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Original Article

Increased expression of *miR-125b* is not regulated by methylation and not associated with prognosis in acute myeloid leukemia

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Abstract: The role of microRNAs is critical to the development and progression of acute myeloid leukemia (AML). The present study was aimed to investigate the expression status of *miR-125b* and the potential clinical relevance in patients with AML. Real-time quantitative PCR was carried out to evaluate the expression level of *miR-125b* in 114 AML patients and the corresponding clinical significance was further analyzed. Compared with the healthy individuals, *miR-125b* expression was significantly over-expressed in AML patients (P<0.001), especially in FAB-M3 subtype. *MiR-125b* promoter was unmethylated in both controls and AML patients. The frequency of *miR-125b* overexpression was much higher in FAB-M3 subtype than other subtypes [94% (16/17) versus 29% (28/97), P<0.001]. No significant differences were obtained in both complete remission (CR) rate and overall survival (OS) time between high *miR-125b* expression group and low *miR-125b* expression group in non-M3 AML patients (P = 0.246 and 0.766, respectively). These findings implicated that *miR-125b* overexpression is associated with AML especially the FAB-M3 subtype but is not a prognostic biomarker in AML. Moreover, *miR-125b* expression is not regulated by its methylation in AML.

Keywords: *MiR-125b*, expression, methylation, acute myeloid leukemia, acute promyelocytic leukemia

Introduction

Acute myeloid leukemia (AML) is a kind of malignant disease that occurs as a consequence of massive leukemic stem cells (LSCs) in bone marrow (BM), which attack the normal haematopoietic progenitors, leading to high mortality [1]. The clinical characteristics and outcome of AML patients are largely influenced by cytogenetic abnormalities such as t(8;21), t(15;17), inv(16)/t(16;16), t(9;22), 11q23, -5/5q-, -7/7q-, and complex karyotypes [2]. Moreover, molecular biological alterations including mutations in *FLT3*, *C/EBPA*, *C-KIT*, *NPM1*, *DNMT3A*, *IDH1/2*, and *N/K-RAS* genes, deregulated gene expressions such as *BAALC*, *MN1*, *EVI1*, *WT1* as well as some microRNAs (miRNAs), can also provide a lot of information for diagnoses and prognosis assessment in AML [3-7].

MiRNAs, a series of small noncoding RNA molecules about 22 nucleotide (nt) in length, act as crucial posttranscriptional regulators of target gene expression through translational repression or transcript cleavage [8]. It is well acknowledged that deregulation of miRNAs may lead to alterations in diverse biological processes (proliferation, differentiation, and apoptosis, etc.) and is tightly related to the initiation and progression of diseases, including almost all types of human cancers as well as leukemia [9, 10]. Moreover, our previous researches have showed the existence of several aberrant miRNAs expression in patients with AML. As examples, the expression of *miR-215* was demonstrated significantly downregulated in AML patients compared with healthy controls with its down-regulation correlated with poor prognosis [11]. In addition, overexpression of *miR-378* was detected in AML and associated with

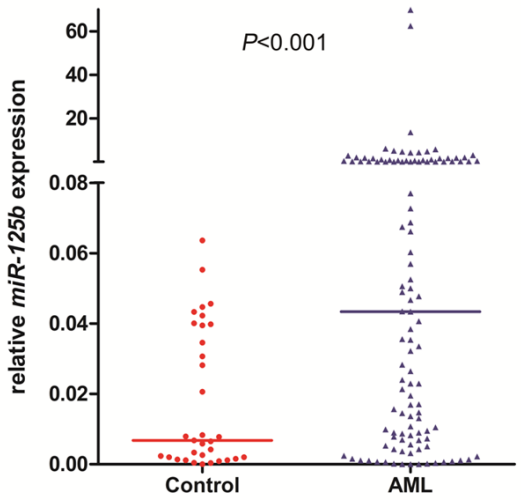

Figure 1. Relative expression levels of *miR-125b* in controls and AML patients.

