Int J Clin Exp Pathol 2015;8(11):14658-14666
www.ijcep.com /ISSN:1936-2625/IJCEP0015860

Original Article

Serum MicroRNA-370 as a potential diagnostic and prognostic biomarker for pediatric acute myeloid leukemia

Xiaofei Lin[1*], Zhengyan Wang[2*], Yumei Wang[3], Weijing Feng[4]

[1]Department of Pediatric Intensive Care Units, Huai'an Maternity and Child Health Hospital of Yangzhou University, Huai'an 223002, Jiangsu Province, P.R. China; [2]Department of Pediatrics, Huai'an Hospital Affiliated of Xuzhou Medical College and Huai'an Second People's Hospital, 62 Huaihai Road South, Huai'an 223002, P.R. China; [3]Department of Child Health Care, Huai'an Maternity and Child Health Hospital of Yangzhou University, Huai'an 223002, P.R. China; [4]Department of Pediatrics, Huai'an First People's Hospital, Nanjing Medical University, 6 Beijing Road West, Huai'an, Jiangsu 223300, P.R. China. [*]Equal contributors.

Received September 8, 2015; Accepted October 22, 2015; Epub November 1, 2015; Published November 15, 2015

Abstract: Background: Controversial data on the expression pattern of microRNA-370 (miR-370) in acute myeloid leukemia (AML) were previously reported. Objective: To clarify the expression pattern of miR-370 and its clinical implications in pediatric AML patients. Methods: Real-time quantitative PCR was performed to detect the expression of miR-370 in both bone marrow mononuclear cells and sera obtained from pediatric AML patients and healthy controls. Results: Compared with healthy controls, the expression levels of miR-370 in the bone marrow and sera of pediatric AML patients were both decreased significantly (both P=0.001). Importantly, serum miR-370 level could efficiently screen pediatric AML patients from healthy controls (Area under receiver operating characteristic curve, AUC =0.993). Then, low serum miR-370 level was significantly associated with French-American-British (FAB) classification subtype M7 subtype (P=0.02) and unfavorable karyotype (P=0.01). Moreover, pediatric AML patients with low serum miR-370 level had shorter relapse-free and overall survivals than those with high serum miR-370 level (both P=0.001). Multivariate analysis further identified serum miR-370 level as an independent prognostic factor for both relapse-free and overall survivals. Interestingly, the prognostic relevance of serum miR-370 level was more obvious in the subgroup of patients with intermediate-risk cytogenetics. Conclusions: MiR-370 expression may be markedly and consistently decreased in pediatric AML patients and in turn contributes to aggressive progression of this malignancy. Serum miR-370 may serve as a potential non-invasive diagnostic/prognostic marker for pediatric AML patients.

Keywords: Pediatric acute myeloid leukemia, microRNA-370, serum, real-time quantitative PCR, prognosis

Introduction

Acute myeloid leukemia (AML) is a malignant disorder of clonal hematopoietic stem cell, characterized by the rapid proliferation of leukemic blasts leading to abnormal accumulation of immature precursors and suppression of growth and maturation of cells involved in normal hematopoiesis [1, 2]. AML accounts for more than 30% of the deaths from pediatric leukemia, although it makes up only 15~20% of pediatric leukemia [3]. The prognosis for AML patients is very variable, ranging from survival of a few days to cure. In the most successful studies, the 5-year disease-free survival in pediatric AML patients is approximately 50% [4]. Based on the World Health Organization (WHO) categorization of AML, cytogenetic and molecular analyses play a crucial role in predicting the remission and survival rates of AML patients [5]. However, it is very difficult to estimate the prognosis of an individual AML patient accurately and the molecular mechanism in the progression of AML is also elusive. It is therefore extremely necessary to identify novel biochemical markers for the early detection and the prediction of treatment response of AML patients.

