Molecular genetic insights in cytogenetically normal pediatric acute myeloid leukemia

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Molecular genetic insights in cytogenetically normal pediatric acute myeloid leukemia

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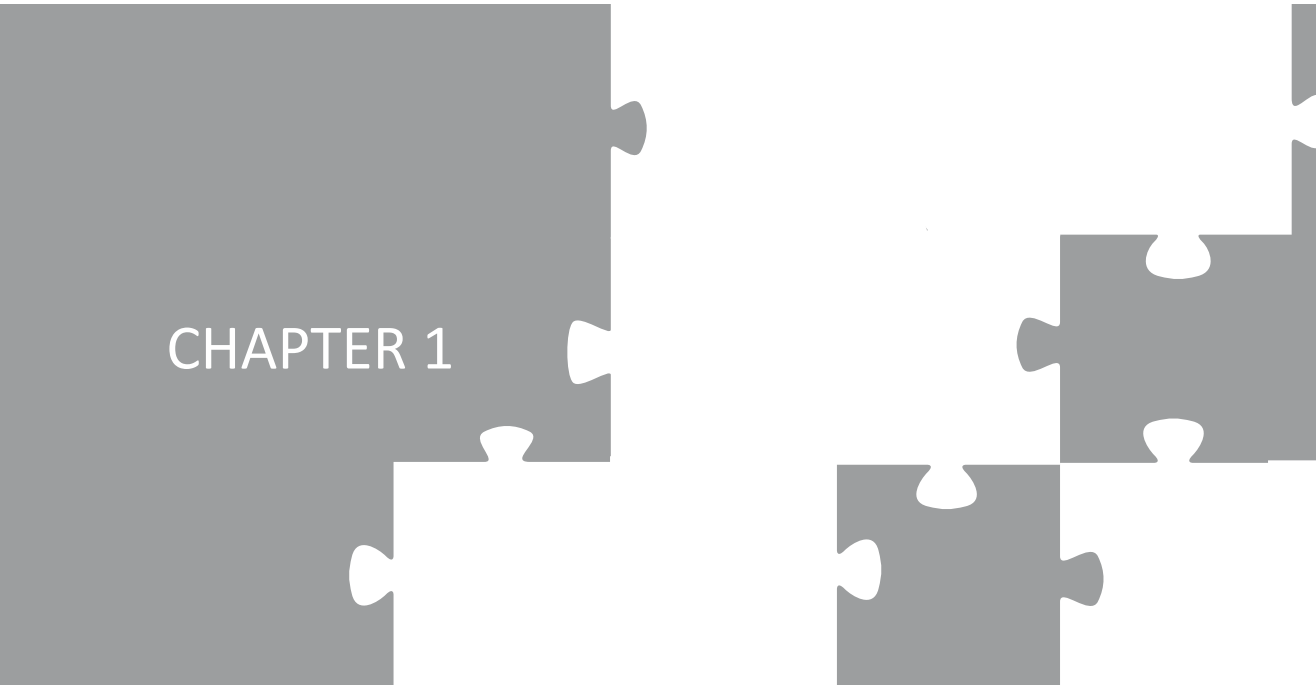
The motive that will conquer cancer
will not be pity nor horror; it will be
curiosity to know how and why...

H.G. Wells 1927

CONTENTS

CHAPTER 1



General Introduction

NORMAL HEMATOPOIESIS AND LEUKEMIA

Hematopoiesis is the process of formation of new blood cells.[1] During embryonic development, the process starts in the yolk sac, in so called blood islands. As development of the embryo progresses, blood cell formation continues predominantly in the liver, and after birth the bone marrow takes over the process of hematopoiesis.

New blood cells continuously need to be generated throughout life, as the majority of mature blood cells have a limited life-span. In a healthy adult, this results in the estimated production of approximately 10^{10} new blood cells per hour.[1] All different types of mature blood cells originate from a pool of self-renewing hematopoietic stem cells (HSC's) that resides in the bone marrow. In a strictly regulated process, the multipotent HSC's can differentiate into either a common myeloid or common lymphoid progenitor cells (Figure 1). While the lymphoid progenitor cells will differentiate and mature into either B-lymphocytes, T-lymphocytes, or natural killer cells, the myeloid progenitor cells will give rise to the other white blood cells (leukocytes) such as granulocytes (i.e. neutrophils, basophils and eosinophils), monocytes/macrophages and mast cells, but also to the red blood cells (erythrocytes) and the platelets (thrombocytes).

Leukemia (in Greek *'leukos'* means white and *'haima'* means blood) is cancer of the blood cells characterized by the uncontrolled proliferation of immature white blood cells (blasts) in the bone marrow. As a result normal hematopoiesis fails and patients suffer from the lack

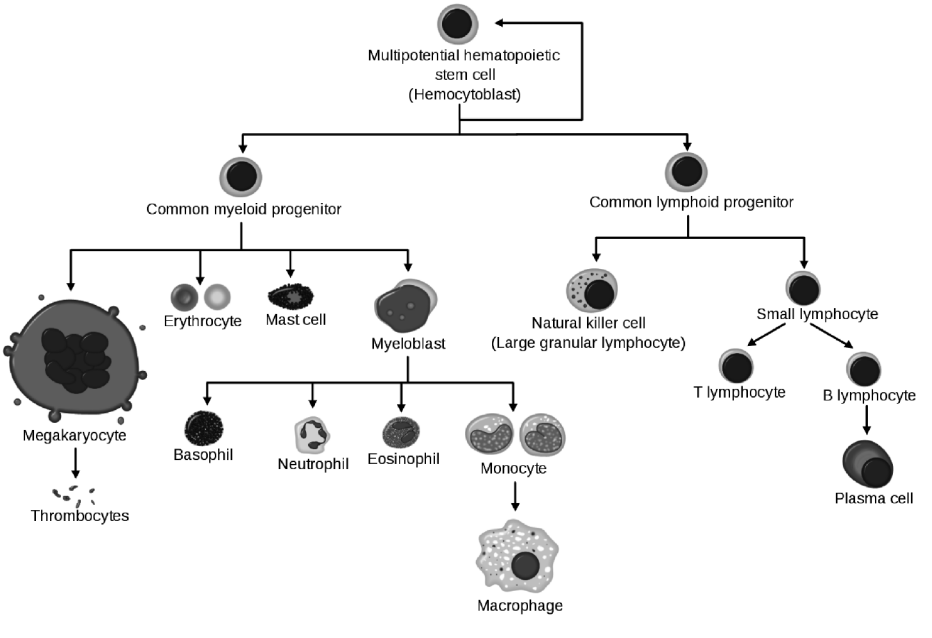


FIGURE 1. Schematic representation of normal hematopoiesis. The multipotent hematopoietic stem cell possesses self-renewal capacity and can differentiate into the different mature blood cells.

of normal blood cells.[1] Leukemia can be divided into an acute and chronic form; acute leukemias are characterized by the clonal expansion of hematopoietic progenitor cells caused by a maturation arrest combined with enhanced proliferation resulting in a fast increase of immature blood cells. In contrast, chronic leukemias result from hyperproliferation without a clear maturation arrest and usually develop slower. A further division can be made depending on the lineage of the progenitor cell that is affected. When the leukemia originates from a lymphoid progenitor cell, it is named a lymphocytic or lymphoblastic leukemia, and when it originates from the myeloid lineage, it is called a myeloid or myelogenous leukemia. This creates the 4 major types of leukemia: acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), chronic myeloid leukemia (CML) and chronic lymphocytic leukemia (CLL).

In children, chronic leukemias are very rare, ALL comprises the largest part (75-80%), and AML accounts for 15-20% of pediatric leukemias.[2]

PEDIATRIC ACUTE MYELOID LEUKEMIA

Epidemiology

AML is actually a disease of the elderly reflected by the median age at diagnosis of AML, i.e. 67 years (SEER Cancer Statistics Registry USA, figures of 2004-2008).[2] After a peak in the incidence of AML in infants (1.6 per 100000 individuals each year), the incidence is low throughout childhood (0.7 per 100000 individuals each year), starts rising in adolescence, and continuously increases during adulthood up to 16.1 per 100000 individuals aged over 65 years each year.[2] AML is slightly more common in males than in females, and geographic and ethnic variation exist in the incidence of AML.[2] In the Netherlands, around 120 children are newly diagnosed with leukemia each year, of which approximately 20-25 children suffer from AML.

The cause of AML is largely unknown, although exposure to environmental factors has been described as a potential cause.[3] Underlying diseases or inherited genetic mutations predisposing to AML exist, such as Fanconi anemia or Down syndrome, and familial germline *CEBPA* and *RUNX1* mutations, but they form a minority.[4-6] Furthermore, AML can be preceded by a myelodysplastic syndrome (MDS) or can occur secondary to previous treatment with irradiation or chemotherapy (alkylating agents or epipodophyllotoxins). AML in children arises in the majority *de novo*, whereas in adult AML treatment-related or AML following MDS occur more frequently.

Diagnosis and classification

The presenting symptoms of AML are variable, but predominantly arise from the inhibition of healthy blood cell formation due to the accumulation of leukemic blasts in the bone marrow. Pallor and tiredness result from anemia, easy bruising and spontaneous bleeding from

TABLE 1. FAB classification of AML and the association with recurrent chromosomal rearrangements.

FAB	Name	% of pediatric AML	Chromosomal rearrangements
M0	Minimally differentiated AML	2-5	
M1	AML without maturation	10-15	t(8;21)(q22;q22)
M2	AML with maturation	25-30	t(15;17)(q22;q12)
M3	Acute promyelocytic leukemia (APL)	5-10	
M4	Acute myelomonocytic leukemia	15-25	inv(16) (p13;q22) / t(16;16)
M4eo	As M4, with bone marrow eosinophilia	10	(p13;q22)
M5	Acute monocytic leukemia	15-25	*MLL*-rearrangements*
M6	Acute erythroid leukemia	1-3	
M7	Acute megakaryoblastic leukemia	5-10	t(1;22)(p13;q13)

* *MLL* indicates *mixed-lineage leukemia* gene

thrombocytopenia, and fever and infections from the lack of normal leukocytes. Blasts can infiltrate organs, such as the liver, spleen and testes causing organomegaly, and the central nervous system, or sporadically present as leukemic masses elsewhere (chloromas). The diagnosis of AML is mainly based on morphology of the peripheral blood and bone marrow aspirates combined with cytochemistry and immunophenotyping of the leukemic blasts for specific markers. In children, a threshold of more than 30% blasts is used to differentiate between AML and advanced MDS, versus 20% in adults.[7] However, when specific chromosomal rearrangements are present, or when it concerns a child with Down syndrome, AML is diagnosed irrespective of the percentage of blasts.

AML is a heterogeneous disease comprising diverse clinical entities reflected by differences in the morphology, underlying genetic aberrations and clinical behavior. Traditionally, AML is classified based on morphology, cytochemistry and immunophenotype according to the French-American-British (FAB) system.[8-9] This system is based on the cell lineage of origin of the AML and the degree of maturation, and ranges from M0 (minimally differentiated AML) to M7 (acute megakaryoblastic leukemia) (Table 1). Nowadays, it is increasingly acknowledged that the underlying genetic aberrations predominantly characterize the AML, and therefore the World Health Organization (WHO) attempted to design a more clinically useful classification, which also incorporated recurrent genetic aberrations and specific clinical features.[10] In 2008, a revised WHO-classification of AML and related neoplasms was presented, which now also included the novel molecular subtypes of AML with mutated *NPM1* and AML with mutated *CEBPA* as provisional entities (Table 2).[11]

Treatment and outcome

The clinical outcome of children with AML has improved significantly over the past decades.[12] Nearly all children with AML died of the disease before the seventies, whereas nowadays most collaborative study group protocols reach long-term survival rates of 60-70%.[13-16] However, these results can only be achieved with very intensive chemotherapy treatment regimens, which result in a relatively high frequency (5-10%) of treatment-related deaths as well as long-term side effects. Moreover, this intensive therapy is only possible when sus-

TABLE 2. The WHO classification of 2008 of AML and related neoplasms.

Acute myeloid leukemia with recurrent genetic abnormalities	AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1*
	AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
	APL with t(15;17)(q22;q12); *PML-RARA*
	AML with t(9;11)(p22;q23); *MLLT3-MLL*
	AML with t(6;9)(p23;q34); *DEK-NUP214*
	AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1*
	AML (megakaryoblastic) with t(1;22)(p13;q13); *RBM15-MKL1*
	Provisional entity: AML with mutated *NPM1*
	Provisional entity: AML with mutated *CEBPA*
Acute myeloid leukemia with myelodysplasia-related changes	
Therapy-related myeloid neoplasms	
Acute myeloid leukemia, not otherwise specified	AML with minimal differentiation
	AML without maturation
	AML with maturation
	Acute myelomonocytic leukemia
	Acute monoblastic/monocytic leukemia
	Acute erythroid leukemia Pure erythroid leukemia Erythroleukemia, erythroid/myeloid
	Acute megakaryoblastic leukemia
	Acute basophilic leukemia
	Acute panmyelosis with myelofibrosis
Myeloid sarcoma	
Myeloid proliferations related to Down syndrome	Transient abnormal myelopoiesis Myeloid leukemia associated with Down syndrome
Blastic plasmacytoid dendritic cell neoplasm	

tained by the substantially improved and rigorous supportive care. Chemotherapy regimens usually consist of a backbone of 4-5 blocks of cytarabine and an anthracycline. Allogeneic stem cell transplantation (allo-SCT) in first complete remission (CR) is not recommended anymore by most study groups, except for selected high-risk patients.[17] In most treatment protocols, patients are stratified into 2 or 3 risk-groups, which are predominantly based on cytogenetic aberrations present in the leukemic blasts at diagnosis and the early response on treatment, measured as blasts in the bone marrow at day 15 or the achievement of CR after 1 course of treatment. In contrast to pediatric ALL, the value of minimal residual disease (MRD)-based stratification in AML is still under investigation as it is more difficult to

find a stable, common marker for the assessment of MRD.[15, 18] Only for the acute promyelocytic leukemia (APL) subtype, characterized by the *PML-RARA* fusion gene, MRD stratification is considered standard care.[19]

Despite the intensive treatment, still approximately 30-40% of pediatric AML patients experience a relapse. The outcome of relapsed AML is poor with approximately only one third of these patients surviving.[20] As further intensification of chemotherapy for pediatric AML is believed not to be feasible, improvement of survival and achievement of lesser toxicity of therapy is likely to come from the development of novel drugs that specifically target the leukemic cell.

Prognostic factors

The response on treatment and the clinical outcome of patients diagnosed with AML is highly dependent on the characteristics of the AML clone. Risk group classification is important as it may avoid overtreatment of patients with a good prognosis and undertreatment of patients with a high chance of relapse. In the past, it has been shown that AML blasts largely differ in their *in vitro* drug sensitivity.[21-22] However, this did not consistently correlate with treatment outcome. Furthermore, the expression of multidrug resistance genes such as MDR1 and BCRP has been related to a clinically resistant phenotype, although a clinical study aimed at MDR1-reversal was negative.[23-26] Nowadays, the cytogenetic aberrations present in the leukemic blasts at diagnosis are considered the main factor in predicting outcome and characterizing the prognostic relevant subtypes of AML. For instance, the chromosomal rearrangements t(8;21)(q22;q22), inv16(p13q22)/t(16;16)(p13q22) and t(15;17)(q22;q12) are strongly correlated with a favorable outcome.[27-28] Other important factors that are associated with prognosis are white blood cell count at diagnosis (WBC), secondary or therapy-related AML, and the early response on treatment.[12, 29] In recent years, mutations or altered expression of certain genes have emerged to correlate with clinical outcome of adult patients with AML, such as mutations in *FLT3*, *NPM1* and *CEBPA*, and the expression of *EVI1*, *BAALC*, *ERG* and *MN1*.[30-35]

GENETICS OF ACUTE MYELOID LEUKEMIA

Unraveling the diversity of genetic aberrations underlying AML is of utmost importance for a better understanding of the pathogenesis of the disease, which will possibly guide the development of novel more targeted therapies. Furthermore, it may improve current risk-group classification as outlined above. AML results from a multistep pathogenesis through the accumulation of different (epi-) genetic aberrations. In 2002, Kelly and Gilliland introduced the two-hit model in which they postulated that at least two cooperative types of events have to occur in a hematopoietic progenitor cell to result in acute leukemia.[36] Type-II aberrations primarily impair normal differentiation, and typically results from aberrations

of hematopoietic transcription factors such as the AML-characteristic fusion genes *PML/RARA*, *AML1/ETO* and *CBFβ/MYH11* that result from the above mentioned chromosomal rearrangements t(15;17), t(8;21) and inv(16)/t(16;16) respectively. The type-II aberrations usually define specific subtypes of AML based on morphology (Table 1) and gene-expression profiling. Type-I aberrations lead to uncontrolled proliferation and/or survival, and are often activating mutations in signal transduction molecules, e.g. *FLT3*/internal tandem duplications (*FLT3*/ITD), and mutations in *KIT* and *RAS*. Evidence for this type-I/-II model is supported by the fact that in AML usually one known type-I aberration and one known type-II aberration are present.[37] In addition, type-I and type-II aberrations are associated in a non-random fashion, suggesting cooperation between specific type-I and type-II aberrations.[37] Furthermore, AML-specific fusion genes can already be found in cord-blood samples, but may cause AML several years later.[38] Lastly, it has been shown in mice models that one aberration is not sufficient to induce leukemia, hence both types of aberrations are mandatory to develop an overt AML. For example, knock-in of *FLT3*/ITD leads to the development of a myeloproliferative disorder but lacks the maturation arrest typical for acute leukemia, whereas only together with co-expression of PML/RARA or CBFβ/MYH11 it resulted in a full-blown AML.[39-42]

Cytogenetics

Conventional karyotyping is the classical way of investigating chromosomal aberrations present in a leukemic cell. The technique is based on the staining of the chromosomes with a dye after arresting the cell during cell division (metaphase) by colchicine. The resulting karyotype, i.e. the number and appearance of the set of chromosomes in one cell, is depicted in a karyogram. At least 20 metaphases are required for a reliable result, although fewer metaphases are also accepted in case of abnormal clone or clones.[43] Karyotyping, which belongs to the field of cytogenetics, is performed in the standardized work-up of patients diagnosed with AML. The failure rate is usually less than 10%.[43] Different recurrent chromosomal (also called cytogenetic) aberrations can be found in AML (Table 3).[27-28, 44-45] These consist of numerical changes, such as trisomy 8 and monosomy 7, as well as structural aberrations, such as translocations, inversions, deletions and amplifications. As the resolution of conventional karyotyping is restricted to 5-10 mega base pairs (Mb) or less in case of a low quality of metaphases, additional analyses with molecular techniques such as fluorescence in situ hybridization (FISH) or reverse transcriptase-polymerase chain reaction (RT-PCR) is desirable to confirm or detect recurrent aberrant fusion genes in AML.[43]

Cytogenetic aberrations are currently the major prognostic factors in AML as outlined above. Presence of the chimeric fusion genes *AML1/ETO*, *CBFβ/MYH11* and *PML/RARA*, confers a favorable prognosis.[27-28] The prognostic relevance of rearrangements of the *MLL* gene located at 11q23 mainly depends on its partner gene.[46] The association of other recurrent cytogenetic aberrations with outcome is depicted in Table 2, but may vary between study groups as prognostic relevance also depends on the given treatment.

For most recurrent fusion genes in AML, there is experimental or theoretical evidence

TABLE 3. Recurrent cytogenetic aberrations in pediatric and adult AML.

Cytogenetic aberration	Involved gene(s) / fusion gene	% ped AML	% adult AML	Prognostic relevance
None	-	20-25	45	Intermediate
t(15;17)(q22;q21)	*PML-RARA*	6-10	8	Favorable
inv(16)(p13q22)/ t(16;16)(p13;q22)	*CBFβ-MYH11*	6-9	5	Favorable
t(8;21)(q22;q22)	*AML1-ETO*	12-15	6	Favorable
t/inv(11q23)	*MLL*-diverse partner genes	16-23	3	Dependent on partner gene
t(6;9)(p23;q34)	*DEK-NUP214*	1-2	1	Unfavorable
t(9;22)(q34;q11)	*BCR-ABL*	<1	1	Unfavorable
t(1;22)(p13;q13)	*RBM15-MKL1*	<1	0	Intermediate / Unfavorable
t(7;12)(q36;p13)	*HLXB9-ETV6*	1	0	Unfavorable
t(8;16)(p11;p13)/ inv(8)(p11q13)	*MOZ-CBP/-TIF2*	1	NA	Unknown
inv(3)(q21q26)/ t(3;3)(q21;q26)	*EVI1*	<1	1-2	Unfavorable
abn(12p)*	?	2-4	3	Unfavorable
abn (17p)*	?	2-3	2	Intermediate / Unfavorable
del(9q)*	?	3-5	2	Intermediate
+21*	?	5-6	2	Intermediate
+8*	?	9-13	9	Intermediate
-Y*	?	4-5	4	Intermediate
-7/7q-*	?	2-7	8	Unfavorable (in ped AML restricted to -7)[47]
-5/5q-*	?	1-2	7	Intermediate / Unfavorable
complex (≥3 abn)	?	8-15	11	Intermediate / Unfavorable

Based on references [27-28, 44-45, 48]; ped indicates pediatric. *percentage reflects the mentioned aberration as sole aberration or in combination with other cytogenetic aberrations.

that they impair differentiation, which classifies them as type-II aberrations. However, for the other cytogenetic aberrations the affected gene or oncogenic mechanism of the resulting aberration still need to be resolved. The frequency of several recurrent cytogenetic aberrations differs significantly between pediatric and adult AML (Table 2). Moreover, some aberrations are exclusively present in pediatric AML such as t(1;22)(p13;q13) in infants with megakaryoblastic (FAB-M7) AML and t(7;12)(q36;p13) in infant AML.[49-50]

Cytogenetically normal AML

A significant group of AML cases lacks aberrations using conventional karyotyping, and is also referred to as cytogenetically normal AML (CN-AML), which represents 20-25% of pe-

diatric AML cases, and approximately 45% of adult AML cases (Table 3).[27-28, 51] The discovery of the driving oncogenic events such as gene mutations or other aberrations in this subtype of AML is especially needed to find targets for the development of novel therapies and to determine prognostic factors for this group of patients. Despite having a normal karyotype, CN-AML is a very heterogeneous group of AMLs. This is reflected by the different prognostically relevant gene mutations that have been discovered in adult CN-AML recent years, as described in more detail below.[51]

Molecular genetics

When further zooming into the level of DNA, several pathogenic single gene mutations are known to occur in AML. These mainly comprise activating mutations in signal transduction molecules, representing the type-I aberrations, including mutations in the receptor tyrosine kinases *FLT3* (i.e. internal tandem duplications (ITD) and tyrosine kinase domain mutations (TKD)) and *KIT*, and mutations in *RAS*-signaling pathway components, i.e. *N-RAS, K-RAS* and *PTPN11*. All these mutations lead to constitutive activation resulting in uncontrolled proliferation of the leukemic cell. They can be identified by diverse molecular techniques, such as direct sequencing and fragment length analyses. At the time of start of the work described in this thesis a comprehensive study of pediatric AML showed that *FLT3*/ITD was present in 11% of cases, *N-* or *K-RAS* mutations in 18%, and *KIT* mutations in 11%.[30, 37] *KIT* mutations were predominantly present in core-binding factor (CBF)-AML [i.e. AML with t(8;21) or inv(16)/t(16;16)]. However, 60% of pediatric AML cases lacked one of the investigated type-I aberrations, and in the small number of CN-AML cases included in that series this percentage was 43%.[37] *FLT3*/ITD represents a well-known poor prognostic marker in adult as well as pediatric AML.[30] However, there is evidence that the poor prognostic impact might be restricted to cases with a high ratio of mutant/wild type ratio *FLT3*.[52] The influence of *KIT* mutations on the outcome of CBF-AML patients is still controversial.[53-54]

In adult AML, it was recently discovered that specific single gene mutations could act as type-II aberrations and herewith define novel distinct groups of AML with clear prognostic significance.[34] These mutations were indeed mutually exclusive with the other 'fusion gene' type-II aberrations, and interestingly, predominantly found in the CN-AML group. A great discovery in 2005 were mutations in *Nucleophosmin* or *NPM1*, which were found to comprise 25-35% of all adult AML cases and even 50-60% of adult CN-AML cases.[55] Four base pair insertions in exon 12 coding for the C-terminus disturb the nucleolar localization signal and creating a nuclear export signal that dislocates the NPM1 protein into the cytoplasm, also referred to as NPMc.[56] Mutations in the transcription factor C*CAAT/enhancer-binding protein alpha* (*CEBPA*) were identified in 2001, and impair the function of this important granulocytic differentiation factor.[57-58] They were present in 5-14% of adult AML cases, and the majority carried two different mutations, referred to as *CEBPA* double mutants.[59] Importantly, both *NPM1* mutations and *CEBPA* double mutations displayed distinct subtypes of adult AML,[60-61] and recognized adult AML patients with a favorable outcome. These findings

improved risk-group classification of adult CN-AML patients.[51, 62-63] Furthermore, partial tandem duplications of the *mixed-lineage leukemia* gene (*MLL*-PTD) are known to be present in 3-6% of adult AML cases, and associated with poor outcome.[64-66] The involvement of the *MLL* gene in leukemia is predominantly known from the recurrent *MLL* translocations present in approximately 20% of pediatric AML cases.[67] The frequency of *MLL*-PTD in pediatric AML was still controversial, as large differences in frequency were found, which might be based on technical differences between the studies.[68-70]

When we started the research presented in this thesis, the role of these type-II gene mutations and the landscape of primary genetic aberrations specifically in the pediatric CN-AML subtype were largely unknown.

Genome-wide approaches to further unravel AML

Several high-resolution genome-wide array-based techniques have become available in the last decade to further study AML. Small chromosomal copy number alterations such as amplifications and deletions below the detection limit of conventional karyotyping can now be made visible by high resolution array-based comparative genome hybridization (A-CGH) and single nucleotide polymorphism arrays (SNP-A) at a resolution up to 0.7 kilo base pairs (kb). Furthermore, the latter technique is also able to identify chromosomal regions with copy number neutral loss of heterozygosity (also referred to as (segmental) uniparental disomy (UPD)) as a result of mitotic recombination. This means that a chromosomal region was lost, but copied again from the remaining chromosome including potential pathogenic gene mutations. During the work described in this thesis it became clear that UPD occurs approximately in 10-20% of adult AML, and often involves mutated genes.[71-73] Furthermore, small copy number alterations appeared to be relatively infrequent in AML compared to other malignancies, including ALL, and herewith AML is considered as a relatively genomically stable disease.[74-75]

Microarray-based gene-expression profiling (GEP) is a technique to measure the expression levels of thousands of genes at once using a single micro-array chip. It proved to be a powerful tool for classification and prediction of tumor subtypes *(class prediction)*, identification of novel subtypes *(class discovery)*, and providing insight in the pathobiology of a disease and its subtypes *(class comparison)*.[76-77] In pediatric and adult AML gene expression profiles could predict the major type-II defined subtypes of AML such as t(8;21), inv(16), t(15;17) and *MLL*-rearrangements with a very high accuracy.[68, 78-81] Furthermore, novel subtypes of leukemia were determined based on similarities in gene expression profiles, such as the *BCR-ABL*-like group of patients that harbored a similar expression profile as the patients characterized by the *BCR-ABL* fusion gene in pediatric ALL.[82] Moreover, in adult AML patients a novel distinct subtype characterized by epigenetic *CEBPA* silencing was discovered using GEP.[83] Other GEP applications include for example the study for its ability to predict prognosis and the correlation with drug resistance or sensitivity.[79, 84-87]

The development of next-generation or high-throughput sequencing technologies in the

past years have paved the way to discover the complete genetic landscape of diseases. Targeted re-sequencing of candidate genes, such as all kinase-coding genes,[88] did not lead to the expected discovery of many novel recurrent mutations in AML. In contrast, the publication of the first two completely sequenced whole AML genomes in 2008 and 2009, which identified recurrent isocitrate dehydroxygenase 1 (*IDH1*) mutations in adult AML, is expected to be the start of the discovery of novel pathogenic mutations in AML.[89-90] As whole genome sequencing has become relatively affordable nowadays but still goes along with enormous datasets, restriction of sequencing to the complete coding region of the genome (whole exome sequencing) might be a valuable alternative for now.[91]

In this thesis we applied A-CGH, GEP and exome sequencing to further dissect the pediatric CN-AML subtype.

OUTLINE OF THIS THESIS

In pediatric AML there is a need for novel therapies as still 30-40% of patients experience a relapse. Intensification of the current treatment strategies is not feasible due to treatment-related mortality and morbidity, and to further improve survival rates other therapeutic options are needed. To direct the development of novel therapies specifically targeting the leukemic cells, molecular insight into the heterogeneity and pathobiology of AML is necessary. Therefore, the aim of this thesis was to unravel the molecular genetic aberrations underlying pediatric CN-AML.

The work described in this thesis is divided in two parts. In part one, we started to characterize pediatric CN-AML for the recently described type-II gene mutations in adult AML. As in 2005 it was discovered that mutations in the *NPM1* gene were present in more than half of adult CN-AML cases, we first started to investigate the frequency, characteristics and prognostic relevance of *NPM1* mutations in a large pediatric AML series **(chapter 2)**.Thereafter, we focused on the important myeloid transcription factor *CEBPA* in **chapter 3**, in which we not only screened for *CEBPA* gene mutations, but also investigated the frequency of *CEBPA* silencing by promoter hypermethylation. Furthermore, we used gene expression profiling of cases with different *CEBPA* aberrations to determine their differences and similarities at the transcriptome level. In **chapter 4** we applied the novel technique multiplex ligation-dependent probe amplification (MLPA) to determine the frequency of *MLL*-PTD in pediatric AML, which was subject to debate. In an integrative analysis described in **chapter 5,** we combined and analyzed the different established type-I and type-II aberrations together in one large pediatric series to have the power to investigate the associations between the different aberrations, their relation with clinical characteristics and their prognostic value.

In the second part of this thesis, we and others applied high-resolution genome-wide techniques to discover novel aberrations underlying pediatric CN-AML. Using A-CGH profiling we identified via the detection of one case with a *Wilms tumor 1 (WT1)* gene deletion, the

frequent involvement of *WT1* gene mutations, and their poor prognostic impact in pediatric AML **(chapter 6)**. In **chapter 7** other aberrations of the *WT1* gene, i.e. a single nucleotide polymorphism (SNP) in the hotspot mutated region and overexpression of the gene were assessed for their prognostic relevance. A-CGH profiling also led us to the discovery of the frequent involvement of the cryptic *NUP98/NSD1* translocation in pediatric CN-AML, which is described in **chapter 8**. Furthermore in this chapter, we analyzed gene-expression profiles of the cases of this novel identified subtype to get more insight into their pathobiology. Complete sequencing of the first two whole AML genomes in the world unraveled the presence of mutations in the metabolites isocitrate dehydroxygenase (IDH) 1 and 2 in adult AML. We determined the frequency of these mutations in pediatric AML, and investigated their influence on gene expression signatures in **chapter 9**. In **chapter 10** we performed a pilot study with a novel next-generation sequencing approach, that targets the whole coding region of the genome (i.e. whole exome sequencing), in a paired germline –tumor sample of one pediatric CN-AML patient to uncover novel mutations and to determine the genetic landscape of mutations in a pediatric AML exome. **Chapter 11** comprises the general discussion of this thesis and provides future perspectives.

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PART ONE

CHAPTER 2

Favorable prognostic impact of *NPM1* gene mutations in childhood acute myeloid leukemia, with emphasis on cytogenetically normal AML

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ABSTRACT

Nucleophosmin (*NPM1*) mutations occur frequently in adult cytogenetically normal AML (CN-AML) and confer favorable outcome. We investigated the frequency and prognostic significance of *NPM1* mutations in childhood AML (n=298), specifically focusing on the CN-AML subgroup (n=100). Mutations were found in 8.4%, and clustered significantly in the CN-AML subgroup (22%). No mutations were found in patients below the age of 3 years; in CN-AML there was an increasing incidence above this age. In the overall group, *NPM1* mutations conferred an independent favorable prognostic impact on event-free survival (pEFS 66 vs. 39%; p=0.02), which did not translate into a significantly better overall survival (pOS 68 vs. 56%; p=0.30). However, when the favorable cytogenetic subgroups [inv(16) and t(8;21)] were excluded from the *NPM1* wild-type group the difference in pOS was borderline statistically significant (pOS 68 vs. 45%; p=0.07). In the CN-AML cohort, *NPM1* mutations were an independent prognostic factor on 5-year pEFS (80 vs. 39%; p=0.01), and pOS (85 vs. 60%; p=0.06), which was not influenced by *FLT3*/ITD. However, in *NPM1* wild-type CN-AML, *FLT3*/ITD-positive patients had a significantly worse outcome (pEFS 48 vs. 18%; p<0.001). We conclude that *NPM1* mutations confer a favorable prognosis in childhood AML and in CN-AML in particular.

INTRODUCTION

Nucleophosmin (NPM1), encoded by the *NPM1* gene localized at chromosome 5q35, is a multifunctional nucleo-cytoplasmic shuttling protein that is predominantly localized in the nucleoli. The protein functions in different cellular processes, such as ribosome biogenesis, centrosomal duplication, maintenance of genomic integrity and the ARF-p53 tumor-suppressor pathway.[1-4] *NPM1* mutations, which occur in exon 12, encoding for the C terminus of the NPM1 protein, have recently been described in adult acute myeloid leukemia (AML).[5] They consist of net insertions of four nucleotides, affecting at least one of the tryptophan residues at amino acid positions 288 or 290. Loss of these tryptophan residues, as well as the creation of an additional nuclear export signal motif, causes delocalization of the protein to the cytoplasm.[6-7] This aberrant cytoplasmic localization of the NPM1 protein can easily be detected by immunocytochemistry.[5]

NPM1 mutations are present in approximately 50-60% of adult cytogenetically normal (CN-AML) samples,[5,8-14] among several other newly identified molecular abnormalities.[15] They confer a favorable clinical outcome in this subgroup of patients.[8,12,14] However, this favorable prognosis is lost in patients whose AML cells harbor both a mutation in *NPM1* as well as an internal tandem duplication in the *FLT3* gene (*FLT3*/ITD).[8-9,12,14]

CN-AML is less frequent in children than in adults, and accounts for ± 20 to 25% of childhood AML cases.[16] This CN-AML subgroup lacks favorable prognostic cytogenetic aberrations, leading to stratification in the intermediate or high-risk arm of risk-adapted pediatric AML treatment protocols. Recent studies from the Childhood Oncology Group (COG), reporting results from the POG-9421 protocol, and from the AIEOP (Associazione Italliana di Ematologica e Oncologica Pediatrica) on their AML02 study, showed *NPM1* mutations in 6.5 to 8% of childhood AML cases.[17-18] However, in studies from the Japanese Childhood AML Cooperative Study Group and a Taiwanese study, lower frequencies of 0 to 2.1% were found.[10,19] *NPM1* mutations in children also cluster in the CN-AML subgroup, but the frequencies described range from 0 to 27%.[10,17-20] To date, only the COG analyzed the prognostic significance of *NPM1* mutations in childhood AML, showing favorable outcome for children with *NPM1*-mutated AML lacking a *FLT3* mutation.[18] However, this report does not focus on the CN-AML subgroup.

We therefore screened 298 childhood AML samples to determine the overall frequency of *NPM1* mutations. Besides a survival analysis in the total cohort of *de novo* childhood AML patients, we performed a subgroup analysis on CN-AML patients to analyze the prognostic significance of *NPM1* mutations in this subgroup specifically. *NPM1* mutations were found to confer an independent favorable event-free survival (pEFS), but not significantly better overall survival (pOS) in childhood AML overall. However, in childhood CN-AML, *NPM1* mutations were found to confer an independent favorable prognostic impact for both parameters.

MATERIAL AND METHODS

Patient samples

Viably frozen bone marrow or peripheral blood samples from children with AML were provided by the Dutch Childhood Oncology Group (DCOG, The Hague, The Netherlands) and the AML-Berlin-Frankfurt-Münster Study Group (AML-BFM SG, Hannover, Germany). The institutional ethical review board approval for these studies had been obtained according to local law and regulations. Each study group performed a central review of the morphological and cytogenetic classification, as well as clinical follow-up of these patients. Leukemic samples were routinely investigated for cytogenetic abnormalities by standard chromosome-banding analysis, and screened for recurrent non-random genetic abnormalities characteristic for AML, including t(15;17), inv(16), t(8;21) and *MLL* gene rearrangements, using either reverse transcription-PCR and/or fluorescent *in situ* hybridization (FISH).

After thawing, contaminating non-leukemic cells were removed using immunomagnetic beads as described earlier.[21] Blast percentages were assessed morphologically on May-Grünwald-Giemsa-stained cytospin slides.

Mutational screening data from a part (n=75) of the patients have been reported earlier elsewhere.[20]

Treatment protocols

Patients were treated according to subsequent German and Dutch childhood AML treatment protocols between 1982 and 2005. The survival analysis was, however, restricted to the de novo AML patients treated according to the following consecutive protocols (AML-BFM/DCOG 87, AML-BFM/DCOG 93/92-94, DCOG 97, AML-BFM 98 and AML-BFM 04), to reduce variability in treatment regimens, and because this comprised the majority of patients (n=264, see results section). Details of these treatment protocols and overall outcome data have been published earlier, with the exception of study AML-BFM 04, which is ongoing.[22-24] 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.

Mutational analysis of *NPM1* and *FLT3*/ITD

Mutational analysis of *NPM1* and *FLT3*/ITD was performed both in Rotterdam and in Dresden. As a quality control, 20 samples, enriched for CN-AML samples, were assessed blinded in both laboratories. No discrepancies in results were found.

Genomic DNA was extracted from the leukemic cells using TRIzol reagent (Invitrogen Life Technologies, Breda, the Netherlands) or Qiagen Blood DNA kits (Qiagen, Hilden, Germany), according to the manufacturer's instructions.[25] In Rotterdam, exon 12 of the *NPM1* gene was PCR amplified using the following primers NPM1-F: 5'-CTGGTGGTAGAATGAAAAATAGAT-3' and NPM1-R: 5'-GGCAGGGACATTCTCATAG-3', situated respectively in intron 11 and 3'-UTR.

Purified PCR products were directly sequenced using the described reverse primer. *NPM1* mutants without an unambiguously identified sequence were confirmed by cloning using the TOPO-TA cloning kit (Invitrogen Life Technologies), followed by direct sequencing. In Dresden, samples were analyzed with PCR amplification followed by GeneScan analysis, as reported earlier.[8] Sequencing was performed for confirmation when a mutation was identified. Detection of *FLT3*/ITD was performed on genomic DNA by PCR amplification followed by GeneScan analysis or by gel electrophoresis and additional GeneScan analysis in case of a positive *FLT3*/ITD.[25-27]

Statistical analysis

To compare categorical variables we used χ^2 analysis, and Fisher's exact test in case of small numbers. The nonparametric Mann-Whitney *U*-test was applied for continuous variables.

To assess outcome, the following parameters were used: CR rate, pEFS (defined as time between diagnosis and first event, including relapse, death of any cause, failure to achieve remission or second malignancy), pOS (defined as time between diagnosis and death from any cause), and the cumulative incidence of relapses (CIR) (defined as time between diagnosis and relapse). 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's test. Prognostic factors were examined by multivariate Cox regression analysis.

P-values of ≤ 0.05 were considered statistically significant (two-tailed testing).

RESULTS

Study population

Of the 298 newly diagnosed AML cases whose cells were available to us, we successfully screened 297 samples for *NPM1* mutations. One sample failed because of unsuccessful harvesting of DNA. The successfully screened cohort included 286 patients with *de novo*, 10 with secondary and 1 with Down's syndrome AML. Patient characteristics are shown in Table 1. Survival analysis was restricted to patients with *de novo* AML, and in addition the following patients were excluded: patients treated according to other protocols (n=7), patients lost-to-follow-up (n=3), treatment without curative intent (n=1), isolated myelosarcoma (n=3) and PML-RARα (n=8). We compared the characteristics of this cohort (n=264) with the other not-investigated patients who were treated according to the same study protocols and after excluding the same subgroups (n=871). There were no significant differences in French-American-British (FAB) subtypes or karyotype distribution between these two groups. However, in our study cohort, there were significantly less females (40 vs. 50%; p=0.007), and less children below 2 years of age (19 vs. 37%; p<0.001), but they had higher white blood cell counts at diagnosis (WBC) (WBC <20x10^9/l: 34 vs. 61%; p<0.001).

TABLE 1. Clinical characteristics of the 297 successfully screened pediatric AML patients, divided by *NPM1* mutational status.

	All patients	*NPM1*-mutated	*NPM1* wild-type	*P* value
Number (%)	297	25 (8.4%)	272 (91.6%)	
Age, median (year)	9.6	11.0	9.5	0.06
<3 yr, n(%)	55	0 (0%)	55 (100%)	
3-10 yr, n(%)	103	11 (11%)	82 (89%)	0.04
≥10 yr, n(%)	138	14 (10%)	124 (90%)	
Sex (% female)	43%	64%	41%	0.03
WBC, median x10^9/l	38.0	39.3	37.7	0.89
(range)	(0.0-585.0)	(5.2-230.0)	(0.0-585.0)	
FAB classification: N(%)				0.02
M0	14 (5%)	0 (0%)	14 (5%)	
M1	36 (12%)	7 (28%)	29 (11%)	
M2	74 (25%)	10 (40%)	64 (24%)	
M3	12 (4%)	0 (0%)	12 (4%)	
M4	70 (24%)	7 (28%)	63 (23%)	
M5	69 (23%)	0 (0%)	69 (25%)	0.004[1]
M6	2 (1%)	0 (0%)	2 (1%)	
M7	7 (2%)	0 (0%)	7 (3%)	
Other	7 (2%)	0 (0%)	7 (3%)	
Unknown	6 (2%)	1 (4%)	5 (2%)	
FLT3/ITD (n=276), N(%)	53 (19%)	10 (40%)	43 (17%)	0.006
Median allelic ratio,	0.68	0.86	0.66	0.10
(range)	(0.17-2.48)	(0.50-2.33)	(0.17-2.48)	
Median ITD length,	54	45	55	0.51
(range)	(20-150)	(20-90)	(21-150)	

WBC indicates white blood cell count at diagnosis; FAB, French-American-British morphology classification; *FLT3*/ITD, internal tandem duplication of the *FLT3* gene; N and n indicates number of cases

[1] FAB M5 in *NPM1*-mutated vs. wild-type cases; p=0.004

The cohort used for survival analysis included 63 samples of patients with CN-AML. Subsequently, we identified another 37 *de novo* CN-AML samples from patients treated on the same therapy protocols from various cell banks. Patient characteristics of these 100 CN-AML samples are shown in Table 2. This cohort was used to further describe the prognostic impact of *NPM1* mutations in the subgroup of CN-AML.

We also successfully tested 46 paired diagnosis-relapse samples.

Type of NPM1 mutations

All detected *NPM1* mutations consisted of net insertions of four nucleotides, resulting in a shift of the reading frame with loss of at least one of the two tryptophans, and creation of a nuclear export signal (Table 3). The predominant type of mutation was a CATG insertion (type B, 40%), followed by a TCTG insertion (type A) in 20% of samples. Two novel mutations were identified, consisting of an insertion of 10 plus a deletion of 6 nucleotides, and an

TABLE 2. Clinical characteristics of the 100 pediatric cytogenetically normal AML patients (CN-AML) included in this study, divided by *NPM1* mutational status.

	All CN-AML patients	*NPM1*-mutated	*NPM1* wild-type	*P* value
Number (%)	100	20 (20%)	80 (80%)	
Age, median (year)	9.8	11.0	9.1	
<3 year	17	0 (0%)	17 (100%)	0.05
3-10 years	34	7 (21%)	27 (79%)	
≥10 years	49	13 (27%)	36 (73%)	0.06
Sex (% female)	56%	60%	53%	0.16
WBC, median x10^9/l	36.0	32.3	38.5	
(range)	(0.7-534.6)	(2.3-230.0)	(0.7-534.6)	0.81
FAB classification: N(%)				
M0	4 (4%)	0 (0%)	4 (5%)	
M1	22 (22%)	7 (35%)	15 (19%)	
M2	30 (30%)	6 (30%)	24 (30%)	
M3	2 (2%)	0 (0%)	2 (3%)	0.52
M4	26 (26%)	6 (30%)	20 (25%)	
M5	10 (10%)	0 (0%)	10 (13%)	
M6	3 (3%)	1 (5%)	2 (3%)	
M7	1 (1%)	0 (0%)	1 (1%)	
Other	2 (2%)	0 (0%)	2 (3%)	
Unknown	0 (0%)	0 (0%)	0 (0%)	0.21[1]
FLT3/ITD, N(%)	35 (35%)	10 (50%)	25 (31%)	
Median allelic ratio,	0.63	0.73	0.52	0.12
(range)	(0.10-2.48)	(0.10-2.33)	(0.17-2.48)	0.09
Median ITD length,	60	61	60	
(range)	(20-150)	(20-102)	(21-150)	0.84

WBC indicates white blood cell count; FAB, French-American-British morphology classification; *FLT3*/ITD, internal tandem duplication of the *FLT3* gene; N and n indicates number of cases

[1] FAB M5 in *NPM1*-mutated vs. wild-type cases; p=0.21

insertion of 12 plus deletion of 8 nucleotides in one single patient each, both affecting only the tryptophan residue 290.

NPM1 mutations in the genetic subgroups

Overall, *NPM1* mutations were identified in 8.4% (25 of 297) of childhood AML samples. No mutations were detected in the patients with secondary or Down's syndrome AML.

NPM1 mutations were not randomly distributed over the different cytogenetic subgroups within AML (Figure 1), but showed a significant clustering in the CN-AML cases (15 of 67 (22.4%)) compared with cases with cytogenetic aberrations (5 of 201 (2.5%); p<0.001). No mutations were found either in the favorable karyotype subgroups t(8;21), inv(16) or t(15;17), or in the *MLL*-rearranged AML cases. However, within the complex karyotype subgroup, 1 of 12 (8.3%) samples was mutated, as were 4 of 58 samples (6.9%) in the group with 'other cytogenetic abnormalities'. These 5 cases with *NPM1* mutations and cytogenetic

TABLE 3. Distribution of the types of *NPM1* gene mutations, the DNA-sequence and predicted protein, as identified in this study, categorized according to Falini et al.[28]

Type	Number (%)	DNA sequence *NPM1* gene - exon 12	Protein
WT	-	gatctctg ---- gcagt ---- ggaggaagtctctttaagaaaatag	286-DLWQWRKSL
A	6 (20%)	gatctctg **tctg** gcagt ---- ggaggaagtctctttaagaaaatag	286-D**L**CLA**V**EE**V**S**L**RK
B	12 (40%)	gatctctg **catg** gcagt ---- ggaggaagtctctttaagaaaatag	286-D**L**CMA**V**EE**V**S**L**RK
D	3 (10%)	gatctctg **cctg** gcagt ---- ggaggaagtctctttaagaaaatag	286-D**L**CLA**V**EE**V**S**L**RK
Gm	1 (3%)	gatctctg **cagg** gcagt ---- ggaggaagtctctttaagaaaatag	286-D**L**CRA**V**EE**V**S**L**RK
Km	3 (10%)	gatctctg **ccgg** gcagt ---- ggaggaagtctctttaagaaaatag	286-D**L**CRA**V**EE**V**S**L**RK
4	1 (3%)	gatctctg **cttg** gcagt ---- ggaggaagtctctttaagaaaatag	286-D**L**CLA**V**EE**V**S**L**RK
14	1 (3%)	gatctctg ---- gcagt **tatt ttccc**aagtctctttaagaaaatag	286-D**L**WQC**C**SQ**V**S**L**RK
DD-5	1 (3%)	gatctctg **tcag** gcagt ---- ggaggaagtctctttaagaaaatag	286-D**L**CQA**V**EE**V**S**L**RK
Novel	1 (3%)	gatctctg ---- gc**tcc gatt tgc**ggaagtctctttaagaaaatag	286-D**L**WLR**F**AEVS**L**RK
Novel	1 (3%)	gatctctg ---- gcagt **atct gggggccc**tctctttaagaaaatag	286-D**L**WQY**L**GA**L**S**L**RK

WT indicates wild-type (NM_002520); tryptophan residues (W) indicated with an underscore; creation of leucine-rich NES motif indicated in bold

abnormalities carried the following non-recurrent aberrations; patient 1) a hyperdiploid karyotype reported as 60,XXX,+3,+5,+5,+8,+9,+9,+13,+13,+17,+18,+Mar1,+Mar2,+Mar3; patient 2) a del(Y)(q11); patient 3) an add(4)(p); patient 4) a del(9)(q) and patient 5) a t(7;12)(p12;p13). Of 29 samples, karyotypic information was lacking, and five of these samples (17.2%) appeared to be mutated. Of these 29 cases, we were able to screen 20 samples for nonrandom abnormalities such as core-binding factor abnormalities and *MLL* gene rearrangements using molecular methods such as FISH or PCR. These nonrandom abnormalities were not found in this subgroup.

There was an increased frequency of *FLT3*/ITD positivity in *NPM1*-mutated AML (40%) compared with *NPM1* wild-type AML (17%; p=0.006) (Table 1). However, no significant differences were seen between the two groups regarding the *FLT3*/ITD characteristics, such as the allelic ratio (AR) or the ITD length. There was no correlation between the type of *NPM1* mutation and *FLT3*/ITD positivity.

Paired diagnostic-relapse samples

The 46 tested diagnosis-relapse pairs included three cases with mutated *NPM1* and 43 cases with wild-type *NPM1*. The three *NPM1*-mutated cases carried the same mutation at relapse, and none of the 43 *NPM1* wild-type cases gained a mutation at relapse; so no clonal instability of *NPM1* mutations was observed in this set of patients.

Relationship between *NPM1* mutations and clinical characteristics

NPM1 mutations were not found below the age of 3 years, but showed a steady frequency

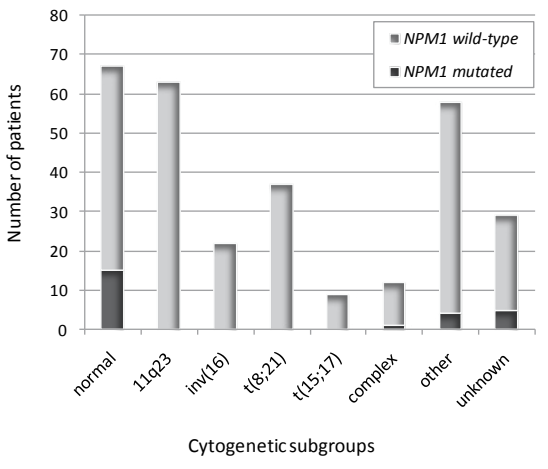

FIGURE 1. Differences in the frequency of *NPM1* mutations (n=25) in the various cytogenetic subgroups of childhood AML (n=297).
NPM1 mutations mainly clustered in the CN-AML subgroup, and only a few cases carried cytogenetic abnormalities. However, no mutations were found in the favorable risk cytogenetic subgroups nor in the *MLL*-rearranged childhood AML cases.

of 10-11% in children aged 3 years and above (Table 1). However, analysis restricted to the CN-AML subgroup showed a trend for an increasing frequency above the age of 3 years; 0% below the age of 3 years; 21% in the age group 3 years or older but below 10 years, and 27% in children aged 10 years or older (p=0.06) (Table 2). There was no clear correlation between the type of mutation and age.

There was no difference in WBC between *NPM1*-mutated (median $39.3x10^9$/l) and *NPM1* wild-type AML samples (median $37.7x10^9$/l; p=0.89). *NPM1*-mutated AML patients were more likely to be female (64%) compared with wild-type patients (41%; p=0.03). The FAB subtypes were not equally distributed between the two groups (p=0.02). This difference was mainly because of the absence of the FAB M5-subtype in the *NPM1*-mutated cases (0 of 25 vs. 69 of 272; p=0.004). This absence of the FAB M5-subtype in *NPM1*-mutated AML was also seen in the CN-AML subgroup.

Prognostic impact of *NPM1* mutations in childhood AML

The median follow-up time for survivors was 36.5 months (38.7 months for *NPM1*-mutated and 36.2 months for wild-type patients; p= 0.76). There were no significant differences in the pEFS between the various protocols (p=0.58). The frequency of SCT in CR1 was 15.2%, and it did not differ significantly either between *NPM1*-mutated (8.0%) and wild-type patients (15.9%; p=0.39), or between *FLT3*/ITD-positive (21.7%) and -negative patients (13.5%; p=0.16).

NPM1-mutated AML patients had an excellent CR rate (92.0%). None of them was refractory to induction therapy, but two patients were lost because of an early death. The CR rate did not differ significantly from the wild-type cases (84.5%; p=0.31). *NPM1*-mutated AML patients had a significantly better 5-year pEFS (66±10%) compared with wild-type patients (39±4%; p=0.02) (Figure 2A). However, this did not translate in a better 5-year pOS (68±10% vs. 56±4%, respectively; p=0.30) (Figure 2B). However, when excluding the favorable prog-

nostic subgroup (defined as AML with inv(16) or t(8;21)) from the *NPM1* wild-type group, *NPM1*-mutated AML patients had a better 5-year pOS (68±10% vs. 45±4%) that was borderline significant (p=0.07; or p=0.03 when the difference was tested as point estimate at 5 years).

FLT3/ITD was a significantly poor prognostic factor for pEFS as well as for pOS (*FLT3*/ITD-positive vs. *FLT3*/ITD-negative cases: 5-year pEFS 25±9% vs. 44±4%; p=0.05 and 5-year pOS 42±8% vs. 63±4%, respectively; p=0.04). When analyzing outcome according to the combined *NPM1* and *FLT3*/ITD status, we did not find a negative influence on outcome of *FLT3*/ITD in the *NPM1*-mutated patients (*FLT3*/ITD-positive vs. -negative cases, 5-year pEFS 79±13% vs. 58±13%; p=0.29, and 5-year pOS 74±16% vs. 64±13%, respectively; p=0.44) (Figures 2C and D). However, *FLT3*/ITD was a significantly poor prognostic factor for the *NPM1* wild-type AML patients (*FLT3*/ITD-positive vs. -negative cases, 5-year pEFS 11±9% vs. 43±4%; p<0.001, and 5-year pOS 33±9% vs. 63±4%, respectively; p=0.004)

No significant differences in effect on outcome were found between the different types of *NPM1* mutations (data not shown); however, the numbers were small.

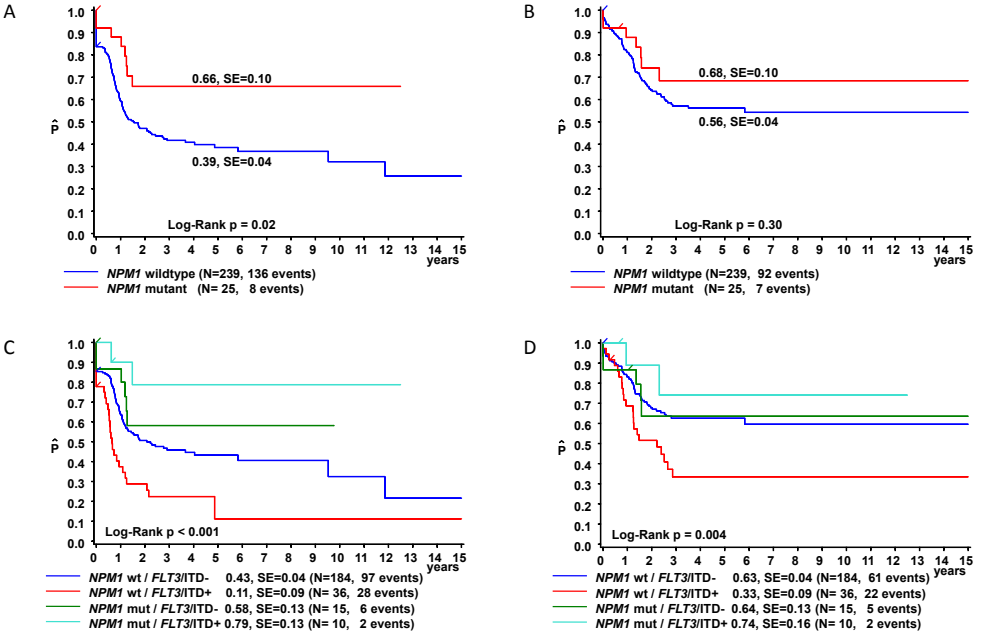

FIGURE 2. Survival curves of all childhood AML patients with and without *NPM1* mutations, and according to the combined *NPM1* and *FLT3*/ITD status.