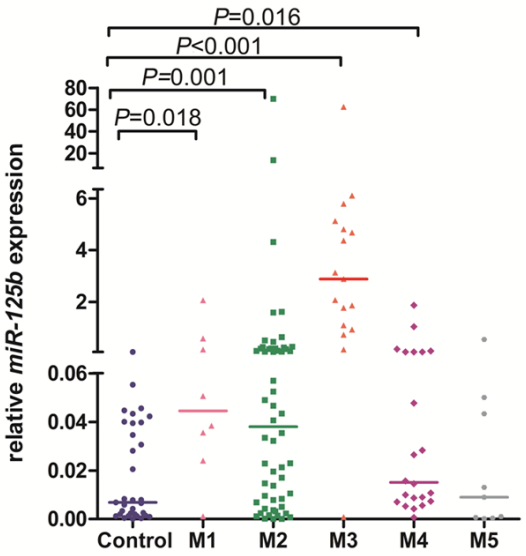

Figure 2. Relative expression levels of *miR-125b* in AML according to FAB classification and controls.

poor prognosis in AML, which might be useful as an indicator for predicting the clinical outcome of AML patients [12]. The increasing expression level of *miR-24* was associated with AML patients with t(8;21) and might serve as a therapeutic target for the treatment of AML with t(8;21) [13].

MicroRNA-125b (*miR-125b*), the mammalian homolog of the C. elegans heterochronic microRNA lin-4, is highly conserved in numerous species from nematodes to human, with the ability to function as either a tumor suppressor or a promoter in human diseases [14]. *MiR-125b* is composed of two homologs, which is verified to be transcribed from two loci located on chromosomes 11q23 (*hsa-miR-125b-1*) and 21q21 (*hsa-miR-125b-2*) [15]. Increasing amounts of data have demonstrated that the aberrant expression of *miR-125b* was associated with tumorigenesis and tumor progression in diverse types of cancer. However, the function role of *miR-125b* during tumorigenesis and its expression pattern in cancer remain controversial. It was downregulated in some solid tumors such as the breast cancer [16], osteosarcoma [17], ovarian cancer [18], and bladder cancer [19], but was upregulated in lung cancer [20], glioma [21] and prostate cancer [22]. Moreover, hypermethylation of *miR-125b* was identified in several solid tumors including breast cancer, ovarian cancer and colorectal cancer [23-25]. However, few studies revealed *miR-125b* expression and methylation pattern in hematological malignancies. The purpose of this present study was to investigate the expression and methylation status of *miR-125b*, and further to determine their clinical relevance in patients with AML.

Materials and methods

Patients and samples

This study was approved by the Ethics Committee and Institutional Review Board of the Affiliated People' Hospital of Jiangsu University, China. A total of 114 patients and 33 healthy controls participated in this study gave their written informed consent before BM sample collection. The diagnosis and classification of AML patients were based on the French-American-British (FAB) and World Health Organization (WHO) classification system as well as cytogenetic analysis [26-29]. Karyotypes were analyzed by conventional R-banding method and karyotype risk was classified according to the precious report [29]. The group used as controls consisted of healthy donors with no clinical symptoms of cancer or other diseases.

RNA isolation and cDNA synthesis

Total RNA was reverse transcribed to cDNA by using the mirVana miRNA isolation kit (Ambion, Austin, TX, USA) to extract RNA and the MiScript Reverse Transcription Kit (Qiagen, catalog no. 218061) to synthesize cDNA. The procedure

