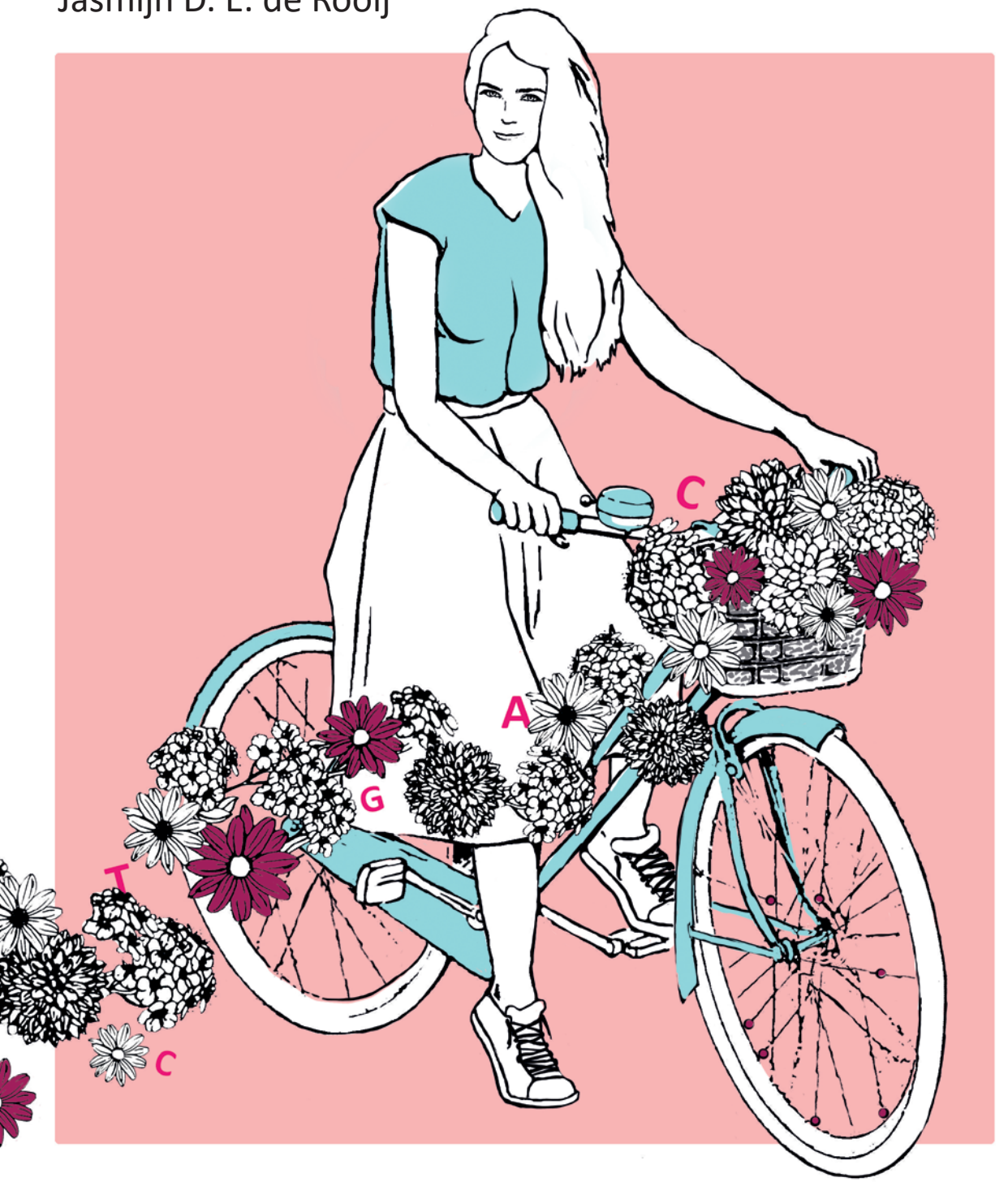
MOLECULAR GENETIC ABERRATIONS AND THEIR PROGNOSTIC RELEVANCE IN PEDIATRIC ACUTE MYELOID LEUKEMIA

Jasmijn D. E. de Rooij



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Moleculaire genetische afwijkingen en hun prognostische relevantie in acute myeloïde leukemie bij kinderen

Jasmijn D.E. de Rooij

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Contents

Chapter 1
General introduction

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Jasmijn D. E. de Rooij, C. Michel Zwaan and Marry van den Heuvel-Eibrink

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General introduction

Epidemiology of AML

In children, the most frequently occurring hematological malignancies include acute leukemias, of which 80% are classified as acute lymphoblastic leukemia (ALL) and 15%–20% as acute myeloid leukemia (AML). Chronic leukemias are exceedingly rare in childhood and mainly comprise chronic myeloid leukemia (CML) and juvenile myelomonocytic leukemia (JMML).

