Involvement of the *MLL* Gene in Adult T-Lymphoblastic Leukemia

Seval Türkmen,[1,2] Bernd Timmermann,[3] Gesine Bartels,[4] Daniela Gröger,[4] Claus Meyer,[5] Stefan Schwartz,[4] Claudia Haferlach,[6] Harald Rieder,[7] Nicola Gökbuget,[8] Dieter Hoelzer,[8] Rolf Marschalek,[5] and Thomas Burmeister[4]*

[1]Labor Berlin, Abteilung Tumorzytogenetik, Berlin, Germany
[2]Institut für Medizinische Genetik und Humangenetik, Charité, CVK, Berlin, Germany
[3]Max-Planck-Institut für Molekulare Genetik, Berlin, Germany
[4]Medizinische Klinik für Hämatologie, Onkologie und Tumorimmunologie, Charité, CBF, Berlin, Germany
[5]Institut für Pharmazeutische Biologie, Diagnostikzentrum für akute Leukämie (DCAL), Goethe-Universität, Frankfurt, Germany
[6]MLL Münchner Leukämie Labor, München, Germany
[7]Institut für Humangenetik und Anthropologie, Heinrich-Heine-Universität, Düsseldorf, Germany
[8]Medizinische Klinik II, Goethe-Universität, Frankfurt, Germany

While the *MLL* "recombinome" is relatively well characterized in B-cell precursor acute lymphoblastic leukemia (BCP ALL), available data for adult acute T-lymphoblastic leukemia (T-ALL) are scarce. We performed fluorescence in situ hybridization (FISH) for an *MLL* split signal on 223 adult T-ALL samples obtained within the framework of the German Multicenter ALL 07/2003 therapy trial. Three biphenotypic leukemias (T-ALL/AML) were also included in the analysis. Samples showing any alteration by FISH were further investigated to characterize the *MLL* aberration. In addition, they were investigated for common genetic lesions known in T-ALL. Twenty-two cases (9.5%) showed an abnormal *MLL* signal by FISH analysis. Most of these appeared to be deletions or gains but in five cases (2.1%) a chromosomal translocation involving the *MLL* gene was identified. The translocation partners and chromosomal breakpoints were molecularly characterized. Three T-ALLs had an *MLL-AF6*/t(6;11) and two biphenotypic leukemias had an *MLL-ELL*/t(11;19). The chromosomal breakpoints in two of the *MLL-AF6*-positive cases were located outside the classical *MLL* major breakpoint cluster known from BCP ALL. In conclusion, the spectrum of *MLL* translocation partners in adult T-ALL much more resembles that of AML than that of BCP ALL and thus the mechanisms by which *MLL* contributes to leukemogenesis in adult T-ALL appear to differ from those in BCP ALL. Proposals are made for the diagnostic assessment of *MLL* fusion genes in adult T-ALL.

INTRODUCTION

Chromosomal translocations of the *MLL* gene on 11q23 are found in 5–10% of acute B-lineage or myeloid leukemias. While the *MLL* "recombinome" in adult B-cell precursor acute lymphoblastic leukemia (BCP ALL) is molecularly relatively well characterized (Burmeister et al., 2009; Meyer et al., 2009), the data available for acute T-lymphoblastic leukemia (T-ALL) are scarce. Most data are based on conventional cytogenetic analyses which may underestimate the frequency and do not provide molecular insight in the resulting fusion genes. In 2002, Hayette and coworkers at the Centre Léon Bérard in Lyon investigated 47 adults with T-ALL by fluorescence in situ hybridization (FISH) and Southern blot analyses and found four cases with *MLL* translocations (Hayette et al., 2002). The most interesting aspect of this work was—besides the high frequency of *MLL* aberrations (8%)—the fact that in two of the four cases the chromosomal breaks occurred outside the major breakpoint cluster (mbcr) region of the *MLL* gene known from BCP ALL. The authors hypothesized that the frequency of *MLL* aberrations in T-ALL might be underestimated when using RT-PCR methods optimized for detecting fusion genes arising from chromosomal breaks in the *MLL* mbcr. No systematic investigation of a larger patient cohort regarding this issue has since been pursued. To get a better understanding of the involvement of *MLL* in adult T-ALL, we thus investigated a larger cohort of adult T-ALL patients using FISH, various RT-PCRs, and long-distance inverse PCR (LDI PCR). The

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*Correspondence to: Thomas Burmeister, Charité, CBF, Medizinische Klinik für Hämatologie, Onkologie und Tumorimmunologie, Hindenburgdamm 30, 12200 Berlin, Germany.
E-mail: thomas.burmeister@charite.de

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respective fusion transcripts and chromosomal breakpoints were characterized and the immunophenotypic and genetic features and clinical courses of the *MLL*+ patients are reported.

MATERIALS AND METHODS

Patient Samples

All patient samples had been obtained during routine diagnostic procedures within the framework of the German adult ALL therapy study 07/2003 (ClinicalTrials.gov Identifier: NCT 00198991). Patients had given their written consent for further scientific investigation and our study complied with the principles set forth in the Declaration of Helsinki.

