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Regulation of DNA methylation and tumor suppression gene expression by miR-29b in leukemia patients and related mechanisms

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Abstract. – OBJECTIVE: Leukemia is characterized as a kind of malignant clonal disease in hematological stem cells. The study showed an abnormal level of DNA methylation in leukemia cells, which further led to an abnormal expression of hematological genes. This study investigated the role of miR-29b on the modulation of DNA methylation and tumor suppressor gene expression in leukemia patients.

PATIENTS AND METHODS: A total of 21 leukemia patients were recruited for the collection of monocytes. Methylation levels of promoter sequence of ESR1 and p15 genes were analyzed by methylation assay kit combined with DHPLC. DNA microarray and qRT-PCR were used to measure microRNA expressional profile, and bioinformatics plus luciferase reporter assay confirmed target gene of miR-29b. After transfection with miR-29b, promoter methylation levels of ESR1 and p15 gene were measured. Protein expressions of DNMT1 DNA (cytosine-5)-methyltransferase 1 (DNMT1), DNA (cytosine-5)-methyltransferase 3A (DNMT3A) and DNA (cytosine-5)-methyltransferase 3B (DNMT3B) were quantified.

RESULTS: The methylation levels of the promoter region of ESR1 and p15 genes in monocytes of leukemia patient were significantly elevated ($p < 0.05$). DNA microarray and qRT-PCR confirmed the down-regulation of miR-29b ($p < 0.05$). Luciferase reporter assay revealed DNMT1, DNMT3A and DNMT3B as target genes of miR-29b. MiR-29b transfection inhibited the expressions of DNMT3A and DNMT3B in Kasumi-1 cells ($p < 0.05$), and promoter methylation levels of estrogen Receptor 1 (ESR1) and p15 gene were decreased ($p < 0.05$).

CONCLUSIONS: In leukemia cells, hyper-methylation existed in the promoter region of tumor suppressor gene. The methylation was enhanced in gene DNMT1, DNMT3A and DNMT3B via the reduction of miR-29b in leukemia tumor cells.

Key Words:
Leukemia, MiR-29b, Methylation, Mononuclear cells, Tumor suppressor genes.

Introduction

Leukemia is a kind of malignant disease that occurs in hematological stem cells[1]. Clonal leukemia cells are abundantly aggregated in bone marrow and other hematological tissues due to dysregulated proliferation, differentiation disorder and impeding of apoptosis. They infiltrate other non-hematological tissue/organs and affect normal hematological functions[2]. Clinical manifestation of leukemia includes anemia, hemorrhage, infection, and swelling of liver, spleen and lymph node[3]. In China, the incidence of leukemia ranks 7th among all malignant tumors[4]. Epidemic survey showed that the population of leukemia patients was becoming younger in recent years. As the most common childhood cancer, it severely threatens health of younger generation[5]. With rapid progression in biomedical technology and molecular biology, pathogenesis of certain subtypes of leukemia has been elucidated, and some patients have been cured by transplantation with hematological stem cell or targeted drugs. However, large amounts of patients cannot present complete recovery from the disease. Therefore, further studies on pathogenesis mechanism of different subtypes of leukemia are still the focus for clinicians[6].

Currently, large amounts of studies showed close correlation between abnormal DNA methylation and occurrence or progression of human malignant tumors[7]. Cancer cells can inhibit the

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expressions of tumor suppressor genes via enhancing methylation level of these genes, and decrease methylation levels of oncogenes, thus potentiating their transcriptional activity[8]. Abnormal DNA methylation is one of the important features of malignant tumor cells[9]. In leukemia patients, hypo-methylation of whole genome was found in conjunction with hyper-methylation in CpG island in certain gene promoters in leukemia patients. These genes are mostly related with cell behaviors including cell proliferation, mitosis, cell cycle modulation, apoptosis and adhesion[10]. The investigation of regulatory mechanism underlying abnormal DNA methylation in leukemia patients may provide novel insights for clinical treatment[11].