MicroRNAs (miRNAs) are a family of small non-coding RNA molecules with 18-25 nucleotides in length and function as a class of negative

Table 1. Association of serum miR-370 level with clinical characteristics of 106 pediatric acute myeloid leukemia patients

Clinical variables	No. of patients (%)	Serum miR-370 (n, %)		P
		Low	High	
Gender				
Male	58 (54.72)	30 (51.72)	28 (48.28)	NS
Female	48 (45.28)	28 (58.33)	20 (41.67)	
Age (year)				
>6	40 (37.74)	25 (62.50)	15 (37.50)	NS
≤6	66 (62.26)	33 (50.00)	33 (50.00)	
Leukocyte (/μL)				
>10,000	66 (62.26)	36 (54.55)	30 (45.45)	NS
≤10,000	40 (37.74)	22 (55.00)	18 (45.00)	
FAB classification				0.02
M0	3 (2.83)	0 (0)	3 (100.0)	
M1/M2	62 (58.49)	26 (41.94)	36 (58.06)	
M3	10 (9.43)	6 (60.0)	4 (40.0)	
M4/M5	21 (19.81)	16 (76.19)	5 (23.81)	
M7	10 (9.43)	10 (100.00)	0 (0)	
Extramedullary disease				
Absent	80 (75.47)	45 (56.25)	35 (43.75)	NS
Present	26 (24.53)	13 (50.00)	13 (50.00)	
Cytogenetics				
Favorable	35 (33.02)	20 (57.14)	15 (42.86)	NS
Intermediate	52 (49.06)	25 (48.08)	27 (51.92)	
Unfavorable	19 (17.92)	13 (68.42)	6 (31.58)	
Day 7 response to treatment				
Favorable	65 (61.32)	23 (35.38)	42 (64.62)	0.01
Unfavorable	41 (38.68)	35 (85.37)	6 (14.63)	

'NS' refers to the difference without statistical significance.

regulators of post-transcriptional gene expression through directly targeting the 3'-untranslated regions (3'-UTR) of target mRNAs, leading to the degradation or translation suppression of target mRNAs [6]. miRNAs are highly conserved in the genomes of invertebrates, vertebrates and plants [7]. Physiologically, miRNAs play an important role in various biological processes including development, embryogenesis, proliferation, differentiation, organogenesis, and apoptosis [8]. Pathologically, aberrant expression of miRNAs has been observed in many diseases, including cancer [9]. Increasing studies have shown that the dysregulation of miRNAs may contribute to the aberrant activation of oncogenes and the inactivation of tumor suppressor genes in human carcinogenesis [10]. Since miRNAs are stable in blood and their expression patterns have tissue specificity, miRNA expression profiles in serum may be used as non-invasive biomarkers to classify specific cancers [11, 12]. Especially in the research of leukemia, recent studies have highlighted the potentials of aberrant expression patterns and functional abnormalities in various miRNAs as prognostic markers or therapeutic targets for AML [13, 14].

Growing evidence show that miR-370 may function either as a tumor suppressor or as an oncogene in various human cancer types, implying its crucial roles during tumor development and progression. García-Ortí et al. [15] in 2012 performed an integrative analysis including data from high resolution SNP arrays, mRNA expression arrays, and miRNA expression profiles in 16 myeloid cell lines and highlighted that miR-370 expression was upregulated in AML cells, which could increase cellular proliferation and colony formation. In contrast, Zhang et al. [16] in the same year reported that the downregulation of miR-370 expression was a frequent event in both leukemia cell lines and primary leukemic cells from patients with de novo AML. They also confirmed that miR-370 might function as a tumor suppressor which could inhibit the cell cycle progression of AML cells. Taking into account the limited and controversial data regarding the involvement of miR-370 in human AML, we here aimed to clarify the expression pattern of miR-370 and its clinical implications in pediatric AML patients.