Flow Cytometry Immunophenotyping

low cytometry immunophenotyping was performed using routine methods. Samples were immunologically classified as early (pre/pro) T, thymic T, or mature T ALL, as previously described (Burmeister et al., 2006).

FISH

FISH investigations were performed on cytospin samples using routine methods and the Vysis LSI MLL Dual Color Break apart Rearrangement probe (Abbott, Chicago, IL) comprising one 350 kb SpectrumGreen-labelled and one 190 kb SpectrumOrange-labelled probe. The first probe covers the centromeric 5′ part of the *MLL* gene locus up to exon 7 and the second probe covers the telomeric 3′ part of the *MLL* locus beginning from exon 7. Cases showing gains of the *MLL* locus were further investigated by FISH with the CEP11(D11Z1) SpectrumOrange Probe (Abbott).

RNA/DNA Isolation and cDNA Synthesis

RNA was isolated using either the Trizol method (Invitrogen, Darmstadt, Germany) and cDNA was synthesized using Ready-To-Go™ You-Prime First-Strand Beads (GE Healthcare, Munich, Germany). DNA was isolated using the PureGene method (QIAGEN, Hilden, Germany).

RT-PCRs for *MLL* Fusion Genes

RT-PCRs for different *MLL* fusion genes were all performed in a multiplex PCR format. The following three *MLL* primer were designed (with exon location, numbering according to Nilson et al., 1996): mll-7 TGCCTTCCAAAGCCTACC TGCAGAAGC (7), mll-13 TCTCACTGTGTCA TGATTGCGCCAAGCT (14), and mll-20 ACCA ACTCCTCTGCATCCTCCTACACCACCA (20). The three *MLL* primers were combined with one antisense primer located in a putative partner gene. The following partner genes were investigated: *ENL* (*MLLT1*), *AF10* (*MLLT10*), *AF6* (*MLLT4*), *ELL*, *AF4* (*AFF1*), *AF17* (*MLLT6*), and *TET1*. For some of these genes more than one primer/PCR was used. The primer sequences were the following (gene name, NCBI exon location): enl-2 TCTGGGCTTGGGGAAGCTGTC (*ENL*, 2), enl-6 GCTCCCGGTGCTCCTTGGT CACC (*ENL*, 6), enl-8 CTCCTCGCCTGACGA AGAGT (*ENL*, 8), X-7 GATCATATGACCTAT TAGATCCCCGTTTGCTC (*AF10*, 7), X-10 TC CTGGGGAGACTGCACTGAACTTCCA (*AF10*, 10), X-14 TCCCGAGCCAGATACTACATTTG GAAACGA (*AF10*, 14), X-19 TGAGGGCTCTT ACTGCTGTTCAAGGAATC (*AF10*, 19), af6-2 TTGAGTGGTGGCAGTACTAGAGACCCGAAT (*AF6*, 2), af6-7 TCAGAATGCTGGGCTC CAG GAGGAAGC (*AF6*, 7), ell-4 TGTATGCTGCC CAGGCAGTCCAGGTGA (*ELL*, 4), af4-9 TGT CACTGAGCTGAAGGTCG (*AF4*, 9), af17-10 AGAAGTCAGGGGAGCTCTGCAGGGA (*AF17*, 10), af17-13 GTCCCCCGACACCGGAAGCTGC AGT (*AF17*, 13), tet1-10 ACGACCTTCCTTGC TGCCAAGCCGAC (*TET1*, 10), and tet1-12 AG GAGCGGATGGCATCAGCGAATAAGT (*TET1*, 12). Each sample was thus analyzed with 15 multiplex PCRs. Previously identified positive patient samples served as positive controls in the PCRs, wherever available. The HotStarTaq Mastermix (QIAGEN) was used with the following cycler program: 95°C 15 min, 35 cycles (95°C 30 sec, 63°C 30 sec, 72°C 1 min). PCR results were analyzed on an 1% agarose gel.

RT-PCRs for *TLX1*, *TLX3*, and *NUP214-ABL1*

Expression of *TLX3* was investigated by quantitative real-time PCR and *NUP214-ABL1* was investigated by a conventional 6-primer multiplex PCR as described previously (Burmeister et al., 2006).

Analysis for *NOTCH1* Mutations

NOTCH1 mutations were analyzed by sequencing PCR products generated by three different PCRs. The PCR primer combinations were the following: notch26F ACTGCGACCAGGGCTGC AACAGCGC/ notch26R ATGGGGTCCAGCTC

CCTCCGCCGC, notch27F GCGGGGGAGGAG GAAGCCTCGGGTC/ notch27R ATGGCACCC CCTGCAGGCAGAGCCTG, and notch34F AG GGGCCCTGAATTTCACTGTGGGCG/ notch 34R ATGCGGGCGATCTGGGACTGCATG.

LDI PCR for *MLL* Breaks in the *MLL* Major Breakpoint Cluster Region

The LDI-PCR to identify breaks in the *MLL* major breakpoint cluster region was performed at the DCAL Frankfurt/Main as described in detail previously (Meyer et al., 2005).

