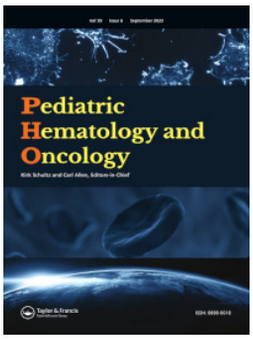

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MicroRNA-199a deficiency relates to higher bone marrow blasts, poor risk stratification and worse prognostication in pediatric acute myeloid leukemia patients

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ABSTRACT

MicroRNA-199a (miR-199a) inhibits the progression of several hematological malignancies and enhances the sensitivity to chemotherapy in acute myeloid leukemia (AML), but its clinical role in AML needs further investigation. This study aimed to explore the correlation of miR-199a with clinical features and prognosis in pediatric AML patients. Totally, 71 pediatric AML patients were enrolled. Their bone marrows (BMs) before and after one course of treatment were collected. Besides, 30 pediatric patients with nonmalignant hematological disease who underwent BM examination were enrolled with their BMs collected. miR-199a expression was detected by reverse transcription-quantitative polymerase chain reaction. miR-199a expression was lower in pediatric AML patients than in controls ($p < 0.001$). Meanwhile, downregulated miR-199a expression was correlated with the occurrence of FLT3-ITD mutation ($p = 0.023$), higher BM blasts ($p = 0.037$), poor NCCN risk stratification ($p = 0.012$) and unfavorable Chinese Medical Association risk stratification ($p = 0.002$) but not associated with other clinical features. Additionally, downregulated miR-199a expression was correlated with lower complete response (CR) rate after one course of treatment ($p = 0.036$). Interestingly, after treatment, miR-199a expression was increased in patients who achieved CR ($p < 0.001$), but remained unchanged in those who didn't achieve CR ($p = 0.163$). Moreover, downregulated miR-199a expression was correlated with shorter event-free survival ($p = 0.021$); meanwhile, it showed a trend of associating with poor OS ($p = 0.055$), which was not statistically significant. In this series, decreased expression of miR199a was associated with inadequate treatment response and worse OS in pediatric AML patients, indicating its potential as a prognostic biomarker for pediatric AML.

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Complete response; event-free survival; microRNA-199a; overall survival; pediatric acute myeloid leukemia

Introduction

Pediatric acute myeloid leukemia (AML), the second most common leukemia occurring in childhood, accounts for 15%–20% of pediatric acute leukemia.[1–3] Although great

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efforts have been made to improve the management of pediatric AML (including chemotherapy, hematopoietic stem cell transplantation, targeted therapy and supportive care), pediatric AML still has a relatively unsatisfying prognosis among all pediatric leukemias with a 5-year overall survival (OS) of 60%.[4] Currently, NCCN risk stratification for pediatric AML is based largely on adult AML stratification strategies, with few biomarkers to inform specific risks for children and young adults with AML.[5,6] Therefore, it is urgent to find novel prognostic markers and risk stratification factors for pediatric AML patients to improve their prognosis.

MicroRNA-199a (miR-199a) is a family member of non-coding RNAs, which plays a key role in maintaining normal homeostasis and regulating various disease pathogenesis.[7] Recent studies show that miR-199a suppresses the progression of multiple hematological malignancies.[8–10] However, the clinical involvement of miR-199a in AML, especially the pediatric AML, is seldom reported.

Hence, the current study aimed to investigate the correlation of miR-199a with disease risk, clinical features, treatment response and survival profile in pediatric AML patients.

Methods

Subjects

From August 2016 to August 2020, a total of 71 pediatric patients with *de novo* AML who treated in our hospital were reviewed in this study. The screening criteria were (i) diagnosed as AML according to the World Health Organization classification of AML;[11] (ii) less than 16 years old; (iii) had available complete response (CR) data of one course of treatment; (iv) had available bone marrow (BM) specimens before induction treatment and after one course of treatment. The exclusion criteria were: (i) diagnosed as acute promyelocytic leukemia (APL); (ii) complicated with other cancers, hematological malignancies or BM failure syndromes at diagnosis; (iii) received chemotherapy or radiotherapy therapy at or before AML diagnosis. In addition, 30 pediatric patients with nonmalignant hematological disease who received examination of BM specimens were reviewed in the study as controls. The exclusion criteria for AML patients were also appropriate for the controls. This study was approved by the Institutional Research Ethics Committee.

