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Original Article

Decreased *miR-218* expression predicts unfavorable prognosis in de novo acute myeloid leukemia

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Abstract: Purpose: This study was aimed to investigate the expression status of *miR-218* and its clinical relevance in patients with acute myeloid leukemia (AML). Methods: *MiR-218* expression was detected using real-time quantitative PCR in 106 AML patients and 25 controls. Results: *MiR-218* expression was significantly down-regulated in AML compared to controls (*P=0.001*). *MiR-218* low-expressed patients had significantly shorter overall survival (OS) than *miR-218* high-expressed patients in all AML (median 3.5 and 7 months, respectively, *P=0.013*) and AML with normal karyotype (median 3 and 6 months, respectively, *P=0.030*). Multivariate analysis confirmed low *miR-218* expression as an independent risk factor not only in all AML (*P=0.013*) but also in cytogenetically normal AML (*P=0.040*). Conclusions: Our findings indicate that down-regulated *miR-218* is a common event and predicts unfavorable prognosis in de novo AML patients.

Keywords: *MiR-218*, AML, prognosis

Introduction

Acute myeloid leukemia (AML) is a clonal hematopoietic malignant disease which is characterized by the accumulation of immature myeloid progenitor cells in the bone marrow (BM) and peripheral blood. Somatic gene mutations, nonrandom chromosomal translocations, genetic abnormalities, and epigenetic alterations play important roles in the pathogenesis of AML [1, 2]. Recently, dysregulation of microRNAs (miRNAs) expression is also involved in cancer occurrence and development including leukemia [3]. Moreover, abnormal expression of miRNAs could also provide helpful information for the prognosis of AML [4, 5].

MiRNAs are small noncoding RNA molecules that consist of 18-22 nucleotides with the function in regulating gene expression and diverse physiologic functions like cell proliferation, cell differentiation, and cell apoptosis and so on [6-13]. An increasing number of studies revealed that *microRNA-218* (*miR-218*) acted as a tumor suppressor gene by targeting many oncogenes related to proliferation, invasion, and apoptosis [14-19]. Moreover, reduced *miR-218* expression was shown in several solid tumors including gastric cancer, breast cancer, and colorectal cancer [14-19]. Furthermore, in some cancers, *miR-218* dysregulation also correlated with clinical staging, metastasis, and prognosis [20-22]. However, the status of *miR-218* expression in AML remains unknown. This study was aimed to investigate the expression status of *miR-218* and its clinical relevance in de novo AML patients.

Materials and methods

Patients and samples

This study included 106 patients who had a diagnosis of de novo AML at the Affiliated People's Hospital of Jiangsu University. BM was collected from all patients after providing written informed consent. The diagnosis and classification of AML patients were based on the French-American-British (FAB) and World Health Organization (WHO) criteria (blast ≥20%). The study was approved by the Institutional Review Board of the Affiliated People's Hospital of

Table 1. Correlation between *miR-218* expression and patients parameters

Patient's parameters	Status of *miR-218* expression		
	Low (n=56)	High (n=50)	*P*
Sex, male/female	35/21	31/19	1.000
Median age, years (range)	59 (25-93)	51 (20-86)	0.006
Median WBC, $\times 10^9$/L (range)	13.8 (0.3-197.7)	8.4 (0.5-528.0)	0.455
Median hemoglobin, g/L (range)	82 (40-138)	68.5 (34-131)	0.062
Median platelets, $\times 10^9$/L (range)	32 (5-399)	35 (3-136)	0.759
BM blasts, % (range)	45.5 (1-94.5)	40 (3-97.5)	0.463
FAB			0.275
M1	4	0	
M2	27	25	
M3	10	9	
M4	13	10	
M5	2	5	
M6	0	1	
WHO			0.171
AML with t (8;21)	6	9	
APL with t (15;17)	10	8	
AML without maturation	5	0	
AML with maturation	22	18	
Acute myelomonocytic leukemia	12	10	
Acute monoblastic and monocytic leukemia	1	4	
Acute erythroid leukemia	0	1	
Karyotype classification			0.105
Favorable	16	17	
Intermediate	36	25	
Poor	2	7	
No data	2	1	
Karyotype			0.431
Normal	29	20	
t (8;21)	6	9	
t (15;17)	10	8	
Complex	3	7	
Others	6	5	
No data	2	1	
Gene Mutation			
C-KIT (+/-)	2/52	2/48	1.000
FLT3 ITD (+/-)	6/49	9/41	0.404
NPM1 (+/-)	6/44	5/45	1.000
C/EBPA (+/-)	4/50	10/37	0.081
CR (+/-)	30/24	22/23	0.525

WBC, white blood cells; FAB, French-American-British classification; AML, acute myeloid leukemia; CR, complete remission; +: positive; -: negative.