LDI PCR for *MLL* Breakpoints Between *MLL* exons 21 and 24

In cases with a chimeric mRNA transcript indicative of a breakpoint between *MLL* exons 21 and 24, that is, outside the *MLL* bcr, a new LDI PCR method was designed to identify the breakpoint. The LDI PCR was performed using the restriction enzyme *Mfe*I and conditions exactly as published recently in a different context (Burmeister et al., 2011). However, different primers were chosen: mll-i19r AGGCCAGCTACATGGC TGTACCACACATGT (in intron 19, antisense) in combination with mll-21f CCCAGGCATAG AAGACAATAGACAGTGTGCGT (in exon 21, sense; for breaks between *MLL* exons 21 and 23) and mll-i19r in combination with mll-23f GCCA ACGACATCGGGATTTGATCAAAGGCGA (for breaks 3′ downstream of *MLL* exon 23).

DNA Sequencing

DNA sequencing of *NOTCH1*, the *MLL* fusion transcripts, and the atypical *MLL* breakpoints was performed at the MPI Köln using routine methods on a 3730xl ABI sequencer. Sequencing of the three typical *MLL* breakpoints was done at the DCAL Frankfurt on an ABI sequencer (Life-Technologies, Carlsbad, CA).

Multiplex Ligation-Dependent Probe Amplification (MLPA®)

MLPA® analysis was performed using the Salsa MLPA Kit P335-A4-ALL-IKZF1 (MRC-Holland, Amsterdam, The Netherlands) for the following genes/genomic regions: *IKZF1*, *PAX5*, *CDKN2A/B*, *ETV6*, *RB1*, *BTG1*, *EBF1*, Xp22.33/Yp11.32, and *RB1* (Schwab et al., 2010). Sample preparation, analysis, and acquisition of results were done using the recommended methods. Analysis was performed on an ABI capillary sequencer (Applied Biosystems). Data were analyzed using the Coffalyser Microsoft Excel spreadsheet (MRC-Holland).

RESULTS

Cytospins were prepared from a randomly selected cohort of 223 adult (i.e., aged $\geq$ 15 years) ALL patients. The immunophenotypes (43 mature T, 57 early T, and 123 thymic T) of the samples approximately matched their normal distribution. The median age of the patients was 35 years (range: 16–83 years) and the male/female ratio was 165/58. All samples showed a high blast count (all >40%, in most cases >70–80%) as revealed by flow cytometry. Three cases of biphenotypic acute leukemia were also included in the analysis because they showed a T-ALL immunophenotype with myeloid antigens (one thymic T-ALL and two early T-ALLs). Cytogenetic data were not available at the start of the project but were later obtained in selected cases. FISH investigations for an *MLL* split were performed by means of routine methods using the commercial Vysis MLL Break-Apart probe. Cases that showed any abnormalities in this FISH investigation (structural or numerical) were further investigated by different PCRs. The results are shown in Table 1. An abnormal *MLL* FISH result was observed in 22 cytospin samples (9.7% of all). Four cases (3, 8, 9, and 21; numbering according to Table 1) showed an *MLL* split, suggestive of a translocation, while six revealed loss of one *MLL* locus (*MLL* monosomy, 1, 4, 13, 17, 20, and 22). In one of these cases (4), the loss was apparently subclonal. One patient (10) showed gain of the 5′ end of *MLL*. The remaining 11 cases (2, 5, 6, 7, 11, 12, 14, 15, 16, 18, and 19) showed gains (3 or 4 copies) of the *MLL* locus, with two of these alterations being subclonal (6 and 16). All 22 patients were successively investigated by RT-PCR for *MLL-ENL*, *MLL-AF6*, *MLL-AF10*, *MLL-ELL*, *MLL-AF4*, *MLL-AF17*, and *MLL-TET1*. PCR primers in *MLL* and in the partner genes were chosen in such a way that they covered a wide area of different exons to also identify unusual chromosomal break locations. *TET1* was investigated because it was recently identified as *MLL* translocation partner in B precursor ALL (Burmeister et al., 2009). All other genes have previously been reported in childhood and/or adult ALL. The PCR investigations revealed a *MLL* translocation in five cases.