The incidence of AML in infants is 1.5 per 100,000 individuals per year, subsequently the incicidence decreases to 0.9 per 100,000 individuals aged 1–4 and 0.4 per 100,000 individuals aged 5–9 years, after which it gradually increases into adulthood, up to an incidence of 16.2 per 100,000 individuals aged over 65 years.[1] The underlying cause of AML is unknown, and childhood AML generally occurs *de novo*. In adult and elderly patients, AML is often preceded by myelodysplastic syndrome (MDS), but in childre n, the occurrence of AML preceded by clonal evolution of preleukemic myeloproliferative diseases, such as MDS or JMML, is rare. Germline affected individuals, such as those with Fanconi anemia or Bloom syndrome, have an increased risk for developing AML as a secondary malignancy.[2, 3] Recently, germ-line mutations in several genes, such as *TP53*, *RUNX1*, *GATA2* and *CEBPA*, have been found in families with an unexplained high risk of AML, suggesting a familial predisposition to develop AML, but these cases are rare.[4-8]

Children with Down syndrome classically present with a unique megakaryoblastic subtype of AML, typically following a transient myeloproliferative disorder in the neonatal period, which is characterized by somatic mutations in the *GATA1* gene. The leukemic cells of patients with Down syndrome are usually highly sensitive to chemotherapy with an exceptional high survival rate, and therefore it is pivotal to treat these patients with adjusted treatment protocols.[9] In addition, AML may occur following previous radiotherapy or chemotherapy containing alkylating agents or epipodophyllotoxins, as secondary neoplasm. These are typically characterized by either *KMT2A*-rearrangements (formerly known as *MLL*) or by monosomy 7.[10, 11]

Diagnostic Approach and Classification

AML is a heterogeneous disease with respect to morphology, immunophenotyping, cooperating underlying genetic abnormalities, as well as clinical behavior. The standard diagnostic process of AML is based on a combination of morphology, cytochemistry, immunophenotyping, cytogenetic and molecular characterization of the leukemic blasts derived from the bone marrow or peripheral blood.[12] Each AML patient can be risk-classified into a clinically relevant subgroup. The previously used morphology-based French-American-British (FAB) classification is nowadays replaced by the World Health Organization (WHO) classification, which also takes karyotype and molecular aberrations into account (Table 1.1).[13, 14] Cytochemistry, morphology and immunophenotyping is generally used to distinguish AML from ALL, which further classifies pediatric AML according to the cell lineage of origin and differentiation stage at which the differentiation arrest occurs. Especially for the diagnosis of FAB-types, M0 and M7 immunophenotyping is indispensable.[12, 15] The majority of chromosomal abnormalities is detected by conventional karyotyping and

complemented with fluorescent in situ hybridization (FISH) or reverse transcriptase PCR to detect relevant (cryptic) translocations, fusion genes or loss of chromosome material.[16] Nonetheless, the WHO classification is not fully applicable for pediatric AML, since it groups together specific genetic aberrations with clearly different oucome, and alocates ~44%, being the largest subgroup, as 'AML not otherwise specified'.[17]

