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Clinical relevance of *Wilms tumor 1* gene mutations in childhood acute myeloid leukemia

Iris H. I. M. Hollink,[1] Marry M. van den Heuvel-Eibrink,[1] Martin Zimmermann,[2] Brian V. Balgobind,[1] Susan T. C. J. M. Arentsen-Peters,[1] Marielle Alders,[3] Andre Willasch,[4] Gertjan J. L. Kaspers,[5] Jan Trka,[6] Andre Baruchel,[7] Siebold S. N. de Graaf,[8] Ursula Creutzig,[9] Rob Pieters,[1] Dirk Reinhardt,[2] and C. Michel Zwaan[1]

[1]Department of Pediatric Oncology/Hematology, Erasmus MC–Sophia Children's Hospital, Rotterdam, The Netherlands; [2]AML-BFM Study Group, Department of Pediatric Oncology/Hematology, Medical High School, Hannover, Germany; [3]Clincial Genetics, Academic Medical Center, Amsterdam, The Netherlands; [4]Department of Hematology and Oncology, University Children's Hospital of Frankfurt, Frankfurt, Germany; [5]Department of Pediatric Oncology/Hematology, VU University Medical Center, Amsterdam, The Netherlands; [6]Pediatric Haematology/Oncology, Second Medical School, Charles University Prague, Prague, Czech Republic; [7]Hematology, Saint-Louis Hospital, Paris, France; [8]Department of Pediatric Oncology/Hematology, Radboud University Medical Center, Nijmegen, The Netherlands; and [9]AML-BFM Study Group, University Children's Hospital, Münster, Germany

Wilms tumor 1 (_WT1_) mutations have recently been identified in approximately 10% of adult acute myeloid leukemia (AML) with normal cytogenetics (CN-AML) and are associated with poor outcome. Using array-based comparative genome hybridization in pediatric CN-AML samples, we detected a _WT1_ deletion in one sample. The other _WT1_ allele was mutated. This prompted us to further investigate the role of _WT1_ aberrations in childhood AML. Mutations were found in 35 of 298 (12%) diagnostic pediatric AML samples. In 19 of 35 (54%) samples, more than one _WT1_ aberration was found: 15 samples had 2 different mutations, 2 had a homozygous mutation, and 2 had a mutation plus a _WT1_ deletion. _WT1_ mutations clustered significantly in the CN-AML subgroup (22%; _P_ < .001) and were associated with _FLT3_/ITD (43 vs 17%; _P_ < .001). _WT1_ mutations conferred an independent poor prognostic significance (_WT1_ mutated vs wild-type patients: 5-year probability of overall survival [pOS] 35% vs 66%, _P_ = .002; probability of event-free survival 22% vs 46%, _P_ < .001; and cumulative incidence of relapse or regression 70% vs 44%, _P_ < .001). Patients with both a _WT1_ mutation and a _FLT3_/ITD had a dismal prognosis (5-year pOS 21%). _WT1_ mutations occur at a significant rate in childhood AML and are a novel independent poor prognostic marker. (Blood. 2009;113:5951-5960)

Introduction

Childhood acute myeloid leukemia (AML) is a heterogeneous disease characterized by different recurrent cytogenetic aberrations that, together with early treatment response, determine the current risk-group classification in childhood AML.[1,2] Over the past decades, the prognosis of childhood AML patients has improved significantly, now reaching long-term survival rates of approximately 60%.[3] Further intensification of chemotherapy is not expected to improve survival any further, as the current regimens are already very intensive, resulting in relatively high frequencies of treatment-related death, as well as long-term side effects.[1] Instead, new therapeutic strategies are needed. Drugs that target specific aberrations in the leukemic cells are of advantage, not only for improving prognosis, but also as they may cause less side effects. Therefore, the identification of new genetic aberrations that play a role in the pathogenesis of pediatric AML is warranted. Such aberrations may also improve classification and risk-group stratification.

In childhood AML, approximately 20% to 25% of cases at diagnosis cannot be characterized by karyotypic aberrations and are referred to as cytogenetically normal AML (CN-AML). In adults, this subgroup is larger (approximately 40%-50%), and several molecular aberrations have been identified in this subgroup, such as mutations in *NPM1*, *CEBPα*, partial tandem duplications of the *MLL* gene (*MLL*-PTD), as well as internal tandem duplications of the *FLT3* gene (*FLT3*/ITD). These molecular aberrations are also found in childhood AML, but in a smaller percentage of childhood CN-AML compared with adult CN-AML. In part, this is due to a 2-fold lower frequency of *NPM1* mutations in children (20%-25%)[4-6] compared with adults (50%-60%).[7] Thus, in a relatively large cohort of childhood CN-AML, the underlying genetic aberrations are still unknown.

To identify novel abnormalities in childhood CN-AML, we performed array-based comparative genome hybridization (array-CGH), and found an 11p13 deletion that included the *Wilms tumor 1* (*WT1*) gene in 1 of 43 patients (Figure 1A). The remaining *WT1* allele carried a truncating mutation (Figure 1B). This observation focused our interest on *WT1* aberrations in childhood AML, as only limited data on this topic are available.[8,9]

The *WT1* gene is known to be overexpressed in leukemias and, therefore, is used as a marker for minimal residual disease (MRD) detection, as well as a target for immunotherapy.[10] The gene encodes for a zinc-finger-motif–containing transcription factor involved in the regulation of growth and differentiation. Although the precise role of the WT1 protein in hematopoieses is currently

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Figure 1. *WT1* aberrations in patient no. 1885 detected by array-CGH and sequencing. (A) The left part of the figure shows a chromosome 11 ideogram and corresponding oligonucleotide array-CGH plot of the ratio of patient no. 1885 DNA and control DNA (blue tracing) versus the dye-swap experiment (red tracing) from an array-CGH experiment (105-K oligonucleotide array-CGH platform; Agilent Technologies, Palo Alto, CA). A cryptic 11p13 deletion of approximately 2.5 Mb is found, which area includes the *WT1* gene. The right part of the figure zooms in on the deleted area and presents the genes located in this area. (B) Sequence analysis of patient no. 1885 showing a c.901C→T non-sense mutation on the remaining *WT1* allele.

unknown, it has been hypothesized to act both as an enhancer of cellular quiescence in hematopoietic stem cells and as an inducer of cellular differentiation in more committed precursor cells.[10,11] *WT1* mutations cause translation of an aberrant protein with loss of normal function and might therefore result in stem cell proliferation and blocking of differentiation, thereby contributing to leukemogenesis. Recently, *WT1* mutations were found in approximately 10% of the adult CN-AML subgroup and were reported to be associated with treatment failure and a poor prognosis.[12-14]

To determine further the role of *WT1* aberrations in childhood AML, we searched for these aberrations in a large, well-characterized cohort of childhood AML patients. *WT1* mutations were found in 12% of diagnostic childhood AML samples and appeared to confer independent prognostic significance.

