Int J Clin Exp Pathol 2015;8(5):4321-4331
www.ijcep.com /ISSN:1936-2625/IJCEP0007814

Original Article

The 5' flanking region of *miR-378* is hypomethylated in acute myeloid leukemia

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Received March 11, 2015; Accepted April 24, 2015; Epub May 1, 2015; Published May 15, 2015

Abstract: Background: Aberrant expression of *miR-378* has been observed in various malignancies including acute myeloid leukemia (AML). However, the mechanism regulating of *miR-378* expression remains unknown. This study was aimed to investigate *miR-378* methylation and to explore its clinical significance in AML. Methods: Methylation status of *miR-378* 5'-flanking region was investigated by real-time quantitative methylation-specific PCR (RQ-MSP) and bisulfite-sequencing PCR (BSP). The expression of *miR-378* was evaluated by real-time quantitative PCR (RQ-PCR). The correlation between expression of *miR-378* and 5'-flanking region methylation was analyzed using 5-aza-2'-deoxycytidine (5-aza-dC) treatment. Results: *miR-378* 5'-flanking region was significantly hypomethylated in AML patients compared to controls (median 0.109 vs. 0.058) (P=0.048). *miR-378* expression was correlated with *miR-378* 5'-flanking region in leukemic cell line treated with 5-aza-dC, but not in AML patients. The level of *miR-378* hypomethylation significantly increased in M2 subtype compared to other subtypes. Moreover, patients with t(8;21) harbored the highest level of *miR-378* hypomethylation. However, there was no significant difference in overall survival between patients with high and low *miR-378* hypomethylation. The association of *miR-378* expression with methylation was not observed in AML patients, but *miR-378* expression in THP-1 line was increased while methylation status of *miR-378* 5-flanking region was decreased after 5-aza-dC treatment. Conclusions: Our findings suggest that *miR-378* is reactivated by demethylation after 5-aza-dC treatment. 5'-flanking region of *miR-378* is hypomethylated in AML especially in those with t(8;21).

Keywords: *miR-378*, hypomethylation, acute myeloid leukemia, 5-aza-dC

Introduction

MicroRNAs (miRNA) are a group of small non-coding RNAs that regulate gene expression by inducing degradation or repressing translation of targeted mRNAs [1, 2]. It is well-known that miRNAs play pivotal roles in regulating cellular activities, including development, proliferation, differentiation, and apoptosis. Since the first direct link between miRNAs and cancer was made in 2002 when the loss of miR-15 and miR-16 was identified in chronic lymphocytic leukemia with del (13q14) [3], the association between alteration of miRNAs with cancer development and progression has been clear [4, 5]. The mechanisms involved in the control of miRNA expression include mutation, deletion, amplification, loss of heterozygosity, and epigenetic mechanisms [6].

Aberrant DNA methylation linked to silencing of individual miRNA genes has been found in acute myeloid leukemia (AML) [6]. Several miRNAs, such as *miR-212*, *miR-3151*, *miR-181*, and *miR-29b*, have been identified relevant with the outcome of AML [7-10]. However, the prognostic value of more miRNA abnormalities in AML is yet to be determined. Our previous study revealed the overexpression of *miR-378* in AML [11]. We identified that the 5'-flanking region of *miR-378* gene, located on chromosome 5q32, is embedded in a large CpG island containing 212 CpG binucleotides. It remains unclear whether *miR-378* overexpression is regulated by methylation. Moreover, the incidence of aberrant methylation of *miR-378* in AML is also unknown. In this study, we analyzed the methylation pattern of *miR-378* 5'-flanking region in AML patients using a real-time methyl-