TABLE 1. Characteristics of the Patients with *MLL* Aberrations

Nr	Age	Sex	FACS	PCR	Karyotype	FISH	MLPA	*TLX1*	*TLX3*	*NUP214 -ABL*	*NOTCH1*	Clinical Course	WBC ($\times 10^9$/l)
1	24	M	thy	neg	n. a.	nuc ish(MLLx1) [194/200], (D11Z1x2)[200]	Homozygous *CDKN2A/B* deletion, partial *ETV6* deletion, heterozygous *IKZF1* deletion, heterozygous deletion of X-chromosomal genes	neg	**pos**	neg	wt	Alive d2551	76
2	34	M	early T		46,XY,-6,add(14) (q32),+21 [3]	nuc ish(MLLx3) [158/200], (D11Z1x2)[200]	Normal	neg	neg	neg	wt, SNP	Allografted, alive 1634	67
3	43	F	thy	*MLL-AF6*	47,XX, +mar [5]/46,XX [35]	nuc ish(5′MLLx2, 3′MLLx1)(5′MLL con 3′MLLx1) [174/200]	Homozygous *CDKN2A/B* deletion, heterozygous *PAX5* deletion, partial *ETV6* deletion	neg	neg	neg	wt, SNP	Allografted, alive d2488	305
4	38	M	thy	neg	46,XY [26]	nuc ish(MLLx1) [40/200], (D11Z1x2)[200]	Homozygous *CDKN2A/B* deletion, partial *ETV6* deletion	**pos**	neg	neg	**mut**, SNP	Therapy stopped in CR, died d171	49
5	62	F	mature	neg	46,XX [3]	nuc ish(MLLx3) [158/200], (D11Z1x2)[200]	Partial *ETV6* deletion	neg	neg	neg	wt	n. a.	n. a.
6	37	F	early T	neg	46,XX,der(1) t(1;5)(p36;?), del(5)(q15q33), del(6)(q15q22), der(10)t(10;13) (p15;q22),del (12)(p12) [cp 9]	nuc ish(MLLx3) [27/200], (D11Z1x2)[200]	Partial *ETV6* deletion	neg	neg	neg	wt	Allografted, died d573	109
7	40	F	thy	neg	46,XX [4]	nuc ish(MLLx3∼4) [122/200], (D11Z1x3∼4) [117/200]	Normal	neg	neg	neg	wt, SNP	n. a.	n. a.

(Continued)

TABLE 1. Characteristics of the Patients with *MLL* Aberrations (Continued)

Nr	Age	Sex	FACS	PCR	Karyotype	FISH	MLPA	*TLX1*	*TLX3*	*NUP214 -ABL*	*NOTCH1*	Clinical Course	WBC ($\times 10^9$/l)
8	30	M	thy	*MLL-AF6*	46,XY,?del(6)(q24), add(11)(q23), add(19)(p13) [15]	nuc ish(MLL)x2 (5′MLL sep 3′ MLLx1)[154/200]	Homozygous *CDKN2A/B* deletion	neg	neg	neg	wt, SNP	Allografted, alive d1728	48
9	35	F	thy/AML	*MLL-ELL*	46,XX,t(11;19) (q23;p13) [3][a]	nuc ish(′MLL)x2 (5′MLL sep 3′MLLx1)[16/200]	Normal	**pos**	neg	neg	wt, SNP	n. a.	n. a.
10	33	F	thy	*MLL-AF6*	87-91,XXXX [15]/46,XX [5]	nuc ish(5′MLLx4, 3′MLLx2) (5′MLL con 3′MLLx2) [165/200]	Homozygous *CDKN2A/B* deletion, heterozygous *PAX5* and *ETV6* deletion, partial *RB1* amplification	**pos**	neg	neg	wt	Alive d1560	9
11	30	M	thy	neg	92,XXYY,der(1;13), i(17)(q10) [cp 3]	nuc ish(MLLx4) [140/200], (D11Z1x3∼4) [157/200]	homozygous *CDKN2A/B* deletion	**pos**	neg	neg	**mut**	Alive d1340	29
12	27	M	early T	neg	46,XY [15]	nuc ish(MLLx3) [131/200], (D11Z1x3) [119/200]	X-Chromosomal genes amplified	**pos**	**pos**	neg	wt	Allografted, alive d1340	15
13	24	F	early T	neg	46,XX,del(11) (q13) [4]/ 46, XX [14]	nuc ish(MLLx1) [93/200], (D11Z1x2)[200]	Heterozygous *CDKN2A/B* deletion	neg	**pos**	**pos**	wt, SNP	Allografted, alive d835	90
14	24	M	mature	neg	50,XY,+8,+11, +13,+14,-15, +19 [7]	nuc ish(MLLx3) [143/200], (D11Z1x3) [180/200]	Heterozygous *IKZF1* deletion, *RB1* amplification	neg	neg	neg	wt	Alive d1001	39
15	43	M	thy	neg	no metaphases	nuc ish(MLLx3∼4) [150/200], (D11Z1x3∼4) [49/200]	Partial *PAX5* deletion	neg	neg	neg	wt, SNP	Allografted, died d499	20
16	25	M	early T	neg	92,XXYY [3][a]	nuc ish(MLLx3∼4) [22/200], (D11Z1x3∼4) [36/200]	Normal	neg	neg	neg	wt, SNP	Relapsed, alive d532	27
17	19	M	mature	neg	n. a.	nuc ish(MLLx1) [165/200], (D11Z1x3) [26/200]	Homozygous *CDKN2A/B* deletion, partial *IKZF1* deletion	**pos**	neg	neg	wt	Allografted, alive d532	337

(Continued)

TABLE 1. Characteristics of the Patients with *MLL* Aberrations (Continued)