Treatment and Outcome

The clinical outcome of pediatric AML has improved significantly over the past few decades, with current long-term survival rates of ~70% (Table 1.2).[18-28] This improvement is due to intensification of chemotherapeutic regimens, better risk-group stratification, better salvage at relapse and improved supportive care. Risk-group stratification is usually based on (cyto)genetic abnormalities present in the leukemic blasts in combination with early response to treatment, either specified as complete remission (CR) rate after one or two courses or applying minimal-residual disease measurements, which in AML is mainly based on flow-cytometry.[29] The chemotherapeutic regimens consist of 4–5 cycles of intensive chemotherapy, typically including cytarabine combined with an anthracycline. The added value of hematopoietic stem cell transplantation (SCT) in newly-diagnosed pediatric AML is under discussion, as in general, the occurrence of procedure-related deaths needs to be counterbalanced by the reduction in relapse risk. The procedure-related deaths are dependent on the intensity of the prior induction chemotherapy as well as on the condition regimen which is adjusted to the HLA-barrier between the donor and the recipient. SCT in first CR is therefore currently only recommended for a selected subset of high risk cases in most European protocols.[12, 30, 31] SCT plays a more prominent role in most North-American treatment protocols. Currently, several trials include minimal residual disease (MRD) levels after Courses 1 or 2 to allocate patients to the high risk group, and with that treatment including SCT.[32]

Despite intensive treatment, ~30% of the pediatric patients relapse, and outcome is poor following relapse, reflected by the ~30%–40% of patients surviving in the largest and most recent series reported to date.[33, 34]

Nevertheless, the high frequency of treatment-related deaths (5%–10%), both in treatment protocols for newly-diagnosed, as well as for relapsed disease, and the occurrence of long-term side effects, such as anthracycline-induced cardiomyopathy, illustrate that further intensification of chemotherapy seems hardly feasible.[35] Therefore, knowledge on the molecular and genetic background is of utmost relevance in order to detect novel, leukemia and patient-specific treatment targets.

Relevant Molecular and Genetic Aberrations in Pediatric AML

AML is thought to arise from at least two classes of cooperating genetic events.[36] Type I abnormalities result in increased, uncontrolled proliferation and/or survival of the leukemic cell and are often activating mutations of genes involved in signal transduction pathways, such as *FLT3*, *KIT*, *N-RAS*, *K-RAS* and *PTPN11*. Type II abnormalities impair differentiation and mainly result from genetic aberrations in hematopoietic transcription factors, due to, for instance, the AML-characteristic translocations t(8;21)(q22;q22)/*AML1-ETO* and 11q23/*KMT2A* rearrangements or from mutations in single genes, such as *NPM1* and *CEBPA*.[7, 37-40] The most common cytogenetic abnormalities (Type II) in children are t(8;21)(q22;q22), inv(16)(p13.1q22) (together referred to as core binding factor

(CBF)-AML), t(15;17)(q22;q21) and 11q23/*KMT2A*-rearranged abnormalities (Figure 1.1A).[41-44] Together, these account for approximately half of all pediatric AML cases, a much higher frequency than in adults. Some translocations, for example t(1;22)(p13;q13) and t(7;12)(q36;p13), are specific for young children and are rarely or never found in adults[45-52]. Translocations involving hematopoietic transcription factors often lead to dysregulated gene expression, either as a result of the fusion partner itself or the recruitment of different co-factors to the transcription complex. For example, the *KMT2A* gene has histone methyltransferase activity and is part of a chromatin modifying complex. More than 60 fusion partners have been identified in AML, but the breakpoint of the *KMT2A* gene is highly conserved.[53, 54] Fusion proteins lead to a gain of function of the *KMT2A*-complex, resulting in inappropriate histone modification and increased expression of *MEIS1* and, specifically, *HOXA* genes, maintaining a stem-cell phenotype. In addition, the presence of the DOT1L protein, which is recruited into the *KMT2A*-complex, is required for the leukemogenic activity of several *KMT2A* rearrangements and may be a target for treatment.[55, 56]

Approximately 20%–25% of pediatric AML cases are cytogenetically normal.[40, 57] Of interest in these cases, specific Type II mutations and translocations are identified in ~70% of the cases, such as *NPM1* mutations, biallelic *CEBPA* mutations, as well as the cryptic translocations, *NUP98/NSD1*, all invisible with conventional karyotyping and, hence, requiring additional molecular diagnostics (Figure 1.1B).[38, 51, 52, 58]

The combination of the Type I and Type II mutations is not completely random as specific combinations frequently occur. Examples are the Ras pathway mutations, which are often found in combination with *KMT2A*-rearrangements; *KIT* mutations which are mainly found in CBF-AML; and *FLT3*-internal tandem duplications which are often seen in combination with *PML/RARA* and *NUP98/NSD1* (Figure 1.1).[40, 51]

