MicroRNA Expression Deregulated by Aberrant Methylation in B-ALL Mouse Model

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Research Article

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Abstract

The expression of microRNAs (miRNAs) in the serum of B-cell acute lymphoblastic leukemia (B-ALL) patients is abnormal. However, the mechanism is not clear. Recent studies have shown that the methylation state of circulating cell-free DNA (cfDNA) is different between tumor patients and normal people. Therefore, we speculate that abnormal expression of miRNA may be associated with methylation in cfDNA. The aim of this study was to establish a fluorescently labeled B-ALL transplantation animal model, so as to explore the relationship between the serum miRNA expression and the related gene of cfDNA methylation. The expression levels of miRNAs were detected with quantitative real-time PCR (qRT-PCR). The cfDNA methylation levels of related gene were tested by bisulfite sequencing polymerase chain reaction (BSP). The result showed that the expression levels of miR-196b, miR-203, miR-34a-5p, miR-335-3p, miR-34b-5p, miR-615, miR-375-3p and miR-193b-5p in the serum of the model mice were significantly lower than those of the control ($P < 0.05$). The methylation level of miR-196b promoter in cfDNA of model group was significantly lower than that of the control group ($P < 0.05$), and there was no significant difference in miR-203 promoter. The methylation levels of miR-196b and miR-203 coding region in cfDNA of model group were significantly higher than those of the control ($P < 0.05$). The results indicate that CpG island hypermethylation in the miRNA coding region of cfDNA is related to the low expression of miR-196b and miR-203.

Introduction

B-ALL is a hematological malignant disease result from the abnormal proliferation of primitive B lymphocytes in the bone marrow, which is the most commonly diagnosed childhood malignancy [1]. The patients with ALL are often accompanied by the occurrence of extramedullary infiltration, which is the main cause of the recurrence. MiRNA is a kind of non-coding RNA composed of 18–25 nucleotide fragments, which regulates gene expression patterns by targeting specific mRNAs. They are epigenetic regulators of a variety of cell signaling pathways [2]. Data showed the expression of miRNAs in the serum of B-ALL patients are abnormal [3]. Most of miRNA genes are found in the intergenic region, called intergenic miRNA, and some of them are found in the exon and intron regions, called intragenic miRNA [4, 5]. It is generally believed that miRNA genes are co-transcribed with their host genes. Some miRNA genes have independent transcriptional promoters [6, 7]. DNA methylation is a kind of reversible epigenetic change, which is closely related to the progress of malignant tumor diseases. Hypermethylation of DNA causes gene expression to be down-regulated. DNA methylation usually occurs at the cytosine bases of CpG dinucleotides [8], short sequences of 5–10 CpG dinucleotides per 100 bp locate at gene promoters and regulatory regions [9]. It has been observed that most of CpG islands located near miRNA genes are involved in their transcriptional regulation [10]. These suggest that the miRNAs differences in B-ALL may be caused by different methylation status of the CpG island near their gene.

cfDNA is mainly derived from necrotic or apoptotic cells and the active release of intracellular DNA [11]. The content of cfDNA in serum of healthy people is about 1-10ng/mL [12], while was increased in patients with inflammatory diseases or tumor [13]. Recent study has shown that the methylation state of

cfDNA is different between tumor patients and healthy people [14]. Therefore, we speculate that methylated cfDNA that affect miRNA expression may be found in the serum of B-ALL patients.

In this study, we established a fluorescent labeled B-ALL transplantation animal model with the fluorescent labeled cell lines. Meanwhile, we evaluated the expression levels of some miRNAs in serum, and identified the methylation levels of the promoter and the coding region of two miRNA miR-203 and miR-196b in serum cfDNA.

Materials And Methods

Cell Culture and transfection

Mouse B-ALL cell line, L1210, was obtained from the Chinese Academy of Sciences, Shanghai Institutes for Biological Sciences. The cells were maintained in DMEM (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, USA) and 1% penicillin/streptomycin in a humidified atmosphere of 5% CO₂ at 37°C. The lentiviral vector encoding GFP (GenePharma, China) was transfected into L1210 cell lines with polybrene transfection reagent (GenePharma, China) according to the instructions. The expression of GFP was observed under the fluorescence microscope, and subjected to flow cytometric (NovoCyte 2060R, USA) analysis for GFP detection. L1210-GFP cells were cultured under the same condition as L1210 cells.

