Clin. Lab. 2019;65:XXX-XXX

ORIGINAL ARTICLE

MicroRNA-16 Regulates Myeloblastosis Oncogene Expression to Affect Differentiation of Acute Leukemia Cells

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SUMMARY

Background: **This study was designed to evaluate the effects of micro-RNA-16 (miR-16)-regulated expression of myeloblastosis oncogene (MYB) on the differentiation of acute leukemia cells, the expressions of miR-16 and MYB mRNA, and protein in differently differentiated leukemia cells were detected by real-time PCR and western blot.**
Methods: **1,25-Dihydroxyvitamin D3 (1,25 D3) induced monocytic differentiation of HL60 cells, and the resulting changes in miR-16 and MYB expressions were detected. Morphology of the cells induced by 1,25 D3, after being transfection with miR-16 mimics, was observed by Wright-Giemsa staining. The expression of mononuclear cell surface marker CD14 was detected by flow cytometry.**
Results: **Minimum miR-16 was expressed in early-differentiation KG-1a cells, while late-differentiation U937 and THP-1 cells had higher expressions ($p < 0.01$). The expressions of MYB changed oppositely. During the monocytic differentiation of HL60 cells, miR-16 expression showed a time-dependent increase, but MYB expression gradually decreased. Overexpression of miR-16 in HL60 cells promoted 1,25 D3-induced morphological changes and CD14 expression ($p < 0.05$).**
Conclusions: **MR-16 facilitated the monocytic differentiation of leukemia HL60 cells by negatively regulating MYB expression.**
(Clin. Lab. 2019;65:xx-xx. DOI: 10.7754/Clin.Lab.2018.181008)

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Manuscript accepted December 4, 2018

KEY WORDS

micro-RNA-16, myeloblastosis oncogene, acute myelocytic leukemia, cell differentiation

INTRODUCTION

Acute myelocytic leukemia is a malignant clonal disease originating from hematopoietic stem cells, mainly typified as disorders in the differentiation and maturation of bone marrow hematopoietic cells. Blockage of the differentiation of normal hematopoietic cells and leukemia cells is regulated by various factors. Particularly, microRNAs (miRNAs) play important roles as one of the main epigenetic mechanisms [1,2]. As small non-coding, single-stranded RNAs of about 19 - 25 bp, miRNAs mainly regulate target gene expressions on the post-transcriptional level as well as participate in the regulation of various biological processes such as cell

differentiation, proliferation, and apoptosis. Abnormally expressed in leukemia, miR-16 is involved in the progression of this disease by regulating apoptosis- and cell cycle-related genes such as Bcl2, cyclin D1, and MCL1 [3,4]. In addition, miR-16 plays a crucial role in the differentiation of hematopoietic stem cells [5]. Therefore, it may exert key regulatory effects on the onset and progression of leukemia. The myeloblastosis oncogene (MYB), as one of the target genes of miR-16, is the central regulatory factor in the proliferation and differentiation of blood cells, also participating in the onset and progression of B-cell lymphoma [5,6]. However, the roles of miR-16 and its downstream target gene MYB in the regulation of leukemia differentiation remain unclear. The aims of this study were to evaluate the effects of miR-16/MYB on the monocytic differentiation of HL60 cells and to unravel the possible mechanism by which miR-16 blocks leukemia differentiation.

MATERIALS AND METHODS

Ethical approval

The study was approved by the Institutional Ethics Committee of our hospitals, and written informed consent was obtained from all participants.

Materials

miR-16 mimics and negative control (NC) were purchased from ABI (NY, USA). Lipofectamine™ 2000 transfection reagent, Opti-MEM, and primers were bought from Life Technologies (NY, USA). Fetal bovine serum (FBS) and RPMI 1,640 culture medium were obtained from Gibco. RNA extraction reagent TRIzol and RT-PCR kit were provided by TaKaRa (Tokyo, Japan). Wright-Giemsa staining solution and 1,25-dihydroxyvitamin D3 (1,25 D3) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phycoerythrin (PE)-labeled mouse anti-human CD14 antibody and isotype control, mouse anti-human MYB monoclonal antibody and goat anti-mouse secondary antibody were bought from Abcam (MA, USA). Pro-light HRP chemiluminescence detection kit was obtained from Millipore (CA, USA).

