, regulate cell cycle and inhibit cell apoptosis.

2.3 MiR-10a-5p down-regulates SHANK3 expression in NPM1-mut MOLM-14 cells.

We further investigated the potential target gene of miR-10a-5p after confirming that miR-10a-5p could promote the proliferation of NPM1-mut MOLM-14 cells. Firstly, we differentially analyzed the mRNA expression matrix data of AML patients (including NPM1-mut/wt) downloaded from GEO database. Totally, 23 significantly down-regulated mRNAs were screened out (Fig. 3A and Supplementary Table 3). Then, we used the mirDIP database to predict the target mRNAs of miR-10a-5p, which were intersected with the 23 differentially down-regulated mRNAs in GEO, and SHANK3 was identified eventually (Fig. 3B). The expression of SHANK3 in NPM1-mut, NPM1-wt and common MOLM-14 cells was tested, showing that SHANK3 expression in NPM1-mut MOLM-14 cells was significantly lower than that in other two groups (Fig. 3C and 3D). After that, binding site sequences of miR-10a-5p on SHANK3 3’UTR were predicted using the TargetScan database (http://www.targetscan.org/vert_72/) (Fig. 3E). The results of dual-luciferase assay showed that overexpression of miR-10a-5p inhibited the luciferase activity of SHANK3-wt while had no effect on SHANK3-mut (Fig. 3F). Further, western blot and qRT-PCR results demonstrated that overexpression of miR-10a-5p inhibited the expression of SHANK3 in cells (Fig. 3G and 3H). Based on these results, we could find that SHANK3 was a target gene of miR-10a-5p in NPM1-mut MOLM-14 cells.

2.4 MiR-10a-5p affects the proliferation, apoptosis and cell cycle of NPM1-mut MOLM-14 cells by regulating SHANK3
In this part, we firstly detected SHANK3 expression in three groups: NC mimic + oe-NC, miR-10a-5p mimic + oe-NC, and miR-10a-5p mimic + oe-SHANK3. The results of qRT-PCR and western blot showed that SHANK3 expression in the miR-10a-5p mimic + oe-SHANK3 group was significantly increased in comparison with that in the miR-10a-5p mimic + oe-NC group (Fig. 4A and 4B). MTT and colony formation assays showed that miR-10a-5p overexpression remarkably promoted cell viability ($p < 0.05$) and increased cell colony number ($p < 0.01$). However, the promotive effect on cell viability and the number of colonies was decreased when SHANK3 and miR-10a-5p were simultaneously overexpressed (Fig. 4C and 4D). The cell cycle and apoptosis were further measured and it was noted that the cell apoptosis was decreased obviously after miR-10a-5p was overexpressed ($p < 0.05$), and more cells were found to aggregate in S phase. Nevertheless, the cell apoptosis was reversely increased when SHANK3 and miR-10a-5p were simultaneously overexpressed, and cell cycle was then arrest in G0/G1 phase (Fig. 4E and 4F). These findings validated that miR-10a-5p regulated the proliferation, apoptosis and cell cycle of NPM1-mut MOLM-14 cells by targeting the expression of SHANK3.

3 Discussion

MiRNAs are involved in multiple processes during the initiation of AML, and it is essential to understand the role of miRNAs in regulating the biological function of oncogenes or tumor suppressor genes in AML[19, 20]. MiR-10a-5p has been proved to have a regulatory effect on tumor growth in various cancers[21, 22]. However, its biological function and molecular mechanism in NPM1-mutated AML have received little notice. Our research showed that miR-10a-5p was highly expressed in NPM1-mutated AML cells. Functional analysis also revealed that miR-10a-5p overexpression could evidently facilitate NPM1-mutated AML cell proliferation. These findings indicated that miR-10a-5p works as a promoter in NPM1-mutated AML. Debernardl et al. observed that the expression levels of miR-10a-5p, miR-10b, and miR-196a are positively correlated with the expression of HOXA and HOXB in AML patients and actively associated with the occurrence of AML as well[23]. Bosman et al. noted that the expression of miR-10a-5p is significantly higher in adriamycin-resistant AML cell strain HL-60 than that in susceptible cell strain HL-60[24]. In conclusion, we believed that miR-10a-5p serves as a promoter and plays a crucial role in growth of AML.

