Prognostic Significance of Mixed-Lineage Leukemia (MLL) Gene Detected by Real-Time Fluorescence Quantitative PCR Assay in Acute Myeloid Leukemia

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Background: The overall prognosis of acute myeloid leukemia (AML) patients with mixed-lineage leukemia (MLL) gene-positivity is unfavorable. In this study, we evaluated the expression levels of the MLL gene in AML patients.

Material/Methods: We enrolled 68 MLL gene-positive patients out of 433 newly diagnosed AML patients, and 216 bone marrow samples were collected. Real-time fluorescence quantitative PCR (RQ-PCR) was used to precisely detect the expression levels of the MLL gene.

Results: We divided 41 patients into 2 groups according to the variation of MRD (minimal residual disease) level of the MLL gene. Group 1 (n=22) had a rapid reduction of MRD level to $10^{-4}$ in all samples collected in the first 3 chemotherapy cycles, while group 2 (n=19) had MRD levels constantly >$10^{-4}$ in all samples collected in the first 3 chemotherapy cycles. Group 1 had a significantly better overall survival ($p=0.001$) and event-free survival ($p=0.001$) compared to group 2. Moreover, the patients with >$10^{-4}$ MRD level before the start of HSCT (hematopoietic stem cell transplantation) had worse prognosis and higher risk of relapse compared to patients with ≤$10^{-4}$ before the start of HSCT.

Conclusions: We found that a rapid reduction of MRD level to ≤$10^{-4}$ appears to be a prerequisite for better overall survival and event-free survival during the treatment of AML. The MRD levels detected by RQ-PCR were basically in line with the clinical outcome and may be of great importance in guiding early allogeneic HSCT (allo-HSCT) treatment.

MeSH Keywords: Leukemia, Myeloid, Acute • Myeloid-Lymphoid Leukemia Protein • Neoplasm, Residual • Real-Time Polymerase Chain Reaction

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Background

Karyotype is one of the prognostic factors of acute myeloid leukemia (AML) [1,2]. The most common chromosome abnormalities of AML are t(8;21)(q22;q22), t(15;17)(q22;q11~21), inv(16) (p13q22), and t(11q23) [3–6]. Among these, the mixed-lineage leukemia (MLL) gene, located at 11q23, is often disrupted in hematological malignancies [7,8]. MLL gene rearrangements are common cytogenetic abnormalities in AML [9,10]. The MLL gene can be fused with a variety of partner genes by chromosome translocations, and form typical fusion genes in leukenomogenesis of AML [11–14]. Currently, up to 70 different partners of MLL fusion genes have been confirmed, all correlated with high-risk acute leukemia [15,16]. More than 50 MLL partner genes have been cloned and the most frequent translocation involving 11q23 is t(9;11)(p22;q23) in AML, forming MLL-AF9 fusion transcripts [17,18]. Other common translocations include t(6;11)(q27;q23), t(10;11)(p12;q23), t(11;19)(q23;p13.1), and t(11;19)(q23;p13.3), forming MLL-AF6, MLL-AF10, MLL-ELL, and MLL-ENL fusion transcripts, respectively [19–21].

MLL gene-positive AML patients are characterized by unique cytogenetical, molecular, biological, and clinical features. Particularly, MLL-AF9 and MLL-PTD genes are now correlated with worse outcome. MLL fusion genes have a negative impact on the complete remission (CR), overall survival (OS), and event-free survival (EFS) of AML patients [22,23]. Consequently, the early detection and evaluation of minimal residual disease (MRD) of MLL gene in AML patients is of great clinical significance. In the present study we established a real-time fluorescence quantitative PCR (RQ-PCR) assay to quantify the expression levels of 41 MLL gene-positive patients during the therapy. We aimed to investigate the correlation between the expression levels of the MLL gene and the prognosis of AML patients.

Material and Methods

Patients and treatment

We identified 68 MLL gene-positive AML patients by using multiplex-nested polymerase chain reaction (PCR) from 433 newly diagnosed AML patients since April 2008 in the Department of Hematology, the PLA General Hospital, Beijing, China. The positivity rate of MLL gene-positive AML patients was 15.7%. From 41 MLL gene-positive AML patients, 216 bone marrow samples were available for RQ-PCR assay. The patients were diagnosed with AML according to French-American-British (FAB) classifications, standard immunophenotyping, and morphological criteria. All patients had consented to the use of bone marrow samples. Approval was obtained from the Institutional Review Board of the PLA General Hospital.