Jiangsu University. The clinical characteristics of patients were listed in **Table 1**. Bone marrow (BM) form a total of 25 healthy donors was collected as controls. Treatment protocol for AML patients was described previously [20-22].

RNA extraction and reverse transcription

Total RNA was extracted using the mirVana miRNA isolation kit (Ambion, Austin, TX, USA) and reverse transcribed to cDNA using miScript

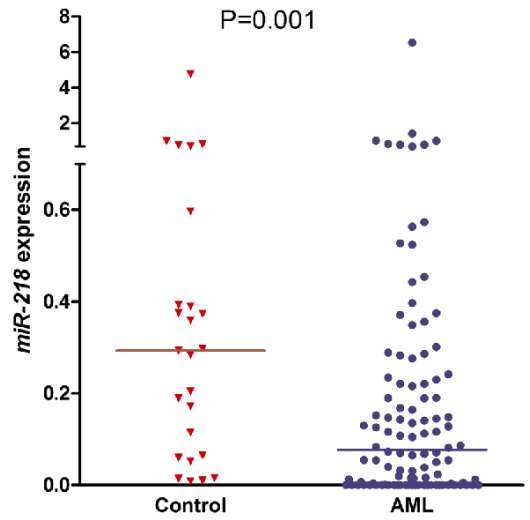

Figure 1. Relative expression levels of *miR-218* in AML patients and controls.

Reverse Transcription Kit (Qiagen, Duesseldorf, Germany).

Real-time quantitative PCR

Real-time quantitative PCR (RQ-PCR) was performed according to the manufacturer's instructions using miScript SYBR green PCR kit (Qiagen, catalog no. 218073) with the manufacturer-provided miScript Universal primer and *miR-218*-specific forward primer (5'-TTGTGCTTGATCTAACCATGT-3') in ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). RQ-PCR amplification consisted of an initial denaturation step of 95°C for 15 min followed by 40 cycles of a denaturation step at 94°C for 15 s, an annealing step at 55°C for 30 s, and an extension step of 72°C for 34 s. At the end of the PCR cycles, melting program (95°C for 15 s, 60°C for 60 s, 95°C for 15 s, and 60°C for 15 s) was performed to validate the specificity of the expected PCR product. The relative expression level of *miR-218* was calculated by the comparative $2^{-\Delta\Delta}$Ct method using U6 small nuclear RNA levels for normalization.

Gene mutation detection

NPM1 and *C-KIT* mutations were detected by high-resolution melting analysis (HRMA) as reported previously [24]. Mutation scanning was performed for PCR products using HRMA with the Light Scanner platform (Idaho Technology Inc., Salt Lake City, Utah). All positive samples were directly DNA sequenced to confirm the results of HRMA. *FLT3* internal tandem duplication (*ITD*) and *C/EBPA* mutations were detected using direct DNA sequencing [25, 26].

Statistical analyses

All statistical analyses were performed using the SPSS 20.0 software package (SPSS, Chicago, IL, USA). Pearson χ^2 analysis or Fisher exact test was employed to compare the difference of categorical variables. Mann-Whitney's *U* test was used to compare the difference of continuous variables. Receiver operating characteristic curve (ROC) and an area under the ROC curve (AUC) were established to assess the value of *miR-218* expression in distinguishing AML patients from normal controls. Overall survival (OS) was measured from the date of diagnosis until the date of death regardless of cause. OS was compared according to the Kaplan-Meier method. To further investigate the effect of *miR-218* expression, a Cox proportional hazards model was constructed, adjusting for potential confounding covariates, using backward elimination. For all analyses, two-tailed *p*-values less than 0.05 were determined statistically significant

Results

MiR-218 expression in AML

We evaluated the level of *miR-218* expression in AML patients and controls. The transcript level of *miR-218* in controls ranged from 0.014 to 4.744 with a median level of 0.293. However, *miR-218* transcript in AML (range 0.000-6.527, median 0.055) was significantly down-regulated compared with controls (*P*=0.001, **Figure 1**).