Data and specimen collection

For AML patients, clinical characteristics were recorded. Risk stratification was referring to the criterion issued by National Comprehensive Cancer Network (NCCN)[12] and the Chinese Medical Association.[13] For specimen collection, BM samples of AML patients before induction treatment and after one course of treatment were collected, then BM samples of controls were also obtained. Additionally, bone marrow mononuclear cells (BMMCs) were separated from BM samples with gradient density centrifugation using Ficoll-Paque PREMIUM (Cytiva, Marlborough, Massachusetts, US) for the subsequent analysis.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay

miR-199a expression was assessed in BMMCs of all subjects by RT-qPCR assay. First of all, total RNA was extracted using TRIzol™ Reagent (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Then reverse transcription was carried out with Quick Master Mix (Toyobo, Osaka, Kansai, Japan). In addition, qPCR was performed using SYBR® Premix DimerEraser™ (Takara, Dalian, Liaoning, China). miR-199a relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method and U6 was used as the internal reference. The sequences of primers are listed in Supplementary Table 1.

Assessment of CR and survival data

Patients received standard regimen for induction therapy, with 3 days of an anthracycline (idarubicin, 10–12 mg/m^2) and 7 days of cytarabine (100–200 mg/m^2). CR after one course of treatment was recorded and assessed according to the European LeukemiaNet guideline.[14] As for patients who were considered as relapsed/refractory, the FLAG/DNX regimen (Fludarabine/cytarabine/granulocyte colony-stimulating factor/liposome daunorubicin) was administered. Patients were followed up regularly, and the median follow-up duration was 21.0 months ranging from 2.0 to 45.0 months. In addition, diseases status was recorded for calculation of event-free survival (EFS) and overall survival (OS).[14]

Statistical analysis

Data analysis and figure plotting were completed using SPSS 20.0 (IBM Corp., Armonk, New York, USA) and GraphPad Prism 6.01 (GraphPad Software Inc., San Diego, California, USA). Pediatric AML patients were classified according to the quartile of miR-199a expression, in which 0–25th, 25th–50th, 50th–75th and 75th–100th quartile of miR-199a expression were classified as quartile 1, quartile 2, quartile 3 and quartile 4 groups, respectively. Comparison between two groups was determined by Mann–Whitney *U* test or Wilcoxon signed-rank test, accordingly. The correlation test was analyzed by linear-by-linear association test and Spearman's rank correlation test. EFS and OS were illustrated using Kaplan–Meier curve and determined by log-rank test. p value < 0.05 was considered statistically significant.

Results

Clinical characteristics of pediatric AML patients

The mean age was 6.7 ± 2.8 years in pediatric AML patients (Table 1). In regard to gender, there were 38 (53.5%) females and 33 (46.5%) males. There were 4 (5.6%), 35 (49.3%), 8 (11.3%) and 24 (33.8%) patients with AML of M1, M2, M4 and M5 subtypes by French-American-British (FAB) classification, respectively. More detailed information of pediatric AML patients are listed in Table 1.

Table 1. Clinical characteristics.

	Pediatric AML patients ($N = 71$)
Demographic characteristics	
Age (years), mean ± SD (range)	6.7 ± 2.8 (1.0–15.0)
Gender, No. (%)	
Female	38 (53.5)
Male	33 (46.5)
Height (cm), mean ± SD	118.4 ± 18.2
Weight (kg), mean ± SD	23.9 ± 9.0
FAB classification	
M1, No. (%)	4 (5.6)
M2, No. (%)	35 (49.3)
M4, No. (%)	8 (11.3)
M5, No. (%)	24 (33.8)
Cytogenetic features	
NK, No. (%)	21 (29.6)
t(8; 21), No. (%)	11 (15.5)
CK, No. (%)	10 (14.1)
Inv(16) or t(16; 16), No. (%)	8 (11.3)
−7 or 7q-, No. (%)	5 (7.0)
t(9; 22), No. (%)	3 (4.2)
t(9; 11), No. (%)	2 (2.8)
+8, No. (%)	2 (2.8)
11q23, No. (%)	1 (1.4)
Others non-defined, No. (%)	8 (11.3)
Molecular abnormalities	
FLT3-ITD mutation, No. (%)	19 (26.8)
NPM1 mutation, No. (%)	16 (22.5)
CEBPA mutation, No. (%)	12 (16.9)
WT1 mutation, No. (%)	11 (15.5)
Biochemical indexes	
WBC (x10^9/L), median (IQR)	30.5 (19.2–46.7)
BM blasts (%), median (IQR)	73.0 (61.0–83.0)
Risk stratification	
NCCN risk stratification, No. (%)	
Favorable	25 (35.2)
Intermediate	21 (29.6)
Poor	25 (35.2)
Chinese Medical Association risk stratification, No. (%)	
Low risk	8 (11.3)
Intermediate risk	32 (45.1)
High risk	31 (43.7)
CR after 1st course, No. (%)	44 (62.0)

