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Original Article

Expression of miR-34a in bone marrow of adult acute lymphoblastic leukemia decreases multidrug resistance in cells

Bo Han[1*], Ping Sui[2*], Wei Shao[3], Zenglei Han[4]

[1]Department of Hematology, Haici Medical Group Affiliated to Medical College of Qingdao University, Qingdao 266000, Shandong, China; [2]Department of Oncology of The Affiliated Yantai Yuhuangding Hospital of Qingdao University, Yantai 264000, Shandong, China; [3]Department of Oncology, Haici Medical Group Affiliated to Medical College of Qingdao University, Qingdao 266000, Shandong, China; [4]Department of Pathology, Municipal Hospital of Qingdao, Qingdao 266011, Shandong, China. [*]Equal contributors and co-first authors.

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Abstract: Objective: To investigate the expression of miR-34a in bone marrow of adult acute lymphoblastic leukemia and its relationship with drug resistance. Methods: The bone marrow samples of 47 cases of newly diagnosed adult acute lymphoblastic leukemia were collected. Rt-PCR were used to detected the expressions of miR-34a in bone marrow samples, CCRF-CEM cells and resistant strains CEM-C1 cells. Cell proliferation inhibition rate (%) and resistance index (RI) were calculated. Results: In this study, the relative expression of miR-34a at newly diagnosis was significantly lower than complete remission and the control group ($P < 0.05$). The expression of miR-34a in CEM-C1 cells was significantly lower than the CCRF-CEM cells ($P < 0.05$). The relative expression in CCRF-CEM cells transfected with miR-34a inhibitor was (3.14±1.15) significantly lower than the miRNA inhibitor negative control group ($P < 0.05$). The relative expression of miR-34a in CEM-C1 cells transfected with miR-34a mimic was (5.06±1.73) significantly higher than the negative control miRNA mimics ($P < 0.05$). Conclusion: The expression of miR-34a was down-regulated in bone marrow samples of adult acute lymphoblastic leukemia. It might be associated with the recurrence and drug resistance of ALL.

Keywords: Acute lymphoblastic leukemia, miR-34a, drug resistance

Introduction

Acute lymphoblastic leukemia (ALL) is a malignant disease caused by abnormal hyperplasia of T or B lymphocytes in the bone marrow, which accounts for 20~30 percent of adult leukemia [1]. Currently, the use of bone marrow transplantation and multi-agent chemotherapy significantly improved the recovery rate of ALL after treatment. However, poor efficacy was observed in a few individuals [2], and the basic cause of leukemia cell tolerance is unclear [3]. Therefore, the mechanisms and characteristics of leukemia cell tolerance in refractory ALL patients need to be investigated.

MicroRNA (miRNA) is a non-coding single stranded RNA, which plays an important role in cell proliferation, differentiation, apoptosis and tumor formation and development [4]. Studies have shown that miRNA is intimately associated with the susceptibility of tumor cells to chemotherapeutic drugs [5]. MiR-34a is expressed less frequently in the marrow of multiple myeloma phase III patients than in phase I. It is also related to the expression of Th17 related-cytokines [6]. Furthermore, miR-34a was expressed abnormally in the tumor cells of colonic carcinoma and large B-cell lymphomas (DLBCLs) [7, 8]. We detected miR-34a in the marrow of ALL patients, and determined its role in the tolerance of leukemia cells, to provide basic clinical data.

Materials and methods

Patients and clinical samples

Forty-seven patients who were diagnosed for the first time with ALL and treated at our hospital during October 2012 to December 2014 included 31 males and 16 females, with a mean

age of 45.4±6.1 years (SD) (range 19 to 53 years, median 42 years). The bone marrow samples were collected when the patient was newly diagnosed and recurred or completely recovered. According to the treatment outcome, the newly diagnosed-complete remission group comprised 26 pairs of specimens, and the newly diagnosed-recurrence group included 21 pairs of specimens. Data were analyzed by comparing pairwise differences.

The treatment outcomes and types were identified according to the adult ALL diagnostic and treatment consensus of China. Based on immunophenotyping, 18 cases of T-ALL, and 29 cases of B-ALL were distinguished. Based on FAB rules, 13 cases of L1, 23 cases of L2, and 11 cases of L3 were found. All the treatment regimens were designed according to the consensus on the diagnosis and treatment of adult acute lymphoblastic leukemia in China. At the same time, we collected 11 cord blood samples donated by healthy mothers during the postnatal period, representing the healthy control group. The study was approved by the Human Research Ethics Committee of our hospital and informed consent of patients and their families was obtained.

