Risk assessment by monitoring expression levels of partial tandem duplications in the *MLL* gene in acute myeloid leukemia during therapy

Martin Weisser
Wolfgang Kern
Claudia Schoch
Wolfgang Hiddemann
Torsten Haferlach
Susanne Schnittger

Background and Objectives. A partial tandem duplication within the *MLL*-gene (*MLL*-PTD) can be found in 8% of all patients with karyotypically normal acute myeloid leukemia (AML), a group in which polymerase chain reaction-(PCR) based minimal residual disease analysis has not, so far, been possible.

Design and Methods. A sensitive real-time PCR assay to quantify *MLL*-PTD transcripts was established and expression ratios assessed in diagnostic and follow-up samples. The prognostic significance of *MLL*-PTD expression levels was evaluated in 145 *MLL*-PTD positive patients at diagnosis and in 44 patients during and after treatment.

Results. Paired samples from 16 patients evaluated at diagnosis and relapse for the presence of the *MLL*-PTD were analyzed in parallel and all samples were positive at both time points. Overall, 173 samples from 44 patients were analyzed during follow-up (median sample number: 4/patient (range 2-17)). Nineteen patients were evaluable for MRD within the first 2 months, 15 patients within 4 months, and 19 patients within 6 months after the start of therapy. A ≥ 2 log reduction of *MLL*-PTD expression in comparison to < 2 log reduction within 2, 4, and 6 months after start of therapy was found to be significantly associated with longer overall survival (p=0.029, p=0.007, and p=0.022, respectively). A molecular relapse was detected in 2 cases, in each case preceeding clinical manifestation by 35 days.

Interpretation and Conclusions. These data suggest that *MLL*-PTD is a stable marker and can be used as a prognostically important marker of MRD in patients with karyotypically normal AML.

Keywords: risk assessment, expression levels, *MLL* gene, AML.

Haematologica 2005; 90:881-889

From the Laboratory für Leukemia Diagnostics, Medical Department III, Klinikum Grosshadern, Ludwig-Maximilians-University, Munich, Germany

Correspondence:
Martin Weisser, Laboratory for Leukemia Diagnostics, Medical Department III, Klinikum Grosshadern, Marchionini Str. 15, 81377 Munich.
E-mail: martin.weisser@med.uni-muenchen.de

Cytogenetic and molecular genetic aberrations provide important insights into the biology and pathogenesis of acute myeloid leukemia (AML). Furthermore, they can serve as specific markers for detection and quantification of minimal residual disease (MRD). The clinical significance of quantifying MRD has recently been proven for *AML1-ETO*, *CBFB-MYH11* and *PML-RARA* positive AML.[1-4] It has been shown that the expression levels of *AML1-ETO*, *CBFB-MYH11*, and *PML-RARA* at diagnosis and the reduction of transcript levels during the first 3-4 months of anti-leukemic therapy have a significant impact on event-free and overall survival.[1] The information drawn from MRD analysis is being increasingly used for the assessment of prognosis and individual treatment decisions e.g. whether to escalate or reduce the intensity of treatment. However, fusion genes as follow-up markers are available in less than 25 % of AML.

The *MLL* (mixed lineage leukemia) gene located on chromosome 11q23 is frequently disrupted in human hematologic malignancies.[5,6] Translocations involving 11q23 lead to fusion genes containing the *MLL* gene fused to 50 different chromosomal sites (for a review see *http://www.infobiogen.fr/services/chromcancer/Anomalies/11q23ID1030.html* and Dimartino *et al.*).[7] In addition, a partial tandem duplication of the *MLL* gene (*MLL*-PTD) has been described as another genetic mechanism of leukemogenesis.[8-11] At the molecular level the *MLL*-PTD usually spans exons 2 to 6, 2 to 7, and 2 to 8, or exons 3-9, exons 3-10, exons 3-11, or exons 3-10 and exons 3-11, according to the nomenclature of Nilson *et al.*[12] *MLL*-PTD have been detected in about 10% of AML with normal karyotypes.[8,10,13-15] and were associated

with a poor prognosis.[13,14,16] Very low levels of *MLL*-PTD expression have also been detected in healthy controls.[17] Beside its significance as a prognostic marker at diagnosis *MLL*-PTD may be a target for polymerase chain reaction (PCR)-based detection of minimal residual disease. Whether the *MLL*-PTD is a stable and useful MRD marker in AML and whether MRD levels are applicable for the assessment of prognosis in *MLL*-PTD positive AML has been addressed in the present work for the first time. Using real-time reverse transcription-PCR analysis we assessed the level of *MLL*-PTD transcripts at diagnosis in 145 patients with proven *MLL*-PTD positive AML and also followed-up 44 of these patients with quantitative PCR during the course of anti-leukemic therapy. The transcript levels were then correlated to the clinical course of the disease.

Design and Methods

Screening for MLL-PTD

An *MLL*-PTD was found in 218/3901 (5.6%) of newly diagnosed cases of unselected AML. (Schnittger *et al., unpublished data*). It was detected solely in cases with normal and other prognostically intermediately rated karyotypes and was found to have an unfavorable impact on complete remission, overall survival and event-free survival rates in the subgroup with normal karyotypes. This was the rationale for selecting the cases analyzed in the present study, in which the expression of the *MLL*-PTD was assessed in patients with a normal karyotype. In this karyotypically normal subgroup 145/1706 (8.5%) were found to be positive for the *MLL*-PTD. Thus the frequency of this marker is in the same range as that of *PML-RARA*, *CBFB-MYH11*, and *AML1-ETO* and was presumed to be an appropriate target for MRD detection.