All patients underwent standard induction chemotherapy of IA, MA, or DA (IA: idarubicin 8–10 mg/m², MA: mitoxantrone 8–10 mg/m², DA: daunorubicin 45 mg/m², for 3 days), then cytarabine 100 mg/m²/day×7. Patients who achieved hematological complete remission (CR) underwent intensified consolidation and maintenance therapy thereafter. A total of 21 patients received allogeneic hematopoietic stem cell transplantation (allo-HSCT).

RNA extraction, reverse transcription, and RQ-PCR

RNA was extracted from fresh bone marrow cells and reverse-transcribed to CDNA as previously described [24]. Reverse transcription and RQ-PCR were performed using the Veriti® Thermal Cycler (Applied Biosystems, USA) and Stratagene Mx3000P (Stratagene, La Jolla, CA). The primers and probes for the detection of MLL fusion genes in the RQ-PCR were synthesized by Life Technologies (Applied Biosystems, USA) and are listed in Table 1.

RQ-PCR analysis was carried out using TaqMan universal master mix (Applied Biosystems, USA). GAPDH mRNA was used as an internal positive control to confirm the integrity of the extracted RNA and to rectify synthesis of cDNA. The total reaction volume of 20 μL contained 2 μL cDNA samples, 2 μL appropriate primers and probes of MLL fusion genes, 6 μL nuclease-free water, and 10 μL PCR buffer. The amplification was performed as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. A standard curve was produced for MLL fusion genes by a 10-fold serial dilution of 4 different plasmid concentrations. A reference dilution was studied in all assays of RQ-PCR to have the standard curve loaded over the reference sample (Figure 1).

Karyotype analysis

Metaphase chromosomes were karyotyped according to the International System for Human Cytogenetic Nomenclature and then analyzed to demonstrate the aberrations. The bone marrow blast cells underwent cytogenetic analysis after 24-h unstimulated culture.

Statistical analysis

Survival curves were evaluated using Kaplan-Meier analysis to calculate overall survival (OS) from diagnosis to death or last contact for other patients, and event-free survival (EFS) from diagnosis to the date of failure or last follow-up. Differences between groups were analyzed using a 2-sided log-rank test. Statistical significance was considered at p<0.05 on both sides. Cox regression analysis was used to evaluate the prognostic significance of several prognostic factors. Patient age, treatment, and the group division based on variation in expression...
The statistical analyses were performed with SPSS 19.0 for Windows (SPSS, Chicago, IL).

Results

Patients and samples

A total of 41 MLL gene-positive AML patients and 216 bone marrow samples were included in the study. A median follow-up time was 19 months (range, 1–55 months) and the median number of samples was 5 samples (range, 1–15 samples) from diagnosis. The specific characteristics of 41 patients are summarized in Table 2.

| No. | Fusion genes | Primer 5'-3' | Probe |
|-----|--------------|-------------|-------|
| 1   | MLL-PTD      | F CAAGAAAAGAAGTCCAAAAACCA AAGAAAAAGCAGGCCTC | R ACTTCGCACTCTGACCTTTCCACTT |
| 2   | MLL-AF9      | F AAGCAGTGCTGAAGATGGAA ACTTCGCACTCTGACCTTTCCACTT |
| 3   | MLL-Ell     | F TCCAGAGGAGAGGCAAACAGAAAAAAGCAGCCTCAGCAGG |
| 4   | MLL-AF10     | F CAATATAAAAAAGACCTGCTGCAAGA TTCACTATGCGTGCACTTCT |
| 5   | MLL-AF17     | F GTTCCCCAAAACCATCTCTTAGGA TGACCTCTTACCTTCAGGAGG |
| 6   | MLL-AF6      | F GTTCCCCAAAACCATCTCTCTAGGA TGCAAGAATGTTCCAGAGG |
| 7   | MLL-ENL      | F TCCAGAGGAGAGGCAAACAGAAAAAAGCAGCCTCAGCAGG |
| 8   | MLL-CBP      | F TGGGAGATGAGGAGCTCTAGG TGACCTCTTACCTTCAGGAGG |
| 9   | MLL-AF1P     | F GTCCAGAGGAGGAATGAGAA TTCACTATGCGTGCACTTCT |
| 10  | MLL-AF1Q     | F CACTTTGACATCTCAGGACACTCT TTCAATGAGAATGTT |
| 11  | MLL-AFX1     | F GGTCCAGAGGAGAGGCAAACAG AAAAGGCTGCTCCAGG |

Table 1. PCR primers and probes for detection of MLL fusion genes.