(A)+(C) pEFS; (B)+(D) pOS. Patients are divided by their *NPM1* status (A+B), and show a significantly favorable outcome for *NPM1*-mutated AML patient for pEFS, but not for pOS. When excluding the patients with favorable cytogenetics, this difference was borderline statistically significant (see text result section). When subdivided in 4 subgroups according to their combined NPM1 and *FLT3*/ITD status (C+D), no impact on outcome is seen of *FLT3*/ITD in *NPM1*-mutated subgroup. (A color version of this figure can be found in the appendices.)

Prognostic impact in the subgroup of CN-AML

The median follow-up time for survivors was 60.1 months. There were no significant differences in the pEFS between the various protocols (p=0.59). The frequency of SCT in CR1 in CN-AML was 22%, and did not differ significantly either between *NPM1*-mutated (25%) and wild-type patients (21%; p=0.77), or between *FLT3*/ITD-positive (26%) and -negative patients (20%; p=0.51).

The CR rate for the 20 *NPM1*-mutated AML patients was 95.0%; one patient was lost because of a cerebral bleeding. In the 80 wild-type patients, the CR rate was 82.5%; 4 patients died early because of complications, and 10 patients were refractory to induction therapy. However, this difference in CR rate did not reach statistical significance (p=0.29). *NPM1*-mutated AML patients had a significantly better 5-year pEFS (80±9%) than the wild-type patients (40±6%) (p=0.02; Figure 3A). The 5-year pOS for *NPM1*-mutated compared with wild-type patients was 85±8% vs. 59±6%, respectively, (Figure 3B), which was borderline statistically significant (p=0.06). When we tested the difference in survival as a point estimate at 5 years, the difference was statistically significant (p=0.01). The 5-year cumulative incidence of relapse (CIR) was 15±8% for the *NPM1*-mutated vs. 39±8% for the wild-type patients (p(Gray)=0.18). It is interesting to note that survival analysis was influenced by one late event in the *NPM1*-mutated group. This concerned a late relapse that occurred 8 years after initial diagnosis, without a change in FAB type, immunophenotype or karyotype between the diagnosis and relapse sample. The median time to relapse from CR1 was 14.2 months in the four relapsed *NPM1*-mutated cases.

FLT3/ITD-positive AML patients had a 5-year pEFS of 38±9% vs. 52±7% in patients without *FLT3*/ITD (p=0.10). The 5-year pOS was 54±9% vs. 70±6%, respectively (p=0.08). Despite the relatively small numbers, we next analyzed the effect that *FLT3*/ITD had on outcome within the *NPM1*-mutated and *NPM1* wild-type subgroup separately (Figures 3C and D). There were no survival differences between *NPM1*-mutated/*FLT3*/ITD-positive (n=10) versus *NPM1*-mutated/*FLT3*/ITD-negative patients (n=10), in terms of 5-year pEFS (90±9% vs. 70±14%; p=0.62) and pOS (90±9% vs. 80±13%; p=0.54). Within the *NPM1* wild-type subgroup, *FLT3*/ITD-positive (n=25) patients had a significantly worse outcome than *FLT3*/ITD-negative patients (n=55) in terms of 5-year pEFS (18±10% vs. 48±7%; p=0.006) and pOS (40±11% vs. 68±7%; p=0.009). As we did not identify a difference in outcome based on *FLT3*/ITD within the *NPM1*-mutated group, we also subdivided the patients into three rather than four subgroups (Figures 3E and F), that is *NPM1*-mutated AML, irrespective of *FLT3*/ITD status (5-year pEFS 80±9%); *NPM1* wild-type and *FLT3*/ITD-negative AML (5-year pEFS 48±7%) and *NPM1* wild-type AML harboring an *FLT3*/ITD as single abnormality (5-year pEFS 18±10%; p<0.001). The effect of *FLT3*/ITD did not change when analyses were performed with *FLT3*/ITD AR or ITD length instead of the presence of *FLT3*/ITD per se.

Multivariate analysis of prognostic factors

When we included *NPM1* mutations with other risk factors in the Cox model, including age

(utilizing 10 years of age as cutoff value), WBC (using 50x10^9/l as cutoff value), *FLT3*/ITD, and SCT as time-dependent co-variable, we identified *NPM1* mutations as an independent factor for pEFS (risk ratio (RR) 0.38; 95% confidence interval (CI) 0.18-0.80; p=0.01), but not for pOS (RR 0.66; 95% CI 0.30-1.47; p=0.31) (Table 4).

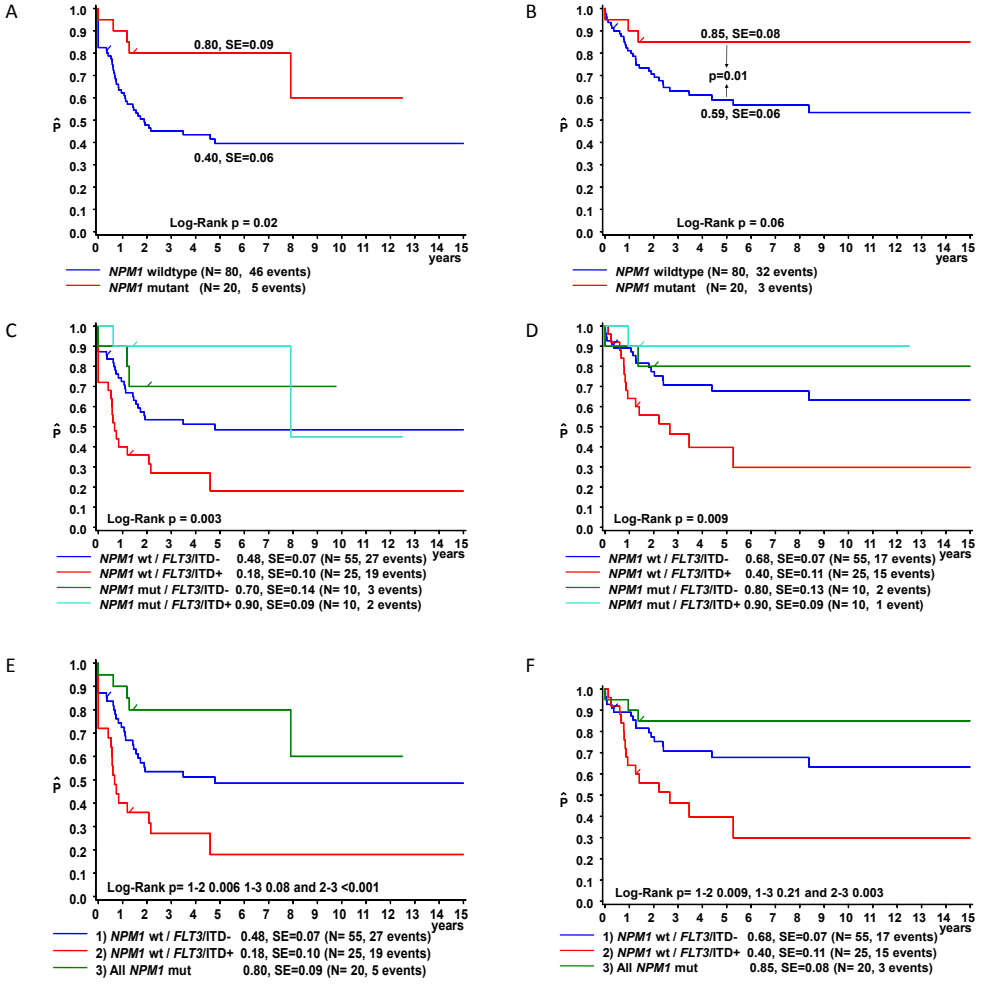

FIGURE 3. Survival curves of the subgroup of CN-AML patients with and without *NPM1* mutations, and according to the combined *NPM1* and *FLT3*/ITD status.

(A)+(C)+(E) pEFS, (B)+(D)+(F) pOS. (A) and (B) shows the curves of patients divided by their *NPM1* status, and *NPM1*-mutated AML patients show a favorable outcome in the CN-AML subgroup. (C) and (D) show the curves when patients are subdivided in 4 groups according to their *NPM1* and *FLT3*/ITD status, which show that *FLT3*/ITD does not abrogate the favorable outcome of the *NPM1*-mutated AML patients. (E) and (F): the patients are now subdivided in the 3 prognostic relevant subgroups according to *NPM1* and *FLT3*/ITD status, i.e. *NPM1*-mutated AML patients have an excellent outcome and *NPM1* wild-type patients can be subdivided based on their *FLT3*/ITD status. (A color version of this figure can be found in the appendices.)

Table 4. Results of multivariate analysis for pEFS and pOS in childhood AML and the subgroup of CN-AML.

(Sub)group	Outcome	Variable	Risk ratio (95% CI)	*P* value
All AML	pEFS	*NPM1*	0.38 (0.18-0.80)	0.01
		FLT3/ITD	1.79 (1.15-2.77)	0.01
		WBC>50	1.31 (0.92-1.87)	0.14
		SCT	0.85 (0.49-1.50)	0.58
		Age>10	0.93 (0.65-1.33)	0.70
	pOS	WBC>50	1.70 (1.10-2.62)	0.02
		FLT3/ITD	1.56 (0.94-2.59)	0.08
		NPM1	0.66 (0.30-1.47)	0.31
		SCT	1.33 (0.73-2.42)	0.36
		Age>10	0.96 (0.62-1.48)	0.85
CN-AML	pEFS	*NPM1*	0.29 (0.11-0.75)	0.01
		WBC>50	1.80 (0.95-3.40)	0.07
		FLT3/ITD	1.58 (0.80-3.11)	0.18
		SCT	0.62 (0.24-1.61)	0.33
		Age>10	0.96 (0.54-1.71)	0.90
	pOS	*NPM1*	0.30 (0.09-1.00)	0.05
		WBC>50	2.15 (0.99-4.66)	0.05
		SCT	0.34 (0.08-1.47)	0.15
		FLT3/ITD	1.56 (0.70-3.47)	0.28
		Age>10	0.98 (0.49-1.94)	0.94

Abbreviations: 95% CI indicates 95% confidence interval; WBC>50, white blood cell count with cutoff value $50x10^9$/l; age>10, age with cutoff value above 10 years old; SCT, stem cell transplantation.

In CN-AML, an *NPM1* mutation was the strongest independent predicting risk factor for pEFS (RR 0.29; 95% CI 0.11-0.75; p=0.01), as well as for pOS (RR 0.30; 95% CI 0.09-1.00; p=0.05) (Table 4). *FLT3*/ITD did not reach significance in the model either for pEFS or for pOS. When we entered *FLT3*/ITD with high ARs (≥0.4) in the model instead of *FLT3*/ITD *per se*, this did not change the results.

DISCUSSION

Our study confirms the observed lower frequency (7-9%) of *NPM1* mutations in childhood AML compared with adult AML (25-35%), as reported earlier by the COG (POG-9421 study) and the AEIOP-AML02 study.[17-18,28] In agreement with studies on adult AML, *NPM1* mutations were mainly observed in the CN-AML subgroup (22%). However this frequency is significantly lower than the observed frequency in adult CN-AML (50-60%). Very low frequencies of *NPM1* mutations (0.0-2.1%) were found in Asian children with CN-AML,[10,19] although

similar age cohorts were studied. This may be because of the ethnic differences in genetic subgroup distribution as has also been suggested for acute promyelocytic leukemia and *PTPN11* mutations.[29-30]

Interestingly, no mutations were found in children below the age of 3 years. Moreover, this frequency appeared to be age-related in the CN-AML subgroup, with an increasing frequency from the age of 3 years onwards. A similar tendency has been described for *FLT3*/ITD mutations.[31] These data suggest that there may be differences in leukemogenesis between CN-AML in very young (<3 years of age) and older pediatric AML patients. As FAB M5 is the predominant FAB subtype in very young children with AML,[32] which are usually characterized by *MLL* rearrangements, this also explains in part our finding of absence of *NPM1* mutations in AML FAB M5. In another pediatric study also, none out of the 18 tested FAB M5 cases harbored an *NPM1* mutation.[17] This is in contrast with adult studies where *NPM1* mutations are predominantly found in myelomonoblastic leukemia's (FAB M4 and M5).

In adult AML, several studies have shown the favorable impact of *NPM1* mutations on prognosis, but there is a paucity of published data on childhood AML, in particular on CN-AML cases. Only the COG reported a favorable outcome for children with *NPM1*-mutated AML lacking *FLT3/*ITD,[18] and they presented a trend toward improved survival for children with *NPM1*-mutated CN-AML (pOS 56 vs. 39%, p=0.15).[33]

In our study on *de novo* childhood AML patients we showed an independent favorable outcome for children with *NPM1*-mutated AML in terms of pEFS; however, this did not translate into a significantly better pOS. This was mainly because of the high salvage rate of patients without *NPM1* mutations after relapse.[34] When analyzing this further it appeared that the high salvage rate was mainly because of patients with favorable-risk AML carrying either a t(8;21) or inv(16) (acute promyelocytic leukemia cases were already excluded from the analysis), but without *NPM1* mutations. When they were excluded from the comparison, the difference in pOS between *NPM1*-mutated and *NPM1* wild-type cases was borderline statistically significant. We therefore feel it is justified to conclude that *NPM1* mutations do confer a favorable outcome in pediatric AML, as also shown by the pediatric study of Brown et al.[18] Our data also suggest that in future prospective studies analysis of *NPM1* mutations should not be restricted to the CN-AML subgroup only, but should be performed in all patients.

When focusing on the CN-AML subgroup, which is the largest pediatric CN-AML cohort studied to date, we detected *NPM1* mutations in ~20% of the cases. Patients with *NPM1*-mutated CN-AML experienced an excellent outcome with 85% survival, and the presence of an *NPM1* mutation was found to be an independent predictor of prognosis in multivariate analysis. The outcome data presented here are significantly better than those reported in other series of *NPM1*-mutated CN-AML, as outcome is usually in the 30-50% range in adults, and survival reached 56% in the pediatric POG-9421 study, which suggests that the clinical outcome of *NPM1*-mutated patients may be treatment-dependent.[8-9,12-14,33]

FLT3/ITD had no significant impact on outcome in the *NPM1*-mutated group in our study.

This is different from the findings in most adult studies on CN-AML, as well as the results from the pediatric POG-9421 study, which reported that *FLT3*/ITD overrules the favorable effect of *NPM1* mutations.[8,9,12,14,18] However, it needs to be kept in mind that our data were based on 20 CN-AML patients with *NPM1* mutations only, and hence confirmation in a larger series is needed. It is interesting to note that a recent large study of the Medical Research Council (MRC) in adult CN-AML, also reported a positive impact of *NPM1* mutations in the *FLT3*/ITD-positive group, irrespective of the *FLT3*/ITD AR.[35] We did not identify differences in the characteristics of *FLT3*/ITD, that is, AR or ITD length, that could explain why *FLT3*/ITD did not have prognostic influence in our *NPM1*-mutated patients. In addition, SCT could not explain the difference in impact; only a small number of our patients were transplanted, and there were no significant differences in the number of patients transplanted in the various subgroups. Moreover, SCT did not have independent prognostic significance in the Cox-model.

No significant differences in outcome were detected between the different types of *NPM1* mutations, although numbers were small. There are currently no data available from adult studies suggesting differences in outcome between type A (which is found in ~80% of adult AML compared with only 20% in our cohort) and the other mutations. Hence, it remains speculative whether the excellent outcome data in *NPM1*-mutated childhood (CN-)AML presented here may be because of differences in the underlying biology of the various *NPM1* mutations.

Our study also has important implications for pediatric (CN)-AML patients with wild-type *NPM1*, as the presence or absence of *FLT3*/ITD clearly distinguished between two subgroups: one characterized by *FLT3*/ITD and a poor prognosis, and one characterized by wild-type *FLT3* and an intermediate prognosis. In multivariate analysis restricted to the *NPM1* wild-type CN-AML patients *FLT3*/ITD was confirmed as the strongest predictor for pEFS (data not shown).

Recently, Mrozek et al.[15] suggested that molecular dissection of adult CN-AML may be relevant for treatment stratification in clinical practice, and they proposed an algorithm related to the molecular abnormalities identified in this subgroup. In this algorithm, patients with *FLT3/*ITD have poor outcome, irrespective of *NPM1* status. Based on our data, the clinical consequences of *NPM1* mutations and *FLT3*/ITD may differ between children and adults, and therefore implementation of this algorithm for children may not be warranted until more pediatric data become available. The first step would be to prospectively validate our results in a larger uniformly treated patient group. This is needed to avoid the potential selection bias and heterogeneity of treatment, which are the obvious drawbacks of a retrospective study. Another potential limitation of our study was that patients were treated on different therapy protocols, although all protocols used the same backbone of cytarabine and anthracycline, and consisted of 4-5 blocks of intensive chemotherapy. Moreover, there was no significant influence on prognosis between the different protocols.

When comparing *NPM1* mutations in the initial diagnosis-relapse pairs, no discrepancies

were found. In particular, in the 43 samples with wild-type *NPM1* at initial diagnosis, no *NPM1* mutations were identified at relapse, suggesting that *NPM1* mutations may be important as early events in AML. We did not identify any *NPM1* mutation losses at relapse, different from what has been found in AML with *FLT3*/ITD.[26] However, only three *NPM1*-mutated diagnosis-relapse pairs could be investigated. This stability may be relevant when using *NPM1* mutations as a marker for MRD studies.

Taken together, our data suggest that *NPM1*-mutated AML, in particular within the subgroup of CN-AML, should be classified as a separate and distinct entity in pediatric AML. Moreover, in patients with *NPM1* wild-type (CN-)AML, *FLT3*/ITD distinguishes between patients with an intermediate and a poor clinical outcome. These data may have important consequences for stratification of AML patients, and particularly patients with CN-AML in future pediatric AML treatment protocols, when they can be prospectively validated.

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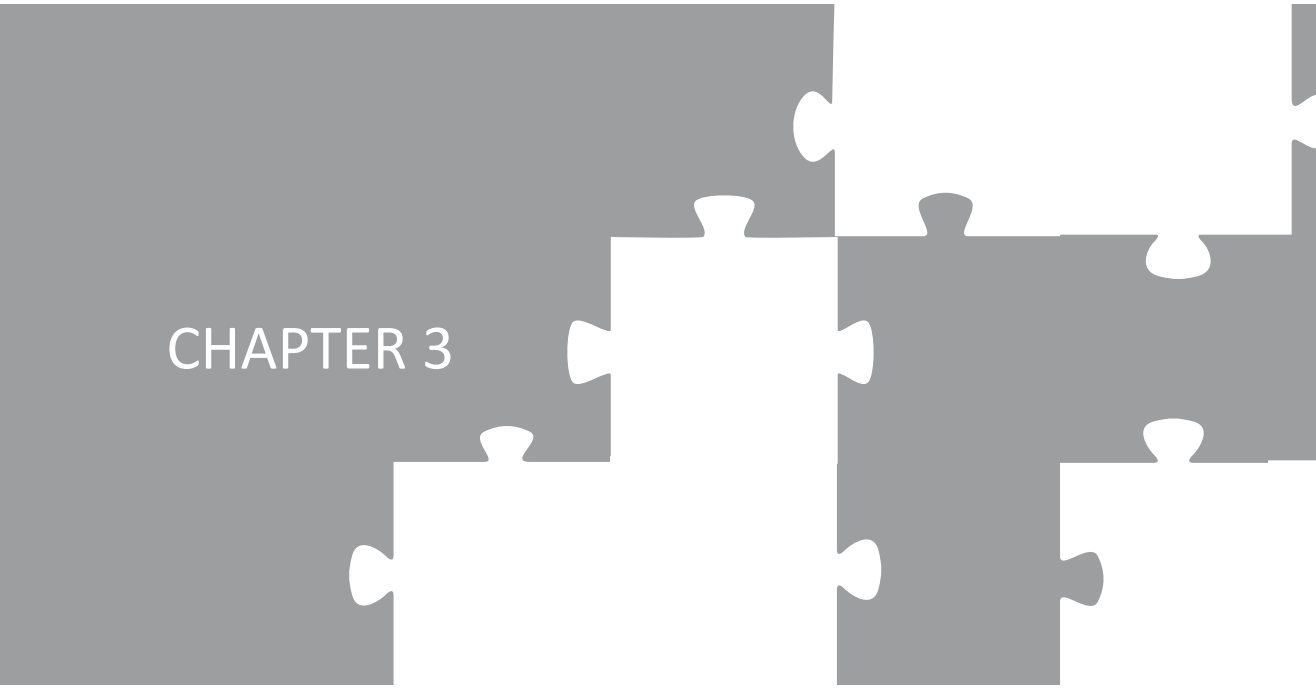
CHAPTER 3

Characterization of *CEBPA* mutations and promoter hypermethylation in pediatric acute myeloid leukemia

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ABSTRACT

Background

Dysfunctioning of CCAAT/enhancer binding protein alpha (C/EBPα) in acute myeloid leukemia (AML) can be caused, amongst others, by mutations in the encoding gene (*CEBPA*) and by promoter hypermethylation. *CEBPA*-mutated AML is associated with a favorable outcome, but this may be restricted to the cases with double mutations in *CEBPA* in adult AML. In pediatric AML, data on the impact of these mutations are limited to one series, and data on promoter hypermethylation are lacking. Our objective was to investigate the characteristics, gene expression profiles and prognostic impact of the different *CEBPA* aberrations in pediatric AML.

Design and Methods

We screened a large pediatric cohort (n=252) for *CEBPA* single and double mutations by direct sequencing, and for promoter hypermethylation by methylation-specific polymerase chain reaction. Furthermore, we determined the gene-expression profiles (Affymetrix HGU133 plus 2.0 arrays) of this cohort (n=237).

Results

Thirty-four mutations were identified in 20 out of the 252 cases (7.9%), including 14 double-mutant and 6 single-mutant cases. *CEBPA* double mutations conferred a significantly better 5-years overall survival compared with single-mutants (79% vs. 25%, respectively; p=0.04), and compared with *CEBPA* wild-type AML excluding core-binding factor cases (47%; p=0.07). Multivariate analysis confirmed that the double mutations were an independent favorable prognostic factor for survival (hazard ratio 0.23, p=0.04). The combination of screening for promoter hypermethylation and gene expression profiling identified five patients with silenced *CEBPA*, of whom four cases relapsed. All cases characteristically expressed T-lymphoid markers. Moreover, unsupervised clustering of gene expression profiles showed a clustering of *CEBPA* double-mutant and silenced cases, pointing towards a common hallmark of abrogated C/EBPα -functioning in these AMLs.

Conclusions

We showed the independent favorable outcome of patients with *CEBPA* double-mutant AML in a large pediatric series. This molecular marker may, therefore, improve risk-group stratification in pediatric AML. For the first time, *CEBPA*-silenced cases are suggested to confer a poor outcome in pediatric AML, indicating that further investigation of this aberration is needed. Furthermore, clustering of gene expression profiles provided insight into the biological similarities and diversities of the different aberrations in *CEBPA* in pediatric AML.

INTRODUCTION

Current risk-group classification in pediatric acute myeloid leukemia (AML) is determined by recurrent cytogenetic aberrations together with early treatment response.[1] However, the majority of patients are stratified in the intermediate risk group, including patients with cytogenetically normal AML (CN-AML), representing 20-25% of all children with AML. Molecular markers with prognostic implications have been identified in pediatric AML, such as internal tandem duplications of the *FTL3* gene (*FLT3*/ITD), and mutations in *NPM1* and the *Wilms tumor 1* (*WT1*) gene, which may further refine risk-group classification.[2-4]

Mutations in *CEBPA*, encoding the CCAAT/enhancer binding protein alpha (C/EBPα), have also been detected in AML.[5,6] C/EBPα is one of the crucial transcription factors for myeloid cell development. Targeted disruption of the *CEBPA* gene results in a selective early block of granulocyte differentiation.[7,8] C/EBPα function is frequently abrogated in AML by mutations, but also by (post-)transcriptional or post-translational inhibition due to dysregulation by oncogenes such as *AML1-ETO*, *CBF-MYH11* and *FLT3/ITD*.[9-13] More recently, epigenetic modification through hypermethylation of the *CEBPA* promoter, resulting in *CEBPA* silencing, has also been reported.[14,15]

Various mutations throughout *CEBPA* have been described, but two locations are most frequently affected.[16-18] N-terminal frame shift mutations are located between the major translational start site and a second ATG further downstream. They result in truncation of the full-length p42 isoform of C/EBPα, while preserving the shorter p30 isoform, which has been shown to inhibit the function of full-length p42.[9] C-terminal mutations are in-frame insertions or deletions located in the basic leucine zipper (bZIP) domain, and impair DNA binding and/or homo- and heterodimerization.[19] The majority of AML patients with *CEBPA* mutations harbor a mutation at both locations (*CEBPA* double mutants), and these are typically on different alleles, resulting in the lack of wild-type C/EBPα p42 expression in these cases.[20,21] However, single *CEBPA* mutations also occur, in which expression of the wild-type product is retained, albeit at lower levels.

CEBPA mutations are found in 5 to 14% of adult patients with AML, and are associated with a favorable outcome in these patients.[5,6,21-24] In contrast, *CEBPA* promoter hypermethylation has been suggested to confer a poor outcome.[15] Pediatric data are available from two studies, showing *CEBPA* mutations in 4.5% and 6% of cases, and only the Children's Oncology Group (COG) reported outcome data according to *CEBPA* status, which confirmed association with a favorable outcome.[25,26] Recently, two adult studies showed that the favorable prognosis was associated uniquely with *CEBPA* double-mutant AML, but not with the presence of a *CEBPA* single mutation.[27,28] In the COG study however, pediatric patients with *CEBPA* single-mutant AML showed a favorable outcome comparable to that of children with double-mutant AML. Pediatric data on *CEBPA* promoter hypermethylation are lacking to date.

Interestingly, adult studies showed a highly characteristic gene expression signature for

CEBPA double-mutant AML, in contrast to that for single-mutant AML.[27] It is also interesting that *CEBPA* promoter hypermethylated cases showed a similar signature to that for the *CEBPA* double-mutants, which is apparently characterized by the lack of C/EBPα functioning.[15]

In this study we investigated the characteristics, expression profiles and impact of *CEBPA* mutations and promoter hypermethylation in a large series of pediatric AML.

DESIGN AND METHODS

Study cohort

Viably frozen bone marrow (BM) or peripheral blood (PB) samples taken at initial diagnosis from 252 children with AML were provided based on availability 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 Hôpital Saint-Louis (Paris, France). In addition, 33 paired initial diagnosis-relapse BM or PB samples, and seven paired initial diagnosis-remission BM samples were provided by the DCOG and AML-BFM-SG. Institutional review board approval for these studies was obtained according to local laws and regulations. Each study group performed a central review of the morphological, immunophenotypic and cytogenetic classifications, and provided data on the clinical follow-up.

After thawing, leukemic cells were isolated from these samples as previously described.[29] Blast percentages were greater than 80% as assessed morphologically on May-Grünwald-Giemsa-stained cytospin slides. Genomic DNA and total cellular RNA were extracted using TRIzol reagent (Invitrogen Life Technologies, Breda, the Netherlands), as described before.[30]

Survival analysis was restricted to patients with *de novo* AML who were treated according to DCOG and AML-BFM-SG studies (i.e. DCOG/AML-BFM 87, DCOG 92/94, DCOG 97, AML-BFM 98 and 04) to reduce treatment variability; these patients accounted for the majority of subjects in our study (n=185). Patients treated according to other protocols (n=43), and, in addition, patients with PML-RARα (n=15) or with secondary AML (n=8) were excluded. Details of the treatment protocols and overall outcome data have already been published, with the exception of the AML-BFM 04 study, which was closed recently. In these protocols, treatment consisted of four or five blocks of intensive chemotherapy, using a standard cytarabine and anthracycline backbone. Stem cell transplantation (SCT) in first complete remission (CR1) was used only in selected high-risk patients. There was no statistically significant difference between the treatment protocols for obtaining CR (p=0.65) or in event-free survival (p=0.41), but for overall survival there was a difference between the protocols (p=0.04). However, patients with *CEBPA* single-mutated AML, double-mutated AML and wild-type AML were equally distributed over the different treatment protocols (p=0.28).

Cytogenetic and molecular analysis

Samples were routinely screened for cytogenetic aberrations using standard chromosome-banding karyotyping, and further analyzed for recurrent non-random genetic aberrations characteristic for AML, including t(15;17), inv(16), t(8;21) and *MLL* gene rearrangements, using reverse transcriptase polymerase chain reaction (RT-PCR) and/or fluorescent *in situ* hybridization (FISH) by each study group. In case of lacking data, RT-PCR or FISH was performed at the laboratory of Pediatric Oncology of the Erasmus MC-Sophia Children's Hospital.

Hotspot regions for mutations of *KIT*, *FLT3, MLL*, *NPM1*, *PTPN11*, *N-RAS, K-RAS* and *WT1* were screened for, as previously described.[2] Regions of *NOTCH1* known to be mutated in T-ALL [heterodimerization domain (HD), exons 26 and 27; proline-glutamate-serine-threonine-rich domain (PEST), exon 34] were also analyzed for the presence of mutations with a (nested) PCR-based direct sequencing approach.[31]

Analysis of *CEBPA* mutations and promoter hypermethylation

Mutation analysis of *CEBPA* was performed as previously described[21], with minor modifications. Primer sequences and PCR conditions are described in Online Supplementary Table S1. Genomic DNA was PCR amplified using specific primers, i.e. primers 1 and 10, and 4 and 8, to cover the whole *CEBPA* gene. Purified PCR products were directly sequenced from both strands using the described primers on an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA). The sequence data were analyzed using CLC Workbench version 3.5.1 (CLC Bio, Aarhus, Denmark).

For methylation analysis of the promoter region of *CEBPA*, genomic DNA was treated with sodium bisulfite using the EZ DNA Methylation kit (Zymo Research, Orange, CA, USA) according to the manufacturer's protocol. The bisulfite-treated DNA was used as a template for methylation-specific PCR (MSP) and unmethylation-specific PCR (USP), which were performed as previously described.[14] MSP and USP products were subsequently separated by gel electrophoresis and visualized with ethidium bromide.

Gene expression profiling and analysis

The integrity of total RNA was checked using the Agilent 2100 Bio-analyzer (Agilent, Santa Clara, CA, USA). Biotinylated cRNA was synthesized, hybridized and processed on the Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's guidelines. Unsupervised clustering analysis was performed and visualized as previously described.[32] Briefly, probe set intensity values were normalized using MAS5.0 software and values less than 30 were set at 30. For each probe set the geometric mean of the intensity values of all samples was calculated. The level of expression of each probe set in every sample was then determined relative to this geometric mean and logarithmically transformed (on a base 2 scale). The transformed expression data were subsequently imported into OmniViz software (OmniViz v3.7, Tewksbury, MA, USA). Pairwise correlations between the gene expression profiles of the 237 samples was calculated with

Pearson's correlation on the basis of 1608 differently expressed probe sets representing the subset of probe sets with a 16-factor increase or decrease relative to the geometric mean.

To test the probe set prediction signatures previously described in adult AML for our pediatric *CEBPA*-silenced cases and *CEBPA* single- and double-mutant cases, normalized probe set intensities for the 237 cases were used in a linear prediction algorithm (linear discriminant analysis; equal prior probabilities, predicting four variables: Partek v6.09.1008, Missouri, USA), with both the described 21- and 9-probe sets.[15,27] Prediction results of samples were visualized using a principal component analysis scatterplot (Partek), and both cases and probe sets were hierarchically clustered using Euclidean distance (Genemaths XT, Applied Maths, Austin, TX USA).

Other statistical analyses

Statistical analyses were performed with SPSS 15.0 (SPSS Inc. Chicago, IL, USA). Variables were compared using the χ^2 or Fisher's exact test for categorical values, the Mann-Whitney *U*-test for continuous variables, and the Kruskal-Wallis test when more than two groups were compared.

To assess outcome, the following parameters were used: CR (defined as less than 5% blasts in the bone marrow, with regeneration of trilineage hematopoiesis plus absence of leukemic cells in the cerebrospinal fluid or elsewhere), probability of event-free survival (pEFS, defined as time between diagnosis and first event, including failure to achieve remission, relapse, death from any cause or second malignancy) and probability of overall survival (pOS, defined as time between diagnosis and death). pEFS and pOS were estimated by the Kaplan-Meier method, and compared using the log-rank test. The independency of prognostic factors was examined by multivariate Cox regression analysis. All tests were two-tailed and *P* values less than 0.05 were considered statistically significant.

RESULTS

Single and double *CEBPA* mutations in pediatric AML

We identified 34 *CEBPA* mutations in 20 out of 252 (7.9%) diagnostic samples from patients by sequencing the entire coding region (Figure 1A, Online Supplementary Table S2). Of these, 13 cases had the combination of an N-terminal frame shift mutation and an in-frame mutation in the bZIP region. One case combined an N-terminal frame shift mutation with a frame shift-causing insertion before the bZIP region. These 14 cases (70%) are henceforth referred to as *CEBPA* double mutants. The other six cases carried a single *CEBPA* mutation: four had an in-frame bZIP mutation and two had frame shift mutations in the TAD2 domain and before the bZIP domain, respectively. The latter two cases do not represent the classical N-terminal mutation, as the C/EBPα p30 isoform is also affected, but because of their functional consequence they were classified as mutations.

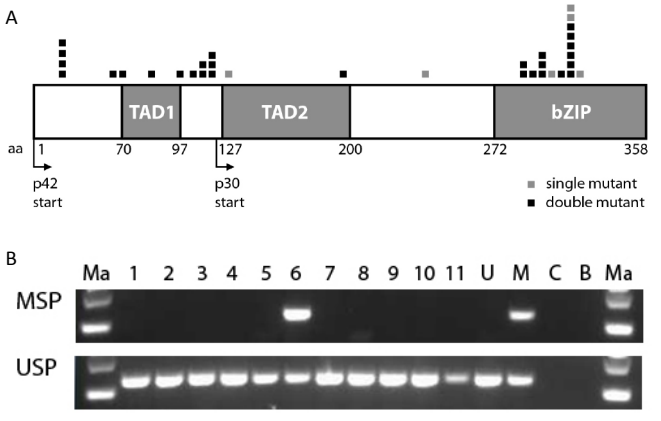

FIGURE 1. *CEBPA* mutations and promoter hypermethylation in pediatric AML cases.
(A) Schematic representation of the *CEBPA* gene and location of the identified mutations.
(B) Representative picture of methylation-specific (MSP) and unmethylation-specific PCR (USP) products of the *CEBPA* promoter separated by 2% agarose gel electrophoresis and visualized with ethidium bromide. Patient sample 6 shows a positive MSP product, indicating *CEBPA* promoter hypermethylation.
Ma: marker, numbers 1-11: patients' samples, U: unmethylated positive control, M: methylated positive control, C: control bisulfite-untreated DNA, B: blank distilled H2O

Cases with an in-frame insertion polymorphism in TAD2[20,33] (n=7), with variation(s) that did not lead to amino acid changes, or with a single amino acid change (situated between the TAD1 and -2 domains) of unknown significance (n=1) were considered as wild-type *CEBPA*.

Characteristics of *CEBPA* single- and double-mutant pediatric AML

The clinical and cell-biological characteristics of the study cohort are shown in Table 1. *CEBPA* double mutations were not present in patients below the age of 3 years. However, the median age of patients with *CEBPA* double-mutant AML (12.3 years) did not differ significantly from that of patients with *CEBPA* single-mutant AML (7.5 years) or with *CEBPA* wild-type AML (9.7 years) (p=0.26). *CEBPA* double mutations occurred exclusively in French-American-British (FAB) types M1 and M2, in contrast to single mutations, which were found in more diverse FAB types (p=0.04). No statistical significant differences were detected between the three subgroups regarding sex and white blood cell count at diagnosis (WBC).

Single and double *CEBPA* mutations did not occur in the favorable cytogenetic subgroups [inv(16), t(8;21) and t(15;17)], or in the *MLL*-rearranged subgroup. *CEBPA* double mutations occurred mainly in CN-AML (57%), but five cases (36%) also carried an additional cytogenetic aberration (Online Supplementary Table S2). In one case (7%) cytogenetic analysis failed, but RT-PCR and/or FISH excluded recurrent cytogenetic aberrations in this case. *CEBPA* single mutations were present in three cases with cytogenetic aberrations (50%), two cases (33%) with a normal karyotype and in one case (17%) cytogenetic analysis failed, but recurrent cytogenetic aberrations were excluded. Additional molecular aberrations were equally

TABLE 1. Characteristics of the 252 pediatric AML patients included in this study, divided by *CEBPA* mutational status.

	All	*CEBPA* single mutation	*CEBPA* double mutation	*CEBPA* wild-type	*P* value
Number	252	6	14	232	
Age, median (years)	9.7	7.5	12.3	9.7	0.26*
<3 yr, n (%)	49	1 (17%)	-	48 (21%)	
≥3 yr, n (%)	203	5 (83%)	14 (100%)	184 (79%)	0.16#
Sex (% female)	45.2%	66.7%	42.9%	44.8%	0.56#
WBC (x10^9/l), median (range)	42 (0-535)	20 (8-535)	60 (6-388)	41 (0-483)	0.69*
FAB, n(%)					0.001#
M0	12 (5%)	1 (17%)	-	11 (5%)	
M1	27 (11%)	2 (33%)	6 (43%)	19 (9%)	
M2	55 (23%)	1 (17%)	8 (57%)	46 (21%)	
M3	20 (8%)	-	-	20 (9%)	
M4	60 (25%)	2 (33%)	-	58 (26%)	
M5	57 (24%)	-	-	57 (26%)	
M6	3 (1%)	-	-	3 (1%)	
M7	8 (3%)	-	-	8 (4%)	
Other	1 (0%)	-	-	1 (0%)	
Unknown	9 (4%)	-	-	9 (4%)	
Karyotype, n(%)					0.049#
t(8;21)	27 (11%)	-	-	27 (12%)	
inv(16)	26 (10%)	-	-	26 (11%)	
t(15;17)	18 (7%)	-	-	18 (8%)	
11q23	49 (19%)	-	-	49 (21%)	
Normal	55 (22%)	2 (33%)	8 (57%)	45 (19%)	
Other	60 (24%)	3 (50%)	5 (36%)	49 (21%)	
Unknown	17 (7%)	1 (17%)	1 (7%)	15 (7%)	
FLT3/ITD, n(%) (n=252)	52 (21%)	1 (17%)	3 (21%)	48 (21%)	0.97#
N- or *K-RAS*, n(%) (n=251)	52 (21%)	1 (17%)	2 (14%)	49 (21%)	0.80#
KIT, n(%) (n=251)	17 (7%)	-	-	17 (7%)	0.45#
MLL-PTD, n(%) (n=244)	5 (2%)	-	-	5 (2%)	0.80#
NPM1, n(%) (n=247)	18 (7%)	-	-	18 (8%)	0.43#
WT1, n(%) (n=250)	27 (11%)	2(33%)	3 (21%)	22 (10%)	0.08#

*Kruskal-Wallis test; #Chi-square test;

frequent in the *CEBPA* single- and double-mutants, and consisted of *FLT3*/ITD, *RAS* and *WT1* mutations, as described in Table 1.

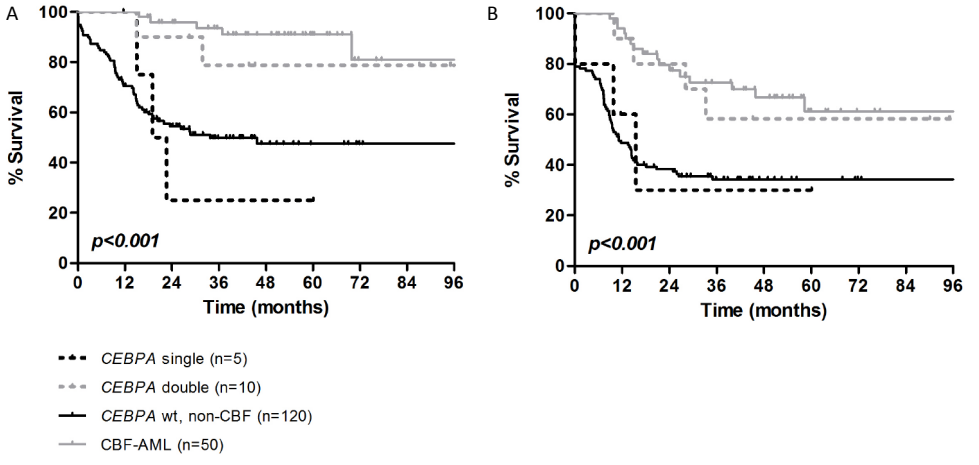

FIGURE 2. Kaplan-Meier survival curves of overall survival (pOS) and event-free survival (pEFS) according to *CEBPA* status in pediatric AML. (A) pOS and (B) pEFS of four subgroups of patients with pediatric AML, that is *CEBPA* single-mutant AML (*CEBPA* single), *CEBPA* double-mutant AML (*CEBPA* double), *CEBPA* wild-type non-core-binding factor (CBF-)AML (*CEBPA* wt, non-CBF) and CBF-AML.

Identification of one case with gain of a single *CEBPA* mutation at relapse

We screened 33 pairs of samples taken at initial diagnosis and relapse, including three *CEBPA*-mutant (2 single and 1 double) and 30 *CEBPA* wild-type cases at initial diagnosis. All three *CEBPA*-mutated cases carried the same mutations at relapse. Of the 30 *CEBPA* wild-type cases at diagnosis, one case (3%) gained an N-terminal frame shift mutation (c.226delG) in *CEBPA* at relapse, i.e. 10 months after diagnosis. The other (cyto)genetic aberrations in this patient (45,X,-X and a *WT1* mutation) were present at diagnosis and relapse.

Frequency of germ-line origin of *CEBPA* mutations

Of 7 patients with *CEBPA*-mutated AML (4 single and 3 double mutants), remission material (bone marrow mononuclear cells taken in full CR) was available. In one case (14%) with *CEBPA* double-mutant AML, the N-terminal frame shift mutation (c.69dupC) was detected in the germ-line material; the second *CEBPA* mutation in this patient, which was located in the bZIP region (c.937_939dupAAG), was somatically acquired in the leukemic cells. An *FLT3*/ITD was also somatically acquired. This patient was diagnosed with AML at the age of 6 years and died 14 months after diagnosis in CCR because of bleeding. It was not possible to test the *CEBPA* mutational status of the parent as they could not be reached. Interestingly, in unsupervised cluster analysis based on gene expression data (Figure 3), this case (#4746) clustered together with the other *CEBPA* double-mutant cases, indicating that, based on gene expression profiles, the leukemia of this patient was comparable with the 'sporadic' *CEBPA* double-mutant AMLs.

TABLE 2. Results of multivariate analysis for overall survival (OS) and event-free survival (EFS).

Outcome	Variable	Hazard ratio (HR)	95% confidence interval	*P* value
OS	Favorable karyotype	0.11	0.04-0.30	<0.001
	CEBPA double mutation	0.23	0.06-0.96	0.04
	NPM1 mutation	0.43	0.17-1.09	0.08
	WBC >50x10E9/l	1.23	0.76-2.10	0.36
	FLT3/ITD	1.28	0.70-2.34	0.42
	Age >10 years	1.07	0.64-1.79	0.79
EFS	Favorable karyotype	0.27	0-15-0.47	<0.001
	NPM1 mutation	0.29	0.13-0.69	0.005
	CEBPA double mutation	0.32	0.12-0.89	0.03
	FLT3/ITD	1.34	0.81-2.23	0.25
	Age >10 years	1.18	0.78-1.78	0.46
	WBC >50x10^9/l	1.03	0.68-1.54	0.90

Prognostic impact of *CEBPA* single and double mutations in pediatric AML

Survival analysis was restricted to 185 patients with *de novo* AML, including five with *CEBPA* single mutations and ten with *CEBPA* double mutations (Online Supplementary Tables S2 and S3). The median follow-up time of the survivors was 4.4 years. All ten patients with *CEBPA* double mutations reached CR (100%), while CR was achieved in four out of the five (80%) patients with *CEBPA* single mutations, as one patient had resistant disease. The CR rate for *CEBPA* wild-type patients (n=170) was 84%.

Patients with *CEBPA* double-mutant AML had a significant better 5-year overall survival compared with those with a *CEBPA* single mutation (5-year pOS 79±13% vs. 25±22%; p=0.04), although the 5-year event-free survival was not significantly different (5-year pEFS 58±16% vs. 30±24%; p=0.16) (Figure 2). In fact, the outcome of patients with *CEBPA* double mutations was comparable to that of patients in the favorable-risk group with core-binding factor (CBF-)AML [inv(16) or t(8;21)], who had a 5-year pOS of 91±4%; p=0.51 and pEFS of 61±9%; p=0.74. Furthermore, patients with *CEBPA* double mutations showed a clear trend to a more favorable outcome than patients with wild-type *CEBPA* after excluding the CBF-AML cases (5-year pOS 47±5%; p=0.07 and pEFS 33±4%; p=0.06, respectively). The impact of additional molecular or cytogenetic aberrations (e.g. *FLT3*/ITD) on the *CEBPA* single- and double-mutated group could not be investigated due to small numbers.

Multivariate analysis, including age, WBC, favorable cytogenetics, *NPM1* mutations and *FLT3*/ITD, showed that the presence of a *CEBPA* double mutation was an independent favorable prognostic factor for pOS (HR 0.23; p=0.04) as well as pEFS (HR 0.32; p=0.03) (Table 2). *CEBPA* single mutations were not included in the multivariate analysis as a factor because of the small number of cases.

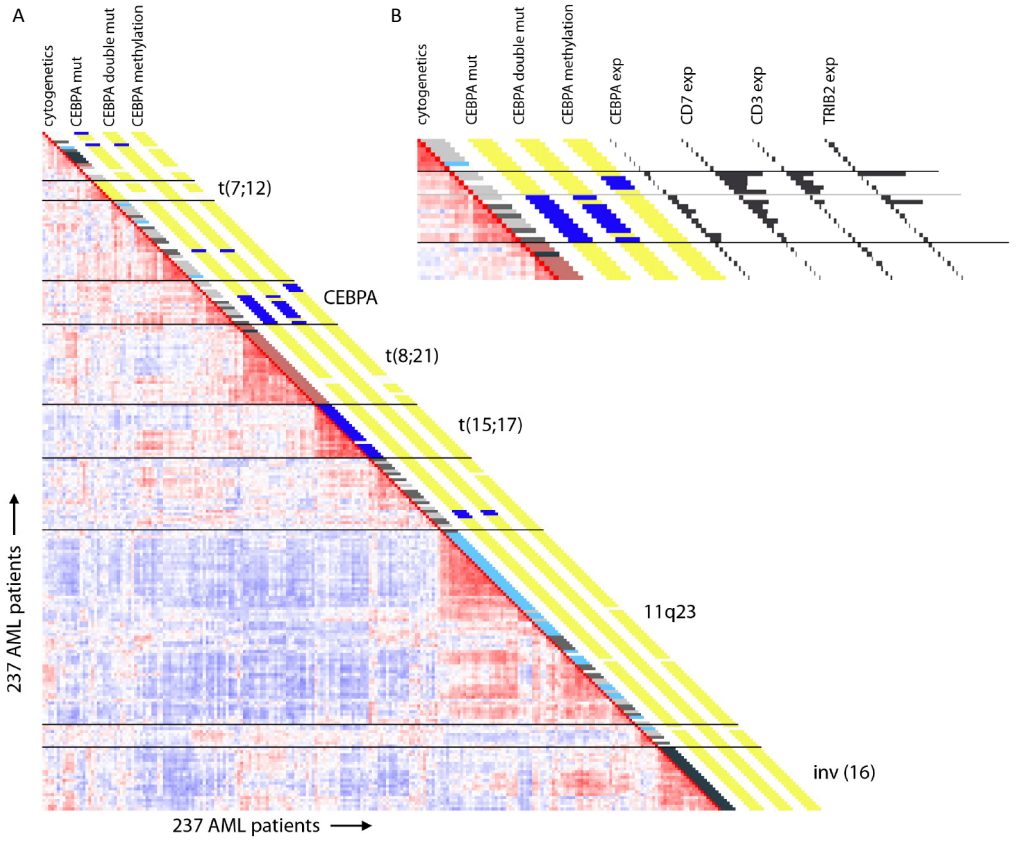

FIGURE 3. Unsupervised clustering of gene expression data revealed clustering of cases with aberrant *CEBPA* predominantly in one cluster in pediatric *de novo* AML.

(A) Pair wise correlations between gene expression profiles of 237 *de novo* pediatric AML samples, calculated on the basis of 1608 probe sets (cutoff: 16-fold), are displayed in a correlation plot. Colors of boxes represent the Pearson's correlation coefficient with a color gradient ranging from deep blue for a negative correlation, to vivid red for a positive correlation. Distinct clusters of samples, which can be recognized by the red blocks showing high correlation along the diagonal, are observed. The first column to the right of the plot indicates the major cytogenetic subgroup the samples belong to [dark blue: inv(16), pale red: t(8;21), bright blue t(15;17), yellow: t(7;12), light blue: 11q23, dark grey: normal cytogenetics, light grey: other cytogenetic aberrations and white: failure]. Clustering of these cytogenetic subgroups is seen. The second, third and fourth column represent presence (blue) or absence (yellow) of a *CEBPA* mutation, *CEBPA* double mutation and *CEBPA* promoter methylation, respectively. The majority of *CEBPA* mutant cases aggregated together with all *CEBPA* hypermethylated cases in one main cluster.

(B) An enlarged view of the correlation plot focusing on the *CEBPA* main cluster is shown. The order of the patient samples in this cluster from top to bottom is as follows: #4728, #5033, #3496, #3451, #4736, #3439, #5041, #5061, #4746, #5063, #5047, #4396, #4445, #4747, #5013. Additionally, four histograms show the expression of *CEBPA* (204039_at), *CD7* (214551_s_at), *CD3D* (213539_at) and *TRIB2* (202478_at). Within the *CEBPA* main cluster, two sub-clusters based on expression of *CEBPA* can be identified and are separated by a grey line, i.e. *CEBPA* low or absent expression (n=5), including the three *CEBPA* promoter methylated cases, and *CEBPA* high expression with the *CEBPA* mutant cases (n=10). The five silenced *CEBPA* cases are further characterized by high *CD3* and *CD7* expression. Silenced case #4728 and single-mutated case #5041 show high *TRIB2* expression, which has been shown to inhibit C/EBPα p42. (A color version of this figure can be found in the appendices.)

Table 3. Characteristics of the 5 *CEBPA*-silenced AML cases, including 3 cases with *CEBPA* promoter hypermethylation.

ID	MSP	Age (yr)	Sex	WBC ($x10^9$)	FAB	Karyotype	Mutation	Protocol	Follow-up
#3451	+	6.3	M	196.0	M0	46,XY,del(17)(p12p13) or add(17)(p11)[2]	*WT1, N-RAS*	DCOG97	Relapse, 11.7 mo
#3496	+	11.3	F	NA	M5	NA	*WT1, K-RAS*	DCOG97	Relapse, 8.2 mo
#5033	+	10.3	F	NA	M0	46,XX,t(3;4)(q11-12;p15-16[6]/ 47,XX,t(3;4)(q11-12;p15-16), +mar[12]/46,XX[2]	none	LAME	CCR, 8.5 yrs (SCT at 8 mo)
#4728	-	9.7	M	2.4	M0	47,XY,inv(12)1?3,q1?3),+19[6]/ 46,XY[14]	none	DCOG97	Relapse, 38.4 mo
#4736	-	8.5	F	2.9	M4	46,XX,del(7)?(p13;p21)[1], idem + t(3;7)?(p25;p15)[11], idem + del(11)?(p11p14)[13]	none	DCOG97	Relapse, 7.3 mo

MSP indicates methylation-specific PCR for *CEBPA* promoter hypermethylation: + positive, - negative;
NA, not available; WBC, white blood cell count; FAB, French-American-British; mo, months after diagnosis.

Aberrant *CEBPA* promoter hypermethylation in pediatric AML

Methylation-specific PCR could be performed in 237 cases and revealed hypermethylation of the *CEBPA* promoter region in only three cases (1.3%) (Figure 1B). As expected, *CEBPA* gene expression (determined with probe set 204039_at, and depicted in Figure 3) was down-regulated in these cases. The characteristics of these three *CEBPA*-promoter hypermethylated cases are shown in Table 3. *CEBPA* promoter hypermethylation was also present in the relapse material (n=2) from these patients, demonstrating clonal stability of the hypermethylation pattern.

Unsupervised analysis reveals clustering of *CEBPA* mutant and hypermethylated cases

Unsupervised cluster analysis of 237 cases with *de novo* AML showed distinct clusters (Figure 3A). Cases with *CEBPA* mutations and promoter hypermethylation predominantly clustered together, and are referred to as the main *CEBPA* cluster. This cluster contained 15 cases in total, including eight double-mutants and all three promoter hypermethylated cases. Of interest, two cases with *CEBPA* single mutations were also present in this main *CEBPA* cluster, despite the fact that these cases are expected to have wild-type expression of C/EBPα p42, in contrast to *CEBPA* double-mutant and promoter hypermethylated cases. Interestingly, extremely high *TRIB2* expression (probe set 202478_at) was present in one of these *CEBPA* single-mutant cases (#5041), which may explain C/EBPα p42-inhibition of its remaining allele, as *TRIB2* directly inactivates C/EBPα p42.[34]

Furthermore, the two remaining cases in the *CEBPA* main cluster had low *CEBPA* gene expression, and clustered closely with the three cases with *CEBPA*-promoter hypermethyla-

tion, despite the fact that we did not detect hypermethylation in these cases using MSP (Figure 3B, Table 3). Of note, one of these cases also had with very high *TRIB2* expression (#4728). So, taken together, five cases with silenced *CEBPA* were found among 237 cases with *de novo* pediatric AML (2.1%). Four of these cases experienced a relapse, and only one patient was in continuous CR after a SCT (Table 3).

Clearly, a common gene expression signature was shared for all the cases in the CEBPA main cluster, which was confirmed when comparing this cluster with all others (Online Supplementary Figure S1, Online Supplementary Table S4).

T-cell characteristics of *CEBPA*-silenced cases in pediatric AML

As *CEBPA*-silenced cases were reported to express T-cell lineage genes and *NOTCH1* mutations in adults, we next investigated T-cell characteristics and screened for *NOTCH1* mutations in our five pediatric cases with silenced *CEBPA*.[15] Flow cytometry data revealed high CD7 expression in all five cases besides myeloid (CD33/CD13 and CD11b) and stem-cell markers (CD34 and CD117) (Online Supplementary Table S5). One case weakly expressed cCD3 (#5033), but expression of other T-cell antigens was not seen. However, high mRNA expression of *CD3* (*CD3Z*: 210031_at, *CD3G*: 206804_at and *CD3D*: 213539_at) was seen in all cases (Online Supplementary Table S5). Also high *LCK* expression, which is a well-known T-lineage marker (probe set 204891_s_at)[35], was found in all five cases. We did not detect *NOTCH1* mutations in the HD or PEST domain in our five *CEBPA*-silenced cases.

Prediction of *CEBPA* double-mutant and -silenced cases using adult AML gene signatures

We utilized previously established gene prediction signatures in adult AML, based on 21 and 9 probe sets for *CEBPA* double-mutant and *CEBPA*-silenced cases, respectively.[15,27] Of the 12 *CEBPA* double-mutant cases, ten were correctly predicted using the 21-probe set-containing signature, one was predicted as being a single mutant, and one as a silenced case (sensitivity 83%, specificity 99%) (Online Supplementary Table S6A). Visualizing these results, it can be seen that the double-mutant cases form a main cluster apart from the wild-type cases (Online Supplementary Figures S2A and S2B). However, three *CEBPA* single-mutant cases (one predicted as a double mutant), with the single mutation located in the bZIP region, also clustered with the *CEBPA* double-mutant cases.

The 9-probe set-signature for *CEBPA*-silenced cases predicted three of our five silenced cases, but also one *CEBPA* wild-type was false positively recognized (Online Supplementary Table S6B, Online Supplementary Figures S2C and S2D). This resulted in a low sensitivity (60%) of these probe sets for the prediction of *CEBPA*-silenced cases in our pediatric series.

DISCUSSION

In this study we investigated *CEBPA* aberrations in pediatric AML to determine their fre-

quency and prognostic impact, and also to gain further insight into the biology of pediatric AML with *CEBPA* aberrations. We detected *CEBPA* mutations in 7.9% of pediatric AML cases, which is comparable to the reported frequency in adult AML (5-14%)[9,21-24], and the two available pediatric series from Taiwan (6%) and North-America (4.5%).[25,26] Seventy percent of *CEBPA*-mutated cases carried a double mutation, which is in agreement with previous studies reporting that the majority of *CEBPA*-mutated cases carried double mutations, typically affecting both alleles.[20,21]

Recently, two reports on adult series postulated that *CEBPA* single- and double-mutant AML are different entities, as a favorable outcome was associated uniquely with *CEBPA* double mutants.[27,28] Moreover, cases with double mutations were characterized by a specific gene expression signature, in contrast to those with *CEBPA* single mutations. This is further sustained by recently published from mouse models that showed an efficient synergistic effect of the two different *CEBPA* mutations on leukemic transformation.[36-38] Single *CEBPA* mutations are, however, believed to predispose the pre-leukemic initiating cell to subsequent acquirement of secondary (epi)genetic mutations necessary before development of full-blown AML.[37-38] Despite small numbers, we observed differences, both in presenting characteristics as well as in prognosis, between *CEBPA* single- and double-mutant AML. With regards to presenting characteristics, *CEBPA* double-mutant AML did not occur in very young patients, was restricted to FAB M1/M2 subtypes, and had the strongest association with CN-AML. However, the frequency of additional molecular aberrations was not higher in *CEBPA* single-mutant cases than in *CEBPA* double-mutant cases.

