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Low frequency of *MLL*-partial tandem duplications in paediatric acute myeloid leukaemia using MLPA as a *novel* DNA screenings technique

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ABSTRACT

Mixed-lineage leukaemia (*MLL*)–partial tandem duplications (PTDs) are found in 3–5% of adult acute myeloid leukaemia (AML), and are associated with poor prognosis. In adult AML, *MLL*–PTD is only detected in patients with trisomy 11 or internal tandem duplications of *FLT3* (*FLT3*-ITD). To date, studies in paediatric AML are scarce, and reported large differences in the frequency of *MLL*–PTD, frequently utilising mRNA RT-PCR only to detect *MLL*–PTDs. We studied the frequency of *MLL*–PTD in a large cohort of paediatric AML ($n = 276$) and the results from two different methods, i.e. mRNA RT-PCR, and multiplex ligation-dependent probe amplification (MLPA), a method designed to detect copy number differences of specific DNA sequences. In some patients with an *MLL*-rearrangement, *MLL*–PTD transcripts were detected, but were not confirmed by DNA-MLPA, indicating that DNA-MLPA can more accurately detect *MLL*–PTD compared to mRNA RT-PCR. In paediatric AML, *MLL*–PTD was detected in 7/276 patients (2.5%). One case had a trisomy 11, while the others had normal cytogenetics. Furthermore 4 of the 7 patients revealed a *FLT3*-ITD, which was significantly higher compared with the other AML cases ($p = 0.016$). In conclusion, using DNA-MLPA as a *novel* screenings technique in combination with mRNA RT-PCR a low frequency of *MLL*–PTD in paediatric AML was found. Larger prospective studies are needed to further define the prognostic relevance of *MLL*–PTD in paediatric AML.

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1. Introduction

Cure rates in paediatric acute myeloid leukaemia (AML) are currently in the 50–70% range, and cytogenetic abnormalities and early response to treatment are the most important factors for treatment stratification.[1] The *mixed-lineage leukaemia* (*MLL*)-gene, localised on chromosome 11q23, plays an important role in the development of both AML and acute lymphoblastic leukaemia (ALL). The *MLL*-gene encodes for a DNA-binding protein that is involved in the methylation and acetylation of histones. These are required for maintaining normal gene expression, especially of the *HOX*-genes, which play a role in the development of leukaemia.[2–4]

To date more than 50 different translocation partners of the *MLL*-gene have been discovered.[5,6] In paediatric AML survival in *MLL*-rearranged AML is dependent on the translocation partner. We recently identified in a large retrospective collaborative study that *t*(1;11)(q21;q23) was associated with a favourable outcome, whereas *t*(10;11)(p12;q23), *t*(10;11)(p11.2,q23) or *t*(6;11)(q27;q23) was associated with a poor outcome.[7]

In 1994, a partial tandem duplication (PTD) of the *MLL*-gene was discovered in a sample taken from an adult AML patient characterised by normal cytogenetics.[8] These *MLL*–PTDs consist of an in-frame repetition of *MLL* exons, which seems to be the result of mispairing of repetitive regions with high homology. Although it has been suggested that the leukaemogenic mechanism for *MLL*–PTD is different from that of *MLL*-rearrangements, mouse model studies have shown that the same *HOX*-genes are affected, which are known to be dysregulated in *MLL*-rearranged leukaemias.[2,9]

In adult AML, *MLL*–PTD was detected in 3–10% of patient samples, using RT-PCR on either the transcript (mRNA) or the genotype (DNA) level (summarised in Table 1).[10–19] In some series *MLL*–PTD was associated with poor outcome.[10,11,14] In adult AML, *MLL*–PTD was mutually exclusive with most other molecular-genetic aberrations, except for trisomy 11 and internal tandem duplications of *FLT3* (*FLT3*-ITD).[12]

So far, information on the incidence and prognostic relevance of *MLL*–PTD in paediatric AML is limited and large differences in the frequency have been reported.[13,20,21] Shimada and colleagues found a frequency of 13% for *MLL*–PTD in 158 paediatric AML cases. In addition, *MLL*–PTD was associated with adverse outcomes. Ross and colleagues detected a frequency of 10% in 130 paediatric AML cases, whereas Shih and colleagues reported a frequency of only 0.9% in 123 paediatric AML cases.