Animal model

40 ICR mice (20 male and 20 female) aged 4 weeks (13.87 ± 3.44 g) were reared in clean and ventilated environment for a week to adapt to the environment. The model group (n = 20) was intraperitoneally injected cyclophosphamide (2 mg / (kg·d)) three days before modeling, then injected with 1.0×10^7 L1210-GFP cells via tail vein. The control group (n = 20) was injected with equal volume of PBS. After 30 days, the fluorescence signal intensity of peripheral blood mononuclear cells was tested by flow cytometry (ACEA, USA). Spleen sections were cut at 5 µm using a microtome (LEICA, Germany) and stained with Hoechst33258 (Sigma, USA). All experimental procedures were approved by the Institutional Animal Care and Use Committee of Beihua University, Jilin, P.R. China.

Quantitative Real-time PCR (qRT-PCR)

According to the manufacturer's instruction, the miRNA was isolated from L1210 cell lines and serum of mice by miRcute miRNA Isolation Kit (Tiangen, China). The cDNA was synthesized by Prime Script RT Reagent Kit (TaKaRa, Japan). The qRT-PCR was conducted with the SYBR Premix Ex Taq Kit (TaKaRa, Japan). The primers used are given in Table 1. U6 was treated as internal control to quantify the expression levels of miRNAs by $2^{-\Delta\Delta Ct}$ method.

Table 1
The sequences of miRNA primers and BSP primers used in this study.

Primer	Sequence(5'-3')	GenBank accession
mus-U6-F	AGCCAGGGAGACTGATGCTA	NM_015816.4
mus-U6-R	AGGTCCAGCCTTACTGCTCA	
mus-miR-196b-5p	TAGGTAGTTTCCTGTTGTTGGG	NR_029912.1
mus-miR-203	GTGAAATGTTTAGGACCACTAGA	NR_029590.1
mus-miR-34a-5p	TGGCAGTGTCTTAGCTGGTTGT	NR_029751.1
mus-miR-335-3p	TTTTTCATTATTGCTCCTGACC	NR_029900.1
mus-miR-320-5p	GCCTTCTCTTCCCGTTCTTCC	NR_029802.1
mus-miR-615	TCCGAGCCTGGGTCTCCCTCT	NR_030526.1
mus-miR-375-3p	TTTGTTTCGTTGGCTCGCGTGA	NR_029876.1
mus-miR-193b-5p	CGGGGTTTTGAGGGCGAGATGA	NR_030549.1
mus-miR-34b-5p	AGGCAGTGTAATTAGCTGATTGT	NR_029655.1
mus-miR-196b	GTAGGAAATGTTTTTTTGTGTTTTGG	NR_029912.1
-promoter-L		
mus-miR-196b	AAATTATACCCTCCCTCCTTTTACA	
-promoter-R		
mus-miR-196b	AAAGGTGGATTTTAATAATAGGAAATTATT	NR_029590.1
-coding region-L		
mus-miR-196b	CCTCTTCATCCACCATAAAATTAAA	
-coding region-R		
mus-miR-203	ATTGTATTGGAAGGGAATTTTAAAG	NR_029590.1
-promoter-L		
mus-miR-203	TTCTACTTCCCAAACCTAAACCTCT	
-promoter-R		
mus-miR-203	GTGAAATGTTTAGGATTATTAGATT	NR_029590.1
-coding region-L		
mus-miR-203	CTCTACACTCCTACCAAACCTAC	NR_029590.1
-coding region-R		

Bisulfite sequencing PCR (BSP)

According to the manufacturer's instructions, the DNA was isolated from L1210 cell by TIANamp Genomic DNA Kit (Tiangen, China). The serum cfDNA of the mice was isolated by Serum Circulating DNA Kit (Tiangen, China). The cell genomic DNA and the serum cfDNA were treated with bisulfite solution (2.5mol/L, pH = 5.0) for the bisulfite conversion according to the data [15]. The modified DNA was subjected to PCR amplification. The primers are shown in Table 1 and Fig. 1. The purified PCR products were cloned into pTG19-T Vector (TaKaRa, Japan). The individual bacterial colonies were sequenced at least 20 colonies from each sample. The final sequence results were processed by BiQ Analyzer.

Statistical analysis

Data are expressed as mean $\pm$ standard deviation ($\pm$ SD). The expression levels of miRNA and DNA methylation among each group were tested by *t*-test using SPSS version 16.0 software. Differences were considered to be statistically significant at $P < 0.05$.

Results

Characteristics of fluorescently labeled B-ALL mouse model

After transfected with lentivirus and screening, uniform green light of L1210-GFP cells was observed with the fluorescence microscope and flow cytometry (Fig. 2a, 2b). Thirty days after transplantation, flow cytometry results showed that the green fluorescence intensity of peripheral blood mononuclear cells in the model group was higher than that of the control group (Fig. 2c). After dissection, splenomegaly could be seen in the model group (Fig. 2d). The weight of spleen in the model group (0.19 ± 0.03 g) was significantly higher than that of the control group (0.14 ± 0.03 g) ($P < 0.05$). There was no significant difference in the weight of heart, kidney and liver (Fig. 2e). The leukemic cell infiltration was observed under fluorescence microscope in the model group (Fig. 2f).

