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Original Article

Upregulation of miR-224 predicts poor prognosis in patients with pediatric acute myeloid leukemia

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Abstract: Dysregulation of miR-224 has been shown to be involved in cancer tumorigenesis and progression of several cancer types. However, its expression patterns in tumors are controversial. The aim of this study was to determine the expression and clinical significance of miR-224 in pediatric acute myeloid leukemia (AML). Expression levels of miR-224 in bone marrow mononuclear cells were detected by real-time quantitative PCR in 104 patients with newly diagnosed pediatric AML. The results showed that miR-224 expression was significantly upregulated in the bone marrow of pediatric AML patients when compared with controls (P<0.001). Also, miR-224 upregulation occurred more frequently in French-American-British classification subtype M7 than in other subtypes (P=0.007). Regarding to cytogenetic risk, the levels of miR-224 in pediatric AML patients with unfavorable karyotypes were dramatically higher than those in intermediate and favorable groups (P=0.028). Moreover, Kaplan-Meier analysis showed that pediatric AML patients with high miR-224 expression tended to have shorter disease free and overall survivals. In multivariate analysis stratified for known prognostic variables, high miR-224 expression was identified as an independent prognostic factor for both disease free and overall survivals. In conclusion, our data indicated that the upregulation of miR-224 was associated with advanced clinical features and poor prognosis of pediatric AML patients, suggesting that miR-224 upregulation may serve as an unfavorable prognostic biomarker in pediatric AML.

Keywords: Pediatric acute myeloid leukemia, miR-224, real-time quantitative PCR, prognostic

Introduction

Acute myeloid leukemia (AML), as a heterogeneous disease, is characterized by the uncontrolled proliferation of monocytic, granulocytic, megakaryocytic, or rarely, erythroid blast cells [1]. Accumulating studies have indicated that numerous of different cytogenetic and molecular abnormalities may be involved in tumorigenesis and tumor progression of AML. The distinct feature of leukemogenesis in AML is differentiation arrest and proliferative advantage of myeloid progenitors. Although AML makes up only 15%-20% of pediatric leukemia, it still accounts for more than 30% of deaths from leukemia [2]. Dose-intensive treatment by induction chemotherapy and allogeneic stem cell transplantation with a matched related donor has been considered as effective treatment for pediatric AML, however, the majority of patients without an appropriate related donor need to receive continuous chemotherapy. Of note, pediatric AML has different response to therapy and prognosis when compared to adult AML. The relapse remains a major cause of failure and the clinical outcome of pediatric AML is still poor. In the most successful studies, the 5 year disease free survival in pediatric AML patients is about 50% [3]. Thus, it is necessary to identify novel and effective molecular markers for pediatric AML in order to improve prognostic evaluation levels and to develop more appropriate therapeutic approaches.

MicroRNAs (miRNAs), which are small non-coding RNAs, regulate the translation of specific protein-coding genes by translational repression or degradation of the complementary mRNA [4]. As master regulators of gene expression, miRNAs are involved in modulating diverse biological processes, such as metabolism, sur-

Table 1. Correlations between miR-224 expression and clinical features of pediatric AML patients (n=104)

Variable	Number of cases	miR-224 expression		*P* value
		Low (n=52)	High (n=52)	
Gender				0.353
Male	56	29	27	
Female	48	23	25	
Age (years)				0.722
<7	41	22	19	
≥7	63	30	33	
Leukocyte (10^9/L)				0.315
<10	29	17	12	
≥10	75	35	40	
Hemoglobin (g/L)				0.585
<80	65	33	32	
≥80	39	19	20	
Platelet count (10^9/L)				0.408
<50	58	30	28	
≥50	46	22	24	
FAB classification				0.007*
M1-M6	94	51	43	
M7	10	1	9	
Extramedullary disease				0.269
Absent	80	42	38	
Present	24	10	14	
Cytogenetics				0.028*
Favorable	35	22	13	
Intermediate	46	28	18	
Unfavorable	23	2	21	
Day 7 response to treatment				0.072
Favorable	65	35	30	
Unfavorable	39	17	22	

*$P<0.05$.

