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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 $\leq 10^{-4}$ 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 $\leq 10^{-4}$ before the start of HSCT.

Conclusions: We found that 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 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 leukemogenesis 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/m^2, MA: mitoxantrone 8–10 mg/m^2, DA: daunorubicin 45 mg/m^2, for 3 days), then cytarabine 100 mg/m^2/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 reversely 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

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

No.	Fusion genes	5'-3'	
		Primer	Probe
1	*MLL-PTD*	F CAAGAAAAGAAGTTCCCAAAACCA R ACTTCGCACTCTGACTTCTTCATCT	AAGAAAAAGCAGCCTCC
2	*MLL-AF9*	F AAGCAGTGCTGCAAGATGAGAA R CCTCCTCATTGTCATCAGAATGC	ATGTCAGAATCTACAATGGA
3	*MLL-ELL*	F TCCAGAGCAGAGCAAACAGAAA R GGTTACAGAACTGACATGCTGAGAGT	CCGCCCAAGTATC
4	*MLL-AF10*	F CAATATAAAGAAGCAGTGCTGCAAGA R TTCACTATCGCTGCCATCACTT	AGAAAATGTCAGAATCTAC
5	*MLL-AF17*	F GTTCCCAAAACCACTCCTAGTGA R TTCATCTGATTCCTCCTCATTGTC	CAAGAAAAAGCAGCCTC
6	*MLL-AF6*	F GTTCCCAAAACCACTCCTAGTGA R TTGCAAAGTTTCCAGCAGCTT	CAAGAAAAAGCAGCCTC
7	*MLL-ENL*	F TCCAGAGCAGAGCAAACAGAAA R GGCGTATGTATTGCTGTCAAAGG	CGCCCAAGTATCCCT
8	*MLL-CBP*	F TGGGAGATGGGAGGCTTAGG R TGACTTCTTCATCTGAGCCAAAAC	ATCTTGACTTCTGTTCCTATAA
9	*MLL-AF1P*	F GTCCAGAGCAGAGCAAACAGAA R TTCTTCATCTGAGCCAAAACCTAA	AAAGTGGCTCCCCGCC
10	*MLL-AF1Q*	F CACTTTGAACATCCTCAGCACTCT R TTCTTCATCTGAGCCAAAACCTAA	TCCAATGGCAATAGTTC
11	*MLL-AFX1*	F GGTCCAGAGCAGAGCAAACAG R GATTCCTCCTCATTGTCATCAGAA	AAAAAGTGGCTCCCCGC

level of *MLL* gene were in multivariate Cox regression analysis. 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. The patients consisted of 10 *MLL-PTD* (39%), 8 *MLL-AF9* (15%), 7 *MLL-ELL* (14%), 6 *MLL-AF10* (12%), 2 *MLL-AF17* (4%), 2 *MLL-AF6* (4%), 2 *MLL-ENL* (4%), and 1 each of *MLL-CBP* (2%), *MLL-AF1P* (2%), *MLL-AF1Q* (2%), and *MLL-AFX1* (2%) (Figure 2). All positive AML samples were confirmed by karyotype, sequencing, or FISH analysis.

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 $\leq 10^{-4}$ 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. 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.