Materials and methods

Patients and tissue samples

The present study was approved by Huai'an First People's Hospital Ethics Committee. Prior informed consent was obtained from the patients for the collection of specimens in accordance with the guidelines of Huai'an First People's Hospital, China. All specimens were

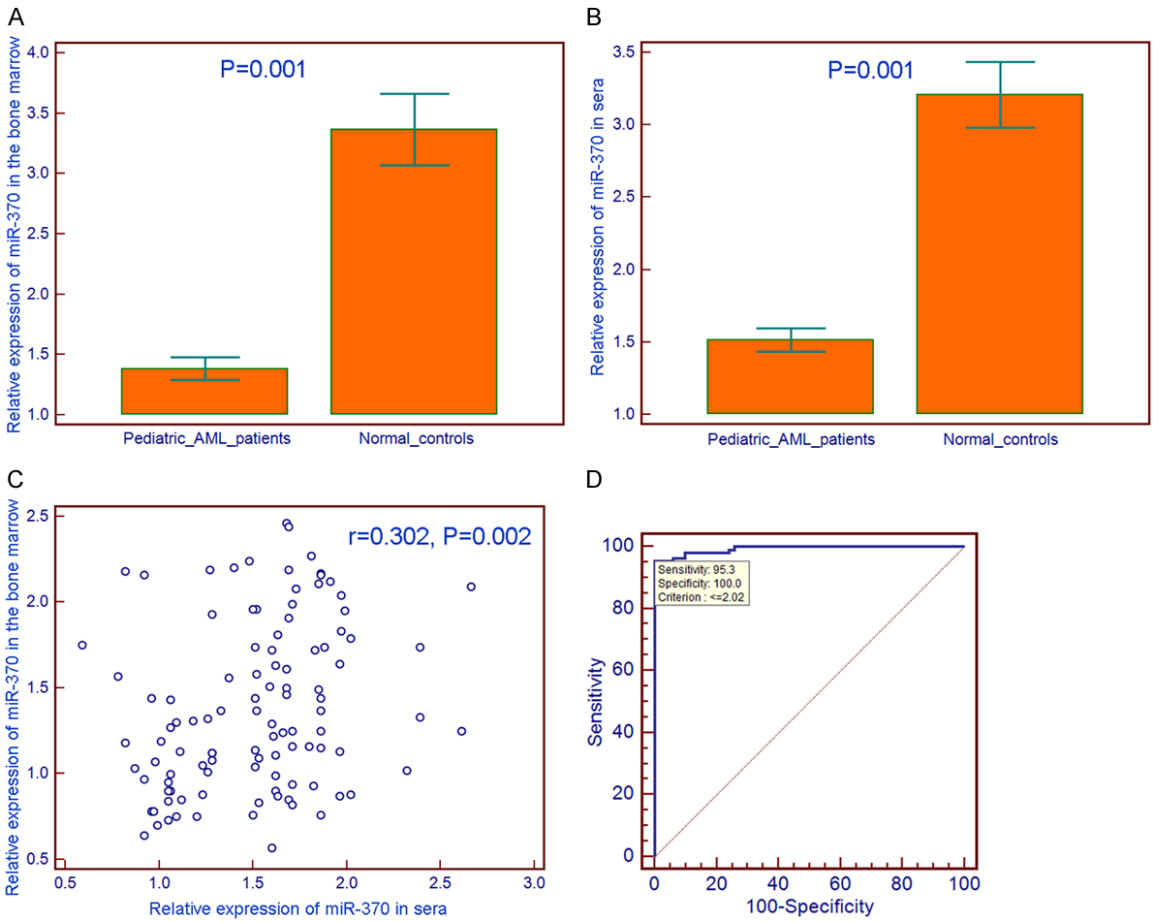

Figure 1. Decreased expression of microRNA (miR)-370 in pediatric acute myeloid leukemia (AML) patients. A. Compared with normal controls, miR-370 expression in the bone marrow of pediatric AML patients were significantly decreased (AML vs. normal: 1.38±0.48 vs. 3.36±0.63, P=0.001). B. Serum miR-370 level in pediatric AML patients were dramatically lower than that in healthy controls (AML vs. normal: 1.51±0.41 vs. 3.21±1.80, P=0.001). C. Spearman's correlation analysis showed that the expression levels of miR-370 in the bone marrow of pediatric AML patients were closely correlated with those in patients' sera (Spearman's correlation: r=0.302, P=0.002). D. ROC curve analysis illustrated that the serum miR-370 level was a potential biomarker for screening pediatric AML patients from healthy controls with the area under the ROC curve (AUC) of 0.993, and the serum miR-370 level at 2.02 was the clear cutoff value to screen pediatric AML patients from healthy controls. Based on this cutoff value, the sensitivity and specificity of the serum miR-370 level for distinguishing AML was 95.30% and 100.00%, respectively.

handled and made anonymous according to the ethical and legal standards.

