miR-181a down-regulates MAP2K1 to enhance adriamycin sensitivity in leukemia HL-60 cells

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Abstract. – OBJECTIVE: MAPK kinase 1 (MEK1), also known as MAP2K1, plays a role in activating extra-cellular signal-regulated protein kinase/mitogen-activated protein kinase (ERK/MAPK) signaling pathway to regulate cell proliferation and apoptosis. The abnormal expression of MAP2K1 is associated with leukemia. Bioinformatics analysis showed the targeted relationship between microRNA-181a (miR-181a) and the 3'-UTR of MAP2K1. This study aimed to investigate the role of miR-181a in regulating MAP2K1 expression, the effects on leukemic cell proliferation, apoptosis, and adriamycin (ADM) resistance.

MATERIALS AND METHODS: Dual luciferase reporter gene assay was applied to confirm the targeted relationship between miR-181a and MAP2K1. ADM resistant cell line HL-60/ADM was established. MiR-181a and MAP2K1 expressions were detected. HL-60/ADM cells were cultured in vitro and divided into two groups, including miRNA-Normal control (miR-NC) group and miR-181a mimic group. MAP2K1, phosphorylated MAP2K1 (p-MAP2K1), and phosphorylated ERK (p-ERK) protein expressions were tested. Cell apoptosis was assessed with flow cytometry. Cell proliferation was determined using EdU staining.

RESULTS: There is a targeted regulatory relationship between miR-181a and MAP2K1 mRNA. miR-181a expression was significantly lower, while MAP2K1 mRNA and protein expressions were markedly higher in HL-60/ADM cells than HL-60 cells (p<0.05). Transfection of miR-181a mimic markedly reduced expressions of MAP2K1, p-MAP2K1, and p-ERK in HL-60/ADM cells, enhanced cell apoptosis, and weakened cell proliferation compared to miR-NC (p<0.05).

CONCLUSIONS: MiR-181a reduction and MAP2K1 elevation were related to ADM resistance in leukemia cells. Up-regulation of miR-181a expression inhibited leukemia cell proliferation, induced apoptosis, and reduced ADM resistance via targeting MAP2K1 expression and ERK/MAPK signaling pathway.

Key Words: Leukemia, ADM, Drug resistance, miR-181a, MAP2K1.

Introduction

Leukemia is a group of heterogeneous hematopoietic stem cell malignant clonal diseases caused by differentiation block, apoptosis arrest, and malignant proliferation at different stages of hematopoietic stem/progenitor cells. Chemotherapy is an important method for treating leukemia. However, the emergence of drug resistance is one of the key factors that limit the efficacy of chemotherapy and affect the survival and prognosis of patients¹². MAPK kinase 1 (MEK1), also known as MAP2K1, phosphorylates and activates extracellular signal regulated kinases (ERK) protein, thereby activating ERK/mitogen activated protein kinase (MAPK) signaling pathway. It was showed that the expression and functional activity of MAP2K1 are associated with the development, progression, and drug resistance of leukemia¹³-³. MicroRNAs are a type of endogenous small non-coding single-stranded RNA. They participate in the biological process, such as cell survival, proliferation, apoptosis, and migration, by complementary binding with the 3'-UTR of target gene to promote target gene mRNA degradation or inhibit mRNA translation. The abnormal expression and function of miRNA in chemotherapy resistance have attracted more and more attention⁶⁻⁷. MiR-181a is a highly studied miRNA that is related to the occurrence, progression, and drug resistance of various tumors, such as lung cancer⁴, thyroid cancer⁵, cervical cancer⁶⁰, and prostate cancer⁴¹. It was found²⁻⁵ that miR-181a is involved in the regulation of biological processes, such as proliferation, apoptosis, cycle and drug resistance of leukemia cells. Bioinformatics analysis showed the targeted relationship between miR-181a and 3'-UTR of MAP2K1. This study investigated the role of miR-181a in regulating MAP2K1 expression, affecting leukemic cell proliferation, apoptosis, and adriamycin (ADM) resistance.