With regards to prognosis, only patients with *CEBPA* double mutations were associated with a favorable outcome with a 5-year overall survival of 79%. Those with single mutations had a relatively poor outcome in our series (5-year pOS 25%). In fact, the outcome of *CEBPA* double-mutant cases was comparable to that of the subgroup with the favorable CBF-AML. This is in agreement with the results of the pediatric COG study,[25] which showed an overall survival of approximately 80% at 5 years for patients with *CEBPA* double mutations. Multivariate analysis confirmed the independent prognostic significance of *CEBPA* double mutations, which points to its potential as marker for further refinement of risk-group stratification in pediatric AML, when validated in prospective series. The mechanism of the relative drug sensitivity of the *CEBPA* double-mutated cases remains to be elucidated.

The outcome of cases with single mutations in the series reported by the COG (5-year pOS 85%) was similar to that of the cases with double mutations. Despite the fact that the numbers of single-mutant cases in both series were small, we hypothesize the difference in outcome between our studies may be based on the underlying biology of the type of the single mutation (i.e. a mutation at the N-terminus, bZIP region or other location) as different leukemogenic capacities have been associated with the different types of mutations,[37] or by different cooperating genetic events. Further studies of the single-mutant group in pediatric and adult AML are clearly warranted to determine the impact of the different mutation types and cooperating genetic aberrations.

Germ-line *CEBPA* mutations have been discovered in familial AML, in which the N-terminal mutation is present in the germ-line, and frequently a *CEBPA* bZIP mutation is somatically acquired as a second hit to develop AML.[39,40] The frequency of germ-line mutations in adult AML with *CEBPA* mutations was estimated at 11% (2 cases out of 18).[41] Here we showed a similar frequency: one out of seven cases with *CEBPA*-mutated AML had a germ-line mutation. In our pediatric case we also found an N-terminal mutation in the germ-line, and a somatically acquired bZIP mutation. The occurrence of AML in both children and adults with germ-line *CEBPA* mutations illustrates a variable latency time.

Promoter hypermethylation of *CEBPA* was present at a low frequency in our pediatric series. These hypermethylated cases indeed showed *CEBPA*-silencing and, utilizing unsupervised clustering of gene expression data, clustered together with the double-mutant cases. Two other cases with *CEBPA*-silencing without promoter hypermethylation were detected; these two cases clustered together with the hypermethylated cases. The mechanism of gene silencing in these two cases still has to be elucidated, and might be hypermethylation in other regions of the promoter,[42] silencing by other epigenetic processes or by binding of microRNAs. All five *CEBPA*-silenced cases had T-lymphoid characteristics beside their myeloid and stem cell markers. However, no *NOTCH1* mutations were detected, which may be due to the small number of cases as *NOTCH1* mutations were present in only 50% of adult cases with silenced *CEBPA*.[15] In contrast to the favorable outcome of patients with *CEBPA* double mutations, four out of the five *CEBPA*-silenced cases experienced a relapse. Interestingly, the patient not relapsing received a stem cell transplant. Cases with silenced *CEBPA* due to hypermethylation might potentially benefit from the use of demethylating agents.

Clustering of *CEBPA*-silenced and double-mutant cases points towards a common feature of C/EBPα inactivation in these leukemias. Cases in this *CEBPA* main cluster clearly shared a specific gene expression profile. However, the main cluster can also be divided in two subclusters, separating the double-mutant and silenced cases, which might underlie biological factors influencing drug resistance and thereby the difference in prognosis between the two subgroups. Differences in methylation profiles have already been shown between these two subgroups.[43] Interestingly, two *CEBPA* single-mutant cases, which are expected to have full-length C/EBPα p42 expression of the unaffected allele, also aggregated in this cluster. In one of these cases, high *TRIB2* expression was detected, which is known to directly inactivate the C/EBPα p42 isoform.[34] Complete C/EBPα p42 inactivation of the wild-type allele is hereby established and clustering with cases of AML without functional C/EBPα p42 can be explained. The mechanism of the expected C/EBPα p42 inactivation in the other single-mutant case remains to be elucidated.

We tried to predict *CEBPA* double-mutant and silenced cases in our pediatric series based on a prediction signature derived from adult studies. A high sensitivity and specificity was reached for *CEBPA* double-mutants, although one single-mutant was also falsely predicted, and two single-mutant cases clustered with the double-mutant cases. These three cases did, however, carry a mutation in the bZIP region, which was previously shown to have a ten-

dency towards a *CEBPA* double-mutant gene expression profile.[27] Prediction of the *CEBPA*-silenced cases was difficult due to a low sensitivity.

In conclusion, we showed the independent favorable outcome of patients with *CEBPA* double-mutant AML in a large series of pediatric AML. Hence, *CEBPA* double mutations may improve risk-group stratification in pediatric AML, if these data are validated in prospective series. For the first time, *CEBPA*-silenced cases are suggested to confer a poor outcome in pediatric AML, warranting further investigation of this *CEBPA* aberration. Furthermore, clustering of gene expression profiles provided insight into the biological similarities and diversities of *CEBPA* aberrations in pediatric AML.

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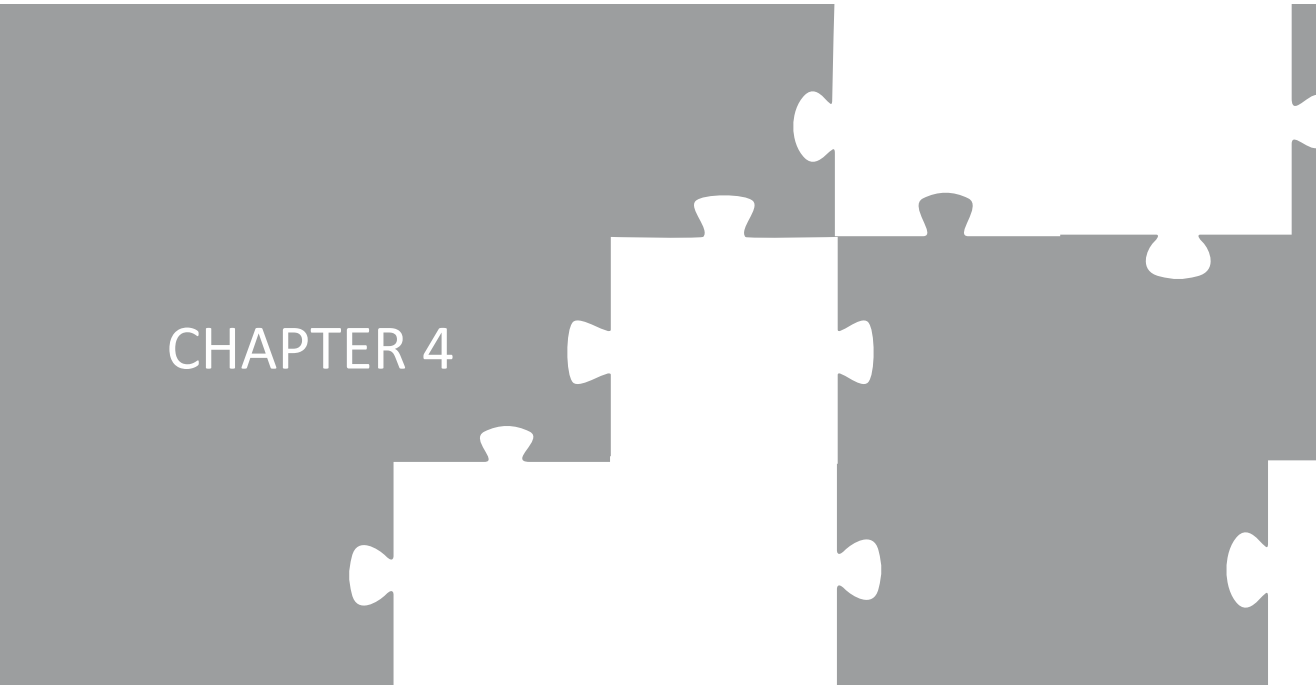
CHAPTER 4

Low frequency of *MLL*-partial tandem duplications in pediatric 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 pediatric 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 pediatric AML (n = 276) combining 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 pediatric 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 an *FLT3*/ITD, which was significantly higher compared to 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 pediatric AML was found. Larger prospective studies are needed to further define the prognostic relevance of *MLL*-PTD in pediatric AML.

INTRODUCTION

Cure rates in pediatric acute myeloid leukemia (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 Leukemia (MLL)*-gene, localized on chromosome 11q23, plays an important role in the development of both AML and acute lymphoblastic leukemias (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 leukemia.[2-4]

To date more than 50 different translocation partners of the *MLL*-gene have been discovered.[5-6] In pediatric 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 favorable outcome, whereas t(10;11)(p12;q23), t(10;11)(p11.2,q23), and t(6;11)(q27;q23) were 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 characterized 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 leukemogenic 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 leukemias.[2,9]

In adult AML, *MLL*-PTD was detected in 3-10% of patient samples, using reverse transcriptase polymerase chain reaction (RT-PCR) on either the transcript (mRNA) and/or the genomic (DNA) level (summarized 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 pediatric 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 pediatric AML cases. In addition, *MLL*-PTD was associated with an adverse outcome. Ross and colleagues detected a frequency of 10% in 130 pediatric AML cases, whereas Shih and colleagues reported a frequency of only 0.9% in 123 pediatric 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 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,

TABLE 1. Summary of published studies on *MLL*-PTD in adult and pediatric acute myeloid leukemia.

Study	No. of patients	Adult/Pediatric	*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 + Soutern Blot
Steudel et al.[11]	956	Adult	5.0	mRNA RT-PCR + Soutern Blot
Libura et al.[17]	185	Adult	3.2	Soutern 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	Pediatric	10.0	mRNA RT-PCR
Shih et al.[13]	123	Pediatric	0.9	Multiplex PCR
Shimada et al.[21]	158	Pediatric	13.0	mRNA RT-PCR

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 pediatric 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.

MATERIAL AND METHODS

Patients

Viably frozen diagnostic bone marrow or peripheral blood samples from 276 newly diagnosed pediatric AML patients were provided by the Dutch Childhood Oncology Group (DCOG), the AML 'Berlin-Frankfurt-Münster' Study Group (AML-BFM SG), the Czech Pediatric Haematology Group (CPH), and the St. Louis Hospital in Paris, France. Informed consent was obtained after Institutional Review Board approval according to local laws 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, leukemic cells were isolated by the depletion of contaminating cells as previously described.[27] All the resulting samples contained >80% leukemic cells, as determined morphologically by May-Grünwald-Giemsa (Merck, Darmstadt, Germany)-stained cyto-

TABLE 2. Primer sequences DNA-MLPA *MLL*-PTD

Probe location	Probe sequence
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 indicates forward probe; RV, reverse probe

spins. The purified leukemic cells were used for DNA and RNA extraction, and a minimum of $5x10^6$ leukemic cells were lysed in TRIzol reagent (Invitrogen Life Technologies, Breda, the Netherlands) and stored at -80°C. Genomic DNA and total cellular RNA were isolated as described before.[28]

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 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 external control according to the manufacturer's protocol (MRC Holland, Amsterdam, the Netherlands) (Table 2). The patient samples were analyzed according to the manufacturer's protocol. Briefly, genomic DNA was denaturated and hybridized 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 (version 1.5), the peak patterns obtained were compared to those of three 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 three controls (Online Supplementary Figure S1).

We also performed mRNA RT-PCR to detect *MLL*-PTD transcripts, to allow comparison with the DNA-MLPA results, using *MLL*-654c (5'-AGGAGAGAGTTTACCTGCTC-3') as forward primer and *MLL*-5.3 (5'-GGAAGTCAAGCAAGCAGGTC-3') as reverse primer.[29]

Validation of DNA-MLPA on a different patient cohort

The DNA-MLPA method to detect *MLL*-PTDs was validated in an independent adult leukemia cohort (23 AML, 2 ALL and 1 myelodysplastic syndrome), 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 (Online Supplementary Table S1).

Cytogenetic and molecular analysis

The pediatric 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* hybridization (FISH). *NPM1*, *CEBPA*, *N-RAS*, *K-RAS*, *PTPN11*, *KIT* and *FLT3* mutational screening were done as previously described, and included mutational hotspots only.[31-35]

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 *P* values less than 0.05 were considered significant.

RESULTS

Frequency of *MLL*-PTD using DNA-MLPA in pediatric 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 six patients, *MLL*-PTD mRNA expression was confirmed with

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
Age, years (median, range) (n=271)	9.8 (0.1-18.8)	7.5 (4.8-18.0)	0.74[§]
Sex, N (%) (n=276)			0.70[#]
Male	150 (56)	3 (43)	
Female	119 (44)	4 (57)	
WBC, $x10^9$/l (median, range) (n=231)	40 (0-585)	97 (45-170)	0.08[§]
FAB, N (%) (n=276)			0.15[#]
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[#]
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 **	81 (30)	3 (43)	
FLT3/ITD, N (%) (n=253)			0.02[#]
No	208 (85)	3 (43)	
Yes	38 (16)	4 (57)	

[#] Chi-square/Fisher Exact test; [§] Mann-Whitney *U*-test; ** See Table 4 for further details.

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 six cases both DNA-MLPA and mRNA RT-PCR were positive for *MLL*-PTD as described above. In seven 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.

Characteristics of patients with *MLL*-PTD

The characteristics of the seven patients with *MLL*-PTD are described in Tables 3 and 4. None

TABLE 4. Patient characteristics of the *MLL*-PTD-positive patients.

ID	Age (years)	Sex	WBC (10^9/l)	FAB	Karyotype	*MLL*-PTD	Allelic ratio	Mutation
#4375	7	M	68.1	M1	47,XY,+11	ex2-ex8	1.5	*N-RAS*
#4412	7	M	120.4	M1	46,XY	ex2-ex7	1.5	*FLT3*/ITD
#4721	14	F	44.8	M1	46,XX	ex2-ex8	1.5	*FLT3*/ITD
#4127	11	F	72.9	M2	46, XX	SB, RT-PCR pos	NA	*FLT3*/TKD
#4378	7	F	169.9	M4	NA	ex2-ex8	2	*FLT3*/ITD
#4367	4	F	NA	M0	NA[a]	ex2-ex9	1.5	-
#5026	18	M	133.0	M4	NA[b]	ex2-ex8	2.5	*FLT3*/ITD

Abbreviations: M indicates male; F, female; ex, exon; SB Southern Blot; pos, positive; NA, not available.
[a] No *MLL*-rearrangement or t(8;21) detected; [b] No inv(16) detected.

of the patients harbouring an *MLL*-rearrangement, t(8;21), inv(16) or t(15;17) revealed an *MLL*-PTD. In one patient with an *MLL*-PTD a trisomy 11 was found, while the other six cases were found among patients with normal cytogenetics (CN-AML) (n=3) or among patients in whom cytogenetic analysis failed (n=3).

FLT3/ITD was present in four out of seven patients with *MLL*-PTD, while one patient showed a mutation in the kinase domain of *FLT3* and another showed a mutation in *N-RAS*. There was a 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 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 vs. 40x10^9/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 133.0x10^9/l and 169.0x10^9/l, respectively.

Comparison of *MLL*-PTD with *MLL*-rearranged AML

We also compared the patients characterized 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 vs. 6.2 years, p=0.074) and median WBC at diagnosis (96.7 vs. 61.0x10^9/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<0.001, Table 5).

Clinical outcome in pediatric 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 pediatric AML cases. Only two out of seven patients with an AML harbouring an *MLL*-PTD were in first continuous complete remission (CCR) after three years. Another two patients initially achieved CR, but one patient died after hematopoietic

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

	MLL-rearranged patients	*MLL*-PTD-positive patients	*P* value
Age, years (median, range) (n=75)	6.2 (0.1-18.8)	7.5 (4.8-18.0)	0.07 [§]
Sex, N (%) (n=75)			0.70 [#]
Male	38 (56)	3 (43)	
Female	30 (44)	4 (57)	
WBC, x10^9/l (median, range) (n=64)	61 (1.2-585)	97 (45-170)	0.35 [§]
FAB, N (%) (n=76)			<0.001[#]
M0	3 (4)	1 (14)	
M1	2 (3)	3 (43)	
M2	1 (1)	1 (14)	
M3	-	-	
M4	9 (13)	2 (29)	
M5	50 (72)	-	
M6	-	-	
M7	1 (1)	-	
Other/unknown	3 (4)	-	
FTL3/ITD, N (%) (n=74)			<0.001[#]
No	65 (97)	3 (43)	
Yes	2 (3)	4 (57)	

[#] Chi-square/Fisher Exact test; [§] Mann-Whitney *U*-test

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.

DISCUSSION

In this large pediatric 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 pediatric AML series as summarized in Table 1. The higher frequency in these two pediatric 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.

In contrast, 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 pediatric 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, *MLL*-PTD transcripts 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 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 the two methods, i.e. DNA-MLPA and mRNA RT-PCR, are together 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 hypothesized 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 mutation), 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 *KIT* and t(8;21) or inv(16), further supporting the hypothesis put forward by Gilliland and colleagues.[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 200bp downstream of 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 pediatric AML, so far no conclusive results are available for the outcome of *FLT3*/ITD in *MLL*-PTD because of 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 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 pediatric AML. *MLL*-PTD was related to a more immature phenotype compared to *MLL*-rearranged AML, which mostly presents with a FAB-M4 or -M5. These differences in differentiation-arrest could indicate differences in the leukemogenesis of both types of aberrations.

Although both types of aberrations in *MLL* show overexpression of *HOX*-genes, we recently

showed that 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 pediatric 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 leukemogenesis is not clear, these patients might benefit from 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 leukemia.[9] However, leukemic blast cells of adult patients with *MLL*-PTD, which are present on one allele only, do not express 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, treatment of *MLL*-PTD-positive cases with DNMT and HDAC inhibitors resulted in induction of the expression of 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 pediatric AML. DNA-MLPA provided the opportunity to investigate allelic ratios, and two of the six patients showed a relative allelic ratio of more than two, indicating the presence of more than two *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 above $100x10^9$/l, which is a well-known risk factor for poor outcome in pediatric AML.

In conclusion, the frequency of *MLL*-PTD in pediatric AML is low and may have been overestimated in earlier studies. In this study, we screened the largest pediatric 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 pediatric AML. Larger prospective studies are necessary to define the prognostic relevance of *MLL*-PTD in pediatric AML.

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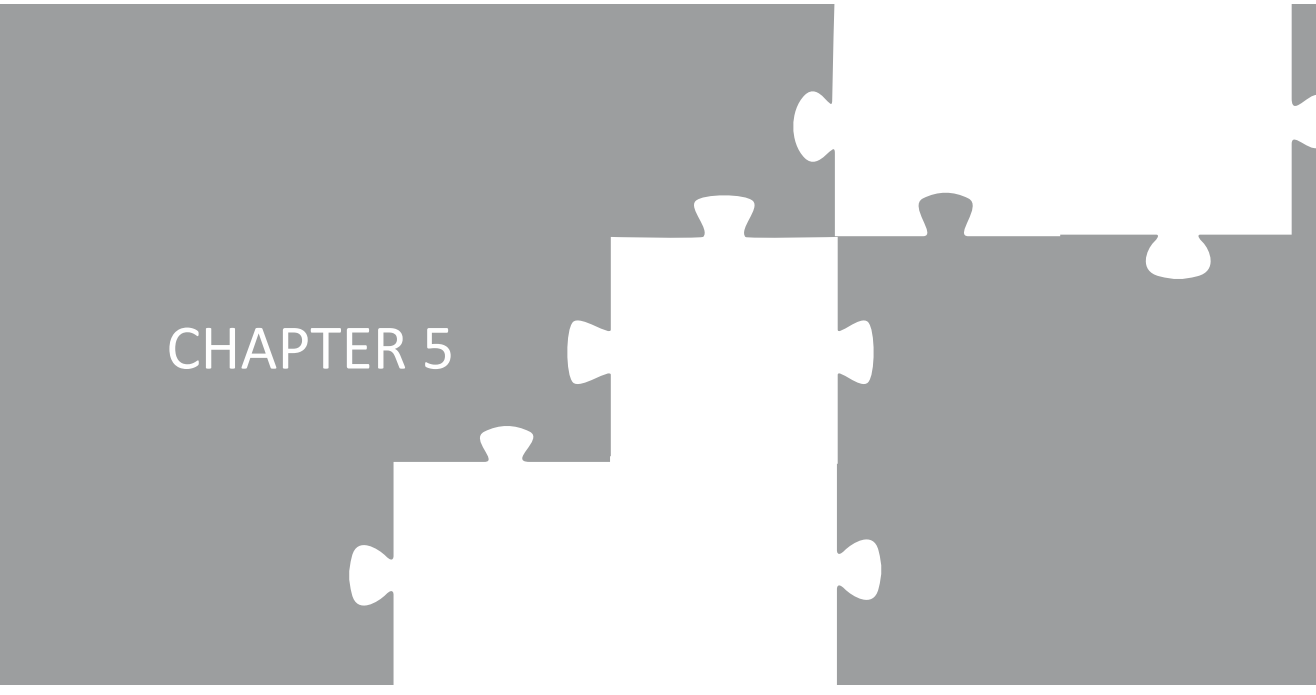
CHAPTER 5

Integrative analysis of type-I and type-II aberrations underscores the genetic heterogeneity of pediatric acute myeloid leukemia

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ABSTRACT

Background

In pediatric acute myeloid leukemia (AML), several studies have described the various type-I or type-II aberrations and their relationship with clinical outcome. However, a recent comprehensive overview of these genetic aberrations in one large pediatric AML is lacking.

Design and Methods

We studied the different genetic aberrations, their associations and their impact on prognosis in a large pediatric AML series (n=506). Karyotypes were studied, and hotspot regions of *NPM1*, *CEPBA*, *MLL*, *WT1*, *FLT3*, *N-RAS, K-RAS*, *PTPN11* and *KIT* were screened for mutations of available samples. The mutational status of all type-I and type-II aberrations was available in respectively 330 and 263 cases. Survival analysis was performed in a subset (n=385) treated on consecutive AML-Berlin-Frankfurt-Münster Study Group and Dutch Childhood Oncology Group treatment protocols.

Results

Genetic aberrations associated with specific clinical characteristics, e.g. significant higher diagnostic white blood cell counts in *MLL*-rearranged, *WT1*-mutated and *FLT3*/ITD-positive AML. Furthermore, the distribution of these aberrations differed significantly between children below and above the age of 2 years. Non-random associations, e.g. *KIT* mutations with core-binding factor-AML, and *FLT3*/ITD with respectively t(15;17)(q22;q21), *NPM1*- and *WT1*-mutated AML were observed. Multivariate analysis revealed that 'favorable karyotype' (i.e. t(15;17)(q22;q21), t(8;21)(q22;q22) and inv(16)(p13q22)/t(16;16)(p13;q22)), *NPM1* and *CEBPA* double mutations were independent factors for favorable event-free survival. *WT1* mutations combined with *FLT3/*ITD showed the worst outcome for 5-year overall survival (22±14%) and 5-year event-free survival (20±13%), although it was not an independent factor in multivariate analysis.

Conclusions

Integrative analysis of type-I and type-II aberrations provides insight in the frequencies, non-random associations and prognostic impact of the various aberrations, reflecting the heterogeneity of pediatric AML. These aberrations are likely to guide the stratification of pediatric AML and may direct the development of targeted therapies.

INTRODUCTION

Acute myeloid leukemia (AML) accounts for 15-20% of pediatric leukemias.[1] Despite intensification of chemotherapy over the last decades, only approximately 60-70% of children with AML are cured.[2] AML is not a single disease entity but very heterogeneous, which is reflected by differences in morphology, immunophenotype as well as cytogenetic and molecular aberrations.[3] Moreover, recurrent (cyto)genetic aberrations are important prognostic factors in pediatric AML which are used for risk-group stratification by an increasing number of study groups.[4-5]

Gilliland et al. hypothesized that the development of AML at least requires two types of genetic events.[6] Type-I aberrations occur as mutations in hotspots of specific genes involved in signal transduction pathways (*FLT3*, *KIT*, *N-RAS*, *K-RAS* and *PTPN11*), which lead to uncontrolled proliferation and/or survival of leukemic cells. Type-II aberrations are often chromosomal rearrangements of transcription factors resulting in the translation of fusion proteins leading to impaired differentiation of the leukemic cells (including *PML-RARA* [t(15;17)(q22;q21)]*, AML1-ETO* [t(8;21)(q22;q22)]*, CBFB-MYH11* [inv(16)(p13q22)/t(16;16)(p13;q22)] and 11q23/*MLL*-rearrangements). This hypothesis was further strengthened by observations from mouse models that one aberration is not sufficient to induce leukemia, but that cooperative events are needed to develop frank leukemia. For example, knock-in of *FLT3*/ITD leads to the development of a myeloproliferative disorder but lacks the maturation arrest typical for acute leukemia,[7] whereas co-expression with inv(16)(p13q22) or t(15;17)(q22;q21) resulted in AML.[8-9]

In pediatric AML, the individual type-I or type-II aberrations and their relationship with clinical outcome have been described by several studies.[4-5,10-16] However, a comprehensive overview of the associations and the prognostic impact of type-I and type-II aberrations in one large cohort of pediatric AML patients is lacking. Furthermore, novel molecular genetic aberrations in pediatric AML, such as mutations in the *CEBPA*, *NPM1* and *WT1* genes, as well as partial tandem duplications in the *MLL* gene (*MLL*-PTD) have been identified in the last decade.[11,13,17] The prognostic impact of these newly identified aberrations together in one large pediatric AML series have not been presented so far. Identifying prognostic factors in pediatric AML may lead to improved risk-group stratification, and hence may have direct impact on current and future treatment protocols. Secondly, specific leukemogenic aberrations may guide development of targeted therapy approaches for selected patient groups.

Therefore, we performed a study on type-I and type-II aberrations in the largest pediatric AML series so far, including their associations with clinical characteristics and outcome.

DESIGN AND METHODS

Study cohort

This study included 506 pediatric patients with *de novo* AML, of whom data were provided by the Dutch Childhood Oncology Group (DCOG), the AML-'Berlin-Frankfurt-Münster' Study Group (AML-BFM SG), the Czech Pediatric Hematology (CPH) group, as well as the St. Louis Hospital in Paris, France. Institutional review board approval for these studies and informed consent was obtained according to local laws and regulations. Each study group performed central review of the morphology, according to the WHO/FAB classification.[18] Clinical and cell-biological data, including cytogenetic results, were obtained from these study groups and institute.

Survival analysis was restricted to a subset of 385 AML patients who received treatment according to DCOG/AML-BFM 87, DCOG 92-94/AML-BFM 93, AML-BFM 98, AML-BFM 04 and MRC-12/15 protocols to reduce treatment variability. Details of these treatment protocols and overall outcome data have been previously published.[19-24] Treatment consisted of four to five blocks of intensive chemotherapy, using a standard cytarabine and anthracycline backbone. Stem cell transplantation in first complete remission was performed only in a small number of selected high-risk patients.

DNA and RNA isolation

After thawing of the available viably frozen bone marrow and peripheral blood samples, leukemic cells were isolated and enriched as previously described.[25] Blast percentages were confirmed morphologically on Cytospin slides stained with May-Grünwald-Giemsa (Merck, Darmstadt, Germany). Subsequently, leukemic cells were lysed in TRIzol reagent (Invitrogen Life Technologies, Breda, the Netherlands). Genomic DNA and total cellular RNA were isolated according to the manufacturer's protocol with minor modifications.[26]

Cytogenetic analysis

Leukemic samples were routinely investigated for cytogenetic aberrations by G-, Q-, or R-banded karyotyping, and were screened for recurrent non-random genetic aberrations characteristic for AML, including *MLL*-rearrangements, inv(16)(p13q22)/t(16;16)(p13;q22), t(8;21)(q22;q22) and t(15;17)(q22;q21), using either reverse transcriptase polymerase chain reaction (RT-PCR) and/or fluorescent *in situ* hybridization (FISH) by each study group. In case of lacking data but available material, these aberrations were screened for by the Laboratory of Pediatric Oncology/Hematology at Erasmus MC-Sophia Children's Hospital, Rotterdam, the Netherlands. In addition, patients under the age of 18 months were screened for the presence of t(7;12)(q36;p13) by FISH. The probes used were five cosmid clones covering the breakpoints in the *ETV6* gene and a PAC clone (RP5-1121A15) containing the *HLXB9* gene.[27]

Definition of cytogenetic groups

The cytogenetic groups, including 11q23/*MLL*-rearrangements, inv(16)(p13q22)/t(16;16)(p13;q22), t(8;21)(q22;q22), t(15;17)(q22;q21) and t(7;12)(q36;p13) were defined by the presence of the specific aberration, independent of accompanying aberrations. A complex karyotype was defined as the presence of three or more aberrations in a single clone, and without any AML-specific aberrations. Monosomy 7 and trisomy 8 were defined as the loss of chromosome 7 and a gain of chromosome 8, respectively, but without any AML-specific aberration and without a complex karyotype. The cytogenetically normal (CN-AML) group included those with a normal karyotype without any aberrations. The 'other' group included all samples not categorized in the groups mentioned above, with the exception of the 'unknown' group, which included cases with failed karyotyping but without any AML-specific aberration as screened for with FISH or RT-PCR.

Definition of gene mutations as type-I and type-II aberrations

Screening for gene mutations was carried out based on availability of material. Mutations were determined in the hotspot regions of *NPM1* (n=337), *CEPBA* (n=282), *MLL* (i.e. partial tandem duplications (PTD; n=309), *WT1* (n=330), *FLT3* (i.e. internal tandem duplications (ITD; n=372) and tyrosine kinase domain mutations (TKD; n=330), *N-RAS* and *K-RAS* (n=353), *PTPN11* (n=350) and *KIT* (n=368), as previously described.[13-14,28-32] This resulted in screening of all type-I aberrations in 330 cases and all type-II aberrations in 263 cases. A complete list of screened regions per gene, primers and PCR conditions is provided in Online Supplementary Table S1. The 'fusion gene' type-II aberrations, including *MLL*-rearrangements, t(8;21)(q22;q22), inv(16)(p13q22)/t(16;16)(p13;q22), t(15;17)(q22;q21) were mutually exclusive with *NPM1* mutations*, CEBPA* double mutations and *MLL*-PTD aberrations, which suggests that these latter mutations might be considered as type-II aberrations. This is further strengthened by evidence that these aberrations result in a maturation arrest; targeted disruption of C/EBPα results in a selective early block of granulocyte differentiation,[33] while *NPM1* mutations and *MLL*-PTD disrupt the controlled expression of *HOX*-genes resulting in impaired differentiation of the hematopoietic cells.[34-36] *NPM1* mutations, *CEBPA* double mutations and *MLL*-PTD aberrations were therefore considered to be type-II aberrations, whereas mutations in *FLT3*, *N-/K-RAS*, *PTPN11* and *KIT* were considered to be type-I aberrations. As the leukemogenic mechanism of *WT1* mutations still needs to be elucidated,[37] these mutations were arbitrarily categorized as type-I aberrations for purposes of this study, because they overlapped with the type-II-defined subtypes.

Statistical analysis

Calculations were performed using SPSS 17.0 (SPSS Inc. Chicago, USA) or SAS 9.1 (SAS Institute, Cary, USA). Categorical variables were compared using $\chi 2$ analysis or Fisher's exact test, and for continuous variables, the non-parametric Mann-Whitney *U*-test was used. Probabilities of overall survival (pOS) and event-free survival (pEFS; events were defined as

failure to achieve complete remission (CR), occurrence of relapse, occurrence of secondary malignancy, or death from any cause) were estimated by the method of Kaplan and Meier, and compared using the log-rank test. Cumulative incidence of relapses (CIR) (with other events and death while in CR as competing events) were constructed by the method of Kalbfleisch and Prentice and compared by Gray's test.[38] For survival analysis, only those groups were included containing more than ten cases; otherwise they were included in the 'other' group. Multivariate analyses were performed using the Cox proportional Hazard model. P-values below 0.05 were considered significant (two-tailed testing).

RESULTS

Study cohort

Characteristics of the study cohort are presented in Table 1. The sex distribution was 57% male vs. 43% female. The median age was 8.7 years (yrs) (range 0-18 yrs), and the distribution according to the age categories <2 yrs, 2-9 yrs and ≥10 yrs was 18%, 38% and 44% respectively. The median white blood cell count at diagnosis (WBC) was $34x10^9$/l (range 0-$585x10^9$/l). FAB-M2, -M4 and -M5 were the most common morphological subtypes in this cohort (23%, 24% and 24% respectively). The distribution for sex, age, WBC and FAB morphology was comparable with the AML-BFM trials [i.e. the AML-BFM 93 (n=471) and 98 (n=473) trials], indicating that our cohort is representative for pediatric AML (Online Supplementary Tables S2 and S3). The 385 pediatric AML cases included in the survival analysis had a 5-year pEFS and pOS of 42±3% and 60±3%, respectively. These survival rates are in a similar range as the studies previously published.[39]

Characteristics of pediatric AML with specific cytogenetic subtypes

Patients were assigned to the following cytogenetic groups: *MLL*-rearranged AML (24%; 122/506), t(8;21)(q22;q22) (13%; 64/506), inv(16)(p13q22)/t(16;16)(p13;q22) (10%; 48/506), t(15;17)(q22;q21) (6%; 28/506), t(7;12)(q36;p13) (1%; 7/506), t(6;9)(p23;q34) (1%; 7/506), complex karyotype (6%; 30/506), monosomy 7 (1%; 6/506), trisomy 8 (2%; 12/506), CN-AML (17%; 84/506), and 'other karyotype' (13%; 65/506) (Table 1A). In 7% (33/506) of the cases conventional karyotyping failed, and neither RT-PCR or FISH led to classification of these patients. Those cases were therefore assigned to the 'unknown' cytogenetic group. An overview of the cytogenetic group assignment and the mutational status of the investigated genes of all individual patients are provided in Online Supplementary Table S4. No difference in sex distribution was identified between the different cytogenetic groups (Table 1A). Patients with t(8;21)(q22;q22) presented with a significantly lower WBC (median $13x10^9$/l; p<0.001), and *MLL*-rearranged AML patients with a significant higher WBC (median $63x10^9$/l; p=0.001), compared with the other cytogenetic groups (Table 1A). The median ages of children with *MLL*-rearranged AML (3.7 yrs; p<0.001), with t(7;12)(q36;p13) (0.3 yrs;

TABLE 1A. Overview of baseline clinical characteristics per cytogenetic group.

Cytogenetic aberration	Frequency n (%)	Age (yr)				Sex			WBC (x10^9/l)			
		n	Median	Range	*P* value*	n	% F	*P* value*	n	Median	Range	*P* value
MLL-rearrangements	122 (24.1)	122	3.7	0.0-17.3	**<0.001**	122	38	0.16	96	63	1-585	**0.001**
t(8;21)(q22;q22)	64 (12.6)	64	11.5	2.6-18.5	**<0.001**	64	42	0.85	57	13	2-320	**<0.001**
inv(16)(p13q22)	48 (9.5)	48	10.5	0.7-17.3	0.13	48	46	0.71	42	68	3-234	0.10
t(15;17)(q22;q21)	28 (5.5)	28	10.2	1.9-17.7	**0.03**	28	54	0.26	24	26	1-247	0.36
t(7;12)(q36;p13)	7 (1.4)	7	0.3	0.2-1.5	**<0.001**	7	71	0.25	6	55	14-227	0.38
t(6;9)(p23;q34)	7 (1.4)	7	12.8	10.3-14.9	**0.03**	7	43	1.00	4	96	24-120	0.21
Complex (≥3 aberrations)	30 (5.9)	30	2.5	0.1-14.4	**<0.001**	30	47	0.70	24	21	3-320	0.24
Monosomy 7	6 (1.2)	6	10.3	4.2-14.1	0.70	6	33	0.70	3	55	3-66	0.76
Trisomy 8	12 (2.4)	12	12.0	1.0-16.8	0.11	12	33	0.48	8	77	8-302	0.13
CN-AML	84 (16.6)	84	10.7	0.1-18.8	**0.01**	84	41	0.57	73	43	1-535	0.15
Other	65 (12.9)	65	8.8	0.0-18.4	0.89	64	48	0.37	54	29	1-452	0.27
Unknown	33 (6.5)	33	9.0	0.7-18.0	0.19	32	47	0.67	26	37	0-483	0.49
All cases	506 (100)	506	8.7	0.0-18.8	-	504	43	-	417	34	0-585	-

Abbreviations: CN indicates cytogenetically normal; WBC, white blood cell count at diagnosis; yr, years; %F, percentage female.

* *P* values refer to the comparison of the variable between the genetic subgroup vs. all others; bold *P* values are statistically significant.

TABLE 1B. Overview of baseline clinical characteristics per molecular aberration.

Molecular aberration (number tested)	Frequency %	Age (yr) n	Median	Range	*P* value	Sex n	%F	*P* value	WBC (x10^9/l) n	Median	Range	*P* value
NPM1 mut (n=337)	7.7	26	11.0	3.6-18.8	**0.03**	26	58	0.14	25	43	5-230	0.83
CEBPA double mut (n=282)	6.0	17	12.0	4.0-18.5	0.08	17	59	0.15	16	60	6-388	0.38
MLL-PTD (n=309)	2.3	7	7.5	4.8-18.0	0.64	6	50	0.70	5	73	45-170	0.10
WT1 mut (n=330)	8.8	29	9.2	1.9-17.8	0.47	29	38	0.52	26	86	3-354	**0.03**
FLT3/ITD (n=372)	18.0	67	10.1	1.6-18.8	**0.008**	66	46	0.88	59	74	5-535	**<0.001**
FLT3/TKD (n=330)	2.7	9	11.2	2.0-16.9	0.25	9	44	1.00	8	46	7-320	0.96
RAS-pathway (n=348)	21.6	75	9.6	0.1-16.9	0.98	75	37	0.14	61	42	3-483	0.54
N-RAS mut (n=353)	16.1	57	9.6	0.1-16.9	0.82	57	39	0.31	46	42	4-483	0.85
K-RAS mut (n=353)	3.7	13	11.3	5.5-16.9	0.06	13	15	0.03	10	118	3-225	0.09
PTPN11 mut (n=350)	2.0	7	4.2	0.6-13.5	0.26	7	57	0.71	6	32	4-115	0.60
KIT mut (n=368)	8.4	31	10.3	0.2-16.7	0.35	31	39	0.58	29	37	2-234	0.48

Abbreviations: mut indicates mutation, WBC white blood cell count at diagnosis, yr years, %F percentage female.

* *P* values refer to the comparison of the variable between the mutated vs. the wild-type cases; bold *P* values are statistically significant.

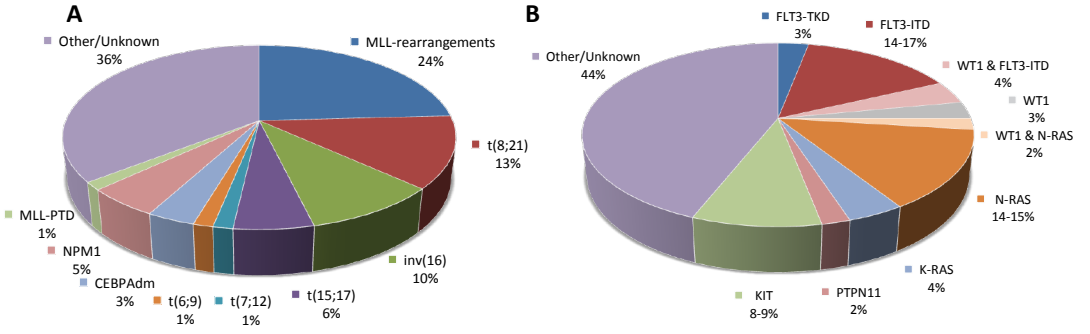

FIGURE 1. Distribution of the different type-I and type-II aberrations in pediatric AML. The heterogeneity of pediatric AML is reflected by the presence of the different type-I and type-II genetic aberrations. However, in a large amount of cases the type-II (A) or type-I (B) aberrations have not yet been identified. (A color version of this figure can be found in the appendices.)

p<0.001) and with a complex karyotype (2.5 yrs; p<0.001) were all significantly lower compared to the other cytogenetic groups. In contrast, children with t(8;21)(q22;q22) (11.5 yrs; p<0.001), t(15;17)(q22;q21) (10.2 yrs; p=0.03), and t(6;9)(p23;q34) (12.8 yrs; p=0.03) were significantly older compared to the other cytogenetic groups (Table 1A).

Characteristics of pediatric AML patients with type-II gene mutations

The following frequencies of gene mutations considered as type-II aberrations were found: *NPM1* (8%; 26/337), *CEBPA* double mutations (6%; 17/282) and *MLL*-PTD (2%; 7/309) (Table 1B). These aberrations were mainly present in patients with CN-AML, and were mutually exclusive with all other type-II aberrations. No differences were found for WBC or sex between patients carrying any of these aberrations and those without the indicated type-II aberration. Patients with *NPM1*-mutated AML were significantly older (median 11.0 yrs; p=0.03) compared to their wild-type counterparts (Table 1B). In 180/506 (36%) cases, neither one of the type-II gene mutations in *NPM1*, *CEBPA* and *MLL*, or one of the 'fusion gene' type-II aberrations [i.e. *MLL*-rearrangements, t(8;21)(q22;q22), inv(16)(p13q22)/t(16;16)(p13;q22), t(15;17)(q22;q21), t(7;12)(q36;p13) and t(6;9)(p23;q34)] were identified (Figure 1A). This percentage, however, is probably slightly lower (estimated approximately 33%) as we could only screen 118 of these 180 cases for *NPM1*, and 101 of the 180 cases for *CEBPA* and *MLL*-PTD mutations (Table 2).

Characteristics of pediatric AML patients with type-I aberrations

For the classical type-I aberrations [i.e. mutations in *FLT3*, *N-* and *K-RAS*, *PTPN11* and *KIT*], we found the following frequencies: *FLT3/*ITD (18%; 67/372), *FLT3/*TKD (3%; 9/330), *N-RAS* (16%; 57/353), *K-RAS* (4%; 13/353), *PTPN11* (2%; 7/350) and *KIT* (8%; 31/368) (Table 1B). *WT1* mutations, which were arbitrarily categorized as type-I aberrations for this study, were found in 9% (29/330). Together, we identified type-I aberrations in 185/330 (56%) cases

TABLE 2. Overview of the association between type-I and type-II aberrations in pediatric AML.

	Type-I aberrations*								
	FLT3/ITD (n=372)			*FLT3*/TKD (n=330)			*KIT* (n=368)		
Type-II aberrations	mut (%)	mut (n)	wt (n)	mut (%)	mut (n)	wt (n)	mut (%)	mut (n)	wt(n)
MLL-rearrangements	3	2	93	1	1	80	4	4	92
t(8;21)	5	2	40	6	2	31	31	14	31
inv(16)	5	2	37	3	1	34	28	11	29
t(15;17)	64	14	8	10	2	19	-	0	22
t(7;12)	-	0	7	-	0	7	14	1	6
t(6;9)	33	2	4	-	0	6	-	0	6
NPM1 mut	39	10	16	-	0	25	4	1	23
CEBPA double mut	18	3	14	-	0	17	-	0	17
MLL-PTD	57	4	3	17	1	5	-	0	6
Other/unknown type-II**	25	28	83	2	2	97	-	0	105
All cases	18	67	305	3	9	321	8	31	337

Abbreviations: mut indicates mutations; wt, wild type

* type-I aberrations were mutually exclusive, except for *FLT3*/ITD & *N-RAS* (n=2), *FLT3*/TKD & *N-RAS* (n=1), *KIT* & *N-RAS* (n=1), *N-RAS* & *PTPN11* (n=1) and *N-RAS* & *K-RAS* (n=1).

** the following numbers were screened for molecular type-II aberrations in the other/unknown type-II group: *CEBPA* (n=101/180), *NPM1* (n=118/180) and *MLL*-PTD (n=101/180).

****WT1* mutations overlap with *FLT3*/ITD (n=12), *FLT3*/TKD (n=3), *N-RAS* (n=6) and *K-RAS* (n=1).

(Figure 1B). Regarding sex distribution of the different type-I aberrations, *K-RAS* mutations were significantly associated with the male sex (85% vs. 54% of *K-RAS* wild-type cases; p=0.03). *FLT3*/ITD-positive and *WT1*-mutated AML cases had a significant higher WBC (median $74x10^9$/L; p<0.001, and $86x10^9$/L; p=0.03, respectively) compared to their wild-type counterparts. Patients with *FLT3*/ITD-positive AML had a significant higher median age (10.1 yrs; p=0.008) compared to their wild-type counterparts (Table 1B).

Non-random associations between type-I and type-II aberrations in pediatric AML

An overview of the associations between the type-I and type-II aberrations is presented in Table 2. Although *FLT3*/ITD mutations were identified in almost all type-II defined subtypes, the majority (42%) was restricted to cases with t(15;17)(q22;q21), *MLL*-PTD and *NPM1* mutations, in which respectively 64%, 57% and 39% harbored an *FLT3*/ITD (Figure 2). Of note, *FLT3*/ITD was also simultaneously present in 41% of the *WT1*-mutated cases. For *FLT3*/TKD mutations, which were far less frequent in pediatric AML, the highest frequencies were found in the *MLL*-PTD (17%) and t(15;17)(q22;q21) (10%) subtypes. *KIT* mutations associated clearly with core-binding factor AML (CBF-AML) [i.e. t(8;21)(q22;q22) and inv(16)(p13q22)/t(16;16)(p13;q22)] (p<0.001). They were observed in 31% of t(8;21)(q22;q22) cases and in 28% of the inv(16)(p13q22)/t(16;16)(p13;q22) cases (Figure 2). Mutations in

TABLE 2. Continued (from left to right).

Type-I aberrations*											
N-RAS (n=353)			*K-RAS* (n=353)			*PTPN11* (n=330)			*WT1* (n=330)***		
mut (%)	mut (n)	wt (n)	mut (%)	mut (n)	wt (n)	mut (%)	mut (n)	wt(n)	mut (%)	mut (n)	wt(n)
18	17	76	8	7	86	1	1	90	1	1	71
11	4	32	3	1	35	-	0	37	-	0	37
19	7	30	8	3	34	3	1	36	3	1	36
-	0	22	-	0	22	5	1	21	5	1	21
-	0	7	-	0	7	14	1	6	-	0	4
33	2	4	-	0	6	-	0	6	33	2	4
23	6	20	-	0	26	-	0	24	-	0	24
12	2	15	-	0	17	-	0	17	18	3	14
17	1	5	-	0	6	-	0	6	-	0	6
18	18	85	2	2	101	3	3	100	20	21	84
16	57	296	4	13	340	2	7	343	9	29	301

N-RAS, K-RAS and *PTPN11*, together combined as RAS-pathway activating mutations, showed an equal distribution among the patients with different type-II aberrations when taken together. The exception was the t(15;17)(q22;q21) subtype, in which no RAS-pathway mutations were observed, except for only one case with a *PTPN11* mutation. Interestingly, *K-RAS* mutations, which were four times less frequent than *N-RAS* mutations, occurred preferably in *MLL*-rearranged AML and in CBF-AML, while *N-RAS* mutations were most prevalent in t(6;9)(p23;q34) and *NPM1*-mutated AML. In *MLL*-rearranged AML, 43% carried one of the investigated type-I aberrations, of which the majority were RAS-pathway aberrations (33%; Figure 2). *WT1* mutations were predominantly present in t(6;9)(p23;q34)-AML (33%), *CEBPA* double-mutant AML (18%) and the subtype with 'other/unknown' type-II aberrations (20%).

The distribution of genetic aberrations is highly correlated with age in pediatric AML

We investigated the frequency of genetic subtypes, according to the age categories 0-2 yrs, 2-5 yrs, 5-10 yrs, 10-15 yrs, and 15 yrs and older (Online Supplementary Figures S1A and S1B). The largest differences in genetic aberrations were detected between children <2 yrs and children ≥2 yrs of age, and therefore we focused further on these two age groups (Online Supplementary Figures S1C and S1D). In children <2 yrs, significant higher frequencies of *MLL*-rearrangements and complex karyotypes were detected when compared to children ≥2 yrs (51 vs. 18%; p<0.001, and 13 vs. 4%; p=0.001, respectively). Furthermore, the youngest age category enclosed all seven patients with t(7;12)(q36;p13) (p<0.001). In contrast, in this age category t(8;21)(q22;q22) was not observed, but this translocation was found in 15% (p<0.001) of the children ≥2 yrs. t(15;17)(q22;q21) was only present in a single case <2 yrs

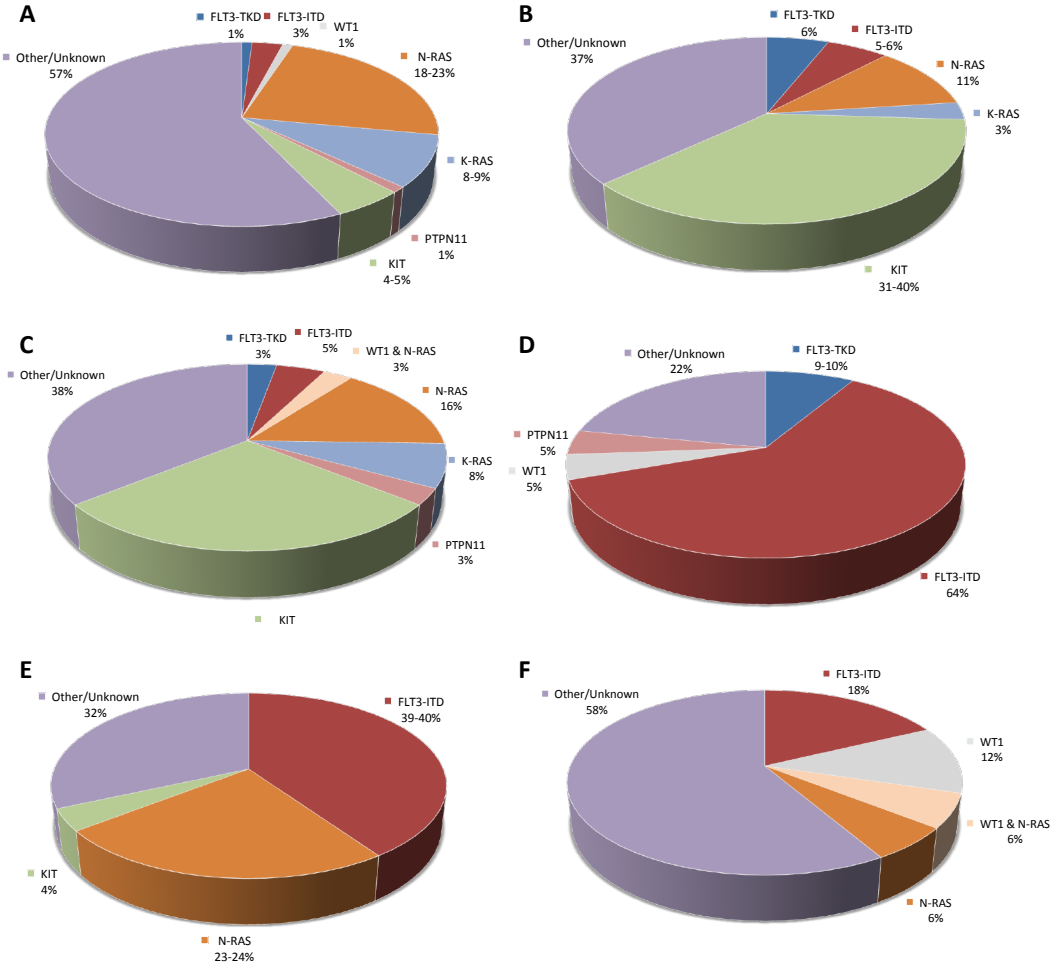

FIGURE 2. Type-I aberrations per type-II-defined subtype. Distribution of the different type-I aberrations according to the different type-II-defined subtypes including >10 cases, i.e. (A): *MLL*-rearrangements, (B): t(8;21), (C): inv(16), (D): t(15;17), (E): *NPM1*-mutated and (F): *CEBPA* double mutated AML. (A color version of this figure can be found in the appendices.)

(1.9 yrs; 1%), but occurred significantly more often in children ≥2 yrs (7%; p=0.04). Furthermore, in children ≥2yrs, a higher frequency of CN-AML was found compared to children <2 yrs (respectively 19 vs. 6%, p=0.002) (Online Supplementary Figure S1C). *NPM1*, *CEBPA* and *MLL*-PTD aberrations were not detected in any of the patients <2 yrs of age. In both age categories (<2 yrs and ≥2 yrs) the percentage of 'other/unknown' type-II aberrations was about one third of the AML's, but the distribution of the different type-II aberrations was clearly different (Figure 3A).

In children ≥2 yrs, a significantly higher frequency of *FLT3*/ITD was found (21 vs. 3% in children <2 yrs; p=0.001), and a trend was observed for a higher frequency of *WT1* muta-

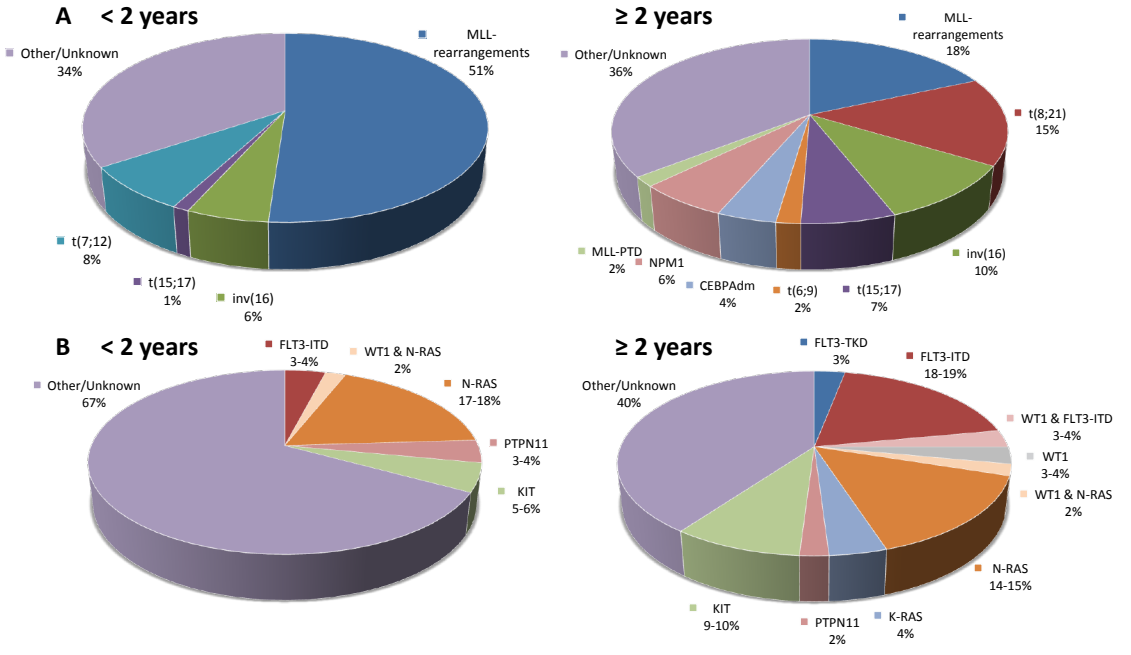

FIGURE 3. Distribution of the different type-I and type-II aberrations according to age. The largest differences in the frequencies of type-II (A) and type-I (B) aberrations were found between children older and younger than 2 years. (A color version of this figure can be found in the appendices.)

tions in patients ≥2 yrs of age (10 vs. 2% in children <2 yrs; p=0.06). Furthermore, all *FLT3*/TKD (n=9) and *K-RAS* mutations (n=13) were found in children ≥ 2 yrs, although this did not reach statistical significance. In contrast, both age categories enclosed almost similar frequencies of *N-RAS* (15-20%), *PTPN11* (2-3%) and *KIT* mutations (5-10%) (Figure 3B, Online Supplementary Figure S1D). When *RAS*-pathway aberrations were taken together, this pathway was affected in a similar frequency in both age categories (22 vs. 21%, in children < 2 yrs and ≥2 yrs of age, respectively). In children <2 yrs, 67% of the cases did not harbor one of the investigated type-I aberrations (i.e. *FLT3*/ITD, *FLT3/*TKD, *N-RAS*, *K-RAS*, *PTPN11, KIT* or *WT1*) vs. only 40% in children ≥2 yrs (p<0.001). The difference between these age categories could largely be explained by the frequency of *FLT3*/ITD, which was only sporadically found in children <2 yrs (Figure 3B).