Abnormal expression of micorRNA is another feature of malignant tumor cells[12]. MicroRNA can modulate cell proliferation, division and invasion potency[13]. For example, proliferation and metastasis of colorectal carcinoma is closely correlated with miR-200[14]. Previous study identified the important role of miR-29b in hematological stem cell differentiation. Moreover, researchers have found abnormal expression of miR-29b in leukemia patients, thus speculating close correlation between miR-29b and occurrence and progression of leukemia[15]. This study aimed to investigate the regulatory function of miR-29b on DNA methylation and tumor suppressor gene expression in leukemia patients and related mechanisms.

Patients and Methods

Monocytes Separation

A total of 21 acute myelocytic leukemia (AML) patients who received treatment in Huzhou Central Hospital from April 2015 to April 2016 were recruited, including 13 males and 8 females (average age = 11.3 ± 6.2 years). All patients did not receive any treatment before admission. Another cohort of 10 healthy volunteers was recruited as the control group, which presented similar sex ratio and average age with disease group. Bone marrow fluid samples (0.2 mL) were collected via iliac bone puncture after focal anesthesia. Bone marrow fluid was mixed with heparin (7:3 ratio) and was separated for human monocytes precipitated by hydroxyethyl starch (Merck, Temecula, CA, USA). In brief, sample fluid was centrifuged at 2000 g for 10 min to remove plasma in the supernatant. Equal volume of saline was then added and mixed with 6% hydroxyethyl starch at 1:4 ratio. After incubation at 40 min room temperature for precipitation, the supernatant was centrifuged at 2000 g for 5 min. Cell precipitation was then washed twice in saline. Obtained monocytes were cultured in serum-free medium, which contained granulocyte-macrophage clonal stimulating factor (50 ng/mL), IL-3 (20 ng/mL), IL-6 (20 ng/mL) and stem cell factor (100 ng/mL). The study protocol was approved by the Research Ethics Committee of Huzhou Central Hospital, and all patients gave their informed consent before study commencement.

MiRNA Expression Profile Analysis

Monocytes were extracted for total RNA using RNAprep pure Tissue Kit (Qiagen, Hilden, Germany). Tail tag and biotin label were attached to miRNA in total RNA samples using FlashTag Biotin HSR kit (Affymetrix, Santa Clara, CA, USA). Labeled samples were loaded onto miRNA4.0 microarray and were scanned in GCS3000 microarray scanner (Affymetrix, Santa Clara, CA, USA). Raw data were input into ExpressionConsole software (Affymetrix, Santa Clara, CA, USA) for calculating averaged values with replicates and standard deviation after deducing baseline level. Data were then normalized and tested in independent sampling manner. A statistical significance was defined when $p < 0.01$.

qRT-PCR

Firstly, primers for qRT-PCR were designed based on miR-29b sequence (GeneBank access No.: NR_029517, Table I). Using total RNA extracted from HEK293 cells as the control, qRT-PCR was performed to quantify miR-29b expression level. MirVanat qRT-PCR miRNA test kit (Ambion, Thermo Fisher Scientific, Waltham, MA, USA) was used for qRT-PCR reaction in Real-time PCR cycler (Bio-Rad, Hercules, CA, USA) under following conditions: 95°C for 3 min, followed by 40 cycles each containing 95°C for

Table I. qRT-PCR primer sequence.

Name	Sequence
miR-29b-F	5'-AGCTGGTTTCATATGGTGGTTTAGA-3'
miR-29b-R	5'-CACTGATTTCAAATGGTGCTAGACA-3'
U6-F	5'CTCGCTTCGGCAGCACA3'
U6-R	5'AACGCTTCACGAATTTGCGT3'

15 s, and 60°C for 30 s. Built-in software of PCR cycler (V2.02) was used for analysis using U6 gene as internal reference. Results were presented by $2^{-\Delta\Delta Ct}$ approach[13].