AML, acute myeloid leukemia; SD, standard deviation; FAB, French-American-British; NK, normal karyotype; CK, complex karyotype; FLT3-ITD, the internal tandem duplication representing the most common type of FMS-like tyrosine kinase 3; NPM1, nucleophosmin 1; CEBPA, CCAAT/enhancer binding protein alpha; WT1, Wilms' tumor 1; WBC, white blood cell; IQR, interquartile range; BM, bone marrow; NCCN, the National Comprehensive Cancer Network; CR, complete response.

Correlation of miR-199a expression with clinical characteristics

Downregulated miR-199a expression correlated with the occurrence of FLT3-ITD mutation ($p = 0.023$, Table 2). Besides, downregulated miR-199a expression was associated with higher BM blasts ($p = 0.037$). Additionally, downregulated miR-199a expression correlated with poor NCCN risk stratification ($p = 0.012$). Regarding the Chinese Medical Association risk stratification, downregulated miR-199a expression was associated with high-risk stratification ($p = 0.002$). Nevertheless, downregulated miR-199a expression did not correlate with other clinical characteristics, such as age, gender and FAB classification.

Table 2. Correlation between miR-199a expression and clinical characteristics.

	miR-199a expression				
	Quantile 1 ($n=18$)	Quantile 2 ($n=17$)	Quantile 3 ($n=18$)	Quantile 4 ($n=18$)	p value
Age (years), mean ± SD	7.7 ± 3.3	6.3 ± 3.1	6.1 ± 2.0	6.9 ± 2.5	0.532
Gender, No. (%)					0.492
Female	11 (61.1)	8 (47.1)	11 (61.1)	8 (44.4)	
Male	7 (38.9)	9 (52.9)	7 (38.9)	10 (55.6)	
FAB classification, No. (%)					0.909
M1	3 (16.7)	1 (5.9)	0 (0.0)	0 (0.0)	
M2	8 (44.4)	6 (35.3)	10 (55.6)	11 (61.1)	
M4	2 (11.1)	1 (5.9)	1 (5.6)	4 (22.2)	
M5	5 (27.8)	9 (52.9)	7 (38.9)	3 (16.7)	
FLT3-ITD mutation, No. (%)	7 (38.9)	6 (35.3)	5 (27.8)	1 (5.6)	0.023*
NPM1 mutation, No. (%)	3 (16.7)	3 (17.6)	3 (16.7)	7 (38.9)	0.140
CEBPA mutation, No. (%)	3 (16.7)	4 (23.5)	4 (22.2)	1 (5.6)	0.388
WT1 mutation, No. (%)	5 (27.8)	2 (11.8)	3 (16.7)	1 (5.6)	0.106
WBC ($x10^9$/L), median (IQR)	32.0 (24.0–54.1)	26.7 (14.2–64.4)	33.2 (24.6–55.3)	23.0 (8.8–43.5)	0.089
BM blasts (%), median (IQR)	78.5 (65.3–89.5)	79.0 (72.0–91.0)	62.0 (55.0–80.5)	71.0 (63.8–78.0)	0.037*
NCCN risk stratification, No. (%)					0.012*
Favorable	4 (22.2)	3 (17.6)	10 (55.6)	8 (44.4)	
Intermediate	4 (22.2)	7 (41.2)	4 (22.2)	6 (33.3)	
Poor	10 (55.6)	7 (41.2)	4 (22.2)	4 (22.2)	
Chinese Medical Association risk stratification, No. (%)					0.002*
Low risk	0 (0.0)	0 (0.0)	4 (22.2)	4 (22.2)	
Intermediate risk	7 (38.9)	8 (47.1)	7 (38.9)	10 (55.6)	
High risk	11 (61.1)	9 (52.9)	7 (38.9)	4 (22.2)	

miR-199a, microRNA-199a; SD, standard deviation; FAB, French-American-British; FLT3-ITD, the internal tandem duplication representing the most common type of FMS-like tyrosine kinase 3; NPM1, nucleophosmin 1; CEBPA, CCAAT/enhancer-binding protein alpha; WT1, Wilms' tumor 1; WBC, white blood cell; IQR, interquartile range; BM, bone marrow; NCCN, the National Comprehensive Cancer Network.
*$p = 0.05$.