In the present study, bioinformatics analysis and luciferase reporter assay elucidated that miR-10a-5p could target and regulate SHANK3. Furthermore, miR-10a-5p was negatively correlated with SHANK3 in expression in NPM1-mutated AML cells. SHANK3 gene codes multidomain scaffold proteins containing 1,747 amino acids, which mainly express in post synaptic density (PSD) of excitatory neuron[25]. Besides, SHANK3 is a susceptibility gene of multiple mental disorders, such as schizophrenia and bipolar disorder, etc[26]. However, there are few studies on the role of SHANK3 gene in tumor and leukemia. Our research findings revealed that SHANK3 overexpression could markedly reverse the promotive effect of overexpressed miR-10a-5p on the proliferation of NPM1-mutated AML cells. This finding showed that the regulatory effect of miR-10a-5p on NPM1-mutated AML cells may partially performed by targeting SHANK3.
All in all, our experiments verified the positive regulatory effect of miR-10a-5p on NPM1-mutated AML cells, specifically, miR-10a-5p can promote the proliferation, inhibit the apoptosis and regulate the cell cycle of NPM1-mutated AML cells via targeting SHANK3. The finding above not only provides a deep understanding about the role of miR-10a-5p in AML, but also lays a foundation for exploring new targeted therapies against AML.

**Declarations**

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Availability of data and materials**

The data used to support the findings of this study are included within the article. The data and materials in the current study are available from the corresponding author on reasonable request.

**Competing interest**

The authors declare no conflicts of interest.

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None.

**Authors' contributions**

All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agreed to be accountable for all aspects of the work.

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Figures
Figure 1

MiR-10a-5p is significantly up-regulated in NPM1-mutated AML tissue and cells. (A) Volcano plot of the differentially expressed miRNAs in AML from GEO database (Red represents up-regulated miRNAs, and green represents down-regulated miRNAs); (B) Box plots of the 10 differential miRNAs in NPM1-mutated AML patients; (C) qRT-PCR was used to detect miR-10a-5p expression in NPM1-mut, NPM1-wt and common MOLM-14 cells; *** p<0.0001.

Figure 2
MiR-10a-5p overexpression regulates NPM1-mut MOLM-14 cell proliferation, apoptosis and cell cycle. (A) qRT-PCR was used to detect miR-10a-5p expression in NPM1-mut MOLM-14 cells transfected with miR-10a-5p mimic or NC mimic; (B) MTT was used to detect cell viability of NPM1-mut MOLM-14 cells in two groups; (C) Colony formation assay was used to detect the proliferation of NPM1-mut MOLM-14 cells in two groups; (D) Flow cytometry was used to detect the effect of miR-10a-5p overexpression on cell apoptosis; (E) Flow cytometry was used to detect the effect of miR-10a-5p overexpression on cell cycle; * p<0.05.

Figure 3

MiR-10a-5p targets to regulate SHANK3. (A) Volcano plot of the differentially expressed mRNAs of AML patients downloaded from GEO database (Red for up-regulated mRNAs and greed for down-regulated mRNAs); (B) Intersection of the differentially down-regulated mRNAs in GEO and the target genes of miR-10a-5p predicted by the TargetScan database; (C-D) qRT-PCR and western blot were used to detect SHANK3 expression in NPM1-mut, NPM1-wt and common MOLM-14 cells; (E) Putative binding sites between SHANK3 and miR-10a-5p; (F) Dual-luciferase assay was used to detect the targeting relationship
between miR-10a-5p and SHANK3; (G-H) qRT-PCR and western blot were used to detect SHANK3 expression in NPM1-mut MOLM-14 cells after overexpressing miR-10a-5p; * p<0.05.

Figure 4

MiR-10a-5p affects the proliferation, apoptosis and cell cycle of NPM1-mut MOLM-14 cells by regulating SHANK3. (A) qRT-PCR was used to detect mRNA expression of SHANK3 in NC mimic+oe-NC, miR-10a-5p mimic+oe-NC, and miR-10a-5p mimic+oe-SHANK3 groups; (B) Western blot was used to detect protein expression of SHANK3 in each group; (C) MTT assay was used to detect the proliferation of NPM1-mut MOLM-14 cells in each group; (D) Colony formation assay was used to detect the colony formation ability of cells in each group; (E) Flow cytometry was used to detect the apoptosis of cells in each group; (F) Flow cytometry was used to detect the cell cycle of cells in each group; * p<0.05.
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