A total of 106 pediatric AML patients, including 58 boys and 48 girls, were collected from the Department of Pathology, Huai'an First People's Hospital, China. All the patients were younger than 18 years of age (median 6 years). The diagnosis of AML was made according to a morphologic assessment of the Wright-Giemsa stained smears of the bone marrow aspirates along with special stains and immunophenotyping by flow cytometry. Laboratory investigation included conventional and molecular cytogenetic analyses. The median leukocyte count at diagnosis was 20,606/μL (range 420-352,906/μL). According to the French-American-British (FAB) classification, 3 patients had AML M0, 62 had M1/M2, 10 had M3, 21 had M4/M5 and 10 had M7. Among 26 patients with extramedullary disease, 21 patients had chloroma (scalp in 10 patients, orbit in 6 patients and skin in 5 patients), and 5 patients had a central nervous system involvement of leukemic cells. The clinical characteristic of 106 pediatric AML patients was summarized in **Table 1**.

All pediatric AML patients 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

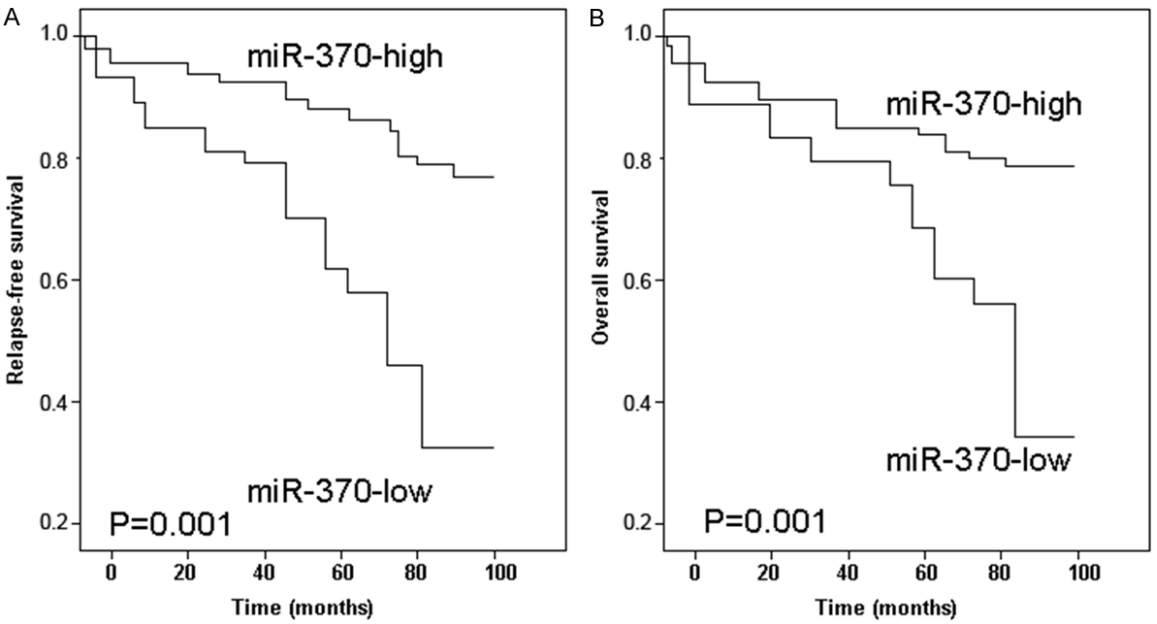

Figure 2. Kaplan-Meier curves of relapse-free survival (RFS, A) and overall survival (OS, B) of pediatric AML patients stratified by the serum miR-370 levels. Patients with low serum miR-370 levels had shorter RFS and OS than those with low levels (both P<0.001).