Prediction of Functional Target of miR-29b

Bioinformatics software TargetScan Release 5.1 (www.targetscan.org) was used to predict miR-29b function. Possible targets of miR-29b were confirmed by luciferase reporter gene. Based on prediction results, 3'UTR sequence of DNMT1 (GeneBank ID NM_001130823), DNMT3A (NM_175629) and DNMT3B (NM_005892) genes were obtained for designing primers (Table II). By PCR amplification, 3'-UTR sequence of DNMT1, DNMT3A and DNMT3B were obtained, and were inserted to the downstream of firefly luciferase gene coding region of pmirGLO plasmid to construct pmirGLO-DNMT1, pmirGLO-DNMT3A and pmirGLO-DNMT3B plasmids, which were used to transfect HEK293 cells. Those cells with successful transfection were transfected with miR-29b mimic to potentiate miR-29b activity in cells. By successful transfection, cells were incubated for 48 h to analyze fluorescent strength. Dual luciferase reporter gene analysis system (Promega, Madison, WI, USA) and MicroLumar Plus LB96V spectrometry (Berthold, Bad Wildbad, Germany) was used for fluorescent intensity analysis.

Cell Transfection of miR-29b

MiR-29b mimic or miR-29b inhibitor was used to up-regulate or suppress miR-20b expression in leukemia cell line Kasumi-1. MiR-29b mimic and miR-29b inhibitor were purchased from Gimma Pharma (Shanghai, China). Negative control (NC) sequence was employed in parallel. Liposome INTERFERin™ transfection kit (Polyplus transfection, New York, NY, USA) was used for cell transfection assay. Cells were resuscitated and cultured until log-growth phase. Cells were digested in trypsin, counted and diluted in fresh medium, and were inoculated into 96-well plate for 24 h incubation before transfection, following manual instruction of test kit.

DNA Methylation Assay

To quantify methylation status of promoter region of ESR1 tumor suppressor gene and p15 in monocytes, we firstly utilized QIAmp DNA Mini Kit (Qiagen, Hilden, Germany) to extract total DNA from monocytes. Using normal human genomic DNA as the control group, EZ DNA methylation kit (Zymo Research, Irvine, CA, USA) was used for methylation modification of genomic DNA. Modification products were purified and collected using YM-100 kit (Millipore, Billerica, CA, USA). Primers were then designed based on promoter sequence of ESR1 and p15 gene (Table III for primer sequences). After PCR amplification, methylation modified ESR1 and p15 gene promoter sequences were obtained. PCR amplification products were analyzed in DHPLC (WAVE, Transgenomic Inc, Waltham, MA, USA) and Trangenomic DNAseq chromatographic column. Partial denature temperature of samples were determined by WAVE Navigator software based on experimental data. Promoter sequence with different methylation level was differentiated based on averaged partial denature temperature.

Western Blot

Cells after incubation were collected and mixed with protein lysis buffer. The supernatant was collected for electrophoresis, and was transferred to polyvinylidene difluoride (PVDF) membrane under electric field. Western blot was used to test protein expression levels related with methylation, including DNMT1, DNMT3A, DNMT3B. PVDF membrane was blocked in 5% defatted milk powder for 1 h at 37°C. After TBST washing, primary antibody working solution (anti-DNMT1, anti-DNMT3A, anti-DNMT3B and anti-β-actin, CST, Danvers, MA USA, 1:200 dilution) (Abcam, Cambridge, MA, USA) was added for 4°C overnight incubation. Excess primary

Table II. PCR amplification primer sequences.

Name	Sequence
DNMT1-F	5'-GGAGGAGGAAGCTGCTAAGG-3'
DNMT1-R	5'- GGTTTATAGGAGAGATTTATTTG-3'
DNMT3A-F	5'-GCTCTAGACGAAAAGGGTTGGACATC-3'
DNMT3A-R	5'-GCTCTAGAGCCGAGGGAGTCTCCTTT-3'
DNMT3B-F	5'-GCTCTAGATAGGTAGCAACGTGGC-3'
DNMT3B-R	5'-GCTCTAGAGCCCCACAAAACTTGTC-3'

Table III. PCR amplification primer sequence.

Name	Sequence
p15-F	5'-AGGTTTGTAGGTTTATAGGTTTTT-3'
p15-R	5'-TCTACTCTCTCTAACAAATCATAATAAT-3'
ESR1-F	5'-GGGTAGAATTAGAGTAGTTTTGTGTT-3'
ESR1-R	5'-AAAATTCTTTACTCCCAAAAATAAC-3'

antibody was washed by TBST. Horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG secondary antibody (1:1000) (Abcam, Cambridge, MA, USA) was added for incubation 1 h at room temperature. After TSBT washing, the membrane was developed in freshly prepared DAB substrate for 10 min in the dark, and was quenched in distilled water. Western blot results were analyzed by gel imaging system to quantify integrity gray values of all protein bands[14].