Cell culture and transfection

All cells were provided by the China Center for Type Culture Collection. HL60 cells were cultured in RPMI 1640 complete medium containing 20% FBS in a 5% CO_2 atmosphere at 37°C, and then those in the logarithmic growth phase were used for further experiments. Before transfection, HL60 cells were resuspended in Opti-MEM, inoculated onto 6-well plates at the density of 4 x 10^6/well, and centrifuged at 1,000 r/minute for 20 minutes. Then 100 pmol miR-16 mimics/NC and 5 μL of Lipofectamine™ 2000 were added into 250 μL of Opti-MEM, left still at room temperature for 5 minutes, mixed, left again at room temperature for 20 minutes and then added to the 6-well plates to transfect the cells for 6 hours. Afterwards, 1 mL of RPMI 1640 complete medium was added, and cells were cultured an additional 48 hours. HL60 cells were then transfected with miR-16 mimics/NC. Twelve hours after the culture medium was refreshed, 100 nmol/L 1,25 D3 was added to induce monocytic differentiation.

Real-time PCR

The above cells were collected, RNA was extracted using Trizol, and then reverse-transcribed into cDNA. Specific reverse transcription primers: RT primer for internal reference U6:
5'-AACGCTTCACGAATTTGCGT-3';
RT primer for miR-16:
5'-TAGCAGCACGTAAATATTGGGCGAATATTTA CGTGCTGCTATT-3'.
By using RT-PCR product as the template, real-time PCR was performed under the following conditions: pre-denaturation at 95°C for 30 seconds, denaturation at 94°C for 10 seconds, annealing at 55.8°C for 20 seconds, and extension at 72°C for 30 seconds, 40 cycles in total. The experiment was conducted in triplicate. U6 snRNA was used as the internal reference for relative quantitation. Primers for real-time PCR: has-miR-16 (forward primer:
5'-ACACTCCAGCTGGGTAGCAGCACGTAAATA-3';
reverse primer:
5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAG TTGAGCGCCAATA-3'),
U6 snRNA (forward primer:
5'-CTCGCTTCGGCAGCACA-3';
reverse primer:
5'-AACGCTTCACGAATTTGCGT-3').
Relative expression was calculated by $2^{-\Delta\Delta Ct}$.

Western blot

Cells in the logarithmic growth phase were collected, and total protein was extracted to determine the concentration by the Bradford method. The protein extraction solution (100 μg) was separated by 12% separating gel and transferred onto a PVDF membrane. The gel was blocked with mouse anti-human MYB monoclonal antibody (diluted by 1:600, mixed in 5% skimmed milk) at 4°C overnight, then incubated with HRP-labeled goat anti-mouse IgG secondary antibody (diluted by 1:900) at room temperature for 1.5 hours, washed with TBST, and finally color-developed by chemiluminescence reagent. With β-actin as the internal reference, western blot bands were analyzed quantitatively by Quantity One® 1-D analysis.

Wright-Giemsa staining

The cells that had been induced by 100 nmol/L 1,25 D3 for 72 hours were collected, washed twice with 0.01 mol/L PBS, coated onto sections, air-dried, stained by Wright-Giemsa reagent for 5 minutes, rinsed with double-distilled water, color-separated and air-dried to observe the morphology under an optical microscope.