Patients

The blood and bone marrow samples from patients were analyzed at our Laboratory for Leukemia Diagnostics. All cases were diagnosed as having AML according to the FAB and WHO classifications.[18-20] All patients were treated according to the AMLCG92 and AMLCG99[21,22] trials. The studies adhered to the declaration of Helsinki and were approved by the ethics committees of the participating institutions prior to the initiation of the studies. Each patient provided informed consent. Patients were selected according to *MLL*-PTD positivity by RT-PCR, confirmed by Southern blot analysis as described previously,[13] or by real time PCR. The sizes of the amplified PCR products were 240 bp (exon 9/3), 380 bp (10/3), and 460 bp (11/3). Cases of *de novo*, secondary and therapy-related AML were included (n=123, n=18, and n=4, respectively). In total, samples taken at primary diagnosis from 145 patients were available. Samples from 44 of these 145 patients were sent for follow-up assessment. A median of 4 follow-up samples (range 2-17) per patient were sent and the median follow-up time was 184 days (range 16-1588). For the present analysis we defined 3 intervals from the start of therapy: interval 1 was from 0-2 months, interval 2 was from 2-4 months and interval 3 was from 4-6 months. In addition, a joint analysis of intervals 2 plus 3 (2-6 months) was performed.

In detail, MRD was evaluated in 19 of 44 patients in interval 1, 15 in interval 2, and 19 in interval 3. MRD analysis was performed in 26 patients during the combined interval (2-6 months). The patients' characteristics are shown in Table 1.

Table 1. Characteristics of all *MLL*-PTD positive patients analyzed in the study.

MLL-PTD positive patients at diagnosis	*N=145*
Median age (years)	63 y (range 24-89)
Sex	82 m / 63 f
FAB	
M0	5
M1	30
M2	56
M4	25
M5	5
M6	9
Not evaluated	15
De novo AML	123
sAML	18
tAML	4
MLL-PTD transcript types	
exon3/exon9*	84
exon3/exon10*	9
exon3/exon11*	9
exon3/exon10 and exon3/exon11*	43

**Nomenclature according to Nilson et al.*[12]

Nucleic acid isolation

Mononuclear cells were obtained by Ficoll density gradient centrifugation. From each sample, mRNA from 5×10^6 human cells was isolated according to the Magna Pure LC mRNA protocol for human cells (Roche Diagnostics, Mannheim, Germany) and eluted in 30 µL H_2O.

cDNA synthesis

Using Superscript II (Invitrogen, San Diego, California) randomly primed cDNA was synthesized from 5 µL of mRNA from the diagnostic samples, corresponding to approximately 0.8×10^6 cells in a 50 µL reaction. All 30 µL of mRNA from follow-up sam-

ples were transcribed into cDNA in order to gain a higher sensitivity.

Quantitative real time PCR

Quantitative PCR was performed using the LightCycler® System (Roche Diagnostics, Mannheim, Germany) applying hybridization probes as the detection format. PCR was performed using 2 µL mastermix (LightCycler Fast Start DNA Master Hybridization Probes, Roche Diagnostics Mannheim, Germany), 4 mM $MgCl_2$, 0.25 µM of each 3´and 5´ fluorescent hybridization probe, 0.5 µM of each 3´ and 5´ primer (TibMolBiol, Berlin, Germany), 2 µL of cDNA and water to a final volume of 20 µL. Amplification occurred after initial incubation at 95°C for 10 min in a three-step cycle procedure (denaturation at 95°C for 1s, ramp rate 20°C/s, annealing at 64°C for 10 s, ramp rate 20°C/s, and extension at 72°C for 26 s, ramp rate 2°C/s) for 45 cycles.

Primers and hybridization probes to amplify and quantify the *MLL*-PTD fusion transcripts were forward: 6.1: GTCCAGAGCAGAGCAAACAG, reverse: E3AS ACACAGATGGATCTGAGAGG, *MLL*-sensor: CTTTTCTTTTGGTTTTTGTTTTACAG-FL, *MLL*-anchor: Red640-TGGGCGGGGAGCCACTTTTTTC-P. The expression of *MLL*-PTD was normalized against the expression of the control gene *ABL* to adjust for variations in RNA quality and efficiencies of cDNA synthesis. The expression ratios are given as: 100×*MLL*-PTD/*ABL*. *ABL* expression was quantified according to Emig *et al.*[23]

Sensitivity of the assay

To compare the efficiency and sensitivity of the PCR for the three different fusion types, serial dilution experiments of *MLL*-PTD positive in *MLL*-PTD negative cDNA of individual patients were performed and efficiencies calculated from the slope of the standard curves. As assessed using three different samples per fusion type the efficiencies of the assays for exon 3/9, exon 3/10, exon 3/11, and exon 3/11 with alternative splicing of exon 3/10 were 1.96, 1.96, 2.01, and 2.00, respectively. These were in the same range as the efficiency of the *ABL* standard (1.99). Thus, *ABL* and *MLL*-PTD estimations of a specific sample were done in the same run with use of an *ABL* plasmid standard curve.

The final sensitivity for all fusion types was 1:100-1:10000 depending on the patients' initial transcription ratio (Figure 1A).

Statistical analysis

Survival curves were calculated for overall survival and event-free survival from the start of therapy using Kaplan-Meier estimates. Survival curves were compared using a double-sided log rank test. Results were significant at a level of $p<0.05$ at both sides. The normalized expression levels, the initial white blood cell counts, and the initial blast percentage were used as co-variates for Cox regression analysis and correlated to overall survival and event-free survival by Spearman's rank correlation. The SPSS 12.0 for Windows software package (SPSS, Chicago, IL, USA) was used for the statistical analysis.

