Down-regulation of JARID1B expression inhibits cell proliferation, induces apoptosis and blocks cell cycle in human acute lymphoblastic leukemia cells

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Abstract. – OBJECTIVE: Acute lymphocytic leukemia (ALL) is a multi-factorial blood disease with unknown pathogenesis. Histone H3K4 methylation was significantly reduced in ALL patients, whereas jumonji AT-rich interactive domain 1B (JARID1B) was the specific demethylase of H3K4me. This study explores the expression level of JARID1B in ALL patients and down-regulated JARID1B expression in ALL cells to explore the function of JARID1B in ALL.

PATIENTS AND METHODS: JARID1B mRNA expression level in ALL patients was detected by Real-time PCR. The peripheral blood mononuclear cells from healthy volunteers were selected as control. JARID1B shRNA was transfected with MOLT-4 cells and BALL-1 cells. JARID1B protein expression and H3K4me2 and H3K4me3 levels were detected by Western blot assay. Cell proliferation was assessed by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay. Cell apoptosis and cell cycle were determined by flow cytometry. Bcl-2, Bax, Procaspase 3, and cyclin P21 expressions were evaluated by Western blot assay.

RESULTS: JARID1B mRNA expression in primary bone marrow cells from ALL patients was significantly higher than that of healthy volunteers (p<0.05). The levels of histone H3K4me3 and H3K4me2 were up-regulated after JARID1B shRNA transfection. JARID1B shRNA significantly inhibited the proliferation of MOLT-4 and BALL-1 cells, induced apoptosis, and blocked cell cycle in G0/G1 phase compared with the control group (p<0.05).

CONCLUSIONS: JARID1B is highly expressed in ALL. Down-regulating its expression inhibited leukemia cell proliferation, promoted apoptosis, and blocked cell cycle in G0/G1 phase through histone H3K4 methylation. JARID1B is an oncogene in ALL.

Key Words: JARID1B, H3K4, Histone methylation, Acute lymphocytic leukemia.

Introduction

Acute lymphoblastic leukemia (ALL) is currently considered as B-line or/and T-line lymphoid progenitor cell abnormal proliferation and apoptosis caused by a variety of factors, leading to clonal cell proliferation. However, it is currently still unknown about the exact mechanism. Traditional genetics believed that histone only plays a role in maintaining the folding structure of DNA, whereas recent studies1-3 found that the different modification state of histone terminal can change chromatin structure and development, which further affects active protein binding in the chromatin DNA to regulate DNA-related gene transcription.

Histone methylation is a type of histone modification, usually referring to the methylation for N-terminal arginine or lysine residues of H3 and H4 histone4. Histone methylation and demethylation were catalyzed by histone methyltransferase and histone demethylase, respectively. At present, dozens of histones demethylase have been found5. Jumonji AT-rich interactive domain 1B (JARID1B) (also known as KDM5B or PLU1) is a newly discovered histone demethylase that specifically reduces the degree of H3K4 methylation, thus inhibiting the transcription of the corresponding genes6. JARID1B shows high specificity in normal human tissues, mainly in spermatogonia of normal adult testes and cell meiosis7,8. In the previous study9, we found that histone H3K4 methylation level was significantly reduced in patients with acute leukemia compared with non-leukemia patients and healthy controls. Since JARID1B was H3K4me-specific demethylase, whether its expression was also changed in ALL? This investigation explores the expression level of JARID1B in ALL patients and down-regulated JARID1B expression

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in ALL cells to explore the function of JARID1B in ALL. Therefore, the aim is to provide new targets for the treatment of ALL.

Patients and Methods

Sample Collection
A total of 30 cases of ALL patients were enrolled from September 2011 to December 2012 in Zhangzhou Affiliated Hospital of Fujian Medical University, Zhangzhou, China. The mononuclear cells were extracted from the bone marrow. There were 18 males and 12 females with a median age at 38.5 (15-73) years old. All patients were in line with Franch-American-Britain (FAB) and morphology-immunology-cytogenetics-molecular biology (MICM) international typing diagnostic criteria, including 24 cases in Common B, 1 case in Pre-B, 3 cases in Pro-B, and 2 cases in Mature B. Another 12 healthy volunteers were selected as the control group, including 7 males and 5 females with the median age of 27.5 (23-36) years old. This study was approved by the Medical Ethics Committee in Zhangzhou Affiliated Hospital of Fujian Medical University Zhangzhou, China.