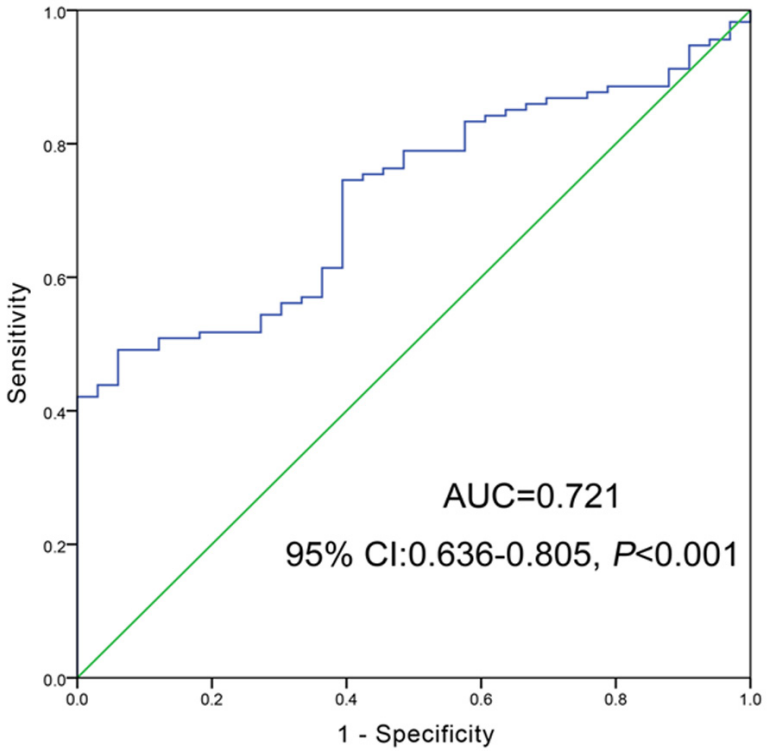

Figure 3. ROC curve analysis using *miR-125b* for discriminating AML patients from normal controls.

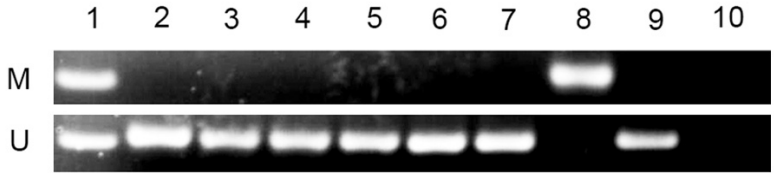

Figure 4. MSP results of *miR-125b* in normal controls and AML patients. U and M represent PCR results using primer sets for unmethylated and methylated *miR-125b*, respectively. 1: Gene Ruler™ 100 bp DNA ladder. 2-3: normal control; 4-7: AML samples; 8-9: positive control; 10: ddH_2O.

mentioned above was performed according to the manufacturer's protocols.

Real-time quantitative PCR (RQ-PCR)

Real-time quantitative PCR was performed to detect the expression level of *miR-125b* transcript using miScript SYBR green PCR kit (Qiagen, Duesseldorf, Germany) with the manufacturer-provided miScript universal primer and miRNA-specific forward primer (5'-CCCTGAGACCCTAACTTGTG-3') in an ABI 7500 Thermo cycler (Applied Biosystems, Foster City, CA, USA). The PCR conditions were 94°C for 15 min, followed by 40 cycles at 94°C for 15 s, 55°C for 30 s and 70°C for 30 s. The relative expression of *miR-125b* was calculated by the comparative $2^{-\Delta\Delta Ct}$ method with U6 small nuclear RNA levels used for normalization. All experiments were performed at least in triplicate.

DNA isolation, methylation-specific PCR (MSP)

Genomic DNA Purification kit (Gentra, Minneapolis, MN, USA) was used to isolate genomic DNA with CpGen ome DNA Modification Kit (Chemicon, Ternecula, Canada) used to modify. The primer sequences for the methylated (M) miR-125b promoter were 5'-TTAAAGGTTTTAAAGAATTCGTAGC-3' (forward) and 5'-CAAACTATCATTTAATAAACACGAA-3' (reverse), and for the unmethylated (U) miR-125b promoter were 5'-AAAGGTTTTAAAGAATTTGTAGTGG-3' (forward) and 5'-CCAAACTATCATTTAATAAACACAAA-3' (reverse). PCR conditions were 95°C for 5 min, 40 cycles for 10 s at 95°C, 30 s at 55°C (M or U), 30 s at 72°C followed by a final 7 min extension step at 72°C. Amplification was carried out in a 9600 Perkin-Elmer Thermal Cycler (PerkinElmer Inc., Ramsey, MN, USA). PCR products were analyzed on 2% agarose gels and visualized under UV illumination, with both positive and negative controls included.