Evaluation of miR-218 expression as a potential differentiating marker

ROC curve revealed that the level of *miR-218* expression could be available as a potential biomarker for differentiating AML from controls with an AUC of 0.706 (95% CI=0.601-0.811, *P*=0.001, **Figure 2A**). At the cut-off value of 0.068, the sensitivity and the specificity were 60% and 80%, respectively. Moreover, ROC curves also pointed out that *miR-218* level might act as a valuable biomarker in cytoge-

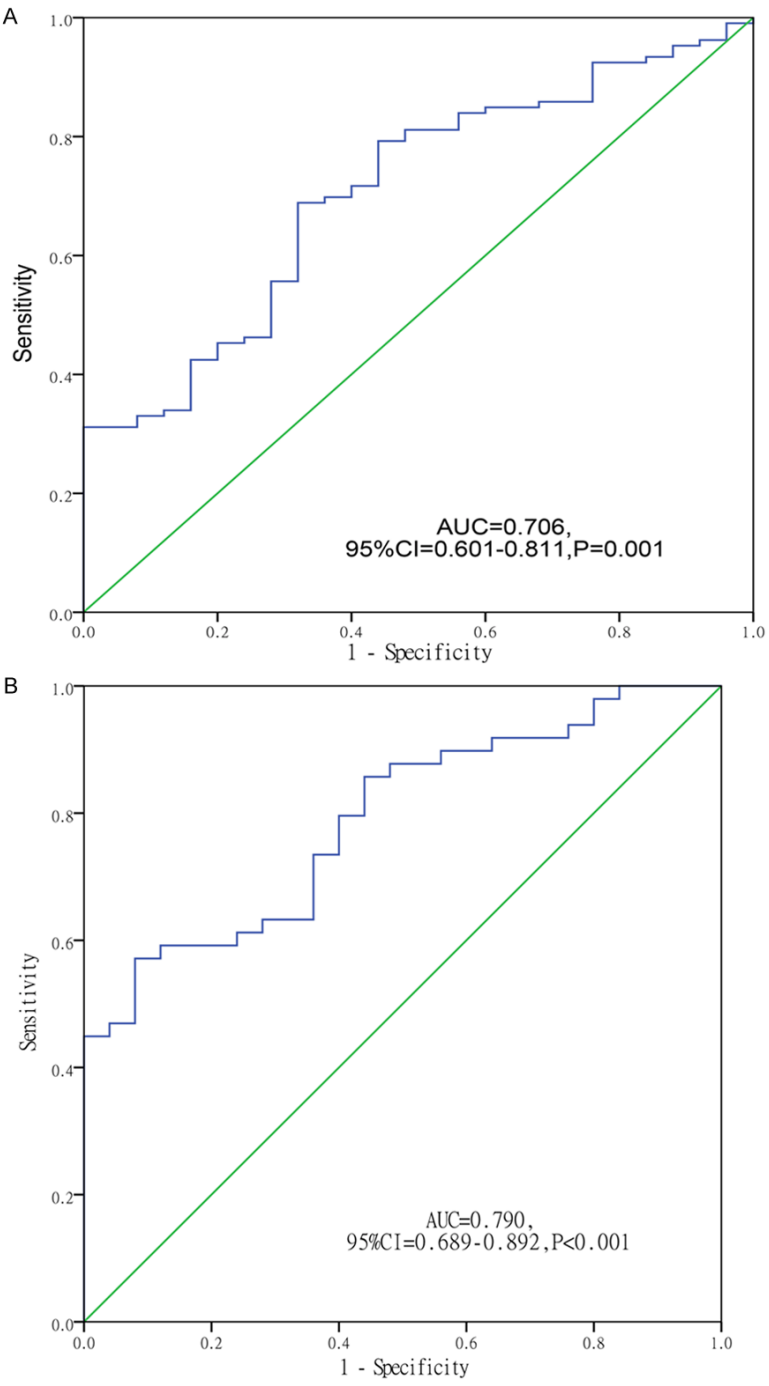

Figure 2. A. ROC curve analysis using *miR-218* for discriminating AML patients in all patients. B. ROC curve analysis using *miR-218* for discriminating AML patients incytogenetically normal patients.

netically normal AML (CN-AML) (AUC=0.790, 95% CI: 0.689-0.892, *P*<0.001) (**Figure 2B**).

Clinical and laboratory characteristics of AML

According to the set cut-off value of 0.068, this cohort of 106 AML patients was divided into two groups: low *miR-218* expression (≤0.068) and high *miR-218* expression (>0.068). There were no significant differences in gender, white blood cells, hemoglobin, platelet count, percentage of BM blasts, WHO or FAB classifications, and gene mutations between the two groups (**Table 1**). However, the patients with low *miR-218* expression had significantly older age than those with high *miR-218* expression (*P*= 0.006, **Table 1**).

