Dysregulation of miR-200s clusters as potential prognostic biomarkers in acute myeloid leukemia

Jing-dong Zhou1,2†, Liu-chao Zhang3†, Ting-juan Zhang1,2, Yu Gu1,2, De-hong Wu4, Wei Zhang1,2, Ji-chun Ma2,5, Xiang-mei Wen2,5, Hong Guo2,5, Jiang Lin2,5* and Jun Qian1,2*

Abstract

Background: Increasing studies showed that miR-200 family (miR-200s) clusters are aberrantly expressed in multiple human cancers, and miR-200s clusters function as tumor suppressor genes by affecting cell proliferation, self-renewal, differentiation, division and apoptosis. Herein, we aimed to investigate the expression and clinical implication of miR-200s clusters in acute myeloid leukemia (AML).

Methods: RT-qPCR was performed to detect expression of miR-200s clusters in 19 healthy donors, 98 newly diagnosed AML patients, and 35 AML patients achieved complete remission (CR).

Results: Expression of miR-200a/200b/429 cluster but not miR-200c/141 cluster was decreased in newly diagnosed AML patients as compared to healthy donors and AML patients achieved CR. Although no significant differences were observed between miR-200s clusters and most of the features, low expression of miR-200s clusters seems to be associated with higher white blood cells especially for miR-200a/200b. Of the five members of miR-200s clusters, low expression of miR-200b/429/200c was found to be associated with lower CR rate. Logistic regression analysis further revealed that low expression of miR-429 acted as an independent risk factor for CR in AML. Based on Kaplan–Meier analysis, low expression of miR-200b/429/200c was associated with shorter OS, whereas miR-200a/141 had a trend. Moreover, multivariate analysis of Cox regression models confirmed the independently prognostic value of miR-200b expression for OS in AML.

Conclusions: Expression of miR-200a/200b/429 cluster was frequently down-regulated in AML, and low expression of miR-429 as an independent risk factor for CR, whereas low expression of miR-200b as an independent prognostic biomarker for OS.

Keywords: miR-200, Expression, Prognosis, Acute myeloid leukemia

*Correspondence: linjiangmail@sina.com; qianjun0007@hotmail.com
†Jing-dong Zhou and Liu-chao Zhang contributed equally to this work
1 Department of Hematology, Affiliated People’s Hospital of Jiangsu University, 8 Dianli Rd., Zhenjiang 212002, Jiangsu, People’s Republic of China
2 Laboratory Center, Affiliated People’s Hospital of Jiangsu University, 8 Dianli Rd., Zhenjiang 212002, Jiangsu, People’s Republic of China
Full list of author information is available at the end of the article

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Background

Acute myeloid leukemia (AML) is a highly heterogeneous malignant hematological disorder with complex molecular pathophysiology. Although the treatment strategies against AML have been updated in the past decades, the majority of patients eventually succumb to relapse after induction chemotherapy [1]. Clinical outcome of AML remains unsatisfactory especially in those with specific karyotypes/biomarkers such as inv(3)(q21q26.2), t(6;9) (p23; q34), 11q abnormalities other than t(9;11), -5/ del(5q), -7, TP53 mutations, FLT3-ITD mutations, C-KIT mutations, WT1 overexpression, and BAALC overexpression [2–4]. The development of effective therapeutic options against AML relies on mechanistic understanding of AML biology, especially in molecular regulators of AML pathogenesis and molecular predictor of AML prognosis [5].

MicroRNAs, a class of small (19–22 nucleotides) single-stranded RNAs, negatively regulate various genes by targeting 3′-untranslated region (3′-UTR) of mRNAs, thereby facilitating translational silencing or degradation of targeted genes [6]. Mounting evidences have implicated that microRNAs play crucial roles in regulating many fundamental and biological processes including cancer development [7]. Moreover, microRNAs have been reported as novel biomarkers for diagnosis and prognosis, and regarded as potential therapeutic targets in AML [8]. For instance, recent studies implicated that several microRNAs such as miR-216b, miR-362-5p, miR-217, and miR-193b were prognosis-related predictors in AML and may involve in AML biology [9–12].