Methods

Patient samples

Viably frozen bone marrow (BM) or peripheral blood (PB) samples taken at diagnosis from 298 children with AML were provided by the Dutch Childhood Oncology Group (DCOG; The Hague, The Netherlands), the AML-Berlin-Frankfurt-Münster Study Group (AML-BFM-SG; Hannover, Germany, and Prague, Czech Republic), and the Saint-Louis Hospital (Paris, France). In addition, 39 paired diagnostic-relapse samples (BM or PB) and 5 paired diagnostic-remission samples (BM) were provided by the DCOG and AML-BFM-SG. Institutional review board approval for these studies had been obtained from Erasmus MC-Sophia Children's Hospital according to local laws and regulations, and informed consent was obtained accordance with the Declaration of Helsinki. Each national study group

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performed central review of the morphologic and cytogenetic classification, as well as clinical follow-up. For a complete list of the individual study participants in the DCOG and AML-BFM studies, see the Appendix (available on the *Blood* website; see the Supplemental Materials link at the top of the online article).

After thawing of the samples, contaminating nonleukemic cells were eliminated as previously described.[15] Blast percentages were assessed morphologically on May-Grünwald-Giemsa–stained cytospin slides. Genomic DNA and total cellular RNA were extracted from leukemic cells using TRIzol reagent (Invitrogen, Breda, The Netherlands) as described before.[16]

Samples were routinely screened using standard chromosome banding analysis by the national study groups and were analyzed further in our laboratory for recurrent nonrandom genetic abnormalities, including t(15;17), inv(16), t(8;21), and *MLL* gene rearrangements, using reverse transcription polymerase chain reaction (RT-PCR) and/or fluorescent in situ hybridization.

Treatment protocols

WT1 mutation analysis was performed on samples obtained from patients who were treated between 1987 and 2005, according to consecutive AML-BFM-SG/DCOG and to Leucámie Aiquë Myéloïde Enfant (LAME) collaborative childhood AML treatment protocols. The survival analysis was, however, restricted to the patients included in the AML-BFM-SG/DCOG cohorts, to reduce the variability in treatment regimens, and because this comprised the majority of patients (n = 232). Details of the treatment protocols included in the survival analysis and overall outcome data have been previously published, with the exception of study AML-BFM 04, which is ongoing.[17-19] In these protocols, treatment consisted of 4 to 5 blocks of intensive chemotherapy, using a standard cytarabine and anthracycline backbone. Stem cell transplantation (SCT) in CR1 was used only in selected high-risk patients.

Mutation analysis of *WT1*

For mutation analysis of exons 7 through 10 of the *WT1* gene, genomic DNA was PCR-amplified using specific primers described in Table S1. The following PCR conditions were used: 2 minutes at 50°C, 10 minutes at 95°C, 40 cycles of 15 minutes at 95°C and 1 minute at 60°C, and an extension step of 10 minutes at 72°C. Purified PCR products were directly sequenced from both strands using the described primers. The sequence data were analyzed using CLC Workbench version 3.5.1 (CLC Bio, Aarhus, Denmark). *WT1* mutants without an unambiguously identified sequence or containing more than one *WT1* mutation were confirmed by cloning using the TOPO-TA Cloning kit (Invitrogen). Plasmid DNA from multiple isolated clones was purified and directly sequenced from both strands.

To explore the presence of mutations in the other exons of the *WT1* gene, exons 1-6 were screened in a cohort of 68 patients, which included 28 patients with *WT1* mutations in exons 7-10 and 40 patients with wild-type *WT1* for exons 7-10. For mutation analysis of exons 1-6, the purified DNA was subjected to 41 cycles of PCR using a touchdown approach of 30 minutes at 94°C, 30 minutes at 66°-54°C (1 × 66°C, 2 × 64°C, 3 × 62°C, 4 × 60°C, 5 × 58°C, 6 × 56°C, and 20 × 54°C) and 30 minutes at 72°C using (M13-tagged) primers also described in Table S1. PCR products were directly sequenced unidirectionally using M13-primers and analyzed using Codoncode aligner (Codoncode, Dedham, MA). Mutations were confirmed by an independent amplification of the fragment and direct sequencing of both strands. This method used for analysis of exons 1 to 6 has originally been developed for mutation detection in nephroblastomas.

Expression analysis of mutated *WT1*

To confirm the expression of mutated *WT1*, cDNA of *WT1* exon 7-mutated samples was PCR-amplified using specific primers described in Table S1. PCR and direct sequencing conditions were the same as for *WT1* mutation analysis on genomic DNA as described above.

For quantitative expression analysis, quantitative RT-PCR of mutated *WT1* samples was performed as described before.[20,21]

Multiplex ligation-dependent probe amplification

Multiplex ligation-dependent probe amplification (MLPA) was used to detect small deletions in the *WT1* gene. MLPA analysis was performed using the SALSA MPLA Kit P118 (MRC Holland, Amsterdam, The Netherlands). SALSA P118 consists of one reaction mixture containing probes for all *WT1* exons and 20 control probes in other regions of the genome. The exact location of the MLPA probes can be downloaded from the MRC Holland website (http://www.mrc-holland.com). MPLA was carried out according to the manufacturer's protocol. Electrophoresis of MLPA-PCR products was done on an ABI-Prism 310 (Applied Biosystems, Foster City, CA). Data analysis was performed by exporting the peak areas to Microsoft Excel. Sample-related and peak-related differences in PCR and electrophoresis efficiency were corrected by first calculating the peak area relative to the sum of peak areas per sample and subsequently calculating each normalized peak area relative to the mean of that peak in control samples. Peak heights outside the range 0.7 to 1.3 times the control peak height were considered abnormal, with those below 0.7 representing deletions, and those above 1.3 representing duplications. Several control samples were included in each MLPA test.