Nr	Age	Sex	FACS	PCR	Karyotype	FISH	MLPA	*TLX1*	*TLX3*	*NUP214 -ABL*	*NOTCH1*	Clinical Course	WBC ($\times 10^9$/l)
18	30	M	thy	neg	n. a.	nuc ish(MLLx4) [81/200], (D11Z1x4) [72/200]	Normal	neg	neg	neg	wt, SNP	Alive d209	24
19	31	F	early T	neg	92,XXXX,+der(X) t(X;17)(q26;q21), del(11)(q13q25)x2, del(17)(q21q25) x2 [2]/46,XX, del(11) (q13q25) [2]	nuc ish(MLLx4) [100/200], (D11Z1x4) [81/200]	Homozygous *CDKN2A/B* deletion, partial *PAX5* and *RB1* deletion	**pos**	**pos**	neg	wt	Allografted, alive d164	4
20	55	M	mature	neg	n. a.	nuc ish(MLLx1) [195/200], (D11Z1x2)[200]	Heterozygous *ETV6* and *PAX5* deletion, homozygous *CDKN2A/B* deletion, amplification of X-chromosomal genes	neg	neg	**pos**	wt	Allografted, alive d175	72
21	63	F	early T /AML	*MLL-ELL*	no metaphases	nuc ish(MLL)x2(5′MLL sep 3′MLLx1)[180/200]	Heterozygous *EBF1* and *RB1* deletion, partial *ETV6* deletion	neg	neg	neg	wt, SNP	n. a.	n. a.
22	24	F	mature	neg	n. a.	nuc ish(MLLx1)[176/200], (D11Z1x2)[200]	*RB1* amplification	neg	neg	neg	wt, SNP	Allografted, died d140	18

[a]karyotype at relapse.

Abbreviations: M, male; F, female; PCR: PCR analysis (RT-PCR, LDI PCR) for *MLL* fusion genes; FACS: immunophenotype as determined by flow cytometry; thy, thymic T-ALL; early T, early T-ALL; mature, mature T-ALL; WBC, white blood count (at diagnosis); wt, wild-type (unmutated); mut, mutated; SNP, single nucelotide polymorphism rs10521.

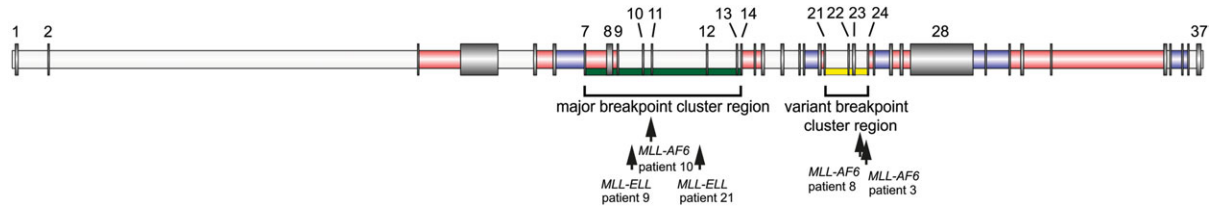

Figure 1. *MLL* gene with the classical major breakpoint cluster region and the variant breakpoint region, as observed in T-ALL. The exons are in three different reading frames and the corresponding introns are thus displayed in three different colors. The exon numbering scheme includes the 99 bp exon 2, which is omitted in the NCBI numbering scheme (Nilson et al., 1996). The locations of the chromosomal breaks in the detected five cases are indicated by arrows (patient numbering as in Table 1). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Two cases of biphenotypic/biclonal leukemia (9 and 21) showed an *MLL-ELL* chimeric transcript, while three genuine T-ALL cases (3, 8, and 10) revealed an *MLL-AF6* transcript. The two *MLL-ELL*-positive cases showed a fusion of *MLL* exons 9 and 11 to *ELL* exon 2. Two of the three *MLL-AF6*-positive cases showed atypical exon fusions, that is, a fusion of *MLL* exon 23 to *AF6* exon 2, suggestive of a chromosomal break outside the *MLL* major breakpoint cluster region between exons 7 and 14. The third *MLL-AF6*-positive case showed a fusion of *MLL* exon 10 to *AF6* exon 2. All the 22 FISH-abnormal cases were subjected to LDI PCR as described previously to further exclude any rearrangement in the *MLL* major breakpoint cluster region (Meyer et al., 2005). This LDI PCR disclosed the respective chromosomal breakpoints in *MLL* in the two t(11;19)-positive and in the *MLL-AF6*-positive case with involvement of *MLL* exon 10 (Fig. 1). In the latter case, the break on chromosome 6 was located 5′ of the first *AF6* exon, thus resulting in a "spliced fusion" *MLL-AF6* transcript with skipping of the first *AF6* exon, as known from a number of *MLL* fusion genes (Meyer et al., 2009). This LDI PCR did not reveal a positive result in the two *MLL-AF6*-positive cases with involvement of *MLL* exon 23. To also identify the breaks in these two cases, a new LDI PCR was developed to identify chromosomal breaks between *MLL* exons 21 and 24. This region was chosen because exons 21–23 are in the same reading frame and chromosomal breaks in *MLL* introns 21–23 may result in a similar type of functional chimeric transcript (Fig. 1). This LDI PCR detected the two atypical breakpoints. All FISH-abnormal cases were investigated with this new LDI PCR but no additional cases were identified. All five chromosomal break sequences were submitted to the EMBL nucleotide database (Acc. No. HE805693-HE805697). The three chromosomal breaks in the major breakpoint cluster region were located in known "hot spots" (Burmeister et al., 2009), while the two "atypical" breaks were located in *MLL* intron 23 at nucleotides 387/388 and 614/615. Since the reciprocal alleles were not identified, no conclusion could be drawn about the possible break mechanism.