Bisulfite sequencing PCR (BSP)

The primer sequences for bisulfite modified *miR-125b* were 5'-TTTATTTTTAGTTTGATGAGGAAAG-3' (forward) and 5'-CACCAAACTATCATTTAATAAACAC-3' (reverse). The BSP was carried out at 95°C for 5 min, 40 cycles for 10 s at 95°C, 30 s at 58°C, 72°C for 30 s, and followed by a final 7 min extension step at 72°C. The PCR products were analyzed on 2% agarose

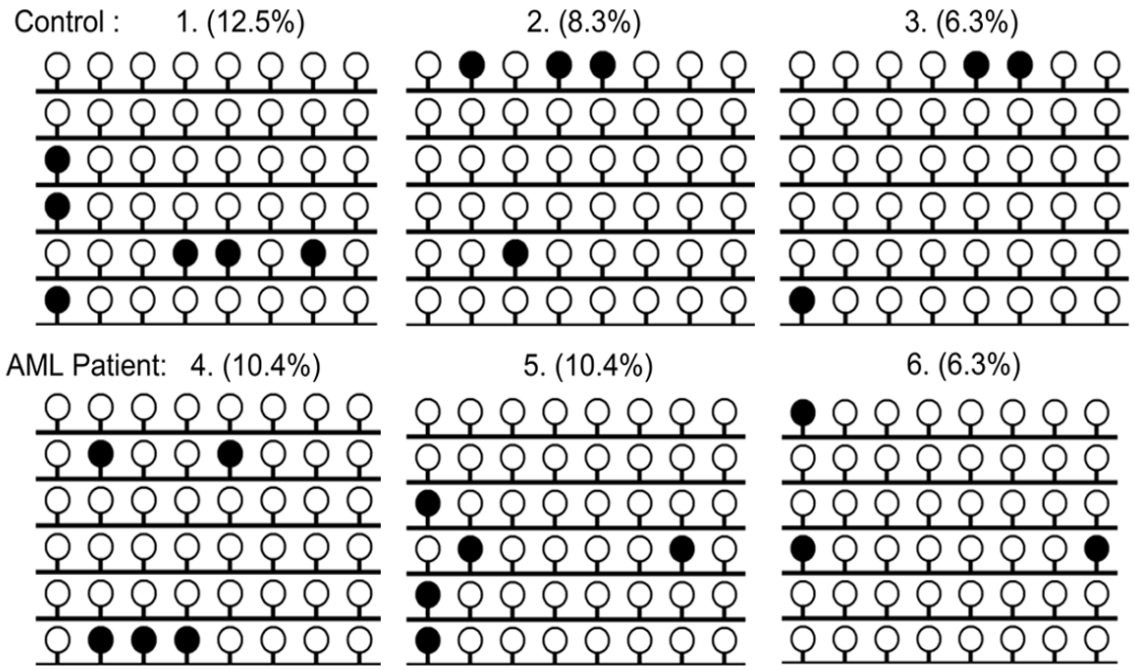

Figure 5. Methylation density of *miR-125b* in normal controls and AML patients. White cycle: unmethylated CpG dinucleotide; Black cycle: methylated CpG dinucleotide. 1-3: controls; 4-6: AML patients.

gels. The PCR products were purified and cloned into pMD19-T Vector (Takara, Tokyo, Japan), then transfected into DH5A competent cells (Vazyme, Carlsbad, CA, USA). Six clones from each sample were sequenced (BGI Tech Solutions Co., Shanghai, China).

Gene mutation detection

The detections of *IDH1/2*, *DNMT3A*, *N/K-RAS*, and *U2AF1* mutations were reported previously [30-33]. The detections of *NPM1* and *C-KIT* mutations were performed using PCR and high-resolution melting analysis (HRMA). All positive samples determined byHRMA were then confirmed by direct DNA sequencing. *FLT3* internal tandem duplication (*ITD*) and *C/EBPA* mutations were detected using direct DNA sequencing [34, 35].

Statistical analyses

Statistics were performed using Social Sciences (SPSS) 20.0 software package (SPSS, Chicago, IL). Mann-Whitney's *U* test was performed to compare the *miR-125b* expression level in BM of AML patients and healthy controls. With regard to the correlation of AML clinical features with *miR-125b* expression level, Mann-Whitney's *U* test together with Pearson's chi-square analysis or Fisher's exact test were employed to compare intergroup differences. Survival was analyzed according to the Kaplan-Meier method and Cox regression. Differences were considered statistically significant when a two-sided *P* value was less than 0.05.