Results

Expression levels of MLL-PTD at primary diagnosis and in healthy controls

As has been reported previously[17] *MLL*-PTD transcripts are detectable at low levels in healthy controls. To quantify this *normal background*, *MLL*-PTD expression levels were analyzed in 8 bone marrow (BM) and 50 peripheral blood (PB) samples from healthy volunteers. The median expression level in BM was 0.047 (range 0-0.08) and in PB 0.019 (range 0-0.08). Consequently, levels of ≤ 0.08 in AML follow-up samples were defined to be *normal*. The median *MLL*-PTD expression level in the 145 patients with proven *MLL*-PTD was 137 (range 10–1255). In addition, transcript levels were quantified in 49 *MLL*-PTD negative patients. The median *MLL*-PTD expression level among these patients was 0.017 (range 0.097) as shown in Figure 1B.

Effect of initial MLL-PTD expression level on overall and event-free survival

The outcome of 145 *MLL*-PTD positive AML patients was analyzed with regard to their level of *MLL*-PTD expression at diagnosis. The median overall and event-free survival of the total group was 205 days and 167 days, respectively, demonstrating a slightly worse outcome than that in patients with AML and a normal karyotype. This was in line with previous data on outcome in *MLL*-PTD-positive AML.[13,14] Thus, the patients included in this MRD study are representative of patients with *MLL*-PTD-positive AML (Figure 2). Separating patients according to whether their expression levels were above the 50th percentile (249) or above the 75th percentile (532) of the median expression level from all patients at diagnosis as thresholds (median: 137) did not result in different overall survival (median 290 vs. 65 days, p=0.126, and median 218 vs. 151 days, p=0.497, respectively), or event-free survival (median 227 vs. 182 days, p=0.907 and median 227 vs. 205 days, p=0.902, respectively). In addition, a Cox regression analysis using the initial *MLL*-PTD level as a co-variate showed no significant impact on overall survival (p=0.679) or event-free survival (p=0.344). Age above

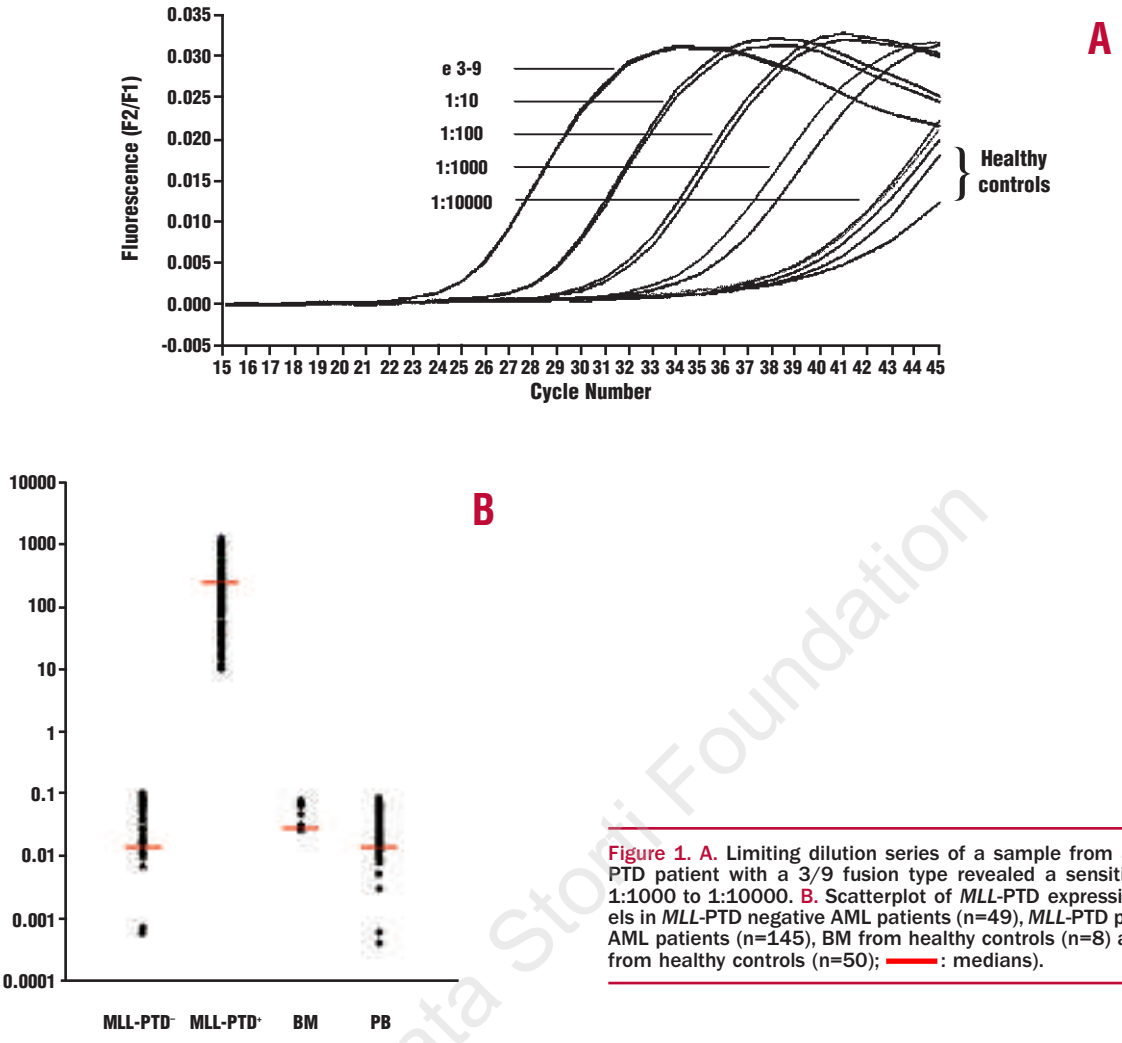

Figure 1. A. Limiting dilution series of a sample from a *MLL*-PTD patient with a 3/9 fusion type revealed a sensitivity of 1:1000 to 1:10000. B. Scatterplot of *MLL*-PTD expression levels in *MLL*-PTD negative AML patients (n=49), *MLL*-PTD positive AML patients (n=145), BM from healthy controls (n=8) and PB from healthy controls (n=50); ——: medians).