Clinical outcome of pediatric AML according to type-I and type-II aberrations

Survival analysis was performed only for the type-I and type-II-defined subtypes containing more than ten cases. For the type-II aberrations, this included *MLL*-rearrangements, t(8;21)(q22;q22), inv(16)(p13q22)/ t(16;16)(p13;q22), t(15;17)(q22;q21), as well as *NPM1*- and *CEBPA*-double mutated cases. All other cases were grouped together as 'other/unknown' type-II aberrations (Table 3A). The Kaplan-Meier curves showed large differences between the different type-II aberrations for 5 yr-pOS, 5 yr-pEFS, and 5 yr-CIR (Figures 4A, 4B and 4C).

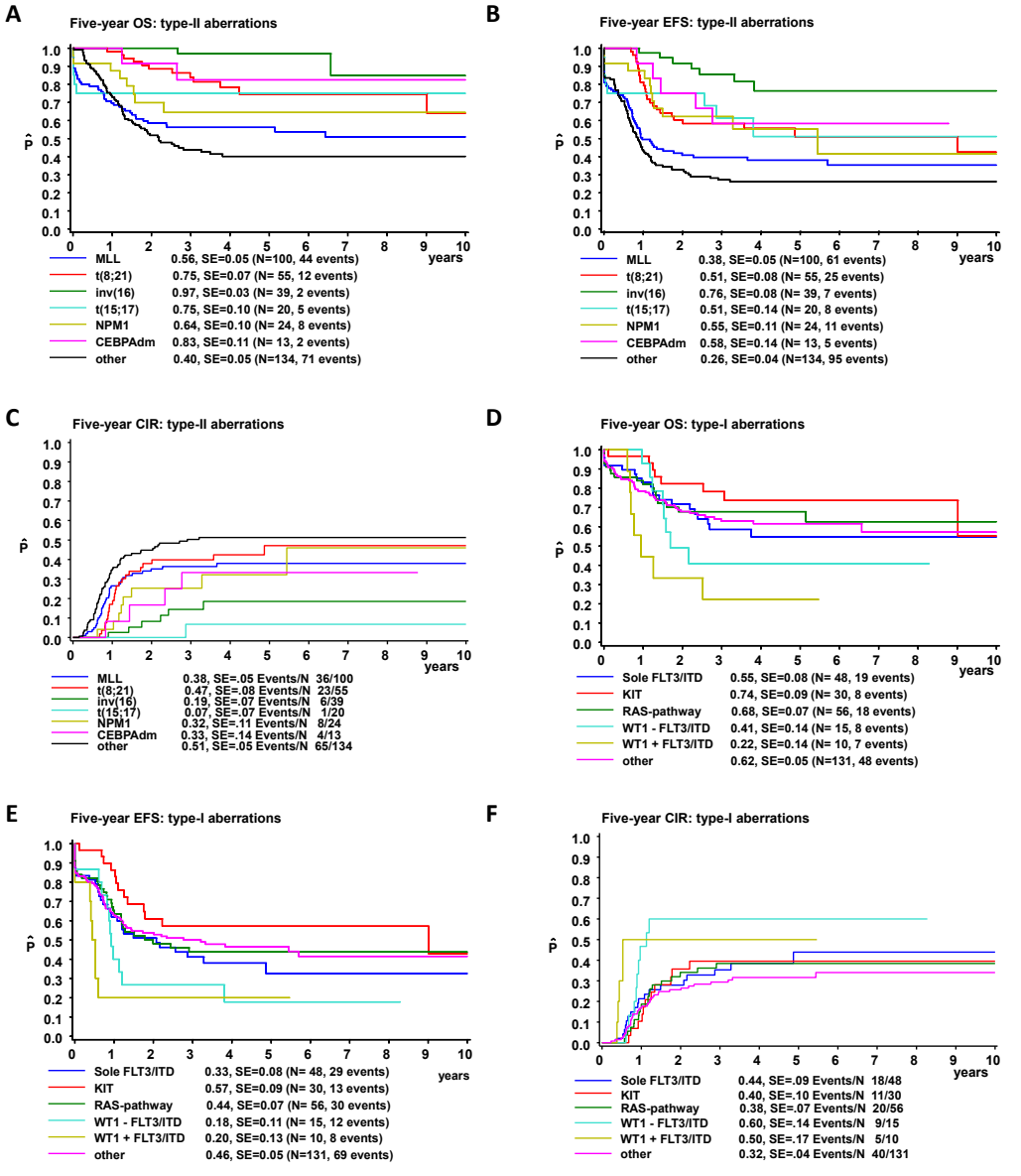

FIGURE 4. Survival analysis of the type-I and type-II aberrations in pediatric AML. Kaplan-Meier estimates for pOS (A+D), pEFS (B+E) and CIR (C+F) for the different type-II and type-I aberrations, respectively. (A color version of this figure can be found in the appendices.)

Patients carrying an inv(16)(p13q22)/t(16;16)(p13;q22)-AML showed the most favorable outcome with a 5 yr-pOS, 5 yr-pEFS, and 5 yr-CIR of 97±3%, 76±8% and 19±7%, respectively. *MLL*-rearranged AML and the group with 'other/unknown' type-II aberrations showed the worst outcome with 5 yr-pOS of 56±5% and 40±5%, respectively, 5 yr-pEFS of 38±5% and 26±4%, respectively, and 5 yr-CIR of 38±5% and 51±5%, respectively. Interestingly, cases

TABLE 3A. Univariate analysis of survival parameters in pediatric AML.

	pEFS			pOS		
	Hazard Ratio	95% CI	*P* value	Hazard Ratio	95% CI	*P* value
Type-II aberration						
MLL-rearrangement	1.3	1.0-1.8	0.06	1.4	1.0-2.0	0.05
t(8;21)(q22;q22)	0.7	0.4-1.0	0.05	0.4	0.2-0.8	0.005
inv(16)(p13q22)	0.2	0.1-0.4	<0.001	0.1	0.0-0.4	0.001
t(15;17)(q22;q21)	0.7	0.3-1.3	0.24	0.7	0.3-1.7	0.44
NPM1 mutation	0.7	0.4-1.2	0.19	0.8	0.4-1.7	0.61
CEBPA double mutation	0.5	0.2-1.2	0.13	0.3	0.1-1.2	0.10
Type-I aberration						
FLT3/ITD	1.3	0.9-1.9	0.17	1.3	0.9-2.0	0.21
WT1 mutation	2.1	1.3-3.4	0.002	2.0	1.2-3.5	0.01
WT1 mutation & *FLT3*/ITD	1.1	1.0-1.1	0.009	1.1	1.0-1.2	0.007
RAS-pathway mutation	1.0	0.7-1.4	0.89	0.8	0.5-1.4	0.47
KIT mutation	0.6	0.4-1.1	0.11	0.6	0.3-1.3	0.18
WBC						
>50x10^9/L	1.4	1.0-1.8	0.03	1.6	1.2-2.3	0.006
Age						
≥2 years	0.9	0.7-1.3	0.72	0.8	0.6-1.2	0.37
≥10 years	0.9	0.7-1.1	0.31	0.9	0.6-1.2	0.47

Abbreviations: pEFS indicates probability of event-free survival; pOS, probability of overall survival; 95% CI, 95% confidence interval; WBC, white blood cell count at diagnosis

TABLE 3B. Multivariate analysis of survival parameters in pediatric AML

	pEFS			pOS		
	Hazard Ratio	95% CI	*P* value	Hazard Ratio	95% CI	*P* value
Type-II aberration						
Favorable karyotype	0.3	0.2-0.5	<0.001	0.2	0.1-0.5	<0.001
NPM1 mutation	0.4	0.2-0.9	0.02	0.6	0.3-1.4	0.21
CEBPA double mutation	0.3	0.1-0.8	0.02	0.2	0.1-0.9	0.03
MLL-rearrangement	1.2	0.7-1.9	0.58	1.5	0.9-2.6	0.16
Type-I aberration						
FLT3/ITD	1.2	0.8-2.0	0.40	1.3	0.7-2.3	0.43
WT1 mutation	1.7	0.8-3.3	0.14	1.7	0.8-3.8	0.17
WT1 mutation & *FLT3*/ITD	1.0	0.9-1.1	0.62	1.0	0.9-1.1	0.78
WBC						
>50x10^9/L	1.2	0.8-1.7	0.40	1.5	0.9-2.3	0.09
Age						
≥10 years	1.2	0.9-1.8	0.27	1.3	0.8-1.9	0.32

Abbreviations: pEFS indicates probability of event-free survival; pOS, probability of overall survival; 95% CI, 95% confidence interval; WBC, white blood cell count at diagnosis; favorable karyotype includes t(8;21)(q22;q22), inv(16)(p13q22) and t(15;17)(q22;q21).

with a t(8;21)(q22;q22) had a relatively high 5 yr-CIR of 47±8%, which seemed to be related to concurrent *KIT* mutations, although numbers were too small to draw definitive conclusions. Cases with a t(15;17)(q22;q21) only had a 5 yr-CIR of 7±7%. For survival analysis of the type-I aberrations, cases with various *RAS*-pathway aberrations were combined. *WT1*-mutated AML cases were analyzed according to their *FLT3*/ITD status. All other cases were grouped together as 'other/unknown' type-I cases for the analysis. The Kaplan-Meier curves showed differences in 5 yr-pOS, 5 yr-pEFS, and 5 yr-CIR for the different type-I aberrations (Figures 4D, 4E and 4F). Cases with a combined *WT1* mutation and *FLT3/*ITD showed the worst prognosis with 5 yr-pOS of 22±14%, 5 yr-pEFS of 20±13%, and 5 yr-CIR of 50±17%.

Independent prognostic factors in pediatric AML

In order to reduce the number of variables in a multivariate Cox proportional hazard model, CBF-AML and t(15;17)(q22;q21) were grouped together as the variable 'favorable karyotype' (Table 3B). The following variables, which were significant in univariate analyses and/or commonly used in pediatric AML (WBC>50 and age ≥10 years), entered the model: 'favorable karyotype', *MLL*-rearrangements, *NPM1* mutations, *CEBPA* double mutations, *FLT3*/ITD, *WT1* mutations, the combination of *WT1* mutation plus an *FLT3*/ITD, WBC and age ≥10 years. This model identified favorable karyotype (hazard ratio (HR) 0.3, p<0.001), *NPM1* mutations (HR 0.4, p=0.02) and CEBPA double mutations as (HR 0.3, p=0.02) as independent prognostic factors for pEFS. For pOS, favorable karyotype was an independent prognostic factor (HR 0.2, p<0.001). Furthermore, *CEBPA* double mutations (HR 0.2, p=0.03) independently predicted favorable pOS.

DISCUSSION

Genetic unraveling of pediatric AML provides a basis for improvement of risk-group stratification. Furthermore, specific genetic aberrations may direct the development of targeted therapy approaches. The low incidence of pediatric AML makes it difficult to describe the relevance of these aberrations, and published data often focus on only one specific aberration. This study describes for the first time a large cohort of pediatric AML cases characterized for various cytogenetic and molecular genetic aberrations, enabling to comprehensively study non-random associations, and their correlation with clinical characteristics and outcome.

We confirmed the non-random associations previously described between the different types of aberrations, e.g. *KIT* mutations with CBF-AML and *FLT3*/ITD with t(15;17)(q22;q21).[40-41] Moreover, *FLT3*/ITD significantly associated with *NPM1*-mutated (39%) and with *WT1*-mutated AML (41%). It is interesting to note that the association of *FLT3*/ITD was not correlated with a specific type of *NPM1* or *WT1* mutation (data not shown). As AML is likely to result from a multistep pathogenesis, it is conceivable that *FLT3*/ITD and *WT1* mutations are associated with additional aberrations, and recently it has been shown that their

combination is frequently present in the rare subtype of adult AML harboring *NUP98*-rearrangements.[42] Overall, the majority of type-I aberrations displayed an unequal distribution over the different type-II-defined subtypes. Although *MLL*-rearranged AML harbored one of the lowest frequencies of type-I aberrations (43%), mutations in the RAS-signaling pathway interestingly represented the vast majority in *MLL*-rearranged AML.

Striking differences in genetic subtypes were found between children younger and older than 2 years at diagnosis of AML. Very young children with AML were characterized by a high frequency of *MLL*-rearrangements (51%), as previously reported.[5] Furthermore, they were characterized by a higher frequency of complex karyotypes, the exclusive presence of t(7;12)(q36;p13), and low frequencies or even total absence of t(8;21)(q22;q22), t(15;17)(q22;q21) and CN-AML. Moreover, the increasing incidence of CN-AML in childhood is continued into adulthood, in which CN-AML is present in approximately 45% of AML cases, whereas *MLL*-rearrangements are rare in adult AML.[43] We did not observe differences in outcome between the age categories <2 yrs and ≥2 yrs. A recent large German study showed in more detail that adolescents (13-21 years) had a slightly inferior outcome compared to younger children, but no difference was seen between infants (0-2 years) and young children (2-13 years).[44] Although this does not suggest a benefit of different treatment strategies based on age, it is conceivable that the biological differences may lead to different treatment strategies for these age categories in the future.

Besides the different frequencies of several cytogenetic aberrations between pediatric AML and adult AML as described above, type-II gene mutations also displayed different frequencies *within* pediatric AML as well as *between* pediatric and adult AML. *NPM1* mutations, *CEBPA* double mutations and *MLL*-PTD did not occur in children below the age of 2 years. In line with this observation, *NPM1* mutations and *MLL*-PTD are less frequent in pediatric compared to adult AML (5-8% and 1-3% vs. 35% and 3-6%, respectively). In contrast, *CEBPA* double mutations display relatively similar frequencies (3-6% vs. 4-10%) between children and adults. With regard to type-I aberrations, pediatric AML cases harbor less frequently *FLT3*/ITD and *FLT3*/TKD, but RAS-pathway aberrations (*PTPN11*, *N-RAS* and *K-RAS* mutations) and *KIT* mutations have comparable frequencies.[45] *WT1* mutations seem to occur at a higher frequency in pediatric vs. adult AML, i.e. 8-12% vs. approximately 5-7%, respectively.

Different type-I and type-II aberrations clearly had an impact on clinical outcome. In addition to the established favorable prognostic cytogenetic group including t(8;21)(q22;q22), inv(16)(p13q22)/t(16;16)(p13;q22), and t(15;17)(q22;q21), the type-II gene mutations *NPM1* and *CEBPA* double mutations conferred independent prognostic relevance in pediatric AML among the other prognostic factors. Herewith the established favorable cytogenetic risk group in pediatric AML can be extended with the molecular aberrations *NPM1* and *CEBPA* double mutations, and will now comprise approximately 35-40% of pediatric AML cases. *MLL*-rearrangements did not have impact on clinical outcome, which is in concordance with our recent report that not *MLL*-rearrangements per se, but the specific *MLL*-translocation partners independently predict prognosis.[46] Regarding the type-I aberrations,

WT1 mutations and the combination of a *WT1* mutation and *FLT3*/ITD characterized poor prognostic subgroups in univariate analyses. These aberrations could not be shown to have independent prognostic significance in multivariate analyses, which might be influenced by the small numbers. We previously showed that this group with combined *FLT3*/ITD plus a *WT1* mutation had a dismal 5-year survival of 21%,[13] and this was confirmed by a large pediatric AML study from the COG.[12] *FLT3*/ITD did not confer prognostic value in our study, which might be influenced by the mutant/wild-type ratio, which has been previously shown to largely impact on the prognostic impact,[16] or by its association with other -favorable- aberrations such as t(15;17)(q22;q21) and *NPM1* mutations. The investigation of the impact of the different type-I aberrations *within* specific subtypes of AML was restricted by small numbers, although *KIT* mutations seemed to be associated with the relatively high relapse rate in t(8;21)(q22;q22). However, recently, Pollard et al. showed in a large COG series that *KIT* mutations lacked prognostic significance in pediatric CBF-AML in contrast to adult CBF-AML.[47-48] This shows that further risk-stratification in pediatric AML based on genetic aberrations has to be further validated by prospective pediatric studies.

Our study has implications for diagnostics in pediatric AML, and on the basis of their frequency, impact on outcome, and possible target for therapy, we would currently suggest to screen for the fusion genes t(8;21)(q22;q22), inv(16)(p13q22)/t(16;16)(p13;q22), t(15;17)(q22;q21), and *MLL*-rearrangements (specifically t(6;11)(q27;q23), t(10;11)(p11.2/p12;q23) and t(1;11) (q21;q23)),[46] and for *NPM1*, *CEBPA*, *WT1* and *FLT3*/ITD mutations, as well as *KIT* mutations in CBF-AML.

Current pediatric AML treatment protocols consist of very intensive chemotherapy regimens, thereby inducing considerable toxicity. To further improve outcome in pediatric AML, new treatment strategies are needed. Different compounds targeting type-I aberrations are currently in development. The poor prognostic group combining an *FLT3*/ITD and a *WT1* mutation may potentially benefit from simultaneously targeting these aberrations. Activated *FLT3* can be targeted by compounds such as midostaurin, lestaurtanib, sorafenib, and other multi-targeted tyrosine kinase inhibitors.[49-50] However, so far monotherapy with these agents in adult AML have shown limited clinical activity.[51-52] In combination with chemotherapy, up-regulation of FLT3-ligand might be a newly identified resistance mechanism.[53] Moreover, a recent randomized placebo-controlled trial of sorafenib did not show benefit for patients in the experimental arm.[54] Whether this strategy turns out to be successful needs therefore to be awaited. Compounds targeting *WT1* mutations are currently not available. Still, high expression of the *WT1* gene is found in most AML cases, and all *WT1*-mutated cases show high *WT1* expression.[55] Immunotherapy using a WT1-peptide vaccine is being developed, and a phase-2 trial in adult AML showed promising results.[56] In general, due to the different cooperating genetic events in AML, monotherapy as with imatinib in CML (where a single fusion gene drives the disease) seems not feasible,[49] and combinations of inhibitors may be required to efficiently kill the leukemic cells.

Intriguingly, in approximately 44% and 33-36% of pediatric AML cases, none of the investi-

gated type-I or type-II aberrations respectively were present. It needs to be mentioned that we may have missed mutations outside the screened hotspot regions, although from previous studies we expect them to be relatively rare. Furthermore, *RUNX1* mutations were not determined, but a recent report suggest they are infrequent events in pediatric AML.[57] In the last decade, efforts have been made to identify the remaining genetic aberrations with high-throughput screening techniques, e.g. by genome-wide copy number analyses (using high resolution array-comparative genome hybridization (A-CGH) and single nucleotide polymorphism (SNP)-arrays), and by re-sequencing candidate genes such as all kinase-coding genes.[28,58-60] Although the former led to the discovery of *ASXL1* and *TET2* mutations, it also revealed that AML harbored only a small number of genomic alterations compared with other cancers.[60] High-throughput sequencing of the first whole genomes of adult AML identified mutations in the metabolites *IDH1* and *IDH2*, and recently in the DNA methyltransferase gene *DNMT3A*, which both appeared to frequently present in adult AML.[61-62] Interestingly, in concordance with the hypothesis that AML results from a multistep pathogenesis, these aberrations might add an additional class of mutations as aberrant *TET2*, *IDH1* and *-2* and *DNMT3A* have been shown to affect the epigenetic landscape of AML. Recent studies however indicated that these mutations might be rare in pediatric AML,[63-65] stressing the need for separate pediatric studies to discover the remaining genetic aberrations, including aberrations in miRNA-coding genes or in methylation of genes or their promoter regions.

In conclusion, the heterogeneity of pediatric AML is reflected by the presence of different age-dependent and clinically relevant genetic aberrations, enabling to identify prognostic relevant groups. In addition, several non-random associations between genetic aberrations are present. The addition of these aberrations will help us to stratify pediatric AML and to direct further development towards targeted therapies.

ACKNOWLEDGEMENTS

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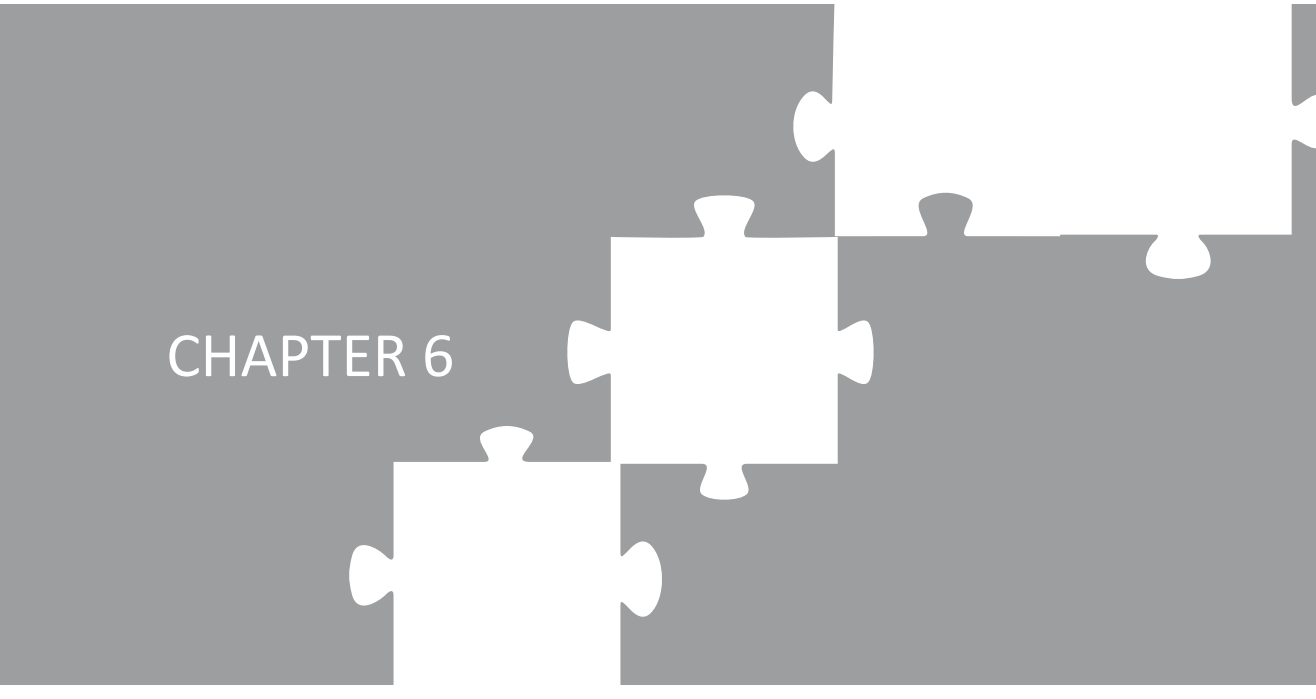
PART TWO


CHAPTER 6

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

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ABSTRACT

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 two different mutations, two had a homozygous mutation, and two had a mutation plus a *WT1* deletion. *WT1* mutations clustered significantly in the CN-AML subgroup (22%; p<0.001) and were associated with *FLT3*/ITD (43 vs. 17%; p<0.001). *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=0.002; 5-year probability of event-free survival (pEFS) 22 vs. 46%, p<0.001; and cumulative incidence of relapse or nonresponse (CIR) 70 vs. 44%, p<0.001). Patients with both a *WT1* mutation and an *FLT3*/ITD had a dismal prognosis (5-year pOS 21%). In conclusion, *WT1* mutations occur at a significant rate in childhood AML and are a novel independent poor prognostic marker.

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*, *CEBPA*, 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 when 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 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]

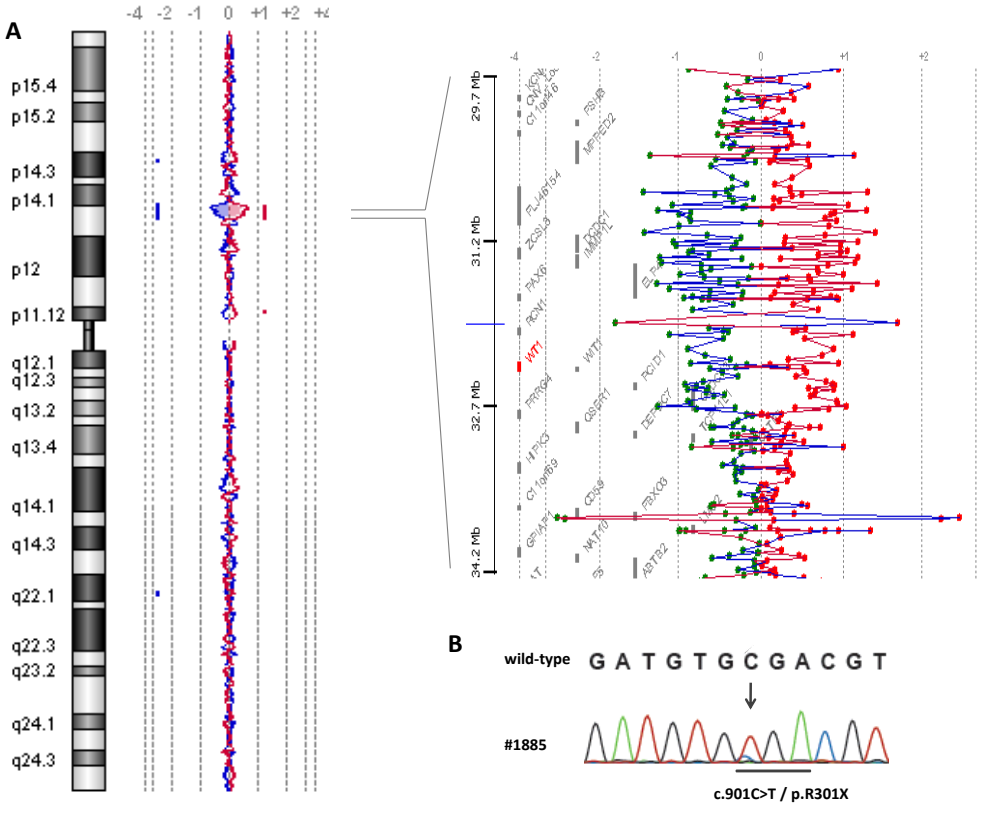


FIGURE 1. *WT1* aberrations in patient #1885 detected by array-CGH and sequencing.
(A) The left part of the figure shows a chromosome 11 ideogram and corresponding oligonucleotide array-CGH plot of the ratio of patient #1885 DNA and control DNA (blue tracing) versus the dye-swap experiment (red tracing) from an array-CGH experiment (105K oligonucleotide array-CGH platform, Agilent technologies, Palo Alto, USA). 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 #1885 showing a c.901C>T nonsense mutation translating into a termination codon on the remaining *WT1* allele.
(A color version of this figure can be found in the appendices.)

To determine 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 according to local laws and regulations, and informed consent was obtained in accordance with the Declaration of Helsinki. Each national study group 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 Online Supplementary Methods.

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 Life Technologies, 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 transcriptase polymerase chain reaction (RT-PCR) and/or fluorescent *in situ* hybridization (FISH).

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 four to five blocks of intensive chemotherapy, using a standard cytarabine and anthracycline backbone. Stem cell transplantation (SCT) in first complete remission (CR1) was used only in selected high-risk patients.

Mutation analysis of *WT1*

For mutation analysis of exons 7 to 10 of the *WT1* gene, genomic DNA was PCR-amplified using specific primers described in Online Supplementary Table S1. The following PCR conditions were used: 2 minutes at 50°C, 10 minutes at 95°C, 40 cycles of 15 seconds 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 to 6 were screened in a cohort of 68 patients, which included 28 patients with *WT1* mutations in exons 7 to 10, and 40 patients with wild-type *WT1* for exons 7 to 10. For mutation analysis of exons 1 to 6, the purified DNA was subjected to 41 cycles of PCR using a touchdown approach of 30 seconds at 94°C, 30seconds at 66-54°C (1x 66°C, 2x 64°C, 3x62°C, 4x60°C, 5x58°C, 6x 56°C and 20x 54°C) and 30 seconds at 72°C using (M13-tagged) primers also described in Online Supplementary 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 Online Supplementary 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 (MLPA)

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/pages/p118pag.html). 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, USA). 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 *CEBPA*, *FLT3*/ITD and TKD, *NPM1*, *N-RAS, K-RAS*, *PTPN11* and *KIT*, was performed as previously described.[6,22-25] The de-

tection 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 to 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, USA).

Statistical analysis

To compare categorical variables we used $\chi 2$ analysis and the Fisher's 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: 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's test. The independence of prognostic factors was examined by multivariate Cox regression analysis. *P* values less than 0.05 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 PML-RARα (n=15), secondary AML (n=8), treatment without curative intent (n=1), and patients lost to follow-up (n=3) were excluded. The *WT1*-mutated and *WT1* wild-type AML patients were equally distributed over the different treatment protocols (p=0.43) and there was no significant difference between the treatment protocols in terms of pEFS and pOS (respectively, p=0.75 and p=0.38). The patient characteristics of the cohort used for survival analysis are shown in Online Supplementary Table S2.

***WT1* mutation analysis**

We first analyzed the samples for mutations in the hotspot areas exons 7 to 10 of the *WT1*

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

	All cases	*WT1*-mutated cases	*WT1* wild-type cases	*P* value
Number (%)	298	35 (11.7%)	263 (88.3%)	
Median age, yr	9.7	9.2	9.7	0.34
<3 yr, n (%)	60	1 (2%)	59 (98%)	
≥3 and <10 yr, n (%)	93	17 (18%	76 (82%)	0.008
≥10 yr, n (%)	145	17 (12%)	128 (88%)	
Sex (% female)	46.6%	48.6%	46.4%	0.81
Median WBC, x10^9/l (range)	38.0 (0.0-534.6)	57.2 (2.5-332.0)	34.1 (0.0-534.6)	0.007
FAB, n (%)				0.60
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%)	<0.001
Median allelic ratio, (range)	0.59 (0.21-2.33)	0.69 (0.32-1.25)	0.58 (0.21-2.33)	0.22
Median ITD length, (range)	54 (18-209)	51 (18-90)	55 (20-209)	0.44
N- or *K-RAS* (n=282), n (%)	63 (22%)	8 (25%)	55 (22%)	0.70
KIT (n=283), n (%)	18 (6%)	0 (0%)	18 (7%)	0.24
CEBPA (n=250), n (%)	20 (8%)	5 (19%)	15 (7%)	0.03
MLL-PTD (n=251), n (%)	6 (2%)	0 (0%)	6 (3%)	1.00
NPM1 (n=293), n (%)	24 (8%)	0 (0%)	24 (9%)	0.09

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

gene. A total of 48 *WT1* gene mutations were detected in 34 of the 298 (11.4%) samples. These mutations predominantly clustered in two areas in exon 7 (n=41), but were also found in exon 8 (n=1) and exon 9 (n=4) (Table 2). In two 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), 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

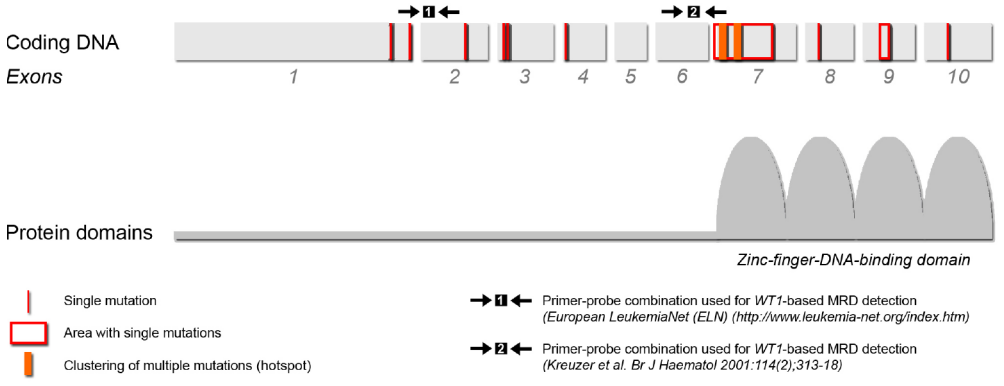


FIGURE 2. Location of the *WT1* gene mutations and their overlap with MRD detection areas.
The location of the *WT1* gene mutations detected in initial diagnosis and relapse childhood AML samples is schematically presented according to the exon structure of the *WT1* gene. Two areas of mutational hotspots 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. (A color version of this figure can be found in the appendices.)

of the *WT1* mutations are schematically presented 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 three cases, silent mutations, which do not result in amino acid changes, were found. In six 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 or 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

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

No*	Mutation(s)**	Exon	Type of mutation	Protein level***	More than one allele affected
1	c.905delGinsCC c.902_938dup37 c.943T>C	7 7 7	frame-shift frame-shift missense	 p.Ser316Pro	yes
2	c.901C>T del11p13, including WT1^	7	nonsense	p.Arg301X	yes
3	c.934_935insA c.979T>C	7 7	frame-shift missense	 p.Tyr327His	yes
4	c.[920_932del13;934C>G]	7	frame-shift		no
5	c.524C>A c.584_585insCCGG	2 3	nonsense frame-shift	p.Ser175X	unknown
6	c.938C>A c.935_939dupCGGTC	7 7	nonsense frame-shift	p.Ser313X	yes
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 + frame-shift	p.Arg301X	yes
11	c.933delA c.1188_1189ins17	7 9	frame-shift frame-shift		yes
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 c.926_935del10ins12	7 7	frame-shift frame-shift		yes
17	c.905_906insTT c.895-1_901GGATGTGC> CAACGGG	7 7	frame-shift frame-shift + affects splice site		yes
18	c.[937_938insG] + [937_938insG]	7	frame-shift		yes
19	c.905_906ins17 c.924_925insGG	7 7	frame-shift frame-shift		yes
20	c.934_935insG	7	frame-shift		no
21	c.924_925insGGTT c.938_939insG	7 7	frame-shift frame-shift		yes
22	c.[933delA; 937_939delTCG;1012T>C] c.937_940dupTCGG	7 7	frame-shift frame-shift		yes
23	c.593delC c.901delCinsGG	3 7	frame-shift frame-shift		unknown

TABLE 2. Continued

No*	Mutation(s)**	Exon	Type of mutation	Protein level***	More than one allele affected
24	c.398insT c.907_908insAT	1 7	frame-shift frame-shift		unknown
25	c.[901_902insG;935G>A]	7	frame-shift		no
26	c.1173_1174insA	9	frame-shift		no
27	c.442_442+2GGT>TTG c.901_902insG	1 7	affects splice site frame-shift		unknown
28	c.1072_1073insC del 11p13, including WT1^^	8	frame-shift		yes
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 c.938C>A c.1006A>G	intron 6/7 7 7	affects splice site nonsense missense	 p.Ser313X p.K336G	yes
34	c.898_929del32	7	frame-shift		no
35	c.900_901insG	7	frame-shift		no

*No indicates patient number; **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.

different between AML samples harboring a *WT1* mutation compared with *WT1* wild-type samples (respectively, 8/35 (23%) vs. 64/263 (24%)).

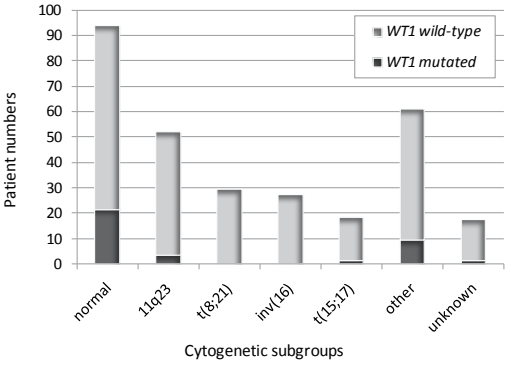
WT1 microdeletions

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

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

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 [t(8;21) and inv(16)], and few were detected in the t(15;17) and in the *MLL* gene rearranged childhood AML cases. Mutations were also found in the 'other karyotype' subgroup, but at a lower frequency (14.8%) than in the CN-AML subgroup (22.3%).



(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 two 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 two mutations, and three samples each had three 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 two 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, two cases with a homozygous mutation, and two 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 nine *WT1*-mutated and nine *WT1* wild-type samples. The median expression (normalized to ABL copies x10000) was $4.1x10^3$ (range, $9.1x10^2$ - $3.6x10^4$) in the *WT1*-mutated samples, which was comparable with the expression measured in the wild-type samples ($2.9x10^3$; range, $4.8x10^2$ - $7.7x10^3$). Three of the nine *WT*-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.

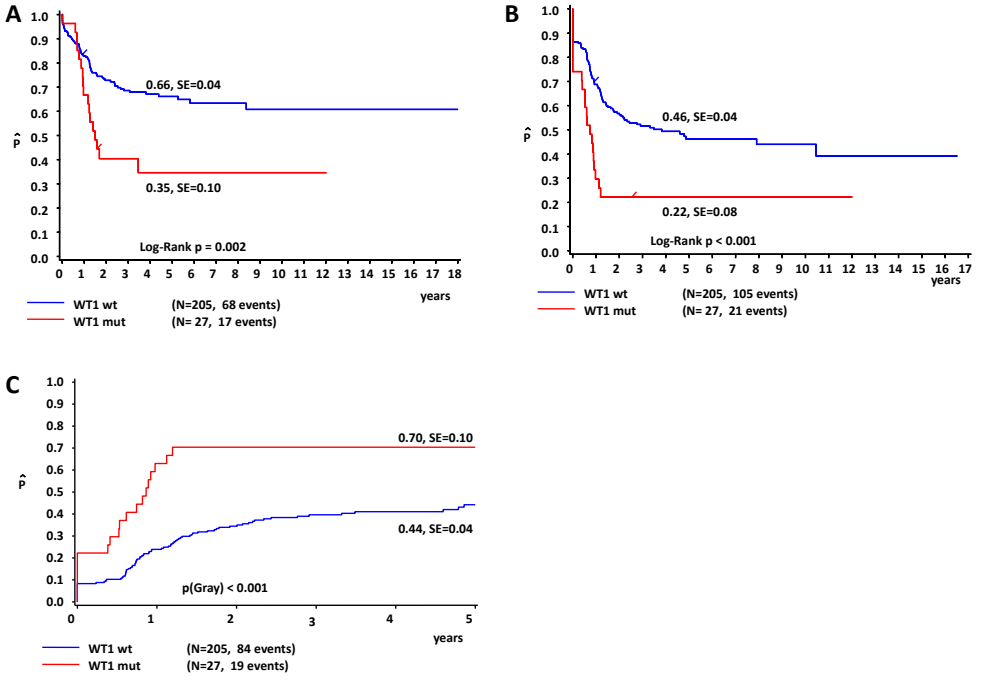


FIGURE 4. Survival curves of childhood AML patients with and without *WT1* mutations.
Kaplan-Meier estimates for 5-year pOS (A) and pEFS (B), showing log-rank *P* values. The 5-year CIR is depicted in (C) and was analyzed according to the Kalbfleisch and Prentice method using Gray's test for statistical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

Paired diagnostic-remission and diagnostic-relapse samples

The *WT1* mutations were not detectable in the remission material of five patients with *WT1*-mutated AML, which suggests 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 two 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%) *WT1* wild-type samples gained a *WT1* mutation at relapse. Two of these patients in fact gained two 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 ≥3 and <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=0.008). The white blood cell count (WBC) at diagno-

Table 3. Five-year OS (A), EFS (B) and CIR (C) according to the combined *WT1* and *FLT3*/ITD mutational status.

A

5-year OS ± SE(%) (n)		*WT1*		*P* value
		wild-type	mutant	
FLT3/ITD	negative	67±4% (n=176)	49±14% (n=14)	0.20
	positive	63±9% (n=29)	21±12% (n=13)	<0.01
P value		0.61	0.09	

B

5-year EFS ± SE(%) (n)		*WT1*		*P* value
		wild-type	mutant	
FLT3/ITD	negative	48±4% (n=176)	29±12% (n=14)	0.07
	positive	34±10% (n=29)	15±10% (n=13)	<0.01
P value		0.32	0.16	

C

5-year CIR ± SE(%) (n)		*WT1*		*P* value
		wild-type	mutant	
FLT3/ITD	negative	42±4% (n=176)	64±15% (n=14)	0.04
	positive	55±13% (n=29)	77±14% (n=13)	0.02
P value		0.27	0.18	

Abbreviations: SE indicates standard error; *WT1, WT1* mutational status (wild-type or mutant); *FLT3*/ITD, *FLT3*/ITD status (positive or negative).

sis was significantly higher in *WT1*-mutated AML patients (median 57.2x10^9/l) than in *WT1* wild-type AML patients (median 34.1x10^9/l; p=0.007). No significant differences between the two groups were found for sex (p=0.81) or for FAB type distribution (p=0.60).

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<0.001). 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, within the subgroup with 'other cytogenetic aberrations' (defined as all other cytogenetic aberrations than the mentioned subgroups), nine 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<0.001), and a *CEBPA* mutation (19 vs. 7%, respectively; p=0.03; Table 2). *WT1* and *NPM1* mutations were mutually exclusive. *WT1* mutations and *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=0.03). However, no difference in *CEBPA* mutations (17% of the *WT1*-mutated vs. 15% of

TABLE 4. Results of multivariate analysis for OS, EFS and RFS.

Outcome	Variable	Hazard ratio (95% confidence interval)	*P* value
OS	Other karyotype*	2.83 (1.68-4.77)	<0.001
	FLT3/ITD	1.89(1.07-3.34)	0.03
	Favorable karyotype	0.39 (0.15-0.96)	0.04
	WT1	1.79 (1.02-3.14)	0.04
EFS	Other karyotype*	2.33 (1.49-3.64)	<0.001
	WT1	2.05 (1.24-3.38)	0.005
	FLT3/ITD	1.82 (1.14-2.92)	0.01
	Favorable karyotype	0.74 (0.41-1.33)	0.31
RFS	Other karyotype*	2.44 (1.48-4.02)	<0.001
	WT1	2.44 (1.42-4.17)	0.001
	FLT3/ITD	1.99 (1.19-3.34)	0.009
	Favorable karyotype	0.84 (0.44-1.58)	0.58

WT1 mutations were tested in a Cox regression model with other well-known risk-factors in childhood AML including favorable cytogenetics [t(8;21, inv(16)], other abnormal karyotypes and *FLT3/*ITD. When including age above 10 years, WBC ≥50x10^9/l, and SCT as time-dependent co-variable the estimates for *WT1* mutations were similar and the *p* values for these three parameters were all above 0.10.

* 'Other karyotype' indicates all other cytogenetic aberrations than the well-known childhood AML subgroups, i.e. normal karyotype and the favorable karyotypes inv(16) and t(8;21).

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=0.01).

The study population included eight patients with secondary AML. Two of these patients (one with AML after a preceding MDS and one with 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=0.89).

The CR rate did not differ significantly between patients with *WT1*-mutated and *WT1* wild-type AML (74.1 vs. 86.3% respectively; p=0.09). However, RD was significantly more frequent in the former group (23.1 vs. 8.8%, respectively; p=0.03). Patients with *WT1*-mutated AML had a significantly worse pOS, pEFS and CIR than *WT1* wild-type AML patients (5-year pOS 35±10% vs. 66±4%; p=0.002; pEFS 22±8% vs. 46±4%; p<0.001; and CIR 70±10% vs. 44±4%; p_{Gray}<0.001, 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=0.02). 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 affected allele (n=14) (data not shown).

As *WT1* mutations were frequently associated with *FLT3*/ITD, we analyzed the impact on

outcome according to the combined mutational status (Table 3). 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% ($p<0.01$) for *WT1* wild-type cases. In the *FLT3*/ITD-negative subgroup, these figures were 49±14% vs. 67±4% ($p=0.20$). 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%; $p=0.05$). There also was a trend for RD to occur more frequently in patients with *WT1*-mutated AML (22 vs. 6%; $p=0.07$). *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%; $p=0.03$, pEFS 32±11% vs. 49±6%; $p=0.02$ and CIR 58±13% vs. 41±7%; $p_{Gray}=0.06$, 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 $50x10^9$/l as cutoff value), cytogenetic subgroups (favorable karyotype [inv(16) and t(8;21)], normal karyotype and other karyotype), *FLT3*/ITD and SCT as time-dependent co-variable. 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; $p=0.041$), pEFS (HR 2.05; 95% CI 1.24-3.38; $p=0.005$) as well as for CIR (HR 2.44; 95% CI 1.42-4.17; $p=0.001$) (Table 4). Inclusion of *NPM1* and *CEBPA* mutations in the model did not change the hazard ratios 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 10-13%.[12-14,28] However, these series were restricted to AML cases with normal cytogenetics (CN-AML). We found a higher percentage (22%) in this particular AML subgroup. It therefore seems that there is a higher frequency of *WT1* gene mutations in childhood AML

when 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 age 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, two other molecular aberrations that also occur frequently within the CN-AML subgroup (*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 two risk factors (i.e. *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 the 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 *CEBPA* mutations; this association disappeared in the CN-AML subgroup. As *CEBPA* mutations are also known to cluster in 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 two 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, also expression was picked up in three samples with mutations exactly located at the binding site of the reverse 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 the 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 an *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.

ACKNOWLEDGEMENTS

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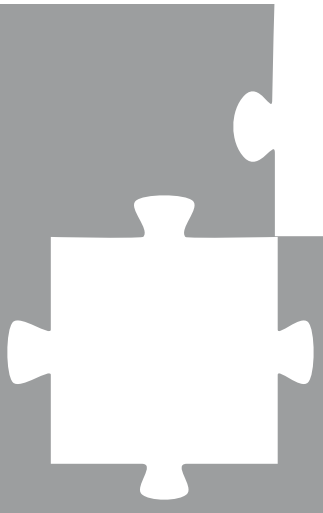
CHAPTER 7



No prognostic impact of the *WT1* gene single nucleotide polymorphism rs16754 in pediatric acute myeloid leukemia

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TO THE EDITOR

With great interest, we read the article by Damm et al,[1] in which the authors describe the prognostic impact of the single nucleotide polymorphism (SNP) rs16754 located in the mutational hotspot of the *WT1* gene in 249 adults with cytogenetically normal acute myeloid leukemia (CN-AML). They found that the presence of one or two minor alleles (*WT1*^{AG}/*WT-1*^{GG}) of SNP rs16754 independently predicted a favorable outcome for these patients, who were intensively treated on two clinical trials (AML SHG 0199 and 0295). This effect was most prominent for high-risk patients (defined as *FLT3* internal tandem duplication (ITD)-positive and/or *NPM1* wild-type). The authors hypothesize that the minor allele of this synonymous SNP might be associated with increased drug sensitivity. No prognostic impact of *WT1* mutations was observed, which is in contrast with most published reports in adult CN-AML.[2-5] Furthermore, *WT1* mRNA expression levels did not predict outcome. Previous reports on the impact of *WT1* expression at diagnosis in AML described conflicting results.[2-5]

We have previously reported the poor prognostic impact of *WT1* mutations in a large series of pediatric AML patients, both in the entire group of patients as well as in those with CN-AML.[6] Moreover, we identified a group with very poor prognosis, characterized by the combined presence of a *WT1* mutation and an *FLT3*/ITD. We also described the presence of SNP rs16754, which was distributed equally between *WT1*-mutated and *WT1* wild-type samples, but we did not report its association with outcome. Furthermore, only a few samples were analyzed for *WT1* mRNA expression in our series. Primed by the paper from Damm et al, we have now performed a full analysis of the characteristics and prognostic impact of *WT1* SNP rs16754 and *WT1* mRNA expression in pediatric AML.

We studied the same cohort of 232 *de novo* pediatric AML bone marrow or peripheral blood samples taken at initial diagnosis, excluding Down syndrome and *PML-RARα* cases.[6] Briefly, patients were intensively treated according to consecutive Dutch Childhood Oncology Group and AML-Berlin-Frankfurt-Münster Study Group treatment protocols between 1987 and 2004. Enrollment of patients with two major alleles (*WT1*^{AA}) versus at least one minor allele (*WT1*^{AG}/*WT1*^{GG}) of SNP rs16754, as well as patients with high versus low *WT1* mRNA expression, were similarly distributed over the treatment protocols. Survival estimates overall survival (pOS) and event-free survival (pEFS) were analyzed by the Kaplan-Meier method and compared using log-rank test, and the cumulative incidence of relapses (CIR) was analyzed by the Kalbfleisch and Prentice method and compared using Gray's test. *WT1* mutations and SNP rs16754 were detected by direct sequencing as described.[6] Affymetrix HGU133 Plus 2.0 microarrays were available for 176 samples. Normalized expression values of probe set 206067_s_at, annotated to the *WT1* gene, showed a good correlation with *WT1* expression values obtained by real-time quantitative polymerase chain reaction[7] (n=34; Spearman's correlation coefficient 0.73; p<0.001). We therefore used normalized expression values of probe set 206067_s_at to determine *WT1* expression. The cases (n=176) were dichotomized at the median *WT1* expression value (median VSN-normalized expres-

TABLE 1. Clinical and molecular characteristics according to *WT1* SNP rs16754 status.

Characteristics	*WT1*$^{AG/GG}$ (n=63)		*WT1*^{AA} (n=169)		*P* value
	n	%	n	%	
Age, years					0.94
Median	9.0		9.7		
Range	0.7-18.5		0.0-18.8		
Sex					0.39
Female	24	38	75	44	
Male	39	62	94	56	
WBC, x10^9/l (n=228)					0.12
Median	31.6		44.0		
Range	0.7-433.0		1.1-534.6		
FAB type (n=230)					0.83
M0	2	3	10	6	
M1	8	13	23	14	
M2	13	21	46	27	
M4	18	30	44	26	
M5	15	25	33	20	
M6	1	2	5	3	
M7	2	3	4	2	
Other	2	3	4	2	
Karyotype					0.41
t(8;21)	9	14	19	11	
inv(16)	6	10	19	11	
11q23	10	16	30	18	
CN-AML	19	30	66	39	
Other	13	21	29	17	
Unknown	6	10	6	4	
NPM1 mut (n=228)	6	10	16	10	0.99
CEBPA mut (n=186)	3	6	12	9	0.76
MLL-PTD (n=187)	1	2	3	2	1.00
FLT3/ITD	8	13	34	20	0.19
RAS mut (n=216)	17	28	35	22	0.36
KIT mut (n=217)	4	7	13	8	1.00
WT1 mut	8	13	19	11	0.76
WT1 mRNA expression (n=176) (VSN-normalized values)					0.60
Median	6.58		6.18		
Range	4.89-8.10		4.45-10.28		

Abbreviations: SNP indicates single nucleotide polymorphism; FAB, French-American-British; CN-AML, cytogenetically normal AML; mut, mutation; VSN, variance and stabilization normalization.

sion 6.27; range, 4.45-10.28).

Sixty-three (27.2%) of the 232 patients carried at least one minor allele of the *WT1* SNP rs16754 (*WT1*^{AG} n=58, *WT1*^{GG} n=5). Three paired remission samples and 10 paired relapse

TABLE 2. Clinical and molecular characteristics according to dichotomized *WT1* mRNA expression.

Characteristics	*WT1* low expression (n=88)		*WT1* high expression (n=88)		*P* value
	n	%	n	%	
Age, years					0.02
Median	8.6		10.8		
Range	0.0-18.5		0.7-18.8		
Sex					0.21
Female	29	33	37	42	
Male	59	67	51	58	
WBC, x10^9/l (n=174)					0.77
Median	40.6		44.4		
Range	2.0-483.0		1.1-377.6		
FAB type (n=174)					<0.001
M0	4	5	4	5	
M1	7	8	14	16	
M2	19	22	24	28	
M4	18	21	29	34	
M5	34	39	8	9	
M6	3	3	0	0	
M7	0	0	5	6	
Other	3	3	2	2	
Karyotype					<0.001
t(8;21)	14	16	12	14	
inv(16)	6	7	19	22	
11q23	29	33	7	8	
CN-AML	12	14	24	27	
Other	19	22	22	25	
Unknown	8	9	4	5	
NPM1 mut (n=175)	4	5	12	14	0.04
CEBPA mut (n=172)	9	11	2	2	0.03
MLL-PTD (n=170)	0	0	4	5	0.12
FLT3/ITD	9	10	19	22	0.04
RAS mut (n=174)	23	26	15	17	0.14
KIT mut (n=175)	7	8	9	10	0.58
WT1 mut	1	1	12	14	0.002

Abbreviations: FAB indicates French-American-British; CN-AML, cytogenetically normal AML; mut, mutation.

samples were available of patients, who carried the minor allele in their diagnostic sample, but no changes in the presence of the minor allele were found. The frequency of the minor allele discovery was 14.7%, which is comparable to that detected in the adult CN-AML cohort.[1] Patients carrying *WT1*^{AG}/*WT1*^{GG} compared with patients carrying *WT1*^{AA} demonstrated a similar distribution of baseline clinical characteristics and cytogenetic and molecular aberrations (Table 1). No difference was observed for the presence of a *WT1* mutation, as re-

ported before.[10] Furthermore, *WT1* expression levels were similar between the two groups. We did not observe differences in outcome between patients carrying $WT1^{AG}$/$WT1^{GG}$ (n=63) and patients carrying $WT1^{AA}$ (n=169) [complete remission (CR) rate 89% vs. 83%, p=0.30; 5-year pOS 59±7% vs. 64±4%, p=0.51; 5-year pEFS 36±7% vs. 48±4%, p=0.32; and 5-year CIR 48±7% vs. 35±4%, p=0.13, respectively]. When we restricted these analyses to patients with CN-AML, we observed a similar lack of favorable prognostic impact of SNP rs16754 [patients carrying $WT1^{AG/GG}$ (n=19) vs. $WT1^{AA}$ (n=66): CR rate 95% vs. 85%, p=0.44; 5-year pOS 55±12% vs. 67±6%, p=0.42; 5-year pEFS 32±11% vs. 51±6%, p=0.37; and 5-year CIR 60±12% vs. 29±6%, p=0.04, respectively].

Regarding *WT1* mRNA expression at diagnosis, patients with high expression (n=88) were older compared with patients with low expression (n=88). Furthermore, *WT1* expression was related to French-American-British morphology and cytogenetic and molecular aberrations as listed in Table 2. However, we observed no difference in outcome, neither for patients with high versus low *WT1* expression in the entire group of AML patients (n=176; pOS p=0.90, pEFS p=0.69), nor for patients with CN-AML (n=36; pOS p=0.47, pEFS p=0.97).

So, in this large pediatric series in which we previously identified *WT1* mutations as a strong independent poor prognostic marker,[6] we did not observe a favorable prognostic impact of *WT1* SNP rs16754, which is different from what was found in adult CN-AML by Damm et al.[1] This may be explained by differences in treatment as the 5-year overall survival in our pediatric patients is almost 20% higher than that reported by Damm et al in the adult series, reflecting that prognostic factors may disappear when higher survival rates are reached. Another explanation might be differences in molecular aberrations between pediatric and adult AML. However, when we analyzed only *NPM1*/*FLT3*/ITD high-risk patients, in whom the most prominent effect was found by Damm et al,[1] we also did not see a prognostic impact (data not shown). Nevertheless, pediatric and adult CN-AML may differ in cooperating molecular aberrations that are still largely unknown. This concept is sustained by the frequency of *NPM1* mutations that is two-fold lower in pediatric CN-AML (20% to 25%) compared with adult CN-AML (50% to 60%).[8] Other yet unknown underlying aberrations could perhaps therefore also influence the prognostic impact of SNP rs16754.

We observed no impact on outcome of *WT1* mRNA expression, similarly to what was found by Damm et al and in concordance with the largest pediatric series to date, recently published by the Childhood Oncology Group.[6]

In conclusion, no prognostic impact of *WT1* SNP rs16754 or *WT1* mRNA expression at diagnosis was found in pediatric AML. Future prospective pediatric and adult studies on *WT1* SNP rs16754 are warranted, however, to confirm these different observations between children and adults with CN-AML and to elucidate underlying mechanisms.

