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Reduced *miR-215* expression predicts poor prognosis in patients with acute myeloid leukemia

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Abstract

Objective: Abnormal expression of *microRNA-215* has been identified in a variety of solid cancers. However, little is known about the expression pattern of *microRNA-215* in acute myeloid leukemia. This study was to investigate the status of *microRNA-215* expression and further analyze its clinical significance in acute myeloid leukemia.

Methods: Real-time quantitative polymerase chain reaction assay was performed to evaluate the expression level of *microRNA-215* in 113 patients with acute myeloid leukemia. Besides, the relationship between *microRNA-215* levels and clinical and pathological factors was explored.

Results: Compared with the healthy individuals, *microRNA-215* expression in acute myeloid leukemia patients was significantly down-regulated ($P = 0.001$). *MicroRNA-215* low-expressed patients had higher white blood cells than *microRNA-215* high-expressed patients ($P = 0.014$). The incidence of *FLT3/ITD* mutation in the patients with low *microRNA-215* expression was significantly higher than those with high *microRNA-215* expression ($P = 0.025$). *MicroRNA-215* low-expressed patients had significantly shorter overall survival than *microRNA-215* high-expressed patients in both non-M3 acute myeloid leukemia patients and cytogenetically normal patients ($P = 0.017$ and $P = 0.044$, respectively). Meanwhile, multivariate analysis confirmed the adverse prognostic value of *microRNA-215* expression in acute myeloid leukemia patients with non-M3 subtypes.

Conclusions: Our study demonstrates that reduced *microRNA-215* expression is a common event and is associated with poor clinical outcome in acute myeloid leukemia.

Key words: *MiR-215*, acute myeloid leukemia, prognosis, real-time quantitative PCR

Introduction

Acute myeloid leukemia (AML), a common type of hematopoietic malignant disease, is characterized by increased self-renewal, differentiation arrest and malignant proliferation of leukemia progenitor cells, which ultimately interferes with normal production of blood cells (1). Both genetic abnormalities and epigenetic alterations play crucial roles in the above-mentioned pathogenetic process (2). Moreover, it is proven that microRNAs (miRNAs), as a new kind of participators of epigenetic regulation besides DNA methylation and histone modifications, also occupy an indispensable position during leukemogenesis and provide useful prognostic information of AML (3,4).

*MicroRNA*s are a class of small non-coding RNAs that are identified as the critical regulators of gene expression at the post-transcriptional level by binding to the 3′-untranslated region of their target mRNAs and thus generally participate in a variety of biological processes including cell proliferation, differentiation, viability and apoptosis (5,6). Recently, dysregulation of miRNAs has been linked to cancer initiation and progression, which provides a new perspective to understanding of the process of carcinogenesis (7,8). Furthermore, accumulating studies have also identified that aberrant expression of unique miRNAs in pathological processes is closely associated with the diagnosis as well as prognosis of hematological malignancies (9–11).

MicroRNA-215 (*miR-215*), identified from the location on chromosome 1q41, has been proven to be a *p53*-inducible miRNA with the capability of enhancing *p21* levels and mediating cell cycle arrest (12–14). Previous studies showed that *miR-215* could act as a tumor suppressor gene, and down-regulation of *miR-215* was identified in several cancers, such as myeloma (14), nephroblastoma (15), esophageal adenocarcinoma (16), breast cancer (BC) (17) and colon cancer (18,19). However, the status of *miR-215* expression and its prognostic value remain unclear in AML. Thus, our study was aimed to investigate the expression pattern and analyze its clinical significance in the patients with AML.

Patients and methods

Patients and samples

The current investigation was approved by the Ethics Committee and Institutional Review Board of the Affiliated People' Hospital of Jiangsu University, China. After written informed consents were signed, a total of 138 bone marrow samples were collected from 25 healthy people who were the hematopoietic stem cell donors and 113 *de novo* AML patients from January 2008 to August 2015. Based on French–American–British (FAB) and World Health Organization (WHO) criteria combined to immunophenotyping and cytogenetic analysis, the diagnosis and classification of AML patients were established (20–23), including the cases with low-percentage blasts (<20%) in bone marrow with the detection of cytogenetic aberrations, such as t(15;17) (q22;q12). The relevant clinical and laboratory features of the patients are presented in Table 1.