60 years at initial presentation was associated with a significantly worse overall survival (median 509 vs. 65 days, p=0.005). In the cohort analyzed, cytomorphologic subtype according to the FAB-classification, initial leukocyte count, and the percentage of blasts in the diagnostic bone marrow sample had no significant impact on overall survival (p=0.425, p=0.107, and p=0.897, respectively).

Stability of MLL-PTD as molecular marker

To prove the stability of the *MLL*-PTD as a molecular marker, paired samples from 16 patients were analyzed at diagnosis and at relapse. The median time from diagnosis to relapse was 410 days (range 127-800). All 16 patients were positive at both time points with the same *MLL*-PTD transcript type. In addition, it was shown that the expression levels of *MLL*-PTD at relapse were in the range of those in the diagnostic samples with at most one log difference

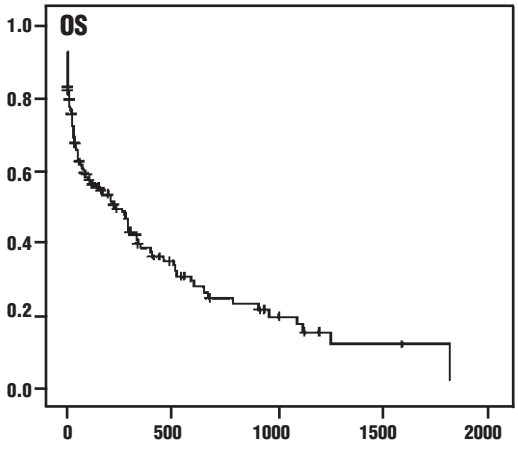

Figure 2. Overall survival of *MLL*-PTD positive patients (n=145, median survival 205 days, censored 51, events 94).

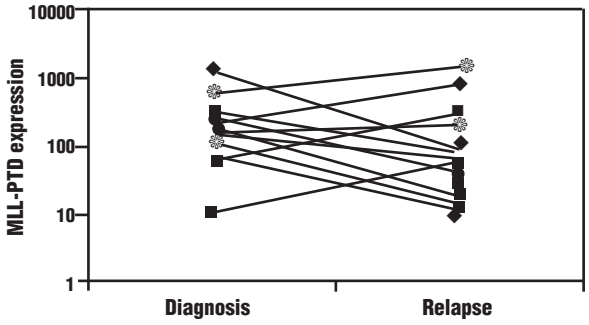

Figure 3. Expression levels of MLL-PTD at diagnosis and at the time of relapse were within a 1 log range in all 16 patients.

Table 2. ***MLL*-PTD expression levels at diagnosis with regard to the involved exon.**

	exon3/exon9 (n=84)	*exon3/exon10 (n=9)*	*exon3/exon11 (n=9)*	*exon3/exon10 and exon3/exon11* (n=43)*
Median	222	125	81	89
Mean	326	247	120	125
Range	11-1255	10-993	26-535	10-646

**Nomenclature according to Nilson et al.*[12]

(Figure 3). The median *MLL*-PTD expression levels at diagnosis and relapse were 139 (range 10-1160) and 72 (range 12-1453), respectively. This analysis was taken as a prerequesite for the use of *MLL*-PTD in follow-up samples to ensure that early detection of relapse is potentially possible.

No impact of exon usage on prognosis

In this study the duplicated region spanned exons 3/9 in 59% of cases and exons 3/10 and/or 3/11 in 41% of all cases. The type of exons involved in *MLL*-PTD had no significant impact on the transcription ratio (Table 2), overall survival (p=0.374) or event-free survival (p=0.599).

Levels of MLL-PTD within 2, 4, and 6 months after the start of therapy

The impact of *MLL*-PTD expression during follow-up on the outcome was evaluated for three different intervals: interval 1 from 0-2 months after the start of treatment, interval 2 from 2-4 months, and interval 3 from 4-6 months. A reduction of at least 2 logs of *MLL*-PTD expression level within 2, 4, and 6 months was a significant prognostic factor for overall survival for all three intervals (interval 1: median survival not reached vs. 205 days, p=0.029; interval 2: 515 vs. 205 days, p=0.007; and interval 3: 665 vs. 344 days, p=0.022). All patients achieving a ≥2 log reduction within the analyzed intervals were in cytomorphological complete remission. Of those patients not achieving a ≥2 log reduction 5/25 patients were in cytomorphological remission. Quantitative PCR and cytomorphology correlated in 80% of cases. In 20% of cases additional information was gained by PCR due to the higher sensitivity of this technique.

For event-free survival a reduction of at least 2 logs within the first 2 months was a significant factor (median survival not reached vs. 167 days, p=0.025, Figure 4A, B) and there was a trend towards improved event-free survival in intervals 2 and 3 (305 vs. 205 days, p=0.078 and 310 vs. 218 days, p=0.386,