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Materials and methods

**Main Reagents and Materials**

Human normal peripheral blood mononuclear cell (PBMC) and promyelocytic leukemia cell HL-60 were purchased from Beijing Beina Biotechnology Co., Ltd. (Beijing, China). HEK293T cells were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Roswell Park Memorial Institute-1640 (RPMI-1640), optional essential medium (Opti-MEM) and fetal bovine serum (FBS) were purchased from Gibco BRL. Co. Ltd. (Grand Island, NY, USA). TRIzol and Lipofectamine 2000 were purchased from Invitrogen/Life Technologies (Carlsbad, CA, USA). QuantiTect SYBR Green RT-PCR Kit was purchased from Qiagen (Hilden, Germany). MiR-181a mimic, miR-181a inhibitor, and miR-NC were purchased from Ribobio (Guangzhou, China). Rabbit anti-human MAP2K1 and p- MAP2K1 polyclonal antibodies were purchased from Abcam Biotech. (Cambridge, MA, USA). Rabbit anti-human β-actin antibody was purchased from Santa Cruz Biotech. (Santa Cruz, CA, USA). Goat anti-Rabbit IgG (H+L) secondary antibody was purchased from Sangon Biotech. Co. Ltd. (Shanghai, China). Cell Counting Kit-8 (CCK-8), Annexin V/ propidium iodide (PI) cell apoptosis detection kit, and bicinechonic acid (BCA) quantification kit were purchased from Beyotime Biotech. (Shanghai, China). EdU Flow Cytometry Kit was purchased from Sigma-Aldrich (St. Louis, MO, USA). Dual-Glo Luciferase Assay System and pGL3 plasmid were purchased from Promega (Madison, WI, USA). Forma 3131 cell incubator was purchased from Thermo Electron Corp. (Waltham, MA, USA).

**Cell Culture**

PBMC and HL-60 cells were maintained in RPMI-1640 (Roswell Park Memorial Institute 1640) medium containing 10% fetal bovine serum (FBS) and cultured in 37°C and 5% CO₂. The cells in logarithmic phase were used for experiments. This study was approved by the Ethics Committee of Nuclear industry 215 Hospital of Shaanxi Province (Xianyang, Shaanxi, China).

**ADM Drug Resistant Cell Line Establishment**

HL-60 cells in logarithmic phase were treated by ADM from 0.1 μg/ml for 2 weeks. Then, cells kept growing and were treated by increased concentration of ADM up to 1.6 μg/ml when the cells can stably grow in ADM. At last, the cells can be stable passaged in ADM to obtain ADM resistant leukemia cell line HL-60/ADM. HL-60 and HL-60/ADM cells were treated by different concentrations (0, 0.1, 1, 10, 100, and 1000 μg/ml) of ADM for 48 h. Next, the cells were added with CCK-8 to measure the absorbance value (A450). Inhibition rate = (1-A450 in drug group)/A450 in control × 100%. IC50 was calculated using SPSS software. Resistance index (RI) = IC50 of drug resistant cell/IC50 of parent cell.

**Flow Cytometry Detection of Cell Proliferation**

The cells were added with EdU solution at 10 μM in logarithmic phase. After incubated for 2 h, the cells were seeded for 48 h and digested by trypsin. After fixed in paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA), the cells were incubated in 10 μl Triton X-100 (Beyotime Biotech. Shanghai, China) at room temperature and in 500 μl reaction fluid at room temperature avoid of light for 30 min. At last, the cell was tested on FC500 MCL flow cytometry (BD Biosciences, San Jose, CA, USA).