Treatment protocol for AML patients was described previously (24). For non-M3 patients, one or two courses with standard of cytarabine (100 mg/m^2) plus daunorubicin (45 mg/m^2) 7 + 3 induction therapy were given. Patients who achieved complete remission (CR) were given subsequent high- or medium-dose cytarabine-based chemotherapy treatment for consolidation. For patients older than 65 years, CHG protocol (cytarabine 10 mg/m^2 q12 h for 14 days, homoharringtonine 1 mg daily for 14 days and G-CSF 200 g/m^2 for 14 days) was administered. For the patients with acute promyelocytic leukemia (APL), induction therapy consisted of oral all-trans retinoic acid (ATRA) 45 mg/m^2 per day until morphologic CR and intravenous daunorubicin 45 mg/m^2 for 3 days and cytarabine 100 mg/m^2 for 7 days. Patients in CR received three monthly consolidation courses consist of daunorubicin (45 mg/m^2 for 3 days) and cytarabine (100 mg/m^2 for 7 days) as the first, followed by mitoxantrone (8 mg/m^2 per day for 3 days) and cytarabine (100 mg/m^2 for 7 days) as the second, and the homoharringtonine (2 mg/m^2 daily for 7 days) and cytarabine (100 mg/m^2 for 7 days) at the last. Patients who were negative for PML/RARA transcript at the end of consolidation were started on maintenance therapy with oral mercaptopurine (50 mg/m^2 per day), oral methotrexate (15 mg/m^2 per week) and oral ATRA (45 mg/m^2 per day for 15 days every 3 months) over 2 years.

RNA isolation and reverse transcription

The mirVana miRNA isolation kit (Ambion, Austin, TX, USA) was used to extract the total RNA. Reverse transcription was performed to synthesize cDNA using MiScript Reverse Transcription Kit (Qiagen, catalog no. 218061). The operations mentioned above were conducted in accordance with the manufacturer's protocols.

MiR-215 level detection

The primers of *miR-215* transcript used for real-time quantitative polymerase chain reaction (RQ-PCR) were 5′-GCATGACCTA TGAATTGACAGAC-3′ and the manufacturer-provided miScript Universal primer (Qiagen, catalog no. 218061). RQ-PCR was performed using miScript SYBR green PCR kit (Qiagen, catalog no. 218073) in an ABI 7300 Thermo cycler (Applied Biosystems, Foster City, CA, USA). The cycling conditions of the reactions are as follows: 94°C for 15 min for initial denaturation, followed by 40 cycles at 94°C for 15 s for denaturation, 55°C for 30 s for annealing and 70°C for 30 s for extension. Relative expression levels were determined by using the $2^{-\Delta\Delta Ct}$ method from the relevant signals. U6 small nuclear RNA was selected as the endogenous normalizer.

Gene mutation detection

The detections of *IDH1/2*, *DNMT3A*, *NRAS* or *KRAS*, *NPM1*, *C-KIT* and *U2AF1* mutations were reported previously (25–28). All samples determined positive by high-resolution melting analysis (HRMA) were further confirmed by direct DNA sequencing. *FLT3/ITD* and CCAAT enhancer binding protein alpha (*C/EBPA*) mutations were detected using DNA sequencing (29,30).

Statistical analyses

The statistical analyses in this study were performed using Statistical Program for Social Sciences (SPSS) software, version 20.0 (SPSS, Chicago, IL). Mann–Whitney's *U*-test and Pearson's chi-square analysis or Fisher's exact test were used to compare the difference of continuous variables and categorical variables between the groups, respectively. Overall survival (OS), defined as the time between the initial diagnosis and death or the last follow-up, was compared to show any significant associations between *miR-215* expression and the survival of the AML patients according to Kaplan–Meier method and a Cox proportional hazards model was performed further to determine the impact of *miR-215* expression. A two-tailed *P*-value <0.05 was considered to indicate a statistically significant result.