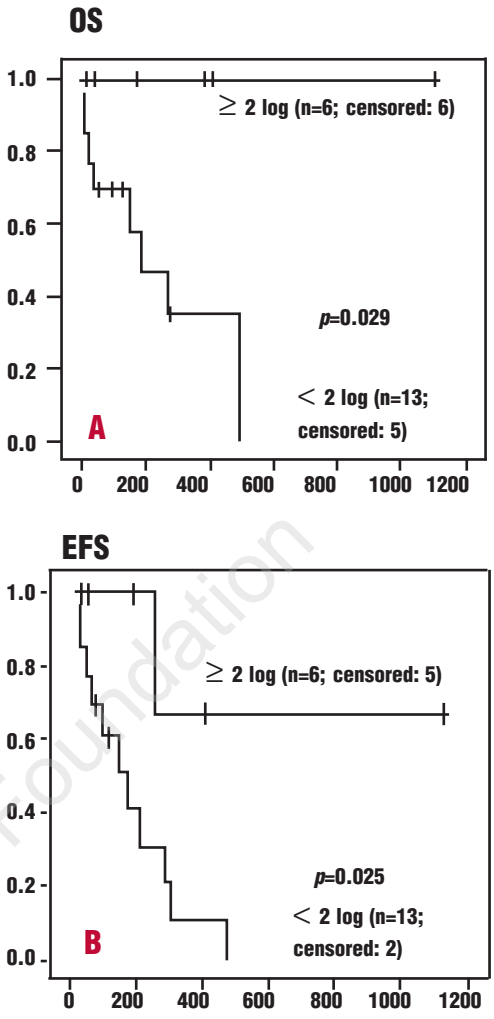

Figure 4. A. Kaplan-Meier plot of overall survival of patients achieving a ≥2 log reduction (n=6, censored: 6) vs. patients achieving a <2 log reduction (n=13, censored: 5) of *MLL*-PTD levels within 2 months after start of therapy. **B.** Kaplan-Meier plot of event-free survival of patients achieving a ≥2 log reduction (n=6, censored: 6) vs. patients achieving a <2 log reduction (n=13, censored: 5) of MLL-PTD levels within 2 months after the start of therapy.

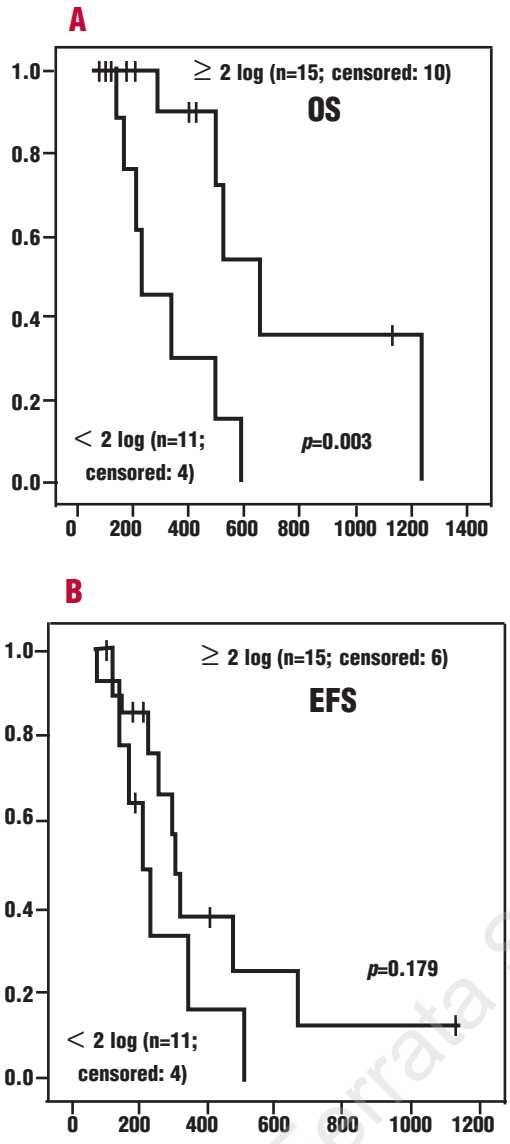

Figure 5. A. Kaplan-Meier plot of overall survival of patients achieving a ≥2 log reduction (n=15, censored: 10) vs. patients achieving a <2 log reduction (n=11, censored: 4) of *MLL*-PTD levels within 2-6 months after start of therapy. **B.** Kaplan-Meier plot of event-free survival of patients achieving a ≥2 log reduction (n=15, censored: 6) vs. patients achieving a <2 log reduction (n=11, censored: 4) of *MLL*-PTD levels within 2-6 months after the the start of therapy.

respectively). As the numbers of patients were rather small for the analyses in intervals 2 and 3, these two intervals were combined. Within this combined interval a ≥2 log reduction of *MLL*-PTD levels was a prognostic factor for overall survival (665 v. 218 days, p=0.003) and there was a trend towards improved event-free survival (310 vs 218 days, p=0.179, Figures 5 A,B). Additional separation of the group with a ≥2 log reduction revealed that a ≥2 and <3 log reduction was achieved by two patients (0 events) in interval

Table 3. Median overall survival and MLL-PTD expression levels at 2, 4 and 6 months after the start of therapy.

	≥ 2 log	*< 2 log*	*p value*
0-2 months (n=19)	n=6	n=13	
Median OS (days)	not reached	205	0.029
MLL-PTD-level			
Median	0.09	102	
Range	0.003-9	11-884	
2-4 months (n=15)	n=10	n=5	
Median OS (days)	515	205	0.007
MLL-PTD-level			
Median	0.18	164	
Range	0-3	23-964	
4-6 months (n=19)	n=12	n=7	
Median OS (days)	665	344	0.022
MLL-PTD-level			
Median	0.04	65	
Range	0-96	2-313	
2-6 months (n=26)	n=15	n=11	
Median OS (days)	665	218	0.003
MLL-PTD-level			
Median	0.04	65	
Range	0-96	2-964	

1, three patients (1 event) in interval 2, two (1 event) in interval 3, and three patients (1 event) in the combined interval (*not shown*). The median survival and median levels of *MLL*-PTD expression for each interval are shown in Table 3. For the patients in whom MRD assessment was performed, the reduction of *MLL*-PTD levels was the only factor significantly influencing the outcome.