Results

Increased expression of miR-125b in AML patients

Compared with healthy controls (range 0.000-0.063, median 0.007), *miR-125b* transcript was markedly up-regulated ranging from 0.000 to 69.835 with a median level of 0.043 in AML patients ($P<0.001$) (**Figure 1**). Since previous study revealed that *miR-125b* was highly expressed in pediatric acute promyelocytic leukemia (APL) [36], we further analyzed *miR-125b* expression among FAB subtypes. Similarly, in our data, *miR-125b* overexpression was identified in FAB-M1/M2/M3/M4 subtypes and with the highest level in FAB-M3 subtypes (**Figure 2**).

Differentiating value of miR-125b expression

In this study, receiver operating characteristic (ROC) curve was performed to evaluate the dis-

Table1. Comparison of clinical characteristics between *miR-125b* expression and whole AML as well as non-M3 patients

Patient's parameters	*MiR-125b* expression in whole AML			*MiR-125b* expression in non-M3 AML		
	Low (n = 70)	High (n = 44)	*P*	Low (n = 70)	High (n = 27)	*P*
Sex, male/female	43/27	25/19	0.696	44/26	17/10	1.000
Median age, years (range)	60 (20-93)	51 (21-87)	0.050	60 (20-93)	61 (29-87)	0.576
Median WBC, $\times10^9$/L (range)	16 (1-582.0)	7 (0.3-136.1)	0.041	14.8 (1.1-528.0)	10.4 (0.8-136.1)	0.497
Median hemoglobin, g/L (range)	76 (34-138)	70 (32-131)	0.228	75 (34-138)	68 (32-131)	0.325
Median platelets, $\times10^9$/L (range)	34 (4-447)	36 (3-264)	0.319	33 (4-447)	42 (3-264)	0.807
BM blasts, % (range)	51.5 (6.0-97.5)	34.8 (1.0-92.0)	0.016	51.8 (6.0-97.5)	56.8 (19.0-92.0)	0.649
FAB			<0.001			0.535
M0	1	0		1	0	
M1	5	3		5	3	
M2	36	20		37	19	
M3	1	16		-	-	
M4	18	4		18	4	
M5	8	1		8	1	
M6	1	0		1	0	
WHO			<0.001			0.087
AML with t(8;21)	15	2		15	2	
APL with t(15;17)	1	16		-	-	
AML without maturation	5	3		5	3	
AML with maturation	22	18		23	17	
Acute myelomonocytic leukemia	19	4		19	4	
Acute monoblastic/monocytic leukemia	7	1		7	1	
Acute erythroid leukemia	1	0		1	0	
Karyotype classification			0.018			0.081
Favorable	15	18		14	2	
Intermediate	46	17		46	17	
Poor	8	6		9	5	
No data	1	3		1	3	
Karyotype			<0.001			0.175
Normal	36	13		36	13	
t(8;21)	15	2		15	2	
t(15;17)	1	16		-	-	
11q23	1	0		1	0	
Complex	8	5		9	4	
Others	8	5		8	5	
No data	1	3		1	3	
Gene mutation						
C/EBPA (+/-)	9/60	6/36	1.000	9/60	6/20	0.343
NPM1 (+/-)	9/60	3/39	0.530	9/60	3/23	1.000
FLT3/ITD (+/-)	10/59	6/36	1.000	10/59	3/23	1.000
C-KIT (+/-)	3/66	0/42	0.288	3/66	0/26	0.559
N/K RAS (+/-)	7/55	1/41	0.139	7/55	1/25	0.427
IDH1/2 (+/-)	4/58	2/40	1.000	4/58	2/24	1.000
DNMT3A (+/-)	7/55	2/40	0.307	7/55	2/24	1.000
U2AF1 (+/-)	2/60	3/39	0.391	2/60	2/23	0.151
CR (+/-)	23/44	25/17	0.017	23/44	13/14	0.246

criminative capacity of *miR-125b* expression. The area under the curve (AUC) was 0.721 (95% confidence interval = 0.636-0.805, *P*<0.001). It revealed that *miR-125b* expression might