Statistical Analysis

All data were tested by analysis of variance (ANOVA) followed by LSD post-hoc test, and results were shown as mean±standard deviation (SD). Comparative analysis was performed by SPSS 20.0 (SPSS Inc., Armonk, NY, USA) in student *t*-test. Significant difference was defined when $p < 0.05$, and extremely significant was identified when $p < 0.01$.

Results

Assay for Methylation in Leukemia Monocytes

Methylation kit combined with DHPLC was used to investigate methylation level of monocytes in all research subjects. WAVE Navigator software was used to analyze methylation percentage of DNA. DHPLC assay was performed under different denature temperatures in promoter regions of ESR1 and p15 genes after amplification. DHPLC assay results were shown in Figure 1. We found the best differentiation index between methylated and non-methylated sequences of two gene promoter regions under 56°C denature temperature. Methylation levels of promoter region for ESR1 and p15 genes were tested as Figure 2. We found that methylation levels of ESR1 and p15 gene promoters were 56.15 ± 23.03% and 61.08 ± 23.07% in leukemia patients, respectively, which were significantly higher than control group levels at 12.77 ± 6.58% and 15.02 ± 5.67% ($p < 0.05$ in both cases).

MiR-29b Expression Assay in Leukemia

Gene microarray approach was used to compare differential expression of microRNA between leukemia and control group. Expression Console software set up the expression of various miRNA in healthy control cells as reference parameters, to analyze differential expression of microRNA in leukemia cells (Figure 3). Compared to NTG cells, significant changes of various miRNAs were found in leukemia cells. We found down-regulation of miR-29b by using qRT-PCR assay (Figure 3). The miR-29b expression was decreased by 72% in leukemia patients compared to that in healthy control group ($p < 0.05$).

Correlation Between miR-29b and Methylation of Gene

Bioinformatics software TargetScan Release 5.1 was used to predict target genes of miR-29b. We found sequence homology between miR-29

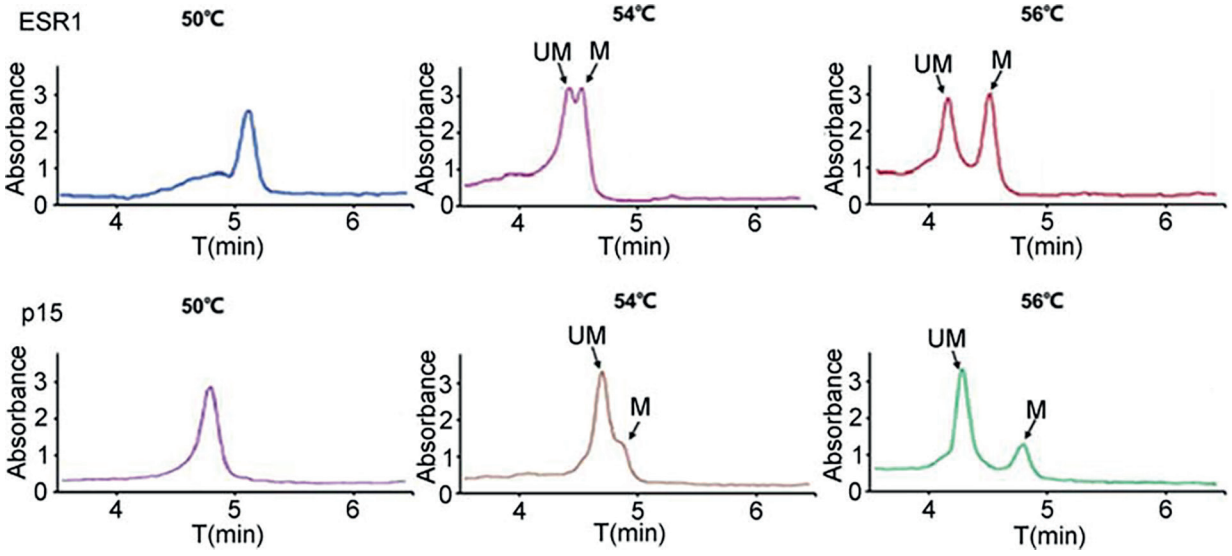

Figure 1. Promoter sequence methylation level of ESR1 and p15 gene by DHPLC. UM, unmethylated sequence; M, methylated sequence.