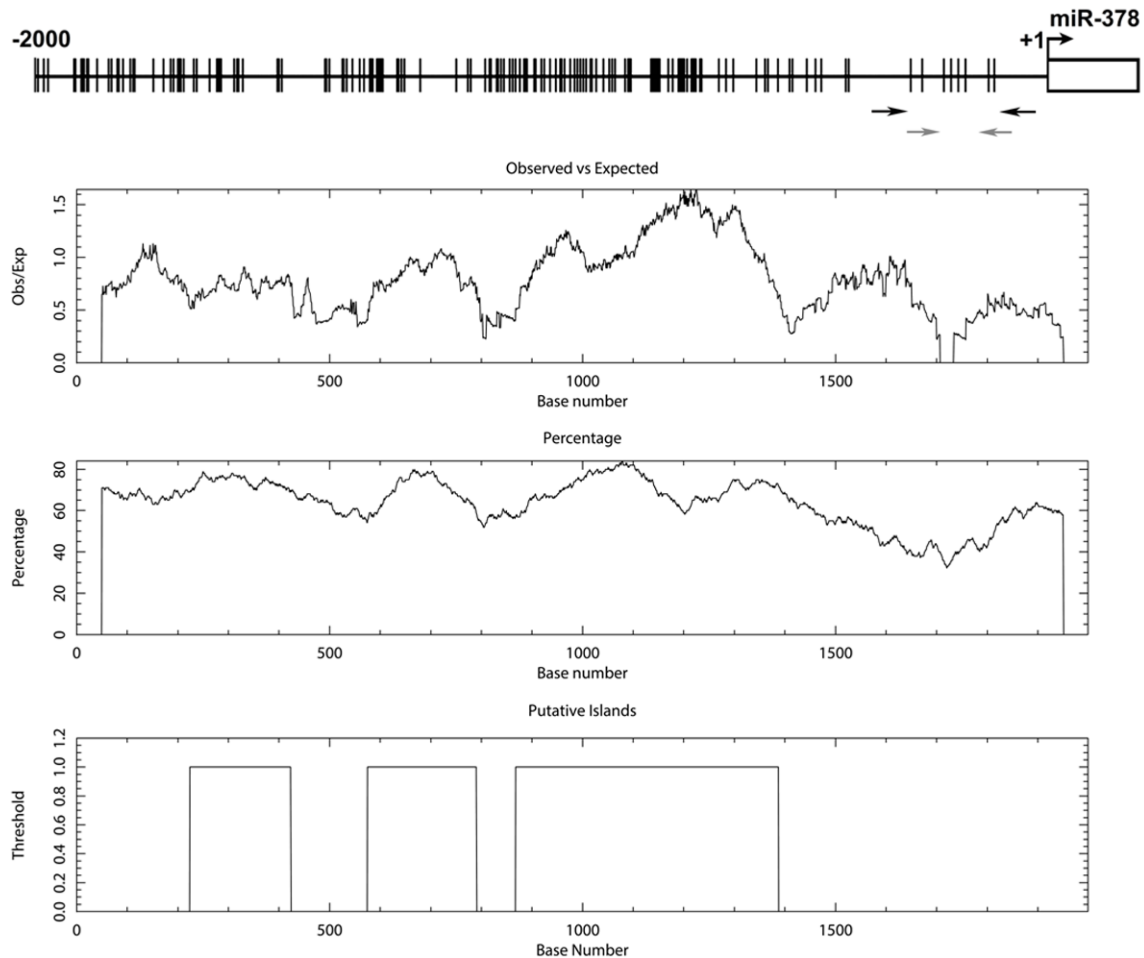

Figure 1. Bioinformatics analysis of the *miR-378* 5'-flanking region on chromosome 5. The vertical lines on the top horizontal line indicate the cytosine resides of CpGs. Numbers in the top panel represent nucleotide positions from *miR-378*. The rectangle indicates the position of the sequence encoding mature *miR-378*. The black arrows indicate the locations of primers used for bisulfite sequencing analysis, and the gray arrows indicate the locations of primers used for RQ-MSP analysis. Second panel represents the distribution of observed/expected ratios of CpG dinucleotides; third panel plots the GC content as a percentage of the total; bottom panel represents the putative 3 CpG islands within the -2.0 kb of analyzed sequence.

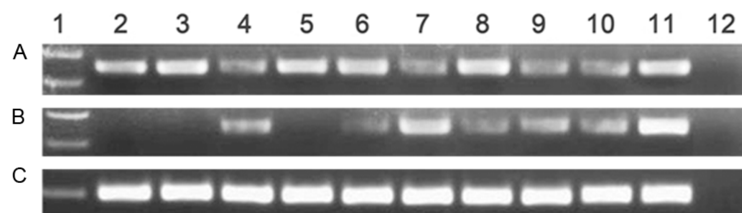

Figure 2. Electrophoresis results of RQ-MSP products in AML patients. A: *miR-378* methylation; B: *miR-378* unmethylation; C: *ALU*. 1: Gene Ruler^{TM} 100bp DNA ladder; 2-3: normal controls; 4-10: AML samples; 11: cloned plasmid; 12: negative control.

ation-specific PCR (RQ-MSP) and evaluated the effect of methylation on patient outcome.

Results

CpG islands in the miR-378 5'-flanking region

We focused on DNA methylation in the *miR-378* 5'-flanking region. Three CpG islands were predicted spanning bp -1775 to -612 (**Figure 1**). The University of California and Santa Cruz Genome Browser (UCSC Genome Browser) (http://genome.ucsc.edu/) and CpG Island Searcher (http://www.cpgislands. com/) also