Main Reagents
TRIzol Reagent was purchased from Invitrogen (Carlsbad, CA, USA). Roswell Park Memorial Institute-1640 (RPMI-1640) cell culture medium was purchased from HyClone (South Logan, UT, USA). RNA reverse transcription kit and Real-time PCR kit were purchased from TaKaRa Corporation (Dalian, China). Annexin-V/PI staining kit and cell cycle kit were purchased from BD (BD Biosciences, San Jose, CA, USA). Dimethyl-Histone H3 (Lys4), Trimethyl-Histone H3 (Lys4), JARID1B antibody, Bcl-2, Bax, Pro-caspase3, P21, β-actin antibodies were purchased from Abcam Biotech. (Cambridge, MA, USA).

Cell Line
ALL cell lines MOLT-4 and BALL were purchased from the Chinese Academy of Sciences Institute of Biochemistry and Cell Biology Shanghai Cell Bank (Shanghai, China).

ALL Patients Bone Marrow and Healthy Volunteers Mononuclear Cell Extraction
A total of 5 ml bone marrow samples were collected from the ALL patients. A total of 15 ml peripheral blood samples were obtained from healthy volunteers and add with a double volume of lymphocyte separation solution (Sigma-Aldrich, St. Louis, MO, USA). Next, the samples were centrifuged at 2500×g and room temperature for 20 min. After centrifugation, the specimen was divided into four layers, including plasma layer, mononuclear cell layer, liquid separation layer, and red blood cell and platelet. The mononuclear cell layer was collected as centrifuged at 2000×g for 5 min. The cell precipitation was stored at -80°C to extract RNA and protein.

Real-time PCR
The RNA was extracted by TRIzol method and reverse transcribed into complementary DNA (cDNA) using RNA reverse transcription kit (Invitrogen, Carlsbad, CA, USA). The Real-time PCR was performed by SYBR Premix Ex TaqII dye method on ABI 7900HT Real-time PCR amplifier (ABI, Foster City, CA, USA). β-actin was selected as loading control. JARID1B primer sequence: forward, 5'-CTCTCTTAGAGGTGCTGTG-3', reverse, 5'-CAGTCTCCTTGCTTTCAG-3'; β-actin primer sequence: forward, 5'-TTCTACAATGAGCTGCGTG-3', reverse, 5'-CTCAAACATGATCTGGGTC-3'. The reaction was performed at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 34 s. mRNA expression level was calculated by $2^{-\Delta\Delta C_t}$ method.

JARID1B Interference Adenovirus Vector
Adenovirus-JARID1B-RNAi-GFP interference adenovirus (JARID1B shRNA) was purchased from Heyuan Biotechnology Company (Shanghai, China). The sequence was GGAGCTATTTCAATTAACTA.

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium Bromide (MTT) Assay
MOLT-4 and BALL-1 cells in logarithmic phase were seeded on 96-well plate at 2 × 10^4/well. The cells were divided into Control, JARID1B shRNA, and neg shRNA groups. The MOI was 80. The cells were cultured at 37°C and 5% CO₂ after transfection. The cells were harvested at 24, 48, 72, and 96 h, respectively. A total of 20 μl MTT (5 mg/ml) was added to each well and incubated for 4 h. After centrifuged at 800 ×g for 10 min, the plate was added with 150 μl DMSO and tested at 570 nm to obtain the absorbance (OD) value. The experiment was repeated for three times.

Flow Cytometry
The cells were seeded in 6-well plate by Roswell Park Memorial Institute-1640 (RPMI-1640).
medium containing 20% fetal bovine serum (FBS) at 2×10⁶/well. The cells were divided into Control, JARID1B shRNA, and neg shRNA groups. The MOI was 80. The cells were cultured at 37°C and 5% CO₂ for 48 h after transfection. After centrifuged at 800×g at room temperature for 10 min, the cells were collected to stain Annexin-V/PI or propidium iodide (PI) (BD Biosciences, San Jose, CA, USA) and flow cytometry was performed (FACSCalibur, BD Company, San Jose, CA, USA).

**Western Blot**

The cell precipitation was washed with pre-cooling phosphate-buffered solution (PBS) twice and lysed by adding 100 μl lysis + 1 μl enzyme inhibitor (Sigma-Aldrich, St. Louis, MO, USA) for 30 min on ice. After centrifuged at 10000 × g at 10°C for 10 min, the protein was quantified by bicinchoninic acid (BCA) protein assay kit (Sigma-Aldrich, St. Louis, MO, USA). The protein was separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis and transferred to membrane. After blocked at room temperature for 2 h, the membrane was incubated with primary antibody at 4°C overnight. After that, the membrane was washed with a Tris-buffered solution (TBS) and incubated with horseradish peroxidase (HRP) conjugated goat anti-mouse secondary antibody at 1:5000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). At last, the membrane was developed and analyzed by AlphaDigiDoc image analysis software (version 7.1; Alpha Innotec, Kasendorf, Germany).