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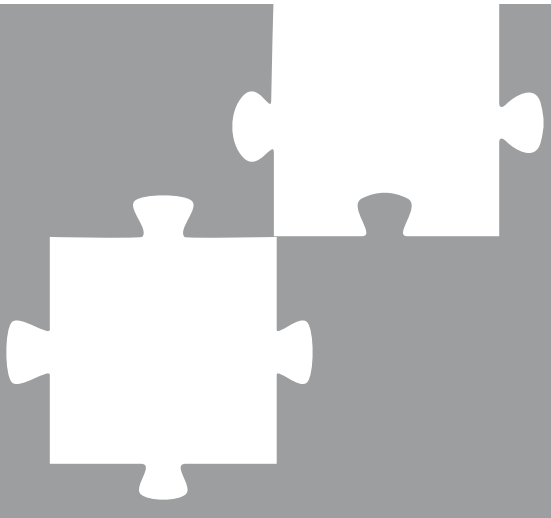
CHAPTER 8



NUP98/NSD1 characterizes a novel poor prognostic group in acute myeloid leukemia with a distinct *HOX* gene expression pattern

Iris H.I.M. Hollink, Marry M. van den Heuvel-Eibrink, Susan T.C.J.M. Arentsen-Peters, Marta Pratcorona, Saman Abbas, Jenny E. Kuipers, Janneke F. van Galen, H. Berna Beverloo, Edwin Sonneveld, Gert-Jan J.L. Kaspers, Jan Trka, Andre Baruchel, Martin Zimmermann, Ursula Creutzig, Dirk Reinhardt, Rob Pieters, Peter J.M. Valk, C. Michel Zwaan

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Supplementary Information accompanies the paper on the Blood website (http://bloodjournal.hematologylibrary.org/)

ABSTRACT

Translocations involving *nucleoporin 98kD* (*NUP98*) on chromosome 11p15 occur at relatively low frequency in acute myeloid leukemia (AML) but can be missed with routine karyotyping. In this study, high-resolution genome-wide copy number analyses revealed cryptic *NUP98/NSD1* translocations in 3 of 92 cytogenetically normal (CN-)AML cases. To determine their exact frequency, we screened more than 1000 well-characterized pediatric and adult AML cases using a *NUP98/NSD1*-specific RT-PCR. Twenty-three cases harbored the *NUP98/NSD1* fusion, representing 16.1% of pediatric and 2.3% of adult CN-AML patients. *NUP98/NSD1*-positive AML cases had significantly higher white blood cell counts (median, $147x10^9$/l), more frequent FAB-M4/M5 morphology (in 63%), and more CN-AML (in 78%), *FLT3*/internal tandem duplications (in 91%) and *WT1* mutations (in 45%) than *NUP98/NSD1*-negative cases. *NUP98/NSD1* was mutually exclusive with all recurrent type-II aberrations. Importantly, *NUP98/NSD1* was an independent predictor for poor prognosis; 4-year event-free survival was below 10% for both pediatric and adult *NUP98/NSD1*-positive AML patients. *NUP98/NSD1*-positive AML showed a characteristic *HOX*-gene expression pattern, distinct from, for example, *MLL*-rearranged AML, and the fusion protein was aberrantly localized in nuclear aggregates, providing insight into the leukemogenic pathways of these AMLs. Taken together, *NUP98/NSD1* identifies a previously unrecognized group of young AML patients, with distinct characteristics and dismal prognosis, for whom new treatment strategies are urgently needed.

INTRODUCTION

Acute myeloid leukemia (AML) is a heterogeneous disease characterized by recurrent genetic aberrations. It is hypothesized that AML results from cooperative but functionally distinct (epi)genetic aberrations.[1-2] Aberrations leading to uncontrolled proliferation and/or survival are classified as type-I, and are often activating mutations in signal transduction molecules, for example, *FLT3/*internal tandem duplications (*FLT3*/ITD). Type-II aberrations primarily block normal differentiation and include the AML-characteristic fusions, for example, PML/RARA, AML1/ETO, and CBFβ/MYH11. In the last decade, novel type-II aberrations were discovered, for example, mutations in *NPM1* and *CEBPA*, and they are mainly found in patients with cytogenetically normal AML (CN-AML).[3-4] Despite this progress, there still is a significant group of AML cases in which the underlying genetic aberrations are unknown.

Type-II aberrations also include translocations of *nucleoporin 98kD* (*NUP98*) located on chromosome 11p15, although they account for less than 1% of AML cases.[5-8] Fusions of *NUP98* to more than 20 different partner genes have been described previously,[5] and they can be divided into homeobox genes (eg, *HOXA9, -C11,* and *-D13)* and nonhomeobox genes (*DDX10*, *NSD1,* and *TOP1)*. NUP98-HOXA9, the most frequent fusion, has aberrant self-renewal capacity, blocks differentiation in transfected myeloid progenitors, and induces AML in mice.[9-10]

Genome-wide approaches proved to be powerful tools to dissect AML molecularly. High-resolution array-based comparative genome hybridization (A-CGH) and single nucleotide polymorphism arrays (SNP-A) identified recurrent copy number aberrations and regions with loss of heterozygosity, although AML seemed to be relatively genomically stable compared with other malignancies.[11-13] Mapping of genetic lesions will improve insight into the AML biology and may ultimately lead to development of new treatments.

In this study, we used high-resolution A-CGH and SNP-A to identify novel genetic aberrations underlying CN-AML. The cytogenetically cryptic *NUP98/NSD1* translocation was initially identified in three cases with these techniques. Subsequently, we performed a comprehensive study of more than 1000 pediatric and adult AML cases, and we identified the *NUP98/NSD1* translocation as a frequent cryptic event within pediatric CN-AML (16.1%) compared with adult CN-AML (2.3%). Moreover, *NUP98/NSD1* seemed to be a novel independent predictor for dismal outcome. *NUP98/NSD1*-positive AML showed a distinct gene expression pattern, including high expression of *HOXB* cluster genes, providing insight into the leukemogenic pathways of these AMLs.

METHODS

Study cohort

Two patient cohorts were included in this study, a pediatric cohort and an adult cohort.

The pediatric cohort (n=293; age, 0-18 years) consisted of children with available frozen bone marrow (BM) or peripheral blood (PB) samples taken at diagnosis that were provided by the Dutch Childhood Oncology Group (DCOG, the Netherlands; n=141), the AML-Berlin-Frankfurt-Münster Study Group (AML-BFM SG, Germany and Czech Republic; n=128) and the Saint-Louis Hospital (Paris, France; n=24). In addition, of three *NUP98/NSD1*-positive cases paired remission and relapse samples were available. The pediatric cohort was representative for pediatric AML, comparing patient characteristics with the AML-BFM98 series (Online Supplementary Table S1), although our cohort included more FAB-M4 and less FAB-M2 cases. Survival analysis was restricted to patients with *de novo* AML enrolled in DCOG and AML-BFM SG studies, excluding PML/RARA cases (n=238). The median follow-up time of survivors was 4.2 years (range, 0.3-22.7 years), and overall probability of event-free survival (pEFS) and probability of overall survival (pOS) for the pediatric cohort were 44±3% and 62±3%, respectively.

The adult cohort consisted of AML patients (n=808; age, 15-77 years, including 20 children aged 15-18 years) treated on consecutive Dutch-Belgian Cooperative Trial Group for Hematology Oncology (HOVON) protocols, with available frozen BM or PB taken at diagnosis.[14] Survival analysis was restricted to patients with *de novo* AML excluding patients with PML/RARA (n=727). The median follow-up time of survivors was 4.2 years (range, 0.5-18.7 years), and overall pEFS and pOS for the adult cohort were 32±2% and 39±2%, respectively.

Morphologic classification and karyotyping were centrally reviewed by each study group. Molecular characterization included mutational analysis of *NPM1*, *CEBPA*, *MLL, FLT3*, *KIT*, *N-RAS, K-RAS*, *PTPN11,* and *WT1* for the pediatric cohort, and *NPM1, FLT3, N-RAS, WT1, IDH1,* and *IDH2* for the adult cohort.[14-20]

Institutional review board approval had been obtained for these studies from Erasmus MC. Informed consent was obtained from the patients in accordance with the Declaration of Helsinki.

Genome-wide copy number analysis

In 45 pediatric CN-AML cases, A-CGH was performed using the human genome CGH Microarray 105K (Agilent Technologies, Palo Alto, USA) according to the manufacturer's protocol.[21] Microarray images were analyzed using feature extraction software (version 8.1, Agilent Technologies), and data were subsequently analyzed with Genomic Workbench (version 5.0.14, Agilent Technologies).

In 47 adult CN-AML samples, SNP-A (Affymetrix 250K NspI DNA-mapping array, Santa Clara, USA) were performed according to the manufacturer's protocol. Genotypes were calculated using BRLMM, copy numbers were assessed using dChipSNP, and data were subsequently visualized in SNPExpress.[22]

Fluorescence *in situ* hybridization.

Split-signal fluorescence *in situ* hybridization (FISH) analysis of *NUP98* was performed on

thawed cytospin slides using the 44 kb overlapping bacterial artificial chromosome (BAC) probes RP11-120E20 and RP11-348A20 (BACPAC Resources Center, Oakland, USA) as described previously.[24] The *NUP98/NSD1* translocation was confirmed using BAC probes RP11-348A20 and RP11-99N22.

Detection of the *NUP98/NSD1* transcript

Presence of *NUP98/NSD1* and the reciprocal *NSD1/NUP98* were determined by reverse transcriptase-polymerase chain reaction (RT-PCR). Primers and cycle conditions are presented in Online Supplementary Table S2. Purified PCR products were directly sequenced on an ABI-PRISM3100 genetic analyzer (Applied Biosystems, Foster City, USA) and analyzed using CLCWorkbench version 3.5.1 (CLC Bio, Aarhus, Denmark). *NUP98/NSD1* was not detected in normal BM controls (n=7).

NUP98/NSD1 transcript levels were measured in duplicate based on the intercalation of SYBR Green (Finnzymes, Woburn, USA) using RT-quantitative (q)PCR (TaqMan chemistry) on an ABI-PRISM-7900HT system (Applied Biosystems) and calculated relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. Amplification efficiency was nearly 100%, and the dissociation curves confirmed amplification of a single product for NUP98/NSD1 as well as GAPDH. The sensitivity of the RT-qPCR, as determined by serially diluting cDNA of a *NUP98/NSD1* case (#4432) in cDNA derived from an *NUP98/NSD1*-negative AML cell line (NB4; DSMZ, Braunschweig, Germany), reached 10^{-3} to 10^{-4}, with an input of only 20ng of cDNA. Transcript levels were calculated relative to #4432 using the standard curve.

Immunofluorescence

Cellular localization of the NUP98/NSD1 fusion protein was examined using immunofluorescence. Thawed cytospin slides with leukemic cells were fixed and permeabilized with ice-cold 96% methanol. After washing with phosphate-buffered saline, slides were incubated with a primary antibody (sc-101546 [α-N-terminal NUP98] or sc-32479 [α-C-terminal NSD1]; Santa Cruz Biotechnology, Santa Cruz, USA). This was followed by incubation with a fluorescently labeled secondary antibody (Dylight 488 goat anti-rat IgG or Dylight 549 rabbit anti-goat, respectively; Jackson ImmunoResearch, West Grove, USA). Cells were visualized with a Zeiss LSM700-microscope (magnification 63x/1.4 NA oil objective), scanned at 2048x2048 pixels in 3 channels (8-bit resolution) and the resulting images were acquired and processed with ZEN2009 Light edition software.

Gene expression profiling

Gene expression profiling data (HGU133 Plus 2.0 microarray; Affymetrix) were available of 274 pediatric cases.[23] Original data are available in the Gene Expression Omnibus repository (http://www.ncbi.nlm.nih.gov/geo; accession GSE17855). Checking RNA quality, microarray processing, data acquisition, and data normalization have been described previously.[23] Differentially expressed genes were calculated using *t* test-based statistics (Bioconductor pac-

kage LIMMA; http://www.bioconductor.org/) in the statistical data analysis environment R (version 2.7.0; http://www.r-project.org/). The *P* values were corrected for multiple testing according to the false discovery rate (FDR)-procedure of Hochberg and Benjaminin (Bioconductor package Multtest). Supervised clustering and principal component analyses were performed using GeneMath XT 1.6.1 software (Applied Maths, Austin, USA). Unsupervised clustering analysis was performed and visualized as described previously.[16,24]

Expression of microRNA-196b and -10a

MicroRNA (miR)-10a and -196b expression was determined in a selection of the pediatric cohort (n=90 and n=84, respectively), reflecting the different genetic subgroups in pediatric AML. miR-expression levels were measured in duplicate on an ABI-PRISM-7900HT system using a stem-loop based RT-qPCR according to the manufacturer's protocol (Applied Biosystems). The threshold was manually set at 0.15, and the comparative cycle threshold (Ct) method was used to calculate the miR-expression levels relative to the endogenous miR-control RNU24.

Statistical analysis

Statistical analyses were performed with SPSS 17.0 (SPSS, Chicago, USA), and SAS/STAT 9.2 (SAS Institute, Cary, USA). Categorical variables were compared using the $\chi 2$ or Fisher's exact test, and continuous variables using the Mann-Whitney *U*-test. To assess outcome, the following parameters were used: complete remission (CR, defined as less than 5% blasts in the bone marrow, with regeneration of trilineage hematopoiesis plus absence of leukemic cells in the cerebrospinal fluid or elsewhere), 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 (defined as time between diagnosis and first event, including failure to achieve remission, relapse, death by any cause or second malignancy), and pOS (defined as time between diagnosis and death). pEFS and pOS were estimated by the Kaplan-Meier method and compared using the log-rank test. CIR curves were constructed by the method of Kalbfleisch and Prentice and were compared using Gray's test. The independency of prognostic factors was examined by multivariate Cox regression analysis. All tests were 2-tailed, and p-values less than 0.05 were considered statistically significant.

RESULTS

Genome-wide copy number profiling identified *NUP98* aberrations

Using high-resolution A-CGH we detected an 11p15-aberration in two of 45 pediatric CN-AML cases, involving the *NUP98* gene. One case harbored a 0.4 Mb duplication involving the 5' part of *NUP98* and the other case a 0.1 Mb deletion of the 3' end (Figure 1A). In both aberrations, the genomic breakpoint was located between the probes in *NUP98* exons 11 and

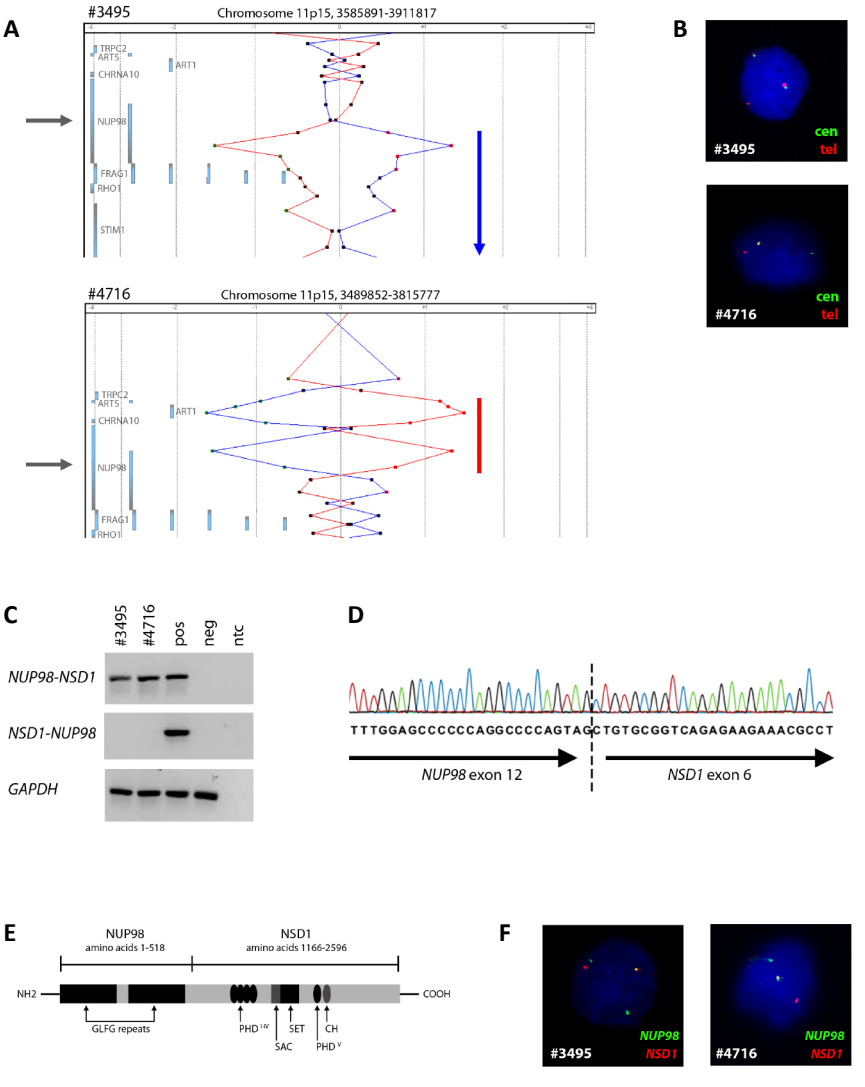

FIGURE 1. Discovery of *NUP98/NSD1* fusion in two CN-AML cases.
(A) Array-CGH profiles of chromosome 11p15 showing the ratio of tumor/control DNA (blue tracing) versus the inverted experiment (red tracing). The profile of patient #3495 (top panel) shows a part of the 0.4 Mb duplication involving the 5' part of *NUP98* (indicated by the blue vertical arrow), and the profile of patient #4716 (bottom panel) shows the 0.1 Mb deletion involving the 3' part of *NUP98* (indicated by the red vertical bar). The horizontal arrows indicate the bar representing the *NUP98* gene. (B) Split-signal FISH analysis of *NUP98* for patients #3495 and #4716 using a partly overlapping green and red probe located in *NUP98* (cen indicates probe situated more centromeric; tel indicates probe situated more telomeric. (C) RT-PCR analysis using *NUP98*- and *NSD1*-specific primers and *GAPDH* primers as internal control reveals a specific *NUP98/NSD1* fusion transcript in patients #3495 and #4716. The reciprocal *NSD1-NUP98* transcript was not detected in both patients (pos indicates positive control [#5007], neg indicates negative control [normal bone marrow] and ntc indicates nontemplate control). (D) Sequence analysis confirmed an identical in-frame fusion between *NUP98* exon 12 and *NSD1* exon 6 in both patients. (E) The NUP98/NSD1 fusion protein will harbor the GLFG-repeats of NUP98, and amongst others the PHD fingers and SET domain of NSD1. (F) Dual-color FISH analysis using a green probe for *NUP98* and a red probe for *NSD1* confirmed the fusion of *NUP98* and *NSD1* at the chromosomal level. (A color version of this figure can be found in the appendices.)

TABLE 1. Individual characteristics of the *NUP98/NSD1*-positive (n=23) and the other *NUP98*-translocated (n=3) AML cases.

ID	*NUP98* parter gene	Cohort	Age (yr)	Sex	WBC ($x10^9$/l)	FAB	Cytogenetic aberations	Aberrant 5q35 or 11p15 on A-CGH or SNP-A	Reciprocal product	Mutations	Therapy protocol	CR	Relapse (RFS in months)	Dead (OS in months)
#0506	*NSD1*	ped	10	M	332.0	M4	normal	no	yes	*FLT3*/ITD + *WT1*	MRC12	+	- (7.2)	+ (7.2) ^
#3397	*NSD1*	ped	15	M	154.4	M5	+8	NA	yes	*FLT3*/ITD	MRC12	+	+ (4.4)	+ (5.6)
#3495	*NSD1*	ped	14	M	57.1	NA	normal	dupl 11p15	no	*FLT3*/ITD	MRC12	+	+ (6.3)	- (6.3) @
#4380	*NSD1*	ped	4	M	7.4	M4	normal	no	no	*N-RAS* + *WT1*	BFM04	+	+ (7.3)	- (64.8) ^
#4417	*NSD1*	ped	17	M	189.0	M2	normal	no	yes	*FLT3*/ITD + *WT1*	BFM04	+	+ (5.3)	+ (30.2) ^
#4432	*NSD1*	ped	6	M	324.0	M2	normal	no	yes	*FLT3*/ITD + *WT1*	BFM04	+	+ (4.9)	+ (8.0)
#4716	*NSD1*	ped	4	M	377.6	M1	normal	del 11p15	no	*FLT3*/ITD	MRC12	+	+ (7.9)	+ (9.4)
#4730	*NSD1*	ped	9	M	121.3	M4	del 9q	NA	yes	*FLT3*/ITD	MRC12	+	+ (8.7)	- (14.4) ^
#4733	*NSD1*	ped	2	M	267.3	M4	normal	no	yes	*FLT3*/ITD	MRC12	+	+ (6.9)	+ (9.9)
#5007	*NSD1*	ped	14	F	NA	NA	del 9q	NA	yes	*FLT3*/ITD + *WT1*	LAME	NA	NA	NA
#5144	*NSD1*	ped	16	F	187.0	M1	normal	NA	yes	*FLT3*/ITD + *WT1*	BFM98	+	+ (4.6)	+ (8.2)
#5166	*NSD1*	ped	13	M	226.0	M1/2	normal	NA	no	*FLT3*/ITD	BFM04	+	-	- (32.0) ^
#6328	*NSD1*	ped	2	M	153.0	M4	normal	no	yes	*FLT3*/ITD	MRC15	+	+ (7.2)	- (31.8) ^
#2176	*NSD1*	adult	41	M	263.4	M4	normal	NA	yes	*FLT3*/ITD	HO04	-		+ (1.1)
#2280	*NSD1*	adult	49	F	6.1	M2	normal	del 11p15	no	*WT1*	HO29	-		+ (2.0)
#2305	*NSD1*	adult	27	M	58.0	M5	normal	no	no	*FLT3*/ITD + *WT1*	HO29	+	+ (11.5)	- (92.3) ^
#4333	*NSD1*	adult	31	M	177.0	M5	normal	NA	no	*FLT3*/ITD	HO42	-		+ (1.5)

TABLE 1 continued.

ID	*NUP98* parter gene	Cohort	Age (yr)	Sex	WBC ($x10^9$/l)	FAB	Cytogenetic aberations	Aberrant 5q35 or 11p15 on A-CGH or SNP-A	Reciprocal product	Mutations	Therapy protocol	CR	Relapse (RFS in months)	Dead (OS in months)
#6463	*NSD1*	adult	63	F	78.0	M5	inv(5) (q1?2q3?4)	NA	no	*FLT3*/ITD	HO43	-		+ (9.2)
#6714	*NSD1*	adult	47	F	140.0	M5	normal	NA	yes	*FLT3*/ITD #	HO04	+	- (19.9)	+ (19.9) ^
#6884	*NSD1*	adult	19	F	126.4	M5	marker *	NA	no	*FLT3*/ITD #	HO04A	+	+ (4.6)	+ (8.5)
#7191	*NSD1*	adult	45	F	131.0	M5	normal	NA	no	*FLT3*/ITD #	HO42	-		+ (10.0)
#11678	*NSD1*	adult	54	F	49.2	M4	normal	NA	yes	*FLT3*/ITD	HO42A	-		+ (3.8)
#13983	*NSD1*	adult	36	M	97.2	M4	normal	NA	yes	*FLT3*/ITD + *WT1*	HO42A	+	+ (3.6)	+ (8.8)
#297	*JARID1A*	ped	1	M	8.4	M7	complex	NA	no	none	MRC12	+	-	- (128.4)
#4096	*TOP1*	ped	12	F	214.0	M4	t(11;20) (p15;q1?2)	NA	yes	*CEBPA*s +*WT1*	MRC12	+	+ (9.8)	+ (19.0)
#6974	*DDX10*	adult	32	M	4.9	M6	+8,inv(11) (p15;q22)	NA	NA	*N-RAS*#	HO29	+	+ (13.9)	+ (22.3)

Abbreviations: ped indicates pediatric; F, female; M, male; NA, not available; WBC, white blood cell count; FAB, French-American-British subtype; del, deletion; dupl, duplication; CR, complete remission; RFS, relapse-free survival; OS, overall survival; *CEBPA*s, *CEBPA* single mutation

* karyotype: 47,XX,+21?/46,XX; # these samples were not tested for *WT1* mutations due to lack of material; @ no follow-up available after relapse

^ these patients received an allo-SCT (#0506, #5166 and #6714 in CR1, and #4380, #4417, #4730, #6328, #2305 after relapse)

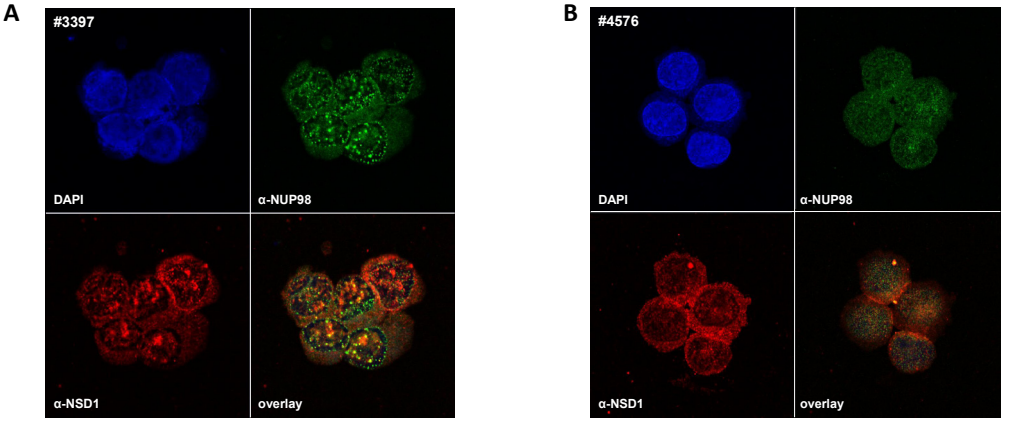

FIGURE 2. Aberrant nuclear-staining pattern of *NUP98/NSD1*-positive samples.
Immunofluoresence of leukemic cells with antibodies directed against the N-terminus of NUP98 (green) and the C-terminus of NSD1 (red) is shown for a *NUP98/NSD1*-positive (A) and a *NUP98/NSD1*-negative patient sample (B). Patient #3397 shows a pattern of nuclear speckles for NUP98 staining as well as NSD1 staining, indicating accumulation of the NUP98/NSD1 fusion protein in aggregates in the nucleus, in contrast to patient #4576. DAPI indicates 4,6-diamidino-2-phenylindole. (A color version of this figure can be found in the appendices.)

13. Because most translocation breakpoints of *NUP98* cluster in introns 11/12 and 12/13,[5] we suspected an unbalanced *NUP98* translocation, and this translocation was confirmed by split-signal FISH in both cases (Figure 1B). To identify the partner genes, we selected candidate genes resulting in cryptic translocations with *NUP98*, including the *nuclear receptor-binding SET domain protein 1* (*NSD1*) gene located on chromosome 5q35, 4 Mb from the telomere. RT-PCR revealed the *NUP98/NSD1* transcript in both cases, and sequence analysis showed an identical in-frame fusion of *NUP98* exon 12 and *NSD1* exon 6 (Figures 1C, 1D and 1E). FISH analysis confirmed these fusions (Figure 1F).

In the adult cohort, high-resolution SNP-A was performed in 47 CN-AML cases. An 11p15-aberration including *NUP98* was detected in one case (Online Supplementary Figure S1). The identical *NUP98/NSD1* fusion, as found in both pediatric cases, was detected in this case.

NUP98/NSD1 and other *NUP98* fusions

To determine the frequency of *NUP98/NSD1*, we screened 1101 AML cases using RT-PCR. The *NUP98/NSD1* transcript was detected in 23 cases (2.1%; Table 1), all carrying the identical in-frame fusion of *NUP98* exon 12 and *NSD1* exon 6. The reciprocal *NSD1/NUP98* transcript was detected in 13 of the 23 cases (57%) only. Three of 10 *NUP98/NSD1*-positive cases analyzed by genomic profiling, demonstrated numerical changes of the *NUP98* or *NSD1* gene adjacent to the breakpoint, indicative of an unbalanced translocation.

We observed an aberrant cellular pattern when staining NUP98 and NSD1 in *NUP98/NSD1*-positive leukemic cells (Figure 2). *NUP98/NSD1*-positive samples showed nuclear speckles,

TABLE 2. Clinical and molecular characteristics of *NUP98/NSD1*-positive vs. -negative cases.

	NUP98/NSD1-positive cases	*NUP98/NSD1*-negative cases	*P* value
Total, N (%)	23 (2.1)	1078 (97.9)	0.001
Pediatric cohort	13 (4.4)	280 (95.6)	
Adult cohort	10 (1.2)	798 (98.8)	
Age (years)			0.004
Median	16.8	40.0	
Range	2.3 – 63.0	0.0 – 77.0	
Sex (%)			0.21
Female	34.8	48.0	
Male	65.2	52.0	
WBC (x10^9/l) (N=1071)			<0.001
Median	146.5	26.2	
Range	6.1 – 377.6	0.3 – 510.0	
FAB type: N (%)			0.26
M0	-	54 (5.0)	
M1	3 (13.0)	193 (17.9)	0.13*
M2	3 (13.0)	260 (24.1)	
M3	-	42 (3.9)	
M4	8 (34.8)	208 (19.3)	0.01 #
M5	7 (30.4)	215 (19.9)	
M6	-	21 (1.9)	
M7	-	11 (1.0)	
RAEB/RAEB-t	-	49 (4.5)	
Other	-	2 (0.2)	
Missing	2 (8.7)	23 (2.1)	
Karyotype: N (%)			0.004
t(8;21)	-	74 (6.9)	
inv(16)	-	91 (8.4)	
t(15;17)	-	39 (3.6)	
11q23	-	87 (8.1)	
CN-AML	18 (78.3)	388 (36.0)	
Other	5 (21.7)	355 (32.9)	
Missing	-	44 (4.1)	
Mutations: N (%)			
*NPM*1 (n=1084)	-	263 (24.8)	0.006
CEBPA (n=268) @	-	15 (5.9)	1.00
MLL-PTD (n=244) @	-	6 (2.6)	1.00
FLT3/ITD (n=1089)	21 (91.3)	234 (22.0)	<0.001
FLT3/TKD (n=1072)	-	88 (8.4)	0.25
N-RAS (n=1069)	1 (4.3)	146 (14.0)	0.35
KIT (n=280) @	-	20 (7.5)	0.61
PTPN11 (n=280)@	-	4 (1.5)	1.00
WT1 (n=680)	9 (45.0)	46 (7.0)	<0.001
IDH1 (n=808)^	-	50 (6.3)	1.00
IDH2 (n=808)^	-	91 (11.4)	0.61

* *P* value represents FAB M1/M2 vs. other FAB type; # *P* value represents FAB M4/M5 vs. other FAB type;
@ only screened in pediatric cohort; ^ only screened in adult cohort

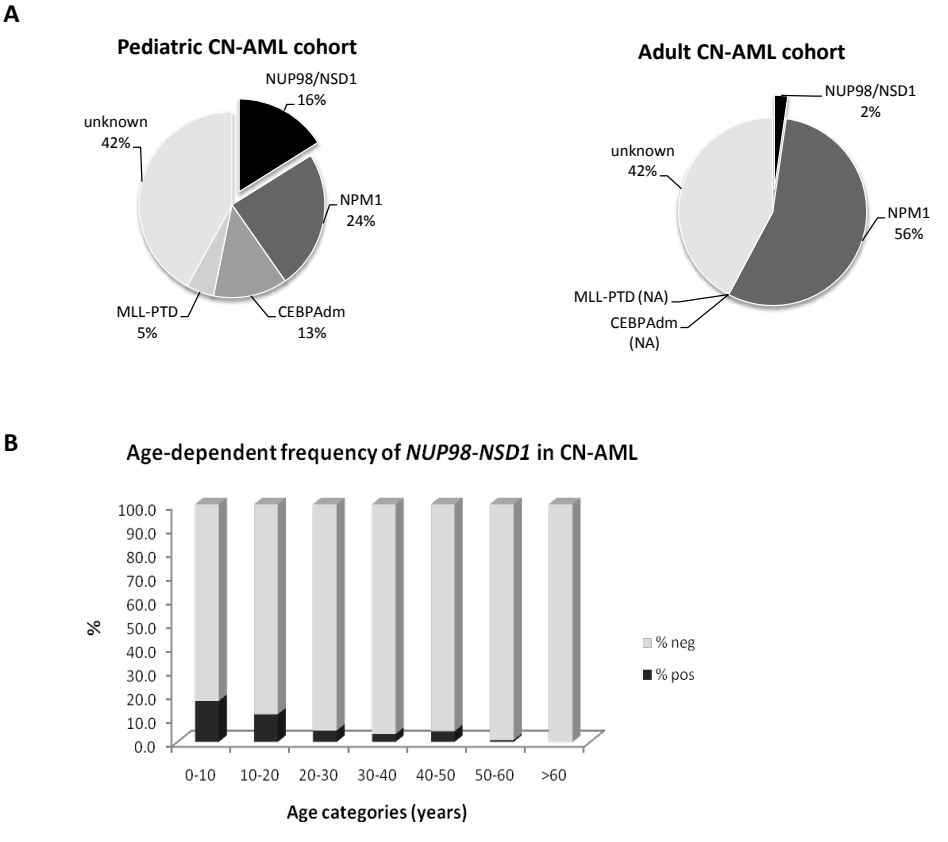

Figure 3. The *NUP98/NSD1* fusion is a frequent event in pediatric CN-AML.
(A) Pie charts showing percentage of the different type II aberrations found in pediatric CN-AML (n=62; left pie chart) and in adult CN-AML (n=344; right pie chart). The mutational analysis of *CEBPA* and *MLL* was not available for the adult cohort. (B) Histogram representing the percentage of the *NUP98/NSD1*-positive cases within the different age categories in CN-AML shows a decreasing frequency of *NUP98/NSD1* with age.

in contrast to *NUP98/NSD1*-negative samples that displayed a fine distribution of NUP98 and NSD1 in the nucleus and cytoplasm. This indicates that the NUP98/NSD1 fusion protein forms nuclear aggregates.

We found four additional AML cases carrying cytogenetically visible 11p15-aberrations, suggesting involvement of *NUP98*. One pediatric case carried the *NUP98/JARID1A* fusion that we described previously.[25] Another pediatric case had a t(11;20)(p15;q1?2), and FISH analysis and RT-PCR confirmed the in-frame *NUP98/TOP1* and reciprocal *TOP1/NUP98* fusion (Online Supplementary Figure S2). One adult case had an inv(11)(p15q22), and RT-PCR showed presence of the in-frame *NUP98/DDX10* (Online Supplementary Figure S2). The second adult case had an inv(11)(p15q13) besides a t(15;17)(q22;q21), but *NUP98* involvement could not be investigated because of lack of material. In addition, nine selected acute

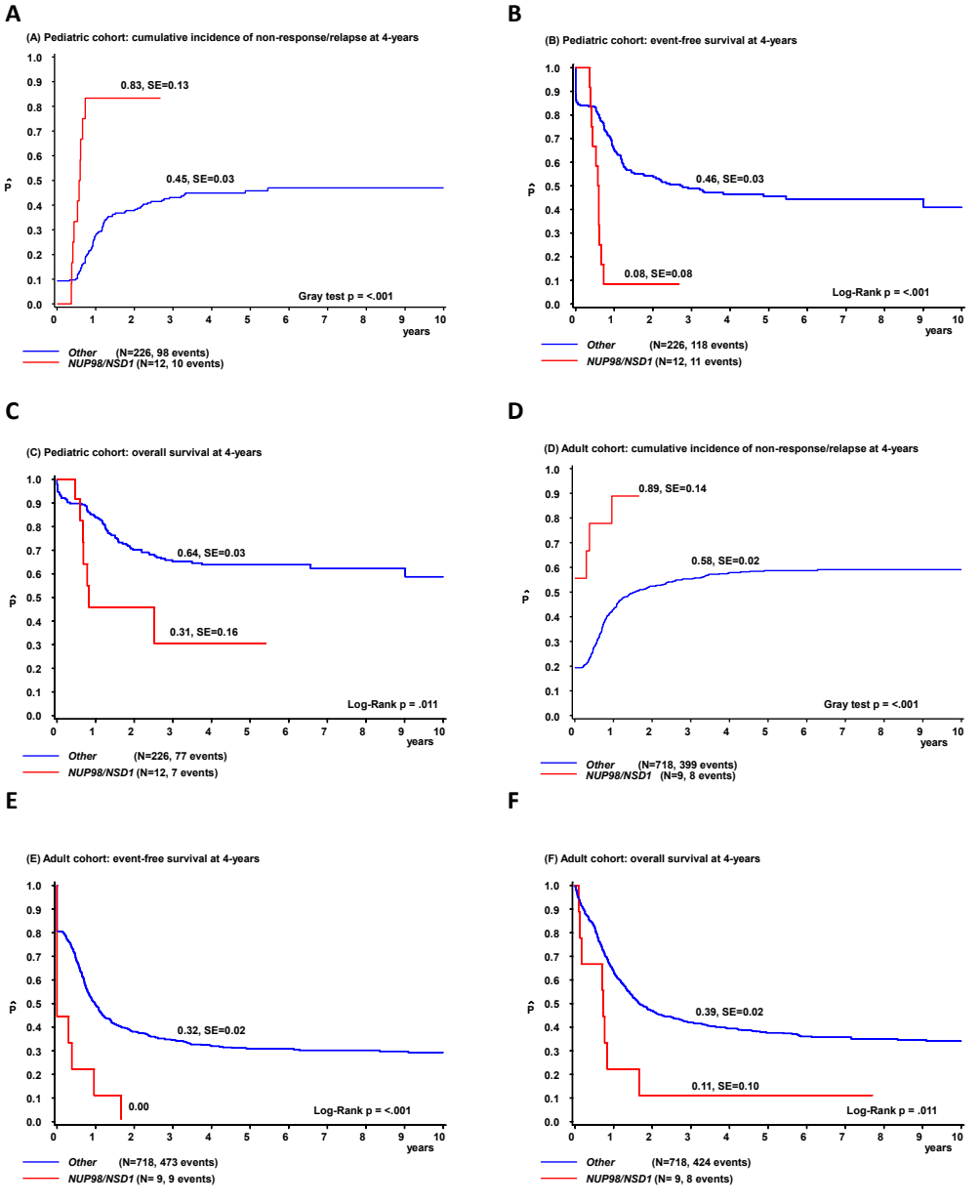

Figure 4. *NUP98/NSD1* confers a poor outcome in pediatric as well as adult AML.
Survival curves of the pediatric cohort (n=238; A-C) and adult cohort (n=727; D-F) depicting the CIR according to the Kalbfleisch and Prentice method (A,D) and Kaplan-Meier estimates of the pEFS (B,E) and pOS (C,F). (A color version of this figure can be found in the appendices.)

lymphoblastic leukemia samples did not harbor *NUP98/NSD1* (see Online Supplementary Methods).

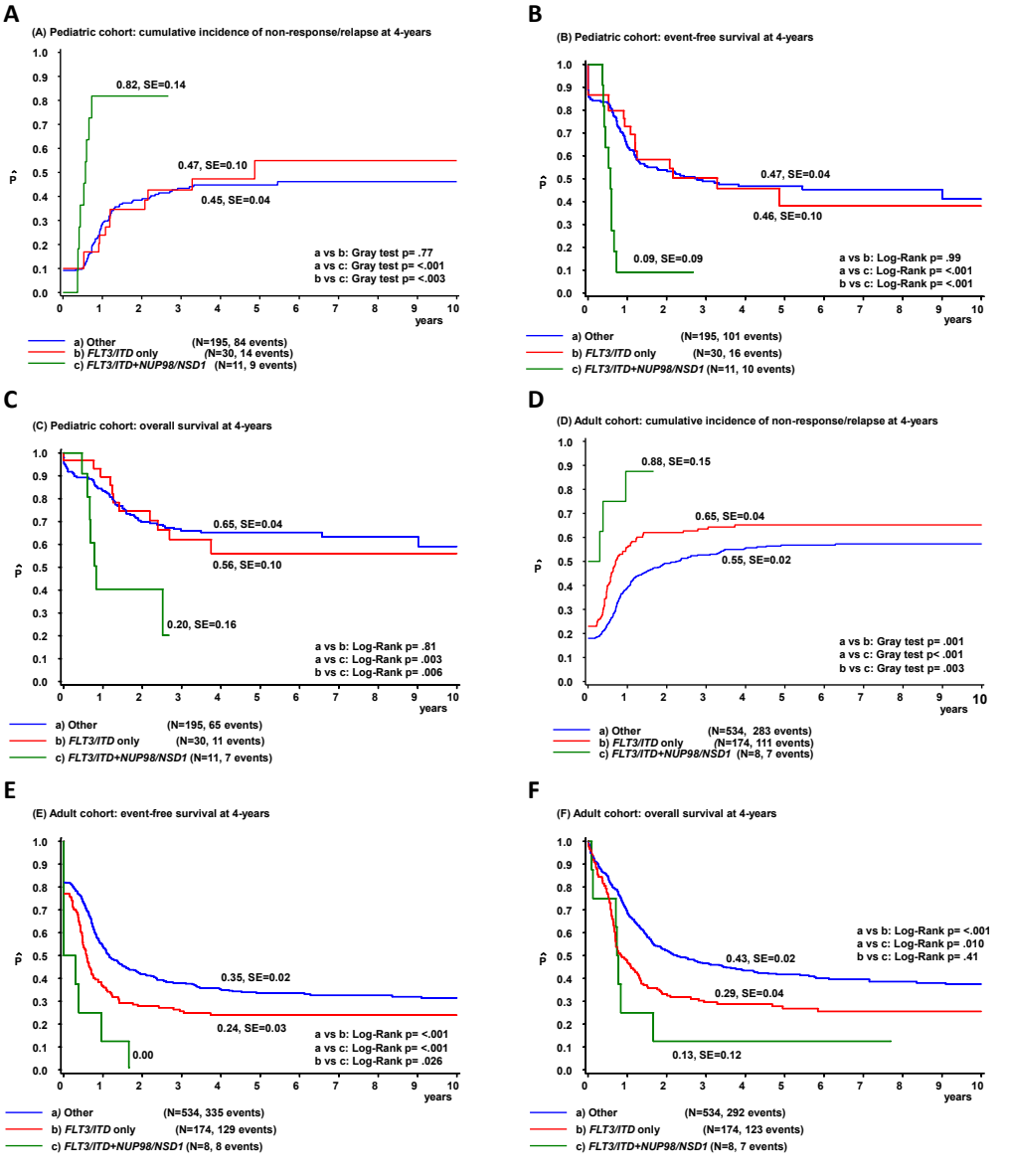

Figure 5. *NUP98/NSD1* clearly identifies a poor prognostic subgroup within pediatric as well as adult *FLT3*/ITD-positive AML. Survival curves of the pediatric cohort (A-C) and adult cohort (D-F) depicting the CIR according to the Kalbfleisch and Prentice method (A,D) and Kaplan-Meier estimates of the pEFS (B,E) and pOS (C,F) according to the *NUP98/NSD1* and *FLT3*/ITD status. (A color version of this figure can be found in the appendices.)

Age-dependency of *NUP98/NSD1*

Thirteen of 313 (4.2%) *NUP98/NSD1*-positive cases were found in children (0-18 years) and 10 of 788 (1.3%) in adults (>18 years; p=0.003). When analyses were limited to CN-AML,

we observed an even higher frequency of *NUP98/NSD1* in children (16.1%) compared with adults (2.3%; p<0.001) (Figures 3A and 3B). Of note, *NUP98/NSD1* cases were not observed in children less than 2 years of age.

Characteristics of *NUP98/NSD1* cases

All but one of the *NUP98/NSD1* cases presented with *de novo* AML (#11678 presented with therapy-related AML; Table 1). *NUP98/NSD1* cases had a higher white blood cell count compared to other cases (median, 147 $x10^9$/l vs. 26 $x10^9$/l; p<0.001) and were associated with FAB-M4/M5 morphology (65.2% vs. 39.2%; p=0.01; Table 2). However, when analyzing the adult and pediatric cohort separately (Online Supplementary Tables S3A and S3B), the association with M4/M5 morphology was only observed in the adult cohort.

Only five of the 23 *NUP98/NSD1* cases showed cytogenetic aberrations (Table 1). *NUP98/NSD1* was mutually exclusive with all type-II aberrations, that is, AML-characteristic fusion genes, *NPM1* mutations, *CEBPA* double mutations, and *MLL*-PTD (Table 2). In contrast, *NUP98/NSD1* was frequently associated with type-I aberrations: 91% of *NUP98/NSD1* cases had an *FLT3*/ITD vs. 22% of *NUP98/NSD1*-negative cases (p<0.001). *WT1* mutations were present in 9 of 20 (45%) *NUP98/NSD1* cases vs. 46 of 660 (7%) of *NUP98/NSD1*-negative cases; p<0.001. One case harbored an *N-RAS* mutation (Tables 1 and 2).

Prognostic relevance of *NUP98/NSD1*

The prognostic impact was analyzed separately in the pediatric and adult cohort. The CR rate of *NUP98/NSD1*-positive cases in the pediatric cohort was 100%, but 10 of the 12 cases relapsed early. The CIR at 4 year was 83±13% for *NUP98/NSD1*-positive vs. 45±3% for negative cases (p<0.001; Figure 4A). *NUP98/NSD1* cases had a poor pEFS and pOS (4-year pEFS 8±8% vs. 46±3%; p<0.001, and 4-year pOS 31±16% vs. 64±3%, p=0.011, for *NUP98/NSD1*-positive vs. -negative cases, respectively) (Figures 4B and 4C). In the adult cohort, six of the 10 *NUP98/NSD1*-positive patients never reached CR, and these cases had a significantly higher CIR, and worse pEFS and pOS than *NUP98/NSD1*-negative cases (4-year CIR 89±14% vs. 58±2%, p<0.001; 4-year pEFS 0% vs. 32±2%, p<0.001; and 4-year pOS 11±10% vs. 39±2%, p=0.011, respectively; Figures 4D, 4E and 4F).

As all but two *NUP98/NSD1*-positive cases carried simultaneously an *FLT3*/ITD, we then analyzed the prognostic relevance of *NUP98/NSD1* versus other patients carrying an *FLT3*/ITD. Among pediatric *FLT3*/ITD-positive (*FLT3*/ITD+) cases (n=41) as well as adult *FLT3*/ITD+ cases (n=182), *NUP98/NSD1* cases had a worse outcome than the other *FLT3*/ITD+ cases (pediatric cohort: 4-year CIR 82±14% vs. 47±10%, p=0.003; 4-year pEFS 9±9% vs. 46±10%, p<0.001; and 4-year pOS 20±16% vs. 56±10%, p=0.006; adult cohort: 4-year CIR 88±15% vs. 65±4%, p=0.003; 4-year pEFS 0% vs. 24±3%, p=0.026; and 4-year pOS 13±12% vs. 29±4%, p=0.41) (Figures 5A-F). When excluding cases with *NPM1* mutations from the *FLT3*/ITD+ group, *NUP98/NSD1* conferred a worse outcome than *FLT3*/ITD+/*NPM1* wild-type cases in the pediatric cohort. The outcome of *NUP98/NSD1*-positive cases and *FLT3*/ITD+/*NPM1*

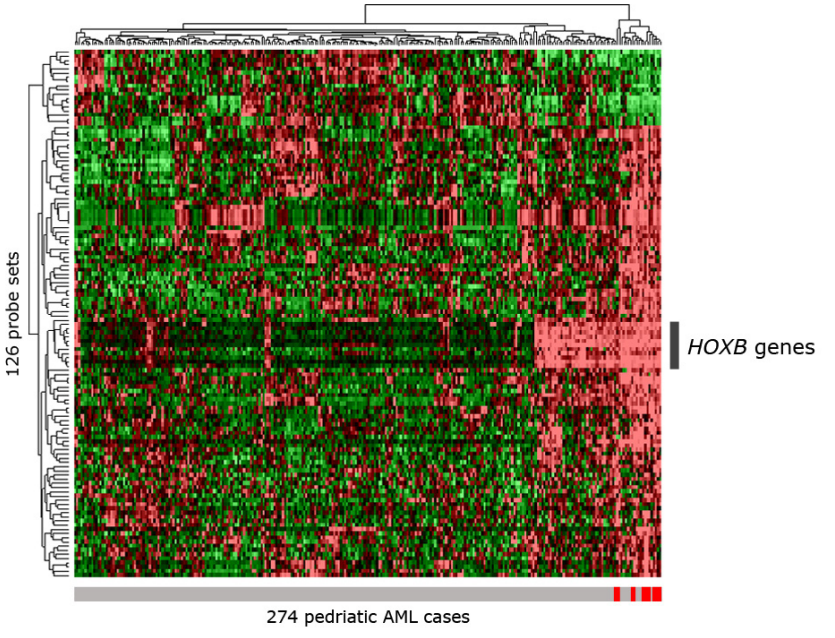

Figure 6. Clustering of 274 pediatric AML cases based on the 126 most discriminative probe sets for *NUP98/NSD1.*

Hierarchical clustering of 274 pediatric AML cases based on the 126 most discriminative probe sets (FDR-adjusted *P* value <0.05) for *NUP98/NSD1* AML is presented in a heat map. The 13 *NUP98/NSD1* cases are indicated with a red vertical bar below the heat map; the other AML cases are indicated with a gray vertical bar. In the heat map, cells represent relative log 2 expression values and have been color-coded on a scale ranging from bright green (-2) to bright red (+2), with black indicating no change relative to the median. Besides clustering of the *NUP98/NSD1* cases, a group of other AML cases show a partly similar gene expression profile mainly based on the highly expressed *HOXB* probe sets, indicated by the gray bar at the right of the heat map. (A color version of this figure can be found in the appendices.)

wild-type cases was both very poor in the adult cohort (Online Supplementary Figures S3A-F).

When all analyses were restricted to CN-AML cases, the impact of *NUP98/NSD1* among the diverse subtypes of the *FLT3*/ITD+ group was less clear, which may be influenced by the small numbers (Online Supplementary Figures S4A-F).

In a multivariate Cox regression model including the variables adult versus pediatric cohort, favorable risk cytogenetics, age (>60 years) and *FLT3*/ITD, *NUP98/NSD1* was an independent poor prognostic factor for the probability of relapse-free survival (pRFS) (Hazard Ratio (HR) 2.6; p<0.001), for pEFS (HR 2.5; p<0.001) and for pOS (HR 1.7; p=0.049; Online Supplementary Table S4).

Regarding the other three *NUP98*-translocated cases, numbers were too small to perform outcome analyses (Table 1).

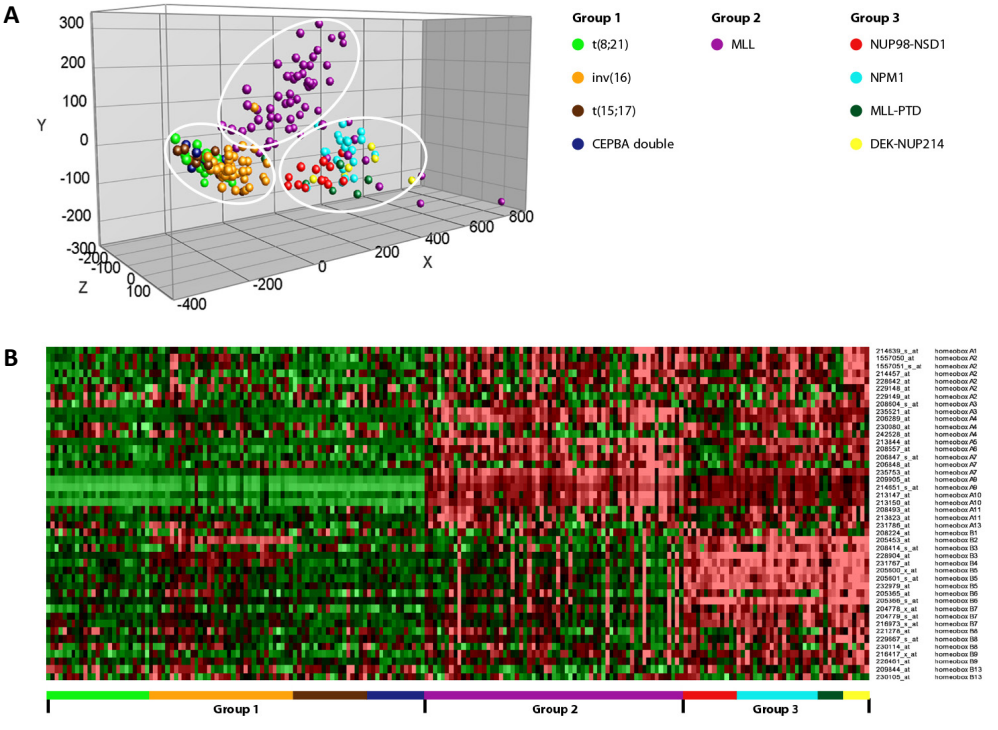

FIGURE 7. Distinct expression pattern of the *HOXA* and -*B* cluster genes in pediatric AML with *NUP98/NSD1*. (A) Principal component analysis (PCA) of pediatric AML subgroups characterized by specific type-II aberrations (n=210) was carried out based on all *HOXA* and -*B*-annotating probe sets present on the HGU133 Plus 2.0 microarray (Affymetrix). Each color-coded circle represents an individual AML case. Three distinct groups are observed in the PCA, which are indicated by the white circles. (B) Heat map showing the expression of all *HOXA* and -*B*-annotating probe sets in which the cells represent relative log 2 expression values and are color-coded on a scale ranging from bright green (-2) to bright red (+2), with black indicating no change relative to the median. The pediatric AML cases are grouped together based on their specific type-II aberration as indicated by the color-coded bars below the heat map, and the probe sets are alphabetically ordered. Three groups with distinct expression patterns are observed, that is, group 1, characterized by low or absent expression of *HOXA* and -*B* genes; group 2, characterized by solely high expression of *HOXA* genes (mainly *HOXA5-A10*); and group 3, characterized by expression of both *HOXA* and -*B* genes (mainly *HOXA5-A10* and *HOXB2-B6*). The latter included the *NUP98/NSD1* cases. (A color version of this figure can be found in the appendices.)

Minimal residual disease (MRD) levels in *NUP98/NSD1*-positive patients

Of three *NUP98/NSD1*-positive patients, BM samples at morphologic remission and relapse were available. In all remission samples, we detected the *NUP98/NSD1* transcript at high levels (10^{-1}-10^{-3}) (Online Supplementary Figure S5). In all relapse samples, *NUP98/NSD1* was present at similar levels compared with diagnosis, indicating clonal stability.

Gene expression profiling

Supervised analysis of gene expression levels of 13 *NUP98/NSD1* vs. 261 other pediatric *de novo* AML cases resulted in 126 discriminative probe sets (FDR-adjusted *P* value <0.05)

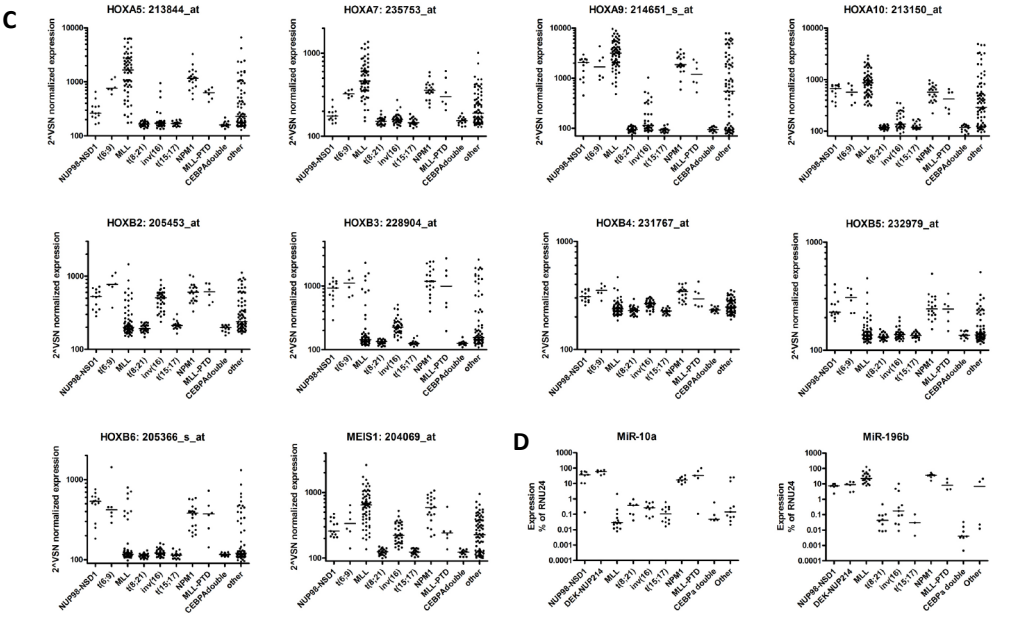

FIGURE 7 continued.

(C) VSN-normalized expression levels of *HOX*-annotating probe sets *A5*, *A7*, *A9* and *A10*, and *B2*, *B3*, *B4*, *B5* and *B6,* and a probe set annotating *MEIS1* were depicted per graph for all pediatric AML cases (n=274) grouped by their specific type-II aberration. *NUP98/NSD1* cases were characterized by high *HOXA9, -A10, -B2, -B3, -B4, -B5*, *-B6,* and *MEIS1* expression. (D) Relative expression levels of miRNA-10a and -196b by stem-loop RT-qPCR in pediatric AML cases (n=90 and n=84, respectively), representing the different genetic subgroups in pediatric AML. Both miRNA-10a and -196b are highly expressed in the 'group 3 members', whereas only miRNA-196b, but not miRNA-10a is highly expressed in the majority of the *MLL*-rearranged cases, correlating exactly with *HOXA* and *-B* expression, respectively.