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. If complete remission (CR) was not achieved after the primary induction chemotherapy regimen, an additional course of induction chemotherapy using high-dose 1-h-D-arabinofuranosylcytosine was given. Once CR 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 stem-cell donor.

As normal controls, bone marrow was collected from 20 patients (12 boys and 8 girls; median age, 9 years; range, 3-18 years) with various diseases but with normal bone marrow morphology as demonstrated by cytological and histological analyses; Serum was collected from 50 healthy volunteers (30 boys and 20 girls; median age, 12 years; range, 5-18 years). Volunteers were all healthy, were not on medication, and had no signs or clinical symptoms of cancer, joint, liver, metabolic or endocrine diseases.

Real-time quantitative RT-PCR for miRNA

Real-time quantitative RT-PCR for miRNA was performed to detect miR-370 expression in bone marrow mononuclear cells and serum. Mononuclear cells were isolated by Ficoll Hypaque density gradient centrifugation of 2 ml bone marrow samples in EDTA from newly diagnosed patients and control samples. Total RNA in bone marrow mononuclear cells and serum were both extracted using a QIAamp RNA Blood kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNU6B were used as control genes to exclude any possible heterogeneous expression in the different AML subtypes. The miR-370 and RNU6B-specific cDNA were synthesized from total RNA using gene-specific primers according to the TaqMan MicroRNA assays protocol (Applied Biosystems, Foster City, CA, USA). The primer sequences were as following: for miR-370: RT 5'-GTC GTA TCC AGT GCA GGG TCC GAG GTG CAC TGG ATA CGA CAC CAG G-3', PCR forward 5'-TGC GGG CCT GCT GGG GTG GAA C-3', PCR reverse 5'-CCA GTG CAG GGT CCG AGG T -3'; for RNU6B: RT 5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA AAA TAT GGA AC-3', PCR forward 5'-TGC GGG TGC TCG CTT CGG CAG C-3', PCR reverse 5'-CCA GTG CAG GGT CCG AGG T-3'. Relative quantifi-

Table 2. Univariate analysis of the impact of variables on relapse-free survival and overall survival in pediatric AML patients

Variable	No. of patients	Relapse-free survival		Overall survival	
		Median (months ± S.D.)	P	Median (months ± S.D.)	P
Cytogenetics					
Favorable	35	Not reached	<0.001	Not reached	<0.001
Intermediate	52	12.61±1.82		22.90±2.12	
Unfavorable	19	3.14±0.53		9.81±1.73	
FAB classification					
M1-M6	96	14.26±2.19	0.01	36.26±3.81	0.008
M7	10	7.92±1.44		20.72±2.52	
MiR-370 expression					
Low	58	6.22±1.18	0.001	15.89±1.26	0.001
High	48	13.67±2.38		39.82±3.82	

cation of target miRNA expression was evaluated using the comparative cycle threshold (CT) method. The raw data were presented as the relative quantity of target miRNA, normalized with respect to RNU6B. Each sample was examined in triplicate. Mean normalized gene expression ± standard deviation (SD) was calculated from independent experiments.

Statistical analysis

The software of SPSS version 16.0 for Windows (SPSS Inc, IL, USA) and SAS 9.1 (SAS Institute, Cary, NC) was used for statistical analysis. When the equal variance test or normality test failed, the Kruskall-Wallis non-parametric test was applied. The correlation of miR-370 expression between bone marrow mononuclear cells and sera was determined by Spearman Correlation analysis. The receiver operating characteristic (ROC) curve was drawn to evaluate the diagnosis value of serum miR-370 level. The Chi-square test was used to show differences of categorical variables. The Kaplan-Meier survival curves were used to determine any significant relationship between the serum level of miR-370 expression and the status of the patients with respect to relapse-free survival (RFS) or overall survival (OS). Differences were considered statistically significant when *P* was less than 0.05.

