The MLL recombinome of adult CD10-negative B-cell precursor acute lymphoblastic leukemia - results from the GMALL study group

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Abstract

*MLL* translocations in adult B-cell precursor (BCP-) ALL are largely restricted to the immature CD10-negative immunophenotypes. *MLL-AF4* is known to be the most frequent fusion transcript, but the exact frequencies of *MLL* aberrations in CD10-negative adult BCP-ALL are unknown. We present a genetic characterization of 184 *BCR-ABL*-negative CD10-negative adult ALL cases (156 cyIg-, 28 cyIg+) diagnosed between 2001 and 2007 at the central diagnostic laboratory of the GMALL study group. Patient samples were investigated by RT-PCR for *MLL-AF4*, *MLL-ENL* and *MLL-AF9* and by long-distance inverse PCR, thus also allowing the identification of unknown *MLL* fusion partners at the genomic level. *MLL-AF4* was detected in 101 (54.9%) and *MLL-ENL* in 11 (6.0%) cases. In addition, rare *MLL* fusion genes were found: two *MLL-TET1* cases, not previously reported in ALL, one *MLL-AF9* and *MLL-PTD* case each, a novel *MLL-ACTN4* and an *MLL*-11q23 fusion. The chromosomal breakpoints were determined in all 118 positive cases revealing two major breakpoint cluster regions in the *MLL* gene. The relative frequency of different *MLL-AF4* transcripts was deduced. Characteristic features of *MLL+* patients were a significantly lower CD10 expression, expression of the NG2 antigen, a higher WBC at diagnosis and female gender. Proposals are made for diagnostic assessment. The clinical studies are registered at [http://www.clinicaltrials.gov](http://www.clinicaltrials.gov) as NCT00199056 and NCT00198991.

Key words

Mixed lineage leukemia gene, *MLL, AF4, ENL, TET1, ACTN4*
Introduction

Molecular aberrations involving the mixed lineage leukemia (MLL) gene on 11q23 are found in 5-10% of acute leukemia cases. In B-cell precursor (BCP) acute lymphoblastic leukemia (ALL) these aberrations are largely restricted to the immature CD10-negative immunophenotypes (pro-B and CD10-negative pre-B). The translocation t(4;11)(q22;q23) with MLL-AF4 (MLL-AFF1) fusion is known to be the most prevalent MLL fusion gene in ALL but precise and reliable data regarding the prevalence of the different MLL fusion partner genes, i.e. the MLL “recombinome” in adult ALL are lacking. Knowledge of the MLL recombinome is warranted since MLL fusions are of interest in detecting minimal residual disease in affected patients and also because controversy exists over whether adult ALL patients with pro-B ALL immunophenotype with or without MLL aberration might have a different prognosis. We report our experience within the framework of the German Multicenter Therapy Trials for Adult ALL (GMALL) between 01/2001-10/2007 at the central diagnostic laboratory of the GMALL study group. We investigated 184 patients with a CD10-negative BCP immunophenotype by RT-PCRs for different MLL fusion genes. Since the chromosomal breakpoints in the MLL gene cluster in a relatively restricted region between exons 8 and 13 (numbering according to Nilsson et al., 1996), encompassing approximately 8.2 kb, we additionally investigated all samples by a recently published long-distance inverse (LDI-) PCR method that also allowed the identification of unknown MLL translocation partners at the DNA level.
Materials and Methods

Patient material

Bone marrow (N=136) and peripheral blood (N=45) samples (N=3 samples unspecified) were obtained for diagnostic purposes within the framework of the GMALL therapy studies 6/99 and 7/03 between 01/2001 and 10/2007. All samples were taken at the time of primary diagnosis and had a high blast count, as revealed by flow cytometry. The genetic investigations were done retrospectively and prospectively on archived residual material. Preparation of samples, immunophenotyping and all RT-PCR investigations were performed at the central diagnostic laboratory of the GMALL study group in Berlin. The samples were obtained within clinical studies that were approved by the institutional ethics committees of all participating institutions. The study design and our investigations complied with the Helsinki Declaration.

Nucleic acid isolation and reverse transcription

Total RNA was isolated using the TRIzol method (Gibco BRL/Invitrogen, Carlsbad, CA, USA) or the RNEasy kit (QIAGEN, Hilden, Germany). Genomic DNA was isolated using the PureGene Kit (Gentra Systems, Minneapolis, MN, USA). Reverse transcription was done using the Ready-To-Go Beads (Amersham Biosciences, Piscataway, NJ, USA) following the protocol as recommended by the supplier.

