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].

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
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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.
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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’-TTGTGC-TTGTACATATG-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^(-ΔΔCt) 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 χ2 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 cyto-
**MiR-218 expression in AML**

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 in cytogenetically normal patients.

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

<table>
<thead>
<tr>
<th>Factor</th>
<th>Hazard ratio (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>0.976 (0.537-1.774)</td>
<td>0.937</td>
</tr>
<tr>
<td>Age</td>
<td>2.144 (1.204-3.818)</td>
<td>0.010</td>
</tr>
<tr>
<td>WBC</td>
<td>1.434 (0.796-2.581)</td>
<td>0.230</td>
</tr>
<tr>
<td>Karyotype classification</td>
<td>2.460 (1.449-4.178)</td>
<td>0.001</td>
</tr>
<tr>
<td>miR-218 expression</td>
<td>0.478 (0.267-0.857)</td>
<td>0.013</td>
</tr>
<tr>
<td>C-KIT mutation</td>
<td>1.018 (0.227-4.565)</td>
<td>0.982</td>
</tr>
<tr>
<td>C/EBPA mutation</td>
<td>1.704 (0.617-4.706)</td>
<td>0.304</td>
</tr>
<tr>
<td>NPM1 mutation</td>
<td>1.489 (0.556-3.983)</td>
<td>0.428</td>
</tr>
<tr>
<td>FLT3-ITD</td>
<td>0.590 (0.223-1.564)</td>
<td>0.289</td>
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Table 3. Multivariate analyses of prognostic factors for overall survival in CN-AML

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

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.

Acknowledgements

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Disclosure of conflict of interest

None.

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