It is likely that the difference in detection methods that were used contributes to the reported differences in the frequency of *MLL*–PTD. *MLL*–PTD was initially discovered using Southern Blot (SB) analysis, but in most subsequent studies the detection was performed with mRNA and DNA RT-PCR. Screening of *MLL*–PTD with SB has its limitations as large amounts of DNA are required to perform SB, and the procedure is laborious. On the other hand, using a nested mRNA RT-PCR approach to detect pathogenic *MLL*–PTD has shown to be not usable, since it detects *MLL*–PTD in healthy individuals and single-round mRNA RT-PCR may also yield false-positive results.[22,23] Another genomic screening method of potential value is the multiplex ligation-dependent probe amplification of DNA (DNA-MLPA), which is already being used as a stand-alone test to rapidly detect aneuploidy in amniotic fluid cells with a high specificity and sensitivity,[24] and reliably detects HER-2/neu amplification in breast cancer.[25] Furthermore DNA-MLPA only needs as little as 100 ng of DNA per patient and is less time consuming. In addition, a large number of patients can be screened at once.

In this study, we screened the largest cohort of paediatric AML cases so far, using both mRNA RT-PCR and DNA-MLPA to accurately detect the occurrence of *MLL*–PTD and the association with other genetic events and prognosis.

2. Material and methods

2.1. Patients

Viably frozen diagnostic bone marrow or peripheral blood samples from 276 newly diagnosed paediatric AML were provided by the Dutch Childhood Oncology Group (DCOG), the AML 'Berlin–Frankfurt–Münster' Study Group (AML-BFM-SG), the Czech Paediatric Haematology Group (CPH) and the St. Louis Hospital in Paris, France. Informed consent was obtained after Institutional Review Board approval according

Table 1 – Summary of the published studies on *MLL*–PTD in adult and paediatric acute myeloid leukaemia.

Study	No. of patients	Adult/paediatric	*MLL*–PTD (%)	Screening method
Schnittger et al.[10]	387	Adult	3.4	Genomic XL PCR
Shiah et al.[18]	81	Adult	11.0	mRNA RT-PCR + Southern Blot
Steudel et al.[11]	956	Adult	5.0	mRNA RT-PCR + Southern Blot
Libura et al.[17]	185	Adult	3.2	Southern Blot
Munoz et al.[19]	93	Adult	10	Genomic XL PCR
Ozeki et al.[16]	181	Adult	10.9	mRNA RT-PCR
Bacher et al.[12]	1881	Adult	5.8	Genomic XL PCR
Olesen et al.[15]	250	Adult	4.0	mRNA RQ-PCR
Shih et al.[13]	865	Adult	6.4	Multiplex PCR
Ross et al.[20]	130	Paediatric	10.0	mRNA RT-PCR
Shih et al.[13]	123	Paediatric	0.9	Multiplex PCR
Shimada et al.[21]	158	Paediatric	13.0	mRNA RT-PCR

to local law and regulations. Each study group performed central morphological review.[26] The collaborative study groups also provided data on the clinical follow-up of these patients.

After thawing, leukaemic cells were isolated by the depletion of contaminating cells as previously described.[27] All the resulting samples contained >80% leukaemic cells, as determined morphologically by May–Grünwald–Giemsa (Merck, Darmstadt, Germany)-stained cytospins. The purified leukaemic cell samples were used for DNA and RNA extraction, and a minimum of 5×10^6 leukaemic cells were lysed in Trizol reagent (Gibco BRL, Life Technologies, Breda, The Netherlands) and stored at −80 °C. Genomic DNA and total cellular RNA were isolated as described before.[28]

2.2. Detection of MLL–PTD

We designed a probe mix for DNA-MLPA analysis containing adjacent probes within exon 2–5 and exon 7–13 of the *MLL*-gene for the detection of common and rare types of *MLL*–PTD. Exon 17 of the *MLL*-gene was used as an internal control. A probe set in the *serpinB2* gene, which is located in a region for which only one copy number variation has been described (http://projects.tcag.ca/variation), was used as an external control according to the manufacturer's protocol (MRC Holland, Amsterdam, the Netherlands) (Table 2). The patient samples were analysed according to the manufacturer's protocol. Briefly, genomic DNA was denaturated and hybridised overnight with a mix of all probes. The adjacent probes were then ligated, so only these sequences were amplified during RT-PCR. Subsequently, these amplified products were separated using capillary electrophoresis. Using Gene Marker v1.5, the peak patterns obtained were compared to those of 3 negative controls to calculate the relative allelic ratios. No inter-assay variability was detected after performing a triple experiment in one assay and for all 3 controls (Supplementary Fig. S1).