vival, differentiation, and apoptosis [4]. The deregulation of expression of miRNAs has been demonstrated to contribute to the multistep processes of carcinogenesis either by oncogenic or tumor suppressor function [5]. Moreover, accumulating evidence indicated that miRNAs are able to be used as effective potential biomarkers for cancer risk, diagnosis and prognosis, even as miRNA-based therapeutic targets with a great interest [6-8]. Especially in the research of leukemia, Zhang and colleagues demonstrated that existing pediatric-associated and prognostic parameter-associated miRNAs could provide therapeutic direction for individual risk-adapted therapy for pediatric leukemia patients [9]. Marcucci et al. suggested that miR-155 expression may be an independent prognostic biomarker for patients with cytogenetically normal AML [10]. Lopotová et al. identified a reciprocal regulatory loop between miR-451 and BCR-ABL as a maintenance mechanism of the leukemic state of chronic myeloid leukemia cells [11]. In the present study, we focus on miR-224, which has been reported to be involved in tumorigenesis and tumor progression of several cancer types, including hepatocellular carcinoma [12], breast cancer [13], pancreatic ductal adenocarcinoma [14] and ovarian cancer [15]. However, its roles in pediatric AML are still unclear. The aim of this study was to investigate the expression and clinical significance of miR-224 in pediatric AML.

Materials and methods

Patients and samples

This study was approved by the Ethics Committee of Shandong Province Qianfoshan Hospital, China. Written informed consent was obtained from all participants. Between February 2007 and December 2009, 104 patients with newly diagnosed pediatric AML according to the French-American-British (FAB) criteria were enrolled in this study from the Department of Hematology at Shandong Province Qianfoshan Hospital, China. All the patients were younger than 18 years of age (mean 7 years) and included 56 males and 48 females. The median leukocyte count at diagnosis was 21.76×10^9/L (range 4.55-362.11×10^9/L). According to the FAB classification, 6 patients had AML M1, 23 had M2, 15 had M3, 17 had M4, 20 had M5, 13 had M6, and 10 had M7. Among 24 patients with extramedullary disease, 18 patients had chloroma (orbit in 9 patients, skin in 4 patients, and scalp in 5 patients,) and 6 patients had a central nervous system involvement of leuke-

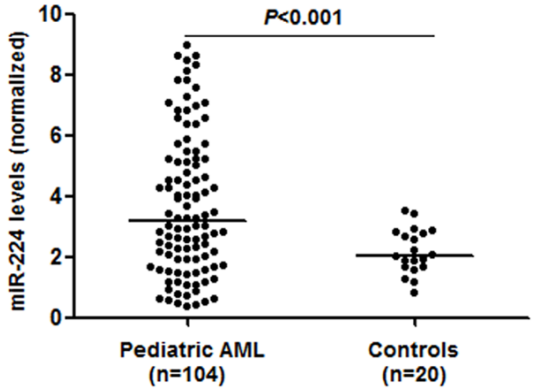

Figure 1. MiR-224 expression was examined using qRT-PCR in the bone marrow of 104 pediatric AML patients and 20 controls, and its expression was normalized to the level of RNU6B in each sample. The line represents the median value.

mic cells. Details of the backgrounds and clinicopathological characteristics of the patients with AML are summarized in **Table 1**.

All 104 patients with AML were treated with 10 days of induction chemotherapy, in which the dose of behenoyl 1-h-D-arabinofuranosylcytosine for the last 3 days was modified according to the bone marrow response on day 7. Discontinuation of the chemotherapy was allowed in patients who experienced sepsis with unstable vital signs before the completion of the induction regimen if at least 7 days of induction chemotherapy had been provided. An additional course of induction chemotherapy using high-dose 1-h-d-arabinofuranosylcytosine was given if complete remission was not achieved after the primary induction chemotherapy regimen. Once complete remission had been achieved, patients with an appropriate stem cell donor received consolidation chemotherapy until the hematopoietic stem cell transplantation. An entire course of consolidation chemotherapy was given in patients without an appropriate related donor.

As controls, bone marrow was collected from 20 patients (range 2-18 years) with various diseases but with normal bone marrow morphology as demonstrated by histological and cytological analyses.

RNA isolation

Mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation of 2 mL bone marrow samples in EDTA from newly diagnosed AML patients and control samples. Total RNA was extracted from mononuclear cells using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The concentration and purity of RNA were measured spectrophotometrically at 260 and 280 nm, whereas RNA integrity was evaluated by agarose gel electrophoresis.