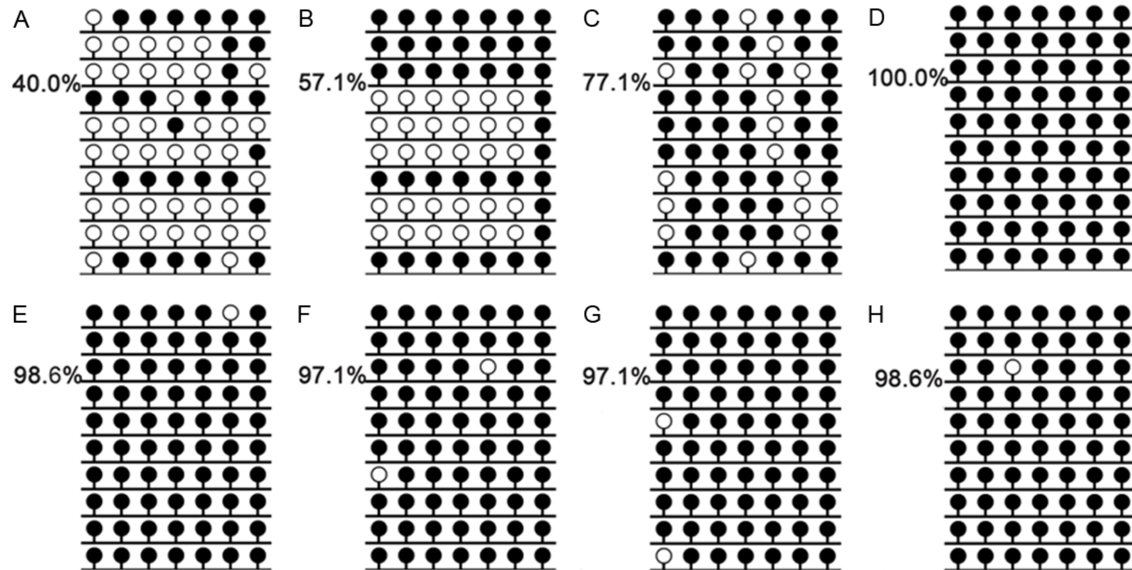

Figure 3. Methylation density of *miR-378* 5'-flanking region identified in two normal controls, three hypomethylated and three methylated AML patients by bisulfite sequencing. Black lollipop: methylated CpG dinucleotide; Blank lollipop: unmethylated CpG dinucleotide. A-C: three hypomethylated samples, A: M2, B: M4a, C: M5b; D-F: three methylated samples, D: M3, E: M1, F: M5b; G-H: normal controls. The results of RQ-MSP and bisulfite-sequencing were significantly negative correlated (*R*=-0.829, *P*=0.042).

Table 1. The sequences of primers used in RQ-MSP and BSP

	Forward (5'→3')	Reverse (5'→3')	Product (bp)
RQ-MSP			
M	GGATGAGTTTTGAGTCGTTT	CCATACAAACCGCTCACTCC	129
U	TAGGATGAGTTTTGAGTTGT	ATAATCCCATACAAACCACT	137
BSP	AGGATTTTTTGGTGATTTTTG	TCACCCTCTAACTACATAATCCC	200

confirmed the presence of CpG islands (data not shown).

Hypomethylation of miR-378 5'-flanking region in AML

The 5'-flanking region of *miR-378* was significantly hypomethylated in AML patients (median 0.109, range 0-20.258) compared to controls (median 0.058, range 0-1.000) (*P*=0.048). The representative electrophoresis results of RQ-MSP products were shown in **Figure 2**. In order to confirm the results of RQ-MSP, we evaluated the methylation density of 7-CpGs in *miR-378* 5'-flanking region in two normal controls, three *miR-378*-hypomethylated and three *miR-378*-methylated AML samples according to RQ-MSP. BSP results of normal controls presented that *miR-378* 5'-flanking region were strong methylated in normal BMNCs. The results of RQ-MSP and bisulfite-sequencing were significantly correlated in AML samples (*R*=-0.829, *P*=0.042) (**Figure 3**).

There was no correlation between *miR-378* hypomethylation with gender, age, and blood parameters. Among FAB subtypes, the level of *miR-378* hypomethylation significantly increased in M2 subtype compared to other subtypes (**Table 2**; **Figure 4**).

Association of miR-378 methylation with karyotypes

Patients with t(8; 21) harbored the highest level of *miR-378* hypomethylation compared to other karyotypes (**Table 2**; **Figure 5**). Due to *miR-378* is located on chromosome 5q32, the level *miR-378* hypomethylation was analyzed in patients with isolated -5/5q- or with concomitant other aberrations (n=5, median 0.708, range 0.144-0.877), which was significant higher than that