We also performed mRNA RT-PCR to detect *MLL*–PTD transcripts, to allow comparison with the DNA-MLPA results, using *MLL*-654c (*AGGAGAGAGTTTACCTGCTC*) as a forward primer and *MLL*-5.3 (*GGAAGTCAAGCAAGCAGGTC*) as a reverse primer.[29]

2.3. Validation of DNA-MLPA on a different patient cohort

The DNA-MLPA method to detect *MLL*–PTDs was validated in an independent adult leukaemia cohort (23 AML, 2 ALL and 1 MDS), whereby Southern Blot analysis for *MLL*–PTD was also performed, as previously described.[30] The positive predictive value, negative predictive value and accuracy of DNA-MLPA were 100%, 89% and 92%, respectively (Supplementary Table S1).

2.4. Cytogenetic and molecular analysis

The paediatric samples were routinely investigated for cytogenetic aberrations by standard chromosome-banding analysis by the collaborative study groups. Moreover, they were screened for recurrent non-random genetic aberrations characteristic for AML, including *t*(15;17), inv(16), *t*(8;21) and *MLL*-rearrangements, using either mRNA RT-PCR or fluorescent in situ hybridisation (FISH). *NPM1*, *CEBPA*, *NRAS*, *KRAS*,

Table 2 – Primer sequences DNA-MLPA *MLL*–PTD.

exon 13 FW	5′-GGGTTCCCTAAGGGTTGGACACAGTGGTCTCATGATTTCT-3′
exon 13 RV	5′-CACTGTGTCATGATTGCGCCATCTAGATTGGATCTTGCTGGCAC-3′
exon 8 FW	5′-GGGTTCCCTAAGGGTTGGAGTGGCTCCCCGCCCAAGTATCC-3′
exon 8 RV	5′-CTGTAAAACAAAAACCAAAAGAAATCTAGATTGGATCTTGCTGGCAC-3′
exon 17 FW	5′-GGGTTCCCTAAGGGTTGGAGATATTGTGAAGATCATTCAAGCAG-3′
exon 17 RV	5′-CCATTAATTCAGATGGAGGACAGCCTCTAGATTGGATCTTGCTGGCAC-3′
exon 10 FW	5′-GGGTTCCCTAAGGGTTGGAGGGAGATGGGAGGCTTAGGAATCTTGA-3′
exon 10 RV	5′-CTTCTGTTCCTATAACACCCAGGGTGGTCTAGATTGGATCTTGCTGGCAC-3′
exon 2 FW	5′-GGGTTCCCTAAGGGTTGGAGCAATTCTTAGGTTTTGGCTCAGATGAAG-3′
exon 2 RV	5′-AAGTCAGAGTGCGAAGTCCCACAAGGTCTTCTAGATTGGATCTTGCTGGCAC-3′
exon 3 FW	5′-GGGTTCCCTAAGGGTTGGAGGAAAAAGGGATCAGAAATTCAGAGTAGTTC-3′
exon 3 RV	5′-TGCTTTGTATCCTGTGGGTAGGGTTTCCAAATCTAGATTGGATCTTGCTGGCAC-3′
SerpinB2 FW	5′-GGGTTCCCTAAGGGTTGGACAGAGAACTTTACCAGCTGTGGGTTCATGCAGC-3′
SerpinB2 RV	5′-AGATCCAGAAGGGTAGTTATCCTGATGCGATTTTCTAGATTGGATCTTGCTGGCAC-3′
exon 4 FW	5′-GGGTTCCCTAAGGGTTGGACGAGGACCCCGGATTAAACATGTCTGCAGAAGAGC-3′
exon 4 RV	5′-AGCTGTTGCCCTTGGCCGAAAACGAGCTGTGTTTCTCTAGATTGGATCTTGCTGGCAC-3′
exon 5 FW	5′-GGGTTCCCTAAGGGTTGGAGAAGATGCTGAACCTCTTGCTCCACCCATCAAACCAA-3′
exon 5 RV	5′-TTAAACCTGTCACTAGAAACAAGGCACCCCAGGAACCTCTAGATTGGATCTTGCTGGCAC-3′
exon 7 FW	5′-GGGTTCCCTAAGGGTTGGAGCCAGCACTGGTCATCCCGCCTCAGCCACCTACTACAGG-3′
exon 7 RV	5′-ACCGCCAAGAAAAGAAGTTCCCAAAACCACTCCTAGTGATCTAGATTGGATCTTGCTGGCAC-3′
exon 9 FW	5′-GGGTTCCCTAAGGGTTGGAGAAAAACCACCTCCGGTCAATAAGCAGGAGAATGCAGGCAC-3′
exon 9 RV	5′-TTTGAACATCCTCAGCACTCTCTCCAATGGCAATAGTTCTATCTAGATTGGATCTTGCTGGCAC-3′
exon 11 FW	5′-GGGTTCCCTAAGGGTTGGACCAAGTCTGTTGTGAGCCCTTCCACAAGTTTTGTTTAGAGGAG-3′
exon 11 RV	5′-AACGAGCGCCCTCTGGAGGACCAGCTGGAAAATTGGTGTTGTCTCTAGATTGGATCTTGCTGGCAC-3′
exon 12 FW	5′-GGGTTCCCTAAGGGTTGGAGCTGGAGTGTAATAAGTGCCGAAACAGCTATCACCCTGAGTGCCT-3′
exon 12 RV	5′-GGGACCAAACTACCCCACCAAACCCACAAAGAAGAAGAAAGTCTGTCTAGATTGGATCTTGCTGGCAC-3′