Main reagents and instruments

The CCRF-CEM cells and the resistant cell line CEM-C1 were purchased from Shanghai Sixin Biotechnology CO., LTD. The fetal bovine serum and RPMI 1640 cell culture medium were purchased from Gibco, USA. The miR-34a primers and reference genes used in this study were synthesized by Sangon Biotech (Shanghai) Co., Ltd. The miRNA inhibitor was purchased from Guangzhou RiboBio Co., LTD. Camptothecin was purchased from Chengdu Likai Chiral Tech Co., Ltd. The Cell Counting Kit CCK-8 was purchased from Sigma, USA. TRIzol RNA extraction kit, reverse transcription PCR kit, and PCR kit were purchased from Invitrogen, USA. The GenePulser Xcell™ electroporation system was purchased from Bio-Rad Laboratories Co., Ltd., USA. The Spectra Max microplate reader was purchased from Molecular Devices, USA. The ABI 7500 real-time PCR instrument was purchased from ABI, USA.

Cell culture and transient transfection

The cell lines were cultured in a humidified chamber at 37°C in 5% CO_2. The CCRF-CEM and CEM-C1 cell lines in the logarithm growth phase at a density of 10^6 cells per well were transiently transfected using electroporation according to the manufacturer's instructions. A 100 pmol miR-34a inhibitor and its negative control were transfected into the CCRF-CEM cell line in a 10 μL system, and 100 pmol miR-34a mimics with a negative control were transfected into the CEM-C1 cell lines in 10 μL. Electroporation was conducted using electric shock once every 35 ms, with 1.3 KV and 25 μF of electric capacity. The cells were harvested after 24 h. The experiment was repeated three times with each group.

Grouping and cell viability

CCRF-CEM and CEM-C1 cell lines in the logarithm growth period were inoculated into 96-well plate at a density of 5×10^4 cells per well, and supplemented with camptothecin at different concentrations (0.00 ng/mL, 0.01 ng/mL, 0.10 ng/mL, 1.00 ng/mL, 10.00 ng/mL, 100.00 ng/mL, and 1000.00 ng/mL). Each concentration set included 3 wells, and the trial was repeated three times. After 22 h, 10 μL of CCK-8 was added to each well, and the absorbance was determined at 450 nm using a microplate reader after 2 h. The inhibition rate of cell proliferation was calculated by the formula, [1- (A450 nm of experiment group - A630 nm of experiment group)/(A450 nm 0f control group - A630 nm of control group)]×100%. The resistance index (RI) was equal to CEM-C1 IC50/CCRF- CEM IC_{50}. In our study, the IC_{50} of CCRF-CEM and CEM-C1 was 2.9 and 3492.00 ng/mL, respectively, with an RI 1460, suggesting that CEM-C1 cell line was resistant to camptothecin.

RNA extraction and real-time PCR analysis

Total RNA was extracted from the bone marrow specimens of patients, healthy controls, and cultured cells, respectively. Considering the limited number of miRNA nucleotides, we designed miR-34a as a stem-loop structure. The primer used for reverse transcription PCR was 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACAACC-3'. The sense and antisense primers for miR-34a were 5'-CAGTGCAGGGTCCGAGGTATT-3' and 5'-TGGCAGTGTCTTAGCTGGTTGTG-3', respectively. U6 was amplified simultaneously as an internal control using sense (5'-CGCTTCGGCAGCACATATA-3') and antisense (5'-TTCACGAATTTGCGTGTCAT-3') prim-

Table 1. Relative expression of miR-34a in the ALL bone marrow samples at different stages of newly diagnosed-complete remission group ($\bar{x} \pm SD$)

Groups	n	Relative Expression of miR-34a
Newly Diagnosed	26	3.28±1.13*,#
Complete Remission	26	7.43±2.54
Control	11	7.34±2.17
F		22.304
P		0.000

Note: Compared with the control group, *$P < 0.05$; compared with complete remission, #$P < 0.05$.

ers. The real-time PCR conditions for the amplification of miR-34a and U6 were as follows: 95°C for 14 min, followed by 35 cycles at 94°C for 15 s and 55°C for 20 s. Fold induction values were calculated using the $2^{-\Delta\Delta Ct}$ method according to the manufacturer's instructions.

Statistical analysis

All the experiments were repeated at least three times. Data were analyzed using SPSS 21.0. Data were expressed as means ± SD. Levene's test was used to test the homogeneity of variance. Differences between two groups were assessed using the two-tailed t-test. Single factor analysis of variance was conducted to test the differences between multiple groups. LSD-t was used to test the differences within the same group. $P < 0.05$ was considered statistically significant.