Table 2. Correlation between *miR-378* methylation and patients' parameters

Parameter	*mir-378* hypomethylation		
	No. of cases	Median (range)	*P*-value
Gender			
Male	80	0.148 (0-20.259)	0.101
Female	50	0.045 (0-4.273)	
Age			
≥60 years	46	0.133 (0-20.259)	0.806
<60 years	84	0.085 (0-4.273)	
WBC	130	17.5 (0.5-528.0)	0.429
FAB			
M1	9	0.035 (0-2.719)	0.126
M2	51	0.183 (0-4.273)	
M3	21	0.031 (0-1.503)	
M4	26	0.204 (0-20.259)	
M5	18	0.082 (0-2.739)	
M6	5	0.012 (0-0.877)	
WHO			
AML with t(8;21)	12	1.611 (0-4.273)	0.024
APL with t(15;17)	21	0.031 (0-1.503)	
AML with 11q23	4	0.397 (0-2.739)	
AML without maturation	6	0.029 (0-0.885)	
AML with maturation	41	0.137 (0-1.307)	
Acute myelomonocytic leukemia	26	0.204 (0-20.259)	
Acute monoblastic and monocytic leukemia	15	0.067 (0-2.635)	
Acute erythroid leukemia	5	0.012 (0-0.877)	
Karyotype classification			
Favorable	33	0.083 (0-4.273)	0.117
Intermediate	75	0.125 (20.259)	
Poor	16	0.397 (0-2.739)	
No data	6	0 (0-1.192)	
Karyotype			
normal	58	0.133 (0-20.259)	0.004
t(8;21)	12	1.611 (0-4.273)	
t(15;17)	21	0.031 (0-1.503)	
11q23	4	0.397 (0-2.739)	
complex	11	0.293 (0-1529)	
others	18	0.076 (0-1.022)	
No data	6	0 (0-0.129)	
C/EBPA mutant			
+	16	0.046 (0-2.719)	0.541
-	113	0.115 (0-20.259)	
NPM1 mutant			
+	16	0.034 (0-20.259)	0.363
-	113	0.115 (0-4.273)	
FLT3 ITD mutant			
+	17	0.022 (0-20.259)	0.075
-	112	0.127 (0-4.273)	

in controls (*P*=0.0045, **Figure 6**). However, There was no difference in *miR-378* hypomethylation between patients with and without -5/5q- (*P*=-0.124).

Association between miR-378 hypomethylation and prognosis

There were 93 patients with available survival data. This cohort of AML patients was divided into two groups according to the median level of *miR-378* hypomethylation. The patients with high level of *miR-378* hypomethylation had similar overall survival (OS) as those with low level of *miR-378* hypomethylation (median 7 months vs. 8 months, *P*=0.558, **Figure 7**). Multivariate analysis identified age and karyotypic risk but not *miR-378* hypomethylation as independent prognostic factors (data not shown).

Association of miR-378 methylation with miR-378 expression

The level of *miR-378* expression was examined in 56 AML patients with available mRNA. No correlation was observed between the level of *miR-378* expression and *miR-378* unmethylation (*R*=0.018, *P*=0.898).

To determine whether *miR-378* expression could be regulated by methylation, THP-1 cells were treated by 5-aza-dC. Obviously, *miR-378* expres-

C-KIT mutant			
+	5	0.026 (0-0.463)	0.357
-	124	0.109 (0-20.259)	
N/K-RAS mutant			
+	14	0.314 (0-2.635)	0.182
-	115	0.087 (0-20.259)	
DNMT3A mutant			
+	13	0.045 (0-1.216)	0.376
-	116	0.120 (0-20.259)	
IDH1/2 mutant			
+	8	0.214 (0-0.932)	0.582
-	121	0.095 (0-20.259)	
CR			
+	41	0.148 (0-20.259)	0.301
-	53	0.136 (0-3.593)	

WBC, white blood cells; FAB, French-American-British classification; AML, acute myeloid leukemia;CR, complete remission; *, percentage was equal to the number of mutated patients divided by total cases in each group.

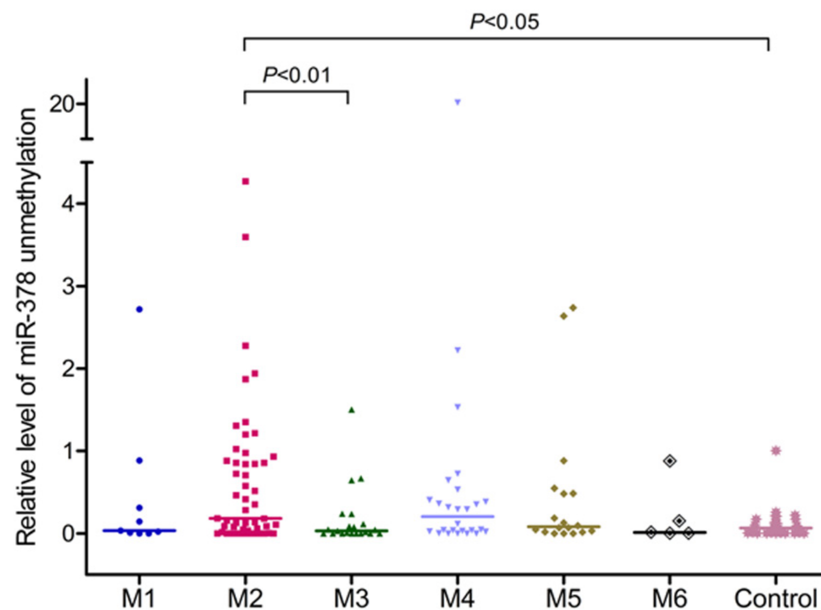

Figure 4. Relative levels of *miR-378* 5'-flanking hypomethylation in AML with different FAB subtypes.

sion was significantly up-regulated after 5-aza-dC treatment (**Figure 8A**). The level of unmethylated *miR-378* 5'-flanking region was increased in a dose-dependent manner in THP-1 cells treated with 5-aza-dC (**Figure 8B** and **8C**).