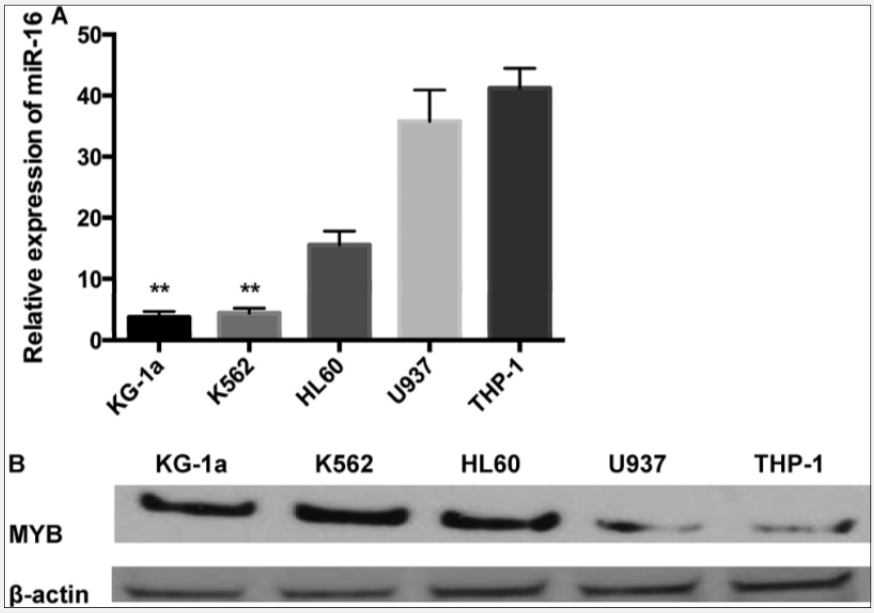

Figure 1. Expressions of miR-16 (A) and MYB (B) in differently differentiated leukemia cells.

**** - p < 0.01, compared with KG-1a cells.**

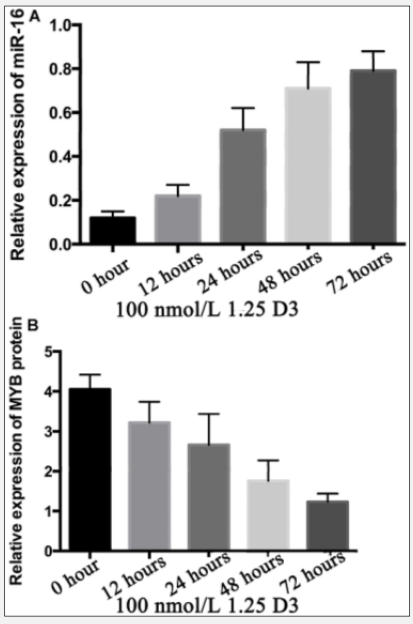

Figure 2. Expressions of miR-16 (A) and MYB (B) in the monocytic differentiation of HL60 cells induced by 1,25 D3.

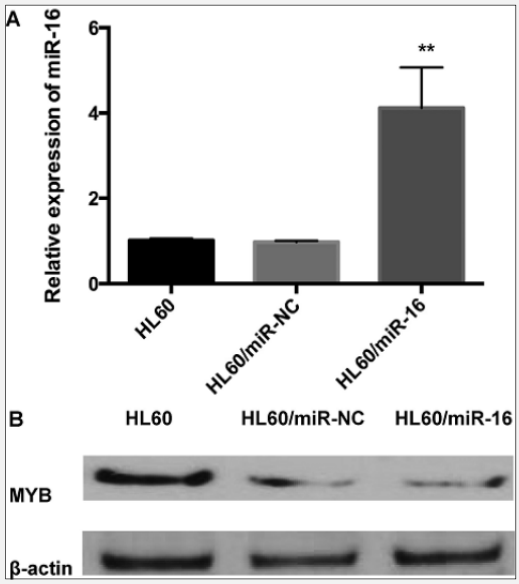

Figure 3. Overexpression of miR-16 inhibited MYB expression in HL60 cells.

(A) miR-16 expression significantly increased after transfection with miR-16 mimics; (B) MYB expression was inhibited after miR-16 was overexpressed. ** - p < 0.01, compared with HL60 and HL60/miR-NC groups.

