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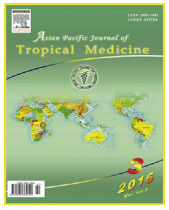

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Onco-microRNA miR-130b promoting cell growth in children APL by targeting PTEN

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ABSTRACT

Objective: To study the expression of microRNA-130b (miR-130b) in children acute promyelocytic leukemia (APL) and its role for regulating PTEN expression.
Methods: A total of 50 children APL marrow tissues and 15 normal marrow tissues between January and December in 2012 were collected into our study. The expression of miR-130b in APL and normal marrow tissues were detected by quantitative real-time polymerase chain reaction. MiR-130b inhibitor was transfected into HL-60 cells. Cell Counting Kit-8 assay and flow cytometry were used to measure cell proliferation and apoptosis, respectively. The expression of PTEN, a potential target of miR-130b, and its downstream genes, *Bcl-2* and *Bax*, in transformed cells were detected by quantitative real-time polymerase chain reaction and western-blot.
Results: The expression of miR-130b was significantly higher in children APL marrow tissues than in normal marrow tissues ($P < 0.05$). Down-regulation of miR-130b could significantly suppress cell proliferation and induce apoptosis in HL-60 cells ($P < 0.05$). PTEN expression was upregulated when miR-130b was knocking-down ($P < 0.05$). As downstream genes of *PTEN*, the expression of *Bcl-2* and *Bax* were regulated as well.
Conclusions: MiR-130b is overexpressed in children APL marrow tissues and associated with cell growth. MiR-130b may promote children APL progression by inducing cell proliferation and inhibiting apoptosis.

1. Introduction

Acute promyelocytic leukemia (APL), classified as M3 in French–American–British classification systems, is a subtype of acute myelocytic leukemia (AML). Globally, APL occupies 7%–27% of all AML types, while in China, this proportion enlarges to 12%–23% [1]. Compared to other AML types, APL has a higher risk of hemorrhage and causes a high mortality [2]. Nowadays, systematical chemotherapies based on all-trans-retinoic acid (ATRA) and arsenic trioxide is the major treatment method [3]. However, both single and combination drug therapies have many disadvantages such as early drug resistance, early recurrence, and serious side-effect [4]. Thus, the treatment outcome and prognosis of APL are still unsatisfied. Hence, the discovery of novel biological therapeutic target is a key for improving the survival of APL patients.

MicroRNAs (miRs) are small (19–25 nt), noncoding RNA molecules that negatively regulate gene expression by interacting with the 3′ untranslated region of targeting mRNA, eventually leading to translational suppression and/or degradation of the targeting mRNA [5,6]. In adult AML patients, the aberrant expression of miR-335 was associated with chemoresistance [7]. In children APL, miR-125b could inhibit cell apoptosis and induce ATRA chemoresistance by targeting BCL2-antagonist/killer 1 [8]. MiR-130b was demonstrated as an important oncogenic miR in many cancers [9]. For example, in hepatocellular carcinoma, miR-130b was up-regulated and associated with large tumor metastasis [10]. It might be suggested that PTEN, a classical tumor suppressor, was a target of miR-130b by bioinformatics study. As a member of phosphatases, PTNE can change PIP3 into PIP2 by phosphorylation, consequently inhibiting the activation of Akt signal [11].

In this study, the expression of miR-130b in children APL marrow tissues was detected and its functions and mechanisms for cell growth *in vitro* were investigated. Finally, we proved that miR-130b was up-regulated in APL marrow tissues and

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HL-60 cells, and it could suppress cell proliferation and induce apoptosis by inhibiting PTEN expression.

2. Materials and methods

2.1. Clinical specimens and cell culture

A total of 50 children APL marrow tissues and 15 normal marrow tissues were collected and stored in liquid nitrogen between January and December in 2012. The range of age was from 7 to 14 year, and the median age was 9 year. The human APL cell line, HL-60, was purchased from T Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Gibco® RPMI 1640 medium (Gibco, USA) supplemented with 10% v/v fetal bovine serum (FBS; Gibco, USA) and maintained in a humidified incubator.