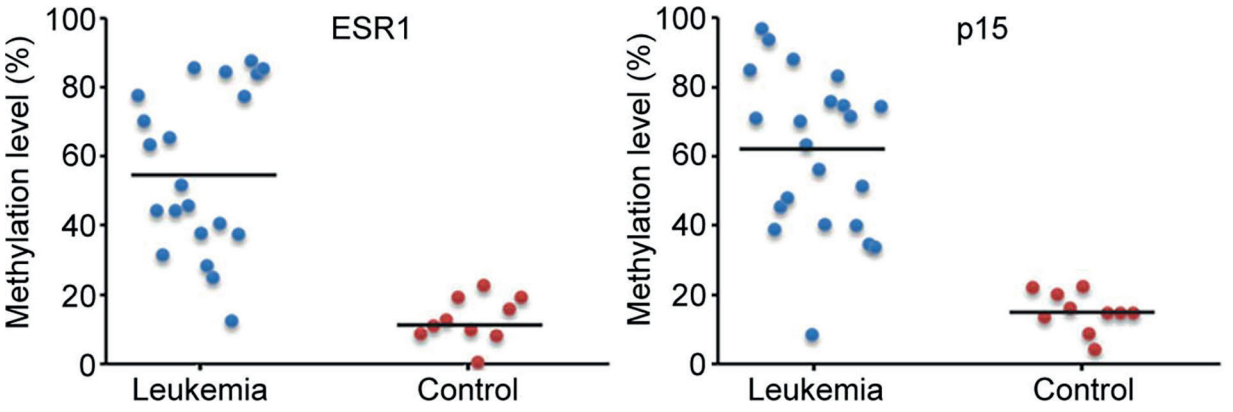

Figure 2. ESR1 and p15 gene promoter methylation level in leukemia patients.

and 3'-UTR of DNMT1, DNMT3A and DNMT3B (Figure 4), thus speculating DNMT1, DNMT3A and DNMT3B as probable target genes of miR-29b. We then constructed luciferase reporter gene expression system for confirmation (Figure 5) and found significantly decreasing fluorescent intensity of cells with miR-29b mimic transfection ($p < 0.05$). These results suggested that 3'UTR of DNMT3A and DNMT3B were functional targets of miR-29b. The change of fluorescent level in DNMT1, however, is insignificant ($p > 0.05$).

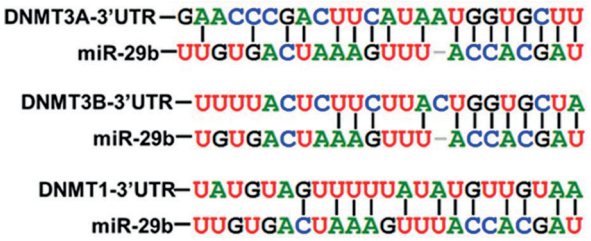

Figure 4. Sequence homology between 3'UTR sequence and miR-29b in methylated related genes.

Effects of miR-29b on Methylated Gene Expression in Kasumi-1 Cells

The transfection with miR-29b mimic up-regulated the level of miR-29b in Kasumi-1 cells. After transfection, cells were continuously cultured until log-growth phase. Western blot was used to test expressional levels of DNMT1, DNMT3A and DNMT3B in addition to β-actin (Figure 6). Comparing between Kasumi-1 cells transfected by miR-29b and control group, DNMT3A and DNMT3B expressions were significantly decreased ($p < 0.05$) whilst DNMT1 expression remained unchanged. These results indicated that in leukemia cells, miR-29b could regulate DNMT3A and DNMT3B expressions, but no significant effects on DNMT1 expression were showed.