**Statistical Analysis**

All data analyses were performed using SPSS19 software (IBM, Armonk, NY, USA). Homogeneity of variance test and normality test were routinely adopted. The data were presented as mean ± standard deviation. 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 between groups. p<0.05 was depicted as statistical significance.

**Results**

**JARID1B highly Expressed in the Primary Bone Marrow Cells from ALL Patients**

To clarify the expression of JARID1B in ALL, we used Real-time PCR to detect the expression of JARID1B mRNA in primary bone marrow cells from 30 primary ALL patients and peripheral blood mononuclear cells from 6 healthy volunteers. JARID1B mRNA in ALL patients was significantly higher than that in the control group (p<0.05, Figure 1).

**JARID1B shRNA Reduced JARID1B Expression and up-Regulated Histone H3K4 Methylation in MOLT-4 and BALL-1 Cells**

JARID1B shRNA recombinant adenovirus was transfected into MOLT-4 cells for 48 h to test the expression of JARID1B mRNA by Real-time PCR. The results showed that the RQ value of JARID1B mRNA was 1.007 ± 0.008 in the JARID1B shRNA group, which was significantly lower than that of control group (p<0.0001). No statistical difference was observed between neg shRNA group and control group (p>0.05). The expression of JARID1B mRNA in BALL-1 cells was detected by Real-time PCR after transfection for 48 h. The results revealed that the RQ value of JARID1B mRNA in the JARID1B shRNA group was 0.084 ± 0.015, which was statistically lower than that of control (p<0.05). JARID1B protein level was decreased in JARID1B shRNA group, while histone H3K4me3 and H3K4me2 expressions were significantly increased compared with control group and neg shRNA group (p<0.05). The results demonstrated that JARID1B shRNA declined the expression of JARID1B gene and elevated the levels of H3K4me3 and H3K4me2 in MOLT-4 and BALL cells (Figure 2).
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JARID1B shRNA inhibited MOLT-4 and BALL-1 cell proliferation

MTT assay was applied to test MOLT-4 cell proliferation. At 48 h after JARID1B shRNA transfection, the OD value was 0.487 ± 0.046 in the JARID1B shRNA group, which was markedly lower than 0.965 ± 0.043 in the control and 0.887 ± 0.076 in the neg shRNA group (p<0.05) (Figure 3A). MTT assay was selected to test BALL-1 cell proliferation. At 48 h after JARID1B shRNA transfection, the OD value was 0.513 ± 0.039 in the JARID1B shRNA group, which was apparently lower than 1.096 ± 0.044 in the control and 1.021 ± 0.043 in the neg shRNA group (p<0.05) (Figure 3B).

JARID1B shRNA induced MOLT-4 and BALL-1 cell apoptosis

Flow cytometry was used to detect MOLT-4 cell apoptosis after JARID1B shRNA transfection. The cell apoptosis rate in the JARID1B shRNA group (28.62 ± 2.23%) was significantly higher than that in the control (2.08 ± 0.82%) and neg shRNA group (3.58 ± 0.93%) (p<0.05) (Figure 4A).

Flow cytometry was performed to determine BALL-1 cell apoptosis after JARID1B shRNA transfection. The cell apoptosis rate in the JARID1B shRNA group (22.52 ± 3.42%) was higher than that in the control (2.72 ± 0.52%) and neg shRNA group (3.27 ± 0.74%) (p<0.05) (Figure 4B).

JARID1B shRNA Arrested MOLT-4 and BALL-1 Cell Cycle in G0/G1 Phase

Flow cytometry was used to detect MOLT-4 cell cycle after JARID1B shRNA transfection. The cell rate in the G0/G1 phase of JARID1B shRNA group (67.54 ± 3.43%) was significantly higher than that in the control (53.54 ± 3.27%) and neg shRNA group (67.54 ± 3.43%) (p<0.05).

Flow cytometry was selected to assess BALL-1 cell cycle after JARID1B shRNA transfection. The cell rate in the G0/G1 phase of JARID1B shRNA group (56.82 ± 2.57%) was significantly higher than that in the control (44.28 ± 3.52%) and neg shRNA group (46.24 ± 3.47%) (p<0.05) (Figure 5).
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The Impact of JARID1B shRNA on Bcl-2, Bax, Procaspase3, and P21 Expressions

Bax and P21 expressions significantly increased, whereas Bcl-2 and Procaspase3 levels significantly down-regulated in MOLT-4 and BALL cells after JARID1B shRNA transfection compared with control (p<0.05) (Figure 6).