2.2. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from marrow tissues and HL-60 cells using TRIZOL® reagent (Invitrogen, USA). The relative expression of *miR-130b* was detected by Bulge-Loop™ miR qRT-PCR Starter Kit (RiboBio, Guangzhou, China). *PTEN*, *Bcl-2* and *Bax* mRNA expression levels were measured by Quant one step qRT-PCR Kit (TIANGEN, Beijing, China). *MiR-130b* primers were purchased from RiboBio Co., Ltd. *PTEN*, *Bcl-2*, *Bax* and *β-actin* qPCR primers were synthesized by Augct Co., Ltd and the sequences were shown as follow: *PTEN* sense 5′-GTAAGGACCAGAGACAAAAAG-3′, anti-sense 5′-CTTTTTTAGCATCTTGTTCTG-3′, *Bcl-2* sense 5′- TTCTTTGAGTTCGGTGGGGTC-3′, anti-sense 5′-TGCATATTTGTTTGGGGCAGG-3′, *Bax* sense 5′- TCCACCAAGAAGCTGAGCGAG-3′, anti-sense 5′-GTCCAGCCCATGATGGTTCT-3′, *β-actin* 5′-CTCCATCCTGGCCTCGCTGT-3′, anti-sense 5′-GCTGTCACCTTCACCGTTCC-3′. The $2^{-\Delta\Delta CT}$ method was used for data calculation.

2.3. MiR transfection

HL-60 cell transfection was performed using the Lipofectamine® 2000 Transfection Reagent (Invitrogen, USA) according to the manufacturer instructions. Briefly, 5×10^5 cells were cultured in six-well culture plates containing 1.5 mL RPMI 1640 per well without FBS. The 100 pmoL miR-130b inhibitor or scrambled-miR inhibitor (RiboBio, Guangzhou, China) was mixed with 5 μL Lipofectamine® 2000 in 500 μL Opti-MEM® I Reduced Serum Medium (Gibco, USA) and incubated for 15 min at room temperature. The complex was then added to the cells and ensured full distribution over all plate surface. After 8 h of incubation, the FBS and antibiotics were added and the cells were incubated for the 24 h, 48 h and 72 h.

2.4. Cell Counting Kit-8 (CCK-8) assay

The viability of cells was assessed by the CCK-8. CCK-8 assay was performed at time intervals 24 h, 48 h and 72 h post transfection. A total of 10 μL CCK-8 solution was added to 5×10^5 HL-60 cells suspended in 100 μL RPMI 1640 medium and incubated for 4 h at 37 °C in dark. Absorbance was measured by using a spectrophotometer at 450 nm.

2.5. Flow cytometry (FCM) analysis

HL-60 cells were collected and diluted into 5×10^5 at time interval 48 h post transfection. Cell cycles and the apoptotic cells were evaluated by Annexin V-FITC and propidium iodine staining (Roche, USA) according to the manufacturer's instruction and analyzed with a FCM.

2.6. Western blot

Cells were washed with cold PBS for twice and broken by RIPA. Lysate was centrifuged at 15 000 r/min for 15 min. The supernatant was obtained and detected the protein content via BCA Kit (Millipore, USA). Proteins were separated by vertical electrophoresis and transferred to PVDF membrane (Millipore, USA). PTEN, Bcl-2, Bax and β-actin antibodies (all purchased from Santa Cruz, USA) were used to detect the proteins expression, respectively. Secondary HRP-conjugated goat anti-rabbit or anti-mouse antibody (Abgent, USA) were used at a 1:5 000 dilution and developed by the ECL Regent (Millipore, USA).

2.7. Statistical analysis

Measurement date were presented as mean ± SD. The SPSS version 13.0 (SPSS, Chicago, USA) was used for two-tailed Student-*t* test and ANOVA. $P < 0.05$ was considered statistically significant.

3. Results

3.1. MiR-130b expression in APL marrow tissues

Analysis of qRT-PCR data demonstrated that the relative expression of *miR-130b* was significantly increased in APL marrow tissues ($n = 50$, 2.198 ± 0.051) compared to normal marrow tissues ($n = 15$, 6.562 ± 0.271) ($P < 0.001$).