FW = forward probe, RV = reverse probe.

PTPN11, *C-KIT* and *FLT3* mutational screenings were done as previously described, and included mutational hotspots only.[31–35]

2.5. Statistical analysis

Statistical analysis was performed using SPSS 15.0 (SPSS Inc. Chicago, USA). Different variables were compared with the Chi-square test or the Mann–Whitney-U test. All tests were two-tailed and a *p*-value less than 0.05 was considered significant.

3. Results

3.1. Frequency of MLL–PTD using DNA-MLPA in paediatric AML and comparison with mRNA RT-PCR

Using DNA-MLPA, which has a 92% accuracy as compared to SB, we detected *MLL*–PTD in 6/275 patients (2.2%). In all the 6 patients, *MLL*–PTD mRNA expression was confirmed with RT-PCR, showing high expression levels of *MLL*–PTD. The patients showed an average relative allelic ratio of the amplified region of at least 1.3 compared to the controls. In one additional patient, DNA-MLPA analysis could not be performed since no DNA was available. However, this patient's sample was considered to harbour an *MLL*–PTD, since mRNA RT-PCR demonstrated *MLL*–PTD transcripts, and SB analysis, which was performed at diagnosis, showed an abnormal *MLL* pattern. In addition, an *MLL*-rearrangement was excluded using split signal FISH analysis (data not shown). Therefore, the total number of patients with *MLL*–PTD was 7/276 (2.5%).

In 226/276 samples mRNA RT-PCR screening for *MLL*–PTD was performed. In 6 cases, both DNA-MLPA and mRNA RT-PCR were positive for *MLL*–PTD as described above. In 7 patients, mRNA RT-PCR detected *MLL*–PTD transcripts without evidence for *MLL*–PTD using DNA-MLPA. Interestingly, these transcripts were only observed in *MLL*-rearranged AML, and not encountered in any of the other 213 AML samples without an *MLL*-rearrangement.

3.2. Characteristics of patients with MLL–PTD

The characteristics of the 7 patients with *MLL*–PTD are described in Tables 3 and 4. None of the patients harbouring an *MLL*-rearrangement, *t*(8;21), inv(16) or *t*(15;17) revealed a *MLL*–PTD. In one patient with an *MLL*–PTD, a trisomy 11 was found, while the other 6 cases were found in patients with normal cytogenetics (CN-AML) ($n = 3$) or in patients in whom cytogenetic analysis failed ($n = 3$).

FLT3-ITD was present in 4 out of 7 patients with *MLL*–PTD, while one patient showed a mutation in the kinase domain of *FLT3* and another showed a mutation in *NRAS*. There was a

Table 3 – Clinical characteristics of *MLL*–PTD-positive patients compared to *MLL*–PTD-negative patients.