Association of miR-199a expression with treatment response

The difference of miR-199a expression between pediatric AML patients and controls was evaluated, which showed that miR-199a expression was lower in pediatric AML patients compared to controls ($p < 0.001$, Figure 1A). The median miR-199a expression was 0.392 (0.232–0.655) and 0.992 (0.674–1.544) in pediatric AML patients and controls, respectively. Besides, downregulated miR-199a expression was correlated with lower CR rate ($p = 0.036$, Figure 1B). Moreover, the expression of miR-199a was detected again after one course of treatment, which suggested that miR-199a expression was increased after treatment in patients who achieved CR ($p < 0.001$, Figure 1C), whereas miR-199a expression was of no difference before and after treatment in patients who didn't achieve CR ($p = 0.163$, Figure 1D). However, no difference was found in miR-199a expression between CR patients (after treatment) and normal controls ($p = 0.077$) (Supplementary Table 2).

Correlation of miR-199a expression with accumulating EFS and OS

Generally, downregulated miR-199a expression correlated with shorter EFS ($p = 0.021$, Figure 2). Subgroup comparison illustrated that patients in the quantile 1 group had

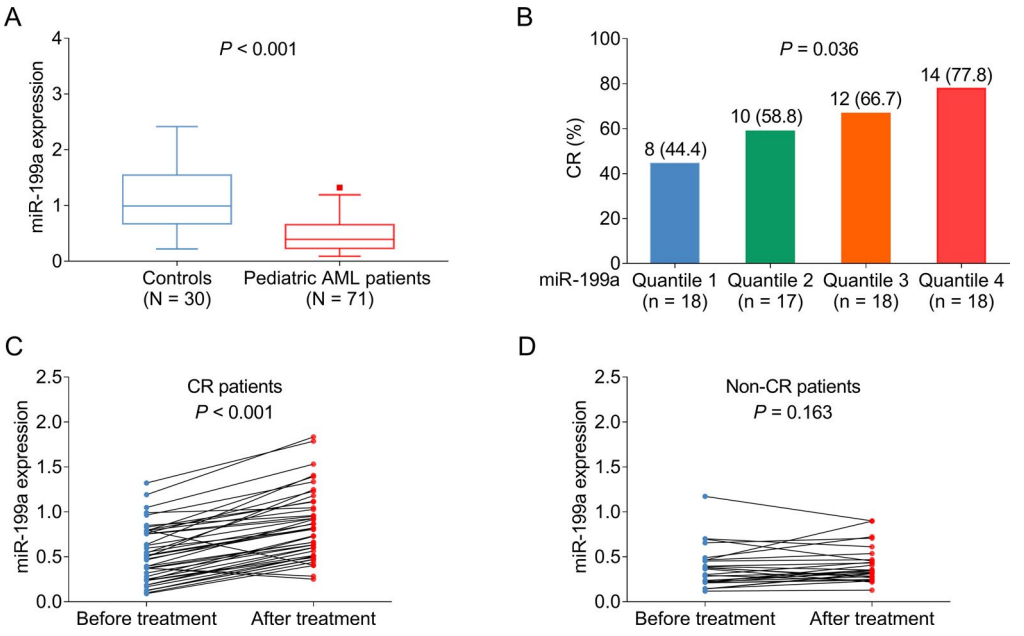
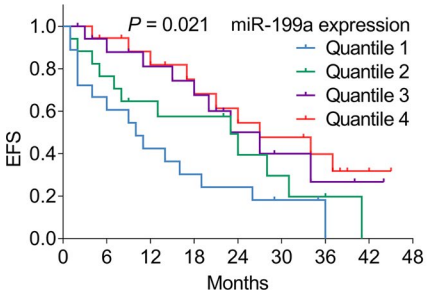
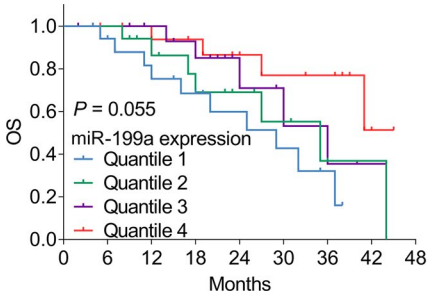