Results

Relative expression of miR-34a in ALL bone marrow samples

In the newly diagnosed complete remission group, the relative expression of miR-34a was significantly lower than in the group with complete remission and the control group ($P < 0.05$). However, the relative expression was not statistically significant between the complete remission and the control groups ($P > 0.05$) (**Table 1**). Further, the relative expression of miR-34a in the newly diagnosed and recurrent cases was significantly lower than in the control group ($P < 0.05$) (**Table 2**). The relative expression of miR-34a in the newly diagnosed cases and recurrent group of patients was not statistically significant ($t = 0.141$, $P = 0.444$).

Relative expression of miR-34a in CCRF-CEM and CEM-C1 cells

The relative expression of miR-34a in CEM-C1 cells was 2.64±1.37), which was significantly lower than in the CCRF-CEM cells (5.14±2.06; $t = 6.693$, $P = 0.000$).

Knock-down of miR-34a increased camptothecin resistance in CCRF-CEM cells

The relative expression of miR-34a in CCRF-CEM cells transfected with miR-34a inhibitor was 3.14±1.15, which was significantly lower than in the miRNA inhibitor negative control group (6.07±2.28; $t = 10.687$, $P < 0.001$). The cell viability of each group cultured with different concentrations of camptothecin was analyzed by the CCK-8 assay (**Figure 1**). The results showed that the IC_{50} of CCRF-CEM cells transfected with miR-34a inhibitor was 2167.00 ng/mL while that of the negative control group was 28.73 ng/mL, and RI was 75.43. Above all, the down-regulation of miR-34a expression increased the camptothecin resistance in CCRF-CEM cells.

Overexpression of miR-34a increased camptothecin resistance in CEM-C1 cells

The relative expression of miR-34a in CEM-C1 cells transfected with miR-34a mimic was 5.06±1.73, which was significantly higher than in the negative control miRNA mimics of CEM-C1 cells (1.84±0.79; $t = 14.760$, $P < 0.001$). The cell viability of each group was analyzed in a CCK-8 assay (**Figure 2**). In CEM-C1 cells transfected with miR-34a mimic, the IC_{50} was 1.27 ng/mL while that of negative controls was 112.57 ng/mL, and the RI was 88.64. In conclusion, the upregulation of miR-34a expression enhanced the susceptibility to camptothecin in CEM- C1 cells.

Discussion

The majority of ALL patients are sensitive to chemotherapeutic drugs initially. However, patients relapse after a period of time and become generally resistant to the original chemotherapy drug. Further, a few patients developed multi-drug resistance, which resulted in chemotherapy failure [9]. Currently, there is no effective treatment for chemotherapy-resistant ALL, resulting in poor prognosis. In recent

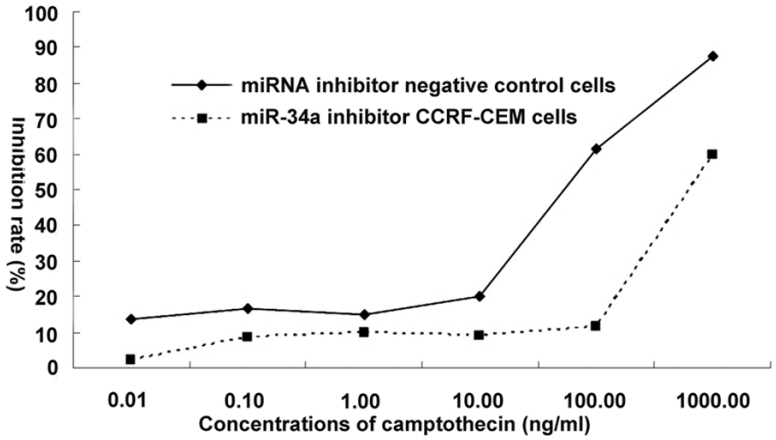

Figure 1. Effects of different concentrations of camptothecin on the proliferation of miR-34a inhibitor in CCRF-CEM cells and miRNA inhibitor-negative control cells (Each experiment was conducted three times with the sample set in triplicate).