The miR-200 family (miR-200s) clusters include five members (miR-200a, miR-200b, miR-200c, miR-141, and miR-429) and can be divided into two clusters (miR-200a/b/429 cluster and miR-200c/141 cluster) based on chromosomal location (chromosome 1p36 and chromosome 12p13) [13]. Numerous studies showed that miR-200s clusters are aberrantly expressed in multiple human cancers, and miR-200s clusters function as tumor suppressor genes by affecting cell proliferation, self-renewal, differentiation, division and apoptosis [14]. Although the tumor-suppressive roles of miR-200s clusters have also been reported in solid tumors with prognostic value [14, 15], the expression and clinical implication of miR-200s clusters in AML remains poorly revealed.

In this study, we investigated expression of miR-200s clusters in AML patients except for acute promyelocytic leukemia (APL), and found that low expression of miR-200s clusters acted as potential prognostic biomarkers in AML.

Methods

Patients and treatment

A total of 98 de novo AML patients except for APL and 19 healthy donors were enrolled in this study. Bone marrow (BM) was collected from all the patients at diagnosis time as well as 35 patients at complete remission (CR) time. AML was diagnosed based on the French–American–British (FAB) and 2016 revised World Health Organization (WHO) criteria [16, 17]. All the patients received chemotherapy as reported [18]. Induction chemotherapy therapy was 1–2 courses of daunorubicin combined with cytarabine. Subsequent consolidation treatment after CR for younger patients included high-dose cytarabine, mitoxantrone with cytarabine, and homoharringtonine combined with cytarabine, whereas for older patients received in an individualized manner decided by the physicians, such as CHG protocol (cytarabine, homoharringtonine, and G-CSF). This study was approved by the Ethics Committee of the Affiliated People’s Hospital of Jiangsu University, and written informed consents were informed and signed by all participants in accordance with the Declaration of Helsinki Principles.

Cytogenetic analysis and mutation detection

BM cells were harvested after 1–3 days of unstimulated culture in RPMI 1640 medium (BOSTER, Wuhan, China) containing 20% fetal calf serum (ExCell Bio, Shanghai, China). Cytogenetics for AML patients were analyzed at the newly diagnosis time by conventional R-banding method and karyotype risk was classified according to reported previously [19, 20]. Hotspot mutations in NPM1, C-KIT, DNMT3A, N/R-K-RAS, IDH1/2, U2AF1, SRSF2 and SETBP1 were detected by high-resolution melting analysis [21–25], whereas mutations in FLT3-ITD and CEBPA were examined by DNA sequencing [26].

RNA isolation and reverse transcription

BM mononuclear cells (BMMNCs) were extracted as reported using Lymphocyte Separation Medium (Absin, Shanghai, China) [27]. According to the manufacturer’s protocols, RNA was extracted from BMMNCs using the mirVana miRNA isolation kit (Ambion, Austin, TX, USA), and was synthesized to cDNA by reverse transcription using MiScript Reverse Transcription Kit (Qiagen, Duesseldorf, Germany).

Real-time quantitative PCR

The level of miR-200s clusters was detected by real-time quantitative PCR (RT-qPCR) using miScript SYBR green PCR kit (Qiagen, Duesseldorf, Germany). The primers were miR-200s specific (Additional file 1: Table S1) and the manufacturer-provided miScript universal primer
The programs for RT-qPCR reactions were performed as reported [28]. U6 small nuclear RNA was selected as the endogenous normalizer detected by RT-qPCR using 2× SYBR Green PCR Mix (Multisciences, Hangzhou, China). Relative miR-200s level was calculated by \(2^{-\Delta\Delta CT}\) method. The healthy donors that possessed the minimal ΔCT between miR-200s (each member) and U6 expression was selected as control, and was defined as 100% expression.

**Statistical analysis**

Mann–Whitney’s U test was carried to compare the difference of continuous variables between two groups, whereas Pearson Chi square analysis/Fisher exact test were applied to compare the difference of categorical variables between two groups. The impact of miR-200s clusters expression on overall survival (OS) was analyzed by Kaplan–Meier analysis, and Cox regression models (univariate and multivariate analyses) were further used to determine the independently prognostic value of miR-200s cluster expression. The effect of miR-200s clusters expression on CR was determined by Logistic regression analysis (univariate and multivariate analyses). All tests were two sided, and \(P<0.05\) was defined as statistically significant. SPSS software 20.0 and GraphPad Prism 5.0 was used to conduct the statistical analyses in this study.