Effect of the reduction of MRD level on overall and event-free survival

We divided 41 patients into 2 groups according to the variation in expression level of MLL gene; 22 patients had a rapid reduction of MRD level to ≤10−4 in all samples collected in the first 3 chemotherapy cycles (group 1), and 19 patients had an MRD level of constantly >10−4 in all samples collected in the first 3 chemotherapy cycles.

In group 1 (n=22), 15 patients were in continuous hematologic complete remission after the chemotherapy, with a median follow-up of 28 months (range, 11–55 months). These patients all survived except for 1 patient who died in complete remission because of graft-versus-host disease (GVHD) at 2 months after allo-HSCT. Five other patients relapsed at a median of 10 months after diagnosis (range, 2–12 months) and survived. One patient relapsed at 7 months after diagnosis and died. The 1 remaining patient is in sustained non-remission state.

In group 2 (n=19), 1 patient relapsed at 7 months after diagnosis and died. The 1 remaining patient is in sustained non-remission state. A >10−4 MRD level was detected in those 6 relapsed patients in the first RQ-PCR assay after relapse and of these, 2 had died by the time of the last RQ-PCR assay.
In group 2 (n=19), the MRD levels of all 19 patients were >10^{-4} at each time point examined during therapy. Among 12 patients who achieved hematologic complete remission, 5 were in continuous hematologic complete remission and 7 had relapsed at a median of 4 months after diagnosis (range, 1–10 months). The remaining 7 patients were all in sustained non-remission state. In group 2, 17 patients died and their last MRD levels were all >10^{-4}. Among these 17 patients, 4 died of GVHD in a relapse state at a median of 6 months after allo-HSCT (range, 2–8 months), 4 died in hematologic complete remission at a median of 6 months after achieve hematologic complete remission (range, 4–7 months), 6 died in sustained non-remission state at a median of 9 months after diagnosis (range, 1–19 months), and 3 died at a median of 2 months after relapse (range, 1–9 months). Of the 2 surviving patients, 1 is in sustained non-remission state and 1 has been in continuous hematologic complete remission for 23 months. We detected a >10^{-4} MRD level in those 7 relapsed patients at the first RQ-PCR assay after relapse, and in those 17 dead patients at the last RQ-PCR assay.

Group 1 had significantly better overall survival (p=0.001) and event-free survival (p=0.001) compared to group 2 (Figure 3). The median overall survival and event-free survival in group

![Amplification curve for standard samples (A) and patient samples (B).](image)

Figure 1. Amplification curve for standard samples (A) and patient samples (B).
The 4-year OS rate and 4-year EFS rate were 90.9% (95%CI 78.9–100%) and 68.2% (95%CI 48.8–87.6%), respectively, in group 1, compared with 10.5% (95%CI 0–24.2%) (p=0.001) and 26.3% (95%CI 6.5–46.1%) (p=0.001), respectively, in group 2. Allo-HSCT was more frequent in group 1 (15/22 vs. 5/19). When censored at the time of allo-HSCT, the differences in overall survival and event-free survival were statistically significant between these 2 groups (p=0.001 and p=0.014, respectively).

**Effect of MRD level before the start of HSCT on overall and event-free survival**

The patients with >10⁻⁴ MRD level before the start of HSCT had worse prognosis and higher risk of relapse compared to patients with ≤10⁻⁴ before the start of HSCT: 4-year OS rate and 4-year EFS rate were both 33.3% (95%CI 0–71.0%) in all 6 patients with a MRD level of >10⁻⁴ before the start of HSCT, compared with 86.7% (95%CI 69.5–100%) (p=0.034) and 73.3% (95%CI 50.9–95.7%) (p=0.030), respectively, in all 15 patients with MRD levels ≤10⁻⁴ before the start of HSCT.