	MLL–PTD-negative patients	*MLL*–PTD-positive patients	*p*-Value
Sex, *N* (%) ($n = 276$)			0.704[a]
Male	150 (56)	3 (43)	
Female	119 (44)	4 (57)	
Age, years (median, range) ($n = 271$)	9.8 (0.1–18.8)	7.5 (4.8–18.0)	0.740[b]
WBC $\times 10^9$/l (median, range) ($n = 231$)	40 (0–585)	97 (45–170)	0.076[b]
FAB, *N* (%) ($n = 276$)			0.146[a]
M0	13 (5)	1 (14)	
M1	27(10)	3 (43)	
M2	55 (20)	1 (14)	
M3	17 (6)	0 (0)	
M4	69 (26)	2 (29)	
M5	71 (26)	0 (0)	
M6	0 (0)	0 (0)	
M7	5 (2)	0 (0)	
Other/unknown	12 (5)	0 (0)	
Cytogenetic abnormalities, *N* (%) ($n = 276$)			<0.001[a]
MLL-rearrangements	69 (26)	0 (0)	
t(8;21)	33 (12)	0 (0)	
inv(16)	29 (11)	0 (0)	
t(15;17)	16 (6)	0 (0)	
Normal cytogenetics	41 (14)	3 (43)	
Trisomy 11	0 (0)	1 (14)	
Other/unknown[c]	81 (30)	3 (43)	
FLT3-ITD, *N* (%) ($n = 253$)			0.016[a]
No	208 (85)	3 (43)	
Yes	38 (16)	4 (57)	

[a] Chi-square/Fisher Exact test.
[b] Mann–Whitney-U test.
[c] See Table 4 for further details.

Table 4 – Patient characteristics *MLL*–PTD.

Patient ID	Age (years)	Sex	WBC (10^9/l)	FAB	Karyotype	*MLL*–PTD	Allelic ratio	Sec abnormality
AML_DE62	7	Male	68.1	M1	47, XY, +11	ex2–ex8	1.5	NRAS
AML_DE129	7	Male	120.4	M1	46, XY	ex2–ex7	1.5	FLT3-ITD
AML_NL97	14	Female	44.8	M1	46, XX	ex2–ex8	1.5	FLT3-ITD
AML_NL182	11	Female	72.9	M2	46, XX	SB, RT-PCR pos	ND	FLT3-TKD
AML_DE68	7	Female	169.9	M4	NA	ex2–ex8	2	FLT3-ITD
AML_DE15	4	Female	NA	M0	NA[a]	ex2–ex9	1.5	–
AML_FR11	18	Male	133	M4	NA[a,b]	ex2–ex8	2.5	FLT3-ITD

NA = not available, SB = Southern Blot, ND = not determined.
[a] No *MLL*-rearrangement or *t*(8;21) detected.
[b] No inv(16) detected.

significantly higher frequency of *FLT3*-ITD in patients with an *MLL*–PTD than in those without *MLL*–PTD ($p = 0.016$) (Table 3). The age of patients with *MLL*–PTD was not different from that of patients without *MLL*–PTD (median 7.5 years and 9.8 years, respectively; $p = 0.72$). Patients with *MLL*–PTD tend to have higher white blood cell counts (WBC) at initial diagnosis than those without *MLL*–PTD (median WBC 97×10^9/l versus 40×10^9/l, respectively; $p = 0.07$). Two *MLL*–PTD patients had a relative allelic ratio of more than 2.0. They presented with a WBC of 133.0×10^9/l and 169.0×10^9/l, respectively.

3.3. Comparison of MLL–PTD with MLL-rearranged AML

We also compared the patients characterised by an *MLL*–PTD with patients with an *MLL*-rearrangement as determined by conventional karyotyping and/or FISH ($n = 69$). There were no significant differences in the sex distribution. Although patients with *MLL*–PTD tend to have a higher median age (7.5 years versus 6.2 years, $p = 0.074$) and median WBC at diagnosis (96.7×10^9/l versus 61.0×10^9/l; $p = 0.345$), these differences were not statistically significant. There was a significant difference in morphology; i.e. most of the *MLL*-rearranged cases had FAB-M5, whereas none of the patients with an *MLL*–PTD were classified as FAB-M5 ($p \leqslant 0.001$, Table 5).

3.4. Clinical outcome in paediatric AML with MLL–PTD

Since the frequency of *MLL*–PTD was low, it was not possible to perform reliable survival analysis in this cohort of 276 paediatric AML cases. Only 2 out of 7 patients with an AML harbouring an *MLL*–PTD were in the first continuous complete remission (CCR) after 3 years. Another 2 patients initially achieved CR; one patient died after hematopoietic stem cell transplantation (HSCT) due to infectious complications, while the other patient relapsed and was salvaged successfully. Another patient had refractory disease and died from progressive disease following two HSCT's. The 6th patient died within 2 days from cerebral haemorrhage. The 7th patient was lost to follow-up.