Molecular remission and prediction of relapse

A molecular remission (defined as expression levels below 0.08) was observed in 19 cases. The mean expression level in molecular remission was 0.003 (median 0, range 0-0.048). For comparison, patients in cytomorphological complete remission without molecular remission (n=6) had a mean expression level of 0.28 (median 0.25, range 0.096-1.08). After conventional chemotherapy 10/44 patients achieved a molecular remission as compared to 9/10 patients after allogeneic transplantation (p=0.00014). A molecular relapse was detected, based on transcript levels increasing by at least 1 log, in 2 patients, in both cases 35 days before cytomorphological relapse. Another 14 cases were assessed at hematologic

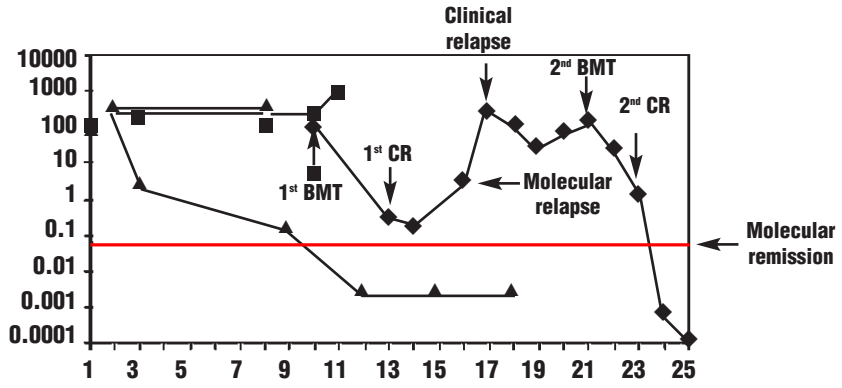

Figure 6. Follow-up of one patient with good response and complete remission (triangles), one patient with therapy resistance (square), and one patient with resistance, BMT, CR*, molecular relapse, clinical relapse, second BMT, second CR* (diamonds). The red line indicates the threshold of complete molecular remission (0.08). **CR: hematologic complete remission.*

relapse, when expression levels had already reached the range found in the diagnostic sample. In these cases the median time between the last follow up sample and the date of relapse was 282 days (range 97-687) and thus was too long for early detection of relapse. The molecular courses of three patients are shown in Figure 6.

Discussion

Quantification of MRD is of increasing interest in AML. So far, fusion genes have been used as targets for PCR-based detection of MRD. However, more than 75% of AML cases lack a specific fusion gene that can be used as a molecular marker for MRD detection. The *MLL*-PTD, an intragene fusion, is detectable in about 8-10% of AML patients with normal karyotypes.[13,16] In the present study we evaluated the applicability of this marker for quantitative real-time PCR-based MRD detection.

MLL-PTD was previously found to be detectable not only in AML but also in the blood and bone marrow of healthy controls.[17] However, in this study we showed that there was a 4 log difference in expression levels between AML and healthy samples. Therefore *MLL*-PTD was regarded as a useful follow-up marker for *MLL*-PTD-positive AML. In the present analysis we included *de novo* as well as secondary and therapy-associated *MLL*-PTD-positive AML, as there are data suggesting that the biology of the disease is determined by genetics rather than by etiology.[24]

Recently, the prognostic significance of expression levels at diagnosis and the reduction of fusion transcript levels has been proven in *PML-RARA*, *CBFB-MYH11*, and *AML1-ETO* positive AML.[1-4] In contrast, it could not be demonstrated that the initial level of *MLL*-PTD expression had an adverse effect on the outcome of *MLL*-PTD positive AML. These discrepancies may be due to the limited number of *MLL*-PTD cases examined or to different biology. Previous studies of AML fusion genes in small numbers of subjects failed to detect differences of outcome with respect to initial transcript levels,[24,25] but a prognostic impact was clearly shown in a larger cohort.[1] Alternatively, the differences in results may be due to different biologies of *MLL*-PTD positive AML and classical fusion gene AML. This is at least true with respect to age and prognostic significance of these subgroups *per se*.[13] The exon fusion type, the FAB subtype, high initial leukocyte count, and the percentage of blasts in the diagnostic sample in this selected cohort did not influence the outcome.

MLL-PTD have been associated with a poor prognosis in our studies[13,14,16] and these findings were confirmed in other series. The key issue of our analysis was the prognostic impact of MRD in this high-risk AML group. A reduction of *MLL*-PTD transcripts of at least 2 logs resulted in a significantly better overall survival in all intervals. Larger numbers of patients and longer follow-up will probably strengthen these data. For those patients achieving a cytomorphological remission, the *MLL*-PTD specific PCR added information on the status of remission because of its higher sensitivity. The sensitivity of our assay was 2-4 logs (depending on initial ratios) which is lower than the sensitivity achieved for CBF-leukemias.[1-4] This is partly due to the *background noise* created by low expression levels in patients in complete molecular remission and present in healthy controls and partly due to the poorer overall prognosis of patients with *MLL*-PTD. A ≥4-log reduction was observed in only seven MLL-PTD patients, all of whom had high initial *MLL*-PTD expression levels (*not shown*).