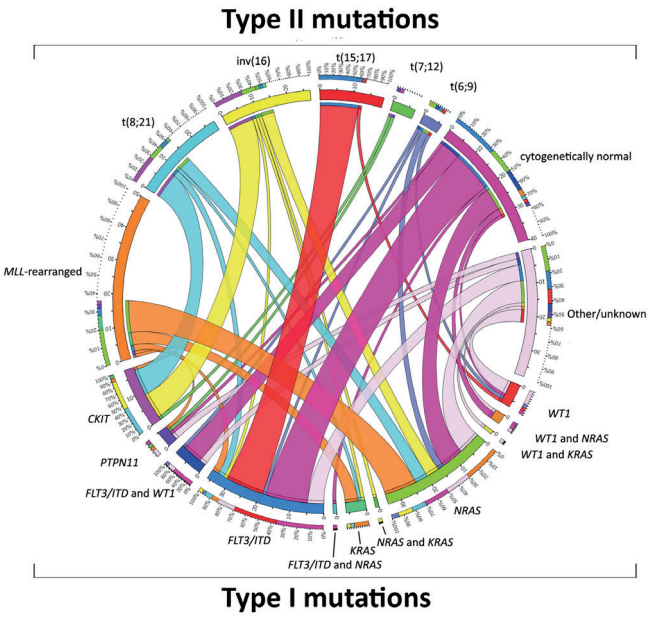
In the study of Hollink *et al*[51] patients were identified with a similar gene expression profile, but unknown aberration. The work shown in this thesis unravels several of these unknowns, with a specific search for type II mutations, to get a better understanding of the underlying biology of pediatric AML.

Mutations in epigenetic regulators, such as *EZH2*, *ASXL1* and *DNMT3A*, add another level of complexity and contribute to both the maturation arrest and proliferative capacity, which are needed to develop AML (Figure 1.2).[58-66] These mutations are more frequent in adult AML, and show the difference in leukemogenesis between AML in adults and children. Still, mutations in specific genes found in adult AML, may be of interest in pediatric AML, such as mutations in *ETV6, BCOR,* or *PHF6*, genes that were screened for mutations in this thesis.

Prognostic Factors and Risk Group Stratification

The most important prognostic factors for the survival of pediatric AML patients are the initial response to treatment and the underlying genetic and molecular aberrations.[12, 68, 69] CBF-AML is a favorable prognostic subgroup.[40, 44, 70] Outcome in *KMT2A*-rearranged AML is variable and depends on the translocation partner. For example, the *KMT2A*-translocation t(1;11)(q21;q23) is associated with a very favorable outcome in pediatric AML. In contrast, poor survival rates were reported in pediatric AML with translocations t(6;11)(q27;q23) and t(10;11)(p12;q23).[76,77] The acute megakaryoblastic leukemias

A



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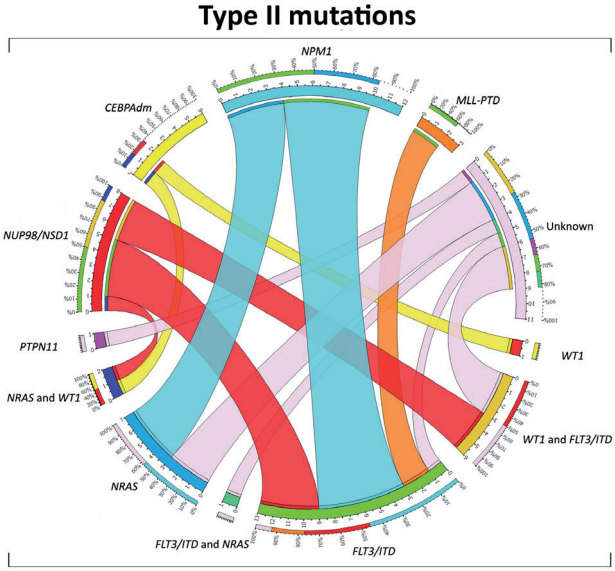