Mutation analysis of other molecular aberrations

Detection of other molecular aberrations, including mutations of *CEBPα*, *FLT3*/ITD and TKD, *NPM1*, *N-RAS* and *K-RAS*, *PTPN11* and *c-KIT*, was performed as previously described.[6,22-25] The detection of *MLL*-PTD was done with PCR as previously described[26] and confirmed with MLPA (MRC Holland). The reaction mixture for MPLA-analysis contained probes for exons 2 through 13 of the *MLL* gene and exon 17 as internal control. A probe in the *serpinB2* gene was used as external control (manuscript submitted; primers available on request). MLPA was performed according to the manufacturer's protocol. Data were analyzed using GeneMarker version 1.5 (SoftGenetics, State College, PA).

Statistical analysis

To compare categorical variables we used χ^2 analysis and the Fisher exact test for small patient numbers. The nonparametric Mann-Whitney U test was applied for continuous variables.

To assess outcome, the following parameters were used: complete remission (CR) rate, resistant disease (RD; defined as failure to achieve remission but excluding patients with early death), probability of event-free survival (pEFS; defined as time between diagnosis and first event, including relapse, death by any cause, failure to achieve remission or second malignancy), probability of overall survival (pOS; defined as time between diagnosis and death from any cause), and the cumulative incidence of relapse or nonresponse (CIR; defined as time between diagnosis and relapse; resistant disease was included as an event on day 0). pEFS and pOS were estimated by the Kaplan-Meier method and compared using the log-rank test. The CIR curves were constructed by the method of Kalbfleisch and Prentice and were compared using Gray test. The independence of prognostic factors was examined by multivariate Cox regression analysis.

P values less than .05 were considered statistically significant (2-tailed testing).

Results

Study population

We screened 298 diagnostic childhood AML samples for *WT1* mutations. The patient characteristics are shown in Table 1. Survival analysis was restricted to the patients who were treated using uniform protocols (n = 232); hence patients treated according to other protocols were excluded (n = 39). In addition, patients with promyelocytic leukemia-retinoic acid receptor α (PML-RARα; n = 15), secondary AML (n = 8), treatment without curative intent (n = 1), and patients lost to follow-up (n = 3) were

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Table 1. Clinical and genetic characteristics of the 298 childhood AML patients included in this study, divided by *WT1* mutational status

	All patients	*WT1* mutated	*WT1* wild-type	*P*
Number (%)	298 (100%)	35 (11.7%)	263 (88.3%)	
Median age, y	9.7	9.2	9.7	.34
$<$ 3 y, n (%)	60	1 (2%)	59 (98%)	
$\geq$ 3 and $<$ 10 y, n (%)	93	17 (18%)	76 (82%)	.008
$\geq$ 10 y, n (%)	145	17 (12%)	128 (88%)	
Sex (% female)	46.6%	48.6%	46.4%	.81
WBC, $\times$ 10^9/L, median (range)	38.0 (0.0-534.6)	57.2 (2.5-332.0)	34.1 (0.0-534.6)	.007
FAB, n (%)				.60
M0	15 (5%)	4 (12%)	11 (4%)	
M1	37 (13%)	6 (18%)	31 (12%)	
M2	66 (23%)	7 (21%)	59 (23%)	
M3	21 (8%)	2 (6%)	19 (8%)	
M4	72 (25%)	8 (24%)	64 (25%)	
M5	61 (21%)	7 (21%)	54 (21%)	
M6	6 (2%)	0 (0%)	6 (2%)	
M7	9 (3%)	0 (0%)	9 (4%)	
Other	2 (1%)	0 (0%)	2 (1%)	
Unknown	9 (3%)	1 (3%)	8 (3%)	
***FLT3*/ITD (n = 298), n (%)**	60 (20%)	15 (43%)	45 (17%)	$<$ .001
Median allelic ratio (range)	0.59 (0.21-2.33)	0.69 (0.32-1.25)	0.58 (0.21-2.33)	.22
Median ITD length (range)	54 (18-209)	51 (18-90)	55 (20-209)	.44
N- or *K-RAS* (n = 282), n (%)	63 (22%)	8 (25%)	55 (22%)	.70
c-KIT (n = 283), n (%)	18 (6%)	0 (0%)	18 (7%)	.24
CEBPα (n = 250), n (%)	20 (8%)	5 (19%)	15 (7%)	.03
MLL-PTD (n = 251), n (%)	6 (2%)	0 (0%)	6 (3%)	$>$ .999
*NPM*1 (n = 293), n (%)	24 (8%)	0 (0%)	24 (9%)	.09

WBC indicates white blood cell count at diagnosis; and FAB, French-American-British morphology classification.

excluded. The *WT1*-mutated and *WT1* wild-type AML patients were equally distributed over the different treatment protocols (P = .43), and there was no significant difference between the treatment protocols in terms of pEFS and pOS (respectively, P = .75 and P = .38). The patient characteristics of the cohort used for survival analysis are shown in Table S2.

WT1 mutation analysis

We first analyzed the samples for mutations in the hotspot areas exons 7-10 of the *WT1* gene. A total of 48 *WT1* gene mutations were detected in 34 of the 298 (11.4%) samples. These mutations predominantly clustered in 2 areas in exon 7 (n = 41), but were also found in exon 8 (n = 1) and exon 9 (n = 4; Table 2). In 2 cases, *WT1* mutations were detected in intron 6-7, located on the splice-acceptor site; these mutations are predicted to disrupt the splice site of exon 7 and are therefore considered pathogenic. Subsequently, a subset of 68 patients was also analyzed for mutations in exons 1 to 6, and additional mutations were found in exon 1 (n = 2), exon 2 (n = 1), and exon 3 (n = 2). The mutations in exons 1, 2, and 3 occurred only in the samples harboring an exon 7 mutation as well, except for one sample that carried both an exon 2 and 3 mutation. Therefore, the total number of mutations detected in the *WT1* gene was 53 mutations in 35 of 298 (11.7%) samples. The locations and hotspots of the *WT1* mutations are presented schematically in Figure 2.

Most of the *WT1* mutations were frame-shift mutations (n = 41) predominantly caused by insertions (range, 1-76 bp) but also by deletions (range, 1-32 bp). Six mutations were non-sense mutations. The frame-shift and non-sense mutations are predicted to result in truncated proteins and, when occurring in exon 7, to the loss of the zinc-finger DNA-binding motif of the WT1 protein. The remaining mutations (n = 6) were missense mutations leading to substitutions of single amino acids. One of these occurred in exon 9 (c.1186G>A), which is a known pathogenic mutation in the Denys-Drash syndrome.[27] The other 5 missense mutations occurred in exon 7, besides a frame-shift mutation in exon 7.