Discussion

Our previous study observed the overexpression of *miR-378* in AML [11]. However, the mechanism underlying *miR-378* upregulation remains unclear. In this study, we identified the large islands in the *miR-378* 5'-flanking region. Further study on primary samples confirmed the aberrant hypomethylation of *miR-378* 5'-flanking region in AML. However, *miR-378* expression was not associated with the pattern of *miR-378* methylation in AML patients. The treatment of THP-1 cell line with demethylating reagent 5-aza-dC indicated that *miR-378* could be reactivated by demethylation. These results suggest that *miR-378* expression may be regulated by other molecular mechanisms besides methylation in leukemia.

Interestingly, although the association of miR-378 expression with methylation was not observed in our cohort of AML patients, this study identified the prevalence of miR-378 hypomethylation in the FAB-M2 subtype, in accordance with our previous findings that the incidence of miR-378 overexpression was highest in the M2 subtype [11]. Further analysis disclosed the high level of miR-378 hypomethylation was associated with the t(8;21) chromosomal aberration, which also confirmed our previous observations [11]. Although miR-378 gene is located on chromosome 5q32, a commonly deleted region in myeloid malignancies, the incidence of miR-378 hypomethylation in this study or miR-378 overexpression reported previously was independent on -5/5q-. Further study is needed to determine the mechanism underlying miR-378 aberrations in the leukemogenesis involved in t(8;21) translocation.

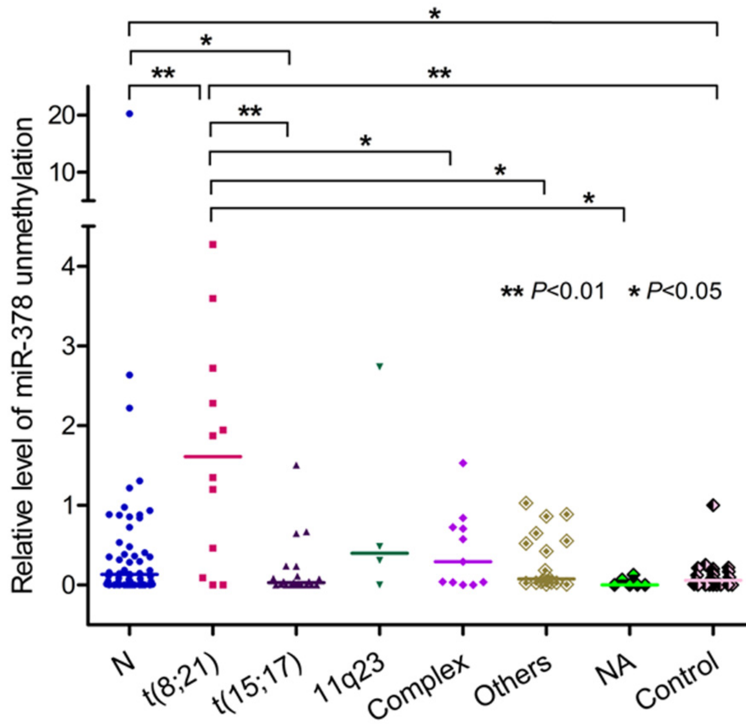

Figure 5. Relative levels of *miR-378* 5'-flanking hypomethylation in AML with different karyotypes.

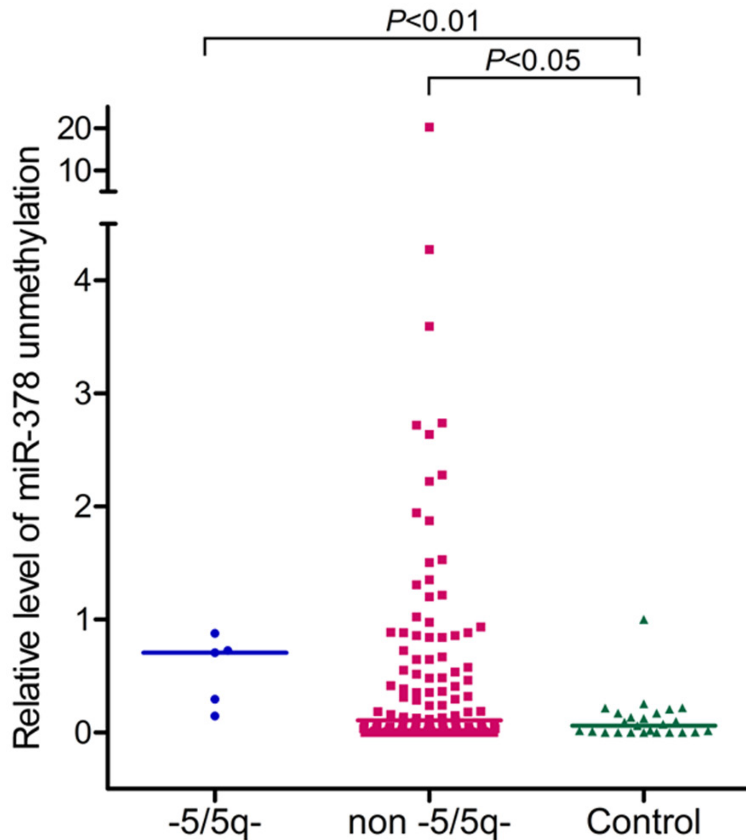

Figure 6. Relative levels of *miR-378* 5'-flanking hypomethylation in AML with and without -5/5q-.