PCR for BCR-ABL, MLL-AF4, MLL-ENL, MLL-AF9

All cDNA samples were investigated for BCR-ABL by two different PCR methods as described recently. These two PCR methods each included an internal control reaction as a control for RNA integrity. In addition, all samples obtained before 2006 were investigated for MLL-AF4 by a nested PCR, as described elsewhere and from
2006 onwards by a single round PCR according to the BIOMED-1 recommendations. PCR for \textit{MLL-ENL} (\textit{MLL-MLLT1}) and \textit{MLL-AF9} (\textit{MLL-MLLT3}) were performed basically as described by Jansen \textit{et al.}, 2007 but with slightly modified \textit{ENL} primers to increase their annealing temperatures (5’-3’):

\begin{align*}
\text{TCTGGGCTTGGGGAAGCTGTC, and CTCCTCGCTGACGAAGAGT.}
\end{align*}

In summary, each patient sample was investigated by 7 different PCRs.

\textbf{PCR methods for other MLL fusion transcripts}

PCR primer pairs for detecting \textit{MLL-TET1} were the following (5’-3’):

\begin{align*}
\text{CCGCCTCAGCCACCTAC / TTCCTTGCTGCAAGCGGACATTCT (for the patient with breakpoint in TET1 intron 8) and GATGCCTTCCAAAGCCTACCTGCAGAAGG / TACTTCAGGTGACGGTCACGTGCAGTACAGTTGACAT for the patient with breakpoint in TET1 intron 11). The reciprocal TET1-MLL transcripts were investigated with the primers CGTACTGTACATGTCAAGGAATTGATCCAGAG / GCCACACTGAGACACAGAAAAGAAAACCAC and GATCTGTGGACTCCATCTGCTGGAA / CTGCGATGATGACAGAGGTTCTTGCC.}
\end{align*}

\text{The expression of MLL-\textit{ACTN4} and \textit{ACTN4-MLL} was detected with the primer pairs CGAAAGCCCGTGGAAAGAGGTG / CGCTCAGCTAGGTAACCAGCT and AGCATGGCAGACTACATGGCCAG / AGATTCCTAGCAGCTCCACCACA, respectively. For the MLL-PTD-positive case, the primer pair CCGCCTCAGCCACCTAC / CTTTTGATCCTTATCCAGATTTGGTCTC was used.}

\textbf{Immunophenotypic analysis}

Immunophenotypic analysis was performed as outlined in detail previously using a FACScan and Cell Quest software (Becton Dickinson, Heidelberg, Germany).
Fluorochrome-labelled monoclonal antibodies were used in dual staining experiments. Cell surface antigens were considered positive when 20% or more cells showed a fluorescence intensity greater than the negative control while the cut-off for cytoplasmic or nuclear antigens (cylg or TdT) was 10%. The diagnosis of a pro-B ALL required expression of at least CD19, CD22 (either membrane or cytoplasmic) and TdT, but negativity for CD10, cytoplasmic IgM (cylg) and surface Ig (sIg). Expression of CD24 was not required for the diagnosis of a pro-B ALL, as CD24 frequently shows attenuated or absent expression in pro-B ALL. CD10-negative pre-B-ALL was diagnosed as outlined by Gleissner et al. (2005). Coexpression of B-lineage and myeloid antigens (CD13, CD33, CD65s, CD15) was confirmed in dual staining experiments in the majority of samples and, in a few samples with limited cell numbers for immunophenotypic analyses, by a ≥ 20% overlap of CD19 positivity and expression of the respective myeloid antigen. The great majority of samples showed a very high cell count and > 50% leukemic cells. Four samples with a lower blast count (CD19-positive cells 27-39%) were excluded from the immunological but not the genetic analysis.

**Long-distance inverse (LDI-) PCR**

LDI-PCR was performed at the DCAL (Frankfurt) as described previously. Briefly, 1µg of genomic DNA was digested with restriction enzymes and re-ligated to form DNA circles before LDI-PCR using *MLL*-specific primers. Restriction polymorphic PCR amplimers were isolated from the gel and subjected to DNA sequence analyses to obtain the patient-specific fusion sequences.