Apart from the 53 pathogenic mutations, we also detected mutations that we considered to be nonpathogenic. In 3 cases, silent mutations, which do not result in amino acid changes, were found. In 6 patients, an identical single base-pair substitution was detected in intron 6-7, which is located 9 nucleotide positions before the start of exon 7 (c.895-9T>C). However, both the lack of a transcript variant in these patients (data not shown) and the analysis of this substitution with regard to the splice-donor and splice-acceptor sites indicated no influence on splicing. These mutations were therefore regarded as nonpathogenic. In one patient, a missense mutation in exon 1 (c.124C>T), causing the substitution of the amino acid proline by a serine, was found. No germ line sample or material of the parents was available to test the leukemic-specific origin of this mutation. However, neither this mutation nor the affected codon has been described before in malignancies. Therefore, we regarded this mutation as an unclassified variant. Several known single-nucleotide polymorphisms (SNPs) were found. One known SNP (c.903A>G) is situated in the mutational hotspot of exon 7 and was detected in 72 of the 298 diagnostic samples (24%). The frequency of this SNP was not different between AML samples harboring a *WT1* mutation compared with *WT1* wild-type samples (respectively, 8/35 [23%] vs 64/263 [24%]).

WT1 microdeletions

To evaluate microdeletions of the *WT1* gene, which might act as a mechanism of knockdown of the other *WT1* allele, we used the MLPA

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Table 2. Characteristics of the *WT1* aberrations detected in the diagnostic samples of 35 childhood AML patients

UPN	Mutation(s)*	Exon	Type of mutation	Protein level†	More than one allele affected?
1	c.905delGinsCC	7	frame-shift		yes
	c.902_938dup37	7	frame-shift		
	c.943T>C	7	missense	p.Ser316Pro	
2	c.901C>T	7	nonsense	p.Arg301X	yes
	del11p13, including WT1‡				
3	c.934_935insA	7	frame-shift		yes
	c.979T>C	7	missense	p.Tyr327His	
4	c.[920_932del13;934C>G]	7	frame-shift		no
5	c.524C>A	2	nonsense	p.Ser175X	unknown
	c.584_585insCCGG	3	frame-shift		
6	c.938C>A	7	nonsense	p.Ser313X	yes
	c.935_939dupCGGTC	7	frame-shift		
7	c.938_939dupTC	7	frame-shift		no
8	c.905delGinsCC	7	frame-shift		no
9	c.934_1009dup76	7	frame-shift		no
10	c.[901C>T;904_905insGA] + [901C>T;904_905insGA]	7	nonsense plus frame-shift	p.Arg301X	yes
11	c.933delA	7	frame-shift		yes
	c.1188_1189ins17	9	frame-shift		
12	c.1186G>A	9	missense	p.Asp396Asn‖	no
13	c.937_940dupTCGG	7	frame-shift		no
14	c.901delCinsGCG	7	frame-shift		no
15	c.937_940dupTCGG	7	frame-shift		no
16	c.905delGinsCC	7	frame-shift		yes
	c.926_935del10ins12	7	frame-shift		
17	c.905_906insTT	7	frame-shift		yes
	c.895-1_901GGATGTGC>CAACGGG	7	frame-shift plus affects splice site		
18	c.[937_938insG] + [937_938insG]	7	frame-shift		yes
19	c.905_906ins17	7	frame-shift		yes
	c.924_925insGG	7	frame-shift		
20	c.934_935insG	7	frame-shift		no
21	c.924_925insGGTT	7	frame-shift		yes
	c.938_939insG	7	frame-shift		
22	c.[933delA;937_939delTCG;1012T>C]	7	frame-shift		yes
	c.937_940dupTCGG	7	frame-shift		
23	c.593delC	3	frame-shift		unknown
	c.901delCinsGG	7	frame-shift		
24	c.398insT	1	frame-shift		unknown
	c.907_908insAT	7	frame-shift		
25	c.[901_902insG;935G>A]	7	frame-shift		no
26	c.1173_1174insA	9	frame-shift		no
27	c.442_442+2GGT>TTG	1	affects splice site		unknown
	c.901_902insG	7	frame-shift		
28	c.1072_1073insC	8	frame-shift		yes
	del 11p13, including WT1§				
29	c.937_940dupTCGG	7	frame-shift		no
30	c.934delCinsGG	7	frame-shift		no
31	c.933_937dupACGGT	7	frame-shift		no
32	c.1168C>T	9	nonsense	p.Arg390X	no
33	c.895-55_895-2del54insCA	intron 6-7	affects splice site		yes
	c.938C>A	7	nonsense	p.Ser313X	
	c.1006A>G	7	missense	p.K336G	
34	c.898_929del32	7	frame-shift		no
35	c.900_901insG	7	frame-shift		no

UPN indicates unique patient number; nd, not done.
*Mutations are described according to the coding DNA sequence (RefSeq NM_0.46642).
†All frame-shift mutations are predicted to produce truncated proteins.
‡Detected by array-CGH and comfirmed by MLPA.
§Detected by MLPA.
‖Mutation is known to be pathogenic in Denys-Drash syndrome.

technique to screen 24 samples (19 *WT1*-mutated and 5 *WT1* wild-type samples) for deletions of exons 1 to 10. The *WT1* deletion already detected by array-CGH (patient 2 in Table 2) was again confirmed by MLPA. One other patient, who appeared to carry a homozygous mutation in exon 8 as detected by sequencing (patient 28 in Table 2), actually harbored a deletion of one *WT1* allele. No other sample carried *WT1* microdeletions. One patient (patient 9 in Table 2) showed a duplication of exon 7 by MLPA. However, this observation could be explained by an exon 7 mutation in this patient, which consisted of a duplication of 76 bp, in which the MLPA probe was exactly situated.