The role of miR-378 in tumorigenesis remains controversial. Several studies indicated the oncogenic function of miR-378 due to enhancing cell survival, reducing apoptosis, accelerating tumor growth, angiogenesis and promoting cell migration and invasion in solid cancers including glioblastoma, non-small cell lung cancer (NSCLC), breast cancer, and nasopharyngeal carcinoma [20-25], whereas other studies suggest that miR-378 functions as a tumor suppressor in gastric and colorectal cancer [26-29]. The occurrence of miR-378 upregulation and miR-378 hypomethylation suggests the potential oncogenic role of miR-378 in AML.

The impact of miR-378 aberration on prognosis has been increasingly explored in cancers. In ovarian cancer, high miR-378 expression was associated with shorter survival after bevacizumab and chemotherapy [30]. Moreover, high miR-378 expression was associated with brain metastasis in NSCLC [21]. However, low miR-378 expression had significantly poorer overall survival in colorectal cancer [28]. The prognostic value of miR-378 expression was not observed in nasopharyngeal carcinoma and renal cell carcinoma [31, 32]. In addition, a genetic variant in pri-miR-378 was associated with increased miR-378 expression and with a better survival in hepatocellular carcinoma [33]. Inconsistent with our previous report in which miR-378 overexpression conferred an unfa-

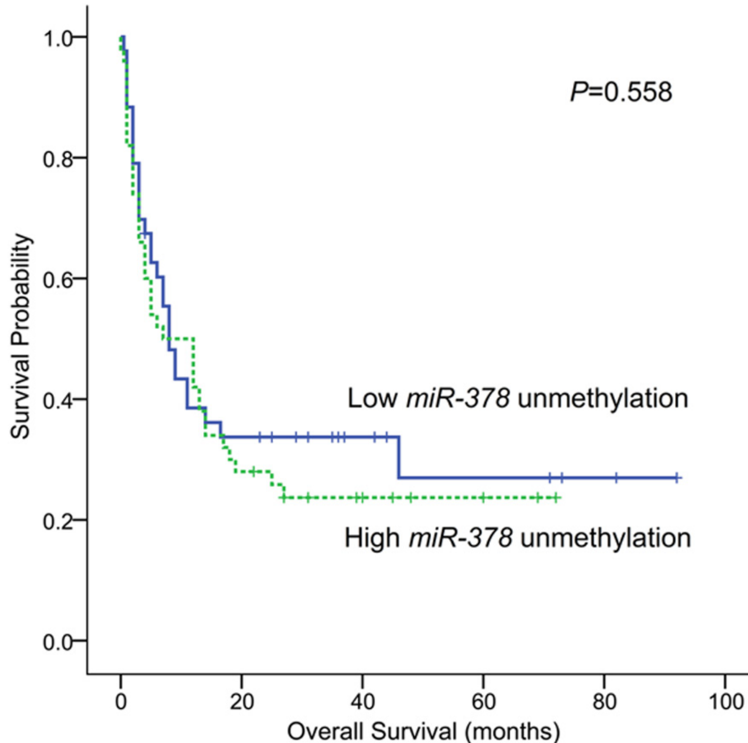

Figure 7. Overall survivals of AML patients.

vorable effect to outcome in AML [11], the present study did not observe the association of survival with miR-378 hypomethylation. Combined with the functional studies in cancers, these results indicate that miR-378 expression is regulated by various mechanisms that play different roles in tumorigenesis and clinical outcome, depending on the tissue- or time- context.

In conclusion, this is the first study on *miR-378* methylation which discloses that *miR-378* is reactivated by demethylation after 5-aza-dC treatment. 5'-flanking region of *miR-378* is hypomethylated in AML especially in those with t(8;21), but is unlikely to provide helpful prognostic information in AML patients.

Materials and methods

Patients and samples

130 patients with primary AML presented at the Affiliated People's Hospital of Jiangsu University were selected for this investigation based on the availability of stored leukemic cells. The diagnosis and classification of AML patients were made according to the revised French-American-British (FAB) classification and the 2008 World Health Organization criteria [12, 13]. Karyotypes were analyzed by conventional R-banding method. Karyotype risk was classified according to reported previously [14]. Treatment protocol was described previously [15]. 25 healthy donors were collected as controls. Written informed consent was obtained from all patients and normal controls. The study was approved by the Ethics Committee of the Affiliated People' Hospital of Jiangsu University. Bone marrow mononuclear cells (BMNCs) were separated by density gradient centrifugation using Ficoll solution and washed twice with PBS.