(Online Supplementary Table S5A). Hierarchical clustering on these probe sets did not group the *NUP98/NSD1* cases exclusively together (Figure 6). A partially similar profile was found in 46 other cases, based on highly expressed *HOXB* cluster genes. Fifteen of these cases carried an *NPM1* mutation, known to be associated with high *HOXB* expression.[26] Unsupervised clustering analysis showed identical results (Online Supplementary Figure S6).

Triggered by this *HOXB* signature, we next investigated the expression pattern of all *HOX* cluster genes among AML groups characterized by specific type-II aberrations. Principal component analysis on all *HOXA* and *-B* probe sets showed distinct clustering of these AML cases in three different groups (Figures 7A and 7B). Group 1 is characterized by low or absent expression of *HOXA* and *-B* genes and included cases with AML1-ETO, CBFβ-MYH11, PML-RARA and *CEBPA* double-mutant AML. Group 2 is characterized by solely high *HOXA* expression and represented the majority of *MLL*-rearranged cases. Group 3 is characterized by both high *HOXA* and *-B* expression and included cases with *NPM1* mutations, cases with *DEK/NUP214,* and the *NUP98/NSD1* cases. *NUP98/NSD1* cases were characterized by high *HOXA9, -A10, -B2, -B3, -B4, -B5,* and *-B6* expression (Figure 7C). No expression of *HOXC* and

HOXD genes was observed. *MEIS1,* a well-known *HOX*-cofactor gene, was also expressed in *NUP98/NSD1*-positive cases. *NUP98/NSD1*-positive cases could be separated from the other 'group 3 members' by lower expression of *HOXA5* and *-A7*. Because miRNA (miR)-196b and -10a are transcriptionally co-regulated with the *HOXA* and *-B* locus, respectively, we investigated their expression in *NUP98/NSD1*-AML. *NUP98/NSD1* cases indeed showed high expression of miR-196b and miR-10a (Figure 7D). Combined high expression of miR-196b and miR-10a was previously reported for *NPM1*-mutated AML,[27] and we indeed also observed high expression for *NPM1*-mutated cases as well as for the other 'group 3 members'.

Further exploration of the most discriminative probe sets for *NUP98/NSD1* showed up-regulation of 108 and down-regulation of 18 probe sets. Besides *HOXB* genes, the up-regulated sets included other cancer-associated transcription factors, such as *VENTX, NKX2-3, UTF1,* and *NFIX,* and two annotated *NRG4*, encoding a ligand for epidermal growth factor receptors (EGFR). Among the down-regulated probe sets were two annotating *STK24* that induces apoptotic pathways. When restricting analyses to CN-AML (n=54), 9 probe sets (FDR-adjusted *P* value <0.05) were discriminative for *NUP98/NSD1* (n=10; Online Supplementary Table S5B) and included *VENTX*, *UTF1,* and *NRG4.* Hierarchical clustering clearly separated *NUP98/NSD1* cases as a distinct group within CN-AML (Online Supplementary Figure S7A). *NUP98/NSD1* cases also clustered together based on discriminative probe sets between *NUP98/NSD1* vs. other *FLT3*/ITD-positive cases, excluding that their expression profile is solely driven by *FLT3*/ITD (Online Supplementary Figure S7B).

DISCUSSION

In this study, we provided evidence that *NUP98/NSD1* is a recurrent translocation characterizing a *novel* clinically relevant group of AML patients. The fusion gene was shown to result from cryptic translocations not visible by conventional karyotyping. The previously reported *NUP98/NSD1* cases were also not observed in the karyogram;[5, 13, 28-33] hence, additional molecular techniques are required to identify these patients at diagnosis. Given the detrimental prognosis, we suggest that *NUP98/NSD1* analysis should be added to the panel of molecular diagnostics in AML.

NUP98/NSD1 represented a frequent event in pediatric CN-AML (16.1%), comparable with the frequency of *NPM1* mutations and *CEBPA* double mutations in this group (Figure 3A). In adult CN-AML, *NUP98/NSD1* was less frequent (2.3%). This age-dependent frequency resembles core-binding factor AML that also occurs more frequently in children. Interestingly, both *NUP98/HOXA9* and *DEK/NUP214* are also typically associated with a younger age.[34-35]

NUP98/NSD1 was mutually exclusive with other type-II aberrations. Wang et al demonstrated that *NUP98/NSD1* inhibited cellular differentiation, establishing *NUP98/NSD1* as a type-II aberration.[36] *FLT3*/ITD was present in the majority of *NUP98/NSD1* cases, suggestive of a novel nonrandom association between type-I and -II aberrations. Furthermore, 45% of

NUP98/NSD1 cases also harbored a *WT1* mutation, although the exact role of *WT1* mutations in leukemogenesis is unresolved. Recent evidence suggests that other *NUP98* fusions are also frequently associated with *WT1* mutations,[34,37] and we previously showed that 33% of *DEK/NUP214* cases also harbored *WT1* mutations.[17] This makes it conceivable that *WT1* mutations have an additive function in *NUP98*- and *NUP214*-rearranged leukemogenesis.

NUP98/NSD1 was identified as an independent factor for dismal clinical outcome. Despite the current intensive treatment regimens, AML cases with *NUP98/NSD1* were either refractory to induction chemotherapy or relapsed within one year of diagnosis. Four-year pEFS rates were below 10% for both pediatric and adult cases. The largest study on *NUP98*-rearrangements to date (n=11) also reported poor outcome for *NUP98/HOXA9* AML cases.[34] Within the unfavorable *FLT3*/ITD+ AML subgroup, cases with *NUP98/NSD1* did worse than patients carrying *FLT3*/ITD without *NUP98/NSD1*. We further subdivided *FLT3*/ITD+ AML cases according to the presence of an *NPM1* mutation. After excluding *NPM1*-mutated cases, *NUP98/NSD1*-positive cases did equally poor as *FLT3*/ITD+/*NPM1* wild-type cases in adult AML, but still significantly worse in pediatric AML. Of note, within CN-AML, the impact of *NUP98/NSD1* among the diverse subtypes of the *FLT3*/ITD+ group was not clear, which might be limited by the small numbers. Three investigated *NUP98/NSD1* cases showed high MRD levels, correlating with the early relapses in these patients. Novel therapeutic strategies are urgently needed for this therapy resistant patient group.

Knowledge of the underlying biology of *NUP98/NSD1* is important as it may identify novel therapeutic targets. Our expression profiles showed up-regulation of oncogenic transcription factors such as VENTX,[38] and of *NRG4*, encoding an EGFR-ligand involved in proliferation,[39] and down-regulation of the proapoptotic gene *STK24*.[40] Moreover, *NUP98/NSD1*-positive AML showed a distinct *HOXA* and *-B* expression signature and concomitant high miR-196b and miR-10a expression. This *HOX* activation pattern was distinct from *MLL*-rearranged cases that were characterized by *HOXA* activation only. *NUP98/NSD1* cases resembled the *HOX* expression pattern of AML with *NPM1* mutations, *DEK/NUP214* and *MLL*-PTD; however, they could be discriminated by lower *HOXA5* and *-A7* expression. NUP98-homeobox fusions bind DNA through the homeodomain of the fusion partner. Recruitment of CREBBP/p300 by the GLFG-repeats of NUP98 results in histone acetylation and subsequent transcriptional activation of target genes.[41] NSD1 does not possess a homeodomain, but Wang et al reported that PHD fingers I to IV of NSD1 allowed binding to the *HOXA7* and *-A9* promoter. Binding capacity to promoters in the *HOXB* cluster was not reported. We observed high *HOXA9* and *-A10* expression in *NUP98/NSD1* patient samples but did not observe high *HOXA5* and *-A7* expression, as seen in *NUP98/NSD1*-transfected progenitors by Wang et al.[36] As transforming capacities of some *HOXA* and *-B* genes are established,[42] it would be of interest to investigate the mechanism and additive effect of high *HOXB* expression in AML with *NUP98/NSD1*. Importantly, Wang et al linked H3K36-methyltransferase activity of the SET domain of NSD1 to *HOXA* activation in *NUP98/NSD1*-transfected progenitors.[36] Therefore, novel therapeutic options might arise from epigenetic studies, because *NUP98/*

NSD1 showed to exert its leukemogenic function through two histone-modifying activities, that is, H3K36-methyltransferase activity and histone acetylation activity of the recruited CREBBP/p300-complex.[36] The latter is probably present in all NUP98 fusions, because the GLFG-repeats, preserved in all *NUP98* translocations, recruit the CREBBP/p300-complex. Therefore, specific histone acetyltransferase inhibitors might be potentially effective in *NUP98*-rearranged AML.[43]

Recently, Takeda et al suggested a novel mechanism by which *NUP98* fusions dysregulate transcription.[44] They showed that *NUP98-HOXA9* and *NUP98-DDX10* inhibited CRM1-dependent nuclear export, resulting in nuclear entrapment of transcriptional regulators, and thereby enhanced transcription of their down-stream targets. We showed that NUP98/NSD1 aberrantly localized in nuclear aggregates, suggesting that this mechanism may also apply for NUP98/NSD1.

In conclusion, the cryptic *NUP98/NSD1* translocation defines a previously unrecognized group of young AML patients with dismal outcome. Routine screening for *NUP98/NSD1* at diagnosis will be essential for proper identification and stratification of these patients.

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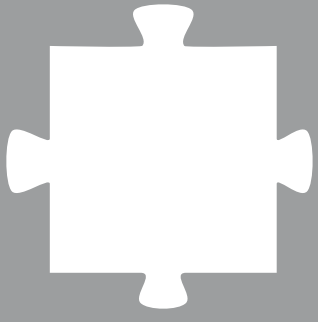
CHAPTER 9



Prevalence and prognostic value of *IDH1* and *IDH2* mutations in childhood AML: a study of the AML-BFM and DCOG study groups

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ABSTRACT

Mutations in the *NADP^{+}-dependent isocitrate dehydrogenase genes 1* and *2* (*IDH1* and *IDH2*) have recently been found in adult acute myeloid leukemia (AML) patients with a prevalence rising up to 33%. To investigate the frequency of *IDH1/2* mutations in pediatric AML, we characterized the mutational hotspot (exon 4) of these genes in diagnostic samples from 460 pediatric AML patients. Our analysis identified somatic *IDH1/2* mutations in 4% of cases (*IDH1* R132 n=8; *IDH2* R140 n=10) and the minor allele of single nucleotide polymorphism (SNP) rs11554137 in 47 children (10.2%). *IDH* mutations were associated with an intermediate age (p=0.008), FAB M1/M2 (p=0.013) and *NPM1* mutations (p=0.001). In univariate analysis, *IDH*^{mutated} compared with *IDH*^{wildtype} patients showed a significantly improved overall survival (OS; P=0.032) but not event-free survival (EFS; P=0.14). However, multivariate analysis did not show independent prognostic significance. Children with at least one minor allele of *IDH1* SNP rs11554137 had similar EFS (P=0.27) and OS (P=0.62) compared with major allele patients. Gene expression profiles of 12 *IDH*^{mutated} were compared with 201 *IDH*^{wildtype} patients to identify differentially expressed genes and pathways. Although only a small number of discriminating genes were identified, analysis revealed a deregulated trypthophan metabolism, and a significant down-regulation of *KYNU* expression in *IDH*^{mutated} cases.

INTRODUCTION

Although treatment results in childhood acute myeloid leukemia (AML) have improved during the last 30 years, there is still a significant number of patients with dismal prognosis.[1-3] To date, analysis of cytogenetic aberrations provide the most important prognostic information at diagnosis of this heterogeneous disease.[4-5] Furthermore, different mutations have been identified in several genes in childhood AML, including *FLT3*[6], *NPM1*[7], *CEBPA*[8-9], and *WT1*[10-11]. Some of these genetic alterations have been associated with treatment outcome and may serve as a basis for better risk assessment and molecularly based therapies.

Recently, by sequencing an AML genome, a somatic mutation was found in codon 132 of the gene for isocitrate dehydrogenase 1 (*IDH1*).[12] The protein encoded by this gene is found in the cytoplasm and peroxisomes. It is an $NADP^+$-dependent isocitrate dehydrogenase and has a significant role in cytoplasmic NADPH production. The same codon of *IDH1* is frequently affected by mutations in malignant glioblastomas.[13] *IDH2* has the same enzymatic activity as *IDH1* but is located in the mitochondrial matrix. *IDH2* mutations were reported in codons R140 and R172 in AML.[14] Mutations of both *IDH1* and *IDH2* provide a new ability of the enzyme to catalyse the NADPH-dependent reduction of alpha-ketoglutarate to 2-hydroxyglutarate (2-HG). Excess accumulation of oncometabolite 2-HG lead to DNA-damage mediated by elevated levels of reactive oxygen species.[14-15] In addition, mutant IDH appears to affect TET2 activity and thereby it induces changes of global and gene specific gene methylation.[16] Discovery of *IDH* mutations led to renewed efforts to decipher the role of altered metabolic processes in cancer.[17] Several studies confirmed the high prevalence of *IDH1* and also *IDH2* mutations in adult AML.[18-23] The prognostic importance of *IDH* mutations has been contradictive in these studies. Furthermore, a single nucleotide polymorphism (SNP) in exon 4 of the *IDH1* gene has been reported to be associated with poor prognosis in adult cytogenetically normal AML (CN-AML).[24]

In contrast to adult AML, only a few reports concerning genetic aberrations of the *IDH* genes have been published in childhood AML. Although in a series of 257 children no *IDH1* mutation was detected[25], *IDH1* mutations were identified in four out of 165 pediatric AML patients in an Italian study.[26] To the best of our knowledge one single case of an 12-year-old boy with an *IDH2* mutation[27] but no studies on the *IDH1* SNP rs11554137 in childhood AML have been reported to date.

Therefore, we performed an analysis of *IDH1/2* mutations and the *IDH1* SNP rs11554137 in 460 childhood AML patients. All patients were intensively treated with consecutive multicenter trials of the AML-Berlin-Frankfurt-Münster (AML-BFM) and Dutch Childhood Oncology Group (DCOG) study groups.

PATIENTS AND METHODS

Patients

Bone marrow (BM) or peripheral blood (PB) samples from initial diagnosis were obtained from 460 patients younger than 18 years with AML (French-American British [FAB] classification M0-M7). A total of 335 patients were treated uniformly within the two multicenter treatment trials AML-BFM 98 (recruitment July 1998 to June 2004, n=68) or AML-BFM 2004 (NCT00111345, start of recruitment 2004, n=267). Details of the AML-BFM 98 and the current AML-BFM 2004 treatment protocols have been reported previously (see also Online Supplementary Figure S2).[1,28-29] The other 125 children with AML were treated between 1987 and 2005 according to consecutive DCOG treatment protocols. Details of these treatment protocols have been published previously; treatment consisted of four to five blocks of intensive chemotherapy, using a standard cytarabine and anthracycline backbone.[30-32] Stem cell transplantation (SCT) in CR1 was used only in selected high-risk patients. The study was approved by the local research ethics committees. Written informed consent from patients, parents, or guardians was obtained. The median follow-up time for patients under follow-up was 2.4 years (range, 0.2 - 10 years).

Cytogenetic analyses

Cytogenetic analyses were carried out and centrally reviewed using standard chromosome-banding analysis, and using reverse transcriptase (RT-)PCR and/or fluorescent *in situ* hybridization for recurrent non-random genetic abnormalities, including t(15;17), inv(16), t(8;21) and *MLL* gene rearrangements as previously reported.[1,5]

Mutational analysis of *IDH1* and *IDH2*

Preparation of mononuclear cells and extraction of genomic DNA were performed as reported previously.[7,10,24,33] The genomic regions of exon four of the *IDH1/2* genes were analyzed in two sets using primers and PCR as described.[19,24] Purified PCR fragments were directly sequenced or were screened for mutations by using denaturing high-performance liquid chromatography. All mutations were confirmed in an independent second experiment by direct sequencing. A total of 122 samples were analyzed by two independent laboratories and showed identical results. Mutation analysis for *NPM1* exon 12 and for *FLT3*/ITD was performed as described.[7,34]

Gene expression profiling

Gene expression data (Human Genome U133 Plus 2.0 Array, Affymetrix) were available for 12 $IDH^{mutated}$ and 201 $IDH^{wildtype}$ cases [including 14 pediatric AML cases (1 $IDH1^{mutated}$ and 13 $IDH^{wildtype}$ patients) from our cell-bank not included in this study as these patients were treated according to different protocols]. Original data files have been deposited previously in the Gene Expression Omnibus repository (http://www.ncbi.nlm.nih.gov/geo) under ac-

cession number GSE17855. Extraction of total cellular RNA, microarray processing, data acquisition and data normalization have been described previously.[35] Differentially expressed genes between *IDH*^{mutated} cases and *IDH*^{wildtype} cases were calculated using *t*-test-based statistics (Bioconductor package LIMMA; http://www.bioconductor.org/) in the statistical data analysis environment R, version 2.7.0 (http://www.r-project.org/). Pathway analysis was performed using Globaltest (Bioconductor package Globaltest) for Kyoto Encyclopedia of Genes and Genomes (KEGG)-based and Gene Ontology (GO)-based gene sets. All *P* values were corrected for multiple testing according to the false discovery rate (FDR) procedure as developed by Benjaminin and Hochberg using Bioconductor package Multtest.[36]

Quantitative RT-PCR

Expression levels of selected genes (*KYNU, S100A8* and *S100A9*) were validated using quantitative RT-PCR (RT-qPCR). Transcript levels were measured in duplicates based on the intercalation of SYBRgreen (Finnzymes, Woburn, USA) on an ABI-PRISM-7900HT system (Applied Biosystems, Darmstadt, Germany), and calculated relative to *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) expression.

Statistical analysis

Event-free survival (EFS) was defined as the time from diagnosis to the date of last follow-up in complete remission or first event. Events were resistance to therapy (nonresponse), relapse, secondary neoplasm, or death from any cause. Failure to achieve remission due to early death or nonresponse was considered as events at time zero. Overall survival (OS) was defined as the time of diagnosis to death from any cause or last follow-up. The Kaplan-Meier method was used to estimate survival rates, differences were compared with the two-sided log-rank test. The Cox proportional hazards model was used for uni- and multivariate analyses. Cumulative incidence (CI) functions for competing events were constructed by the method of Kalbfleisch and Prentice, and were compared with the Gray's test. Results are presented as estimated probability of 5-year EFS (pEFS) and estimated cumulative incidence of relapse (pCIR) with standard error ($\pm$ SE). Differences in the distribution of individual parameters among patient subsets were analyzed using the Fisher's exact test for categorized variables and the Mann-Whitney *U*-test for continuous variables. All statistical analyses were conducted using the SAS program (SAS-PC, Version 9.1; SAS Institute, Cary, USA).

RESULTS

Patient population

Mutations in *IDH1* and *IDH2* were analyzed in pretreatment samples from 460 newly diagnosed AML patients. Comparison of clinical characteristics and outcome for the tested population vs. the population included in the different treatment protocols revealed no signifi-

TABLE 1. Main clinical and biological features of patients with *IDH*-mutated AML.

Patient	*IDH* mutation*	Protein change#	Age, years	Sex	WBC, $x10^9$/l	Cytogenetics	Other mutations	Survival status
1	*IDH1* C394G	R132G	6	F	103	NA	*NPM1*	alive
2	*IDH1* C394T	R132C	9	F	1.8	normal	*NPM1*	alive
3	*IDH1* C394G	R132G	7	M	68	11q23	*MLL*-PTD	alive
4	*IDH1* G395A	R132H	13	M	115	der(3q)	none	alive
5	*IDH1* G395A	R132H	7	M	1.8	t(15;17)(q22;q11)	NA	alive
6	*IDH1* C394T	R132C	15	F	35	NA	none	alive
7	*IDH1* C394T	R132C	4	M	29	t(15;17)(q22;q11)	none	alive
8	*IDH1* G395A	R132H	14	F	45	normal	*FLT3*/ITD, *MLL*-PTD	alive
9	*IDH2* G419A	R140Q	8	F	13.2	t(8;21)(q22;q22)	*FLT3*/ITD	alive
10	*IDH2* G419A	R140Q	9	M	46.7	NA	*NPM1*	alive
11	*IDH2* G419A	R140Q	15	M	78	normal	*NPM1*	alive
12	*IDH2* G419A	R140Q	7	M	120	normal	*MLL*-PTD	alive
13	*IDH2* G419A	R140Q	10	M	66	monosomy 7	*CEBPA*dm	alive
14	*IDH2* G419A	R140Q	10	M	20	t(8;21)(q22;q22)	NA	alive
15	*IDH2* C419T	R140L	7	F	85	NA	*NPM1*	dead
16	*IDH2* G419A	R140Q	14	F	8.6	other	*NPM1*	alive
17	*IDH2* C418T	R140W	10	M	14	t(8;21)(q22;q22)	none	alive
18	*IDH2* G419A	R140Q	6	M	NA	normal	*NPM1, FLT3*/ ITD	alive

Abbreviations: WBC indicates white blood cell count at diagnosis; NA, not available; CEBPAdm, CEBPA double mutation. *The nucleotide sequence variations are designated according to the recommendations of the Human Genome Variation Society (http://www.hgvs.org/mutnomen/). *IDH1* nucleotide numbering uses the first base of the translation start codon as nucleotide _1 on the basis of National Center for Biotechnology Information sequence NM_005896.2. *IDH2* nucleotide numbering uses the first base of the translation start codon as nucleotide _1 on the basis of National Center for Biotechnology Information sequence NM_002168.2.
#The protein changes are theoretically deduced and designated according to the recommendations of the Human Genome Variation Society (http://www.hgvs.org/mutnomen/).

cant differences in induction CR rates, OS or EFS from study entry. Higher diagnostic white blood cell (WBC) counts (P<0.001) were observed in the tested population as compared with the whole population, whereby those tested were older (P<0.001). In addition, the tested population included fewer patients with megakaryocytic leukemia (P=0.002).

Incidence of *IDH1 and IDH2* mutations in AML patients

IDH1 and *IDH2* mutations were found in 18 of 459 (4%; 95% confidence interval (CI) +/- 1.8%) pediatric patients with AML. Eight heterozygous point mutations were detected in exon 4 of *IDH1*, all affecting codon 132. Although a conversion of CGT>TGT leading to a R132C substitution and a conversion of CGT>CAT leading to a R132H were detected in three cases each, the conversion of a CGT>GGT leading to a R132G substitution was observed in two patients.

Eight of the ten patients with *IDH2* mutations showed a conversion of CGG>CAG leading to a R140Q substitution. One patient harboured a CGG>CTG change resulting in a R140L substitution and one patient had a conversion of CGG>TGG resulting in a R140W substitution. No mutations affecting codon R172 of *IDH2* were detected. Furthermore, one missense mutation in codon 116 (AAT>AGT) of *IDH1*, causing the substitution of the amino acid asparagine by serine, was found. Using Polyphen-2 prediction software,[37] the alteration is located in a highly conserved site and is scored as probably damaging. Unfortunately, no germline material was available to test the leukemic-specific origin of this mutation. However, neither this mutation nor the affected codon has been described before. Therefore, we regarded this mutation as an unclassified variant and we excluded this case for all survival analyses. In this cohort, mutations in *IDH1* and *IDH2* were mutually exclusive.

Correlation of *IDH1/2* mutations and *IDH* SNP rs11554137 with clinical features, biological characteristics and other gene mutations

As both *IDH1* and *IDH2* mutants cause the loss of the physiologic enzyme function resulting in elevated 2-hydroxyglutarate levels,[13-14,38] all statistical analyses on prevalence and prognostic influence were performed with the combined mutation status of *IDH1* and *IDH2*. Additionally, a second analysis in which *IDH1* and *IDH2* mutations were each analyzed separately for impact on OS and EFS can be found in the Online Supplement (Online Supplementary Figure S1).

There were no significant differences between $IDH^{mutated}$ and $IDH^{wildtype}$ patients with respect to sex, cytogenetic risk group, central nervous system involvement, hemoglobin levels, WBC count, platelet count, or blast count. However, *IDH* mutations were significantly associated with an intermediate age (2-10 years; 79 vs. 33% in other patients, p=0.008) and with FAB M1/M2 (71 vs. 39%, p=0.013).

A t(8;21) or a t(15;17) was diagnosed in three and two patients, respectively. Five of the 14 $IDH^{mutated}$ patients with available cytogenetics had a normal karyotype (35.7%). *IDH1/2* mutations were significantly more often in patients with concomitant *NPM1* mutations (39 vs. 10%; p=0.001). Other co-occurring mutations were partial tandem duplications in the *MLL* gene (*MLL*-PTD) in three patients and a *CEBPA* double mutation in one patient. The main biological and clinical features of the 18 $IDH^{mutated}$ patients are summarized in Table 1.

In the present pediatric cohort, the minor allele of SNP rs11554137 (SNP+) was detected in 47 of 460 patients (10.2%; 95% CI +/- 2.8%). Thus, its prevalence in the pediatric patients was comparable to adult AML patients (10.9%) and adult controls (12%).[24] All baseline characteristics, cytogenetic aberrations and gene mutations were similarly distributed between SNP+ and SNP- patients.

Influence of *IDH1/2* mutations and *IDH* SNP rs11554137 on treatment response and survival

A total of 17 patients (94%) with *IDH1/2* mutations achieved a CR compared with 387

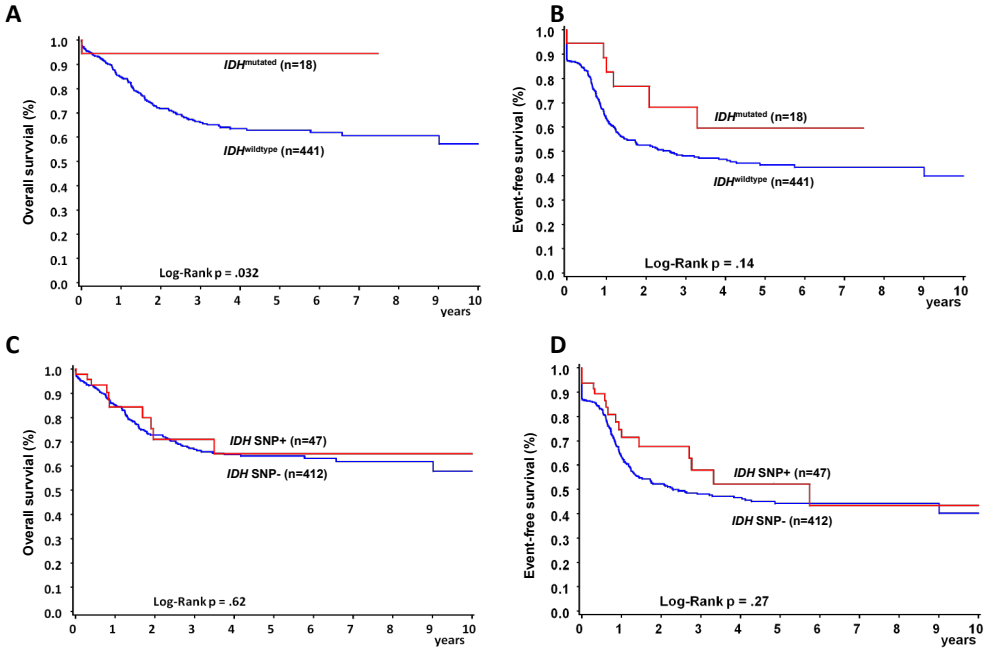

FIGURE 1. Impact of *IDH* mutations and *IDH* SNP rs11554137 on patient outcome. Overall survival (OS) and event-free survival (EFS) according to *IDH* mutation status (A,B); OS and EFS according to *IDH* SNP rs11554137 status (C,D). (A color version of this figure can be found in the appendices.)

IDH1/2^{wildtype} patients (88%; P=.52). In univariate analysis, *IDH*^{mutated} patients compared with *IDH*^{wildtype} patients had a significantly superior OS while no statistical difference was observed for EFS (OS: p=0.032; EFS: p=0.14; Figures 1A and 1B). This was also true when looking at the risk-defined subgroups (data not shown). No difference in achievement of CR (p=0.754) was observed between *IDH* SNP+ and *IDH* SNP- patients. In univariate analysis, SNP+ patients showed no difference for outcome parameters (OS: p=0.62, EFS: p=0.27). A multivariate analysis for OS considering *IDH* mutations status, cytogenetic risk group, WBC, and *NPM1* mutation status revealed no independent prognostic significance for the *IDH* mutation status (HR= 0.49, CI 95% 0.07-3.6, p=0.484; Table 2).

Gene expression profiling of *IDH*^{mutated} cases

We compared the gene expression profiles of *IDH*^{mutated} cases (n=12) with other AML cases (n=201), and identified 18 significantly differentially expressed probe sets (FDR-adjusted p<0.05; Online Supplementary Table S1). These included 3 probe sets annotating the *PAWR* gene (PRKC, apoptosis, WT1, regulator protein), showing up-regulated expression. When focussing on the probe sets with the highest fold change between *IDH*^{mutated} and *IDH*^{wildtype} cases, the two most down-regulated ones annotated the calcium-binding protein encoding genes *S100A8* and *S100A9* (Online Supplementary Table S2). Validation with RT-qPCR corre-

TABLE 2. Multivariate analysis for overall survival.

Variables in the model	Hazard ratio*	95% CI	*P* value
IDH1/2 mutation	0.49	0. 07 – 3.6	0.484
FLT3/ITD	0.92	0.48 – 1.74	0.795
NPM1 mutation	0.79	0.31 – 2.02	0.625
WBC count: above vs. below 100 x10^9/l	1.71	1.05 – 2.79	0.031
Cytogenetic risk group: SR vs. HR	0.19	0.08 – 0.48	<0.001

Abbreviations: CI, confidence interval; WBC, white blood cell count; SR, standard risk; HR, high risk.
*Hazard ratios greater than or less than 1 indicate an increased or decreased risk, respectively, of an event for the first category listed.

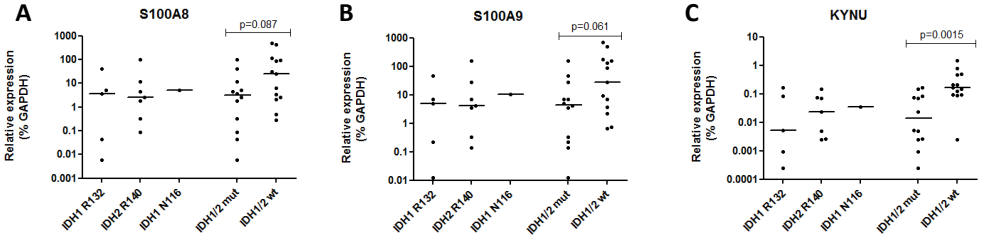

FIGURE 2. Down-regulated expression levels of *S100A8, S100A9* and *KYNU* in *IDH1/2*^{mutated} cases. Relative RT-qPCR expression values are shown for *S100A8* (A), *S100A9* (B) and *KYNU* (C) per *IDH1/2* mutation subtype, and combined as *IDH1/2*^{mutated} cases (indicated as *IDH1/2* mut) vs. the *IDH1/2*^{wildtype} cases (indicated as *IDH1/2* wt).

lated with microarray expression values and showed down-regulated expression of *S100A8* and *S100A9* in *IDH*^{mutated} cases (Figures 2A and 2B). Pathway analysis on KEGG-based gene sets revealed the tryptophan metabolism as the only significantly enriched KEGG-pathway (FDR-adjusted *P* value <0.05). Deregulation of this process in *IDH*^{mutated} cases was sustained by a pathway analysis using GO terms, which showed 25 significantly discriminative GO terms, among which the tryptophan metabolism was clearly enriched (Online Supplementary Table S3). The expression of *KYNU* encoding the enzyme kynureninase, involved in this pathway, was validated by RT-qPCR, and indeed showed a significantly down-regulated expression in *IDH*^{mutated} cases (Figure 2C).

DISCUSSION

In this large cohort of pediatric patients with AML, mutations in the *IDH1* and *IDH2* genes were identified as recurrent mutations in childhood AML. With a frequency of 4% in pediatric AML patients, mutations in the *IDH* genes are substantially less frequent in pediatric as compared with adult AML patients, where the prevalence is up to 33%.[20] The lower frequency of *IDH1* mutations is in accordance to a recent Italian study that found *IDH1* mutations in 2.4% of childhood AML cases.[26] In pediatric AML, mutations in codon R140 of *IDH2*

have only been described in a single case[27] and our findings outline that they have a similar frequency to *IDH1* in that age group. Interestingly, we did not find any mutations affecting codon R172 of *IDH2* in patients with childhood AML. This finding appears to be in line with previous studies in adult AML where 77% of patients with R172 *IDH2* mutations were elderly patients (≥60 years of age).[20]

In the present study, we identified a strong association between *NPM1* and *IDH* mutations that was already described for adult AML patients.[20,24,39-40] Similar to our findings for *IDH* mutations, mutations in *NPM1* also occur less frequently in pediatric AML patients compared with adult patients (8.4 vs. 35%).[7] However, in contrast to adult AML, *IDH* mutations were not associated with a normal karyotype in childhood AML. Interestingly, these mutations were also recurrently observed in patients with good risk cytogenetics (three patients with t(8;21), two patients with t(15;17)). Both types of *IDH* mutations did not occur in children below 3 years of age which affirms the distinctiveness of infant AML.

The prognostic effect of *IDH* mutations in adult AML has been intensively studied but remains a matter of discussion. Although some studies have suggested a negative prognostic effect for different subgroups of CN-AML patients,[18,20-23] others have not found any prognostic effect.[19,24-25,39] Little is known about the prognostic effect of *IDH* mutations in childhood AML. Interestingly, we found a positive prognostic impact of the mutations in childhood for OS (p=0.032). In this line, all *IDH*^{mutated} patients who relapsed are now in complete remission after stem cell transplantation. A multivariate analysis for OS revealed that the *IDH* mutation status did not add new independent prognostic information to the established stratification system. The prognostic evaluation of the mutation is certainly limited by the low number of mutated patients and further studies are needed to complement the prognostic assessment. However, the data clearly points in a different prognostic direction compared with adult AML. Another important difference may be the considerably higher proportion of patients presenting with favorable cytogenetics as compared with adult patients, with more than one third of *IDH*^{mutated} children presenting with t(8;21) and t(15;17). In adult AML, the occurrence of *IDH1/2* mutations in favorable risk patients has been observed only occasionally (<1%).[23,39] In line with our results is a recently reported pediatric AML patient with *IDH2* R140Q mutation who presented with a t(8;21) together with complex chromosomal aberrations. In addition, it has to be kept in mind that dose intensity of induction chemotherapy in pediatric protocols exceeds those in adult studies significantly. Thus, although not formally proven, these two factors might be relevant for the profound differences in the prognostic implications of *IDH* mutations.

We also found a difference between adult and childhood AML when analyzing the prognostic impact of the minor allele of SNP rs11554137 located in exon 4 of *IDH1*. In adult CN-AML patients, this SNP was reported to be associated with poor prognosis.[24] Surprisingly, in the present pediatric cohort SNP+ patients did not show any difference for EFS or OS (Figures 1C and 1D).

To gain biological insight in the contribution of *IDH1/2* mutations to leukemogenesis and

their potential role in drug response in childhood AML, we compared the gene expression profiles of *IDH1/2*^{mutated} AML with other pediatric AML cases. Only 18 discriminative probe sets were identified for *IDH*^{mutated} cases, indicating that these mutations do not drive a strong specific gene expression profile. This could be caused by a stronger profile of the underlying genetic subtypes, although correcting this did not generate more discriminative genes (data not shown). Moreover, cases in the *IDH*^{wildtype} group harbouring *TET2* mutations, which were recently shown to generate similar effects as *IDH1/2* mutations in AML might resemble the *IDH*^{mutated} profile.[16] However, we did observe an affected tryptophan metabolism, and validated down-regulated expression of the enzyme kynureninase, which is involved in the biosynthesis of NAD cofactors from tryptophan. The implication of this altered tryptophan metabolism in *IDH*^{mutated} AML remains to be elucidated. Furthermore, down-regulation of the calcium-binding proteins *S100A8* and *S100A9* was observed in *IDH*^{mutated} cases. A recent study reported that high expression of *S100A8* in AML correlated with poor outcome,[42] which is in concordance with our data showing down-regulated *S100A8* and *S100A9* expression in combination with a favorable outcome in *IDH1/2* mutants. Interestingly, overexpression of *S100A8* and *S100A9* induces prednisone resistance in pediatric ALL, which might also apply for the well-known steroid resistance in AML.[43-44] However, further functional studies have to be done to shed more light on the possible role of these genes in *IDH*^{mutated} AML.

In summary, we identified *IDH1* and *IDH2* mutations as a recurrent but infrequent event in childhood AML. The *IDH1/2* mutation status was not associated with independent prognostic significance. These results further underline the differences in the prevalence and also prognostic importance of molecular aberrations between childhood and adult AML.

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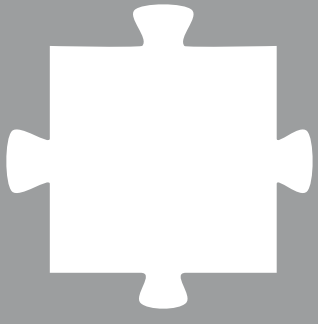
CHAPTER 10



Low frequency of *DNMT3A* mutations in pediatric AML, and the identification of the OCI-AML3 cell line as an in vitro model

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TO THE EDITOR

Recently, next generation sequencing technology has been applied to discover tumor-specific mutations in acute myeloid leukemia (AML) genomes.[1] After the finding of recurrent mutations in the enzyme isocitrate dehydroxygenase 1 (*IDH1*) in AML,[2] two studies recently reported the identification of somatic mutations in the DNA (cytosine-5-)-methyltransferase 3 alpha (*DNMT3A)* gene in adult AML cases.[3-4] Ley et al[3] reported the presence of *DNMT3A* mutations in 22% of *de novo* adult AML cases, after the discovery of this mutation by sequencing a whole AML genome. They found that *DNMT3A* mutations were highly associated with cytogenetically normal (CN-)AML (37% [44 of 120] CN-AML cases). Yan et al[4] detected *DNMT3A* mutations by sequencing the complete coding region of the genome (exome sequencing) of nine AML-M5 samples. They found that these mutations were restricted to the myelomonocytic (French-American-British (FAB)-M4) and monocytic (FAB-M5) AML subtypes, presenting in 13.6% and 20.5% of these cases, respectively. *DNMT3A* mutations were localized in the methyltransferase and the Plant Homeo Domain (PHD) finger domains, and impaired DNMT3A methyltransferase activity, or altered histone H3 affinity *in vitro*. Both studies reported a poor clinical outcome for patients with *DNMT3A*-mutated AML.[3-4]

We identified the presence of a somatic heterozygous R882C mutation in the *DNMT3A* gene by performing exome sequencing of a *pediatric* AML case and confirmed this mutation by Sanger sequencing (Figure 1). This index case concerned an 8-year-old boy with a CN-AML of the FAB-M1 subtype, who is currently in continuous complete remission (CCR) 3.6 years after diagnosis, following treatment according to the AML MRC15-protocol. Furthermore, we identified a *Wilms tumor 1* (*WT1*) R394W mutation in this patient resulting from a missense mutation in exon 9, which was known to be present from previous screening for molecular mutations. This case was also characterized by a large insertion/deletion in *WT1* exon 7 and an internal tandem duplication in the *FLT3* gene (Table 1).

As data on *DNMT3A* mutations in *pediatric* AML were not yet available, we subsequently screened cDNA of a large representative pediatric AML series (n=140; including 34 FAB-M4 and 27 FAB-M5 cases, and including 46 CN-AML cases) from the Dutch Childhood Oncology Group (DCOG) and AML-Berlin-Frankfurt-Münster Study Group (AML-BFM SG) studies for *DNMT3A* mutations in the region including amino acids 460-912, in which all but one of the previously reported mutations were found. Remarkably, only two additional cases with *DNMT3A* mutations were detected; one case harbored a R484W mutation, and the other case harbored a V716F mutation. The characteristics of these cases are presented in Table 1. Of interest, two of the three pediatric cases with *DNMT3A* mutations concerned an AML of the FAB-M1 subtype, which is in contrast with the (myelo)monocytic morphology predominantly found in adult *DNMT3A*-mutated AML cases.[3-4] Unlike the poor clinical outcome of adults with *DNMT3*-mutated AML, all three pediatric cases were in CCR at last follow-up. In conclusion, the estimated frequency of *DNMT3A* mutations in our pediatric AML series is only 2.1% (95% confidence interval (CI) 0%-4.5%), and 6.4% (95% CI 0%-13.4%) in the CN-

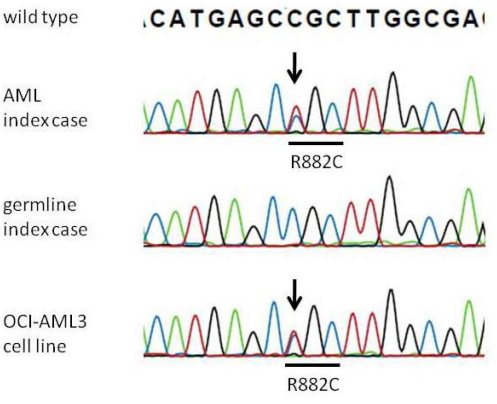

Figure 1. Sequence chromatograms of the *DNMT3A* mutations detected in the pediatric AML index case and the OCI-AML3 cell line. A heterozygous mutation (C>T), changing arginine into cysteine at codon 882, is present in the pediatric AML index case and the OCI-AML3 cell line. The germline sample of the index case did not harbor this mutation. Mutated nucleotides are indicated by arrows. (A color version of this figure can be found in the appendices.)

AML subset. In line with our data, Ho et al[5] recently published that no *DNMT3A* mutations were observed in their pediatric AML series (n=180). Consistent with this low frequency in children, both adult AML studies reported a high mean age (54.9 and 53.1 years, respectively) for *DNMT3A* mutants, indicating that these mutations are associated with a disease onset at advanced age.[3-4] Of note, our frequency might be slightly underestimated as we performed mutational screening on cDNA, and Ley et al[3] showed that 2 of 21 (10%) mutated alleles were not expressed. However, in the study of Yan et al[4] all 23 *DNMT3A*-mutated alleles were expressed.

Furthermore, we screened 12 AML cell lines derived from the German Resource Centre for Biological Material (DSMZ, Germany), and found that the OCI-AML3 cell line harbored a *DNMT3A* R882C mutation (Figure 1). The OCI-AML3 cell line was derived from a 57-year-old male with FAB-M4 AML, and carries an *NPM1* mutation.[6] Hence, this cell line can be used as an *in vitro* model to further study the leukemogenic and drug-resistance aspects of *DNMT3A* mutations in AML.

Our findings further illustrate the large differences in the frequency of genetic aberrations found in pediatric and adult AML. *NPM1* mutations are highly associated with *DNMT3A* mutations in adult AML,[3-4] which is consistent with our finding of a *DNMT3A* mutation in one pediatric *NPM1*-mutated case and in the *NPM1*-mutated OCI-AML3 cell line. In pediatric AML, there is a four to five-fold lower frequency of *NPM1* mutations compared with adult AML,[7] which may partially explain why the frequency of *DNMT3A* mutations is also lower. Moreover, pediatric AML is characterized by a different base pair insertion in *NPM1* as compared with the type of *NPM1* mutations found in adult AML,[7-8] which points towards a different ontogeny of pediatric AML. Furthermore, *IDH1/2* mutations also occurred more frequently in adult *DNMT3A*-mutated cases,[3] but these mutations are, similar as *NPM1* mutations, less frequent found in pediatric AML.[9] None of our pediatric *DNMT3A* mutants carried an *IDH1/2* mutation. Because the recently discovered mutations in AML are less frequent or even absent in pediatric AML, further genome-wide sequencing studies in pediatric AML are warranted separately from adult AML to map genetic aberrations underlying

TABLE 1. Clinical and genetic characteristics of the three *DNMT3A*-mutated pediatric AML cases.

No.	*DNMT3A* mutation	Age, years	Sex	WBC, $x10^9$/l	FAB	Karyotype	Mutations	Therapy protocol	Follow-up
#1	R882C	8.4	M	25.0	M1	CN-AML	*WT1* ex 7/9 + *FLT3*/ITD	MRC-AML15	CCR, 3.6 years
#2	R484W	15.7	F	9.5	M1	CN-AML	*CEBPA* double mutant	AML-BFM04	CCR, 6 mo*
#3	V716F	15.3	M	30.8	M4	CN-AML	*NPM1* + *N-RAS*	AML-BFM04	CCR, 4.8 years

Abbreviations: F/M indicates female/male; WBC, white blood cell count at diagnosis; FAB, French-American-British, CN-AML, cytogenetically normal AML; ex, exons; CCR, continuous complete remission; mo, months.
*This patient was in CCR 6 months after diagnosis, but lost to follow up data after that.

pediatric AML specifically. The discovery of these aberrations is needed to further improve the current stagnated survival rates of pediatric AML, because they provide insight in leukemogenesis and might serve as drugable targets. As genome-wide sequencing studies have become more affordable in recent years, they promise to be a valuable tool to further dissect pediatric AML.

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CHAPTER 11

Summary
General Discussion
Future Perspectives

SUMMARY

Pediatric acute myeloid leukemia is a heterogeneous disease, and comprises diverse clinical entities reflected by differences in the morphology, underlying genetic aberrations and clinical behavior. The progress that has been made in the field of cancer molecular genetics has provided us with techniques to further unravel the molecular genetic basis of pediatric AML. This is warranted as with the current intensive treatment regimens, still 30% of children die of the disease or treatment-related toxicity. Novel treatment options are therefore needed to improve survival rates, and better understanding of the pathobiology of the different unique entities by dissecting the underlying genetic aberrations is likely to guide the development of novel drugs for pediatric AML. In this thesis we specifically focused on the molecular genetic dissection of the group of pediatric AML patients that lacks chromosomal aberrations with conventional karyotyping in their leukemic cells, the so called cytogenetically-normal subtype of AML (CN-AML), as the driving oncogenic lesions were largely unknown in this group. We provide evidence that CN-AML patients represent a heterogeneous population of pediatric AML, characterized by diverse underlying genetic aberrations with distinct prognostic significance.

In the first part of this thesis we assessed the role of the primary molecular genetic aberrations (type-II aberrations) that were recently discovered in adult AML. In **chapter 2** we showed that mutations in *Nucleophosmin* or *NPM1* are present in 8% of all pediatric AML cases, and in 20% of pediatric CN-AML (Figure 1). This frequency is 2-3 times lower than in adult CN-AML, in which *NPM1* mutations comprise 50-60% of the cases. Moreover, the main type of *NPM1* mutations differ between children and adults. *NPM1* mutations characterized a favorable prognostic group of pediatric AML patients regardless of the co-occurrence of the poor prognostic marker *FLT3*/ITD, which was in particular evident in CN-AML.

In **chapter 3** we determined the role of different *CEBPA* aberrations in pediatric AML. Mutational screening revealed *CEBPA* mutations in 8% of all pediatric AML cases: 70% of *CEBPA*-mutated cases carried two individual mutations (i.e. *CEBPA* double mutants), which have previously been shown to occur typically bi-allelic. *CEBPA* double mutations independently conferred a favorable prognosis. The combination of screening for *CEBPA* promoter hypermethylation and unsupervised clustering based on gene expression profiles identified 5 cases with a silenced *CEBPA* gene, accounting for 2% of pediatric AML cases. Moreover, gene expression profiling revealed clustering of *CEBPA*-double mutated and *CEBPA*-silenced cases indicating an identical expression signature of these two subtypes. Of interest, the 5 silenced cases had poor outcome, although larger series are needed to confirm this observation.

In **chapter 4** we showed that using MLPA, a *novel* DNA copy number screenings technique, in combination with mRNA RT-PCR, the *MLL* partial tandem duplication (*MLL*/PTD) was infrequently (2%) found in pediatric AML. *MLL*/PTD-positive AML displayed different characteristics than *MLL*-translocated AML.

In **chapter 5**, we combined the currently known type-I and type-II aberrations in an in-

tegrative analysis resulting in the largest series of pediatric AML to date. The aim was to determine the characteristics, associations and prognostic impact of the various aberrations in one very large series of patients. Genetic aberrations were associated with specific clinical characteristics, e.g. significant higher white blood cell counts at diagnosis in *MLL*-rearranged, *WT1*-mutated and *FLT3*/ITD-positive AML patients. Moreover, the distribution of several genetic aberrations differed significantly between children below and above the age of 2 years. Non-random associations between different type-I and -II aberrations were observed, e.g. *KIT* mutations with CBF-AML, and *FLT3*/ITD with t(15;17)(q22;q21), *NPM1*- and *WT1*-mutated AML. Besides the already established 'favorable karyotype' group within pediatric AML, *NPM1*-mutated and *CEBPA* double-mutated AML were both identified as independent predictors for favorable outcome.

In the second part of this thesis genome-wide screenings techniques were applied to identify novel genetic aberrations that play a role in pediatric CN-AML. In **chapter 6** we identified the frequent involvement of *Wilms tumor 1* (*WT1*) gene aberrations in pediatric AML via the detection of one case with a cryptic deletion of 11p13. Besides one additional case with a whole *WT1* gene deletion, *WT1* aberrations consisted of diverse gene mutations, and both *WT1* alleles were affected in at least 54% of cases. *WT1* aberrations clustered in the CN-AML subtype affecting 22% of cases, and were frequently associated with *FLT3*/ITD. They were identified as a novel marker for poor outcome, and the presence of a *WT1* aberration in combination with *FLT3*/ITD recognized a subgroup with very poor outcome.

Subsequently we analyzed in **chapter 7** the prognostic impact of the *WT1* single nucleotide polymorphism (SNP) rs16754, which is located in the mutational hotspot region, and *WT1* gene expression at diagnosis. Both did not predict clinical outcome.

Using A-CGH we also identified the presence of a chromosomal translocation invisible by conventional karyotyping, i.e. the cryptic *NUP98/NSD1* translocation in 16% of pediatric CN-AML **(chapter 8)**. This fusion gene was mutually exclusive with other type-II aberrations, but frequently associated with the type-I aberration *FLT3*/ITD, and with *WT1* mutations. This translocation was significantly more frequent in pediatric AML than adult AML. In pediatric as well as in adult AML, *NUP98/NSD1* independently predicted dismal outcome, with 5-year event-free survival rates below 10%. Of interest, its prognostic impact was also independent of the poor prognostic factor *FLT3*/ITD. Gene expression profiles demonstrated that *NUP98/NSD1*-positive cases were characterized by high expression of several *HOXA* and *-B* cluster genes, pointing towards *HOX*-driven leukemogenesis in these cases.

In 2009, whole genome sequencing of the second adult AML genome unraveled the presence of mutations in the enzymes *isocitrate dehydroxygenase 1* and *-2* (*IDH1/-2*), which were subsequently linked to CN-AML and poor outcome. Together with our German collaborators, we investigated the characteristics of these mutations in pediatric AML **(chapter 9)**. We found that *IDH1* and *-2* mutations were infrequently present in pediatric AML, non-randomly associated with *NPM1* mutations, and that they did not independently influence outcome. Disruption of these enzymes did not result in a strong discriminative gene expres-

sion signature.

In **chapter 10** we present a pilot study with exome sequencing in one pediatric CN-AML sample together with its matched germline material. We discovered a mutation in the DNA methyltransferase *DNMT3A* and confirmed a *WT1* mutation. Subsequent screening of a pediatric series however revealed that *DNMT3A* mutations are uncommon events in pediatric AML.

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Heterogeneity of pediatric cytogenetically normal AML

In this thesis we showed that a diverse spectrum of recurrent molecular genetic aberrations underlies pediatric CN-AML. These aberrations affect molecules with various functions, which provide us with knowledge on the pathways that are deregulated in AML leukemogenesis. Mutations in *NPM1* delocalize this nucleo-cytoplasmic shuttling protein into the cytoplasm, although the exact mechanism how mutated *NPM1* contributes to development of AML still remains to be elucidated.[1] The majority of *NPM1* mutations occurs in cytogenetically normal leukemias, both in adult and pediatric AML, but the work described in this thesis and other pediatric studies showed that their frequency is two to three times lower in pediatric CN-AML (Figure 1).[2-5] *NPM1*-mutated AML clearly characterizes a distinct entity within AML, regardless of additional cytogenetic aberrations, pointing towards a key role for *NPM1* mutations in those leukemias.[6-8] They are characterized by involvement of different hematopoietic lineages, a specific gene-expression profile, including up-regulation of *homeobox* (*HOX*) *A* and *-B* cluster genes, and a better response to induction therapy and a favorable long-term prognosis.[6, 9] Studies in mice recently provided indeed the first evidence that *NPM1* mutations are primary leukemogenic events in AML, as they by themselves caused delayed-onset AML, and rapid-onset AML together with different 'type-I aberrations'.[10] Mutant NPM1 in hematopoietic stem cells of mice induced *HOX* gene overexpression, increased self-renewal and stimulated myelopoiesis. Taken together, *NPM1* mutations are suggested to be classified as type-II aberrations in AML, although they do not classically induce a differentiation arrest.

An established type-II aberration concerns mutations in the *CEBPA* gene, which encode one of the crucial transcription factors for myeloid cell differentiation.[11] Targeted disruption of *CEBPA* results in a selective early block of granulocyte differentiation.[12-13] Different mechanisms can abrogate *CEBPA* functioning, including a variety of *CEBPA* mutations.[14-16] The majority of *CEBPA*-mutated AML cases harbors *CEBPA* double mutations, which typically affect both alleles and thereby result in complete loss of *CEBPA* wild-type expression.[17-18] Studies in adult AML have proposed that *CEBPA* double mutations define a specific entity, in contrast to cases with a single mutation only, which was based on the association with specific characteristics, e.g. less frequently concurrent genetic aberrations, and a distinctive

gene expression profile.[19-20] Moreover, double mutants were associated with a favorable outcome, but single mutants were not.[20-21] Furthermore, single germline *CEBPA* mutations have been found in familial AML, and the gain of a second somatic *CEBPA* mutation in the AML is a frequent event.[22-23] These data imply that germline *CEBPA* mutations predispose for AML and that additional somatic *CEBPA* mutations or other genetic events contribute to the development of AML. We also found a germline single *CEBPA* mutation and the acquirement of a second *CEBPA* mutation in the leukemic cells of a 6-year-old girl diagnosed with AML, indicating the variable time-latency of AML in cases with germline *CEBPA* mutations. *CEBPA* mutations in pediatric CN-AML were observed in a similar frequency (approximately 15%) as in adult CN-AML (Figure 1), and showed the same relation with favorable outcome, which was restricted to the double mutants.[24] Another large pediatric study conducted by the COG, however, showed favorable outcome for double as well as single mutants,[25] which might be explained by different secondary hits in these *CEBPA* single mutants as compared to our series. In the revised WHO classification of 2008, *NPM1*-mutated AML and *CEBPA*-mutated AML are now recognized as provisional entities within AML.[26] However based on the current data, one might suggest that only *CEBPA* double mutants should be considered to be categorized to this entity. We found that *CEBPA* promoter methylation as a mechanism to abrogate *CEBPA* functioning was infrequent in pediatric AML, and not present in CN-AML.[24] Of interest, the few pediatric cases with *CEBPA* promoter methylation had poor outcome, which was also suggested from a small number of adult *CEBPA* promoter methylated AML cases.[27] Independent series are therefore needed to confirm their poor prognostic impact. Unsupervised analysis of gene expression profiles showed clustering of *CEBPA*-double mutated and promoter methylated cases, indicating overlap in their expression signatures, which confirmed prior observations from our hematology department in adult AML.[27] In contrast, *CEBPA* promoter methylated cases displayed also different features than *CEBPA*-double mutated cases such as an immature morphology with T-lymphoid characteristics, a hypermethylated phenotype, and their potential association with poor outcome. These features point towards differences in pathobiology, which is reflected by the formation of two sub-clusters within their cluster.[27-28]

The third aberration investigated in this thesis, considered as a type-II aberration and known to be associated with trisomy 11 as well as CN-AML, is the partial tandem duplication of the *MLL* gene (*MLL*/PTD), which encodes a histone modifying enzyme.[29-30] Using DNA-MLPA combined with mRNA RT-PCR, we found that *MLL*-PTD is infrequent in all pediatric AML cases, as well as in the subtype of CN-AML (approximately 4%).[31] Translocations of *MLL* represent approximately half of the AML cases in infants, and 20% of all pediatric AML cases.[32] Although *MLL* can translocate with various partner genes, all *MLL* translocations together display a distinctive gene expression profile.[33] We previously showed that cases with an *MLL*/PTD did not cluster together with *MLL*-translocated cases, suggesting that they are a separate entity.[34] Interestingly, in our *HOX*-based clustering of gene expression profiles (chapter 8), we observed that *MLL*/PTD-positive cases were not only characterized by up-

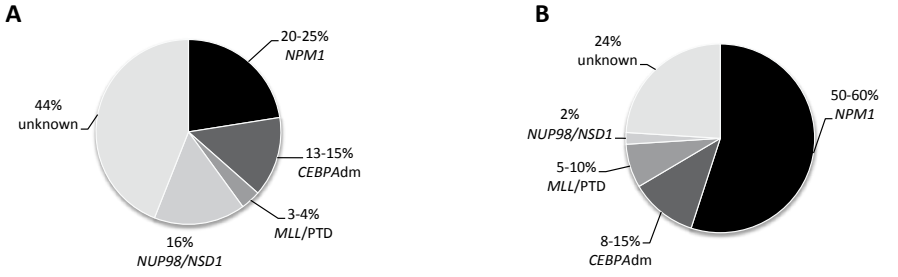

FIGURE 1. The frequency of the different type-II aberrations in pediatric (A) vs. adult (B) CN-AML. Frequencies are obtained from our own data and from literature.

regulation of *HOXA* as the majority of *MLL*-translocated cases, but also by up-regulation of *HOXB* genes. In chapter 4 we showed that *MLL*/PTD-positive AML also displayed different characteristics than *MLL*-translocated AML, such as the association with a higher age at diagnosis, no monocytic morphology, and a high frequency of co-occurrence of *FLT3*/ITD.