Results

Decreased expression of miR-370 in pediatric AML patients

Compared with normal controls, miR-370 expression in the bone marrow of pediatric AML patients were significantly decreased (AML vs. normal: 1.38±0.48 vs. 3.36±0.63, P=0.001, **Figure 1A**). Similarly, the serum miR-370 level in pediatric AML patients were dramatically lower than that in healthy controls (AML vs. normal: 1.51±0.41 vs. 3.21±1.80, P=0.001, **Figure 1B**). In addition, Spearman's correlation analysis showed that the expression levels of miR-370 in the bone marrow of pediatric AML patients were closely correlated with those in patients' sera (Spearman's correlation: r=0.302, P=0.002, **Figure 1C**). Therefore, we further assessed the clinical implications of miR-370 expression in pediatric AML patients using its serum levels.

More importantly, ROC curve analysis illustrated that the serum miR-370 level was a potential biomarker for screening pediatric AML patients from healthy controls with the area under the ROC curve (AUC) of 0.993, and the serum miR-370 level at 2.02 was the clear cutoff value to screen pediatric AML patients from healthy controls. Based on this cutoff value, the sensitivity and specificity of the serum miR-370 level for distinguishing AML was 95.30% and 100.00%, respectively (**Figure 1D**).

Decreased expression of miR-370 associates with aggressive clinical characteristics of pediatric AML patients

To investigate the associations of serum miR-370 level with the clinical characteristics of pediatric AML patients, the median value of serum miR-370 (1.59) expression was used to divide 106 pediatric AML patients into miR-370-low (n=58) and miR-370-high (n=48) expression groups. As shown in **Table 1**, low

Table 3. Multivariate analysis of the impact of variables on relapse-free survival and overall survival in pediatric AML patients

Variable	P	Odds Ratio
Relapse-free survival		
Cytogenetics		
Unfavorable vs. Favorable/ Intermediate	0.01	5.92
FAB classification		
M7 vs. M1-M6	0.06	2.33
MiR-370 expression		
Low vs. High	0.01	5.56
Overall survival		
Cytogenetics		
Unfavorable vs. Favorable/ Intermediate	0.009	7.09
FAB classification		
M7 vs. M1-M6	0.06	2.52
MiR-370 expression		
Low vs. High	0.01	6.29

serum miR-370 level was significantly associated with FAB classification subtype M7 subtype (P=0.02) and unfavorable karyotype (P=0.01). No significant associations of serum miR-370 level with patients' gender and age, leukocyte count, extramedullary disease and day 7 response to treatment were found (all P>0.05, **Table 1**).

Decreased expression of miR-370 predicts unfavorable clinical outcome of pediatric AML patients

All 106 pediatric AML patients received follow-up analysis. The median follow-up duration was 35 months ranged from 10~86 months. Kaplan-Meier curves for RFS and OS stratified according to serum miR-370 levels in pediatric AML patients were shown in **Figure 2**. We found that pediatric AML patients with low serum miR-370 levels had shorter RFS and OS than those with high serum miR-370 levels (both P=0.001, **Figure 2**). In addition, univariate analysis showed that the RFS in the patients with the French-American-British classification subtype M7 (P=0.01, **Table 2**), unfavorable cytogenetic abnormalities (P<0.001, **Table 2**), and low serum miR-370 level (P=0.001, **Table 2**) were all significantly shorter than those with the corresponding controls. However, other clinical characteristics including patients' gender and age, Leukocyte counts, the presence of extramedullary disease, and day 7 response to treatment were not significantly associated with RFS of pediatric AML patients (all P>0.05, data were not shown). Moreover, the French-American-British classification subtype M7 (P=0.008, **Table 2**), unfavorable cytogenetic abnormalities (P<0.001, **Table 2**) and low serum miR-370 level (P=0.001, **Table 2**) were also associated with poor OS. Furthermore, Cox proportional hazards multivariate analysis of the univariate predictors identified cytogenetic abnormalities (P=0.01 and 0.009, respectively) and serum miR-370 level (both P=0.01) as independent prognostic factors for both RFS and OS (**Table 3**).