5-aza-2'-deoxycytidine treatment

The leukemic cell line THP-1 was plated at a density of 1×10^6/ml and was cultured in 5ml RPMI 1640 medium at 37°C in a humidified atmosphere containing 5% CO_2. 5-aza-2'-deoxycytidine (5-aza-dC) (Sigma-Aldrich, Steinheim, USA) diluted in dimethyl sulfoxide (DMSO) was added in four flasks of THP-1 cells once a day at the same time at different final concentrations of 0.1 μM, 1 μM, 10 μM and 50 μM as experimental group. THP-1 cells without treatment were used as the control. All cells were cultured until harvested for extraction of RNA and DNA.

Real-time quantitative PCR

Real time quantitative PCR (RQ-PCR) was performed to detect *miR-378* expression as reported previously [11].

Survey of CpG island in mir-378 5'-flanking region

The 5' sequences flanking the region of genomic DNA (gDNA) encoding *miR-378* on chromosome 5 [strand (+), nucleotides (nt) 149110392-149112413] was surveyed for the presence of CpG islands using cpgplot software (http://

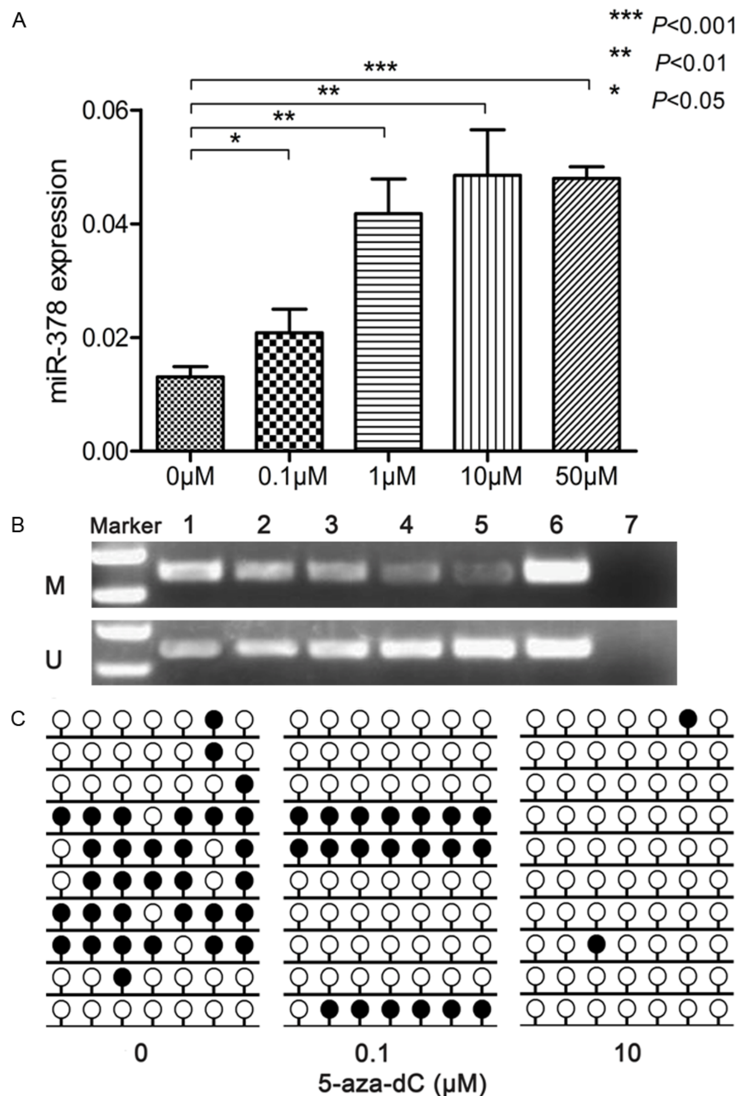

Figure 8. Methylation density of *miR-378* 5'-flanking region in THP-1 cells treated with 5-aza-dC. A: *miR-378* expression; B: RQ-MSP results, 1-5: THP-1 treated with 5-aza-dC (0 μM, 0.1 μM, 1 μM, 10 μM, and 50 μM, respectively), 6: recombinant methylated or unmethylated *miR-378* 5'-flanking region plasmids, 7: ddH20; C: BSP results. black lollipop: methylated CpG dinucleotide; blank lollipop: unmethylated CpG dinucleotide; methylation percentages at 7 CpG sites of *miR-378* 5'-flanking region in THP-1 treated with 0 μM, 0.1 μM and 10 μM of 5-aza-dC were 45.7%, 28.6% and 2.9%, respectively.

emboss.bioinformatics.nl/cgi-bin/emboss/cpgplot). The criteria used were as follows: island size >100 bp, GC percent >50.0%, and ratio of observed (Obs) CpG sites to expected (Exp) CpG sites >0.6).

DNA isolation and bisulfite modification

Genomic DNA was obtained from BMNCs using DNA Purification Kit (Gentra, Minneapolis, MN, USA). 1 μg of genomic DNA was sodium bisulphite-modified as described in manufacturer instruction using the CpGenome™ DNA Modification kit (Chemicon, Ternecula, CA, USA). Modified DNA was used immediately or stored at -80°C at once until analyzed.