Differences of miRNAs in the serum of B-ALL mouse model

The expression of miR-196b, miR-203, miR-34a-5p, miR-335-3p, miR-34b-5p, miR-615, miR-375-3p and miR-193b-5p in the model group were significantly lower than that of the control group ($P < 0.05$), and there was no significant difference in the expression of miR-320-5p (Fig. 3).

Differences of methylation levels in miR-196b and miR-203 promoters

The methylation level of miR-196b promoter in L1210 cells was $99.29 \pm 2.67\%$. The methylation level of miR-196b promoter in cfDNA of model group ($67.86 \pm 44.92\%$) was lower than that of the control group ($94.29 \pm 16.51\%$) ($P < 0.05$) (Fig. 4a). The methylation level of miR-203 promoter in L1210 cells was 86.67

$\pm 10.00\%$, and there was no significant difference between the model group ($86.67 \pm 22.36\%$) and the control group ($86.67 \pm 10.00\%$) (Fig. 4b).

Differences of methylation levels in miR-196b and miR-203 coding regions

The methylation level of miR-196b coding region in L1210 cells was $95.84 \pm 5.89\%$. The methylation level of miR-196b coding region in cfDNA of model group ($97.51 \pm 4.01\%$) was significantly higher than that of the control group ($0.83 \pm 2.62\%$) ($P < 0.05$) (Fig. 4c). The methylation level of miR-203 coding region in L1210 cells was $96.25 \pm 2.31\%$. The methylation level of miR-203 coding region in cfDNA of model group ($100.00 \pm 0.00\%$) was higher than that of the control group ($94.38 \pm 4.17\%$) ($P < 0.05$) (Fig. 4d).

Discussion

In this study, we modified L1210 cells with a lentivirus with fluorescent protein gene, which express green fluorescent protein stably (L1210-GFP). After 30 days of transplantation, the fluorescence intensity of peripheral blood mononuclear cells was increased, and the infiltration of leukemia cells were observed in the spleen sections. These result suggest that the leukemia cells not only proliferate in the blood but also infiltrate the spleen. Among the reported fluorescent tracer tumor models, the fluorescent tracers commonly used to label tumor cells are red fluorescent protein (RFP), GFP and CM-Dil fluorescent dyes [16–20]. After transfected into cells by viral vectors, RFP or GFP genes can be randomly integrated into the chromosome of tumor cells with a long and stable expression characteristic. Researchers have used this method to observe the invasion and metastasis of bladder cancer, esophageal squamous cell carcinoma, and pancreatic cancer cells in vivo [16–18, 20]. CM-Dil fluorescent dye can be directly added to the cell culture medium to combine with the glutathione in the cell to stain the cytoplasm red, which also has the characteristic of long-term non-decolorization. Jalalie et al. used this method to trace the distribution of therapeutic mesenchymal stem cells in ovarian tissue [19]. Compared with common immunohistochemistry, the model labeled with fluorescence is more convenient to observe leukemia cells infiltration in various organs. Our results provide a good animal model for monitoring leukemia extramedullary infiltration.

We evaluated the expression of 9 miRNAs in the serum of this model. Among them, the expression of miR-196b, miR-203, miR-34a-5p, miR-335-3p, miR-34b-5p, miR-615, miR-375-3p and miR-193b-5p were significantly different between the model group and the control group ($P < 0.05$), while the expression of miR-320-5p was no different. This result suggests that most miRNAs were changed in the serum of B-ALL. The present studies revealed that aberrant miR-196b expression appears in multiple hematological malignancies [21, 22]. Bhatia et al. showed a significant down-regulation of miR-196b accompanied by overexpression of the c-myc gene in peripheral blood mononuclear cells derived from B-ALL patients. It is proposed that miR-196b can down-regulate the expression of c-myc and play a role in inhibiting [23]. However, the factors affecting miR-196b expression in B-ALL are not clear. We observed the enrichment of CpG sites in the region near the miR-196b gene by UCSC (<http://genome-asia.ucsc.edu>) and MethylPrimer