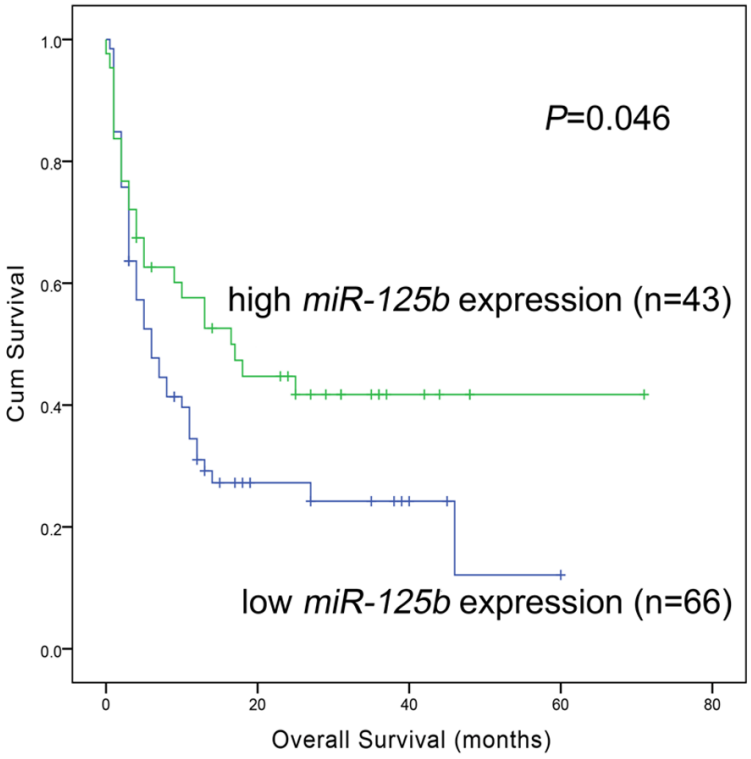

Figure 6. The impact of *miR-125b* expression on overall survival of the whole AML patients.

serve as a promising biomarker in distinguishing AML patients from healthy controls (**Figure 3**).

Methylation status of miR-125b in AML patients

Previous studies have indicated that *miR-125b* was hypermethylated in several solid tumors [23-25]. *MiR-125b* methylation status in AML patients were further determined. Notably, *miR-125b* was unmethylated in both controls and AML patients by MSP (**Figure 4**), which indicated that *miR-125b* expression was not regulated by its methylation in AML. Furthermore, *miR-125b* unmethylation was further confirmed by BSP in three controls and three AML patients (selected randomly) (**Figure 5**).

Association of miR-125b expression with clinical and laboratory characteristics in AML

AML patients expressing *miR-125b* at levels less than 0.077 (determined as the mean plus 3 SD) were assigned to the low-expression group, and those samples with expression equal to or above the value were assigned to the high-expression group. The overexpression of *miR-125b* was identified in 44 (39%) of 114 patients with AML (**Table 1**). No significant differences were observed in sex, hemoglobin (HB), platelets count (PLT), and ten gene mutations between the patients with and without *miR-125b* overexpression (**Table 1**). However, the ages of patients with high *miR-125b* expression were younger than the patients with low *miR-125b* expression (P = 0.050). Moreover, *miR-125b* high-expressed group was associated with both lower white blood cells (WBC) and BM blast count as compared with *miR-125b* low-expressed group (P = 0.041 and 0.016, respectively). In addition, significant differences in *miR-125b* expression were observed among both FAB and WHO subtypes. Among AML subtypes of M0-M6, the frequency of *miR-125b* overexpression was exceptionally higher in M3 subtype than other subtypes [94% (16/17) versus 29% (28/97), P<0.001]. According to WHO classifications, the patients with t(15;17) had the highest frequency of *miR-125b* overexpression in comparison with the others [94% (16/17) versus 30% (28/97), P<0.001]. According to karyotype classification, although no significant difference could be observed, the frequency of *miR-125b* overexpression in patients with favorable karyotype (18/33, 55%) was higher than that in patients with intermediate and poor karyotypes (23/77, 30%) (P = 0.124). Moreover, due to *miR-125b* overexpression was associated with FAB-M3 subtype, we further excluded M3 subtype from our cohort. No significant differences were found between *miR-125b* low-expressed and high-expressed patients in sex, age, blood parameters, FAB/WHO classifications, karyotypes/karyotype classifications, and ten gene mutations (**Table 1**).