Methylation-specific PCR (MSP) and real-time quantitative MSP (RQ-MSP)

RQ-MSP was performed using unmethylation-specific primers (**Table 1**) and *ALU* repetitive sequence on Step One Plus (Applied Biosystems, CA, USA). *ALU* repetitive sequence was used as reference sequence. A final volume of 20 μL of mixture containing 0.4 μM of primers, 10 μM SYBR Premix Ex Taq II, 0.4 μL 50×ROX (Takara, Japan) and 20 ng of modified DNA composed the reaction system. Amplification was carried out at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s, 62°C for 30 s and 72°C for 30 s, and a fluorescence collection step at 75°C for 30 s, followed by a melting program at 95°C for 15 s, 60°C for 60 s, 95°C for15 s, and 60°C for 15 s. Distilled water without DNA was used as negative control as well as recombined methylated and unmethylated *miR-378* plasmids were positive controls for each set of PCR. The normalized ratio ($N_{unmethylation\text{-}miR\text{-}378}$) was calculated in relation to the reference *ALU* sequence [16] and was used to assess the degree of methylation of *miR-378* 5'-flanking region in samples. The standard curves were established using recombined methylated and unmethylated *miR-378* plasmids as well as *ALU* plasmids from 1×10^8 copies/μl to 10 copies/μl before determining the cutoff level of specific fluorescence for the three corresponding sequences in samples. $N_{unmethylation\text{-}miR\text{-}378}$ was calculated according to the following formula.

$N_{unmethylation\text{-}miR\text{-}378} = (E_{unmethylation\text{-}miR\text{-}378})^{\Delta CTunmethylation\text{-}miR\text{-}378\ (control\text{-}sample)} \div (E_{ALU})^{\Delta CTALU\ (control\text{-}sample)}$. CT was the cycle number when the fluorescent signal intensity of detected gene in each reaction reaches to the setting threshold. E was the amplification efficiency of each PCR reaction and was computed as $E=10^{(-1/slope)}$. DNA methylation status of the *miR-378* 5'-flanking region was also determined by MSP using the primers listed in **Table 1**. MSP products were analyzed on 2% agarose gels and visualized under UV illumination after staining with ethidium bromide.

Bisulfite-sequencing PCR (BSP)

To further determine the comprehensive methylation status of the *miR-378* 5'-flanking region in AML patients, bisulfite-modified DNA sequencing PCR was performed in two normal controls, three *miR-378*-hypomethylated and three *miR-378*-methylated AML samples in accordance with the result of RQ-MSP. Each BSP reaction included 25 µL of mixture containing 10×*PCR buffer* (0.25 mM KCl), 6.25 *µ*M of dNTP Mixture, 0.5 *µ*M of primers, 0.75 U of Hot start DNA polymerase (Takara, Tokyo, Japan), and 20 ng of modified DNA. PCR conditions were 98°*C* for 10 s, 40 cycles for 10 s at 98°*C, 30 s at* 56°*C*, and 30 s at 72°C followed by a final 7 min extension step at 72°C. The reaction was performed on iCycler Thermal Cycler (Eppendorf, Hamburg, Germany). PCR products were cloned into pMD®. 19-T Vector (Takara, Dalian, China). 10 independent colonies of each sample were sequenced.

Mutations analysis

NPM1, *C-KIT, IDH1*, *IDH2, N/K-RAS* and *DNMT3A* mutations were detected by high-resolution melting analysis (HRMA) as reported previously [11, 18, 19]. Briefly, genomic DNA samples were amplified using gene-specific primers. Mutation scanning was performed for PCR products using HRMA with the LightScanner™ platform (Idaho, Salt Lake City, Utah). All positive samples were verified by direct DNA sequencing to confirm the results of HRMA. *FLT3* internal tandem duplication (ITD) and *C/EBPA* mutations were detected using direct DNA sequencing [11].

Statistical analysis

Statistical analysis was performed using the SPSS 17.0 software package (SPSS, Chicago, IL, USA). Pearson chi-square analysis and Fisher exact test were carried out to compare the difference of categorical variables between patients group. Mann-Whitney's U-test was carried out to compare the difference of continuous variables between two patient groups. The correlation between the frequency of *miR-378* 5'-flanking methylation and the clinical and hematologic parameters was analyzed with Spearman's rank correlation. Survival was analyzed according to the Kaplan-Meier method and differences in the distribution were evaluated by means of the log-rank test. A *P*-value of less than 0.05 was considered statistically significant.

Acknowledgements

This study was supported by the National Natural Science foundation of China (81270630, 81172592), 333 Project of Jiangsu Province (BRA2013136), Science and Technology Special Project in Clinical Medicine of Jiangsu Province (BL2012056), Science and Technology Infrastructure Program of Zhenjiang (SS2012003), Social Development Foundation of Zhenjiang (SH2013042, SH2013082, SH2014044), Key Medical Talent Program of Zhenjiang City, and Jiangsu Government Scholarship for Overseas Studies.

Disclosure of conflict of interest

None.

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