**Results**

**Expression of miR-200s in AML**

We analyzed miR-200s clusters expression in BM from 19 healthy donors, 98 AML patients, and 35 AML patients achieved CR by RT-qPCR. As presented in Fig. 1, expression of miR-200a/200b/429 clusters but not miR-200c/141 clusters was significantly decreased in AML patients as compared to healthy donors and AML patients achieved CR.

**Relationship between miR-200s and clinical features in AML**

To investigate clinical implication of miR-200s clusters expression, the whole-cohort patients were classified into two groups (high and low miR-200s clusters expression) based on the median level of each member of miR-200s clusters, respectively. We analyzed the association between each member of miR-200s clusters expression and clinic-pathologic features including gender, age,
Table 1 Correlation of miR-200s cluster expression with clinical/laboratory features in AML patients

| Patient's features | miR-200a expression | miR-200b expression | miR-429 expression | miR-200c expression | miR-141 expression |
|--------------------|---------------------|---------------------|-------------------|--------------------|-------------------|
|                    | Low (n = 49)        | High (n = 49)       | P                 | Low (n = 49)        | High (n = 49)     | P                 | Low (n = 49)        | High (n = 49)       | P                 |
| Sex (male/female)  | 36/13               | 23/26               | 0.013             | 32/17              | 27/22             | 0.409             | 31/18              | 28/21             | 0.068             |
| Age (years)        | 58 (21–81)          | 61 (18–87)          | 0.842             | 60 (18–81)         | 59 (18–87)        | 0.831             | 60 (18–81)         | 59 (18–87)        | 0.741             |
| WBC (x 10⁹/L)      | 38.7 (1.3–528)      | 9.6 (1.1–130.2)     | 0.001             | 35.5 (1.3–528)     | 9.6 (1.1–130.2)   | 0.041             | 34.5 (1.1–130.2)   | 13.3 (1.1–130.2)  | 0.099             |
| Hemoglobin (g/L)   | 78 (53–138)         | 76.5 (32–144)       | 0.085             | 80 (53–138)        | 76.5 (32–144)     | 0.330             | 77 (32–138)        | 78 (34–134)       | 0.940             |
| Platelets (x 10⁹/L)| 37 (3–447)          | 47 (4–264)          | 0.558             | 37 (3–447)         | 46 (4–264)        | 0.460             | 36 (3–447)         | 47 (4–264)        | 0.512             |
| BM blasts (%)      | 60% (20–99%)        | 58% (20–95%)        | 0.892             | 63% (20–99%)       | 58% (21–95%)      | 0.651             | 60% (20–98%)       | 59% (20–99%)      | 0.757             |
| FAB sub-types      | M0 1                | 0                   | 0.660             | 0.945              | 0.681             | 0.827             | 0.960             | 0.900             |
|                    | M1 4                | 2                   | 3                 | 3                  | 3                 | 2                 | 3                 | 2                 |
|                    | M2 26               | 24                  | 23                | 27                 | 22                | 28                | 23                | 27                | 24                |
|                    | M4 12               | 15                  | 14                | 13                 | 15                | 12                | 15                | 12                | 15                |
|                    | M5 6                | 6                   | 7                 | 5                  | 8                 | 4                 | 7                 | 5                 | 6                 |
|                    | M6 0                | 2                   | 1                 | 1                  | 1                 | 1                 | 1                 | 1                 | 1                 |
| Karyotypes         | Normal 25           | 28                  | 23                | 30                 | 23                | 30                | 24                | 29                | 24                |
|                    | t(8;21) 5           | 5                   | 5                 | 6                  | 4                 | 5                 | 6                 | 4                 | 6                 |
|                    | +8 2                | 1                   | 2                 | 2                  | 1                 | 2                 | 1                 | 3                 | 0                 |
|                    | −5/5q− 1            | 2                   | 2                 | 2                  | 2                 | 2                 | 1                 | 2                 | 1                 |
|                    | −7/7q− 1            | 1                   | 0                 | 0                  | 1                 | 1                 | 1                 | 1                 | 1                 |
|                    | t(9;22) 1           | 0                   | 1                 | 0                  | 0                 | 1                 | 1                 | 0                 | 1                 |
| Complex 8          | 7                   | 7                   | 6                 | 10                 | 5                 | 8                 | 7                 | 8                 | 8                 |
| Others 6           | 5                   | 6                   | 6                 | 6                  | 6                 | 6                 | 6                 | 6                 | 6                 |
| No data 0          | 1                   | 0                   | 0                 | 1                  | 0                 | 1                 | 0                 | 1                 | 0                 |
| Gene mutations     | CEBPA (+) 7/33       | 6/38                | 0.765             | 5/38               | 8/33              | 0.0376            | 8/36              | 5/35              | 0.555             |
|                    | NPM1 (+) 5/35       | 4/40                | 0.730             | 3/40               | 6/35              | 0.307             | 4/40              | 5/35              | 0.730             |
|                    | FLT3-ITD (+) 6/34    | 3/41                | 0.298             | 6/37               | 3/38              | 0.484             | 6/38              | 3/37              | 0.488             |
Table 1 (continued)