Molecular and Immunphenotypic Characterization

The *MLL*-altered cases were further characterized (Table 1). Cytogenetic data were obtained as far as possible. Numerical *MLL* abnormalities by FISH were not always reflected in the karyotypes. In two of the three *MLL-AF6*-positive cases, no structural alteration of chromosomes 6 or 11 was karyotypically visible (3 and 10). All three T-ALL cases were thymic (CD1a+) T-ALLs, while one bilineage leukemia showed a thymic T-ALL and another an early T phenotype together with myeloid antigens. Of the 11 cases with *MLL* amplification, five showed an early T-cell phenotype (pro/pre T-ALL), three a thymic phenotype, and two a mature phenotype. Three of the six cases with *MLL* monosomy had a mature T-ALL phenotype, two an early T-ALL phenotype, and one a thymic T-ALL phenotype. Residual material was available in four of the five patients with *MLL* translocations (8, 9, 10, and 21; Table 1) and these samples were tested retrospectively by flow cytometry for expression of the NG2 antigen that is positive in ~90% of BCP ALL patients with *MLL* translocation (Burmeister et al., 2009). All four samples were NG2-negative. Aberrant expression of *TLX1* (*HOX11*) and *TLX3* (*HOX11L2*) was detected in six T-ALL and one biphenotypic/biclonal and in four T-ALL cases, respectively. *NUP214-ABL* was detected in two T-ALL cases. *NOTCH1* mutations were detected by sequencing in two T-ALL cases, one with *MLL* deletion and one with *MLL* amplification. Fifteen patients showed the silent single nucleotide polymorphism C^{5094}->T (four of them homozygous).