Taken the frequencies of the above mentioned aberrations together, type-II aberrations underlying pediatric CN-AML were found in approximately 40% of the cases (Figure 1). Moreover, the work described in this thesis adds a novel player to this pie chart, i.e. the cryptic *NUP98/NSD1* translocation that was present in 16% of pediatric CN-AML cases, and mutually exclusive with the other type-II aberrations. Non-cryptic translocations of *NUP98*, which encodes a member of the nucleopore complex, are known to be recurrent but rare events in AML, contributing to less than 1% of all cases. *NUP98* can fuse to more than 20 different partner genes, and transfection of several NUP98 fusions impair normal differentiation in hematopoietic progenitor cells.[35-39] In an *in vitro* model, Wang et al. demonstrated that also the *NUP98/NSD1* fusion inhibited cellular differentiation, thereby establishing it as a type-II aberration.[40] Interestingly, another nucleoporin (NUP214) is also affected in 1% of AML cases by the t(6;9)(p23;q34) translocation creating the *DEK/NUP214* fusion, which might point towards a similar functional contribution of translocated nucleoporins in leukemogenesis.[41]

For the other recurrent genetic aberrations that were identified in the second part of this thesis, their exact role in leukemogenesis and place in the type-I/II model remain to be elucidated. The *Wilms tumor 1* (*WT1*) gene has been attributed tumor suppressor as well as oncogene properties depending on the cellular context.[42] It encodes a transcription factor essential for urogenital development, which is mutated in a subset of Wilms' tumors, a pediatric renal tumor.[43-44] It is known that the majority of AML cases exhibit *WT1* gene overexpression.[45-46] We discovered *WT1* mutations in 22% of pediatric CN-AML (Figure 2), which mainly clustered in exon 7 encoding the first of four zinc finger domains.[47] These mutations are predicted to result in truncated proteins lacking the C-terminal DNA binding zinc fingers. Interestingly, it was reported that mutated transcripts were subjected to nonsense-mediated RNA decay, and truncated proteins could not be detected.[48] However, we did detect the mutations at DNA as well as at RNA level, but how they contribute to leukemogenesis still

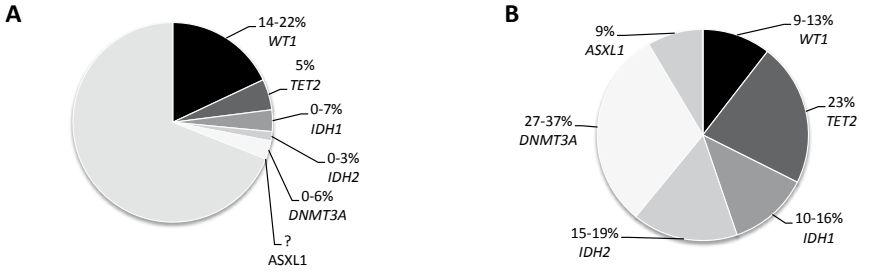

FIGURE 2. The frequency of yet unclassified molecular genetic aberrations in pediatric (A) vs. adult (B) CN-AML. The percentages do not count up to 100%, as the overlap between the different genetic aberrations is not presented as this is not exactly known yet. Frequencies are obtained from our own data and from literature.

needs to be determined.

Another molecule known to be mutated in gliomas and discovered to be affected in AML by whole genome sequencing, is the enzyme isocitrate dehydroxygenase 1 (*IDH1*).[49] We determined that *IDH1* and its family member *IDH2* are far less frequently mutated in pediatric CN-AML than in adult CN-AML (Figure 2). It was demonstrated that mutated *IDH1/2* gained neomorphic enzyme activity resulting in 2-hydroxyglutarate production and consequentially induced global DNA hypermethylation via inhibition of the TET2 enzyme.[50] This study provided evidence that mutated *IDH1/2* inhibited cellular differentiation.[50] However, we found that *IDH1/2* mutations did not display a strong distinctive gene expression profile. This might be caused by a stronger profile of underlying genetic subtypes, or other genetic aberrations exhibiting the same expression profile. One example of the latter are the loss-of-function mutations in the *ten-eleven translocation 2* (*TET2*) gene, that were shown to harbor the same mechanistic properties as mutated *IDH1/2*.[50] This illustrates that functionally different proteins may induce the same leukemogenic effect. *TET2* mutations also appeared to be rare in pediatric AML.[51]

Mutations in the DNA methyltransferase gene *DNMT3A* in pediatric AML were discovered by us using a novel next-generation sequencing approach, i.e. sequencing the complete coding region of the genomes of a tumor and its matched germline sample. Simultaneously, similar screening approaches in adult AML also identified *DNMT3A* mutations, and showed that *DNMT3A* mutations affected methyltransferase activtiy or histone H3 binding capacity of DNMT3A, depending on the mutation location.[52-53] As DNMT3A ensures *de novo* methylation of the genome, mutations subsequently led to global DNA hypomethylation.[53]

These genetic aberrations, i.e. mutated *WT1*, *IDH1/2* and *DNMT3A*, are currently difficult to fit in the type-I/-II model, and one could ask if it will be possible to fit them in this model. These aberrations are mostly seen in addition to the established type-I and -II aberrations, e.g. *WT1* aberrations are frequently observed together with a *NUP98-NSD1* fusion and an *FLT3*/ITD, and *IDH1/2* mutations in *NPM1*-mutated cases. It appears to be more likely that they constitute an additional or several additional layers of genetic aberrations, which fits

in the concept of a multistep pathogenesis model of AML. Interestingly, the above mentioned mutations affect the epigenetic landscape, i.e. DNA methylation status or histone code, which might point towards a commonly affected process in the pathobiology of AML. Other mutated genes that were not investigated in this thesis, but which can also be added to this group, are mutations in *TET2* and additional sex combs-like 1 (*ASXL1*) (Figure 2).[51, 54-55] However, transfection of mutated *IDH1/2* and *TET2* resulted in a maturation arrest of the transfected progenitor cells, which would these mutations categorize as type-II aberrations according to the Gilliland type-I/-II model.[50] In contrast to other type-II aberrations, we showed that *IDH1/2*-mutated cases did not display a distinct expression profile. Their frequent co-occurrence with *NPM1* mutations might also support the presence of additional layer(s) in the development of AML. It might suggest that the different genetic aberrations might not so strictly be distinguished as currently being done in the classical type-I/-II model, i.e. the required disturbance of certain processes for leukemogenesis might be caused by one aberration or gained by several aberrations. Furthermore, one genetic aberration might also affect several leukemogenic processes such as maturation arrest and hyperproliferation as suggested for MLL fusions.

As touched upon above, distinct non-random associations between the different molecular aberrations exist, suggesting cooperation between the specific aberrations in leukemogenesis. This has for example been shown for the *PML-RARA* fusion and *FLT3*/ITD.[56] In addition to the known non-random associations between the different cytogenetic subgroups and specific type-I aberrations, we observed significant associations between the molecular subtypes in pediatric AML: *FLT3*/ITD is significantly associated with *NPM1* mutations, *MLL*-PTD, *NUP98/NSD1* and *WT1* mutations; *IDH1/2* mutations are significantly associated with *NPM1*-mutated AML; and *WT1* mutations had a preference for the nucleoporin-translocated leukemias, i.e. *NUP98*/*NSD1* and *DEK/NUP214*.[3, 31, 47] The next step will be to prove that these aberrations functionally cooperate, and to decipher their distinctive role in leukemogenesis.

In addition to the genetic aberrations, altered gene expression levels may also play important roles in leukemogenesis, although they might also reflect the maturation stage of the affected progenitor cells. However, the expression of several genes has been shown to harbor prognostic significance in adult (CN-)AML, such as the expression levels of *EVI1*, *MN1*, *BAALC*, and *ERG*, which are under investigation by our group in pediatric AML.[57-61]

Pediatric versus adult CN-AML

Notable differences in the genetic aberrations exist between pediatric CN-AML and CN-AML in adults. Although in both groups the same molecular genetic events can be found so far, there are remarkable differences in their frequencies (Figure 1 and 2). Furthermore, it will be interesting to determine if the remaining yet unidentified aberrations will be present in both age cohorts or restricted to one of them. For a few cytogenetic aberrations it is known that they exclusively occur in pediatric AML (i.e. t(7;12) and t(1;22)) or almost exclusively

occur in adult AML (i.e. inv(3)/t(3;3)).[62-64] Furthermore, AML after preceding MDS is rare in pediatric AML. Moreover, a different base-pair insertion in *NPM1* dominated in pediatric AML compared to the type of *NPM1* mutations found in adult AML, which points towards a different ontogeny of pediatric AML.[2-3, 65]

One still could argue that a specific genetic subtype in AML is the same in an adult or a child. Although this might be true, the same genetic subtype does not reach similar outcome rates in children and adults, as the prognosis is generally more favorable in children.[66] This may be due to host factors, such as the relative higher tolerability for intensive chemotherapy in children or differences in pharmacokinetics between adults and children. Other features such as a developing immune system or DNA repair system might also play a role. In addition, there may be differences in leukemia cell-specific factors, such as secondary genetic aberrations, or differences in cellular drug sensitivity as a result of that, or by up-regulation of the *MDR1* phenotype with age.

Moreover, due to the large difference in frequencies of specific genetic subtypes of AML the focus for novel therapies will be different. Therefore, it seems warranted to perform separate studies to elucidate the genetic basis of, and study drug development for, pediatric AML. The latter is also important as children are growing individuals, in which long-term effects side might be different and have more time to develop than in adults.

AML in children below the age of 2 years also seems to form a distinct entity, although it has been shown in the AML-BFM93/98 series that their outcome was not different than older children with AML.[66] AML below 2 years of age is characterized by a high incidence of specific genetic aberrations, such as *MLL*-translocations (~50%) and a complex karyotype (10-15%), and the exclusive presence of the rare translocations t(7;12) and t(1;22). Furthermore, the specific entity of AML in children with Down syndrome also usually present at a very young age. In contrast, significantly less CN-AML cases are present in this age category as compared to older children, as we showed in chapter 5. Furthermore, it is noteworthy that none of the 'molecular genetic' type-II aberrations, i.e. *NPM1* mutations, *CEBPA* double mutations, *MLL*-PTD and *NUP98/NSD1* is found in children below the age of 2 years. This point towards a distinctive ontogeny of infant AML. For *MLL*-translocations, which also represent 80% of infant ALL, there is evidence that solely the translocation by itself can induce a frank leukemia without the necessity of additional hits. Together with the finding that they already arise *in utero* might explain the short latency for *MLL*-rearranged AML and ALL.

Prognostic markers and improvement of risk-group classification

In most collaborative treatment protocols, the majority of CN-AML patients are stratified in the intermediate or standard risk-group, as risk-group classification in pediatric AML is generally based on the presence of certain specific cytogenetic aberrations and the early response on treatment. *FLT3*/ITD is the only used molecular marker up to now, included in the current AML-Berliner-Frankfurt-Münster (AML-BFM) Study Group protocol as high-risk feature. In adult AML it has been acknowledged in the past years that molecular markers

can improve risk-group stratification.[67-69] This thesis and studies by other groups now show that several molecular markers harbor prognostic relevance in pediatric CN-AML that can divide this seemingly homogeneous entity into distinct, prognostic relevant disease entities.

NPM1 mutations and *CEBPA* double mutations independently predicted favorable outcome in our pediatric AML series. This was also found in studies from the Children's Oncology Group (COG, USA) and correlated with the adult data.[4, 25] However, discrepancies were found for the impact of *FLT3*/ITD in *NPM1*-mutated AML. We observed no influence of the presence of *FLT3*/ITD on the favorable impact of *NPM1*-mutated AML, whereas the COG study did report a negative influence of *FLT3*/ITD, which is in concordance with most adult studies.[3-4] Beside small numbers, this might be influenced by differences in the mutant/wild type (allelic) ratio of the *FLT3*/ITDs as it has been shown that this ratio largely determines the prognostic impact of *FLT3*/ITD.[70] These ratios were not reported by the COG, but in our study high allelic ratios had no impact on the outcome of the *NPM1*-mutated patients. Larger pediatric series are therefore needed to ascertain the role of *FLT3*/ITD and its allelic ratio in the *NPM1*-mutated subtype. An important issue in determining prognostic factors is that the impact of markers is highly treatment-dependent. The prognostic influence of markers may disappear when survival increases or treatment differs, as might be the case with *FLT3*/ITD, as slightly higher survival rates were reached in our series compared with the COG study, and adult studies.

CEBPA-double mutated AML was found to favorably impact on outcome.[24] However, in contrast to our and most adult data, the COG did not observe a difference in outcome between *CEBPA* double-mutated and single-mutated AML.[25] Additional aberrations may define outcome of the *CEBPA* single-mutated subtype. In a recent very large series of adult AML, concurrent gene mutations were more frequent and influenced outcome in *CEBPA* single-mutated AML, supporting this hypothesis, and possibly explaining the difference in outcome found for *CEBPA* single-mutated AML between studies.[20]

As an example how this research can be translated into the clinics, the upcoming AML-BFM treatment protocol that is currently in development is planning to incorporate these novel markers into their risk-group classification (personal communication Prof. Dr. D. Reinhardt). AML characterized by *NPM1* mutations and by *CEBPA* double mutations will be added to the favorable risk-group, thereby extending this group from approximately 25% to 40% of the patients. Better risk-group classification may avoid overtreatment of patients with a good prognosis. In contrast, improved risk-group classification may prevent undertreatment of patients with a high chance of relapse.

A novel marker that we identified in this thesis is the cryptic *NUP98/NSD1* fusion, which recognized a very poor prognostic group of pediatric as well as adult AML patients. Only a couple of other *NUP98* translocations have been investigated in adult AML for their prognostic significance. Interestingly, they all conferred poor clinical outcome.[71-72] Moreover, the *DEK/NUP214* fusion is also known to confer poor outcome, and is in many adult treatment protocols stratified into the poor risk-group.[73] *NUP98/NSD1* now awaits further validation

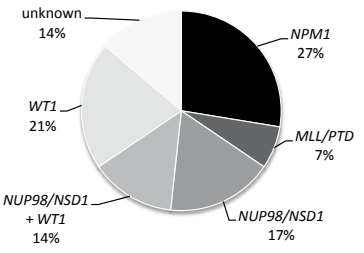

Figure 3. The frequency of type-II and other genetic aberrations in *FLT3*/ITD-positive pediatric CN-AML cases. *IDH1/2*, *TET2* and *DNMT3A* mutations were not yet available for all these cases.

in independent series, but novel therapeutic approaches seem urgently needed for this patient group (which will be discussed in the next paragraph).

Contradictory data exist on the impact of *WT1* gene aberrations in AML; *WT1* gene mutations, *WT1* SNP rs16754 and *WT1* gene overexpression at diagnosis correlated with outcome in some but not in all series.[47, 74-78] These conflicting results are present for pediatric as well as for adult AML cohorts, although there seems consensus on the dismal prognosis of patients with the combination of a *WT1* mutation and an *FLT3*/ITD. Interestingly, this combination frequently occurred together with the *NUP98/NSD1* fusion, which might underlie their poor outcome, and suggesting that the poor outcome is not due to *WT1* mutations and *FLT3*/ITD.

The type-I aberration *FLT3*/ITD is an established poor prognostic marker, which occurs in approximately 45% of pediatric CN-AML cases. However, in this thesis evidence is provided that in pediatric AML the prognosis of *FLT3*/ITD-positive cases might be dependent on the underlying type-II or driving primary genetic aberration(s), which were present in the majority of the *FLT3*/ITD-positive cases in our series (Figure 3). The left-over *FLT3*/ITD-positive cases (i.e. excluding *NPM1*-mutated and *NUP98/NSD1*-positive cases) had a similar outcome as the *FLT3*/ITD wild-type cases, although numbers are small (unpublished data). It is likely that also these cases harbor other yet unidentified primary (epi)genetic aberrations, which might influence outcome, and that 'solely *FLT3*/ITD' does not exist.

Investigating the impact of less prevalent markers in pediatric AML such as mutations in *IDH1/2* and *DNMT3A*, is difficult by the low incidence of pediatric AML. Therefore, international collaboration by combining patient cohorts is of substantial value to determine the impact of these markers in such a rare disease. The recent international retrospective study on *MLL*-translocations in pediatric AML facilitated by the International AML-BFM Study Group is an excellent example of such collaboration.[79]

Besides genetic aberrations, improvement of risk-group classification might also result from other variables such as expression levels of single genes or combined in expression profiles, or from sensitive assessment of minimal residual disease (MRD) levels. As the past decade has yielded a wide scale of novel prognostic markers, it remains important to prove their value in a prospective evaluation, and to examine their independent and additive effect to the established risk-classification system.

Towards subgroup-directed and targeted therapy

Within most current pediatric AML treatment protocols, patients receive therapy based on a backbone of two induction courses consisting of an anthracycline and cytarabine followed by consolidation courses with high-dose cytarabine. Currently only one AML subtype receives specific subgroup-directed therapy, i.e. acute promyelocytic leukemia (APL) harboring t(15;17), which generates the *PML/RARA* fusion. They are successfully treated (survival rates >90%) by the addition of all-trans retinoic acid (ATRA) to the chemotherapy backbone, which activates the retinoic acid receptor (RARA), and herewith overcomes the differentiation block induced by PML-RARA.[80] In addition, AML in children with Down syndrome (DS-AML), which is an unique AML subtype characterized by truncating mutations in the hematopoietic transcription factor GATA1, are treated with reduced dose-intensities, as DS-AML cells displayed relatively sensitive *in vitro* drug-resistance profiles, and because DS children experience excessive toxicity at regular doses. This improved outcome by reducing treatment-related mortality.[81,82] Currently novel non-specific drugs in development for AML include new formulations of old drugs, such as liposomal daunorubicin (DaunoXome®), which is assumed to be less cardiotoxic than conventional anthracyclines, and novel nucleoside analogues such as clofarabine. Another drug in development is Gemtuzumab ozogamycin (Mylotarg®), which is a conjugated monoclonal antibody linked to the cytotoxic compound calicheamicin, and directed against CD33 which is expressed by the majority of AML cases.[83-88]

The enigmatic example in the oncology field of therapy specifically targeting the leukemic cells is imatinib mesylate, a specific tyrosine kinase inhibitor that inhibits the BCR/ABL fusion in chronic myeloid leukemia (CML), which was developed in the late 1990s.[89] Since then, diverse inhibitors have been developed against targets that are mutated or overexpressed in cancers.[90] The main focus for AML has been on inhibitors against deregulated signal transduction molecules or pathways, such as the mutated growth factor receptors *FLT3* and *KIT* and the *RAS* pathway, reflecting the type-I aberrations in AML. Diverse compounds such as FLT3-directed midostaurin and lestaurtinib and the multi-tyrosine kinase inhibitor sorafenib showed promising results in *in vitro* cytotoxicity assays or *in vivo* models, however, so far monotherapy with these agents in AML have shown limited clinical activity.[91-95] Due to the different cooperating events in AML, monotherapy as with imatinib in CML (where a single fusion gene drives the disease) might not be feasible. Moreover, the combination with chemotherapy might reveal novel insights in resistance mechanisms, such as the upregulation of FLT3 ligand during chemotherapy which might hinder the treatment with FLT3 inhibitors.[96] Our and other studies also show that shifts of the mutational status of type-I aberrations frequently occur during disease progression, which need to be kept in mind when targeting these molecules.[97-98]

As the primary genetic events are frequently indispensable for maintaining the malignant clones, but generally believed to be 'undrugable' by conventional small molecule approaches, it may be worthwhile to invest in the development of strategies to target these leu-

kemia-driving genes or their downstream effectors.[99] In this thesis *NUP98/NSD1*-positive AML was identified as a very poor prognostic subtype for which novel therapy options are urgently needed. Recent studies have demonstrated that NUP98 fusions function by transcriptional activation of e.g. *HOX* genes for which the recruitment of the CBP/p300 complex is probably essential.[100] The histone acetyltransferase activity of CBP/p300 is recruited via the N-terminal GLFG repeats of NUP98, which are commonly present in all *NUP98* fusions. Furthermore, for the *NUP98/NSD1* fusion it has been shown that besides the recruited histone acetyltransferase activity, histone H3K36 methyltransferase activity by the SET domain of NSD1 is essential for leukemogenic transformation.[40] Following this, we started to investigate the potential of compounds directed against histone modifying enzymes, specifically histone acetyl transferase (HAT) inhibitors, for this subtype of AML.[101-102] The recent development of histone methylation inhibitors might also be worthwhile investigating for these patients.[103]

Although not frequently present in pediatric AML, patients characterized by promoter methylated *CEBPA* represented another group that might potentially benefit from epigenetic therapies.[24] An adult AML study showed that these patients not only exhibit *CEBPA* promoter methylation, but display a complete DNA hypermethylated DNA phenotype.[28] Therefore DNA methyltransferase inhibitors, which are already in phase II/III trials for myeloid malignancies, might be of specific interest for this group.[104] In addition, *IDH1/2*- and *TET2*-mutated AML might also be groups of interest for these agents. Furthermore, investigation of the global DNA methylation status showed distinct clusters in adult AML cases.[105] DNA methylation profiling is currently performed by our group in pediatric AML, and might reveal additional groups that from a biological point of view may be of potential interest for demethylating agents.

As the majority of AML blasts overexpress *WT1*, including the poor prognostic group harboring *WT1* mutations, another field of interest might be peptide-based and dendritic cell vaccines directed against *WT1*. Phase-I/II trials in adult AML with WT1 vaccinations showed promising results.[106-107]

The value of genome-wide approaches

Genome-wide profiling of copy number alterations (CNAs) using high-resolution A-CGH and SNP-A did not reveal many recurrent acquired CNAs in AML, and provided evidence that AML is a relatively genomically stable disease when compared to other malignancies such as ALL and various solid tumors.[108-111] One exception are the FAB-M6/M7 subtypes which significantly carried more CNAs in pediatric as well as adult AML.[108-109] However, we and others showed that A-CGH and SNP-A are valuable tools to identify somatically mutated genes, and to unravel genes not previously implicated in AML that may be relevant for pathogenesis. We identified the involvement of *WT1* gene aberrations via the detection of one case with a cryptic 11p13-deletion, and discovered the frequent involvement of the cryptic *NUP98/NSD1* translocation via threewo cases with CNAs of NUP98 resulting in unbalanced

translocations.[47] Other studies unraveled the involvement of *TET2* mutations via the detection of a minimally affected region of loss of heterozygosity (LOH) on chromosome 4q24 by SNP-A, and the involvement of *ASXL1* in myeloid malignancies was revealed in a similar way using A-CGH profiling.[112-114] SNP-A profiling has furthermore the ability to reveal regions of segmental uniparental disomy (UPD), which was shown to occur in approximately 10-20% of AML cases, serving as a mechanism to duplicate an oncogenic mutation with loss of the normal wild-type allele.[111, 115-116] These platforms are anticipated to further reveal affected genes in AML.

At the transcriptome level, gene expression profiling (GEP) has already proven its value in cancer research in different areas, such as *class prediction*, *class discovery* and *class comparison*.[117-119] Ross and colleagues firstly showed that GEP is able to predict specific cytogenetic subtypes in pediatric AML.[120] We recently showed that specific cytogenetic subtypes comprising approximately 40% of pediatric AML cases could be predicted with a very high accuracy with a classifier consisting of only 75-probe sets.[34] In contrast, gene expression signatures were less predictive for molecular genetic subtypes in pediatric AML, which was similar for adult AML cases.[121] In unsupervised clustering analysis of our gene expression data the specific cytogenetic subtypes grouped together. In addition, several other distinctive clusters were present for which the common factor or pathway remains to be elucidated. In one of these clusters *CEBPA* double mutations clustered together with all *CEBPA*-promoter methylated cases, confirming a prior observation from our adult hematology department.[27] Two additional cases were present in this cluster besides the *CEBPA*-promoter methylated cases, also characterized by silenced *CEBPA* and sharing the distinctive T-lymphoid characteristics. The origin of these *CEBPA*-silenced leukemias and their prognostic impact remain to be clarified, and comparing their signatures with immature T-ALL cases might be of interest.

NUP98/NSD1-positive AML cases were characterized by a gene expression profile with deregulated *homeobox* (*HOX)* gene expression. Expression of several *HOX* genes promotes self-renewal of hematopoetic stem cells, but down-regulation is necessary to allow differentiation to mature blood cells.[122] Persistent overexpression is expected to give rise to pre-leukemic clones, and has been shown to be potent leukemogenic events, especially together with cofactors such overexpression of *MEIS1*.[123-125] Deregulation of *HOX* genes frequently occur in AML, and we showed that pediatric AML could be separated in three distinct clusters based on only *HOXA* and *-B* gene expression patterns. *NUP98/NSD1*-positive cases represented a part of the *HOXA* and *-B* activated cluster, in which also *NPM1*-mutated, *MLL/PTD*-positive and *DEK/NUP214*-positive AML cases were present. This cluster was distinct from *MLL*-translocated AML, which were, in the majority of cases, characterized by *HOXA* up-regulation only. Investigating these distinct *HOX* expression clusters might reveal the specific mechanisms of action by the driving genetic aberrations. Furthermore, they might reveal novel *HOX*-activating mechanism in cases without the currently known primary oncogenic events.

Taken together, these studies showed that GEP can discover patient groups sharing leu-

kemic features that empowers us to identify novel leukemogenic mechanisms underlying pediatric AML. GEP has also proven to be valuable for predicting prognosis,[126-128] for drug discovery by comparing expression signatures in the so-called C-MAP database of the Broad Institute, which comprises expression signatures of various cancer cell lines treated with a wide variety of bioactive small molecules,[129-130] and for recognizing subsets of patients with highly drug-resistant profiles.[131]

The development of next-generation sequencing has made it possible to get a complete picture of the mutational landscape of an AML genome. Applying high-throughput sequencing to candidate genes, such as genes known to be involved in leukemogenesis or the complete kinome, did not reveal a satisfactory number of novel mutations in AML.[132-133] However, the unbiased genome-wide approach of sequencing the complete AML genome has led to the identification of recurrent *IDH1/2* mutations, which had not been associated with AML before.[49, 134] We performed a pilot study with exome sequencing, and found a *DNMT3A* mutation in pediatric AML, which was also discovered in adult AML by exome as well as whole genome sequencing.[53, 134] It is believed that the application of these techniques to a larger group of AML samples, will accelerate our understanding of the mutational landscape of AML. Furthermore, it was recently reported that novel chimeric fusion genes resulting from cryptic rearrangements can also be found by whole genome sequencing by applying specific data analysis strategies.[135] However, it remains important to distinguish driver from passenger aberrations to understand their contribution to leukemogenesis. The development of high-resolution array-based and next-generation sequencing techniques is also utilized in other fields, such as genome-wide methylation assays, profiling of histone modifications using chromatin immunoprecipitation coupled with whole genome promoter microarray (ChIP-on-Chip) techniques, and miRNA expression profiling, which are currently also applied by our group in pediatric AML. Integration of all those data will be a great challenge, which will need support from the bioinformatic field, but promises to be valuable for the unraveling of AML, and malignancies in general.

CONCLUSION

In this thesis we provided evidence that CN-AML is heterogeneous and characterized by a diverse spectrum of underlying genetic aberrations with distinct prognostic significance. Although we already determined a variety of genetic aberrations, in a large part the cooperating genetic events in leukemogenesis are still unknown to date. The application of advanced molecular techniques, in particular next-generation sequencing, will likely accelerate our insight in the genetic landscape of AML in the near future and lead to the identification of these aberrations. Deciphering the functional consequences of newly identified aberrations will however be a challenge, but is needed in order to develop new therapies to improve the cure rates of pediatric AML. For a rare disease such as pediatric AML international

collaboration will be of utmost importance to implement these findings in clinical practice.

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CHAPTER 12

Nederlandse Samenvatting

NEDERLANDSE SAMENVATTING

Van alle kinderen die leukemie (bloedkanker) krijgen, gaat het in 15 tot 20% om de vorm acute myeloïde leukemie (AML). Bij acute leukemie vermenigvuldigen onrijpe witte bloedcellen zich razendsnel en stapelen zich op in het beenmerg (de bloedfabriek), waardoor de productie van normale witte bloedcellen, rode bloedcellen en bloedplaatjes verstoord raakt. AML is een heterogene ziekte, waarbij de leukemie kan zijn ontstaan uit voorlopercellen voor rode bloedcellen, bloedplaatjes en bepaalde soorten witte bloedcellen, samen genaamd myeloïde voorlopercellen. Momenteel kan met hele intensieve chemotherapie ongeveer 70% van de kinderen met AML worden genezen. Echter bij een deel van de kinderen reageert de AML niet op de therapie (refractaire AML) of komt deze terug (recidief AML). Daarnaast gaat de intensieve chemotherapie gepaard met relatief hoge sterfte (5 tot 10%) en ernstige bijwerkingen ten gevolge van de therapie. Daarom is het niet mogelijk de huidige chemotherapie nog verder te verzwaren (intensiveren). Om de overleving van AML bij kinderen te verbeteren, moet dus worden gezocht naar nieuwe behandel strategieën.

AML is eigenlijk een algemene term die veel verschillende subtypes omvat. Deze subtypes verschillen onder andere in hun onderliggende afwijkingen in het DNA van de leukemiecellen, wat de verschillende eigenschappen van de AML bepaalt en daarmee de respons op huidige therapie en de prognose voor de patiënt. Op dit moment worden de meeste kinderen met AML behandeld volgens een standaard behandelingsschema, waarbij twee of drie risicogroepen worden onderscheiden op basis van enkele DNA afwijkingen in de leukemiecellen en de vroege respons op therapie. Verbetering van de behandeling zou kunnen worden gerealiseerd door de risicogroep indeling te verbeteren op basis van het subtype AML. Daarnaast lijkt de ontwikkeling van doelgerichte therapieën, die specifiek de leukemiecellen aanpakt en niet alle snel delende cellen zoals bij chemotherapie, veelbelovend, omdat hiervan de bijwerkingen mogelijk minder zijn. Hiervoor is inzicht nodig in de onderliggende ziektemakende mechanismen van de leukemiecellen, die veelal worden veroorzaakt door de genetische veranderingen in het DNA van deze leukemiecellen.

De grote technische vooruitgang op het gebied van de moleculaire genetica in de afgelopen jaren biedt de mogelijkheid om steeds verder deze genetische veranderingen in het DNA van de leukemiecellen te ontrafelen. In dit proefschrift wordt ingezoomd op het subtype AML dat bij standaard chromosomen onderzoek geen afwijkingen laat zien, het zogenaamde subtype 'cytogenetisch normale AML' ofwel CN-AML. Deze groep omvat 20 tot 25% van de kinderen met AML. Aangezien in CN-AML de onderliggende drijvende genetische afwijkingen voor het ontstaan van de leukemie grotendeels onbekend waren, was het doel van dit proefschrift hierin inzicht te krijgen, om patiënten met CN-AML beter in te kunnen delen in risicogroepen voor behandeling en om aangrijpingspunten te verkrijgen voor het ontwikkelen van nieuwe behandelingen voor deze patiënten. In deel 1 van dit proefschrift hebben we eerst gekeken naar de frequentie en karakteristieken van DNA veranderingen in genen (stukjes DNA die coderen voor een eiwit), die recent ontdekt zijn in CN-AML bij

volwassen. In deel 2 zijn vervolgens nieuwe moleculair genetische onderzoekstechnieken toegepast om nieuwe genetische afwijkingen en daarmee subtypes binnen CN-AML bij kinderen te ontdekken.

In **hoofdstuk 2** onderzochten we mutaties in het *Nucleophosmin* (*NPM1*) gen, die in 2005 in volwassen AML werden ontdekt. *NPM1* mutaties bleken aanwezig te zijn in 8,4% van de totale groep van kinder-AML, en in 20% in het CN-AML subtype. Deze frequentie was 2 tot 3 keer lager dan in volwassen CN-AML, waar de frequentie van *NPM1* mutaties 50 tot 60% bedraagt. Daarnaast verschilde ook het type *NPM1* mutatie tussen volwassen en kinderen met AML. *NPM1* mutaties karakteriseerden een groep patiënten met een goede prognose. In onze studie was dit onafhankelijk van de mutatie in het *FLT3* gen (*FLT3*/ITD), wat een bekende slechte prognostische factor is binnen kinder-AML.

In **hoofdstuk 3** hebben we gekeken naar de rol van verschillende afwijkingen van het *CEBPA* gen, dat codeert voor een belangrijke myeloïde transcriptiefactor. Een mutatiescreening liet *CEBPA* mutaties zien in 7,9% van AML bij kinderen, en 70% hiervan had twee verschillende *CEBPA* mutaties (*CEBPA* dubbelmutanten). De aanwezigheid van deze *CEBPA* dubbelmutanten was geassocieerd met een goede prognose in tegenstelling tot de aanwezigheid van *CEBPA* enkelmutanten, wat in overeenstemming was met gegevens uit studies in volwassen AML. Daarnaast onderzochten we de uitschakeling van *CEBPA* door verhoogde DNA methylatie (CEBPA promoter hypermetylatie), waardoor het gen niet meer tot expressie kan komen en kan coderen voor het functionele C/EBPα eiwit. Dit bleek met 1,3% zeldzaam te zijn in kinder-AML. In analyses van gen expressieprofielen, welke een profiel weergeven van de expressie van alle genen in AML cellen, clusterden de casussen met CEBPA promotor hypermethylatie, samen met de *CEBPA* dubbelmutanten. Dit duidt op een gemeenschappelijk mechanisme overeenkomstig tussen deze twee AML subtypes met verschillende *CEBPA* afwijkingen.

In **hoofdstuk 4** gebruikten we een nieuwe DNA screeningstechniek (in het Engels 'multiplex ligation-dependent probe amplification' (MLPA)) om de frequentie van de DNA afwijking *MLL*-PTD, dat is een partiële tandem duplicatie van het *MLL* gen, vast te stellen in kinder-AML. Uiteenlopende frequenties van *MLL*-PTD waren in kinder-AML gerapporteerd door verschillende studies, wat mogelijk gebaseerd zou kunnen zijn op het gebruik van verschillende detectiemethoden. Gebruikmakend van MLPA in combinatie met het aantonen van expressie van deze afwijking (met mRNA RT-PCR) bleek *MLL*-PTD slechts in 2,5% van de AML-en bij kinderen aanwezig.

In **hoofdstuk 5** presenteren wij een overzicht van de tot het moment van schrijven bekende genetische afwijkingen in kinder-AML aan de hand van de indeling in de zogenaamde type-I en -II afwijkingen. Type-I afwijkingen zijn de genetische afwijkingen in de leukemiecel die leiden tot de ongeremde groei (proliferatie), en type-II afwijkingen veroorzaken een stop in de uitrijping (maturatie stop) van de leukemiecel. Deze twee typen afwijkingen zijn minimaal nodig voor het ontstaan van leukemie uit een normale cel. De verscheidenheid van kinder-AML wordt gereflecteerd in de verschillende type-I en -II afwijkingen. In dit hoofd-

stuk bevestigden wij in het grootste overzicht van kinder-AML tot nu toe, dat specifieke type-I en -II afwijkingen met elkaar zijn geassocieerd, zoals *FLT3*/ITD die vaak samen gaat met t(15;17), maar ook met het subtype met *NPM1* mutaties. Ook is er een groot verschil tussen de verdeling van de genetische afwijkingen en daarmee de verschillende subtypes van AML bij kinderen onder en boven de twee jaar. Daarnaast karakteriseren de verschillende afwijkingen groepen van kinderen met AML met een zeer uiteenlopende prognose, en in een statistisch model werd de onafhankelijkheid van deze factoren voor het voorspellen van de recidief- en overlevingskans vastgesteld.

Vervolgens pasten wij de techniek van genoombrede 'array-comparative genomic hybridization (A-CGH)' toe op 43 CN-AML patiënten samples. Met deze techniek kunnen met een hoge resolutie veranderingen in het aantal kopieën van alle chromosomen in één leukemie DNA sample gedetecteerd worden. Een casus vertoonde een verlies van een klein stukje van chromosoom 11 (11p13 deletie) met daarin het *WT1* gen, en het *WT1* gen op het andere chromosoom bleek een mutatie te hebben. In **hoofdstuk 6** en **7** hebben we vervolgens de frequentie van diverse *WT1* afwijkingen onderzocht in een grote groep van kinder-AML. Hierin vonden we dat *WT1* mutaties voorkomen bij 12% van de AML-en bij kinderen, en dat deze specifiek associëren met het CN-AML subtype (22%). Ook kwamen ze significant vaker voor samen met een *FLT3*/ITD. In meer dan 40% van de AML-en waren beide *WT1* genen aangedaan. *WT1* mutaties bleken een nieuwe marker te zijn voor een slechte prognose, en in combinatie met de aanwezigheid van een *FLT3*/ITD herkenden ze een groep patiënten met een 5-jaars overlevingskans van slechts 21% met de huidige therapie. Vervolgens keken we ook naar de associatie van een enkele verandering in het *WT1* gen die echter niet leidt tot een ander WT1 eiwit (synonymous single nucleotide polymorphism (SNP) rs16754) (**hoofdstuk 7**), maar wel gelokaliseerd is in de regio waarin de meeste mutaties voorkomen. Daarnaast onderzochten we ook de expressie van het *WT1* gen in deze groep patiënten. In tegenstelling tot volwassen AML, correleerden in onze kinder-AML groep beide markers, dat zijn de aanwezigheid van *WT1* SNP rs16754 en de hoogte van *WT1* expressie, niet met de kans op overleving.

Met de A-CGH techniek vonden we ook twee patiënten met een afwijking van het *NUP98* gen op chromosoom 11p15 (**hoofdstuk 8**). Dit bleek te berusten op een translocatie tussen chromosoom 5 en 11, wat leidt tot een fusie tussen de genen *NUP98* en *NSD1* (*NUP98/NSD1*), welke onzichtbaar is met standaard chromosomen onderzoek (cryptisch). Wij screenden vervolgens ruim 1000 volwassen en kinder-AML samples specifiek op de aanwezigheid van *NUP98/NSD1*, en vonden dat dit fusiegen significant vaker in CN-AML (16%) bij kinderen voorkomt dan bij volwassen CN-AML (2%). *NUP98/NSD1* werd vaak samen gevonden met *FLT3*/ITD en *WT1* mutaties, maar ging nooit samen met andere type-II afwijkingen. Daarnaast toonden we met gen expressieprofielen aan dat *NUP98/NSD1*-positieve AML gekarakteriseerd wordt door hoge expressie van diverse *HOXA* en *-B* cluster genen, wat inzicht geeft in het mechanisme van deze leukemie. De aanwezigheid van *NUP98/NSD1* associeerde met een zeer slechte prognose voor zowel kinderen als volwassenen, met een 5-jaars event-vrije

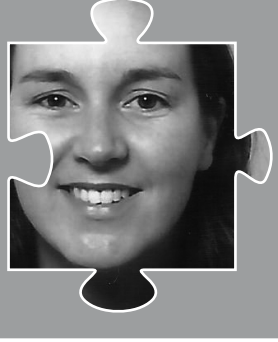
overleving beneden de 10%. Dit was onafhankelijk van de slechte prognostische factor FLT3/ITD. *NUP98/NSD1* identificeert hiermee een niet eerder herkende groep van jonge patiënten met AML met een zeer slechte prognose voor wie nieuwe behandelingsmogelijkheden dringend nodig zijn.

In **hoofdstuk 9** onderzochten wij in samenwerking met met een Duitse onderzoeksgroep de frequentie en karakteristieken van mutaties in de enzymen isocitraat dehydrogenase 1 en 2 (genen *IDH1* en -2) in AML bij kinderen. Deze mutaties werden in 2009 ontdekt bij het compleet in kaart brengen van de volledige volgorde van een genoom van het tweede volwassen AML sample wereldwijd. *IDH1* en *-2* mutaties werden vervolgens geassocieerd met CN-AML en een slechte prognose binnen volwassen AML. Wij vonden dat *IDH1* en *-2* mutaties weinig voorkomen binnen AML bij kinderen, vaak naast een *NPM1* mutatie voorkomen, maar geen relatie hebben met een slechte overlevingskans. Defecte IDH1 of -2 enzymen bleken geen karakteristiek gen expressieprofiel te geven.

In **hoofdstuk 10** presenteren wij een pilotstudie waarin we met de nieuwe techniek 'Exome sequencing' de basenparen volgorde van alle coderende regio's (genen) va het genoom bepaalden van één leukemie DNA sample samen met DNA van normale cellen van deze patiënt. We detecteerden een mutatie in het DNA methyltransferase gen *DNMT3A* en een mutatie in *WT1*. *DNMT3A* mutaties werden recent ook gevonden in volwassen AML (~22%), maar met een screening van een kinder-AML cohort toonden we vervolgens aan dat deze mutaties zeer weinig (2%) binnen kinder-AML optreden.

Concluderend hebben we in dit proefschrift laten zien dat CN-AML bij kinderen niet één ziekte is, maar bestaat uit verschillende subtypes gekarakteriseerd door verschillende onderliggende moleculair genetische afwijkingen die duidelijk subgroepen van patiënten onderscheiden met uiteenlopende overlevingskansen. Hiermee kunnen we een deel van de patiënten nu beter indelen in risicogroepen, en daarnaast geven deze afwijkingen inzicht in de biologie van de AML en daarmee aanknopingspunten voor het ontwikkelen van doelgerichte therapieën. Hoewel we in dit proefschrift al een grote groep van genetische afwijkingen hebben vastgesteld binnen CN-AML bij kinderen, is in een groot deel het volledige plaatje van (epi)genetische afwijkingen leidend tot de AML nog onbekend. Het is waarschijnlijk dat de toepassing van geavanceerde moleculaire technieken, waaronder 'whole genome sequencing', ons inzicht in het genetische landschap van AML in de nabije toekomst flink zal doen toenemen. Het vervolgens onderzoeken van de functionele consequenties van deze afwijkingen voor de leukemiecel zal nodig zijn voor het ontwikkelen van doelgerichte therapieën en daarmee het verbeteren van de overlevingskansen voor kinderen met AML.

ABOUT THE AUTHOR



CURRICULUM VITAE

Iris Helena Inèz Maria Hollink werd op 7 maart 1979 geboren te Hengelo (ov). Na het behalen van haar VWO diploma aan het Lyceum De Grundel te Hengelo in 1997, startte zij met de studie Biologie aan de Rijksuniversiteit Groningen. Een jaar later kreeg zij de mogelijkheid Geneeskunde te gaan studeren aan de Erasmus Universiteit, en verhuisde zij naar Rotterdam. In 2002 sloot ze de doctoraalfase van deze studie af met een wetenschappelijke stage op de afdeling Kinderlongziekten. In de klinische fase van haar opleiding koos ze voor de keuze-coschappen Kindergeneeskunde en Klinische Genetica, en behaalde cum laude haar artsexamen in 2005. Hierna werkte zij een jaar als arts-assistent op de Kinderafdeling van het Reinier de Graaf Gasthuis te Delft. Vanwege haar wetenschappelijke interesse in de genetica startte zij in april 2006 als arts-onderzoeker op het promotieonderzoek *'Molecular genetic insights in cytogenetically-normal pediatric acute myeloid leukemia'* op de afdeling Kinderoncologie/-hematologie van het Erasmus MC - Sophia in Rotterdam. Het resultaat van dit onderzoek is beschreven in dit proefschrift. Aansluitend werkte zij een jaar als post-doc verder aan projecten voortgekomen uit haar promotieonderzoek. In oktober 2011 start zij als arts-assistent op de afdeling Klinische Genetica van het Erasmus MC. Zij woont samen met Michiel van der Horst en hun twee kinderen Merel (2008) en Quinten (2010).

LIST OF PUBLICATIONS

Hollink IHIM[*], Feng Q[*], Danen-van Oorschot AA[*], Arentsen-Peters TCJM, Verboon LJ, Zhang P, de Haas V, Reinhardt D, Creutzig U, Trka J, Pieters R, van den Heuvel-Eibrink MM, Wang J[*], Zwaan CM[*]. Low frequency of DNMT3A mutations in pediatric AML, and the identification of the OCI-AML3 cell line as an in vitro model. Leukemia 2011; Aug 12. [Epub ahead of print] [*]**shared first** and last authorship.

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PHD PORTFOLIO

Summary of PhD training and research

Name PhD student: Iris H.I.M. Hollink
Erasmus MC Department: Pediatric Oncology
Research School: Molecular Medicine (MM)
PhD period: 1 April 2006 – 1 December 2010
Promotor: Prof. Dr. R. Pieters
Co-promotors: Dr. C.M. Zwaan, Dr. M.M. van den Heuvel-Eibrink

1. PhD training	Year	Workload (ECTS)
General courses		
Biomedical English Writing and Communication	2008	4.0
Classical Methods for Data Analysis (CC02) (NIHES)	2007	5.7
Specific courses		
Applied Bioinformatics (MM)	2007	
Microarray Data Analysis using R & Bioconductor Intensive Course (CMSB)	2007	1.4
Basic and Translational Oncology (MM)	2006	1.4
Biomedical Research Techniques (MM)	2006	1.4
Seminars and workshops		
CGC/CBG meeting 'Molecular mechanisms in Cancer', Amsterdam	2010	0.2
Workshop 'Molecular aberrations in acute myeloid leukemia' European Hematology Association (EHA), Cannes, France	2008	1.0
AML-BFM Research Symposium (oral presentation)	2007	1.2
Annual PhD day, Erasmus MC (oral presentation in 2008)	2007,2008	1.2
Annual Pediatric Research Day, Erasmus MC (oral presentations in 2007 and 2010)	2007-2010	2.6
Annual Molecular Medicine Day, Erasmus MC	2007-2009	0.6
Annual Pediatric Oncology Symposium, Erasmus MC	2006-2007,2009	0.6
Presentations		
See 'Seminars and workshops' and '(Inter)national conferences' 7 oral presentations at the weekly Pediatric Research Meetings and Pediatric Oncology Research Meetings	2006-2010	4.0
(Inter)national conferences		
7th Bi-annual I-BFM Leukemia Symposium, Antalya, Turkey (oral presentation)	2010	1.6
4th Dutch Hematology Congress, Arnhem (oral presentation)	2010	1.2
50th ASH Annual Meeting, San Francisco, USA (oral presentation)	2008	2.0
6th Bi-annual I-BFM Leukemia Symposium, Glasgow, UK (poster presentation)	2008	1.6
International Symposium 'Acute Leukemias XII', Munich, Germany (poster presentation)	2008	1.6
49th ASH Annual Meeting, Atlanta, USA (oral presentation)	2007	2.0
39th SIOP Annual Meeting, Mumbai, India (oral presentation)	2007	2.0
18th Annual Meeting of the I-BFM Study Group, Brugges, Belgium (oral presentation)	2007	1.6
5th Bi-annual I-BFM Leukemia Symposium, Noordwijkerhout	2006	0.6

Other		
Travel grant for the 50th and 51th ASH Annual Meeting awarded by the ASH committee	2008,2009	0
Travel grant for the 39th SIOP Annual Meeting, awarded by Erasmus Trustfonds	2007	0.1
Writing grant application KIKA (grant assigned of € 500.000) and writing grant application Sophia Foundation for Medical Research (SSWO)	2009	4.0

2. Teaching	Year	workload (ECTS)
Supervising Master's thesis Supervising Shimaira van Beusekom, student Applied Science- Molecular Research, Avans Hogeschool, Breda, during her 7-month traineeship on 'The importance of ErBb4 and LMTK3 in pediatric AML'	2009-2010	10

Total	**53.6**

DANKWOORD / ACKNOWLEDGEMENTS

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Prof. dr. R. Pieters, beste Rob, dank voor je inspirerende, maar kritische houding tijdens alle besprekingen, waarmee je mij altijd nog verder hebt weten te motiveren voor het onderzoek. Je laagdrempeligheid maakt je een heel prettige promotor!

Mijn copromotor, dr. Zwaan. Beste Michel, toen onze samenwerking begon met een kop koffie die ik tijdens onze eerste bespreking over je bureau gooide en ik later zei dat mijn 'project falling apart' was, had ik niet durven hopen dat het allemaal goed zou komen! Dank voor je introductie in de (inter-)nationale wereld van kinder-AML, je inzet en resultaatgerichtheid voor alle projecten en je bewonderenswaardige snelle correcties van papers. Succes met al je onderzoek binnen en buiten de AML groep!

Mijn tweede copromotor, dr. van den Heuvel-Eibrink. Beste Marry, als tweede co-promotor complementeerde je in de loop van de jaren mijn begeleidingsteam. Dank voor al je input in de projecten! Ik vind het knap hoe je al jouw onderzoeksprojecten naast elkaar in de lucht weet te houden, en daarnaast ook nog altijd zo attent weet te zijn (dank voor alle ontzettend leuke kraamcadeau's!)

Dr. Meijerink, beste Jules, wat ben ik blij dat jij deel uitmaakt van mijn kleine commissie! Ik heb bewondering voor jouw enorme enthousiasme en gedrevenheid voor moleculair genetisch onderzoek, en alle parate kennis die je hebt. Jij maakte me wegwijs op moleculair genetisch gebied, en geen vraag van deze AML promovenda was je ooit te veel.. heel veel waardering, en veel succes met jouw T-ALL groep!

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Berna Beverloo, bedankt voor je inspirerende enthousiasme en het openstaan voor al mijn vragen de afgelopen jaren; het was leuk om samen het laatste jaar aan *NUP98* fusies te werken. En ook leuk dat we nu collega's worden! Vincent van der Velden, bedankt voor jouw enorme hulpvaardigheid en het altijd weer klaarstaan om remissiesamples voor ons op te duiken; ik heb dat erg gewaardeerd!

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Als je als arts uit de kliniek komt en graag lab onderzoek wilt doen, maar geen technische voorkennis hebt, dan ben je heel blij dat Susan bestaat!! Wat waardeer ik al je geduld en hulpvaardigheid om mij op sleeptouw te nemen in dat lab in mijn eerste jaren; daarna konden we samen superenthousiast worden van nieuwe bevindingen, maar ook onze frustraties delen tijdens de vele koffie's in de koffiebar.. wat fijn dat je 'mijn' analist was, en dat je straks als paranimf naast me staat!

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Ook mijn familie pap, mam, Rooz & Frank, Bas & Charlotte, en schoonfamilie Paul, Ineke, Meint & Charlotte, en Roos + Stijn: dank voor al jullie interesse, de vele hand, span- en oppasdiensten als de drukte in ons gezinsleven weer eens uit de hand liep, maar bovenal jullie onvoorwaardelijke steun!

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APPENDICES

COLOR FIGURES

Chapter 2

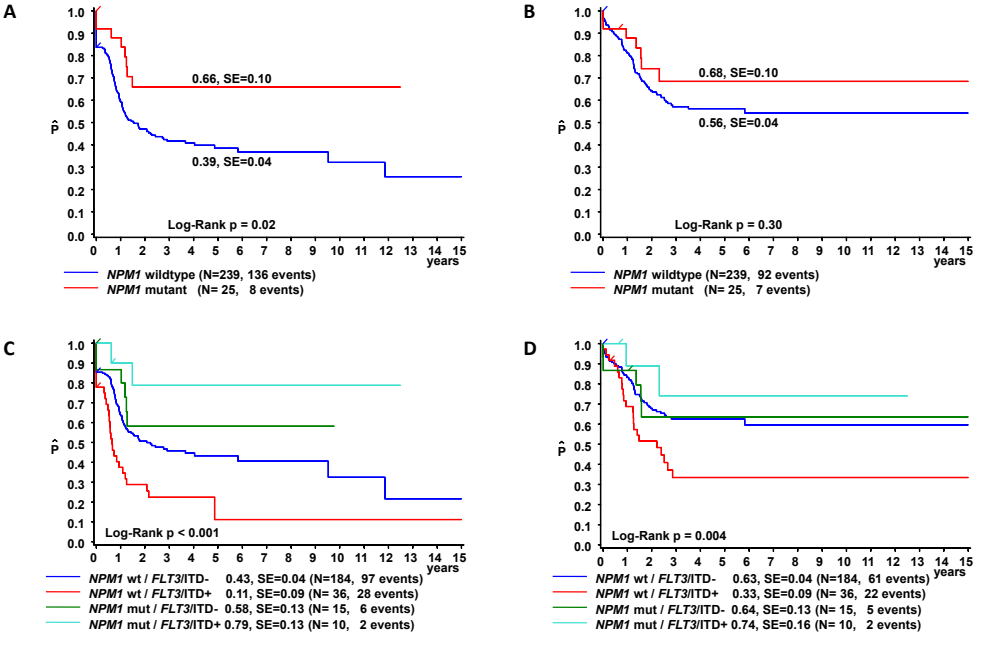

FIGURE 2. Survival curves of all childhood AML patients with and without *NPM1* mutations, and according to the combined *NPM1* and *FLT3*/ITD status.

(A)+(C) pEFS; (B)+(D) pOS. Patients are divided by their *NPM1* status (A+B), and show a significantly favorable outcome for *NPM1*-mutated AML patient for pEFS, but not for pOS. When excluding the patients with favorable cytogenetics, this difference was borderline statistically significant (see text result section). When subdivided in 4 subgroups according to their combined *NPM1* and *FLT3*/ITD status (C+D), no impact on outcome is seen of *FLT3*/ITD in *NPM1*-mutated subgroup.

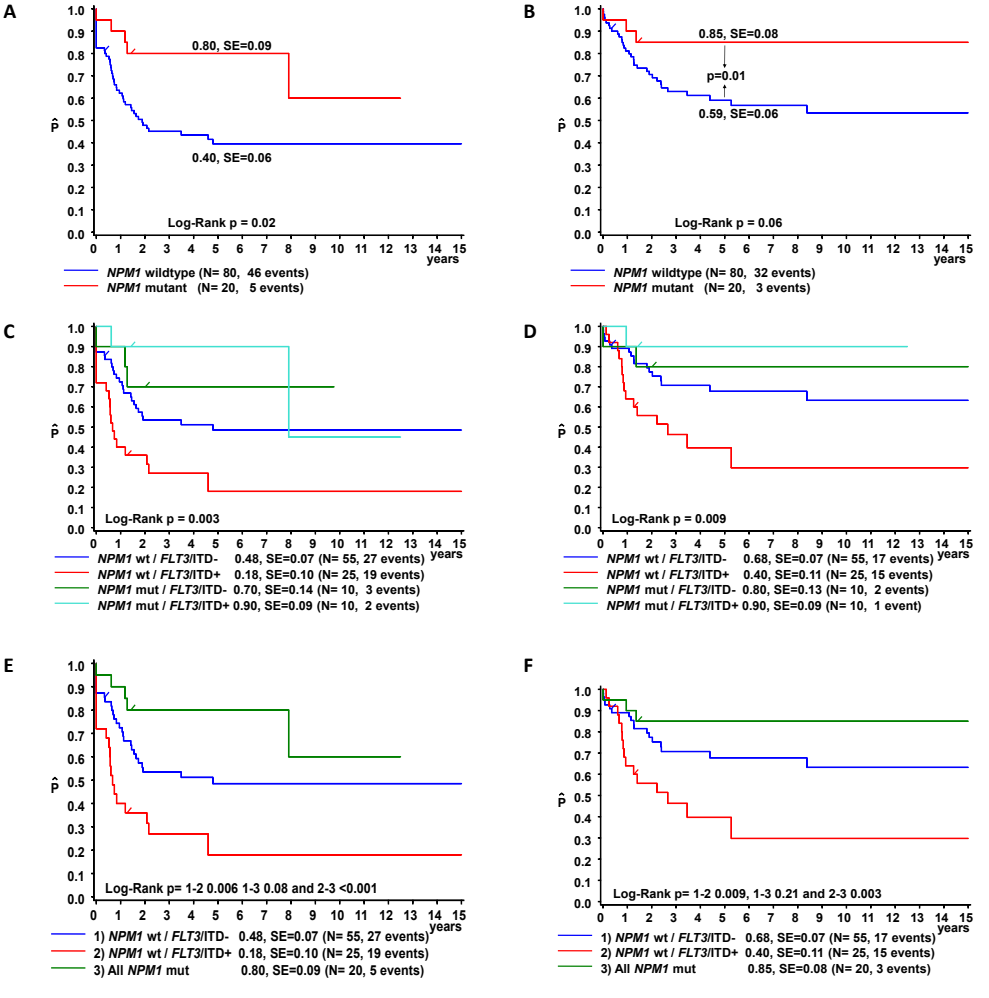

FIGURE 3. Survival curves of the subgroup of CN-AML patients with and without *NPM1* mutations, and according to the combined *NPM1* and *FLT3*/ITD status.