| Patient's features | miR-200a expression | miR-200b expression | miR-429 expression | miR-200c expression | miR-141 expression |
|--------------------|----------------------|----------------------|--------------------|---------------------|--------------------|
|                    | Low (n = 49) | High (n = 49) | P       | Low (n = 49) | High (n = 49) | P       | Low (n = 49) | High (n = 49) | P       | Low (n = 49) | High (n = 49) | P       |
| C-KIT (+)          | 1/39       | 1/43       | 1.000   | 1/42       | 1/40       | 1.000   | 1/40       | 1/42       | 1.000   | 1/42       | 1/40       | 1.000   |
| N/K-RAS (+)        | 4/36       | 6/38       | 0.741   | 3/40       | 7/34       | 0.190   | 6/35       | 4/39       | 0.515   | 6/37       | 4/37       | 0.739   |
| IDH1/2 (+)         | 1/39       | 3/41       | 0.618   | 3/40       | 1/40       | 0.616   | 4/40       | 0/40       | 0.118   | 4/37       | 0/43       | 0.052   |
| DNMT3A (+)         | 4/36       | 2/42       | 0.418   | 4/39       | 2/39       | 0.676   | 4/40       | 2/38       | 0.678   | 3/38       | 3/40       | 1.000   |
| U2AF1 (+)          | 2/38       | 2/42       | 1.000   | 1/42       | 3/38       | 0.354   | 1/43       | 3/37       | 0.343   | 2/39       | 2/41       | 1.000   |
| SRSF2 (+)          | 1/39       | 3/41       | 0.618   | 1/42       | 3/38       | 0.354   | 1/43       | 3/37       | 0.343   | 1/40       | 3/40       | 0.616   |
| SETBP1 (+)         | 2/38       | 0/44       | 0.224   | 2/41       | 0/41       | 0.494   | 2/42       | 0/40       | 0.495   | 2/39       | 0/43       | 0.235   |
| CR (+)             | 16/33      | 23/26      | 0.215   | 14/35      | 25/24      | 0.038   | 14/35      | 25/24      | 0.038   | 15/34      | 24/25      | 0.0098  |

WBC white blood cells, BM bone marrow, FAB French–American–British classification, CR complete remission
Table 2: Univariate and multivariate analyses of variables for overall survival in AML patients