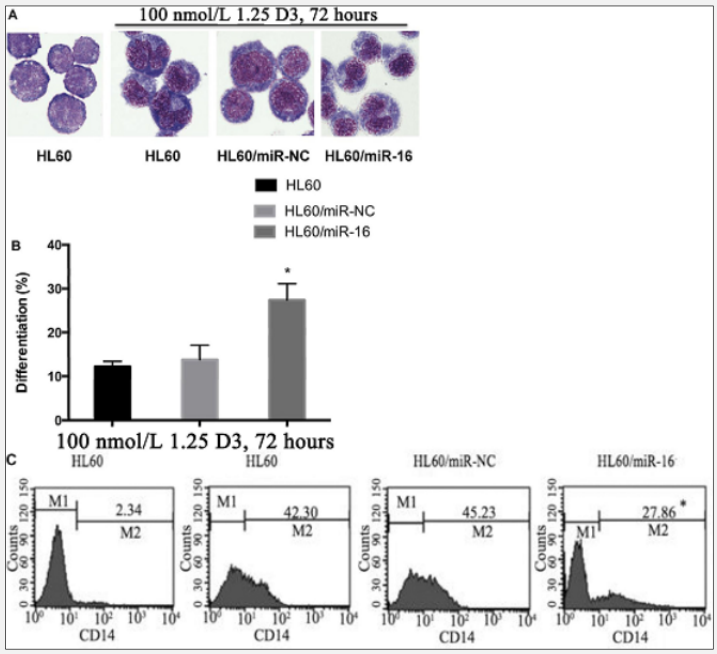

Figure 4. MiR-16 blocked the monocytic differentiation of HL60 cells induced by 1,25 D3.

(A) Morphological changes during cell differentiation (Wright-Giemsa staining, 1,000 x); (B) rates of induced differentiation after 1,25 D3 induction; (C) CD14 expressions detected by flow cytometry. * - p < 0.05, compared with HL60/miR-16 group.

For each section, 400 cells were counted, and those having irregularly distorted or folded nuclei were regarded as differentiated cells to calculate the rate of induced differentiation. Rate of induced differentiation (%) = (number of differentiated cells/400) x 100%. Changes in the rate of 1,25 D3-induced differentiation after miR-16 was overexpressed in HL60 cells were observed.

Detection of CD14 expression by flow cytometry

The cells that had been induced by 100 nmol/L 1,25 D3 for 72 hours were collected, prepared into single cell suspensions ($1 \times 10^6 \sim 2 \times 10^6$), washed twice by pre-cooled 0.01 mol/L PBS (containing 0.2% bovine serum albumin), centrifuged at 1,000 rpm for 5 minutes, incubated with PE-labeled mouse anti-human CD14 antibody at 4°C in dark for 40 minutes, washed twice with 0.01 mol/L PBS, and finally detected by flow cytometry.

Statistical analysis

All data were analyzed by SPSS 13.0. The categorical data were expressed as mean ± standard deviation ($\bar{x} \pm s$), and the numerical data were compared with one-way analysis of variance. $p < 0.05$ was considered statistically significant.

RESULTS

Expressions of miR-16 and MYB in differently differentiated leukemia cells

miR-16 expressions followed an ascending order of early-differentiation KG-1a cells < K562 cells < HL60 cells, while late-differentiation U937 and THP-1 cells had higher expressions ($p < 0.01$, Figure 1A). In contrast, MYB expression was negatively correlated with the differentiation degree of leukemia cells (Figure 1B).

Expressions of miR-16 and MYB in differently differentiated leukemia cells after 1,25 D3 induction

After induction with 100 nmol/L 1,25 D3 for 12 - 72 hours, miR-16 expression in HL60 cells time-dependently increased (Figure 2A), but MYB expression gradually decreased (Figure 2B).

Overexpression of miR-16 inhibited MYB expression in leukemia cells

After transfection with 100 pmol miR-16 mimics for 48 hours, miR-16 expression level in HL60 cells significantly increased, i.e., miR-16 was successfully overexpressed ($p < 0.01$, Figure 3A). Meanwhile, western blot showed that MYB protein expression level evidently reduced after miR-16 was overexpressed (Figure 3B).

Overexpression of miR-16 blocked monocytic differentiation of leukemia cells

After induction with 100 nmol/L 1,25 D3 for 72 hours, all cells underwent monocytic differentiation, mainly manifested as reduced nucleus/cytoplasm ratio as well as irregularly depressed, distorted or folded nuclei. The cells in which miR-16 was overexpressed (HL60/miR-16 group) underwent significant differentiation characteristic changes compared with the untransfected and HL60/miR-NC groups did (Figure 4A). In the meantime, the HL60/miR-16 group had a significantly lower rate of induced differentiation than those of the other two groups ($p < 0.05$, Figure 4B). Flow cytometry showed that the percentage of $CD14^+$ cells in the HL60/miR-16 group (42.30%) was significantly higher than that of the control group ($p < 0.05$, Figure 4C).