Results

Down-regulation of *miR-215* in AML patients

The median level of *miR-215* transcript in controls was 0.019 with a range from 0.006 to 1.000. Compared with controls, we demonstrated

Table 1. Correlation between *miR-215* expression and patients' parameters

Patients' parameters	Low ($n = 47$)	High ($n = 66$)	P
Sex, male/female	32/15	35/31	0.124
Median age, years (range)	57 (20–93)	57.5 (21–87)	0.641
Median WBC, $\times 10^9$/l (range)	18.4 (0.3–197.7)	5.9 (0.5–528.0)	0.014
Median hemoglobin, g/l (range)	76 (40–138)	74 (32–133)	0.108
Median platelets, $\times 10^9$/l (range)	28.5 (3–399)	40 (6–447)	0.058
BM blasts, % (range)	45.5 (1–94.5)	45.8 (3–97.5)	0.622
FAB			0.989
M0	0	1	
M1	4	4	
M2	23	32	
M3	7	10	
M4	10	12	
M5	3	6	
M6	0	1	
WHO			1.000
AML with t(8;21)	7	9	
APL with t(15;17)	7	10	
AML with 11q23 translocation	0	1	
AML without maturation	4	4	
AML with maturation	16	23	
Acute myelomonocytic leukemia	10	13	
Acute monoblastic and monocytic leukemia	3	4	
Acute erythroid leukemia	0	1	
Karyotype classification			0.667
Favorable	14	19	
Intermediate	28	34	
Poor	4	10	
No data	1	3	
Karyotype			0.913
Normal	21	28	
t(8;21)	7	9	
t(15;17)	7	10	
11q23	0	1	
Complex	4	9	
Others	7	6	
No data	1	3	
Gene mutation			
C/EBPA (+/–)	3/42	12/53	0.094
NPM1 (+/–)	6/39	5/60	0.352
FLT3/ITD (+/–)	11/34	5/60	0.025
C-KIT (+/–)	3/42	0/65	0.066
NRAS or *KRAS* (+/–)	2/40	6/55	0.467
IDH1/2 (+/–)	4/38	2/59	0.222
DNMT3A (+/–)	6/36	3/58	0.154
U2AF1 (+/–)	1/41	4/57	0.646
CR(+/–)	17/28	31/32	0.326

WBC, white blood cells; FAB, French–American–British classification; AML, acute myeloid leukemia; CR, complete remission.

a significantly decreased expression of *miR-215* in AML patients (range 0.000–3.713, median 0.008) ($P = 0.001$) (Fig. 1).

Correlation between *miR-215* expression and clinical characteristics in AML

To explore the clinical relevance of *miR-215* expression in AML, receiver operating characteristic curve (ROC) analysis was performed to divide the whole patients into two groups (low *miR-215* expression and high *miR-215* expression) on the basis of the level of *miR-215* expression. ROC analysis showed that at the cut-off value of 0.0054 of *miR-215* expression level, the sensitivity and the specificity were 42% and 100%, respectively (Fig. 2). The area under the curve (AUC) was 0.705 (95% confidence interval = 0.617–0.793, $P < 0.001$). The clinical and laboratory features in AML patients at time of diagnosis with and without *miR-215* low expression are presented in Table 1. As shown in Table 1, lower levels of *miR-215* were associated with a higher white blood cell (WBC) counts ($P = 0.014$). There was no significant association of the *miR-215* expression level between the two groups in other clinical features including sex, age, hemoglobin (HB) counts, platelets (PLT) counts, FAB or WHO classifications and cytogenetic abnormalities ($P > 0.05$).