More importantly, a separate univariate analysis of the intermediate cytogenetic risk group demonstrated that low serum miR-370 level was significantly associated with unfavorable RFS and OS (both P<0.001, **Figure 3**). But no associations were found in the favorable and adverse cytogenetic risk groups (P>0.5 for all comparisons, data were not shown).

Discussion

MiRNAs regulate gene expression, and thus play an important role in critical cellular processes. Dysregulation of miRNAs can cause cancer and miRNA expression signatures have been used to identify cancers. However, information about the expression of miRNAs in pediatric AML is still limited. The current study is the first report on the clinical implications of the novel serum marker miR-370 in pediatric AML patients. We observed the decreased expression of miR-370 in the bone marrow and the serum from pediatric patients with newly diagnosed AML when compared with normal controls. ROC analysis showed that serum miR-370 level could efficiently screen pediatric AML patients from healthy controls. Then, the low serum miR-370 level was dramatically correlated with the aggressive progression of pediatric AML patients. Moreover, both univariate and multivariate analyses demonstrated that serum miR-370 level was independent of other prognostic factors, including the cytogenetic abnormalities, although the influence of cytogenetics might be underestimated because of the small number of our cohort. Furthermore, the prognostic value of serum miR-370 level was espe-

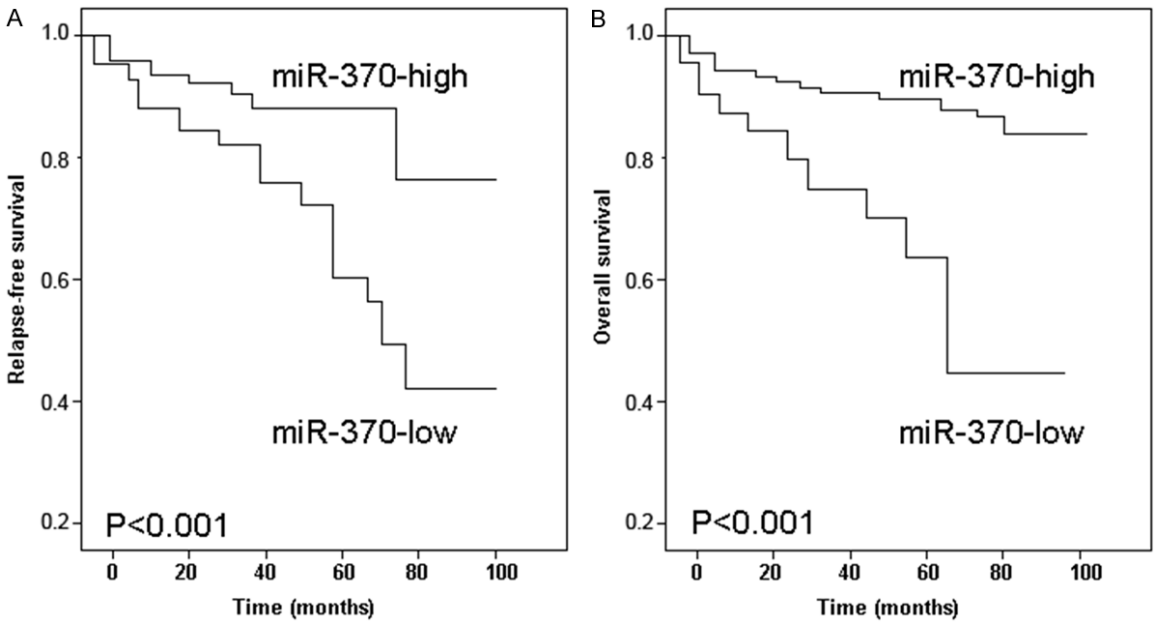

Figure 3. Kaplan-Meier curves of relapse-free survival (RFS, A) and overall survival (OS, B) of pediatric AML patients in the intermediate cytogenetic risk group stratified by the serum miR-370 levels. A separate univariate analysis of the intermediate cytogenetic risk group demonstrated that low serum miR-370 level was significantly associated with unfavorable RFS and OS (both P<0.001).

cially more obvious in the subgroup of patients with intermediate-risk cytogenetics. These findings highlight the diagnostic and prognostic potentials of miR-370 in pediatric AML patients.