Figure 1.1 Distribution of Type I/II abnormalities in pediatric AML. (A) Cooperating type I and Type II mutations in pediatric AML. The circos plot depicts the frequency of the Type II mutations and co-occurrence of Type I mutations in patients with *de novo* pediatric AML. The length of the arch corresponds to the frequency of the Type II mutation and the width of the ribbon with the percentage of patients with a specific Type I mutation or a combination of Type I mutations. FLT3/ITD denotes FLT3 internal tandem duplication. (B) Co operating Type I and Type II mutations in cytogenetically normal AML. The circos plot depicts the frequency of the Type II mutations and co-occurrence of Type I mutations in patients with de novo pediatric cytogenetically normal AML. The length of the arch corresponds to the frequency of the Type II mutation, and the width of the ribbon with the percentage of patients with a specific Type I mutation or a combination of Type I mutations. FLT3/ITD denotes FLT3 internal tandem duplication.

Table 1.1. The WHO classification of acute myeloid leukemia (AML) and related neoplasms.[14]

WHO Classification 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*
	Acute promyelocytic leukemia with t(15;17)(q22;q12); *PML-RARA*
	AML with 11q23 (*KMT2A)* abnormalities
	AML with t(6;9)(p23;q34); *DEK-NUP214*
	AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1*
	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	

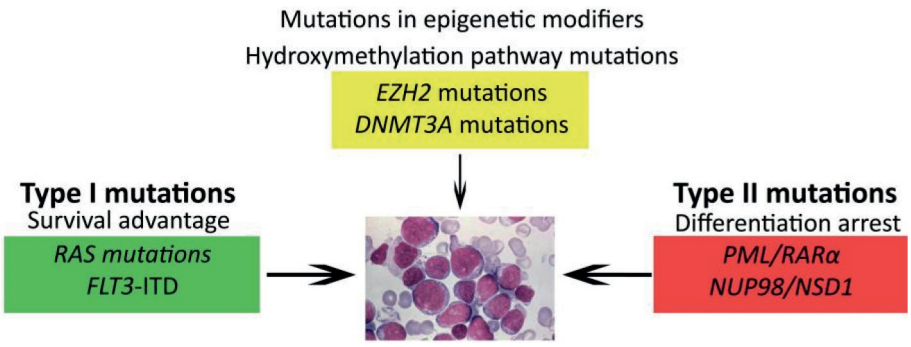

Figure 1.2. Model of cooperating genetic events in AML. Different types of genetic and epigenetic events collaborate in leukemogenesis.

(AMKL, FAB-M7) in non-Down syndrome patients represent a subgroup with poor outcome, with the exception of AMKL harboring t(1;22)(p13;q13), which seems to confer a favorable prognostic group, in contrast to Down syndrome, where AMKL confers a favorable outcome.[9, 52] The t(1;22)(p13;q13), or translocation *RBM15/MKL1*, comprises approximately 10% of the pediatric non-Down Syndrome AMKL cases. Given the poor prognostic outcome, identification of driving aberrations will contribute to risk stratification, and may lead to targeted therapy in the future. In this thesis we unravel more recurrent aberrations, and analyze outcome parameters of this group.

A special subtype of pediatric AML is the cytogenetically normal (CN) AML group, where clinical outcome is highly dependent on the presence of single-gene mutations or cryptic translocations. Of special interest are *NPM1* and bi-allelic *CEBPA* mutations, conferring a favorable prognosis, while the cryptic translocation *NUP98/NSD1* confers a poor prognosis, due to a poor response to treatment and a high risk for relapse, independent of the poor prognostic Type I *FLT3*-itd abnormality.[38, 51, 71]

The described poor prognostic abnormalities in adult AML of chromosomes 3q and 5q and the monosomal karyotypes are extremely rare in children.[28, 72-74] Overexpression of *EVI1* caused by 3q26 abnormalities predicts an adverse outcome in adult AML, but *EVI1* overexpression is not an independent prognostic factor in pediatric AML.[75, 76] The Type I mutations of *WT1* and *FLT3*-itd predict a poor outcome, the latter dependent on the allele ratio, and these mutations are described as events in clonal evolution towards relapse.[77]

Deletion of 7q is described as an intermediate risk in the prognosis in adults, in contrast to the outcome of pediatric AML with a 7q deletion in children. In pediatric patients with a 7q deletion, the outcome seems to be dependent on other cytogenetic abnormalities in the leukemic cell.[44, 78] Monosomy 7 is a well-known poor-prognostic factor and confers a worse outcome.[44, 78] The specific genes contributing to this prognosis may give a better understanding of the leukemogenesis, and is therefore of interest for targeted therapy. In this thesis we elaborate on the genes that may cause the leukemic effect in the monosomy 7 cases.