DISCUSSION

MiRNA-mediated post-transcriptional regulation, which is an important part of the regulatory network for gene expressions in eukaryotes, affects cell growth, development, and differentiation by interacting with transcription factors [7,8]. MiRNAs have been closely associated with tumors stemming from chromosomal abnormalities, epigenetic changes as well as abnormal expressions of key proteins. In most cases, they can inhibit the apoptosis of tumor cells and promote angiogenesis [9-11]. In recent years, many types of miRNAs have been verified to participate in the regulated differentiation of normal hematopoietic cells and leukemia cells, being closely related with the onset and progression of leukemia [12,13]. As a member of the miR-15a/16 family, miR-16 is abnormally expressed in many malignant tumors such as B-cell chronic lymphocytic leukemia, melanoma, and colorectal cancer. By regulating apoptosis- and cell cycle-related genes, miR-16 is involved in cancer onset and progression [3,14]. In addition, its expression gradually increases during the differentiation of hematopoietic stem cells into megakaryocytes, especially in that of mature platelets [5,15]. However, its role in the differentiation of megakaryocytes remains elusive. In this study, miR-16 expression was low in early-differentiation KG-1a cells, while late-differentiation U937 and THP-1 cells had higher expressions. Thus, miR-16 may regulate the differentiation of leukemia cells. Gao et al. reported that miR-16 was up-regulated in AML patients who had complete remission [16]. After *in vitro* monocytic differentiation of HL60 cells was simulated by 1,25 D3 induction [17], miR-16 expression decreased in a time-dependent manner, suggesting that it may participate in the differentiation of leukemia cells. Ultimately, miR-16 was overexpressed by transfection with miR-16 mimics to explore its regulatory effects on leukemia cell differentiation, which suppressed the morphological changes induced by 1,25 D3 and the expression of monocyte surface marker CD14. Our results are consistent with those of Gao et al. who found that overexpression of miR16-1 could enhance ATRA-induced differentiation *in vitro* [16]. Hence, miR-16 indeed played essential roles in blocking the differentiation of leukemia cells.

MYB is a highly conserved transcription factor in many

species, which predominantly regulates the proliferation and differentiation of blood cells [18,19]. Generally, an increase in MYB expression can promote cell proliferation and inhibit the differentiative potential. Contrarily, a decrease in MYB expression blocks cell cycle progression and facilitates the differentiation of hematopoietic stem cells and progenitor cells into mature ones. It is well-documented that miR-16 participates in post-transcriptional regulation by binding non-coding region at the 3'-end of MYB. Similarly, we herein proved that miR-16 managed to regulate MYB protein expression that was positively correlated with the differentiation degree of leukemia cells. Meanwhile, such expression increased with extended time of 1,25 D3 induction. Overexpression of miR-16 suppressed MYB expression and blocked 1,25 D3-induced monocytic differentiation of leukemia cells. Hence, miR-16 may promote *in vitro* differentiation of leukemia cells by targeted inhibition of MYB.

CONCLUSION

In summary, we verified the critical role of miR-16 in leukemia cell differentiation and gave a possible regulatory mechanism. The findings provide valuable theoretical evidence for studying the onset and progression of leukemia, but clinical patient samples are still needed. Induced differentiation therapy based on miR-16 silencing may be of great clinical significance.

Source of Funding:
This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Declaration of Interest:
The authors declare that there is no conflict of interest.

References:

1. Navarro F, Lieberman J. Small RNAs guide hematopoietic cell differentiation and function. J Immunol 2010;184:5939-47 (PMID: 20483778).

2. Ling H, Fabbri M, Calin GA. MicroRNAs and other non-coding RNAs as targets for anticancer drug development. Nat Rev Drug Discov 2013 Nov;12(11):847-65 (PMID: 24172333).