(A)+(C)+(E) pEFS, (B)+(D)+(F) pOS. (A) and (B) shows the curves of patients divided by their *NPM1* status, and *NPM1*-mutated AML patients show a favorable outcome in the CN-AML subgroup. (C) and (D) show the curves when patients are subdivided in 4 groups according to their *NPM1* and *FLT3*/ITD status, which show that *FLT3*/ITD does not abrogate the favorable outcome of the *NPM1*-mutated AML patients. (E) and (F): the patients are now subdivided in the 3 prognostic relevant subgroups according to *NPM1* and *FLT3*/ITD status, i.e. *NPM1*-mutated AML patients have an excellent outcome and *NPM1* wild-type patients can be subdivided based on their *FLT3*/ITD status.

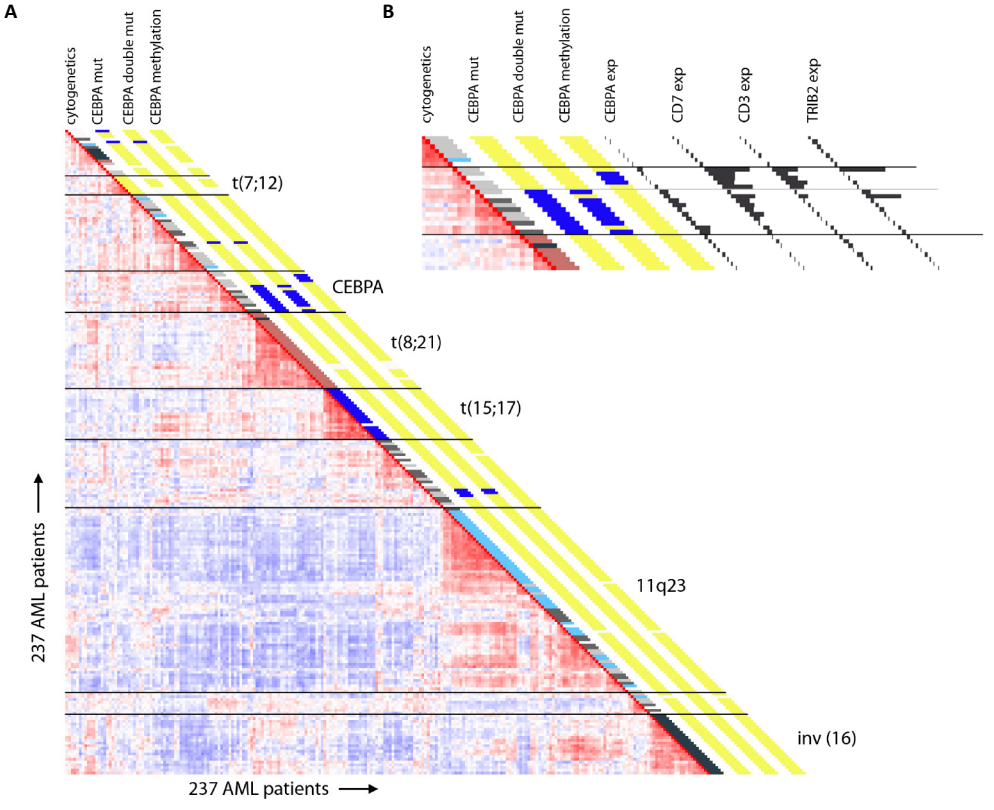

FIGURE 3. Unsupervised clustering of gene expression data revealed clustering of cases with aberrant *CEBPA* predominantly in one cluster in pediatric *de novo* AML.

(A) Pair wise correlations between gene expression profiles of 237 *de novo* pediatric AML samples, calculated on the basis of 1608 probe sets (cutoff: 16-fold), are displayed in a correlation plot. Colors of boxes represent the Pearson's correlation coefficient with a color gradient ranging from deep blue for a negative correlation, to vivid red for a positive correlation. Distinct clusters of samples, which can be recognized by the red blocks showing high correlation along the diagonal, are observed. The first column to the right of the plot indicates the major cytogenetic subgroup the samples belong to [dark blue: inv(16), pale red: t(8;21), bright blue t(15;17), yellow: t(7;12), light blue: 11q23, dark grey: normal cytogenetics, light grey: other cytogenetic aberrations and white: failure]. Clustering of these cytogenetic subgroups is seen. The second, third and fourth column represent presence (blue) or absence (yellow) of a *CEBPA* mutation, *CEBPA* double mutation and *CEBPA* promoter methylation, respectively. The majority of *CEBPA* mutant cases aggregated together with all *CEBPA* hypermethylated cases in one main cluster.

(B) An enlarged view of the correlation plot focusing on the *CEBPA* main cluster is shown. The order of the patient samples in this cluster from top to bottom is as follows: #4728, #5033, #3496, #3451, #4736, #3439, #5041, #5061, #4746, #5063, #5047, #4396, #4445, #4747, #5013. Additionally, four histograms show the expression of *CEBPA* (204039_at), *CD7* (214551_s_at), *CD3D* (213539_at) and *TRIB2* (202478_at). Within the *CEBPA* main cluster, two sub-clusters based on expression of *CEBPA* can be identified and are separated by a grey line, i.e. *CEBPA* low or absent expression (n=5), including the three *CEBPA* promoter methylated cases, and *CEBPA* high expression with the *CEBPA* mutant cases (n=10). The five silenced *CEBPA* cases are further characterized by high *CD3* and *CD7* expression. Silenced case #4728 and single-mutated case #5041 show high *TRIB2* expression, which has been shown to inhibit C/EBPα p42.

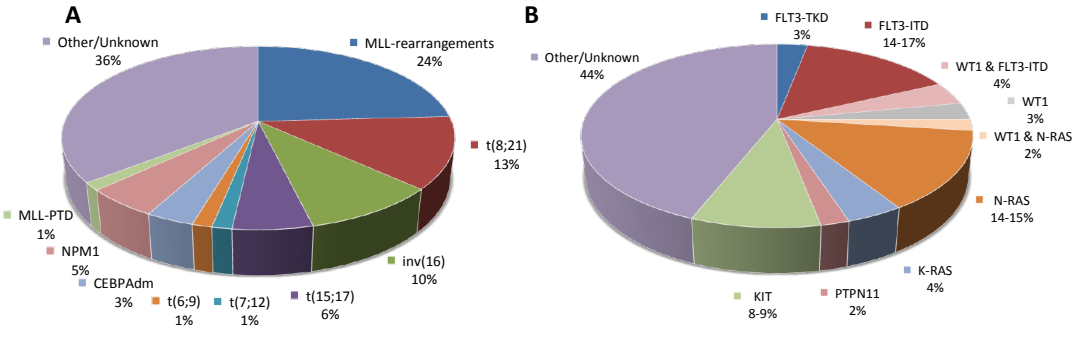

FIGURE 1. Distribution of the different type-I and type-II aberrations in pediatric AML. The heterogeneity of pediatric AML is reflected by the presence of the different type-I and type-II genetic aberrations. However, in a large amount of cases the type-II (A) or type-I (B) aberrations have not yet been identified.

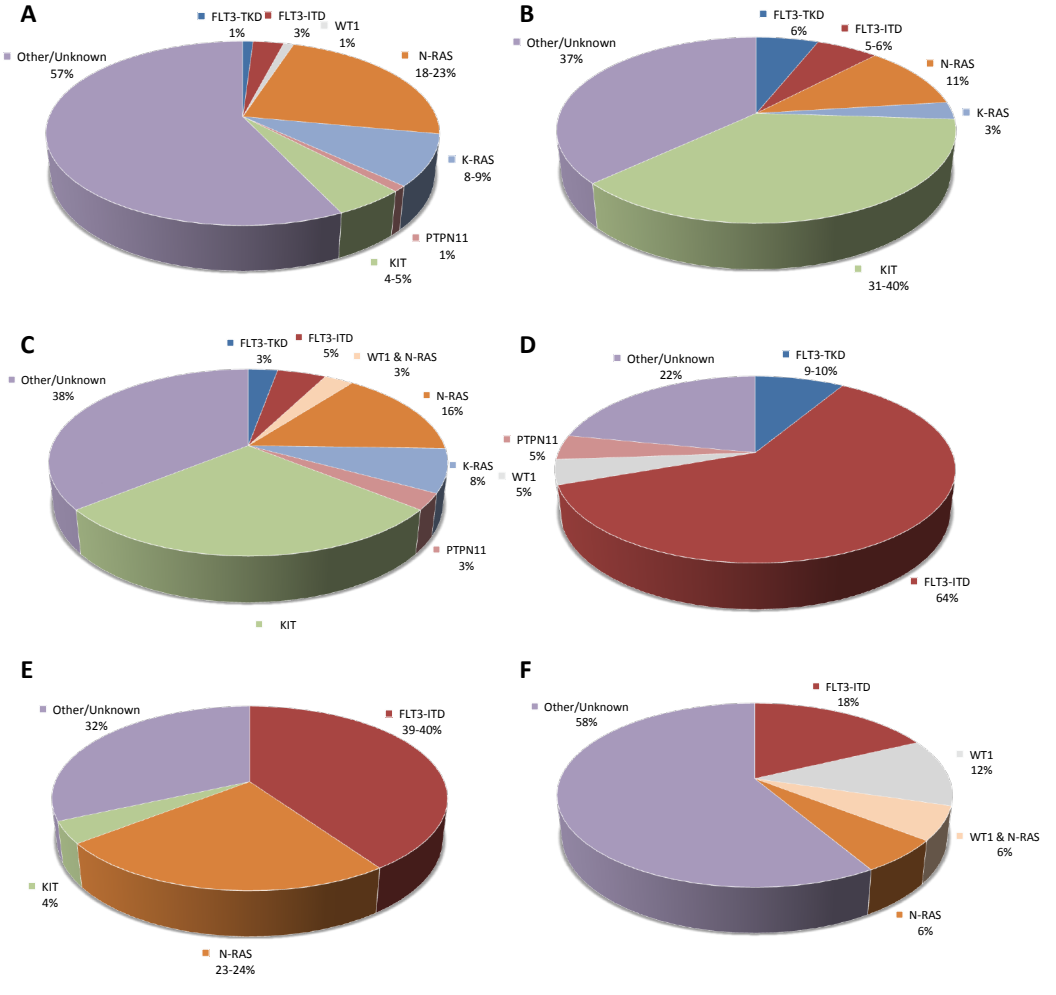

FIGURE 2. Type-I aberrations per type-II-defined subtype. Distribution of the different type-I aberrations according to the different type-II-defined subtypes including >10 cases, i.e. (A): *MLL*-rearrangements, (B): t(8;21), (C): inv(16), (D): t(15;17), (E): *NPM1*-mutated and (F): *CEBPA* double mutated AML.

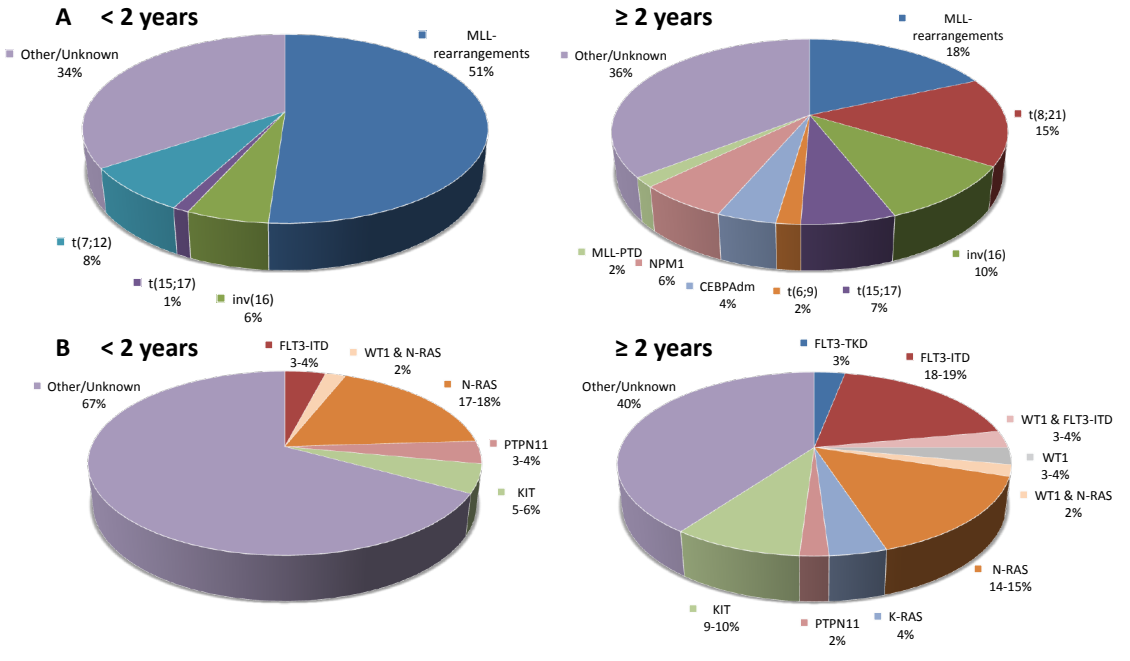

FIGURE 3. Distribution of the different type-I and type-II aberrations according to age. The largest differences in the frequencies of type-II (A) and type-I (B) aberrations were found between children older and younger than 2 years.

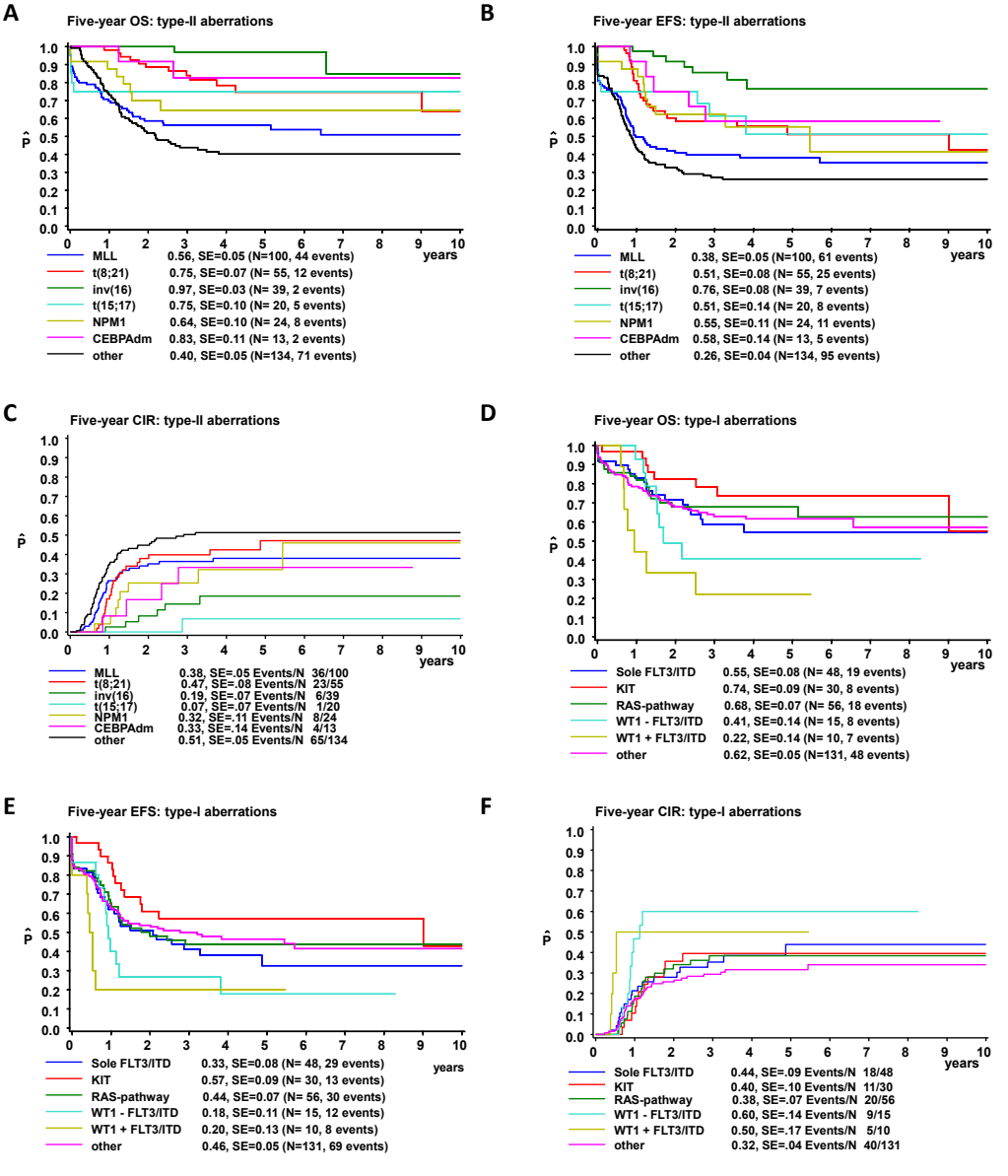

FIGURE 4. Survival analysis of the type-I and type-II aberrations in pediatric AML. Kaplan-Meier estimates for pOS (A+D), pEFS (B+E) and CIR (C+F) for the different type-II and type-I aberrations, respectively.

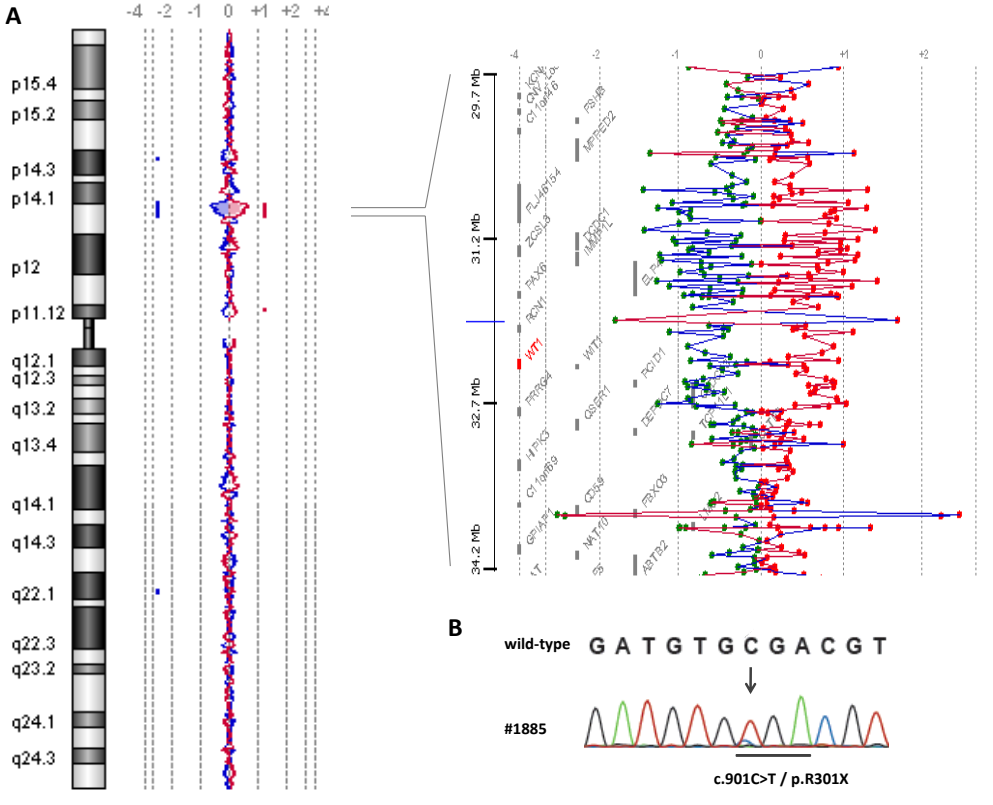

FIGURE 1. *WT1* aberrations in patient #1885 detected by array-CGH and sequencing.
(A) The left part of the figure shows a chromosome 11 ideogram and corresponding oligonucleotide array-CGH plot of the ratio of patient #1885 DNA and control DNA (blue tracing) versus the dye-swap experiment (red tracing) from an array-CGH experiment (105K oligonucleotide array-CGH platform, Agilent technologies, Palo Alto, USA). 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 #1885 showing a c.901C>T nonsense mutation translating into a termination codon on the remaining *WT1* allele.

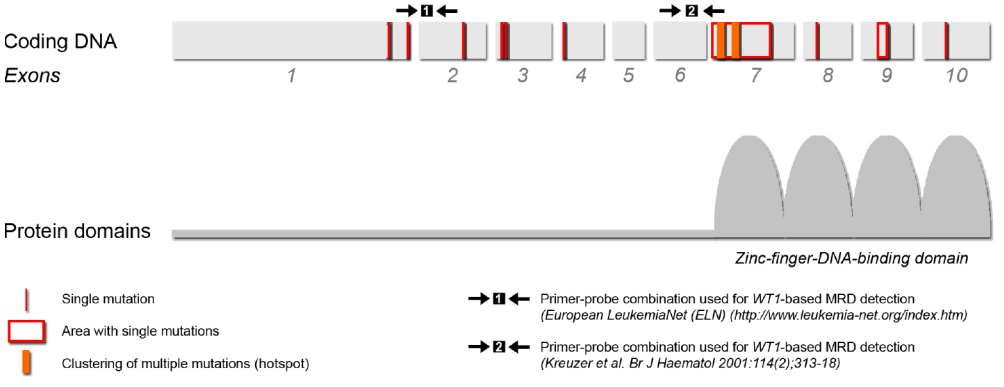

FIGURE 2. Location of the *WT1* gene mutations and their overlap with MRD detection areas.
The location of the *WT1* gene mutations detected in initial diagnosis and relapse childhood AML samples is schematically presented according to the exon structure of the *WT1* gene. Two areas of mutational hotspots 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.

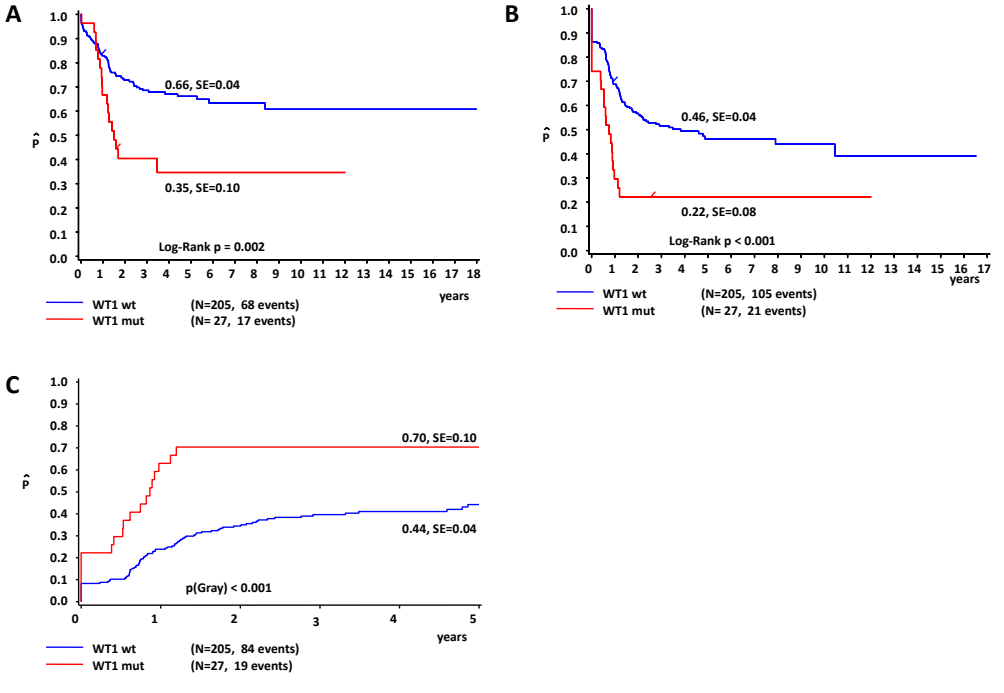

FIGURE 4. Survival curves of childhood AML patients with and without *WT1* mutations.
Kaplan-Meier estimates for 5-year pOS (A) and pEFS (B), showing log-rank *P* values. The 5-year CIR is depicted in (C) and was analyzed according to the Kalbfleisch and Prentice method using Gray's test for statistical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

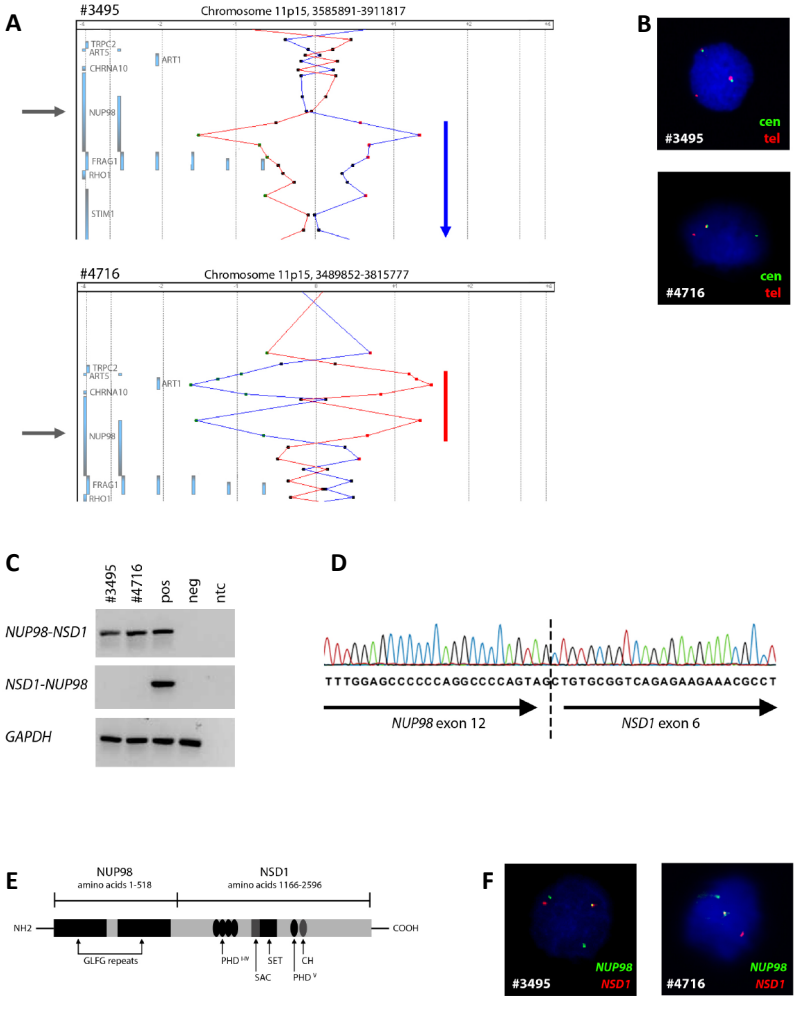

FIGURE 1. Discovery of *NUP98/NSD1* fusion in two CN-AML cases.
(A) Array-CGH profiles of chromosome 11p15 showing the ratio of tumor/control DNA (blue tracing) versus the inverted experiment (red tracing). The profile of patient #3495 (top panel) shows a part of the 0.4 Mb duplication involving the 5' part of *NUP98* (indicated by the blue vertical arrow), and the profile of patient #4716 (bottom panel) shows the 0.1 Mb deletion involving the 3' part of *NUP98* (indicated by the red vertical bar). The horizontal arrows indicate the bar representing the *NUP98* gene. (B) Split-signal FISH analysis of *NUP98* for patients #3495 and #4716 using a partly overlapping green and red probe located in *NUP98* (cen indicates probe situated more centromeric; tel indicates probe situated more telomeric. (C) RT-PCR analysis using *NUP98*- and *NSD1*-specific primers and *GAPDH* primers as internal control reveals a specific *NUP98/NSD1* fusion transcript in patients #3495 and #4716. The reciprocal *NSD1-NUP98* transcript was not detected in both patients (pos indicates positive control [#5007], neg indicates negative control [normal bone marrow] and ntc indicates nontemplate control). (D) Sequence analysis confirmed an identical in-frame fusion between *NUP98* exon 12 and *NSD1* exon 6 in both patients. (E) The NUP98/NSD1 fusion protein will harbor the GLFG-repeats of NUP98, and amongst others the PHD fingers and SET domain of NSD1. (F) Dual-color FISH analysis using a green probe for *NUP98* and a red probe for *NSD1* confirmed the fusion of *NUP98* and *NSD1* at the chromosomal level.

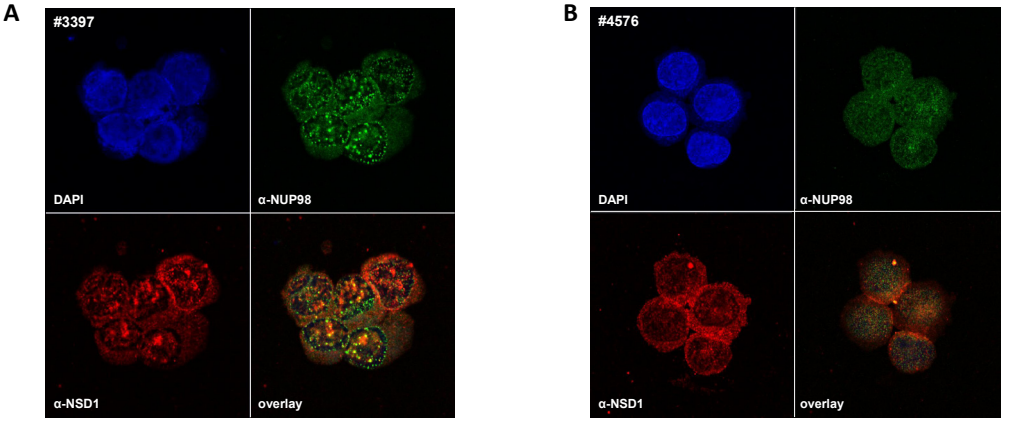

FIGURE 2. Aberrant nuclear-staining pattern of *NUP98/NSD1*-positive samples.
Immunofluoresence of leukemic cells with antibodies directed against the N-terminus of NUP98 (green) and the C-terminus of NSD1 (red) is shown for a *NUP98/NSD1*-positive (A) and a *NUP98/NSD1*-negative patient sample (B). Patient #3397 shows a pattern of nuclear speckles for NUP98 staining as well as NSD1 staining, indicating accumulation of the NUP98/NSD1 fusion protein in aggregates in the nucleus, in contrast to patient #4576. DAPI indicates 4,6-diamidino-2-phenylindole.

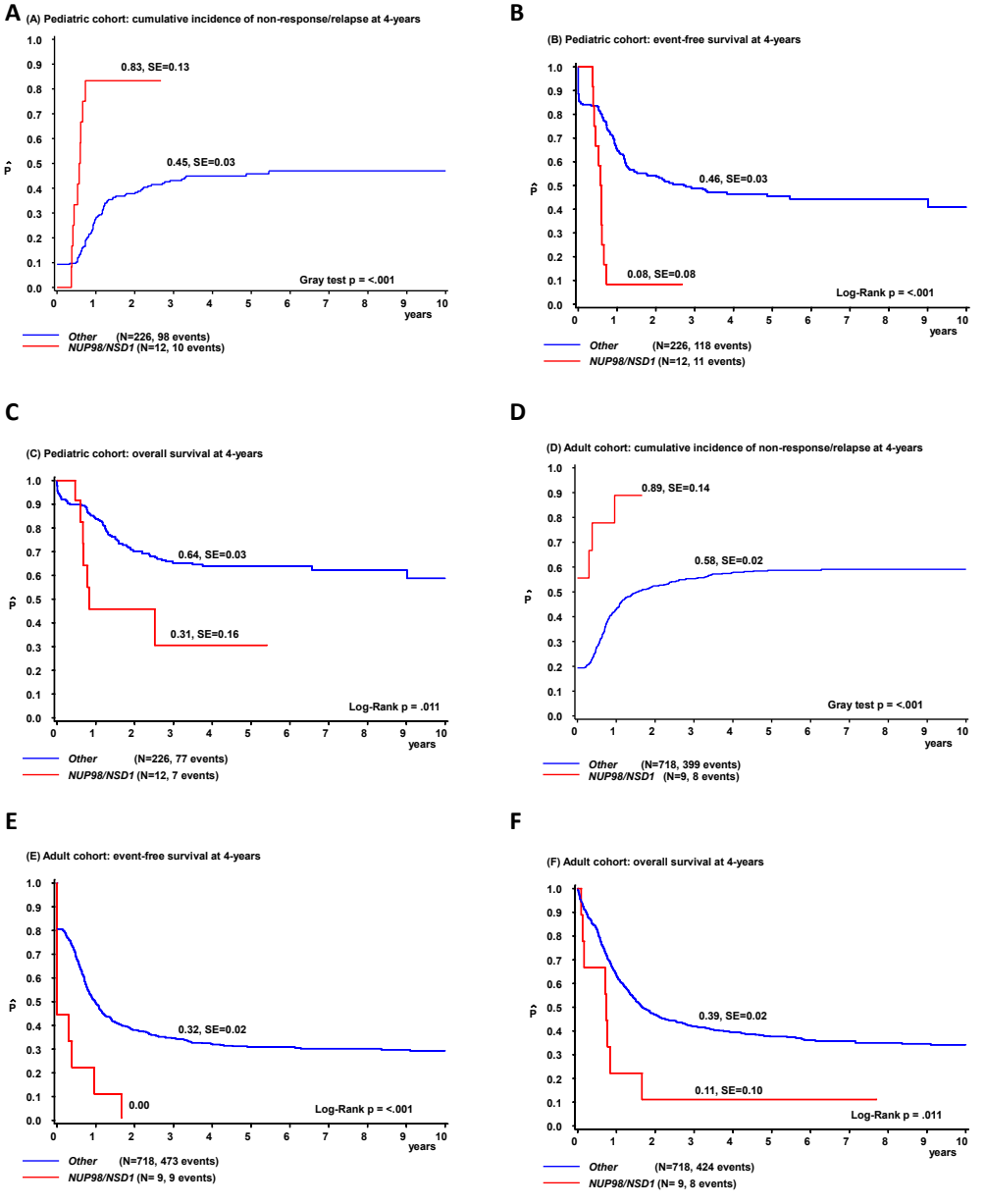

Figure 4. *NUP98/NSD1* confers a poor outcome in pediatric as well as adult AML.
Survival curves of the pediatric cohort (n=238; A-C) and adult cohort (n=727; D-F) depicting the CIR according to the Kalbfleisch and Prentice method (A,D) and Kaplan-Meier estimates of the pEFS (B,E) and pOS (C,F).

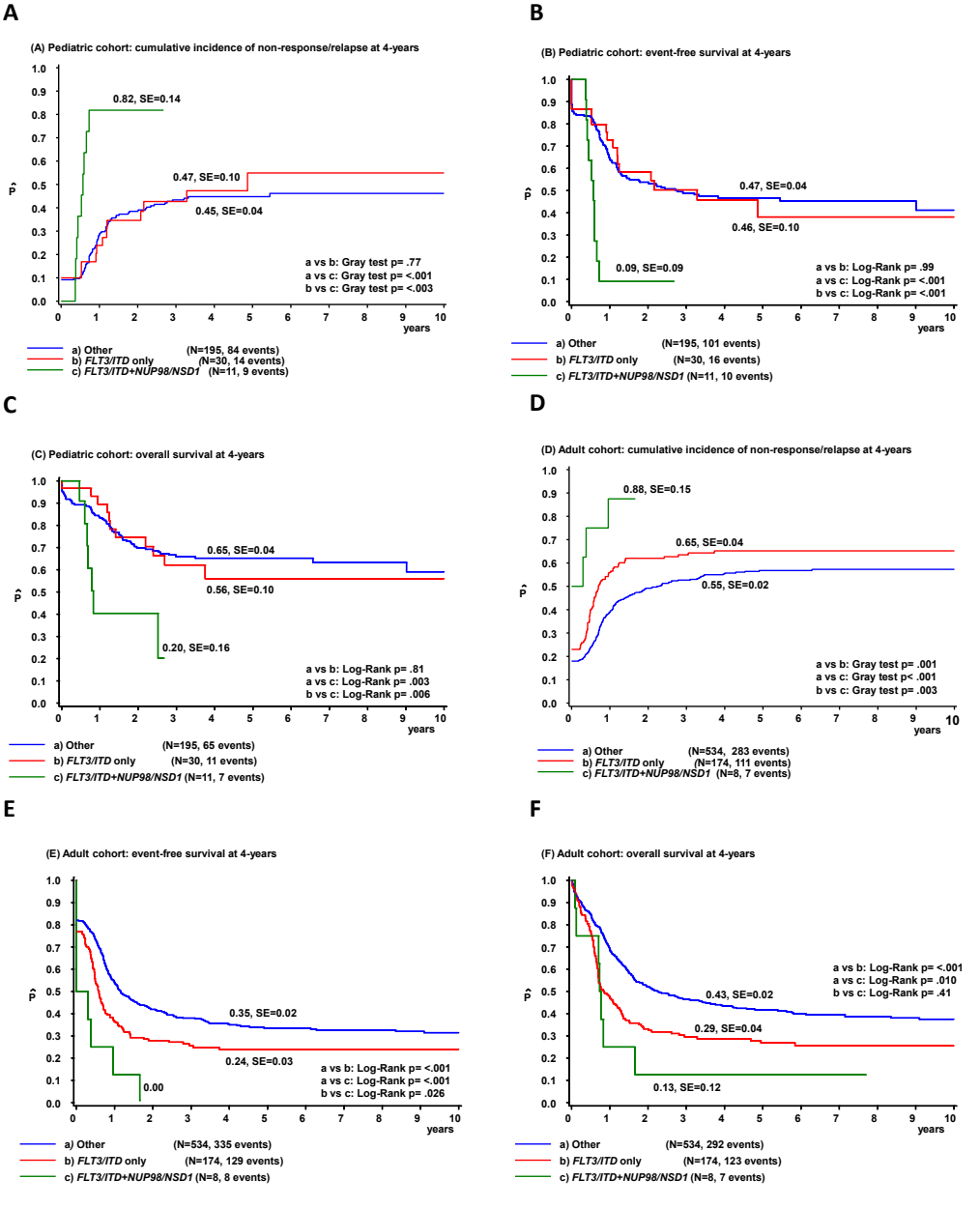

Figure 5. *NUP98/NSD1* clearly identifies a poor prognostic subgroup within pediatric as well as adult *FLT3*/ITD-positive AML. Survival curves of the pediatric cohort (A-C) and adult cohort (D-F) depicting the CIR according to the Kalbfleisch and Prentice method (A,D) and Kaplan-Meier estimates of the pEFS (B,E) and pOS (C,F) according to the *NUP98/NSD1* and *FLT3*/ITD status.

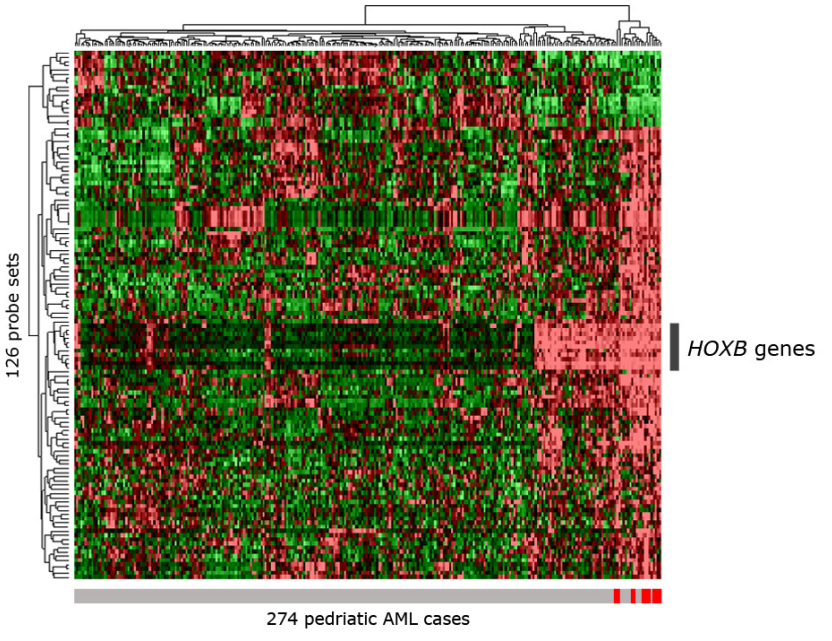

Figure 6. Clustering of 274 pediatric AML cases based on the 126 most discriminative probe sets for *NUP98/NSD1.*

Hierarchical clustering of 274 pediatric AML cases based on the 126 most discriminative probe sets (FDR-adjusted *P* value <0.05) for *NUP98/NSD1* AML is presented in a heat map. The 13 *NUP98/NSD1* cases are indicated with a red vertical bar below the heat map; the other AML cases are indicated with a gray vertical bar. In the heat map, cells represent relative log 2 expression values and have been color-coded on a scale ranging from bright green (-2) to bright red (+2), with black indicating no change relative to the median. Besides clustering of the *NUP98/NSD1* cases, a group of other AML cases show a partly similar gene expression profile mainly based on the highly expressed *HOXB* probe sets, indicated by the gray bar at the right of the heat map.

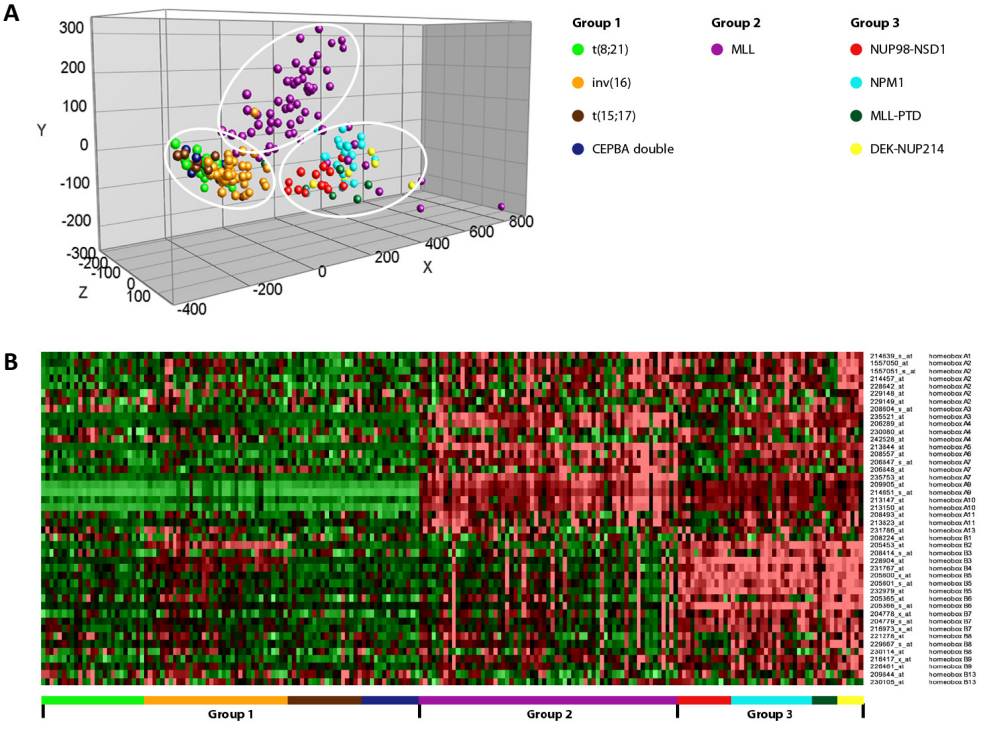

FIGURE 7. Distinct expression pattern of the *HOXA* and -*B* cluster genes in pediatric AML with *NUP98/NSD1*. (A) Principal component analysis (PCA) of pediatric AML subgroups characterized by specific type-II aberrations (n=210) was carried out based on all *HOXA* and -*B*-annotating probe sets present on the HGU133 Plus 2.0 microarray (Affymetrix). Each color-coded circle represents an individual AML case. Three distinct groups are observed in the PCA, which are indicated by the white circles. (B) Heat map showing the expression of all *HOXA* and -*B*-annotating probe sets in which the cells represent relative log 2 expression values and are color-coded on a scale ranging from bright green (-2) to bright red (+2), with black indicating no change relative to the median. The pediatric AML cases are grouped together based on their specific type-II aberration as indicated by the color-coded bars below the heat map, and the probe sets are alphabetically ordered. Three groups with distinct expression patterns are observed, that is, group 1, characterized by low or absent expression of *HOXA* and -*B* genes; group 2, characterized by solely high expression of *HOXA* genes (mainly *HOXA5-A10*); and group 3, characterized by expression of both *HOXA* and -*B* genes (mainly *HOXA5-A10* and *HOXB2-B6*). The latter included the *NUP98/NSD1* cases.

Chapter 9

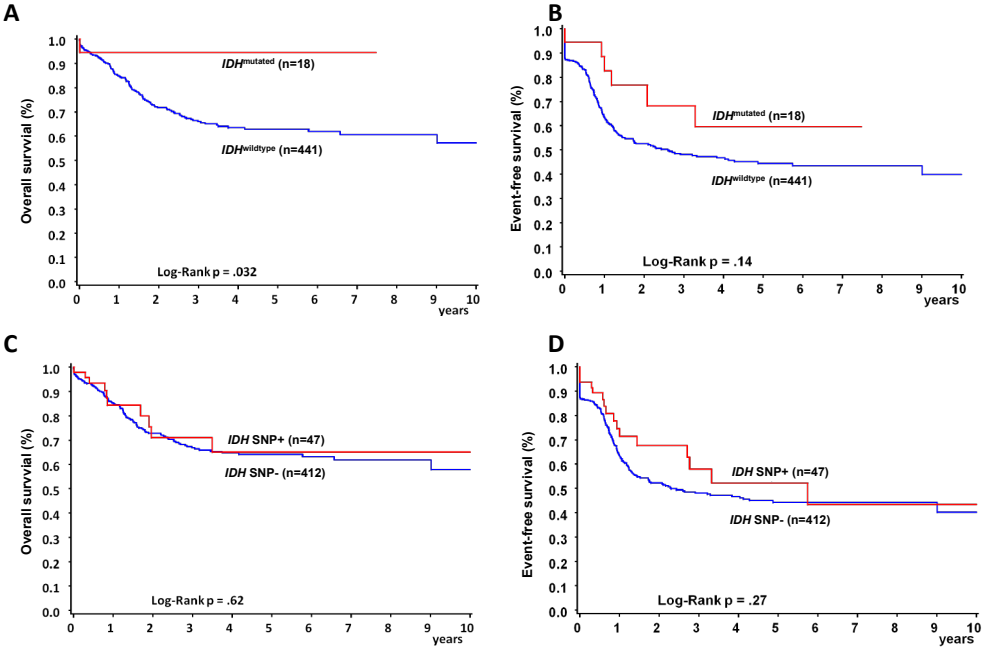

FIGURE 1. Impact of *IDH* mutations and *IDH* SNP rs11554137 on patient outcome. Overall survival (OS) and event-free survival (EFS) according to *IDH* mutation status (A,B); OS and EFS according to *IDH* SNP rs11554137 status (C,D).

Chapter 10

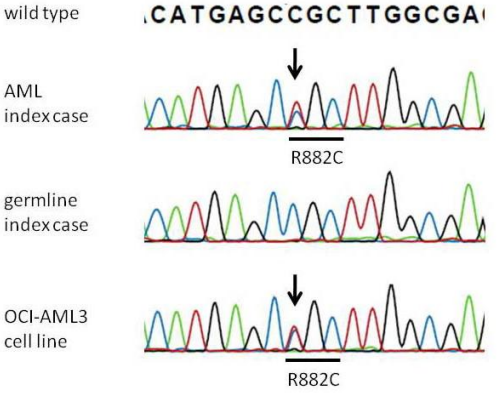

Figure 1. Sequence chromatograms of the *DNMT3A* mutations detected in the pediatric AML index case and the OCI-AML3 cell line. A heterozygous mutation (C>T), changing arginine into cysteine at codon 882, is present in the pediatric AML index case and the OCI-AML3 cell line. The germline sample of the index case did not harbor this mutation. Mutated nucleotides are indicated by arrows.

ABBREVIATIONS

AIEOP	Associazone Italiana Ematologica Oncologica Pediatrica
ALL	Acute lymphoblastic leukemia
AMLP	Acute myeloid leukemia
AML1	Acute myeloid leukemia 1 (gene)
APL	Acute promyelocytic leukemia
A-CGH	Array-based comparative genome hybridization
ATRA	All-*trans* retinoic acid
BAC	Bacterial artificial chromosome
BAALC	Brain and acute leukemia, cytoplasmic (gene)
BFM SG	Berlin-Frankfurt-Münster Study Group
BM	Bone marrow
bZIP	Basic Leucine Zipper domain
CBF	Core-binding factor
CBFB	Core-binding factor, beta subunit (gene)
CD	Cluster of differentiation
CEBPA	CAAT/enhancer binding protein alpha (gene)
C/EBPα	CAAT/enhancer binding protein alpha (protein)
CIR	Cumulative incidence of relapses
CML	Chronic myeloid leukemia
CN-AML	Cytogeneticaly normal AML
COG	Children's Oncology Group
CR	Complete remission
DCOG	Dutch Childhood Oncology Group
DDX10	DEAD (Asp-Glu-Ala-Asp) box polypeptide 10 (gene)
DEK	DEK oncogene (gene)
DHPLC	Denaturing high perfomance liquid chromatography
DNA	Deoxyribonucleic acid
DNMT3A	DNA (cytosine-5)-methyltransferase 3 alpha (gene)
EFS	Event-free survival
ERG	v-ETS erythroblastosis virus E26 oncogene homolog (gene)
ETO	Eight twenty one (gene)
ETV6	ETS variant 6
FAB	French-American-British
FDR	False discovery rate
FISH	Fluorescent *in situ* hybridization
FLT3/ITD	FMS-like tyrosine kinase 3 (gene) internal tandem duplication
FLT3/TKD	FLT3 tyrosine kinase domain mutation
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase (gene)
GEO	Gene Expression Omnibus
GEP	Gene expression profiling
HD	Heterodimerization domain
HAT	Histone acetyltransferase
HMT	Histone methyltransferase
HOXA/B	Homeobox A/B (cluster of genes)
HR	Hazard ratio
HSC	Hematopoietic stem cell
HOVON	Hematology/oncology foundation for adults in the Netherlands
IDH1/2	Isocitrate dehydrogenase 1/2 (gene)
JARID1A	Jumonji, AT rich interactive domain 1A (gene)
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene (gene)
K-RAS	v-ki ras2 Kirsten rat sarcoma viral oncogene homolog (gene)
LAME	Leucémies Aiguës Myéloblastiques de l'Enfant Cooperative Group
LCK	Lymphocyte-specific protein tyrosine kinase (gene)
LIMMA	Linear models for microarray data
LOH	Loss of heterozygosity
MDS	Myelodysplastic syndrome
MEIS1	Meis homeobox 1 (gene)
miRNA	MicroRNA
MLL-PTD	Mixed-lineage leukemia (gene) partial tandem duplication
MN1	Meningioma 1 (gene)

MNX1 (HLXB9)	Motor neuron and pancreas homeobox 1 (gene)
MLPA	Multiplex ligation-dependent probe amplification
MRC	Medical Research Council
mRNA	Messenger RNA
MYH11	Myosin, heavy chain 11 (gene)
NPM1	Nucleophosmin (gene)
N-RAS	Neuroblastoma RAS viral (v-ras) oncogene homolog (gene)
NSD1	Nuclear receptor binding SET domain protein 1 (gene)
NUP98	Nucleoporin 98kD (gene)
NUP214	Nucleoporin 214kD (gene)
OS	Overall survival
PAC	P1-derived artificial chromosome
PCR	Polymerase chain reaction
PEST	Proline (P), glutamic acid (E), serine (S), and threonine (T) domain
PHD finger	Plant Homeo Domain finger
PML	Promyelocytic leukemia (gene)
PTPN11	Protein tyrosine phosphatase, non-receptor type 11 (gene)
RARA	Retinoic acid receptor, alpha (gene)
RFS	Relapse-free survival
RNA	Ribonucleic acid
RT-PCR	Reverse transcription PCR
RT-qPCR	Real-time quantitative PCR
RUNX1	Runt-related transcription factor 1 (gene)
SB	Southern blot
SCT	Stem cell transplantation
SerpinB2	Serpin peptidase inhibitor, clade B (ovalbumin), member 2 (gene)
SNP	Single nucleotide polymorphism
TAD	Transactivation domain
TCR	T-cell receptor
TET2	Tet oncogene family member 2
TOP1	Topoisomerase (DNA) I (gene)
TRIB2	Tribbles homolog 2 (gene)
UPD	Uniparental disomy
VSN	Variance and stabilization normalization
WBC	White blood cell count
WHO	World Health Organization
WT1	Wilms tumor 1 (gene)

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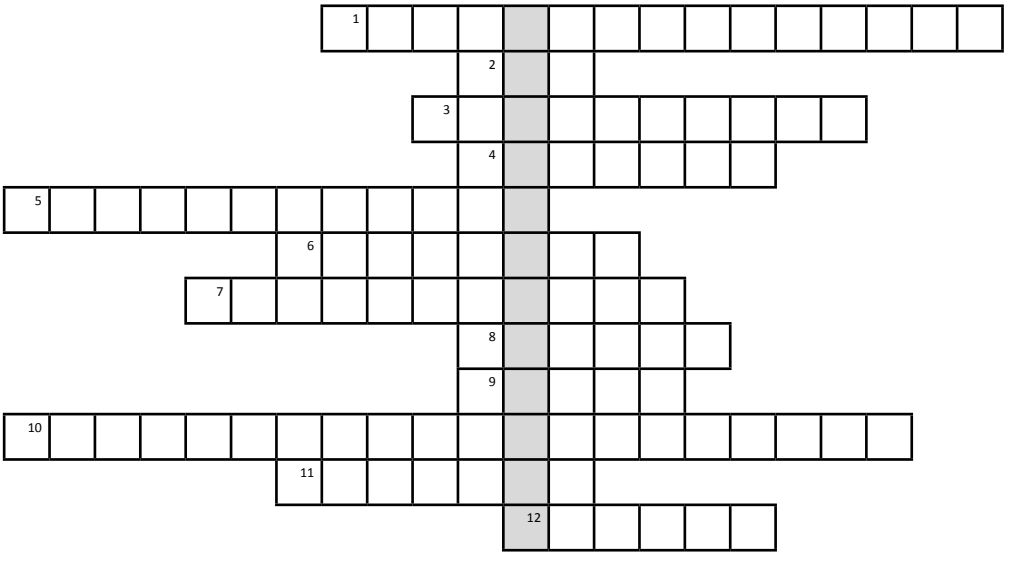

1 Gefeliciteerd, u hebt zojuist ‘Molecular genetic insights in ... normal pediatric acute myeloid leukemia’ uitgelezen
2 Een stukje DNA dat de code bevat voor de productie van een eiwit
3 Fusie-eiwit dat wordt beschreven in hoofdstuk 8, en dat een groep kinderen met AML met een zeer slechte overleving op de huidige therapie identificeert
4 Naamgever van de universiteit in Rotterdam
5 Stad waar het 50e ASH congres is georganiseerd (2 woorden, 3+9)
6 Ziekte die jaarlijks bij ongeveer 120 kinderen in Nederland wordt vastgesteld
7 Specialisme dat zich bezig houdt met afwijkingen in het bloed
8 Statistische maat voor de waarschijnlijkheid dat het gevonden resultaat berust op toeval (Engels)
9 Gen dat wordt beschreven in hoofdstuk 3
10 Type behandeling dat erop gericht is de groei van kankercellen te remmen zonder schade aan te brengen aan gezonde cellen (2 woorden, 12+8)
11 Achternaam van de promotor van dit proefschrift
12 Titel die de auteur van dit proefschrift op 16 november 2011 hoopt te verkrijgen