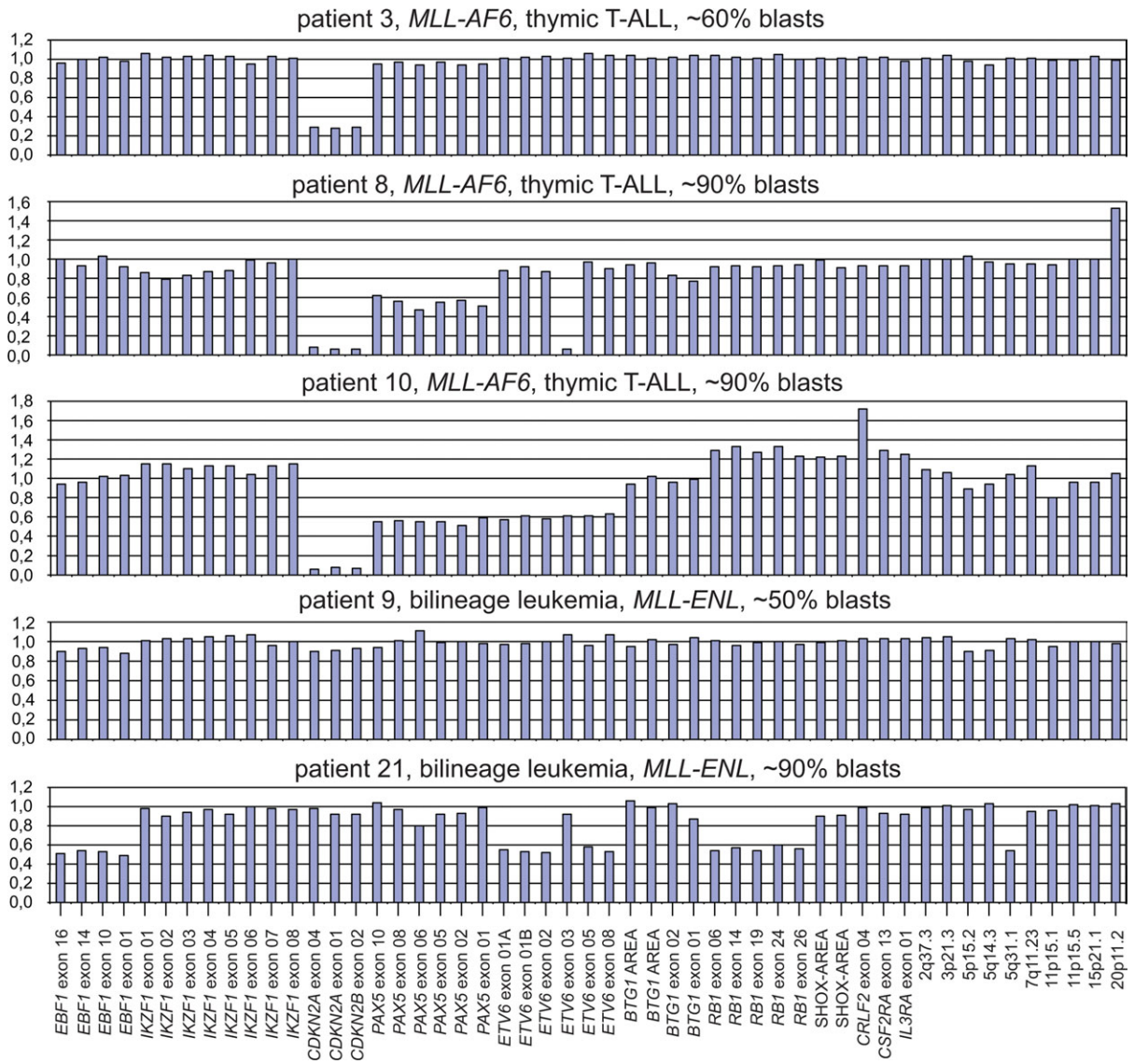

Figure 2. MLPA analysis of the five patients with *MLL* translocations. Patient numbering according to Table 1. The height of the bars corresponds to copy number variations (normal: 1.0 ± 0.2). The blast count as determined by flow cytometry has to be taken into account in interpretation. A blast count of 60% means an admixture of 40% normal cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

To assess submicroscopic deletions or amplifications, each sample was investigated by MLPA. This analysis included the following genes: *EBF1* (exons 1, 10, 14, and 16), *IKZF1* (exons 1-8), *CDKN2A* (exons 2 and 4), *CDKN2B* (exon 2), *PAX5* (exons 1, 2, 5, 6, 8, and 10), *ETV6* (exons 1a, 1b, 2, 3, 5, and 8), *BTG1* (exons 1 and 2), *RB1* (exons 6, 14, 19, 24, and 26), *CRLF2* (exon 4), *CSF2RA* (exon 13), and *IL3RA* (exon 1). In addition, the genomic regions 2q37.3, 3p21.3, 5p15.2, 5q14.3, 5q31.1, 7q11.23, 11p15.1 11p15.5, 15q21.1, and 20p11.2 were investigated with one PCR each (Fig. 2). This analysis showed a homozygous loss of *CDKN2A/B* in nine T-ALL cases, among them the three *MLL-AF6*-positive cases. Two of the *MLL-AF6*-positive cases showed (partial) deletions of *ETV6*, found also in four other cases with numerical *MLL* aberrations. Of the two biphenotypic/biclonal *MLL-ELL*-positive leukemias, one showed a completely normal MLPA pattern while the second showed partial heterozygous deletions of *EBF1*, *RB1*, and *ETV6*.

Clinical Aspects

Treatment and survival data were available for 18 of the T-ALL patients, whereas only few data

were available for the two biphenotypic/biclonal leukemia patients. Regarding the basic clinical characteristics, four of the five patients with *MLL* translocations were female, in accordance with the female preponderance in *MLL*-t BCP ALL (Burmeister et al., 2009), and 11 of the remaining 17 patients with numerical aberrations were male. The average age of the patients was 32 years (range: 19–64). The three *MLL-AF6*-positive T-ALLs had leukocyte counts of 305×10^9/l, 48×10^9/l, and 9×10^9/l and were aged 43, 30, and 33 years at diagnosis. Two of them had been allografted in first remission and all were alive and in complete remission at last follow-up (overall survival of 2488, 1728, and 1560 days). All patients with available clinical data had been treated according to the German Multicenter ALL (GMALL) 07/2003 study protocol. This study comprises two cycles of induction therapy followed by two cycles of consolidation therapy, followed by a reinduction and six additional consolidation cycles. Therapy details have been published previously (Brüggemann et al., 2006). An allogeneic stem cell transplant in first remission is recommended for eligible high-risk patients, for example, those with an unfavorable immunophenotype (early T or mature T ALL) or those with an insufficient reduction in the level of minimal residual disease.

DISCUSSION

Relatively few data exist on the involvement of the *MLL* gene in adult T-ALL. In 1998, the EU Concerted Action Workshop on 11q23 reported six adult T-ALL patients with 11q23 alterations, but only in a minority of cases were molecular techniques used to confirm an *MLL* involvement and few clinical data were provided. The six cases from this series comprised five translocations, three t(11;19)(q23;p13.3), one t(11;17)(q23;q12-21), and one t(4;11)(q21;q23), and one del(11q23) (Harrison et al., 1998; Johansson et al., 1998; Moorman et al., 1998). Three cases with *MLL* aberrations in a series of 56 adult T-ALLs were reported by Moorman et al. (2002), one with a t(11;19)(q23;p13.3) and the other two unspecified. In a large cytogenetic analysis, Marks et al. (2009) reported three adult T-ALL cases with *MLL* translocations out of 216 analyzed, two of them showing a t(11;19)(q23;p13.3) and the third unspecified (one t(11;19) aged 20 years was already reported earlier by Moorman et al. (2002), the other t(11;19) aged 41 years; personal communication A. Moorman). Molecular analyses were not reported. Rubnitz et al. (1999) identified three cases of adult/adolescent T-ALL patients with *MLL* translocations in a cohort of 3578 pediatric and young adult ALL patients. The three patients were 15, 16, and 18 years old and all had a t(11;19)(q23;p13.3) with *MLL-ENL* transcript. One of the patients developed acute myeloid leukemia after 1.2 years, suggestive of biphenotypic leukemia. Fu et al. (2007) reported one 15-year-old T-ALL patient with t(11;19)(q23;p13.3)/*MLL-ENL*. The aforementioned work by Hayette and coworkers revealed four patients with *MLL* rearrangements, three with *MLL-AF6* and one with *MLL-AF10* (Hayette et al., 2002). Cytogenetic analysis of these four showed a complex karyotype without 11q23 involvement in one case and a del(11q23) in three cases. Two of the *MLL-AF6*-positive cases showed atypical in-frame exon fusions (fusion of *MLL* exons 21 and 23 to *AF6* exon 2).