**DNA sequencing**

DNA sequencing was performed mainly at the DCAL (Frankfurt) using standard methods on an ABI sequencer. Selected cases and *MLL* fusion mRNA transcripts
were sequenced at the MPI (Berlin).

Results

Overall, sufficient material from 184 BCR-ABL-negative patients with a CD10-negative BCP immunophenotype was obtained at the central diagnostic laboratory of the GMALL study group that allowed a detailed genetic analysis (Figure 1a). All samples were analyzed for MLL-AF4, MLL-ENL, and MLL-AF9 by RT-PCR. All samples, regardless whether RT-PCR-positive or –negative, were further analyzed by LDI-PCR to identify the chromosomal breakpoint or to disclose/exclude an MLL rearrangement. All chromosomal breakpoint sequences were submitted to the EMBL nucleotide sequence database (accession numbers are listed in the supplementary data). The detection rates for MLL positivity were similar in bone marrow and peripheral blood.

Regarding basic clinical characteristics, MLL-AF4-positive patients had a median age of 46 years (17-81), those with other MLL aberrations had a median age of 54 years (23-72), while MLL-negative patients had a median age of 49.5 years (17-80). The majority of MLL-negative patients were male (37 of 66, 56.1%) while the majority of patients with MLL aberrations were female (MLL-AF4: 65 of 101; 65%; other MLL+: 12 of 17, 70.6%; p=0.011, Fisher’s exact test). MLL-positive patients had a significantly higher WBC at diagnosis (median: 123/nl, range: 0.9-721/nl, N=74), compared to MLL-negative patients (median: 8.33/nl, range: 0.7-660/nl ; N=48; p(2)<0.001, U-test). One hundred and one (54.9%) patients (81 cyIg-, 20 cyIg+) showed an MLL-AF4 fusion transcript. The chromosomal breakpoint in the MLL gene was identified in every case by LDI-PCR and the distribution of breakpoints is shown in Figure 2a. Two distinct breakpoint cluster regions (bcr1 and bcr2) in the MLL gene
could be distinguished. bcr1 encompassed about 3.5 kB from the start of intron 8 up to the first approx. 600 bp of intron 11 and bcr2 included about 200 bp immediately at the 5' boundary of exon 11. Ninety-five percent of breaks occurred within these two regions. Thirteen breaks occurred within MLL exons, one within AF4 exon 3. The relative frequency of MLL-AF4 fusion transcripts was deduced and is shown in Figure 2b. Seventy-six percent of MLL-AF4 transcripts showed a fusion of MLL to AF4 exon 4.

Patients with a breakpoint in bcr2 showed a tendency towards female preponderance and older age at diagnosis, but this finding was not statistically significant. Also, the immunophenotype and WBC did not differ significantly between patients with breakpoints in bcr1 and bcr2.

The second most prevalent fusion gene was MLL-ENL in 11 cases (6.0%; 9 cylg-, 2 cylg+). ENL exon 2 was fused at the cDNA level to MLL exon 9 (2 patients), 10 (2 patients), 11 (6 patients) or 12 (1 patient). However, at the genomic level, 3 breakpoints (EMBL: AM050805, AM949727, AM050803) were found around 30 kB, 25 kB and 25 kb 3' of the first ENL exon indicating a spliced fusion mechanism in these cases. The remaining 8 breakpoints were located in ENL intron 1.

In two patients, a 57-year-old and a 67-year-old female, LDI-PCR analysis revealed a MLL-TET1 fusion with chromosomal breakpoints in MLL intron 10 / TET1 intron 8 and MLL intron 7 / TET1 intron 11, respectively. Both breakpoints on chromosomes 11q23 (MLL-TET1) and 10q21 (TET1-MLL) were determined and the corresponding fusion mRNA transcripts were detected by RT-PCR (Figure 1b). A hitherto unknown spliced cryptic 87 bp exon between TET1 exon 8 and 9 was identified in the first patient. One patient, a 69-year-old female, showed a fusion of MLL to ACTN4 on 19q13 with expression of both fusion mRNA transcripts, MLL-ACTN4 and ACTN4-MLL (Figure 1b). Another patient (female, 66 years) displayed an MLL partial tandem
duplication (PTD) and a second (male, 58 years) showed a fusion of MLL intron 9 to sequences approximately 1.339 MB 3' downstream of MLL, thus indicating a 1.373 MB interstitial deletion on 11q23. No fusion transcripts with the next 3 known same-stranded gene loci (LOC729173, OAF, POU2F3) located 350-450 kB 3' of the breakpoint were detectable by RT-PCR in this latter case and thus there was no evidence of a spliced fusion.