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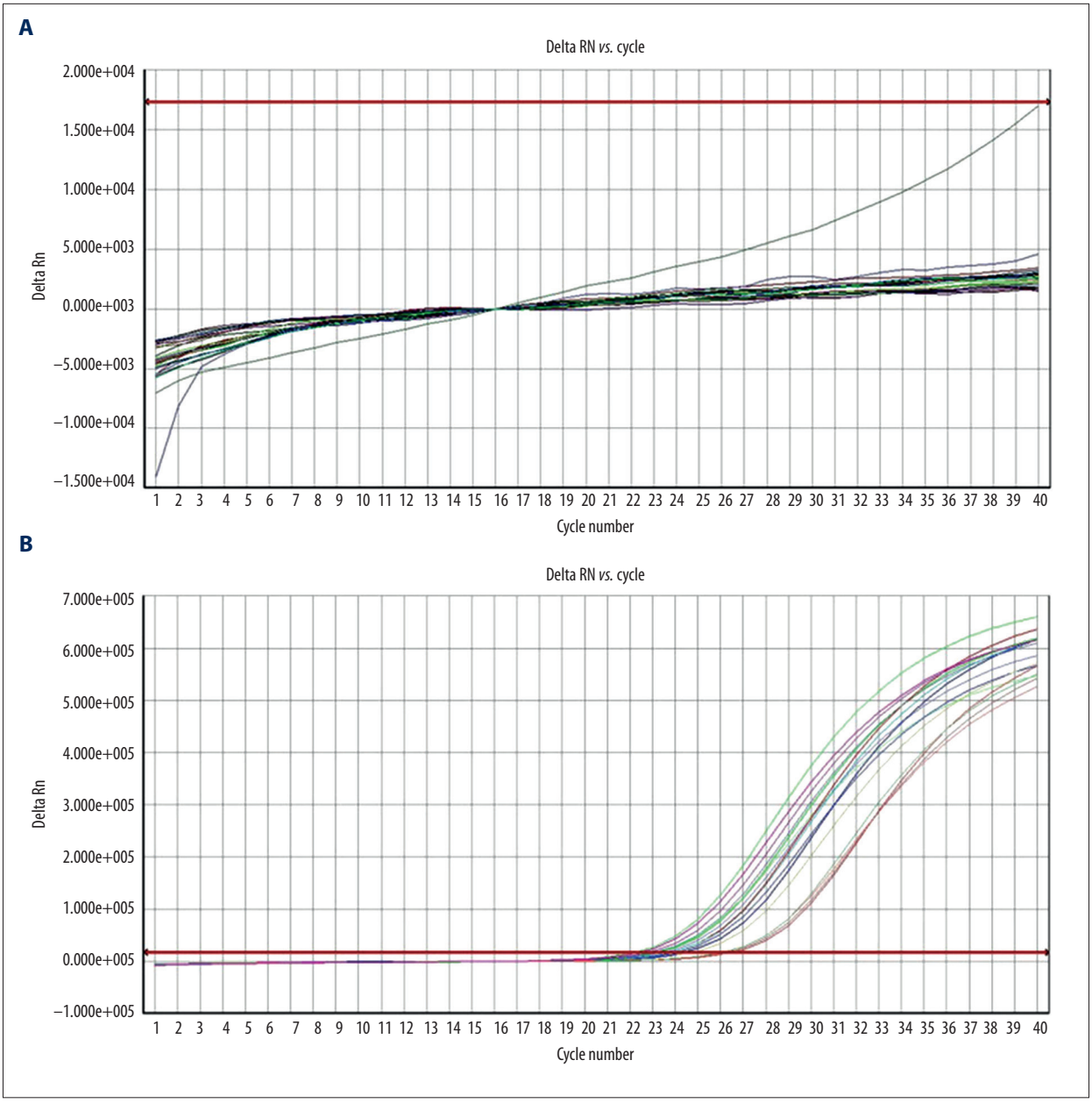

Figure 1. Amplification curve for standard samples (**A**) and patient samples (**B**).

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

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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	216
Median samples (range)	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