| Variables | Complete remission | Overall survival |
|-----------|--------------------|-----------------|
|           | Univariate analysis | Multivariate analysis | Univariate analysis | Multivariate analysis |
|           | OR (95% CI) | P | OR (95% CI) | P | HR (95% CI) | P | HR (95% CI) | P |
| miR-200a | 0.548 (0.242–1.244) | 0.150 | 1.029 (0.285–3.722) | 0.965 | 0.662 (0.415–1.022) | 0.082 | 1.425 (0.651–3.120) | 0.376 |
| miR-200b | 0.384 (0.167–0.885) | 0.025 | 0.823 (0.199–3.401) | 0.788 | 0.511 (0.319–0.819) | 0.005 | 0.524 (0.305–0.902) | 0.020 |
| miR-429 | 0.384 (0.167–0.885) | 0.025 | 0.331 (0.128–0.858) | 0.023 | 0.558 (0.350–0.891) | 0.015 | 0.820 (0.325–2.073) | 0.675 |
| miR-200c | 0.384 (0.167–0.885) | 0.025 | 0.977 (0.149–6.400) | 0.981 | 0.606 (0.380–0.965) | 0.035 | 0.649 (0.190–2.171) | 0.491 |
| miR-141 | 0.460 (0.201–1.050) | 0.065 | 0.594 (0.192–1.833) | 0.364 | 0.695 (0.437–1.104) | 0.123 | 1.152 (0.582–2.279) | 0.684 |
| Age | 4.229 (1.742–10.266) | 0.001 | 4.555 (1.715–12.095) | 0.002 | 2.046 (1.283–3.266) | 0.003 | 1.732 (1.033–2.902) | 0.037 |
| WBC | 2.367 (1.015–5.520) | 0.046 | 1.846 (0.715–4.767) | 0.206 | 2.002 (1.253–3.199) | 0.004 | 1.560 (0.925–2.629) | 0.095 |
| Karyotype | 3.108 (1.338–7.220) | 0.008 | 2.862 (1.164–7.042) | 0.022 | 1.875 (1.295–2.715) | 0.001 | 1.874 (1.210–2.902) | 0.005 |
| CEBPA mutations | 0.526 (0.160–1.731) | 0.290 | 0.870 (0.413–1.829) | 0.713 | 1.200 (0.516–2.793) | 0.672 | 1.200 (0.516–2.793) | 0.672 |
| NPM1 mutations | 0.833 (0.207–3.358) | 0.798 | 0.935 (0.403–2.170) | 0.876 | 0.548 (0.242–1.244) | 0.150 | 1.029 (0.285–3.722) | 0.788 |
| FLT3-ITD mutations | 0.833 (0.207–3.358) | 0.798 | 0.479 (0.066–3.458) | 0.465 | 1.029 (0.285–3.722) | 0.788 | 0.479 (0.066–3.458) | 0.465 |
| C-KIT mutations | 0.673 (0.041–11.150) | 0.783 | 1.311 (0.621–2.770) | 0.478 | 1.311 (0.621–2.770) | 0.478 | 1.311 (0.621–2.770) | 0.478 |
| NXX-RAS mutations | 3.048 (0.605–15.343) | 0.177 | 4.671 (1.637–13.326) | 0.004 | 4.671 (1.637–13.326) | 0.004 | 4.671 (1.637–13.326) | 0.004 |
| IDH1/2 mutations | Undetermined | 0.999 | 1.590 (0.634–3.987) | 0.323 | 1.590 (0.634–3.987) | 0.323 | 1.590 (0.634–3.987) | 0.323 |
| DNMT3A mutations | 1.391 (0.240–8.057) | 0.712 | 2.791 (0.987–7.890) | 0.053 | 2.791 (0.987–7.890) | 0.053 | 2.791 (0.987–7.890) | 0.053 |
| U2AF1 mutations | Undetermined | 0.999 | 1.934 (0.693–5.400) | 0.208 | 1.934 (0.693–5.400) | 0.208 | 1.934 (0.693–5.400) | 0.208 |
| SRSF2 mutations | Undetermined | 0.999 | 0.637 (0.088–4.613) | 0.656 | 0.637 (0.088–4.613) | 0.656 | 0.637 (0.088–4.613) | 0.656 |
| SETBP1 mutations | 0.673 (0.041–11.150) | 0.783 | 0.637 (0.088–4.613) | 0.656 | 0.637 (0.088–4.613) | 0.656 | 0.637 (0.088–4.613) | 0.656 |

OR: odd ratio, HR: hazard ratio, CI: confidence interval. Variables including miR-200s cluster expression (Low vs. High), age (≤ 60 vs. > 60 years), WBC (≥ 30 × 10⁹ vs. < 30 × 10⁹/L), karyotype (favorable vs. intermediate vs. poor), and gene mutations (mutant vs. wild-type). Multivariate analysis includes variables with P < 0.200 in univariate analysis.

We next evaluated the correlation of each member of miR-200s clusters expression with survival. Based on Kaplan–Meier analysis, low expression of miR-200b/429/200c was associated with shorter OS, whereas miR-200a/141 had a trend (Fig. 2). In addition, we also analyzed the impact of composite members of miR-200s clusters expression on OS by Kaplan–Meier analysis as shown in Fig. 3.

Since miR-200s clusters expression was associated with well-established prognostic factor such as WBC counts, we further conducted a Cox regression model adjusting for prognosis-related factors (age, WBC counts, karyotypic classifications, and gene mutations) for OS. Results showed that low expression of miR-200b acted as an independent prognostic biomarker for OS (P = 0.020, Table 2).