MiRNA detection by real-time quantitative RT-PCR (qRT-PCR)

cDNA were generated from 1 μL total RNA using One Step PrimeScript miRNA cDNA Synthesis Kit (Takara, Japan) according to the manufacture's protocol. Real-time PCR was performed in triplicate using SYBR Premix Ex TaqTM II (Takara, Japan) on an ABI 7500 Real-Time PCR System (Applied Biosystems, USA) and the reaction mixtures were incubated at 95°C for 10 s, followed by 40 cycles of 95°C for 5 s and 60°C for 34 s. At the end of the PCR cycles, melting curve analyses were performed in order to validate the specific generation of the expected PCR product. The expression levels of miR-224 were normalized relative to the expression of RNU6B, and were calculated using the $2^{-\Delta\Delta C}$ method [16].

Statistical analysis

The Kruskal-Wallis test or the Mann-Whitney U test was used to compare miRNAs levels between groups. The Kaplan-Meier method was used to estimate survival rates, and the log-rank tests were used to assess survival differences between groups. The influence of each variable on survival was examined by the Cox multivariate regression analysis. All statistical analyses were carried out by using SPSS 13.0 software (IBM, USA). A *P* value less than 0.05 was considered statistically significant.

Results

MiR-224 expression in pediatric AML patients

MiR-224 expression was detected in bone marrow from 104 pediatric AML patients and 20 controls by qRT-PCR. Expression of miR-224 was normalized with RNU6B, and the values obtained were compared. Our data indicated that miR-224 expression in the bone marrow of pediatric AML patients was significantly higher

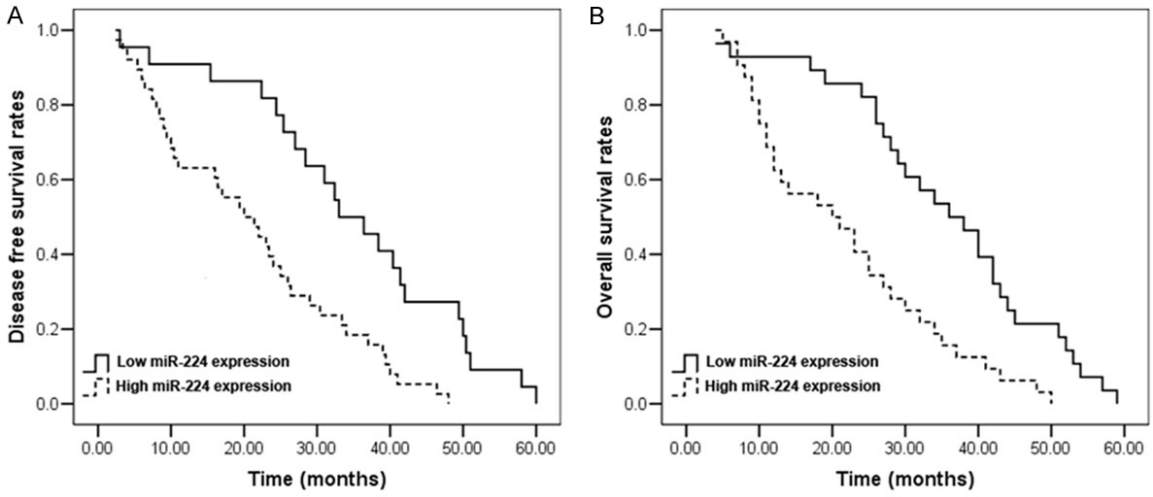

Figure 2. Kaplan-Meier curves for disease free survival (A) and overall survival (B) according to the miR-224 levels. High miR-224 expression was associated with shorter disease free (P=0.023) and overall (P=0.034) survival in pediatric AML patients, respectively.

Table 2. Univariate and multivariate analysis of prognostic parameters in pediatric AML patients by Cox regression analysis