Impact of miR-125b expression on prognosis

Survival analysis was performed in 109 cases with follow-up data to investigate the prognos-

Table 2. Univariate and multivariate analyses of prognostic factors for overall survival in whole AML patients

Prognostic factors	Univariate analyses		Multivariate analyses	
	Hazard ratio (95% CI)	*P* value	Hazard ratio (95% CI)	*P* value
Age (>60/≤60 years)	3.434 (2.120-5.562)	<0.001	1.900 (1.100-3.281)	0.021
WBC (≥30/<30 ×10^9/L)	2.333 (1.587-3.429)	<0.001	1.458 (0.850-2.500)	0.171
Karyotype classification (poor/intermediate/favorable)	5.535 (2.613-11.724)	<0.001	3.403 (1.459-7.940)	0.005
MiR-125b expression (high/low)	0.617 (0.376-1.012)	0.056	0.704 (0.406-1.223)	0.213
C/EBPA mutation (+/-)	1.130 (0.631-2.023)	0.682	-	-
NPM1 mutation (+/-)	1.185 (0.616-2.279)	0.610	-	-
FLT3/ITD mutation (+/-)	0.868 (0.452-1.665)	0.669	-	-
C-KIT mutation (+/-)	0.716 (0.227-2.259)	0.569	-	-
RAS mutation (+/-)	1.713 (0.889-3.303)	0.108	1.258 (0.474-3.340)	0.645
IDH1/2 mutation (+/-)	1.695 (0.881-3.216)	0.114	3.673 (1.333-10.122)	0.012
DNMT3A mutation (+/-)	1.168 (0.567-2.404)	0.674	-	-
U2AF1 mutation (+/-)	2.691 (1.234-5.867)	0.013	2.234 (0.790-6.320)	0.130

tic impact of *miR-125b* overexpression in AML. Notably, patients with high *miR-125b* expression had a higher complete remission (CR) rate than those with low *miR-125b* expression (59% versus 34%, *P* = 0.017) (**Table 1**). However, no definite difference in CR rate between two groups in the cohort of non-M3 patients (*P* = 0.246). Among cytogenetically normal AML (CN-AML) patients, *miR-125b* high-expressed patients had a higher CR rate than those low-expressed patients (*P* = 0.049). Subsequently, Kaplan–Meier analysis showed that AML patients with high *miR-125b* expression had significantly longer overall survival (OS) time (median 13 months) than those with low *miR-125b* expression (median 6 months) (*P* = 0.046) (**Figure 6**). Due to *miR-125b* overexpression correlated with favorable outcome subtype of FAB-M3, Cox regression multivariate analysis was further conducted to assess the prognostic value of *miR-125b* in AML. As expected, *miR-125b* expression was not an independent prognostic biomarker in whole AML patients (*P* = 0.213) (**Table 2**). Moreover, no significant differences between *miR-125b* high-expressed and low-expressed groups in OS were observed not only in non-M3 but also in CN-AML patients (*P* = 0.766 and *P* = 0.157, respectively).

Discussion

MiR-125b was the most studied member in *miR-125* family with a great deal of researches those years supporting the notion that it was closely associated with human cancers. However, conflicting results for *miR-125b* expression have been reported in several cancers. For instance, in breast cancer, *miR-125b* expression was validated down-regulated and methylated in breast cancer and served as a tumor suppressor through suppressing cancer cell proliferation and inducing apoptosis with a series of functions like mediating the *ERBB2* and *ERBB3* pathway, targeting *ETS1* gene and reducing the expression of *MUC1* oncoprotein [23, 37-39]. Conversely, Tang et al. verified that *miR-125b* could also promote metastasis of human breast cancer cells by targeting *STARD13* [40]. Similarly, the dual role of *miR-125b* was also confirmed in prostate cancer [22, 41]. From these results it is noteworthy that the *miR-125b* plays diverse functions in cancers of different tissue origin or cell contexts. Recent years, *miR-125b* in hematopoiesis and its pathological role in leukemogenesis have also drawn widespread attention. In contrast to its tumor-suppressing role in solid cancers, a large body of researches demonstrated that the common upregulation of *miR-125b* in a range of leukemias, such as myelodysplastic syndromes (MDS) [42], AML [42], chronic myeloid leukemia (CML) [43], acute megakaryocytic leukemia (AMKL) [44], B-cell precursor acute lymphoblastic leukemia (BCP-ALL) [45] and transient leukemic (TS) patients with trisomy 21 (Down syndrome) [44]. Meanwhile, several in vitro and vivo assays supported the notion that *miR-125b* possesses tumorigenic potential in hematological system. As examples, overexpression of *miR-125b* in hematopoietic cells could inhibit apoptosis, accelerate