3. Nana-Sinkam SP, Croce CM. MicroRNA in chronic lymphocytic leukemia: transitioning from laboratory-based investigation to clinical application. Cancer Genet Cytogenet 2010 Dec;203(2): 127-33 (PMID: 21156224).

4. Vasilatou D, Papageorgiou S, Pappa V, Papageorgiou E, Dervenoulas J. The role of microRNAs in normal and malignant hematopoiesis. Eur J Haematol 2010 Jan 1;84(1):1-16 (PMID: 19 744129).

5. Kannan M, Mohan KV, Kulkarni S, Atreya C. Membrane array-based differential profiling of platelets during storage for 52 miRNAs associated with apoptosis. Transfusion 2009 Jul;49(7): 1443-50 (PMID: 19389023).

6. Chung EY, Dews M, Cozma D, et al. c-Myb oncoprotein is an essential target of the dleu2 tumor suppressor microRNA cluster. Cancer Biol Ther 2008 Nov;7(11):1758-64 (PMID: 18708755).

7. Agirre X, Martinez-Climent JA, Odero MD, Prosper F. Epigenetic regulation of miRNA genes in acute leukemia. Leukemia 2012 Mar;26(3):395-403 (PMID: 22143672).

8. Babashah S, Sadeghizadeh M, Tavirani MR, Farivar S, Soleimani M. Aberrant microRNA expression and its implications in the pathogenesis of leukemias. Cell Oncol (Dordr). 2012 Oct;35(5): 317-34 (PMID: 22956261).

9. Wu X, Chen BA. Influence of miRNA-155 on lymphoma. Zhongguo Shi Yan Xue Ye Xue Za Zhi 2013 Jun;21(3):806-9 (PMID: 23815945).

10. Jansen F, Yang X, Hoelscher M, et al. Endothelial microparticle-mediated transfer of MicroRNA-126 promotes vascular endothelial cell repair via SPRED1 and is abrogated in glucose-damaged endothelial microparticles. Circulation 2013 Oct 29;128(18): 2026-38 (PMID: 24014835).

11. Ciafre SA, Galardi S, Mangiola A, et al. Extensive modulation of a set of microRNAs in primary glioblastoma. Biochem Biophys Res Commun 2005 Sep 9;334(4):1351-8 (PMID: 16039986).

12. Zheng YS, Zhang H, Zhang XJ, et al. MiR-100 regulates cell differentiation and survival by targeting RBSP3, a phosphatase-like tumor suppressor in acute myeloid leukemia. Oncogene 2012 Jan 5;31(1):80-92 (PMID: 21643017).

13. Pulikkan JA, Dengler V, Peramangalam PS, et al. Cell-cycle regulator E2F1 and microRNA-223 comprise an autoregulatory negative feedback loop in acute myeloid leukemia. Blood 2010 Mar 4;115(9):1768-78 (PMID: 20029046).

14. Willimott S, Wagner SD. Post-transcriptional and post-translational regulation of Bcl2. Biochem Soc Trans 2010 Dec;38(6): 1571-5 (PMID: 21118128).

15. Watanabe Y, Tomita M, Kanai A. Computational methods for microRNA target prediction. Methods Enzymol 2007;427:65-86 (PMID: 17720479).

16. Gao SM, Yang J, Chen C, et al. miR-15a/16-1 enhances retinoic acid-mediated differentiation of leukemic cells and is up-regulated by retinoic acid. Leuk Lymphoma 2011 Dec;52(12):2365-71 (PMID: 21838537).

17. Palma CA, Tonna EJ, Ma DF, Lutherborrow MA. MicroRNA control of myelopoiesis and the differentiation block in acute myeloid leukaemia. J Cell Mol Med 2012 May;16(5):978-87 (PMID: 22225649).

18. Pattabiraman DR, Gonda TJ. Role and potential for therapeutic targeting of MYB in leukemia. Leukemia 2013 Feb;27(2):269-77 (PMID: 22874877).

19. Prouse MB, Campbell MM. The interaction between MYB proteins and their target DNA binding sites. Biochim Biophys Acta 2012 Jan;1819(1):67-77 (PMID: 22067744).