MiR-370 is located within the imprinted DLK1-DIO3 region on human chromosome 14q32, which is regulated by DNA CpG island methylation 200 bp upstream of the mother allele [17]. It was originally cloned from human embryonic stem cells with a very low expression level [18]. The DLK1-DIO3 region has been regarded as a cancer-associated genomic region, suggesting the implication of miR-370 in carcinogenesis [19]. The role of miR-370 in various malignances remains controversial. For example, Chen et al. [20] discovered that miR-370 was down-regulated in endometrioid ovarian cancer cells and miR-370 could suppressed cellular viability and colony formation in IGROV1 and TOV112D endometrioid ovarian cancer cells; Yungang et al. [21] verified that miR-370 might play a tumor suppressive role in laryngeal squamous cell carcinoma by targeting FoxM1; MiR-370 has also been identified as a tumor suppressor in bladder cancer and cholangiocarcinoma cell lines [22, 23]; In contrast, Fan et al. [24] indicated that the overexpression of miR-370 in gastric cancer cells could promote the cell proliferation and anchorage-independent growth, while silencing of miR-370 showed opposite effects; Wu et al. [25] reported that miR-370 expression was significantly upregulated in five prostate cancer cell lines when compared to normal prostatic epithelial cells and the enforced expression of miR-370 could induce proliferation in human prostate cancer cells. From this context, miR-370 may play an important role in human malignancies with a cancer-type dependent manner. In the current study, our data showed the decreased level of miR-370 expression in both bone marrow and serum of from pediatric AML patients, which was in line with the finding of Zhang et al. [15] but not with that of Garcı´a-Ortı´ et al. [16]. The difference might be caused by the heterogeneity of cohorts enrolled in different studies. Since miRNAs are notably stable in blood and their expression patterns appear to be tissue-specific, serum miRNAs have been recognized as good candidates for noninvasive testing for cancer. In order to determine whether miR-370 can be used as a marker for pediatric AML, we also statistically analyzed the correlation of serum miR-370 with clinical characteristics and prognosis in patients with this disease. As the result, serum miR-370 could screen pediatric AML patients from healthy controls with an AUC

value (0.993) approximating 1.0, suggesting that it had a relatively high ability to identify the true pediatric AML patients. Then, the decreased serum miR-370 level was significantly associated with the French-American-British classification subtype M7 and the unfavorable cytogenetic risks. The survival analysis further identified serum miR-370 as an independent prognostic marker for both RFS and OS. More importantly, there may be a significant association between serum miR-370 level and the prognosis in patients of the intermediate cytogenetic risk group, but not in those of the favorable and unfavorable cytogenetic risk groups.

In conclusion, our data offer the convincing evidence that miR-370 expression may be markedly and consistently decreased in pediatric AML patients and in turn contributes to aggressive progression of this malignancy. Serum miR-370 may serve as a potential non-invasive diagnostic/prognostic marker for pediatric AML patients. Although our results gave a high accurate predictive value for our cohort, there are several limitations to this study. For example, the intermediate cytogenetic risk group in our cohort is small in this moderate-sized pediatric sample, and the exact molecular mechanisms on the involvement of miR-370 dysregulation in pediatric AMLs required further investigations.

Disclosure of conflict of interest

None.

Address correspondence to: Weijing Feng, Department of Pediatrics, Huai'an First People's Hospital, Nanjing Medical University, 6 Beijing Road West, Huai'an, Jiangsu 223300, P.R. China. Tel: (86) 517-84952301; Fax: (86) 517-84952301; E-mail: 18752311209@163.com

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