The analysis presented here comprises the largest investigation with focus on *MLL* conducted so far in adult T-ALL in terms of number of investigated patients. Three cases of *MLL-AF6*/t(6;11) were identified among the 223 T-ALL patients. *MLL-AF6* is a well-known aberration in AML, accounting for 5–10% of *MLL*-rearranged cases (Schoch et al., 2003; Meyer et al., 2009). It has very rarely also been reported in childhood T-ALL and infant ALL (Martineau et al., 1998; De Braekeleer et al., 2010). The *AF6* gene has a centromere-to-telomere orientation like *MLL* and thus chromosomal translocations can theoretically occur simply reciprocally. However, as seen from our and the report by Hayette et al. (2002), this translocation may not always be easily detectable by metaphase cytogenetics and may thus require molecular detection methods. A closer look at the aforementioned adult T-ALL case with del(11q23) reported by Harrison et al. (1998) reveals that the patient additionally had an add(6)(q2?) and thus possibly also a cytogenetically unrecognized *MLL-AF6* fusion. RT-PCRs should be designed in a way that also "atypical transcripts" involving *MLL* exons 21–23 are detected. Future research should address the question how MLL-AF6 chimeric proteins resulting from chromosomal breaks between *MLL* exons 21 and 24 differ in their leukemogenic potential, as compared with MLL-AF6 proteins resulting from chromosomal breaks in the *MLL* major breakpoint cluster region (Fig. 1). The former putative proteins are significantly larger and include 1885–1984 instead of 1362–1525 MLL-

derived amino acids. Such "variant" MLL-AF6 proteins would include at least one, possibly even two intact Plant Homeo Domains (PHD, aa 1431–1627 and 1936–1980), which are known structures involved in protein–protein interactions.

Somewhat astonishing was the fact that no cases with *MLL-ENL* transcript were found in our series of patients, since this transcript has been reported repeatedly in previous publications. Nearly all published t(11;19)/*MLL-ENL*-positive non-childhood cases were adolescents (three aged 15, the others aged 16, 17, 18, 19, 20, and 42 years) and thus this type of *MLL* aberration may primarily be found in adolescents, who comprised only a small group in our cohort (fifteen of the 223 patients were aged 19 years or younger). Other *MLL* translocations such as *MLL-AF4*/t(4;11), *MLL-AF17*/t(11;17), and *MLL-AF10*/t(10;11) have to our knowledge only been reported once in the literature in adult T-ALL.

The spectrum of *MLL* translocation found in T-ALL thus differs markedly from that seen in BCP ALL. In adult BCP ALL, *MLL* aberrations are almost exclusively restricted to the $CD10^-$ subset of patients. Sixty-four percent of adult $CD10^-$ BCP ALL patients (i.e., 9% of all B-lineage ALL patients) carry *MLL* aberrations. In 86% and 9% of these cases, *MLL-AF4*/t(4;11) and *MLL-ENL*/t(11;19) are detected, respectively, while other fusion genes are found in the remaining 5% of cases (Burmeister et al., 2009).

In summary, we have characterized the "*MLL* recombinome" in adult T-ALL by investigating a well-characterized cohort of 223 adult T-ALL patients. Three patients were found to be *MLL-AF6*-positive. Two of them showed atypical chimeric *MLL-AF6* transcripts resulting from chromosomal breakpoints outside the *MLL* "major breakpoint cluster region." In two of these cases, the karyotype analysis was not indicative of an *MLL* translocation. All three cases were *NOTCH1*-unmutated, had a homozygous *CDKN2A/B* deletion, and showed a thymic (CD1a+) immunophenotype. In addition, we identified two bilineage leukemia patients who had a thymic T-ALL/AML and early T-ALL/AML phenotype and a *MLL-ELL* chimeric transcript. Flow cytometry showed no NG2 expression in the four evaluable cases. For diagnostic assessment, we suggest that cytogenetic analysis in T-ALL should always include a *MLL* split analysis and those patients showing a *MLL* split signal should further be subjected to PCR analysis to identify the translocation partner and to obtain molecular details on fusion transcripts and chromosomal break locations.

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