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**Table 2. Characteristics of 41 AML patients with MLL rearrangement.**

| Number of MLL fusion gene-positive patients | N=41 (adult 36/teenager 5) |
|-------------------------------------------|---------------------------|
| Median age (years)                        | Adult 48y (range 19–75)   |
|                                           | Teenager 15y (range 13–17)|
| Median follow-up time (months)            | 19 (range 1–55)           |
| Sex                                       | Male 27/Female 14         |
| Median WBC counts (×10⁹/L)                | 8.5 (range 0.15–362)      |
| Median hemoglobin (g/L)                   | 84 (range 52–128)         |
| Median platelet counts (×10⁹/L)           | 69 (range 6–229)          |
| Median bone marrow blasts                 | 37% (range 0.4–97.8%)     |
| Samples                                   | Median samples (range)     |
|                                           | 216                       |
|                                           | 5 (1–15)                  |
| FAB                                       |                           |
| M1                                        | 2                         |
| M2                                        | 10                        |
| M3                                        | 1                         |
| M4                                        | 7                         |
| M5                                        | 13                        |
| M4,M5                                     | 1                         |
| M6                                        | 3                         |
| Others*                                   | 4                         |
| Cytogenetics                              |                           |
| Normal                                    | 8                         |
| Abnormal                                  | 20                        |
| 11q23 abnormalities                       | 3                         |
| +8                                        | 8                         |
| –7/7q-                                    | 3                         |
| Complex karyotype                         | 10                        |
| Unknown                                   | 13                        |
| Treatment                                 |                           |
| Chemotherapy                              | 20                        |
| Allo-HSCT                                 | 21                        |
| Result                                    |                           |
| Survival                                  | 22                        |
| Death                                     | 19                        |

* Others including 1 case each of biphenotypic acute leukemia, MDS-AML, MDS-RAEB, and acute mixed lineage leukemia
Prognostic factor analysis

Univariate analysis was used to assess the prognostic significance of group division based on the variation in expression level of MLL gene (group 1 vs. group 2), age at diagnosis (age above 60 years vs. age below 60 years), sex (male vs. female), diagnostic peripheral WBC counts (≥ 100 x 10^9/L), diagnostic peripheral PLT counts (≥ 50 x 10^9/L), cytogenetics (normal vs. abnormal), and treatment (chemotherapy vs. HSCT). The HR for death for patients in group 1 was 19.762 compared with group 2 (p=0.001). Age above 60 years and chemotherapy patients were univariately associated with worse overall survival (Table 3). Multivariate analysis of the significant univariate prognostic factors (group division, age, and treatment) indicated that group division was the single independent prognostic factor for overall survival.

Figure 2. The proportion of MLL fusion partners in AML patients with MLL rearrangement.

Figure 3. Outcome of AML patients with MLL rearrangement according to MRD risk group. (A) Overall survival; (B) Event-free survival.
### Discussion

It has been demonstrated that RQ-PCR is an effective method for evaluating the prognosis of PML-RARA-, AML1-ETO-, and CBFB-MYH11-positive AML patients [25–30]. In this study, RQ-PCR was used to precisely detect the expression levels of 11 MLL fusion genes in AML patients. We evaluated the applicability in MRD detection of RQ-PCR and correlation with the therapeutic effects and treatment outcome.

A series of MRD detection results showed that regular MRD monitoring of MLL fusion genes using RQ-PCR significantly helps AML patients during the therapy. In this study, patients with a rapid reduction of MRD level to $\leq 10^{-4}$ in all samples collected in the first 3 chemotherapy cycles (group 1) had significantly better overall survival and event-free survival compared to patients with an MRD level of constantly $>10^{-4}$ in all samples collected in the first 3 chemotherapy cycles (group 2; Figure 3). Further analysis showed that the median overall survival and event-free survival in group 1 were significantly longer than in group 2. Moreover, 4-year OS rate and 4-year EFS rate in group 1 were also significantly higher than in group 2. These results suggest that early reduction and a continuous $\leq 10^{-4}$ of the MRD level may be a new determinant to the long-term survival and complete remission in MLL gene-positive AML patients. When censored at the time of allo-HSCT, the differences in overall survival and event-free survival were statistically significant ($p=0.001$ and $p=0.014$, respectively).

Our study showed that 21 patients in group 1 and 10 patients in group 2 achieved complete remission (CR rate was 95.5% and 52.6%, respectively). Only 6 patients relapsed and 7 died in group 1, while 6 patients relapsed and 17 died in group 2 (relapse rates 28.6% and 70%, respectively). A $>10^{-4}$ MRD level was detected in a total of 13 relapsed patients in the first RQ-PCR assay after relapse and in a total of 19 dead patients in the last RQ-PCR assay. This suggests that the MRD results detected by RQ-PCR were basically in line with the clinical outcome of the patients. In addition, we detected molecular relapse in 3 patients before hematologic relapse, indicating that RQ-PCR may be of significance in early prediction of relapse. Some studies have shown that early prediction of molecular relapse and early specific treatment lead to better prognosis [31,32]. However, we still need to clarify whether this is also true for MLL gene-positive AML patients. Twenty patients in our study were in continuous hematologic complete remission, while 15 of them had a rapid reduction of MRD level to $\leq 10^{-4}$ in all samples collected in the first 3 chemotherapy cycles, suggesting it might be critical for prolonged complete remission in MLL gene-positive AML patients.