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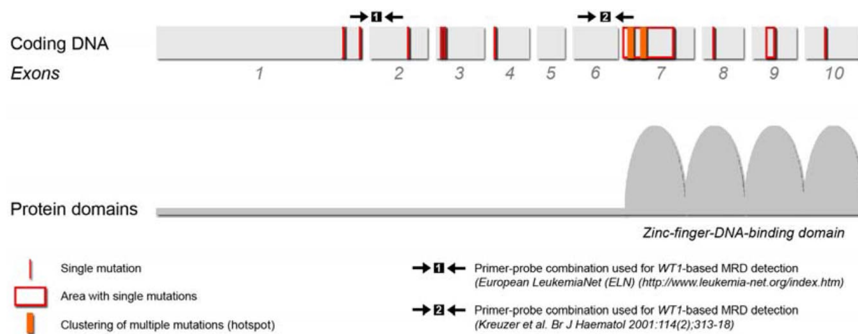

Figure 2. Location of the *WT1* gene mutations and their overlap with MRD detection areas. The location of the *WT1* gene mutations detected in initial diagnosis and relapse childhood AML samples is schematically presented according to the exon structure of the *WT1* gene. Two areas of mutational hotspots were identified in exon 7. Two pairs of primer-probe combinations currently used for *WT1*-based MRD detection are also depicted in the figure and show overlap with the locations of the mutations.

Frequent biallelic involvement of *WT1* aberrations

In 19 of 35 (54%) of the *WT1*-mutated samples, we detected more than one *WT1* aberration. This included either a different *WT1* mutation (n = 15), a homozygous *WT1* mutation (n = 2), or a deletion of the other *WT1* allele (n = 2; Table 2). The *WT1* deletions were detected by array-CGH and MLPA as described above. Of the 2 patients with homozygous mutations, one was screened with MLPA and did not harbor a deletion, but showed homozygosity of all the SNPs in the 11p13 area. This suggests that in this patient a loss of heterozygosity has occurred due to uniparental disomy (UPD) of 11p13. Of the 15 samples with more than one different *WT1* mutation, 12 samples in fact harbored 2 mutations, and 3 samples each had 3 different mutations. In 11 of the 15 cases, we were able to perform cloning of the PCR products followed by direct sequencing to investigate monoallelic or biallelic involvement of the different mutations. This showed more than one affected allele in 10 of 11 cases. The patient (patient 25 in Table 2), who did not show biallelic involvement of the 2 different mutations, had, in addition to a common frame-shift mutation, a missense mutation in exon 7 on the same allele.

In conclusion, we could show that at least 14 of 35 (40%) of the *WT1*-mutated samples had more than one *WT1* allele affected, that is, 11 cases where both alleles were mutated but with different mutations; 2 cases with a homozygous mutation; and 2 cases with a mutation and a deletion of the other *WT1*-allele.

Expression of the mutated *WT1* gene

WT1 transcripts of 16 *WT1*-mutated samples showed the presence of the identical mutations as identified on genomic DNA, indicating that the mutated *WT1* gene is expressed at transcriptional level.

Quantitative analysis of *WT1* expression was performed in 9 *WT1*-mutated and 9 *WT1* wild-type samples. The median expression (normalized to ABL copies $\times$ 10 000) was 4.1×10^3 (range, 9.1×10^2-3.6×10^4) in the *WT1*-mutated samples, which was comparable with the expression measured in the wild-type samples (2.9×10^3; range, 4.8×10^2-7.7×10^3). Three of the 9 *WT1*-mutated samples harbored mutations located exactly at the binding site of the reversed primer used for the quantitative RT-PCR; however, the expression of these samples was still detectable, suggesting that the PCR amplification was relatively unaffected by the mutations in these samples.

Paired diagnostic-remission and diagnostic-relapse samples

The *WT1* mutations were not detectable in the remission material of 5 patients with *WT1*-mutated AML, hence suggesting somatic origin of the *WT1* mutations.

To investigate the clonal stability of *WT1* mutations, we screened 39 diagnostic-relapse pairs. This included 11 mutated and 28 wild-type samples at diagnosis. All 11 *WT1*-mutated diagnostic samples showed the same mutation at relapse. In 2 cases, an additional *WT1* mutation was gained, which was situated on the other *WT1* allele as determined by cloning. No loss of *WT1* mutations was observed. Four of the 28 (14%) samples gained a *WT1* mutation at relapse. Two of these patients in fact gained 2 mutations: in one patient, this concerned mutations in exon 7 and exon 10; in the other patient, in exon 4 and exon 7.

Relationship between *WT1* mutations and clinical and genetic characteristics

The frequency of *WT1* mutations was significantly related to age; below the age of 3 years, only 1 of 60 (2%) cases was *WT1*-mutated; in the age group greater than or equal to 3 years and less than 10 years, we found the highest frequency (17/76 = 18%); and in children at the age of 10 years or above, the frequency decreased (17/145 = 12%; $P = .008$). The white blood cell count (WBC) at diagnosis was significantly higher in *WT1*-mutated AML patients (median 57.2×10^9/L) than in *WT1* wild-type AML patients (median 34.1×10^9/L; $P = .007$). No significant differences between the 2 groups were found for sex ($P = .81$) or for French-American-British (FAB)–type distribution ($P = .60$).

WT1 mutations were not randomly distributed over the different cytogenetic subgroups (Figure 3). Twenty-one of 94 (22.3%) CN-AML cases harbored *WT1* mutations compared with 13 of 187 (7.0%) cases with cytogenetic aberrations ($P < .001$). No mutations were found in the favorable karyotype subgroups t(8;21) and inv(16), and in the t(15;17) and in the *MLL*-rearranged AML cases, only an occasional *WT1* mutation was found. However,

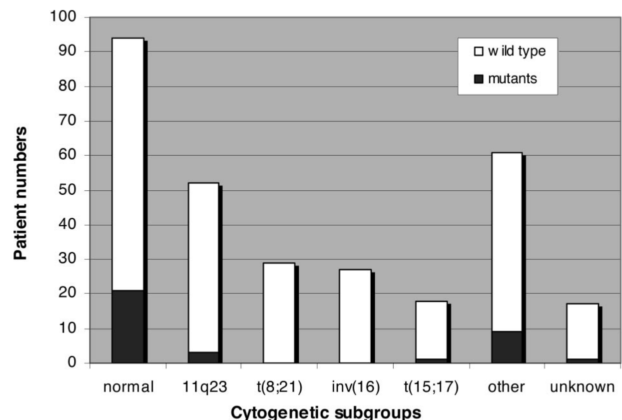

Figure 3. Clustering of the *WT1* gene mutations in the subgroup of childhood CN-AML. *WT1* mutations mainly clustered in the CN-AML subgroup. No mutations were found in the CBF-AML subgroup [ie, t(8;21) and inv(16)], and very few were detected in the t(15;17) and in the *MLL* gene–rearranged childhood AML cases. Mutations were also found in the subgroup with other karyotypes, but at a lower frequency (14.8%) than in the CN-AML subgroup (22.3%).