Among the 10 gene mutations, no one except for *FLT3/ITD* mutation was validated to have a difference between *miR-215* low-expressed and high-expressed patients (Table 1). AML patients with low *miR-215* expression had significantly higher incidence of

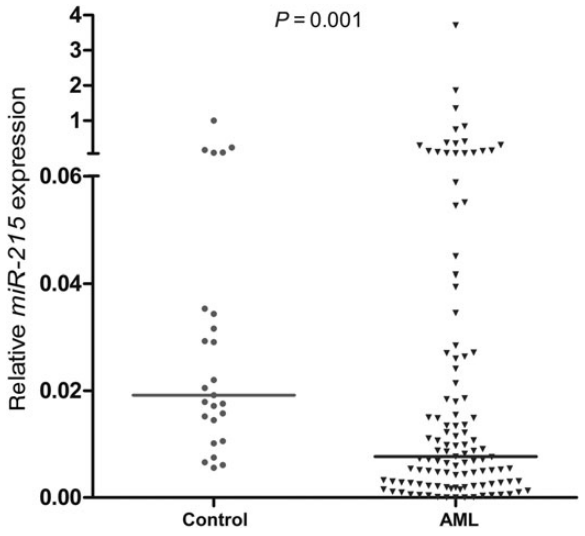

Figure 1. Relative expression levels of *miR-215* expression in acute myeloid leukemia (AML) patients and controls. The level of *miR-215* was significantly lower in AML patients than in healthy controls ($P = 0.001$).

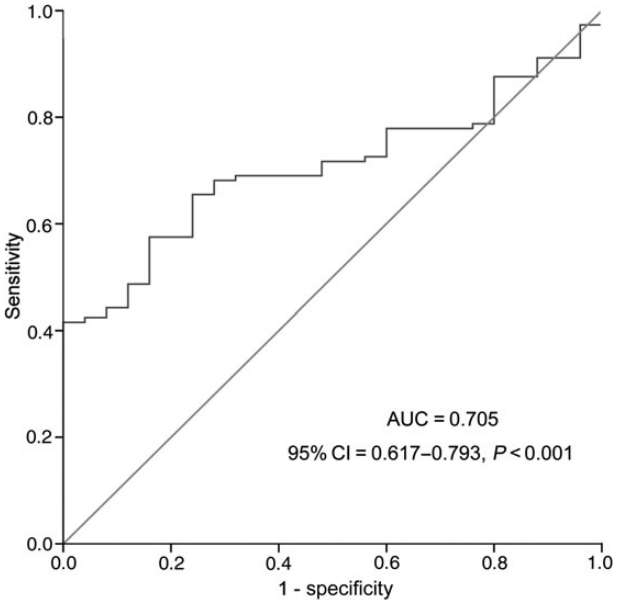

Figure 2. Receiver operating characteristic curve analysis using *miR-215* for discriminating AML patients from normal controls.

FLT3/ITD mutation as compared with those with high *miR-215* expression ($P = 0.025$). Additionally, the relative *miR-215* expression was compared in three groups (*FLT3/ITD*-positive AML, *FLT3/ITD*-negative AML and controls). Analysis of *miR-215* expression by RQ-PCR in AML patients compared with controls validated that *miR-215* to be significantly down-expressed in the *FLT3/ITD*-positive AML patients in comparison to both *FLT3/ITD*-negative AML patients and controls ($P = 0.018$ and $P < 0.001$, respectively) (Fig. 3). Due to the largest number of patients with cytogenetically normal AML (CN-AML), the correlation between gene mutations and *miR-215* expression was further analyzed. However, differences could not be found in the distribution of gene mutations between patients with and without *miR-215* low expression.