Table 5 – Clinical characteristics of *MLL*–PTD-positive patients compared to *MLL*-rearranged patients.

	MLL-rearranged patients	MLL–PTD-positive patients	p-Value
Sex, *N* (%) ($n = 75$)			0.695[a]
Male	38 (56)	3 (43)	
Female	30 (44)	4 (57)	
Age (median, range) ($n = 75$)	6.2 (0.1–18.8)	7.5 (4.8–18.0)	0.074[b]
WBC $\times 10^9$/l (median, range) ($n = 64$)	61 (1.2–585)	97 (45–170)	0.345[b]
FAB, *N* (%) ($n = 71$)			<0.001[a]
M0	3 (4)	1 (14)	
M1	2 (3)	3 (43)	
M2	1 (1)	1 (14)	
M3	0 (0)	0 (0)	
M4	9 (13)	2 (29)	
M5	50 (72)	0 (0)	
M6	0 (0)	0 (0)	
M7	1 (1)	0 (0)	
Other/unknown	3 (4)	0 (0)	
FTL3-ITD, *N* (%) ($n = 70$)			<0.001[a]
No	65 (97)	3 (43)	
Yes	2 (3)	4 (57)	

[a] Chi-square/Fisher Exact test.
[b] Mann–Whitney-U test.

4. Discussion

In this large paediatric AML study, we used DNA-MLPA as a *novel* screenings technique in combination with mRNA RT-PCR. This resulted in a lower frequency of *MLL*–PTD than in two smaller paediatric AML series as summarised in Table 1. The higher frequency in these 2 paediatric AML studies by Shimada and colleagues and Ross and colleagues could be explained by demographic differences. On the other hand it could also be the result of a lack of validation of *MLL*–PTD, as it has been shown that mRNA RT-PCR can give false-positive results.

Contrarily, Shih and colleagues used multiplex PCR on DNA and also showed a low frequency. Combined with our data, this might reflect the true frequency of *MLL*–PTD in paediatric AML. In this study we used DNA-MLPA as a novel method to detect *MLL*–PTD in combination with mRNA RT-PCR. Especially in *MLL*-rearranged cases, transcripts for *MLL*–PTD could be detected with mRNA RT-PCR, as shown in this study and by Shimada et al. In these cases, the high sensitivity of mRNA RT-PCR could be a pitfall in correctly detecting *MLL*–PTD in AML. For example, Schnittger and colleagues were also able to detect the presence of *MLL*–PTD in a subset of normal hematopoietic cells with nested mRNA RT-PCR, whereas SB analysis was negative.

Although DNA-MLPA had a high accuracy of 92% to detect *MLL*–PTD, SB remains the gold standard. Still, DNA-MLPA requires less DNA material, does not require radioactive labelling, provides fast results and can more accurately distinguish *MLL*–PTD from *MLL* translocations. Moreover, it distinguishes all possible variants of *MLL*–PTD, even the rare cases. In this study false-positive results with mRNA RT-PCR were only restricted to *MLL*-rearranged AML cases. However, only 82% of the cases could be screened with mRNA RT-PCR, whereas the remaining 18% still could only be screened for *MLL*–PTD with DNA-MLPA. Therefore, we feel that two methods, i.e. DNA-MLPA together with mRNA RT-PCR, are useful for future diagnostic screening of *MLL*–PTD.

In our series, *MLL*–PTD was found in conjunction with trisomy 11, and mutations in *FLT3* or *RAS*. Gilliland and colleagues hypothesised that the development of AML involves both type-I and type-II mutations. Type-I mutations reflect enhanced proliferation of the hematopoietic cells, whereas type-II mutations lead to impaired differentiation and maturation arrest.[36] *MLL*–PTD mainly clustered with mutations in *FLT3* (type-I mutations), suggesting that there is a non-random association between *MLL*–PTD and *FLT3* mutations. Such non-random associations have been shown for various other subtypes in AML, such as *c-KIT* and *t*(8;21) or inv(16), further supporting the hypothesis put forward by Gilliland and Griffin.[37] The coexistence of both aberrations might indicate an underlying mechanism that could lead to both mutations. It is thought that *MLL*–PTD arises from incorrect homologous recombination of Alu-repeats.[38] However, these repeats are unlikely to be involved in *FLT3*-ITD since the closest repeats are situated 200 bp downstream to exon 14. Still, errors in homologous recombination have been reported, following loop formation within a palindromic hot spot.[39] Although *FLT3*-ITD is a poor prognostic factor in adult and paediatric AML, so far no conclusive results are available for the outcome of *FLT3*-ITD in *MLL*–PTD due to small study populations.