MLL-PTD and *FLT3*-length mutations (*FLT3*-LM) frequently co-exist in AML.[26] Our group has performed many studies using *FLT3* as a follow-up marker; however, real quantitative assays of *FLT3*-LM require more complicated assays with mutation- and patient-specific primers.[27] Thus quantification of *MLL*-PTD may be a more applicable assay for this group. There have also been many reports on the usefulness of *WT1* as a follow-up marker in AML with normal karyotypes. Most of these studies have

shown significant correlation with the clinical course,[28-30] others – mostly non-quantitative studies - have failed to do so.[31,32] Only one study has addressed the issue of risk assessment by quantifying *WT1* levels in follow-up samples.[30] This study examined a small number of follow up samples from a mixed cohort of patients with acute myeloid or lymphocytic leukemia. In contrast to *WT1*, *MLL*-PTD is a leukemia-specific marker and the aim of our study was to evaluate a leukemia-specific follow-up marker for these patients with AML. Immunophenotyping is an another approach that can be used for MRD studies in patients with normal karyoptype AML. However, in contrast to PCR-based technologies, immunophenotyping requires fresh material. Thus, a retrospective analysis like the one presented here, could not have been carried out. In addition, the two methods analyze substantially different properties. While immunophenotyping demonstrates the proportion of cells with a leukemia-associated immunophenotype within a certain number of cells, real-time PCR evaluates the normalized expression levels of target genes within the sample. In principle, the two methods could have different prognostic impacts. In the present study the incidence of molecular remissions was significantly higher after allogeneic stem cell transplantation. This observation is in line with the finding that MRD levels further decreased[3] and the risk of relapse declined after allogeneic transplantation.[33,34] However, given that this observation is based on a small number of patients, it should be interpreted cautiously. A molecular relapse was detected in two patients, in both five weeks before hematologic relapse, on the basis of increasing transcript levels. In 14 patients relapse was not predicted, probably because of the long intervals between analysis of the last follow-up sample and relapse. These results suggest that more frequent MRD controls are necessary to detect patients at high risk of hematologic relapse by increasing expression levels.

Taken together, for the first time real time PCR using a molecular marker was shown to allow risk assessment of AML patients with a normal karyotype. In addition, this is the largest cohort of *MLL*-PTD positive AML published thus far. Despite rather small numbers of follow-up samples significant results were achieved: *MLL*-PTD can serve as a useful target for MRD detection and MRD levels correlate with the outcome of patients. Quantitative assessment of MRD in *MLL*-PTD during the first six months after the start of therapy allows early risk assessment of patients. Later controls potentially allow the early detection of relapses. Future trials that include the option to adjust therapy depending on MRD levels are warranted. Whether early therapy escalation, e.g. allogeneic stem cell transplantation, can improve the outcome of those patients without a sufficient reduction of *MLL*-PTD levels should also be the subject of prospective trials.

MW: analysis of data, writing of the manuscript; WK: immunophenotyping, statistics; CS: cytogenetics; WH: study directors of the clinical trial; TH: cytomorphology, study director; SS: design and analysis of the experiments. All authors were involved in discussing and interpreting the data and writing the manuscript. The authors declare that they have no potential conflicts of interests.

Manuscript received January 11, 2005. Accepted May 24, 2005.

References

1. Schnittger S, Weisser M, Schoch C, Hiddemann W, Haferlach T, Kern W. New score predicting for prognosis in PML-RARA+, AML1-ETO+, or CBFB-MYH11+ acute myeloid leukemia based on quantification of fusion transcripts. Blood 2003;102:2746-55.
2. Buonamici S, Ottaviani E, Testoni N, Montefusco V, Visani G, Bonifazi F, et al. Real-time quantitation of minimal residual disease in inv(16)-positive acute myeloid leukemia may indicate risk for clinical relapse and may identify patients in a curable state. Blood 2002;99:443-9.
3. Guerrasio A, Pilatrino C, De Micheli D, Cilloni D, Serra A, Gottardi E, et al. Assessment of minimal residual disease (MRD) in CBFbeta/MYH11-positive acute myeloid leukemias by qualitative and quantitative RT-PCR amplification of fusion transcripts. Leukemia 2002; 16:1176-81.
4. Marcucci G, Caligiuri MA, Dohner H, Archer KJ, Schlenk RF, Dohner K, et al. Quantification of CBFβ/MYH11 fusion transcript by real time RT-PCR in patients with INV(16) acute myeloid leukemia. Leukemia 2001;15:1072-80.
5. Thirman MJ, Gill HJ, Burnett RC, Mbangkollo D, McCabe NR, Kobayashi H, et al. Rearrangement of the MLL gene in acute lymphoblastic and acute myeloid leukemias with 11q23 chromosomal translocations. N Engl J Med 1993;329:909-14.
6. Gu Y, Nakamura T, Alder H, Prasad R, Canaani O, Cimino G, et al. The t(4;11) chromosome translocation of human acute leukemias fuses the ALL-1 gene, related to Drosophila trithorax, to the AF-4 gene. Cell 1992;71:701-8.
7. Dimartino JF, Cleary ML. MLL rearrangements in haematological malignancies: lessons from clinical and biological studies. Br J Haematol 1999; 106:614-26.
8. Caligiuri MA, Schichman SA, Strout MP, Mrozek K, Baer MR, Frankel SR, et al. Molecular rearrangement of the ALL-1 gene in acute myeloid leukemia without cytogenetic evidence of 11q23 chromosomal translocations. Cancer Res 1994;54:370-3.
9. Schichman SA, Caligiuri MA, Strout MP, Carter SL, Gu Y, Canaani E, et al. ALL-1 tandem duplication in acute myeloid leukemia with a normal karyotype involves homologous recombination between Alu elements. Cancer Research 1994;54:4277-80.
10. Caligiuri MA, Strout MP, Schichman SA, Mrozek K, Arthur DC, Herzig GP, et al. Partial tandem duplication of ALL1 as a recurrent molecular defect in acute myeloid leukemia with trisomy 11. Cancer Res 1996;56:1418-25.
11. Bernard OA, Berger R. Molecular basis of 11q23 rearrangements in hematopoietic malignant proliferations. Genes Chromosomes Cancer 1995;13:75-85.
12. Nilson I, Lochner K, Siegler G, Greil J, Beck JD, Fey GH, et al. Exon/intron structure of the human ALL-1 (MLL) gene involved in translocations to chromosomal region 11q23 and acute leukaemias. Br J Haematol 1996; 93: 966-72.
13. Schnittger S, Kinkelin U, Schoch C, Heinecke A, Haase D, Haferlach T, et al. Screening for MLL tandem duplication in 387 unselected patients with AML identify a prognostically unfavorable subset of AML. Leukemia 2000;