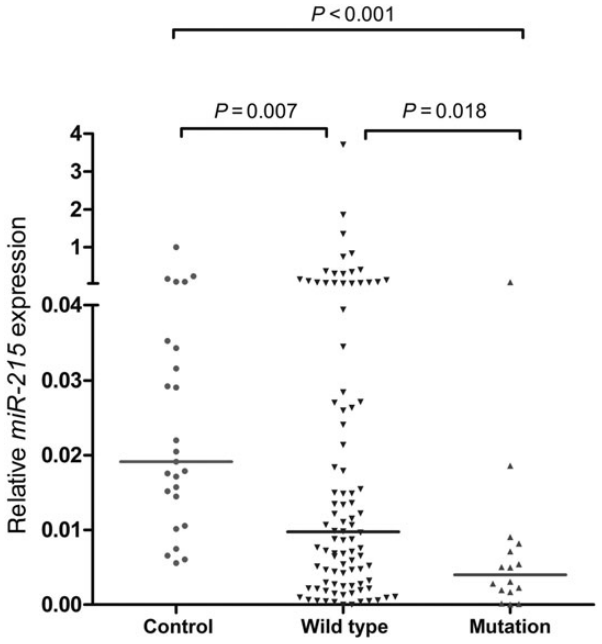

Figure 3. Relative expression levels of *miR-215* in *FLT3-ITD*-positive AML patients (*FLT3/ITD* mutation), *FLT3-ITD*-negative AML patients (*FLT3/ITD* wild-type) and controls. *MiR-215* was significantly down-expressed in the AML patients with *FLT3/ITD* mutation in comparison to both AML patients with *FLT3/ITD* wild-type and healthy controls ($P = 0.018$ and $P < 0.001$, respectively).

Correlation between *miR-215* expression and survival in AML

A total of 108 newly diagnosed patients had the follow-up data. In the whole AML patients, the rate of CR after induction therapy in the *miR-215* low-expressed group (37.8%, 17/45) was similar to the *miR-215* high-expressed group (49.2%, 31/63) ($P = 0.326$). However, among non-M3 patients, the cases with low *miR-215* expression tended to have lower CR rate than those with high *miR-215* expression (28.2 versus 47.3%, $P = 0.086$). There was no significant difference in CR rate between the two groups among CN-AML patients ($P = 0.555$).

Survival data were obtained for 106 AML patients with median follow-up time of 8 months ranging from 1 to 90 months. No significant difference in OS was observed between the groups with low and high *miR-215* expression in the whole cohort of AML patients ($P = 0.258$) (Fig. 4A). However, the non-M3 AML patients with low *miR-215* expression had shorter OS (median 4 months) than those with high *miR-215* expression (median 9 months) ($P = 0.017$) (Fig. 4B). Likewise, among CN-AML patients, the low *miR-215* expression group also had a shorter OS ($P = 0.044$) (Fig. 4C). Moreover, multivariate analysis identified that low *miR-215* expression was an independent prognostic factor besides age, karyotype classifications and *U2AF1* mutation among non-M3 AML patients (Table 2). Among CN-AML patients, multivariate analysis including the same variables except for karyotype classification and *U2AF1* mutation confirmed that low *miR-215* expression tended to predict the adverse prognosis independently (Table 3).

Discussion

Many indicators have been involved in the diagnosis, prognosis and treatment of AML by far, such as karyotypes, mutations in *FLT3*,