Impact of miR-218 expression on outcome of AML patients

A total of 105 patients with follow up data were included in complete remission (CR) analysis. After induction therapy, *miR-218* low-expressed cases showed similar CR rate as compared with *miR-218* high-expressed cases (**Table 1**). Furthermore, no significant differences were also observed between the two groups CR rate in CN-AML patients (*P*=1.000). To investigate the prognostic impact of *miR-218* expression in AML, survival data was obtained for 103 AML patients with mean follow-up time of 10 months (range, 1-57 months). Low *miR-218*-expressing patients had significantly shorter overall survival (OS) than high *miR-218*-expressing patients (median 3.5 and 7 months, respectively, *P*=0.013) (**Figure 3A**). Moreover, the patients with low *miR-218*-expression also had significantly shorter OS than those with high *miR-218*-expression among CN-AML (median 3 and 6 months, respectively, *P*=0.030) (**Figure 3B**).

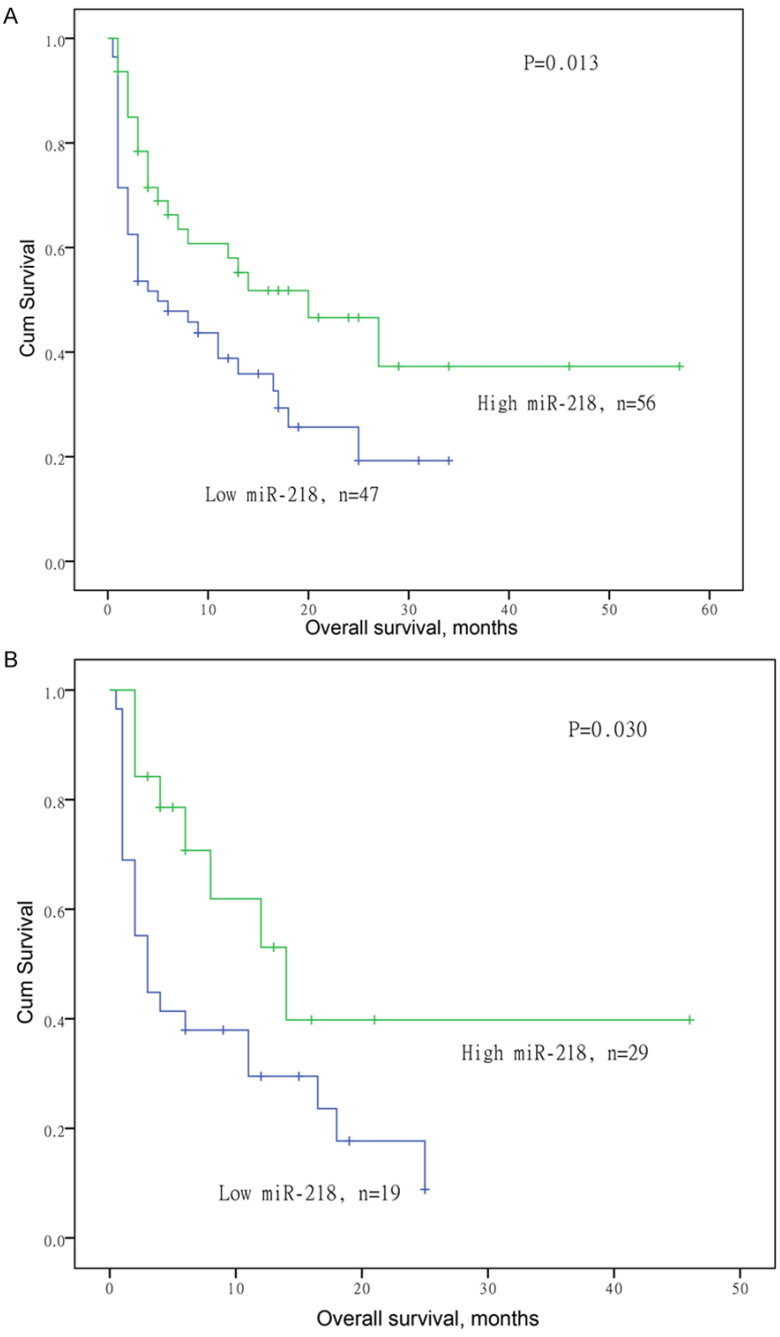

Figure 3. A. Overall survival of AML patients in all patients. B. Overall survival of AML patients incytogenetically normal patients.

A multivariable analysis was conducted to determine if low *miR-218* expression was an independent prognostic factor for OS in AML once the model was adjusted for other characteristics. Variables considered for model inclusion were sex, age (≤ 60 years vs. > 60 years), WBC (< 30×10^9/L vs. ≥ 30×10^9/L), gene mutations, karyotypic classification, and *miR-218* expression. After adjusting for other covariates, *miR-218* expression remained a significant predictor for OS in entire AML (**Table 2**). Additionally, the independent prognostic impact of *miR-218* expression was also identified in CN-AML (**Table 3**).