### Table 3. Cox regression analysis of overall survival for all patients after diagnosis.

| Prognostic factors | No. | Median OS (months) | Univariate | Multivariate |
|--------------------|-----|-------------------|------------|-------------|
|                    |     |                   | HR         | 95%CI       | P   | HR         | 95%CI       | P   |
| Group division     |     |                   |            |             |     |            |             |     |
| Group 1            | 22  | Not reached       | 19.762     | 4.468–87.402| 0.001| 16.636     | 3.626–76.314| 0.001|
| Group 2            | 19  | 8                 |            |             |     |            |             |     |
| Age                |     |                   |            |             |     |            |             |     |
| <60 y              | 30  | 20                | 3.497      | 1.376–8.887 | 0.009| 1.467      | 0.473–4.544 | 0.507|
| ≥60 y              | 11  | 7                 |            |             |     |            |             |     |
| Sex                |     |                   |            |             |     |            |             |     |
| Male               | 27  | 14                | 0.766      | 0.291–2.021 | 0.591| –          | –           | –   |
| Female             | 14  | 19                |            |             |     |            |             |     |
| Peripheral WBC counts |   |                   |            |             |     |            |             |     |
| <100×10⁹/L         | 38  | 19                | 2.852      | 0.651–12.496| 0.164| –          | –           | –   |
| ≥100×10⁹/L         | 3   | 2                 |            |             |     |            |             |     |
| Peripheral PLT counts |   |                   |            |             |     |            |             |     |
| <50×10⁹/L          | 15  | 13                | 0.656      | 0.257–1.675 | 0.378| –          | –           | –   |
| ≥50×10⁹/L          | 24  | 19                |            |             |     |            |             |     |
| Cytogenetics       |     |                   |            |             |     |            |             |     |
| Normal             | 8   | 17                | 1.058      | 0.331–3.378 | 0.924| –          | –           | –   |
| Abnormal           | 20  | 19                |            |             |     |            |             |     |
| Treatment          |     |                   |            |             |     |            |             |     |
| Chemotherapy       | 20  | 9                 | 0.278      | 0.105–0.740 | 0.010| 0.623      | 0.183–2.118 | 0.448|
| HSCT               | 21  | 24                |            |             |     |            |             |     |

*–* Indicates we did not evaluate these prognostic factors by multivariate analysis.
The patients with \( > 10^{-4} \) MRD level before the start of HSCT had worse prognosis and higher risk of relapse compared to patients with \( \leq 10^{-4} \) before the start of HSCT, indicating that a reduction to \( \leq 10^{-4} \) of MRD level should be achieved before the start of HSCT to produce a significantly better clinical outcome.

Furthermore, the multivariate analysis ascertained that the group division based on the variation in expression level of MLL gene was a single independent prognostic factor for overall survival, indicating that the MRD level of the MLL gene is of potential prognostic value in AML patients.

In summary, our study shows that RQ-PCR is an effective and accurate method to quantify and clinically monitor the MRD level of MLL gene-positive AML patients, and might be capable of early detection of molecular relapse of AML. The MRD levels detected by RQ-PCR were basically in line with the clinical outcome and may be of great importance in guiding early allo-HSCT treatment. Furthermore, a rapid reduction of MRD level to \( \leq 10^{-4} \) appears to be a prerequisite for better overall survival and event-free survival during the treatment of AML. The expression levels and variation in expression of PML-RARA-, AML1-ETO-, and CBFB-MYH11-positive AML have been proven to be of prognostic significance [10,30,34–36]. However, more details and more patients need to be studied to validate the present results in MLL gene-positive patients.

**Conclusions**

Our results indicate that a rapid reduction of MRD level to \( \leq 10^{-4} \) appears to be a prerequisite for a better overall survival and event-free survival during the treatment of AML. The MRD levels detected by RQ-PCR were basically in line with the clinical outcome and may be of great importance in guiding early allo-HSCT treatment. Furthermore, we proved that RQ-PCR is an effective method to quantify the MRD level of MLL genes and might be capable of early detection of molecular relapse of AML.

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**Conflict of interest**

The authors state that they have no potential competing interests.

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