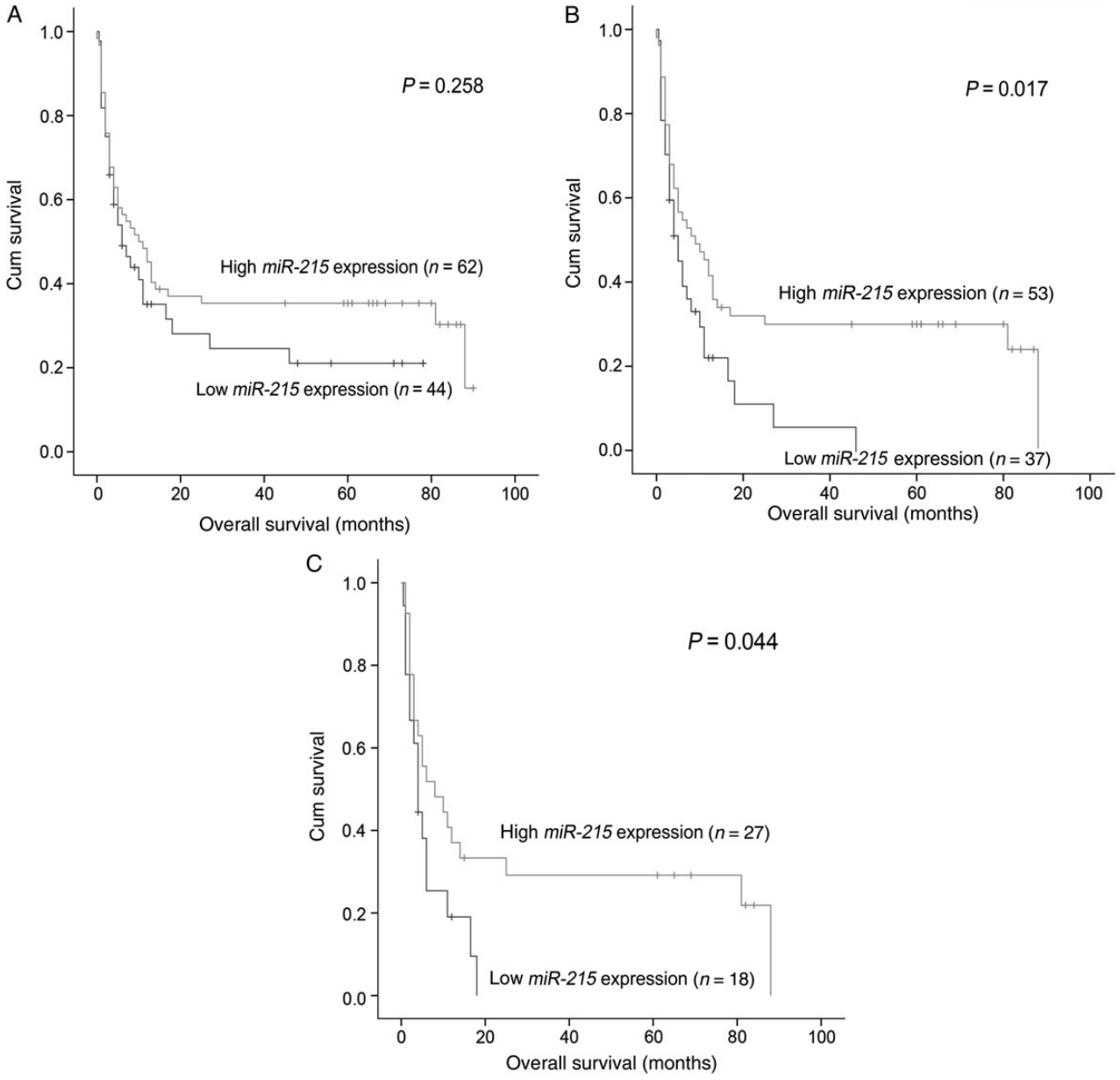

Figure 4. The impact of *miR-215* expression on overall survival of AML patients. (A) All patients; (B) non-M3 patients and (C) cytogenetically normal AML (CN-AML) patients.

Table 2. Multivariate analyses of prognostic factors for overall survival (OS) in 96 non-M3 AML patients

	Hazard ratio (95% CI)	*P* value
Age (>60/≤60 years)	1.988 (1.191–3.320)	0.009
WBC (≥30/<30 $\times 10^9$/l)	1.044 (0.604–1.804)	0.877
Karyotype classification (poor/intermediate/favorable)	2.716 (1.462–5.045)	0.002
miR-215 expression (low/high)	2.309 (1.350–3.953)	0.002
U2AF1 mutation (+/−)	4.261 (1.578–11.506)	0.004

Table 3. Multivariate analyses of prognostic factors for OS in 49 cytogenetically normal AML patients

	Hazard ratio (95% CI)	*P* value
Age (>60/≤60 years)	2.012 (0.984–4.117)	0.056
WBC (≥30/<30 $\times 10^9$/l)	1.290 (0.649–2.564)	0.467
miR-215 expression (low/high)	1.949 (0.951–3.984)	0.068