Discussion

Recently, the function role of *miR-218* in tumorigenesis has been increasingly demonstrated. Recent studies have shown that *miR-218* could increase chemosensitivity and induce apoptosis in gastrointestinal stromal tumor [14, 27, 28]. Furthermore, *miR-218* also was proved to inhibit metastasis and invasion in cervical cancer, pancreatic cancer, liver cancer, and lung cancer [29-31]. In view of the tumor suppressor nature of *miR-218*, an increasing number of studies investigated whether *miR-218* could be considered as a promising biomarker in cancers. Down-regulation of *miR-218* has been found in many cancers such as in bladder, prostate, colorectal, cervical, thyroid, gastric cancers, nasopharyngeal carcinoma (NPC), glioma and so on [15, 32-38]. Moreover, prognostic significance of *miR-218* expression has been revealed in several solid tumors. Huang et al. demonstrated that low expression of *miR-218* was intimately correlated with poor prognosis in small cell carcinoma of the cervix [39]. Li et al. manifested that decreased *miR-218* expression was a prognostically negative biomarker in gastric cancer patients [40]. Peng et al. found that down-regulation of *miR-218* was associated with poor prognosis in oral cav-

Table 2. Multivariate analyses of prognostic factors for overall survival in AML

	Hazard ratio (95% CI)	*P* value
Sex	0.976 (0.537-1.774)	0.937
Age	2.144 (1.204-3.818)	0.010
WBC	1.434 (0.796-2.581)	0.230
Karyotype classification	2.460 (1.449-4.178)	0.001
miR-218 expression	0.478 (0.267-0.857)	0.013
*C-KIT*mutation	1.018 (0.227-4.565)	0.982
C/EBPA mutation	1.704 (0.617-4.706)	0.304
NPM1 mutation	1.489 (0.556-3.983)	0.428
FLT3-ITD	0.590 (0.223-1.564)	0.289

Table 3. Multivariate analyses of prognostic factors for overall survival in CN-AML

	Hazard ratio (95% CI)	*P* value
Sex	0.916 (0.366-2.296)	0.852
Age	2.045 (0.922-4.537)	0.079
WBC	1.614 (0.738-3.527)	0.231
miR-218 expression	0.416 (0.181-0.959)	0.040
C/EBPA mutation	1.924 (0.474-7.812)	0.360
NPM1 mutation	1.231 (0.410-3.697)	0.710
FLT3-ITD	0.709 (0.159-3.161)	0.652

ity squamous cell carcinoma [41]. Moreover, the prognostic value of decreased expression of *miR-218* was also shown in patients with colorectal cancer [42].

Decreased *miR-218* expression was discovered in AML patients especially with t (8;16) [43]. However, the clinical relevance of *miR-218* dysregulation was not described in AML. Karyotypic changes have been introduced into the risk stratification and treatment choice of newly diagnosed de novo AML [44-47]. Normal karyotypes which are classified in the intermediate prognostic category, constitute the largest cytogenetic subset of AML (approximately 45%). Less than half of CN-AML patients are long-term survivors [48-50]. Several genetic mutations had been identified in AML patients with normal karyotypes [51-53]. However, the frequencies of gene mutations are relatively low in AML (<30%). Therefore, new molecular markers are warranted to identify those who are at the risk of poor outcome and to optimize treatment strategies in patients with a normal karyotype. In this study, our data disclosed that reduced *miR-218* expression acted as an independent risk factor both in whole AML and in CN-AML. Prospective studies are needed to confirm and expand our results before *miR-218* expression can be used routinely as a potential marker for risk stratification in de novo AML.

The mechanism regulating *miR-218* expression has not yet been well understood, but it has been proved that the expression level of *miR-218* was associated with the silencing of its host genes, *SLIT2* and/or *SLIT3*. The silencing of *SLIT2* and *SLIT3* was most commonly caused by hypermethylation of CpG islands located on their promoters in a variety of cancers including solid tumor, including nasopharyngeal cancer, cervical cancer, lung cancer and breast cancer [54-57]. Dunwell et al. determined the methylation status of the *SLIT2* gene in chronic lymphocytic leukemia and acute lymphocytic leukemia [58]. A recent study has further disclosed that *miR-218* expression could be regulated by the CpG island methylation of the *miR-218* gene [43].

In summary, our study shows that down-regulated *miR-218* expression is a common event and predicts unfavorable clinical outcome in de novo AML patients.

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Disclosure of conflict of interest

None.

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