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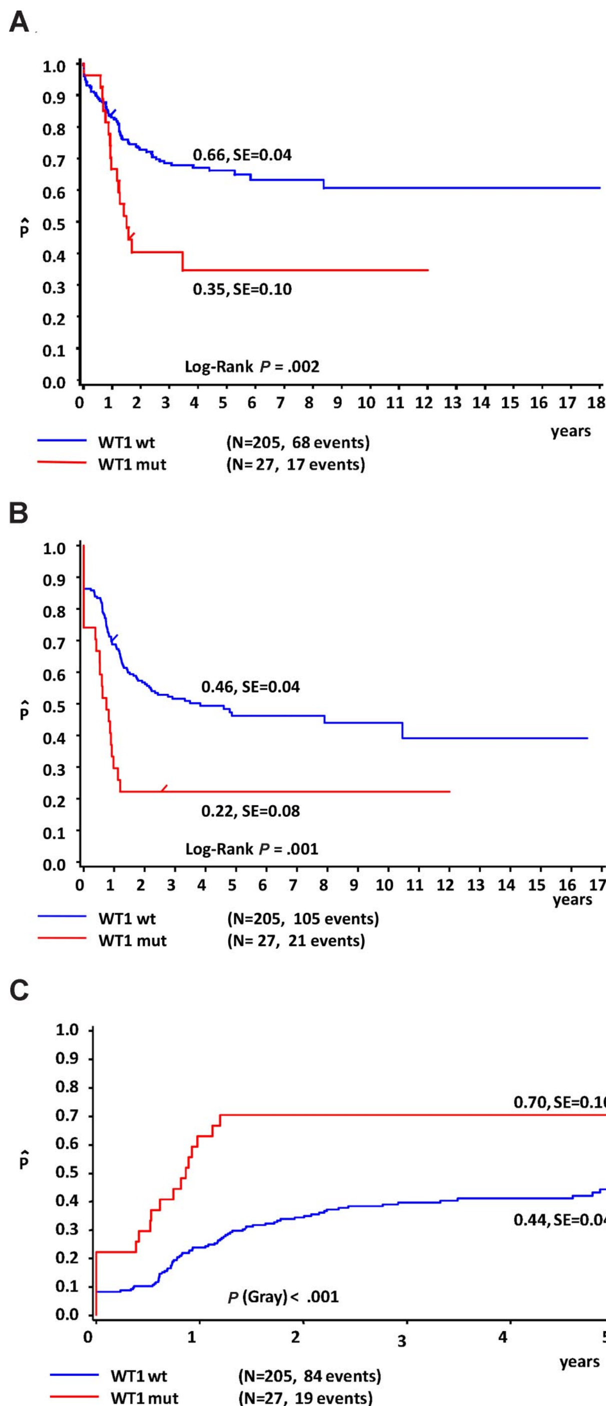

Figure 4. Survival curves of childhood AML patients with and without *WT1* mutations. Kaplan-Meier estimates for 5-year pOS (A) and pEFS (B), showing log-rank *P* values. The 5-year CIR is depicted in (C) and was analyzed according to the Kalbfleisch and Prentice method using the Gray test for statistical significance. *WT1*-mutated AML patients have a significantly worse outcome in terms of OS, EFS, and CIR compared with patients with *WT1* wild-type AML.

within the subgroup with "other cytogenetic aberrations" (defined as all other cytogenetic aberrations than the mentioned subgroups), 9 of 61 (14.8%) samples were *WT1*-mutated. Two of these 9 patients carried a t(6;9)(p23;q34) and another 2 patients carried a 9q-deletion. The other patients in this subgroup did not have cytogenetic aberrations in common.

WT1-mutated patients were more likely to harbor a *FLT3*/ITD (43% vs 17% of the *WT1* wild-type samples; $P < .001$) and a *CEBPα* mutation (19% vs 7%, respectively; $P = .03$; Table 2). *WT1* and *NPM1* mutations were mutually exclusive. *WT1* mutations and *c-KIT* mutations and *MLL*-PTD were also mutually exclusive, however, this was based on small numbers only. Within the CN-AML subgroup, we also identified *FLT3*/ITD to be significantly associated with *WT1* mutations (58% vs 30% for *WT1* mutated and *WT1* wild-type patients, respectively; $P = .03$). However, no difference in *CEBPα* mutations (17% of the *WT1*-mutated vs 15% of the *WT1* wild-type samples; $P = 1.00$) was found in the CN-AML subgroup. *WT1* mutations and *NPM1* mutations were also mutually exclusive in the CN-AML subgroup ($P = .01$).

The study population included 8 patients with a secondary AML. Two of these patients (1 patient with AML after a preceding MDS and 1 therapy-related AML) harbored a *WT1* mutation.

Prognostic impact of WT1 mutations in childhood AML

The median follow-up time for survivors was 4.4 years (4.4 years for *WT1* mutated vs 4.4 years for *WT1* wild-type AML patients; $P = .89$).

The CR rate did not differ significantly between patients with *WT1*-mutated and *WT1* wild-type AML (74.1% vs 86.3%, respectively; $P = .09$). However, RD was significantly more frequent in the former group (23.1% vs 8.8%, respectively; $P = .03$). Patients with *WT1*-mutated AML had a significantly worse pOS, pEFS, and CIR than *WT1* wild-type AML patients (5 years pOS 35 ± 10% vs 66% ± 4%; $P = .002$; pEFS 22 ± 8% vs 46% ± 4%; $P < .001$; and CIR 70% ± 10% vs 44% ± 4%; $P_{Gray} < .001$, respectively; Figure 4). The median time to relapse from CR1 was significantly shorter for the *WT1*-mutated cases (8.5 months) than for the *WT1* wild-type cases (11.4 months; $P = .02$).

Table 3. Five-year OS according to combined *WT1* and *FLT3*/ITD mutational status

FLT3/ITD status	*WT1* mutational status		*P*
	Wild-type	Mutant	
Negative	67% ± 4% (n = 176)	49% ± 14% (n = 14)	.20
Positive	63% ± 9% (n = 29)	21% ± 12% (n = 13)	< .01
P	.61	.09	

Percentages are ±SE.