1 were shorter than in group 2 (8 months and 1 month, respectively). 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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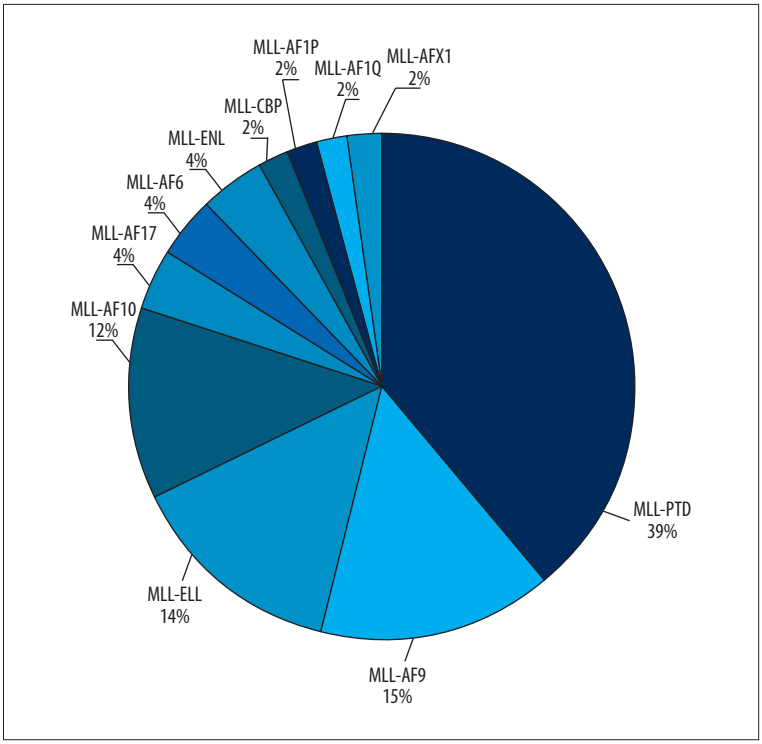

Figure 2. The proportion of *MLL* fusion partners in AML patients with *MLL* rearrangement.

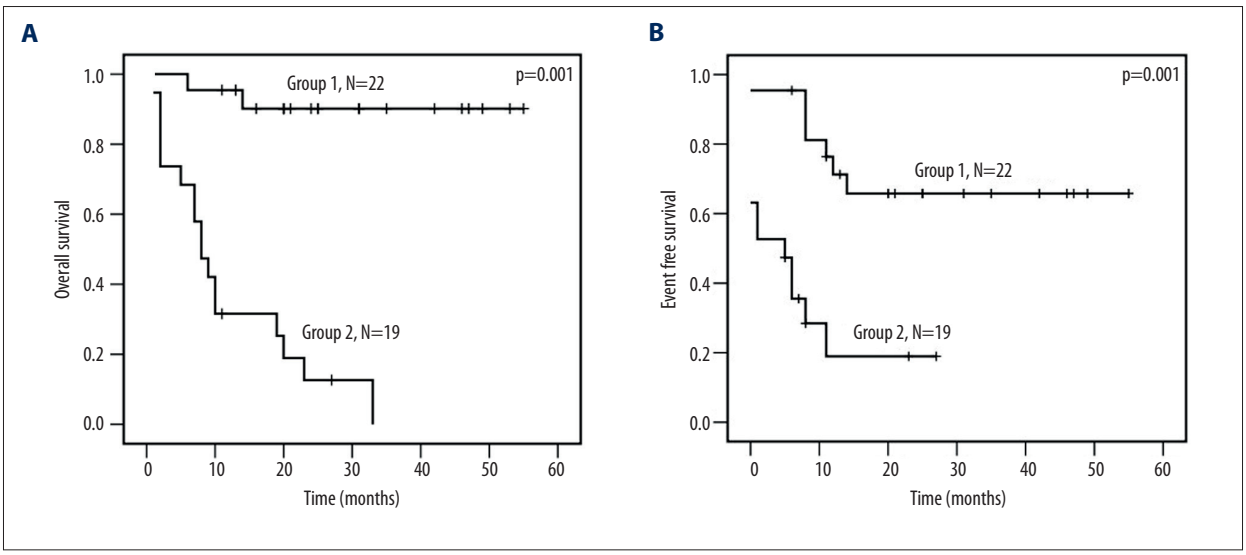

Figure 3. Outcome of AML patients with MLL rearrangement according to MRD risk group. (**A**) Overall survival; (**B**) Event-free survival.

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 (< *vs.* ≥100×10^9/L), diagnostic peripheral PLT counts (< *vs.* ≥50×10^9/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.

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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								
<100×10^9/L	38	19	2.852	0.651–12.496	0.164	–	–	–
≥100×10^9/L	3	2						
Peripheral PLT counts								
<50×10^9/L	15	13	0.656	0.257–1.675	0.378	–	–	–
≥50×10^9/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.

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.

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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, indicating that a reduction to ≤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 ≤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 ≤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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