Table 2. Relative expression of miR-34a in ALL bone marrow samples at different stages of the newly diagnosed-recurrence group ($\bar{x}$ ±*SD*)

Groups	N	Relative Expression of miR-34a
Newly Diagnosed	26	3.57±1.19*,#
Recurrence	26	4.83±1.76*
Control	11	7.34±2.17
F		31.842
P		0.000

Note: Compared with the control group, *$P < 0.05$, compared with complete remission, #$P < 0.05$.

years, studies increasingly focused on the role of miRNA in tumorigenesis and tumor progression as well as drug resistance of tumor cells. Studies suggested that miR-181a knockout in human drug-resistant cell line CEM-C1 significantly enhanced the sensitivity of CEM-C1 cells to chemotherapy [10]. *MiR-34a* is located on human chromosome 1p36. Lou G demonstrated that *miR-34a* acted as a tumor suppressor gene, and interacted with *P53* to regulate tumorigenesis as well as tumor progression, invasion and metastasis [11]. The expression of miR-34a was significantly decreased in patients with chronic lymphocytic leukemia [12]. Our study found that the relative expression of miR-34a in bone marrow samples of newly diagnosed-complete remission group and newly diagnosed-recurrence group patients was significantly lower than in the normal control group, which suggested that patients with ALL poorly expressed miR-34a in bone marrow. Meanwhile, in patients with newly diagnosed and relapsed expression, the expression of miR-34a was significantly higher than in the control group. Patients with complete remission and patients in the control group were not significantly different. Above all, the results further showed that miR-34a may be involved in the incidence and progression of ALL as well as relapse. In addition, the inhibition of miR-34a expression may promote recurrence [13].

In vitro experiments demonstrated that the expression of miR-34a in CEM-C1 cells was significantly lower than in CCRF-CEM cells, indicating that it may be related to drug resistance in ALL cells. We conducted further experiments to show that transfection of miR-34a inhibitor into CCRF-CEM cells significantly decreased the relative expression of miR-34a compared with that of miRNA inhibitors of negative control. The CCK-8 assays showed that the downregulation of miR-34a increased the drug resistance of CCRF-CEM cells to camptothecin, which suggested that the inhibition of miR-34a expression led to drug resistance in CCRF-CEM cells. Transfection of miR-34a mimics into CEM-C1 cells significantly raised the relative expression of miR-34a compared with that of miRNA mimics in the negative control. The growth curves showed that upregulation of miR-34a enhanced the sensitivity to camptothecin in CEM-C1 cells. *In vitro* experiments suggested that downregulation of miR-34a expression was closely related to the development of drug resistance in ALL cells. Inhibition of miR-34a expression accelerated the development of drug resistance. However, the specific regulatory mechanism warrants further investigation.

Above all, the expression of miR-34a was low in adult ALL bone marrow samples, which affected tumor suppression. Further, poor miR-34a expression might be related to ALL recurrence and drug resistance. The inhibition of miR-34a expression reduced the sensitivity to camptothecin in CCRF-CEM cells while the overexpres-

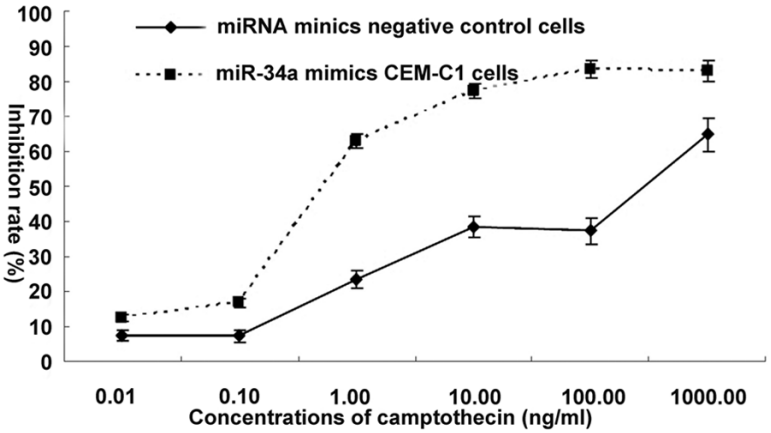

Figure 2. Effects of different concentrations of camptothecin on the proliferation of miR-34a mimic CEM-C1 cells and miRNA mimic negative control cells (Each experiment was repeated three times with the sample set in triplicate).

sion of miR-34a reduced the drug resistance in CEM-C1 cells. Therefore, miR-34a represents a novel therapeutic candidate for the management of relapse, and drug resistance in ALL.

Disclosure of conflict of interest

None.

Address correspondence to: Zenglei Han, Department of Pathology, Municipal Hospital of Qingdao, Jiangsu Road No. 16, Qingdao 266011, Shandong, China. Tel: +86-13969619268; E-mail: hanzenglei@126.com

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