Table 4. Five-year EFS according to combined *WT1* and *FLT3*/ITD mutational status

FLT3/ITD status	*WT1* mutational status		*P*
	Wild-type	Mutant	
Negative	48% ± 4% (n = 176)	29% ± 12% (n = 14)	.07
Positive	34% ± 10% (n = 29)	15% ± 10% (n = 13)	< .01
P	.32	.16	

Percentages are ±SE.

Table 5. Five-year CIR according to combined *WT1* and *FLT3*/ITD mutational status

FLT3/ITD status	*WT1* mutational status		*P*
	Wild-type	Mutant	
Negative	42% ± 4% (n = 176)	64% ± 15% (n = 14)	.04
Positive	55% ± 13% (n = 29)	77% ± 14% (n = 13)	.02
P	.27	.18	

Percentages are ±SE.

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Table 6. Results of multivariate analysis for OS, EFS, and RFS

Outcome/variable	HR (95% CI)	P
OS		
Other karyotype	2.83 (1.68-4.77)	< .001
FLT3/ITD	1.89 (1.07-3.34)	.03
Favorable karyotype	0.39 (0.15-0.96)	.04
WT1	1.79 (1.02-3.14)	.04
EFS		
Other karyotype*	2.33 (1.49-3.64)	< .001
WT1	2.05 (1.24-3.38)	.005
FLT3/ITD	1.82 (1.14-2.92)	.01
Favorable karyotype	0.74 (0.41-1.33)	.31
FS		
Other karyotype*	2.44 (1.48-4.02)	< .001
WT1	2.44 (1.42-4.17)	.001
FLT3/ITD	1.99 (1.19-3.34)	.009
Favorable karyotype	0.84 (0.44-1.58)	.58

WT1 mutations were tested in a Cox regression model with other well-known risk factors in childhood AML including favorable cytogenetics t(8;21), inv16, other abnormal karyotypes, and *FLT3*/ITD. When including age > 10 years, WBC ≥ 50 × 10^9/L, and SCT as time-dependent covariable, the estimates for *WT1* mutations were similar, and the *P* values for these 3 parameters were all > .10.

*Other karyotypes indicates all other cytogenetic aberrations than the well-known childhood AML subgroups, that is, normal karyotype and the favorable karyotypes inv(16) and t(8;21).

Although numbers were small, there were no differences in CR rate, pOS, pEFS, and CIR between *WT1*-mutated patients with one (n = 13) or more than one (n = 14) affected allele (data not shown).

As *WT1* mutations were frequently associated with *FLT3*/ITD, we analyzed the impact on outcome according to the combined mutational status (Tables 3-5). Although numbers are small, we found that, both in the *FLT3*/ITD negative and in the *FLT3*/ITD positive subgroup, *WT1* mutated cases had a worse pEFS and CIR than the *WT1* wild-type cases. However, this only translated in a significant survival disadvantage for the *WT1* mutated AML patients in the *FLT3*/ITD positive subgroup; in the *FLT3*/ITD positive subgroup, the 5-year pOS was 21% (±12%) for patients with *WT1*-mutated AML and 63% (±9%; $P < .01$) for *WT1* wild-type cases. In the *FLT3*/ITD negative subgroup, these figures were 49% (± 14%) versus 67% (±4%; $P = .20$). Conversely, we found no significant impact on outcome of *FLT3*/ITD in the subgroups of *WT1* mutated or wild-type AML patients.

Prognostic analysis restricted to the CN-AML subgroup

In the CN-AML subgroup, the CR rate was worse in the 19 patients with *WT1*-mutated AML (74%) than in the 66 *WT1* wild-type AML patients (91%; $P = .05$). There also was a trend for RD to occur more frequently in patients with *WT1* mutated AML (22% vs 6%; $P = .07$). *WT1* mutated CN-AML patients had lower pOS and pEFS and higher CIR than *WT1* wild-type CN-AML patients (5-year pOS 44% ± 12% vs 69% ± 6%, $P = .03$; pEFS 32% ± 11% vs 49 ± 6%, $P = .02$; and CIR 41% ± 7% vs 58% ± 13%, $P_{Gray} = .06$, respectively).

Multivariate analysis

WT1 mutations were included in a Cox regression model, including age (using 10 years of age as cutoff value), WBC at diagnosis (using 50 × 10^9/L), cytogenetic subgroups [favorable karyotype, ie, inv(16) and t(8;21), normal karyotype, and other karyotype], *FLT3*/ITD, and SCT as time-dependent covariable. We identified *WT1* mutations as an independent adverse risk factor for pOS [hazard ratio (HR) 1.79; 95% confidence interval (CI) 1.02-3.14; $P = .041$], pEFS (HR 2.05; 95% CI 1.24-3.38; $P = .005$), as well as for CIR (HR 2.44; 95% CI 1.42-4.17; $P = .001$; Table 6). Inclusion of *NPM1* and *CEBPα* mutations in the model did not change the HRs for *WT1* mutations. When we entered *FLT3*/ITD with high allelic ratios (cutoff value ≥ 0.4 or the median ≥ 0.66) or *FLT3*/ITD with large ITD length (≥ median 55 bp) in the model, this did not change the results.

Discussion

Our study shows that *WT1* mutations occur in approximately 12% of samples from diagnostic childhood AML patients, which is comparable to the frequency found in a smaller study on childhood AML reporting *WT1* mutations in 4 of 41 patient samples (10%).[8] Recently, several large adult AML series were reported, showing frequencies of *WT1* mutations ranging from 10% to 13%.[12-14,28] However, these series were restricted to CN-AML. We found a higher percentage in this particular AML subgroup (ie, 22%). It therefore seems that there is a higher frequency of *WT1* gene mutations in childhood AML compared with adult AML. Further support for this difference comes from the typical age pattern that was observed, with a peak frequency in children between the ages of 3 and 10 years. Mutations were sporadically found in children younger than 3 years old, and after the age of 10 years, there was a decreasing frequency. Of interest, 2 other molecular aberrations that also occur frequently within the CN-AML subgroup (ie, *NPM1* mutations and *FLT3*/ITD) both show a different age pattern, as their frequency increases with increasing age.[4,6] Although not all patients were screened for *WT1* mutations in exons 1 to 6, we do not expect this to significantly increase the mutation frequency, as these mutations were only sporadically found in absence of a *WT1* exon 7 mutation.