**Dual Luciferase Reporter Gene Assay**

The PCR product of the MAP2K1 3’-UTR full-length fragment or mutant fragment was double-digested and then ligated into the pGL3 vector. After sequencing, the plasmid was designated as pGL3-MAP2K1-WT and pGL3-MAP2K1-MUT. The HEK293T cells were transfected with pGL3-MAP2K1-WT (or pGL3-MAP2K1-MUT) together with miR-181a mimic (or miR-181a inhibitor, miR-NC) by Lipofectamine 2000. After incubated for 48 h, luciferase activity was detected by Dual-Glo Luciferase Assay System kit according to the manual.

**Cell Transfection and Grouping**

HL-60/ADM cells were divided into miR-NC group and miR-181a mimic group. A total of 10 μl of Lip2000, 50 nmol miR-NC, and 50 nmol miR-181a mimic were diluted with 100 μl serum-free Opti-MEM medium, and incubated for 5 min at room temperature, respectively. The mixture was added to the cell culture medium for 72 h. At last, the cells were collected for detection. The cells were seeded in 6-well plate and treated by 1.6 μg/ml ADM for 48 h. The cells were added with EdU solution at 10 μM in logarithmic phase. After incubated for 2 h, the cells were seeded for 48 h and digested by trypsin. After fixed in para-
formaldehyde, the cells were incubated in 100 μl Triton X-100 at room temperature and in 500 μl reaction fluid at room temperature avoid of light for 30 min. At last, the cell was tested on FC500 MCL flow cytometry (BD Biosciences, San Jose, CA, USA).

**Quantitative Real-Time PCR (qRT-PCR)**

The total RNAs from the cells were extracted by using the TRizol regents, which purchased from Beyotime Biotech. (Shanghai, China). Next, the complementary DNA (cDNA) was synthesized by using the SuperScript III first-strand synthesis system (Cat. No. 18080051, Invitrogen/Life Technologies, Carlsbad, CA, USA). QuantiTect SYBR Green RT-PCR Kit (Cat. No. 204243, Qiagen, Hilden, Germany) was used to amplify the targeting genes based on the synthesized cDNAs. The PCR reaction system was composed of 2×QuantiTect SYBR Green RT-PCR Master Mix 10.0 µl, forward primer (0.5 μM) 1.0 µl, reverse primer (0.5 μM) 1.0 µl, Template RNA 2.0 μg, QuantiTect RT Mix 0.5 µl, and ddH₂O. The reaction was performed on Bio-Rad (Mode: CFX96, Bio-Rad Laboratories, Hercules, CA, USA) at 45°C for 5 min and 94°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. The primers for the RT-PCR assay were listed in Table I.

**Western Blot**

Total protein was extracted from the cells by radioimmunoprecipitation assay (RIPA). After quantified by BCA method, a total of 40 μg protein was separated by sodium dodecyl sulphate-polyAcrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane at 250 mA for 100 min. After blocked by 5% skim milk at room temperature, the membrane was incubated in primary antibody at 4°C overnight (MAP2K1 1: 2000, p-MAP2K1 1:1000, and β-actin 1: 10000). After washed by phosphorylate-buffered saline Tween-20 (PBST, Beyotime Biotech. Shanghai, China), the membrane was further incubated in horseradish peroxidase (HRP) conjugated secondary antibody at room temperature for 60 min (1: 15000). At last, the membrane was treated by enhanced chemiluminescence (ECL) Amersham Biosciences (Piscataway, NJ, USA) reagent and developed.

**Cell Apoptosis Detection**

The cells were digested by enzyme and collected. After resuspended in 100 μl binding buffer, the cells were added with 5 μl Annexin V-FITC and 5 μl PI at room temperature avoid of light for 15 min. Next, the cells were tested on flow cytometry.

**Statistical Analysis**

SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) was applied for data analysis. Measurement data were presented as mean ± standard deviation (SD). The Student’s t-test was used to compare the differences between two groups. Tukey’s post-hoc test was used to validate the ANOVA for comparing measurement data among groups. p<0.05 represents a significant difference.