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Disease free survival				
Gender (Male vs. Female)	0.88 (0.56-1.46)	0.673		
Age (<7 vs. ≥7)	0.94 (0.42-1.98)	0.475		
Leukocyte (<10 vs. ≥10)	1.88 (0.76-2.79)	0.383		
Hemoglobin (<80 vs. ≥80)	1.17 (0.57-1.65)	0.547		
Platelet count (<50 vs. ≥50)	1.02 (0.54-1.85)	0.281		
FAB classification (M1-M6 vs. M7)	1.46 (0.72-2.25)	0.036*	1.23 (0.51-2.52)	0.045*
Extramedullary disease (Absent vs. Present)	2.76 (1.25-4.28)	0.093		
Cytogenetics (Favorable + Intermediate vs. Unfavorable)	1.68 (1.05-2.55)	0.023*	1.37 (0.46-2.45)	0.039*
Day 7 response to treatment (Favorable vs. Unfavorable)	2.61 (1.77-3.23)	0.066		
MiR-224 expression (Low vs. High)	3.54 (2.55-5.62)	0.012*	3.08 (1.76-5.02)	0.032*
Overall survival				
Gender (Male vs. Female)	1.09 (0.58-1.56)	0.542		
Age (<7 vs. ≥7)	0.78 (0.51-1.86)	0.332		
Leukocyte (<10 vs. ≥10)	1.47 (0.72-1.68)	0.473		
Hemoglobin (<80 vs. ≥80)	0.88 (0.53-1.29)	0.667		
Platelet count (<50 vs. ≥50)	0.82 (0.56-1.42)	0.318		
FAB classification (M1-M6 vs. M7)	2.38 (1.15-4.52)	0.013*	1.62 (0.72-3.80)	0.041*
Extramedullary disease (Absent vs. Present)	1.78 (1.03-2.76)	0.042*	1.53 (1.17-3.23)	0.058
Cytogenetics (Favorable + Intermediate vs. Unfavorable)	3.13 (1.93-5.11)	0.027*	1.85 (1.08-3.24)	0.036*
Day 7 response to treatment (Favorable vs. Unfavorable)	1.49 (0.69-1.99)	0.254		
MiR-224 expression (Low vs. High)	3.42 (2.35-6.56)	0.015*	3.17 (1.67-4.92)	0.019*

HR hazard ratio, CI confidence interval, *P<0.05.

than those in controls (**Figure 1**, P<0.001). The median expression level of miR-224 (3.05) was used as a cutoff point to divide all 104 AML patients into two groups: AML patients express-

ing miR-224 at levels less than the cutoff value were assigned to the low expression group (median expression value 1.71, n=52), and those samples with expression equal or above the cutoff value were assigned to the high expression group (median expression value 5.26, n=52).

Correlation of miR-224 expression with clinical features of pediatric AML patients

The relationship between miR-224 expression and clinical features in pediatric AML patients was summarized in **Table 1**. The results demonstrated that high miR-224 levels occurred more frequently in FAB classification subtype M7 than in other subtypes (P=0.007). The miR-224 levels in pediatric AML patients with unfavorable karyotypes was also significantly higher than those in intermediate and favorable groups (P=0.028). In contrast, there was no correlation between miR-224 expression and other clinical factors, such as gender, age, leukocyte count, hemoglobin, platelet count, extramedullary disease and day 7 response to treatment (all P>0.05).

Correlation between miR-224 expression and prognosis in pediatric AML patients

All 104 patients with pediatric AML were received follow up analysis. The median follow up duration was 34 months, ranging from 9 to 60 months. The prognostic value of miR-224 expression was investigated using the Kaplan-Meier method and log-rank test. As shown in **Figure 2**, patients with high miR-224 levels had a dramatically lower disease free survival (DFS) or overall survival (OS) rate than that in the low group (27.6% vs. 52.7% for DFS, P=0.023 and 32.8% vs. 61.5% for OS, P=0.034, respectively). Furthermore, univariate Cox proportional hazards regressions model analysis revealed a statistically significant correlation between DFS and miR-224 level (P=0.012), FAB classification (P=0.036) and cytogenetic abnormalities (P=0.023) (**Table 2**). OS was significantly correlated with miR-224 level (P=0.015), FAB classification (P=0.013), extramedullary disease (P=0.042) and cytogenetic abnormalities (P=0.027) (**Table 2**). Parameters significantly related to survival in the univariate analysis were put into the multivariate analysis to identify the independent factors for prognoses. It turned out that miR-224 level still maintained its significance as an independent prognostic factor for DFS (P=0.032) and OS (P=0.019) in pediatric AML patients.

Discussion

AML is an aggressive and metastatic tumor, leading to a high mortality. Finding new molecular targets for its diagnosis, prognosis and treatment has the potential to improve the clinical strategy and outcome of this disease [17, 18]. In this retrospective study of 104 patients with newly diagnosed pediatric AML, there are three significant findings according to our results: firstly, miR-224 expression in the bone marrow from pediatric patients with newly diagnosed AML was significantly higher than those in controls; the increased expression of miR-224 was significantly associated with advanced clinical features of pediatric AML patients; and both univariate and multivariate analyses revealed that the miR-224 level was a significant risk factor affecting the DFS and OS of patients with pediatric AML. These results suggest that miR-224 expression could be a valuable molecular marker of the progression and the prognosis in the patients of pediatric AML. To the best of our knowledge, this is the first study to comprehensively analyze the expression patterns and clinical significance of miR-224 in a large number of pediatric AML patients.