(<http://www.urogene.org/methprimer2/>). BSP results showed that in serum cfDNA of mouse model, the methylation level of miR-196b promoter region was significantly lower than that of the control ($P < 0.05$), while the methylation of miR-196b coding region was significantly higher than that of the control ($P < 0.05$), which is inconsistent with the result of Liu et al.. They showed that the methylation level of the miR-196b promoter region in the bone marrow of chronic lymphocytic leukemia (CLL) patients was higher than that of healthy control [24]. Tsai et al. also found that miR-196b expression was decreased in gastric cancer cells due to hypermethylation of its promoter [25]. The difference is the specimen, we used serum cfDNA as the analysis object, rather than cellular genomic DNA. The fragment of serum cfDNA is not complete, but it is easy to extract and more convenient for monitoring in B-ALL treatment. In addition, Popovic et al. suggest that leukemogenic mixed lineage leukemia (MLL) fusion proteins cause overexpression of miR-196b [26]. Schotte et al. revealed that up-regulation of miR-196b coincides with reduced DNA methylation at CpG islands in the promoter regions of miR-196b in MLL gene-rearranged cases compared to the cases of non-MLL precursor B-cell acute lymphoblastic leukemia and normal bone marrow [27]. These observations suggest that the expression of miR-196b may be MLL-driven in B-ALL, which deserves further studies.

The recent study showed that a positive-feedback loop was found between miR-203 and its target DNA methyltransferases 3b (Dnmt3b) in breast cancer cells. Dnmt3b is a key enzyme in methylation, as restoring miR-203 suppressed Dnmt3b, while knocking down Dnmt3b up-regulated miR-203 [28]. Chim et al. observed that miR-203 was silent by its promoter hypermethylated in a variety of lymphoma cell lines [29]. Our results showed that the miR-203 coding region appears hypermethylated in the serum cfDNA of B-ALL model, whereas the methylation level of the miR-203 promoter region was not significantly different from the control. It may be for the Methyl CpG-binding protein 2 (MeCP2) is prone to bind to the miRNA coding region in mammalian cells, thereby affecting miRNA expression [30]. However, it is still controversial whether the effect of this response on miRNA is increased or decreased.

In conclusion, this study, for the first time, clarified the relationship between the serum cfDNA and miRNA and verified our previous hypothesis. What's more, we observed that the methylated region associated with the expression of miR-196b and miR-203 was coding region rather than promoter in serum cfDNA of B-ALL mouse model.

Conclusion

This study focused on the relationship between the abnormal miRNA expression and the serum cfDNA methylation in fluorescent labeling B-ALL model. Most miRNAs were different between the model group and the control group. Out of these, miR-196b and miR-203 down-regulation are associated with the hypermethylation of related genes coding region in serum cfDNA. Simultaneous evaluation of serum cfDNA methylation level and miRNA will contribute to monitor leukemia better.

Declarations

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Author contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by YDW, YHW, HTH, XYF and TQW. The manuscript was written by YDW and reviewed by LML. All authors read and approved the final manuscript.

Data availability All data generated or analyzed during this study are included in this article.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical approval This Manuscript does not report studies involving human participants. The use of animals and sample collection procedures were in accordance with the ethical standards. Approval was obtained from the Institutional Animal Care and Use Committee of Beihua University.

Informed consent All authors consent to this manuscript submission.

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Figures

Figure 1

The positions of miR-196b and miR-203 a Schematic diagram of the positions of CpG islands along precursor miR-196b and the region of BSP amplification. b Schematic diagram of the position of CpG island along precursor miR-203 and the region of BSP amplification. The CpG island region is indicated as a purple bar. The region of BSP amplification is indicated as a blue bar.

Figure 2

Construction of L1210-GFP cell line and establishment of B-ALL mouse model

a The fluorescence in the L1210-GFP cells were observed under fluorescence inverted microscope.

b After transfected, the fluorescence intensity of cell was higher than the control group.

c The fluorescence intensity of peripheral blood mononuclear cells in the model group was higher than that of the control group.

d The representative mouse spleen of each group.

e The weight of each organ in each group.

f The representative fluorescence staining in spleen of model group. * $P < 0.05$ vs control group

Figure 3

Different expression of miRNAs in each group *P < 0.05 vs control group

Figure 4

Different methylation levels of miR-196b, miR-203 promoter and coding region a The methylation level of miR-196b promoter in cfDNA of model group was significantly lower then the control (P < 0.05). b There was no significant difference between the miR-203 promoter in cfDNA of model group and the control group. c The methylation level of miR-196b coding region in cfDNA of model group was significantly higher then that in the control (P < 0.05). d The methylation level of miR-203 coding region in cfDNA of

model group was significantly higher than the control ($P < 0.05$). Bisulfite genomic sequencing was depicted. Unmethylated (empty circle) and methylated (filled circle) CpG dinucleotides were shown. * $P < 0.05$ vs control group.