**Results**

**The Targeted Regulatory Relationship between miR-181a and MAP2K1**

Bioinformatics analysis showed the complementary binding site between miR-181a and the 3'-UTR of MAP2K1 mRNA (Figure 1A). Dual luciferase reporter gene assay exhibited that miR-181a mimic transfection significantly reduced the relative luciferase activity in HEK293T cells transfected by pGL3-MAP2K1-WT but not by pGL3-MAP2K1-MUT, confirming that MAP2K1 was the target gene of miR-181a (Figure 1B, p<0.05).

**Drug Resistant Leukemia Cells Exhibited Strong ADM Resistance**

CCK-8 assay showed that the proliferative activities of parental HL-60 cells were significantly lower than those of HL-60/ADM cells under the
same dose treatment of ADM. The IC_{50} of HL-60 cells was 1.36±0.11 μg/ml, while it was 18.79±1.26 μg/ml in the drug-resistant HL-60/ADM cells, leading the RI of HL-60/ADM cells at 13.82 (Figure 2A). Under the ADM treatment at 1.36 μg/ml, the apoptotic rate of HL-60 cells reached 25.17%±3.75%, while it was only 2.58%±0.39% in HL-60/ADM cells (Figure 2B).

MiR-181a Reduced, while MAP2K1 Up-Regulated in Drug Resistant Cells

qRT-PCR demonstrated that compared with human normal PBMC cells, miR-181a expression was significantly decreased in HL-60 cells, and its level was obviously lower in drug-resistant HL-60/ADM cells than that of parental HL-60 cells (Figure 3A, p<0.05). qRT-PCR revealed that the MAP2K1 mRNA expression in HL-60 cells was markedly higher than that of PBMC cells (p<0.05), and it was apparently higher in leukemia resistant HL-60/ADM cells compared with the parental HL-60 cells (Figure 3A). Western blot exhibited that compared with PBMC cells, MAP2K1 protein expression in HL-60 cells was significantly up-regulated (p<0.05), and its level in leukemia drug-resistant HL-60/ADM cells obviously enhanced (Figure 3B).

MiR-181a Over-Expression Promoted Leukemia Cell Apoptosis and Reduced ADM Resistance