Discussion

The abnormality of histone methylation is closely related to the occurrence and development of tumor cells. Histone methylation is catalyzed by histone methylase and demethylase synergistically, which is a dynamic modification process. There are many histone methyltransferases expressed abnormally in the tumor. EZH2 protein is the specific histone methylase of H3K27me3, which is found to be over-expressed in prostate cancer, bladder cancer, breast cancer, and many other tumors. Histone methylase NSD2 can activate multiple myeloma-associated oncogenes by increasing H3K36me2 levels, leading to multiple myeloma. H3K9me3 and its specific histone methylase Suv39h1 are found to be elevated in gastric cancer tissues and correlated with clinical stage.

JARID1B is a histone demethylase enzyme found in 2007 that specifically removes the methylation levels of H3K4me, H3K4me2, and H3K4me3. Since histone H3K4 methylation is a marker of gene transcriptional activation, JARID1B can inhibit the transcription of H3K4 by removing H3K4 methylation. JARID1B shows high tissue specificity in normal human tissues, as its low expression in ovaries, lymph nodes, and other tissues, while high level in spermatogonia. JARID1B inhibits the expression of multiple tumor suppressor genes, such as BRCA1, by mediating H3K4 demethylation, thus regulating

Figure 3. JARID1B shRNA inhibited MOLT-4 and BALL-1 cell proliferation. A, MOLT-4 cell line; B, BALL-1 cell line.

Figure 4. JARID1B shRNA induced MOLT-4 and BALL-1 cell apoptosis. A, MOLT-4 cell line; B, BALL-1 cell line. *p<0.05, compared with Control.
Figure 5. JARID1B shRNA arrested MOLT-4 and BALL-1 cell cycle in G0/G1 phase. A, MOLT-4 cell line; B, BALL-1 cell line. *p<0.05, compared with Control.

Figure 6. The impact of JARID1B shRNA on Bcl-2, Bax, Procaspase3, and P21 expressions. A, MOLT-4 cell line; B, BALL-1 cell line. *p<0.05, compared with Control.
cell cycle, cell differentiation, malignant transformation, and tumor formation. JARID1B also suppresses the transcription of related genes to be involved in the occurrence of tumor by recruiting the histone deacetylase to reduce acetylation of local histone. It was found that JARID1B is highly expressed in prostate cancer tissue and related to prostate cancer metastasis and prognosis. Therefore, it is thought that the expression level of JARID1B gene can be used as a prognostic indicator of prostate cancer patients. These studies suggest that JARID1B gene may be an effective target for tumor gene therapy. However, JARID1B exhibits different expression level in different tumor tissues. It plays an oncogene role in most tumor tissues, but inhibits tumorigenesis of malignant melanoma via the absence of expression. Our previous work also found that H3K4me3 methylation was low in mantle cell lymphoma and JARID1B was highly expressed as its specific demethylase. In this work, we also observed that JARID1 gene mRNA is highly expressed in the primary cells of bone marrow from ALL, suggesting it may be related to the pathogenesis of ALL.

In this investigation, the RQ value of JARID1B mRNA was 1.007 ± 0.008 in the JARID1B shRNA group of MOLT-4 cells, which was significantly lower than that of control group. The RQ value of JARID1B mRNA in the JARID1B shRNA group of BALL-1 cells was 0.084 ± 0.015, which was statistically lower than that of control. JARID1B protein level was decreased in JARID1B shRNA group, while histone H3K4me3 and H3K4me2 expressions were significantly increased compared with control group and neg shRNA group. JARID1B shRNA further restrained cell proliferation, enhanced cell apoptosis, and arrested cell cycle in G0/G1 phase. It was in accord with our previous results in HL-60 and Jeko cells. Lin et al adopted drugs to inhibit JARID1B activity, thus to suppress oral cancer invasiveness and enhance radiotherapy sensitivity. In breast cancer MCF-7 cells, Mitra et al silenced JARID1B gene to methylate H3K4, leading to cell cycle-related gene suppression, arrests cell cycle in G0/G1 phase, blocks cell proliferation, and inhibits colony formation, which was thought to be achieved by up-regulating BRCA1. In many stimulus-induced apoptosis, caspase-3 is the main effector protein, while procaspase-3 is the precursor form that cut the structural protein and regulation protein in the nucleus and cytoplasm. The study also found that JARID1B shRNA can down-regulate Bcl-2 levels and increase Bax expression to change the Bcl-2/Bax ratio. It elevated procaspase 3 expression to induce cell apoptosis. P21 protein can inhibit the activity of CDK2, CDK4, and CDK6, so as to arrest cells in G0/G1 phase. Our results demonstrated that JARID1B shRNA can upregulate P21 protein to block cells in G0/G1 phase.

Conclusions

We showed JARID1B is highly expressed in ALL. Down-regulating its expression inhibited leukemia cell proliferation, promoted apoptosis, and blocked cell cycle in G0/G1 phase through histone H3K4 methylation. JARID1B is an oncogene in ALL.

Acknowledgments

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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