14:796-804.
14. Dohner K, Tobis K, Ulrich R, Frohling S, Benner A, Schlenk RF, et al. Prognostic significance of partial tandem duplications of the MLL gene in adult patients 16 to 60 years old with acute myeloid leukemia and normal cytogenetics: a study of the Acute Myeloid Leukemia Study Group Ulm. J Clin Oncol 2002;20:3254-61.
15. Steudel C, Wermke M, Schaich M, Schakel U, Illmer T, Ehninger G, et al. Comparative analysis of MLL partial tandem duplication and FLT3 internal tandem duplication mutations in 956 adult patients with acute myeloid leukemia. Genes Chromosomes Cancer 2003;37:237-51.
16. Caligiuri MA, Strout MP, Lawrence D, Arthur DC, Baer MR, Yu F, et al. Rearrangement of ALL1 (MLL) in acute myeloid leukemia with normal cytogenetics. Cancer Res 1998;58:55-9.
17. Schnittger S, Wormann B, Hiddemann W, Griesinger F. Partial tandem duplications of the MLL gene are detectable in peripheral blood and bone marrow of nearly all healthy donors. Blood 1998;92:1728-34.
18. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR, et al. Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. Br J Haematol 1976;33:451-8.
19. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR, et al. Proposal for the recognition of minimally differentiated acute myeloid leukaemia (AML-MO). Br J Haematol 1991;78:325-9.
20. Jaffe ES, Harris NL, Stein H, Vardiman JW. World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues. Lyon, France: IARC Press. 2001.
21. Buchner T, Hiddemann W, Wormann B, Loffler H, Gassmann W, Haferlach T, et al. Double induction strategy for acute myeloid leukemia: the effect of high-dose cytarabine with mitoxantrone instead of standard-dose cytarabine with daunorubicin and 6-thioguanine: a randomized trial by the German AML Cooperative Group. Blood 1999;93:4116-24.
22. Buchner T, Hiddemann W, Berdel WE, Wormann B, Schoch C, Fonatsch C, et al. 6-thioguanine, cytarabine, and daunorubicin (TAD) and high-dose cytarabine and mitoxantrone (HAM) for induction, TAD for consolidation, and either prolonged maintenance by reduced monthly TAD or TAD-HAM-TAD and one course of intensive consolidation by sequential HAM in adult patients at all ages with de novo acute myeloid leukemia (AML): a randomized trial of the German AML Cooperative Group. The German AML Cooperative Group. J Clin Oncol 2003;21:4496-504.
23. Emig M, Saussele S, Wittor H, Weisser A, Reiter A, Willer A, et al. Accurate and rapid analysis of residual disease in patients with CML using specific fluorescent hybridization probes for real time quantitative RT-PCR. Leukemia 1999;13:1825-32.
24. Schoch C, Kern W, Schnittger S, Hiddemann W, Haferlach T. Karyotype is an independent prognostic parameter in therapy-related acute myeloid leukemia (t-AML): an analysis of 93 patients with t-AML in comparison to 1091 patients with de novo AML. Leukemia 2004;18:120-5.
25. Satake N, Sakashita A, Kobayashi H, Maseki N, Sakurai M, Kaneko Y. Minimal residual disease in acute monocytic leukemia patient with trisomy 11 and partial tandem duplication of MLL. Cancer Genet Cytogenet 1997;96:26-9.
26. Libura M, Asnafi V, Tu A, Delabesse E, Tigaud I, Cymbalista F, et al. FLT3 and MLL intragenic abnormalities in AML reflect a common category of genotoxic stress. Blood 2003;102:2198-204.
27. Schnittger S, Schoch C, Kern W, Hiddemann W, Haferlach T. FLT3 length mutations as marker for follow-up studies in acute myeloid leukemia. Acta Haematologica 2004;112:68-78.
28. Trka J, Kalinova M, Hrusak O, Zuna J, Krejci O, Madzo J, et al. Real time quantitative PCR detection of WT1 gene expression in children with AML: prognostic significance, correlation with disease status and residual disease detection by flow cytometry. Leukemia 2002;16:1381-9.
29. Ostergaard M, Olesen LH, Hasle H, Kjeldsen E, Hokland P. WT1 gene expression: an excellent tool for monitoring minimal residual disease in 70% of acute myeloid leukaemia patients - results from a single-centre study. Br J Haematol 2004;125:590-600.
30. Garg M, Moore H, Tobal K, Liu Yin JA. Prognostic significance of quantitative analysis of WT1 gene transcripts by competitive reverse transcription polymerase chain reaction in acute leukaemia. Br J Haematol 2003;123:49-59.
31. Gaiger A, Schmid D, Heinze G, Linnerth B, Greinix H, Halhs P, et al. Detection of the WT1 transcript by RT-PCR in complete remission has no prognostic relevance in de novo acute myeloid leukemia. Leukemia 1998;12:1886-94.
32. Schmid D, Heinze G, Linnerth B. Prognostic significance of WT1 gene expression at diagnosis in adult de novo acute myeloid leukemia. Leukemia 1997;11:639-43.
33. Zittoun RA, Mandelli F, Willemze R, de Witte T, Labar B, Resegotti L, et al. Autologous or allogeneic bone marrow transplantation compared with intensive chemotherapy in acute myelogenous leukemia. N Engl J Med 1995; 332:217–23.
34. Harousseau JL, Cahn JY, Pignon B, Witz F, Milpied N, Delain M, et al. Comparison of autologous bone marrow transplantation and intensive chemotherapy as postremission therapy in adult acute myeloid leukemia. Blood 1997;90:2978–86.