Not only the non-random association of *MLL*–PTD with *FLT3*-ITD but also with a higher WBC, higher frequency in CN-AML and a morphologically more immature phenotype have previously been described in adult AML. There was no significant difference in the median age between cases with *MLL*–PTD and *MLL*-rearranged cases. Nevertheless it should be emphasised that the youngest patient with an *MLL*–PTD was 4 years old, while 40% of the patients with an *MLL*-rearrangement were younger than 4 years. This may indicate a different age distribution between these two subtypes. Compared to patients with an *MLL*-rearrangement there was a remarkable difference in FAB classification in concordance with the study of Shih et al. in adult and paediatric AML. *MLL*–PTD was related to a more immature phenotype compared with *MLL*-rearranged AML, which mostly present with a FAB-M4 or -M5. These differences in differentiation-arrest could indicate differences in the leukaemogenesis of both types of aberrations.

Although both types of aberrations in *MLL* show overexpression of *HOX*-genes, we recently showed that the gene expression analysis presented a distinct profile for *MLL*-rearranged AML whereas a specific signature for *MLL*–PTD could not be identified.[40] A specific gene expression signature for *MLL*–PTD was also not found in other adult and paediatric AML studies.[20,41] Analyses of larger patient cohorts might contribute to a better understanding of the molecular heterogeneity underlying *MLL*–PTD.

Although the role of *MLL*–PTD in leukaemogenesis is not clear these patients could benefit from the treatment with DNA methyltransferase (DNMT) inhibitors and/or histone deacetylase (HDAC) inhibitors. A recent study has shown that $Mll^{PTD/WT}$ knockin mice are fully viable with modest developmental defects, have aberrant gene expression and altered haematopoiesis, but do not develop leukaemia.[9] However, leukaemic blast cells of adult patients with *MLL*–PTD, which are present on only one allele, do not express the wild type (WT) *MLL*, which is based on epigenetic silencing of the normal allele.[42] This is in contrast to *MLL*-rearranged AML, which does express wild-type *MLL*. Interestingly, the treatment of *MLL*–PTD-positive cases with DNMT and HDAC inhibitors resulted in induction of the WT-*MLL* and selective sensitivity to cell death compared with *MLL*–PTD-negative cases with normal expression of WT-*MLL*.[42]

Given the low frequency of *MLL*–PTD in this study, it is difficult to draw conclusions on the prognostic impact of *MLL*–PTD in paediatric AML. DNA-MLPA provided the opportunity to investigate allelic ratios, and two of the 6 patients showed a relative allelic ratio of more than 2, indicating the presence of more than 2 *MLL*–PTDs. Since *MLL*–PTD has been shown to be restricted to one chromosome,[43] the high allelic ratio is most likely the result of a double PTD within the same allele. Interestingly, these two patients presented with a WBC $> 100 \times 10^9$/l, a well-known risk factor for poor outcome in paediatric AML.

In conclusion, the frequency of *MLL*–PTD in paediatric AML is low and may have been overestimated in earlier studies. In this study, we screened the largest paediatric AML cohort so

far, using DNA-MLPA as a novel screening method for *MLL*–PTD in combination with mRNA RT-PCR, and revealed a frequency of only 2.4% in paediatric AML. Larger prospective studies are necessary to further define the prognostic relevance of *MLL*–PTD in paediatric AML.

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Authorship section

B.V.B. and I.H.I.M.H. designed and performed research and wrote the paper; E.W., S.S.N.G., D.R., J.S. and A.B. made this research possible by collecting patient samples and characteristics in their own study groups and providing additional information; H.B.B. and G.E.G. performed the SB analysis for the adult leukaemias and provided clinical characteristics. M.M.H.-E., C.M.Z. and R.P. designed and supervised research and wrote the paper.

Conflict of interest statement

None declared.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2010.02.019.

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