Discussion
In the current study, we for the first time investigated expression of miR-200s clusters in AML, and revealed that most of the members of miR-200s clusters were down-regulated in de novo AML patients. Recently, Li et al. revealed that introduction of a pre-miR-200c reduced the expression of ZEB2 protein and inhibited the proliferation of human leukemia cell lines (HL-60, AML).
MOLM-13, and THP-1), and mouse miR-200c significantly impaired the proliferation of mouse leukemia cells [29]. Taken together, these results emphasized the crucial role of miR-200s clusters in leukemogenesis. Although the biological role of miR-200s clusters in AML was less studied, tumor suppressor roles of miR-200s clusters have been identified in a variety of human solid cancers, such as bladder cancer, gastric cancer, colorectal cancer, breast cancer, ovarian cancer, endometrial cancer, pancreatic cancer, gliomas, hepatocellular carcinoma, and lung cancer [14, 30]. The miR-200s clusters were reported as key inhibitors of epithelial-to-mesenchymal transition by directly targeting transcriptional repressors of E-cadherin, ZEB1, and ZEB2 [13]. Moreover, miR-200s clusters also played crucial roles in the repression of cancer stem cells self-renewal and differentiation, modulation of cell division and apoptosis, and reversal of chemoresistance [14, 30]. Notably, in some other hematological malignancies, expression or biological role of miR-200s clusters has been preliminary studied. For instance, Choi et al. reported that miR-200c was decreased in patients with myelodysplastic syndrome (MDS) [31]. González-Gugel et al. revealed that down-regulation of mmu-miR-30a and mmu-miR-141 as well as hsa-miR-193b clearly contributed to enhance the expression of Smoothed (SMO) gene in mouse and human lymphomas and, subsequently, to activate the GLI/Hh signalling [32].

In addition to basic research before, it has been noted that low expression of miR-200s clusters could correlate with adverse clinical outcome and serve as a prognostic biomarker for various cancer patients [15]. Although the potential prognostic value of miR-200s clusters in several human cancers remains controversial, a recent meta-analysis demonstrated that lower tissue expression of miR-200s clusters’ members were associated with poor OS and progression-free survival, whereas lower expression of circulating miR-200s clusters’ members were correlated with favorable prognosis [15]. From our study, we showed the negative effect of low expression of miR-200s clusters on AML chemotherapy response and survival. Moreover, multivariate analysis showed that low expression of miR-429 as an independent risk factor for CR, whereas low expression of miR-200b as an independent prognostic biomarker for OS in AML. Due to some
limitations in this study (such as patients numbers, treatment regimens, and single center), prospective studies are needed to verify our results before miR-200s clusters expression could be used routinely as a promising biomarker for risk stratification in AML.

**Conclusion**

Expression of miR-200a/200b/429 cluster was frequently down-regulated in AML, and low expression of miR-429 as an independent risk factor for CR, whereas low expression of miR-200b as an independent prognostic biomarker for OS.

**Additional file**

Additional file 1: Table S1. The primer sequences for miR-200s clusters.

**Abbreviations**

AML: acute myeloid leukemia; 3′-UTR: 3′-untranslated region; APL: acute promyelocytic leukemia; BM: bone marrow; CR: complete remission; BMMNCs: BM mononuclear cells; FAB: French–American–British; WHO: World Health Organization; RT-qPCR: real-time quantitative PCR; OS: overall survival; WBC: white blood cell.

**Authors’ contributions**

JQ and JL conceived and designed the experiments; JZ and LZ performed the experiments; JZ and TZ analyzed the data; YG, WZ and DW collected the clinical data; JM, XW and HG offered technique support; JZ wrote the paper. All authors read and approved the final manuscript.

**Author details**

1. Department of Hematology, Affiliated People’s Hospital of Jiangsu University, 8 Dianli Rd., Zhenjiang 212002, Jiangsu, People’s Republic of China. 2. The Key Lab of Precision Diagnosis and Treatment of Zhenjiang City, Zhenjiang, Jiangsu, People’s Republic of China. 3. Jingjiang College of Jiangsu University, Zhenjiang, Jiangsu, People’s Republic of China. 4. Department of Hematology, The Third People’s Hospital of Kunshan City, Kunshan, Jiangsu, People’s Republic of China. 5. Laboratory Center, Affiliated People’s Hospital of Jiangsu University, 8 Dianli Rd, Zhenjiang 212002, Jiangsu, People’s Republic of China.
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