Our study identifies the presence of *WT1* mutations as a novel poor prognostic risk factor for childhood AML. The 5-year overall survival for *WT1*-mutated AML patients was only 37% compared with 67% for the *WT1* wild-type AML patients. This was due to a significant increase in the number of events in the *WT1*-mutated subgroup, including both primary resistant disease, which was found in 23% of *WT1*-mutated AML patients, as well as an increased frequency of relapse. This was observed both in the overall group as well as in the CN-AML subset; although some of the differences did not reach statistical significance in the CN-AML subgroup. Multivariate analysis showed that *WT1* mutations had strong prognostic significance, independent from other well-known risk factors in childhood AML, with a 2.4-fold increased risk to relapse despite current intensive chemotherapy. When we combined 2 risk factors (ie, *WT1* mutations and *FLT3*/ITD), a very poor risk group was detected with a 5-year overall survival of only 21%. This indicates an additive effect of carrying both mutations with respect to outcome. Our data need to be prospectively validated, given the retrospective nature of this cell-bank study. However, comparable results regarding outcome have been reported in most, but not all, studies in adults with CN-AML.[13,14,28]

In almost half of the patients *WT1* mutations are associated with a *FLT3*/ITD, which is a well-known poor prognostic factor in childhood AML, especially in case of a high mutant/wild-type allelic ratio (AR).[29] Moreover, a recent study also showed that the ITD length has prognostic impact in childhood AML.[30] We did not find differences in the ARs and ITD lengths of the associated *FLT3*/ITDs between *WT1*-mutated and wild-type AML patients. *WT1* and *NPM1* mutations were mutually exclusive and, respectively, predict for poor and excellent outcome in the CN-AML subgroup.[4] However, when including *NPM1* mutations in the multivariate analysis, *WT1* mutations still had independent prognostic significance. In the overall cohort, *WT1* mutations were also associated with *CEBPα* mutations; this association disappeared in the CN-AML subgroup. As *CEBPα* mutations are also known to cluster in

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CN-AML, the observed association in the overall group may be biased. The identification of *WT1* mutations adds to the recognition of CN-AML as a heterogeneous subgroup with various molecular abnormalities in childhood AML, which may be important for future classification and risk-group stratification in childhood CN-AML.

Mutations in the *WT1* gene are localized in 2 hotspot areas in exon 7. However, we also detected mutations in other exons. The mutational hotspots are in agreement with the location of mutations found in previous AML studies, but mutations in exons 4 and 10 have not been reported before.[8,9,13,14,28] Of interest, the affected mutated areas overlap with different primer-probe combinations used for *WT1*-based MRD detection, as reported in the literature.[21,31,32] One of these combinations was used in our study for the expression analysis of the *WT1* mutants.[21] All *WT1*-mutated samples showed presence of mRNA expression. Surprisingly, expression was also picked up in 3 samples with mutations exactly located at the binding site of the reversed primer, indicating no large effect of these mutations on the PCR amplification, and therewith *WT1*-based MRD detection. However, a previous case report did show a negative effect on the expression level of *WT1* in a sample in which a mutation was gained at the primer binding site. Another potential problem for using *WT1* as an MRD marker may be the gain of mutations at relapse, which was observed in 14% of our relapse samples. Future studies on serial samples taken in the course of the disease need to assess the impact of mutations on MRD detection based on *WT1* expression levels.

At least 43% of the patients with *WT1*-mutated AML had more than one affected *WT1* allele in their leukemic cells. This frequency may be higher, as not all *WT1*-mutated samples could be screened for additional mutations in exons 1 to 6. Furthermore, to search for additional second hits in the *WT1*-mutated AML patients, we also screened for small *WT1* deletions using MPLA, but only one extra patient was revealed with a deletion besides a mutation. So far, we were able to detect a second affected allele in approximately half of the *WT1*-mutated AML patients. Sequence analysis of the cDNA of mutated samples showed expression of the mutated as well as the wild-type transcript in samples with one affected allele. This either suggests a dominant or dominant-negative mechanism of the mutated WT1 protein in the subset of patients with only one affected allele, or other mechanisms of silencing of the wild-type allele such as silencing on RNA level. Epigenetic silencing is unlikely as the wild-type allele is expressed. Of interest, there was no apparent difference in outcome between patients with one affected allele versus those with more than one affected allele. Until now, the precise function of WT1 in hematopoiesis is still unclear, as well as whether *WT1* acts as an oncogene or tumor suppressor gene in AML, which issue was recently reviewed by Yang et al[33] Further research is needed to elucidate the underlying functional mechanisms of the mutated WT1 protein and the pathways involved leading to therapy resistance in these patients.

In conclusion, our study showed the presence of *WT1* mutations in 12% of diagnostic childhood AML samples and clustering of these mutations in the CN-AML subgroup (22%). *WT1* mutations are a novel independent poor prognostic factor in childhood AML. In particular, patients with AML carrying both a *WT1* mutation and a *FLT3*/ITD have a very poor outcome of only 21% 5-year survival in this series. Further exploration of the biology of *WT1*-mutated AML is necessary to better understand the mechanisms of therapy resistance. This may reveal new potential therapeutic targets for these patients who suffer from a very poor outcome despite current intensive chemotherapy.

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Authorship

Contribution: I.H.I.M.H., M.M.v.d.H.-E., and C.M.Z. designed the study; G.J.L.K., J.T., A.B., S.S.N. de G., U.C., and D.R. contributed patient samples and clinical data; I.H.I.M.H., B.V.B., S.T.C.J.M.A.-P., M.A., and A.W. performed the laboratory research; I.H.I.M.H., M.M.v.d.H.-E., R.P., and C.M.Z. analyzed and interpreted the data; M.Z. performed statistical analysis; M.M.v.d.H.-E., A.W., G.J.L.K., J.T., and R.P. reviewed the manuscript; and I.H.I.M.H. and C.M.Z. wrote the manuscript.

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Correspondence: C. Michel Zwaan, Erasmus MC–Sophia Children's Hospital, Department of Pediatric Oncology and Hematology, Dr Molewaterplein 60 3015 GJ, Rotterdam, The Netherlands; e-mail: c.m.zwaan@erasmusmc.nl.

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