MiRNAs are small non-coding RNA molecules that regulate gene expression post-transcriptionally by binding to the 3'untranslated regions of target mRNAs, thus playing a crucial role in regulating protein expression [4]. Numerous studies have shown that aberrant miRNA expressions correlate with development and progression in a variety of human diseases, including cancer [5]. Their association with tumor genesis indicates their potential as effective biomarkers for cancer risk, diagnosis and prognosis, even as miRNA-based therapeutic targets [6-8]. To our interests, recent studies have witnessed dysregulation of multiple miRNAs in AML. For example, Rücker et al. found that the p53-miR-34a axis played important roles in the disease progression of complex-karyotype AML [19]. Schwind et al. showed that overexpression of miR-181a in AML patients was significantly correlated with a high percentage of blasts in the blood, high hemoglobin levels at diagnosis, M1/M2 phenotype, no extramedullary disease, NPM1 wild-type

alleles, CEPBα mutation, and a favorable outcome [20]. The miR-29b-1/29a cluster located at 7q32 has been identified as a region deleted in therapy-related AML [21]. These findings showed that deregulation of miRNAs may play a vital role in the tumorigenesis and progression of AML. More extensive investigations are still required to elucidate the roles of miRNAs in the development of AML so as to identify those miRNAs that may serve as novel prognosis markers or as therapeutic targets for AML.

MiR-224, as a potential tumor related miRNA, has been reported to be involved in tumorigenesis and tumor development [22, 23]. Notably, the roles of miR-224 in different cancers are quite contradictory as it can behave either as an oncogene or a tumor suppressor gene, depending on the tumor type examined. For example, Zhang and colleagues reported that miR-224 expression was significantly increased in hepatocellular carcinoma tissues and cell lines, and that miR-224 contributed to the malignant potential such as cell proliferation, migration, and invasion in hepatocellular carcinoma [12]. Lu et al. demonstrated that high miR-224 expression may be an independent poor prognostic factor for human glioma [24]. In breast cancer cell lines, Huang et al. found that the overexpression of miR-224 may play an important role in metastasis of cancer cells to the bone by directly suppressing RKIP [13]. In addition to these, the upregulation of miR-224 was also shown in aggressive pancreatic ductal adenocarcinoma [14], colon cancer [25], and bladder cancer [26]. In contrast, a downregulation of miR-224 has been observed in lung cancer [27], prostate cancer [28], ovarian cancer [15], and oral carcinoma [29]. However, the expression of miR-224 in pediatric AML and its roles in pediatric AML progression are not unclear. In this study, using 104 clinical newly diagnosed pediatric AML patients and 20 controls, we provided the first evidence that miR-224 expression is markedly increased in newly diagnosed pediatric AML patients. In addition, we found that the increased expression of miR-224 was significantly associated with the FAB classification subtype M7 and the unfavorable cytogenetic risks, which raises the possibility that miR-224 might have an important role in the development or pathogenesis of pediatric AML. Moreover, the most important finding of our study was that pediatric AML patients with high miR-224 expression had a poorer prognosis than those with low miR-224 expression, indicating that high miR-224 level is a biomarker of poor prognosis for patients with pediatric AML. Cox proportional hazards model adjusted for known prognostic variables such as FAB classification and cytogenetic abnormalities proved that miR-224 was an independent prognostic biomarker for pediatric AML. Thus, miR-224 could be used as a molecular prognostic marker additive to the known prognostic indicator, identifying patients who are more likely to have higher risk of death, thus, should receive more aggressive treatment. Of course, as the sample size in this study is not large enough, further multicenter studies should be necessary to testify the prognostic values of miR-224 expression.

In conclusion, our findings suggest for the first time that the upregulation of miR-224 may be one of the molecular mechanisms involved in the tumorigenesis and progression of pediatric AML. Since its correlation with poor DFS and OS, miR-224 upregulation may be used as an unfavorable prognostic biomarker in pediatric AML. Further studies are still needed to investigate the precise molecular mechanism of miR-224 dysregulation in pediatric AML and illustrate whether miR-224 may be used as a potential therapeutic target for the treatment of pediatric AML patients.

Disclosure of conflict of interest

None.

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