proliferation, as well as induce abnormal self-renewal in non-stem cells in vitro and of the hematopoietic compartment caused leukemia in mouse models [46-48]. Taken together, these results indicated a direct oncogene role of *miR-125b* in leukemias of different cell origin.

In the present study, we confirmed that *miR-125b* transcript was significantly elevated in AML especially in FAB-M3 subtype, which was consistent with the results of previous studies [36, 49-51]. However, the exact possible mechanism of *miR-125b* function in FAB-M3 subtype was still not be revealed including among adult AML patients. A research found that *miR-125b* had the ability to interfere with primary human CD34+ cell differentiation, and inhibit terminal (monocytic and granulocytic) differentiation in leukemic HL60 and NB4 cell lines [42], indicating its function in blocking differentiation in APL. Further study with large-scale adult APL patients should be performed to determine the significance of *miR-125b* up-regulation in the pathogenesis of M3 AML.

Moreover, our study further found *miR-125b* was unmethylated in both controls and AML patients. These results indicated that *miR-125b* methylation was not the mechanism regulating *miR-125b* expression in AML. Nevertheless, different from the result in our study, hypermethylation of *miR-125b* has been found in several solid cancers such as breast cancer and colorectal cancer [23, 25], in which methylation has been shown to partially account for decreased *miR-125b* expression. Moreover, abnormal expression of *miR-125b* was found to participate in the development of breast cancer via affecting proliferation, G1 cell-cycle arrest of the cancer cells [23]. Chen et al. disclosed the clinical relevance of *miR-125b* methylation in colorectal cancer, in which hypermethylation of *miR-125b* is a potential biomarker for clinical outcome [25]. These results suggested that *miR-125b* may play different roles in solid tumors and hematological malignancies.

Although high *miR-125b* expression was significantly associated with higher CR rate and favorable OS in Kaplan-Meier analysis, Cox multivariate analysis failed to disclose the prognostic impact of *miR-125b* expression in whole AML patients. These results indicated that *miR-125b* overexpression was associated with FAB-M3 subtype in affecting prognosis and was not an independent prognostic predictor in AML. Moreover, no significant difference was obtained between two groups when FAB-M3 subtype was excluded in patients. A recent study reported that over-expressed *miR-125b* group showed worse event-free survival (EFS) than low-expressed *miR-125b* group, while OS was not significantly different between those two groups in non-M3 AML patients [52]. High expression of *miR-125b* was closely correlated with treatment response, as well as relapse in APL [50]. The differences in AML subtype distribution may account for the conflicting results obtained from our data.

Recent reports presented *miR-125b* was associated with some gene mutations in leukemia. Bousquet et al. figured out that *miR-125b* could accelerate the oncogenicity of *BCR-ABL* in vivo [48]. Additionally, a higher expression status of *miR-125b* in *FLT3* mutation was checked out in AML while its up-regulation was not found in patients harboring an *NPM1* mutation, which give a clue that it appears to be specific to cytogenetically distinct subsets of adult AML [53]. The deregulated expression of *miR-125b* is dependent on the transcription factor *C/EBPA* in AML [54]. However, in this study we did not observe the association of *miR-125b* overexpression and gene mutations in both whole AML patients and non-M3 AML patients, which might be due to the small size of patients with mutations in our cohort.

In conclusion, our study indicated that *miR-125b* overexpression is associated with FAB-M3 subtype and is not a prognostic biomarker in AML. Moreover, *miR-125b* expression is not regulated by its methylation in AML.

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Disclosure of conflict of interest

None.

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