3.2. Down-regulation effect of miR-130b inhibitor on miR-130b expression in HL-60 cells

In order to elucidate the biological significance of miR-130b, negative control miR inhibitor (NC inhibitor, $n = 8$, 1.000) or miR-130b inhibitor ($n = 8$, 0.331 ± 0.016) was transfected into HL-60 cells. After 48 h post-transfection, miR-130b was significantly down-regulated in HL-60 cells ($P < 0.001$).

3.3. Effects of inhibition of miR-130b on suppressing viability and inducing apoptosis of HL-60 cells

Overexpression of miR-130b could effectively inhibit viability of HL-60 cells compared to control group ($P < 0.01$). Moreover, BrdU incubation assay showed that miR-130b could significantly repress DNA synthesis of HL-60 cells (135.37 ± 6.43 *vs.* 57.39 ± 4.20, $P = 0.002$). FCM tested the influence of miR-130b for cell apoptosis. Compared to NC miR group, miR-130b could effectively increase apoptotic cell percentage (13.352 ± 1.981 *vs.* 24.482 ± 1.943, $P = 0.003$).

3.4. PTEN as a downstream target of miR-130b in APL

To investigate the possible molecular mechanisms, the mRNA level of *PTEN*, a possible target of *miR-130b*, predicted by bioinformatics retrieval, in APL marrow tissues, was firstly detected, and Person correlation test was used to analyze the association between *miR-130b* and *PTEN* mRNA. It could be seen clearly that a negative correlation existed between *miR-130b* and *BMI-1* mRNA ($r = -0.571$, $P < 0.001$). Moreover, it was also confirmed that the expression of PTEN were up-regulated in miR-130b inhibitor group than in NC group ($P < 0.001$). As downstream genes of *PTEN*, the expression of *Bcl-2* and *Bax* were also regulated ($P < 0.001$). Expression of *Bcl-2* was decreased, and the expression of *Bax* was increased ($P < 0.001$).

4. Discussion

APL is a special subtype of AML. High risk of bleeding and DIC after chemotherapy by anthracyclines are the main features of APL. Identification of *PML/RARα* fusion gene promoted the application of ATRA and arsenic trioxide [12]. APL became the first human cancer which could be treated with its tumor specific antigen [13]. However, novel and safe therapeutic molecules are still needed to be found to further improve patients' survival.

As a novel and effect therapeutic targets, miRs can play double-edged sword roles in cancer, and their aberrant expressions are related to many malignant features [14]. MiR-92a was reported to suppress cell growth of HL-60 cells by silencing P63 expression [15,16]. MiR-21 is a famous onco-miR, and down-regulating miR-21 could enhance HL-60 cell apoptosis induced by cytarabine [17]. MiR-130b was reckoned as a prognostic evaluation biomarker and a vital tumor promoter in many cancers. In ESCC, high expression of miR-130b was associated with higher TNM stage and poorer prognosis, moreover, miR-130b enhanced proliferation, migration, and invasion in human ESCC cells by targeting PTEN [18].

In our study, it was found that the expression of miR-130b in APL marrow tissues was significantly higher than in normal marrow tissues. That meant this differential expression of miR-130b in APL marrow tissues might play an important regulation function for APL progression. *In vitro*, the proliferation inhibiting effect was detected after knocking down miR-130b expression, and cell apoptosis was also induced by miR-130b inhibitor.

PTEN is located at 10q23.3 and encodes a dual-specificity phosphatase with lipid and protein phosphatase activities [19]. PTEN dephosphorylates is an important activator of Akt named PI(3,4,5)P3, which controls cell proliferation, apoptosis, and many other functions [20]. Inhibition of PTEN results in increased levels of activated p-Akt and consequently regulates *Bcl-2*/*Bax* expression [21]. Studies indicated that PTEN is a potential target of miR-221/222, miR-22 and miR-144 in different malignancies [18]. In this study, it was demonstrated that PTEN was a direct target of miR-130b by qRT-PCR and western blot, thus promoting *Bcl-2* expression and reducing *Bax* expression. This phenomenon may partly explain the effect of miR-130b for HL-60 cells proliferation and apoptosis.

The results of our study indicate that expression of miR-130b is up-regulated in patients with diagnosed APL. To the best of our knowledge, this is an interesting study to demonstrate that miR-130b is a tumor promoter by directly targeting PTEN in APL.

Conflict of interest statement

We declare that there is no conflict of interest.

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