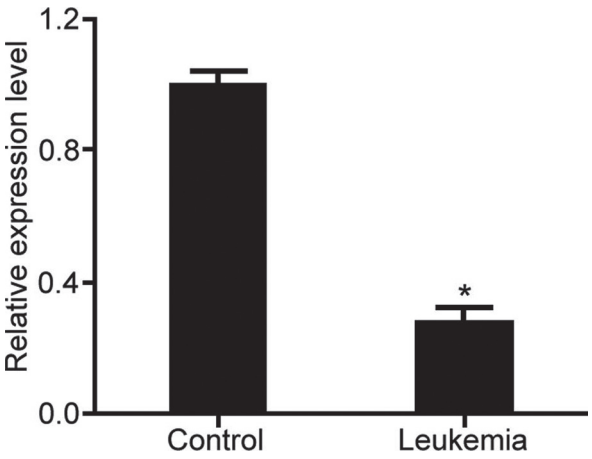

Figure 3. qRT-PCR for miR-29b expression in leukemia cells. *$p < 0.05$ compared to control group.

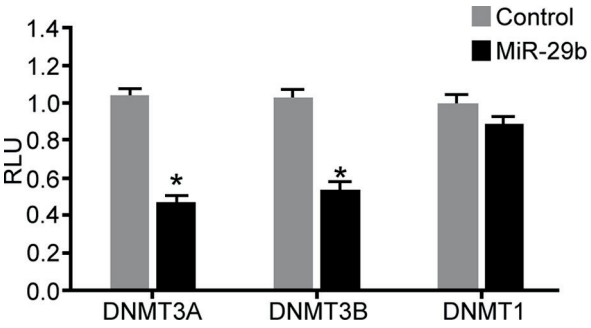

Figure 5. Luciferase reporter gene assay. *$p < 0.05$ compared to control group.

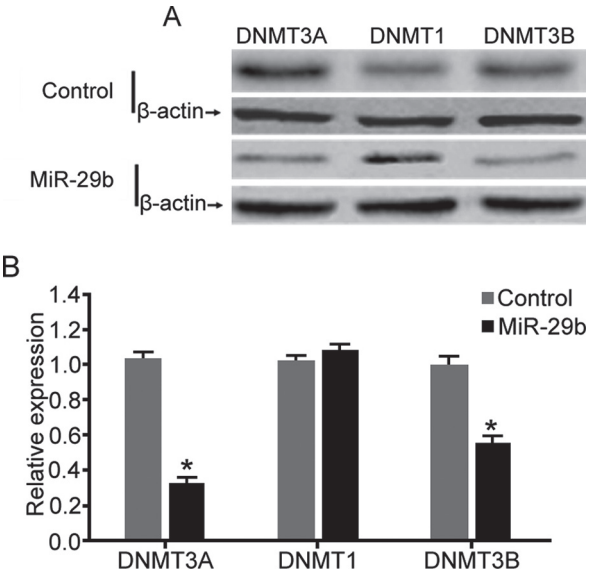

Figure 6. Western Blot for effects of miR-29b on gene methylation of Kasumi-1 cells. ***(A)*** Western blot for methylation related gene expression level in Kasumi-1 cells; ***(B)*** Effects of miR-29b on methylation gene expression in Kasumi-1 cells. *$p < 0.05$ compared to control group.

DNA Methylation Level

After successful transfection with miR-29b mimic, we tested methylation levels in promoter sequence of ESR1 and p15 genes in Kasumi-1 cells via methylation assay kit in conjunction with DHPLC approach, to evaluate the effect of miR-29b on DNA methylation level in leukemia cancer cells. As shown in Figure 7, we found that promoter methylation ratio of ESR1 and p15 genes decreased by 71.8 ± 18.6% and 80.1 ± 20.3% in Kasumi-1 cells transfected with miR-29b mimic ($p < 0.05$). These results showed that miR-29b significantly suppressed cellular DNA methylation level in leukemia cancer cells.

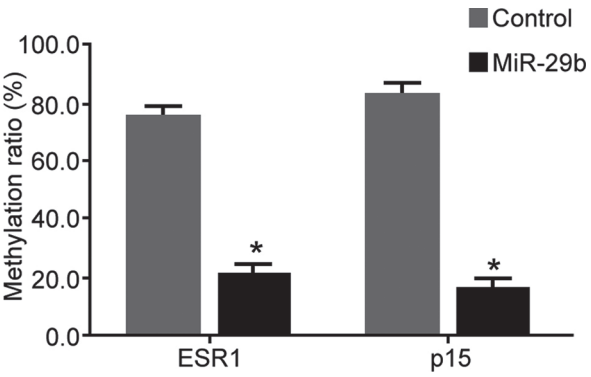

Figure 7. Methylation ratio of promoter sequence of ESR1 and p15 genes. *$p < 0.05$ compared to control group.