The immunophenotypic analysis showed an MLL aberration in 118 (96 cyIg-, 22 cyIg+) patients. The following antigens were significantly less frequently expressed in MLL-positive patients (Fisher’s exact test, cut-off 20%, \( p_{(2)}<0.01 \)): CD13 (6.3% versus 26%, \( N=180 \)), CD33 (4.4% vs 24.6%, \( N=177 \)), while the following antigens were significantly more frequently expressed in MLL-positive patients: NG2 (92.2% vs 15%, \( N=175 \)), CD65s (62.4% vs 21.7%, \( N=177 \)). MLL-positive patients had a significantly lower CD10 expression than MLL-negative patients (median 1.5% versus 3.5%, U test, \( p_{(2)}<0.0001 \)). Positive (P) and negative (N) predictive values for MLL positivity were calculated for a number of variables: NG2+ (P=93.0%, \( N=85.0 \)), CD65s+ (P=86.0%, \( N=52.7 \)), CD15+ (P=83.1%, \( N=44.9 \)), female gender (P=72.6%, \( N=47.4 \)). The combination of different parameters did not yield a better positive predictive value without a decrease in negative predictive value.

Thirteen out of 15 evaluable patients with other MLL aberrations other than MLL-AF4 were NG2-positive.

All breakpoint sequences were submitted to the EMBL/Genbank/DDBJ database.
Accession numbers are listed in the supplementary data to this manuscript.

**Discussion**

This study evaluated a large number of BCR-ABL-negative CD10-negative BCP-ALL
patients with respect to genetic aberrations of the *MLL* gene. In 101 patients (54.9%) an *MLL-AF4* fusion was detected by RT-PCR. In all cases the chromosomal breakpoint in the *MLL* gene could be identified by LDI-PCR thus underlining the reliability of this method for identifying translocations in the *MLL* bcr. Two separate breakpoint cluster regions (bcr1 and bcr2) could be delineated (Figure 2a). Ninety-nine breaks (83%) were located in the 3.5 kB bcr1 and 12 (10.2%) in the 200 bp bcr2. Different mechanisms have been proposed to explain the clustering of breaks in bcr1, e.g. involvement of Alu element mediated recombination events, factors involved in VDJ recombination, topoisomerase II binding sites, scaffold attachment regions, apoptosis-mediated cleavage of DNA and a role for bound RNA polymerase II in bcr2 (briefly reviewed in Meyer *et al.*, 2006). However, all these proposed mechanisms may explain breaks in bcr2, but not in bcr1. The repair mechanism leading to the chromosomal translocation is most likely a NHEJ mechanism as outlined by Reichel 1998.

Eleven patients (6.0%) showed an *MLL-ENL* fusion which is roughly the same prevalence as previously reported in children using cytogenetics. For adult ALL only very few data concerning prevalence and prognostic impact of *MLL-ENL* are available. All patients revealed a fusion of *ENL* exon 2 to *MLL* exons at the RNA level. Three spliced fusions with chromosomal breakpoints far 3' upstream of *ENL* were detected by LDI-PCR.

Two patients had an *MLL-TET1* fusion. *TET1* (*CXXC6*) was first identified as fusion partner of *MLL* in an AML case with trilineage dysplasia and t(10;11)(q22;q23). Two other AML cases with *MLL-TET1* were described by Lohrsbach *et al.* 2003 and Shih *et al.* (2006). The reported fusion transcripts in these 3 cases showed a fusion of *MLL* exons 8 and 9 to *TET1* exon 9, while in the case reported by Shih the *TET1* exon involved was not specified. Our two patients are the first reported ALL
patients with this fusion gene. One patient showed a fusion of MLL exon 9 to TET1 exon 9 and the other a new MLL exon 7 / TET1 exon 12 fusion. Both transcripts, MLL-TET1 and TET1-MLL, were detectable which is in line with the previously reported expression of TET1 in lymphatic tissue. In the first patient an additional spliced TET1 exon was identified. The putative oncogenic mode of action of these fusion genes is unknown as likewise is the physiological role of TET1. TET1 encodes a nuclear 2136 amino acid protein with a zinc-binding CXXC domain. Ono et al. (2002) found various TET1 transcripts expressed in fetal heart, lung and brain and in adult skeletal muscle, thymus and ovary. Lohrsbach and coworkers (2003) detected TET1 transcripts in most organ tissues with the notable exception of adult brain, small intestine, pancreas and uterus.