C-KIT, *NPM1* and *C/EBPA* genes (23,31). However, the numbers of adults with normal cytogenetical AML are >40%, and the frequencies of gene mutations are relatively low in AML actually (<30%) (32). For most AML patients, especially for those who failed to be characterized by relevant cytogenetic or molecular changes, difficulty still exists to estimate their survival and prognosis. Accordingly, it is quite necessary for finding more valuable biomarkers to improve our understanding of the biology of leukemia and identify those who tend to have adverse prognosis as well as optimize treatment strategies in CN-AML

patients. Nowadays, considerable progress has been made in identifying, characterizing and applying new molecular markers (33,34), including miRNAs that were known to be dysregulated during oncogenesis. Due to their advantages of easy detection and less degradation, a growing number of miRNAs has been reported to be biomarkers involved in tumor progression, diagnosis, prognosis and so on (35–37). Here, we focused on *miR-215*, whose overexpression could induce cell cycle arrest, cell detachment and apoptosis in a partially but not completely *p53*-dependent pathway (13). *MiR-215* was also identified as a putative anti-oncogenic miRNA through modulating a number of genes: up-regulated *miR-215* causes a decrease in clonogenicity and mediates the repression of cell cycle and stemness genes downstream of *CDX1* by targeting *BMI1* in colon cancer (18,38), decreased cell migration and invasion in renal cell carcinoma (RCC) by targeting *SIP1/ZEB2* and *MMP3* (39); increased apoptosis by targeting *XIAP* in non-small cell lung cancer (40). However, up-regulation of *miR-215* can also be observed in several studies such as gastric cancer, cervical cancer and hepatocellular carcinoma (41–43). These data may reflect that *miR-215* played distinct roles in many types of cancers with particular tissue origin. Moreover, lots of reports have found that the expression pattern of *miR-215* is associated with the clinical outcome of several cancers (44). Karaayvaz et al. found that low expression of *miR-215* was correlated with poor prognosis in colon cancer (19). Khella et al. demonstrated that decreased *miR-215* expression was a negative prognostic biomarker in RCC (39). Zhou et al. verified that the down-regulation of *miR-215* was an unfavorable prognostic factor in BC (17). Prognostic significance of *miR-215* expression has been revealed in many solid tumors, which suggests that it could serve as a potential role of prognostic biomarker and thereby create a more predictable future when refers to the clinical treatment of patients.

To our best knowledge, this is the first time that reports the clinical significance of *miR-215* expression in AML. Our study presented that down-regulation of *miR-215* expression was a common event in AML. In this study, *miR-215* low expression was validated to be associated with shorter OS in non-M3 AML patients and CN-AML patients and was proven to be an adverse prognostic factor in non-M3 AML. We also confirmed in multivariate analysis that *miR-215* can serve as a significant and independent predictor of AML. Taken together, these results indicated that *miR-215* functioned as a tumor suppressor in AML as well and *miR-215* might be an important modulator involved in AML development. Obviously, our results deserve further studies to be confirmed before *miR-215* expression can be used routinely as a potential biomarker for risk stratification in AML.

Previous studies have reported that *FLT3/ITD* mutation, known as a poor prognostic factor in leukemia, was associated with higher peripheral WBC counts and a higher bone marrow blast percentage (45). Furthermore, several miRNAs have already been reported to be associated with *FLT3/ITD*. For example, expression of *miR-155* was overexpressed and involved in the pathogenesis of AML with *FLT3/ITD* mutation (11,46). Additionally, *miR-16* repression could participate in the process of up-regulating *Pim-1* oncogene, a regulator of *FLT3/ITD* signaling in *FLT3/ITD* expressing cells (47). Our study showed that *miR-215* was down-regulated in AML patients with *FLT3/ITD* mutation and the patients with low *miR-215* expression had a high incidence of *FLT3/ITD* mutation. The exact relationship between down-regulation of *miR-215* and *FLT3/ITD* mutation need further experimental studies.

However, there still exits some limitations such as the small number of patient cases, potential non-applicability of the used cut-off value and some missing data for gene mutations in this study. Of course, more samples with the follow-up data are surely worthy to be employed to take insight into the more accurate clinical significance of *miR-215* in AML.

In summary, down-regulated *miR-215* expression is a common event in *de novo* AML patients and might act as an independent risk factor for prognosis in non-M3 AML.

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Conflict of interest statement

None declared.

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