Figure 1. miR-199a expression in AML patients and controls (A); miR-199a expression in control subjects and pediatric AML subjects (B); miR-199a expression before and after treatment in pediatric AML patients achieving CR after one course of treatment (C); miR-199a expression before and after treatment in pediatric AML patients without CR after one course of treatment (D). miR-199a: microRNA-199a; CR: complete response; AML: acute myeloid leukemia.



miR-199a expression	*P* value
Quantile 1 vs. Quantile 2	0.217
Quantile 1 vs. Quantile 3	0.026
Quantile 1 vs. Quantile 4	0.007
Quantile 2 vs. Quantile 3	0.344
Quantile 2 vs. Quantile 4	0.126
Quantile 3 vs. Quantile 4	0.719

Figure 2. Correlation of miR-199a expression with accumulating EFS in pediatric AML patients. miR-199a: microRfNA-199a; EFS: event-free survival; AML: acute myeloid leukemia.



miR-199a expression	*P* value
Quantile 1 vs. Quantile 2	0.440
Quantile 1 vs. Quantile 3	0.184
Quantile 1 vs. Quantile 4	0.009
Quantile 2 vs. Quantile 3	0.416
Quantile 2 vs. Quantile 4	0.097
Quantile 3 vs. Quantile 4	0.296

Figure 3. Correlation of miR-199a expression with accumulating OS in pediatric AML patients. miR-199a: microRNA-199a; OS: overall survival; AML: acute myeloid leukemia.

poor EFS compared with patients in the quantile 3 group ($p = 0.026$) and patients in the quantile 4 group ($p = 0.007$). However, no correlation was discovered in miR-199a expression with accumulating OS ($p = 0.055$, Figure 3), whereas subgroup comparison illustrated that patients in the quantile 1 group had shorter OS compared with patients in the quantile 4 group ($p = 0.009$).

Discussion

In the present study, miR-199a expression was lower in pediatric AML patients compared with controls. A possible explanation could be that the downregulated miR-199a expression could reflect differential cell proliferation in AML cells compared to control myeloid cells. In the current study, downregulated miR-199a expression correlated with increased BM blasts, unfavorable NCCN risk stratification. These observations could be consistent with an association of miR-199a expression with FL3-ITD, a negative prognostic factor according to NCCN risk stratification in AML patients.[12] Thus, downregulated miR-199a expression is linked to poor risk stratification. Additionally, according to the Chinese Medical Association risk stratification, worse CR after one course of treatment correlated with higher risk stratification.[13]

In this study lower miR-199a expression was associated with decreased CR after one course of treatment and inferior EFS with a trend toward decreased OS. Additionally, miR-199a expression was slighted decreased in CR patients (after treatment) compared to controls. Possible explanations for these observations could include a role for miR-199a in inhibiting protective autophagy as a possible mechanism of overcoming chemotherapy resistance via dysregulating damage regulator autophagy modulator 1 and suppressing WNT2 signaling.[9,10,15] Another possible explanation for these results is the nature of this study that included 71 pediatric AML subjects and associated impact on study power. miR-199a overexpression is slightly correlated with OS. Further, mi-RNA expression relative to OS could be impacted by downstream factors in patients who relapse including response to therapy and variable disease courses of the miR-199a with OS is weakened. Finally, miR-199a could have differential expression in myeloid cells at different stages of differentiation, impacting comparable mi-RNA levels between CR patients (after treatment) and controls. This assumption still needs further validation.

This is discussed already in our study. (1) We merely enrolled 71 pediatric AML patients. The relatively small sample size might cause low statistical power. (2) The follow-up period was comparatively short, thus a longer follow-up duration was needed. (3) The underlying mechanism of miR-199a as an anti-leukemia gene in pediatric AML remained elusive in our study. Thus, further studies were needed.

The results from this study support miR-199a for potential biomarker of disease risk and response to therapy in pediatric AML which merits validation in prospective clinical trials.

Disclosure statement

The authors report no conflict of interest.

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