One patient displayed a MLL-ACTN4 fusion. ACTN4 on chromosome 19q13 is an actin-filament cross-linking protein. Mutations in ATCN4 or ATCN4 deficiency lead to focal and segmental glomerulosclerosis. ACTN4 appears to be expressed nearly ubiquitously and this is in line with the finding in our patient that not only the MLL-ACTN4 but also the reciprocal ACTN4-MLL fusion transcript could be detected. ACTN4 has not been associated with malignant diseases yet.

One patient showed a partial internal duplication of the MLL gene (MLL-PTD) with duplication of exons 3-10 at the mRNA level. MLL-PTD is a fairly common aberration in AML and detected in 5-8% of cases but rarely, if ever, found in ALL.

The distribution of MLL aberrations differs markedly from that recently reported in infant (i.e. age <1 year) ALL, where MLL aberrations were detectable by split FISH, RT-PCR or analysis of the MLL breakpoint region in 79% of 124 cases. Forty-one percent of these infants showed an MLL-AF4, 18% an MLL-ENL and 11% an MLL-AF9 fusion. Twelve patients (10%) with other fusion partners were detected: AF10 (N=3), EPS15 (N=2), SELB (N=1), MSF (N=1), LAF (N=1). Four MLL split fusion
rearrangements remained unresolved at the molecular level. The frequency of *MLL* rearrangements decreased with age and there were associations of different *MLL* aberrations with certain immunophenotypes. Almost all *MLL-AF4*-positive infant patients had a pro-B ALL immunophenotype while in *MLL-ENL*- and *MLL-AF9*-positive patients other immunophenotypes were found as well. Pro-B ALL immunophenotype (N=89) was associated with *MLL* rearrangement in 94% and CD10-negative pre-B ALL immunophenotype (N=4) in 100% of infant cases. For diagnostic assessment we suggest that all CD10-negative BCP ALL patients should be investigated for *MLL-AF4* and *MLL-ENL*. The MoAb 7.1 for the NG2 antigen should be included in the antibody panel for flow cytometry and patients showing NG2 positivity should be subjected to further analysis by LDI-PCR or panhandle PCR to detect a possible cryptic *MLL* rearrangement. In this way more than 99% of all *MLL* rearrangements in adult ALL should be identified and thus might serve as markers for risk stratification and targets for MRD assessment.

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**Author’s contributions**
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Tables

Table 1: Basic clinical data of the patients.

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<th>N</th>
<th>Age [median] (years)</th>
<th>Gender [m/f]</th>
<th>WBC [range] (/nl)</th>
<th>NG2+</th>
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<td>101</td>
<td>17-81 (46)</td>
<td>36/65</td>
<td>141 [0.9-721], N=64</td>
<td>94%</td>
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<tr>
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<td>5/12</td>
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<td>17-80 (49.5)</td>
<td>37/29</td>
<td>8.33 [0.7-660], N=48</td>
<td>15%</td>
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Figures

Figure 1: The *MLL* recombinome of adult ALL

**a.** The diagram shows the distribution of *MLL* fusion genes in 184 adult CD10-negative BCP ALL patients.
b. The agarose gel shows transcripts arising from rare MLL fusions. One patient had an MLL-ACTN4 fusion, two others an MLL-TET1 fusion. In the first MLL-TET1-positive patient additional bands were visible, caused by the splicing of 90 bp TET1 exon 9 and a cryptic 87 bp exon in TET1 intron 8. In all three patients the reciprocal transcript was also detectable.
Figure 2: Chromosomal breakpoints in the MLL gene

a. Distribution of the 118 chromosomal breakpoints between exons 7 and 13 of the MLL gene. Breakpoint locations are denoted as “•”. The upper row shows the 17 breakpoints in patients with MLL aberrations other than MLL-AF4, the lower those 101 with MLL-AF4. Two distinct breakpoint cluster regions (bcr) can be distinguished. All sequences have been submitted to the EMBL/Genbank/DDBJ database (accession numbers listed in the supplement).
b. Relative frequency of different MLL-AF4 mRNA transcripts as deduced from the chromosomal breakpoint locations.
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