Discussion

Currently various studies have demonstrated major difference of microRNA expression between cancer cells and normal cells, including miR-21, miR-10b and miR-29b[16]. Commonly accepted opinions believed that cancer cells can modulate proliferation, invasion and apoptosis via modulating microRNA expression. For example, miR-10b participates in invasion and metastasis of breast cancer cells[17]. Some researchers believed that abnormal methylation level of genomic DNA was correlated with abnormal expression of microRNA in cancer cells[11]. In this study, we found down-regulation of miR-29b in leukemia cells, along with the increasing methylation level in tumor suppressor gene in cancer cells, proving that miR-29b could modulate gene expression via methylation regulation. Therefore we proposed that miR-29b down-regulation induced expression of methylation genes, facilitated methylation of tumor suppressor gene promoter, and inhibited tumor suppressor gene expression during the growth and metabolism of cancer cell.

Currently, various studies showed abnormal expression of microRNA in cancer cells. However, the cause of differential expression of microRNA in malignant tumor cells is still debatable[18,19]. Increasing evidence demonstrated that microRNA participated in regulating DNA methylation process, and microRNA expression level was under the effect of DNA methylation. Chakrabarti et al[19] found partial up-regulation of microRNA in tumor cells after being treated with demethylation reagent, thus speculating the effect of methylation or other irreversible modification on its transcriptional regulatory sequence. In addition, expression product of microRNA target gene can regulate expression of certain microRNA in a negative feedback manner[19]. This study found that miR-29b could regulate the expression of methylation-related genes in leukemia cancer cells, eventually modulating methylation level in cellular DNA, as consistent with other scholars' studied regarding the correlation between microRNA and DNA methylation[18,19].

DNA methylation causes epigenetic changes in human and leads to certain diseases[20]. DNMT1, DNMT3A and DMNT3B belong to DNA methyl transferase family, and can catalyze methylation reaction of CpG island in DNA sequence[21]. When hyper-methylation occurred in promoter

sequence of certain tumor suppressor genes such as ESR1 and p15 genes, gene transcription was terminated, eventually leading to abnormal cell growth or metabolism in the favor of cancer development[22]. Patel et al[23] found that expression of tumor suppressor gene was suppressed due to DNA over-methylation in leukemia cells. Garzon et al[24] also demonstrated that abnormal DNA methylation in leukemia cells was due to up-regulation of methylation related genes including DNMT1, DNMT3A and DNMT3B, as consistent with our study.

Nowadays the incidence of leukemia has gradually elevated in younger population, and severely affects public health[5]. Increasing evidence indicated that abnormal DNA methylation status, especially hyper-methylation of tumor suppressor gene, played critical roles in occurrence and progression of leukemia, diagnosis of mini residual disease (MRD), prediction of disease condition and guiding treatment. Methylation interference drugs have been developed to treat leukemia in clinical trials. Of note, this study found possible regulatory role and mechanism of miR-29b in DNA methylation of leukemia cells, probably providing novel insights for treating leukemia based on methylation modulation strategy. However, the analysis approach of methylation used in this study only revealed methylation level of few genes, and cannot analyze or demonstrate at genomic level, the limitation of which also included the small sample size of research subject. Currently the ratio of 5-methyl-2-deoxycytidine and 2'-deoxyguanosine by LC-MS was determined to analyze methylation level at whole-genome level[25]. Future study thus may analyze more objects and malignant tumor samples to reveal the role of miR-29b in methylation of other human malignant tumors.

Conclusions

In monocytes from leukemia patients, significantly high levels of methylation in promoter sequence of tumor suppressor genes, ESR1 and p15, were observed in parallel with down-regulation of miR-29b in leukemia cancer cells. Further correlation analysis between miR-29b expression and cellular DNA methylation revealed that miR-29b regulated the expressions of tumor suppressor genes DNMT3A and DNMT3B via the modulation of methylation.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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