qRT-PCR showed that miR-181a mimic transfection significantly up-regulated miR-181a expression and declined MAP2K1 mRNA level in HL-60/ADM cells compared with miR-NC group (Figure 4A, p<0.05). Western blot demonstrated that miR-181a mimic transfection significantly reduced MAP2K1, p-MAP2K1, and p-ERK1 protein levels in HL-60/ADM cells (Figure 4B, p<0.05). Flow cytometry revealed that transfection of miR-181a mimic markedly enhanced cell apoptosis, while inhibited cell proliferation in HL-60/ADM cells (Figure 4C, D, p<0.05).
ERK/MAPK signaling pathway is widely expressed in various tissues and cells, and can regulate a variety of biological processes, such as cell proliferation, cycle, apoptosis, migration, and invasion\(^1\),\(^2\),\(^16\),\(^17\). Over-activation of ERK/MAPK signaling pathway is closely related to the occurrence, progression, and metastasis of various tumors, such as oral cancer, esophageal cancer, and lung cancer\(^18\),\(^19\). MAP2K1 is a tyrosine/threonine (Tyr/Thr) dual-specific protein kinase that specifically phosphorylates ERK protein to activate ERK/MAPK signaling pathway\(^21\),\(^22\). It was showed that the expression and functional activity of MAP2K1 are associated with the development, progression, and drug resistance of leukemia\(^3\),\(^4\),\(^5\). It was found that miR-181a is involved in the regulation of biological processes, such as proliferation, apoptosis, cycle and drug resistance of leukemia cells\(^12\),\(^13\),\(^14\). Bioinformatics analysis showed the targeted relationship between miR-181a and the 3'-UTR of MAP2K1. This study investigated the role of miR-181a in regulating MAP2K1 expression, affecting leukemic cell proliferation, apoptosis, and ADM resistance. In this study, dual luciferase reporter gene assay exhibited that miR-181a mimic transfection significantly reduced the relative luciferase activity in HEK293T cells transfected by pGL3-MAP2K1-WT but not by pGL3-MAP2K1-MUT, confirming that MAP2K1 was the target gene of miR-181a. CCK-8 assay showed that the proliferative activities of parental HL-60 cells were significantly lower than those of HL-60/ADM cells under the same dose treatment of ADM. Compared with human normal PBMC cells, miR-181a expression was significantly decreased, while MAP2K1 level was markedly enhanced in HL-60 cells, and their changes amplitude was significantly higher in drug-resistant HL-60/ADM cells than that of parental HL-60 cells, suggesting that miR-181a down-regulation was associated with MAP2K1 over-expression and participated in the regulation of leukemia cell ADM resistance. Schwind et al\(^23\) showed that the treatment response, overall survival rate, and disease-free survival rate of patients with higher expression of miR-181a were significantly better than those with lower expression of miR-181a. Zhu et al\(^14\) adopted gene expression profiling assay and found that the expression of miR-181a in peripheral blood of leukemia patients was abnormally decreased compared with healthy controls, which was related to poor survival and prognosis. Li et al\(^15\) revealed that the expression of miR-181a in drug-resistant leukemia cells was significantly lower than that of the parental leukemia cells. All of the above studies indicated that miR-181a functions as a tumor suppressor gene in leukemia, which was similar to our results. This study further explored whether miR-181a is involved in the regulation of drug resistance in leukemia HL-60 cells. Transfection of miR-181a mimic markedly reduced the expressions of MAP2K1, p-MAP2K1, and p-ERK in HL-60/ADM cells, enhanced cell apoptosis, and weakened cell proliferation. It was showed that the down-regulation of miR-181a was involved in
the regulation of drug resistance in leukemia cells, while the increase of miR-181a inhibited the expression of MAP2K1, decreased the activity of ERK/MAPK pathway, restrained cell proliferation, promoted apoptosis, and reduced drug resistance. Huang et al. found that abnormal expression of miR-181a was associated with leukemia, and over-expression of miR-181a in leukemia cells significantly inhibited cell proliferation, attenuated clonal formation ability, promoted apoptosis, alleviated tumorigenicity in animals, and reduced cell resistance mainly through targeted inhibition of KRAS, NRAS, and MAPK1 expressions. Zhu et al. observed that increasing the expression of miR-181a obviously enhanced the sensitivity of leukemia cells to the anti-tumor drug fludarabine, resulting in a significant apoptosis enhancement through targeting BCL-2, MCL-1, and XIAP. Li et al. demonstrated that transfection of miR-181a inhibitor in leukemia cells markedly promoted cell proliferation and survival, and reduced the sensitivity to the anti-tumor drug daunorubicin (DNR). Transfection of miR-181a mimic apparently facilitated the apoptosis of drug-resistant leukemia cells, weakened cell proliferative capacity, and decreased DNR resistance by targeting Bcl-2 gene expression. Fei et al. revealed that miR-181a upregulation in leukemia cells can inhibited cell proliferation, arrested cell cycle in G2 phase, and promoted apoptosis by targeting Ras-related protein Ral-A (RalA). All of the above studies reported the role of miR-181a in the regulation of drug sensitivity in leukemia cells. This work combined the targeting regulation between miR-181a and...
and MAP2K1, revealing that miR-181a targeted inhibited MAP2K1 expression, decreased ERK/MAPK pathway activity, promoted leukemia cell apoptosis, and reduced ADM resistance. However, whether miR-181a regulating MAP2K1 is related to the drug resistance of leukemia patients is still unclear.

Conclusions

We revealed that MiR-181a reduction and MAP2K1 elevation were related to ADM resistance in leukemia cells. Up-regulation of miR-181a expression inhibited leukemia cell proliferation, induced apoptosis, and reduced ADM resistance via targeting MAP2K1 expression and ERK/MAPK signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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