Genomic Approaches to Unravel the Biology of Pediatric AML

In order to provide more insight into the heterogeneity and biology of AML, apart from using candidate-gene approaches including split-signal FISH, PCR and multiplex ligand-dependent probe amplification (MLPA), genome-wide approaches have been recently employed, although the success rate is variable. Array-based comparative genomic hybridization (array-CGH) and single-nucleotide polymorphism (SNP) arrays identified several regions with loss of heterozygosity and recurrent copy number variations (CNVs), albeit with low frequency in AML.[79] These CNVs included aberrations in the *WT1*, *NF1* and *TET2* genes, the latter being more common in adults than in children.[39, 60, 62, 80]

Gene expression profiling could predict the well-known cytogenetic subtypes of AML with high accuracy, although its value for diagnostic purposes remains limited, since most aberrations can be identified with conventional karyotyping.[81-84] Nevertheless, novel genes involved in the pathogenesis of pediatric AML subtypes were identified using this method, such as *BRE* and *IGSF4.*[85, 86]

In addition to discovering novel gene mutations, next generation sequencing, such as RNA-sequencing and whole exome sequencing, has also proven to be a

powerful tool in the study of the clonal evolution of both adult and pediatric AML.[87, 88] By comparing the mutational spectrum of diagnosis-relapse pairs, it was shown that the founding clone gained novel mutations and evolved into the relapse clone. Moreover, minor subclones present at diagnosis can survive chemotherapy, gain mutations and present as dominant clones at relapse, illustrating their leukemia-driving capacity. Therapeutic targeting of novel identified mutations to prevent relapse may provide an improved outcome for selected patients.[89, 90] In this thesis we combined candidate-gene approaches and next generation sequencing, in order to find new and/or recurrent aberrations in our pediatric AML cohort.

Towards Optimized Therapy

The translation from molecular aberrations towards targeted therapy might be able to improve outcome in the next few decades, next to other therapies such as immunotherapy. Since further intensification of current chemotherapy treatment seems not feasible in pediatric AML, due to high morbidity and mortality rates, new therapeutic approaches that are more tumor-specific and cause less severe side effects are urgently needed. Some new compounds directed at specific molecular targets have already been investigated in early clinical trials in pediatric AML.[32]

International collaboration, which has been pursued over the last few decades on the levels of the International Berlin-Frankfurt-Munster Study Group (BFM-SG), Innovative Therapies for Children with Cancer (ITCC), European Network for Cancer Research in Children and Adolescents (ENCCA), Therapeutic Advances in Childhood Leukemia (TACL) and Childhood Oncology Group (COG), has been proven successful in clinical and biological studies and will speed up efforts to enhance therapeutic options and the availability of novel agents for individual pediatric AML patients.[12, 26, 52, 78, 91, 92]

Outline of this thesis

The work described in this thesis focused on characterizing pediatric AML in the search for unknown aberrations, determine new outcome parameters and understanding the biological changes caused by specific molecular aberrations.

In **part 1**, the research focusses on the driving aberrations and their prognostic value in pediatric AMKL. **Chapter 2** describes the identification and analyses of the frequency of *NUP98/KDM5A* as a recurrent event in pediatric AMKL, occurring in approximately 10% of all pediatric AMKL. In **chapter 3** we focused on the identification of *RB1* deletions as a specific event in *NUP98/KDM5A* translocated pediatric AMKL patients, and unraveled the biologic effect of the co-occurrence of these two aberrations using various techniques. Furthermore, we report on data using RNA-sequencing and DNA-sequencing to identify aberrations in pediatric AMKL, summarized in **chapter 4**. We were able to screen 98 pediatric AMKL samples, with international collaborators from among others the St. Jude Research Hospital (Memphis, TN, USA), the BFM-SG, the AIEOP (Bologna, Italy), and DCOG. In **chapter 5**, the results are shown of another international collaboration, with samples derived from the AIEOP, the COG, the BFM-SG and DCOG, we analyzed outcome parameters in pediatric AMKL, of the most recurrent (cryptic) cytogenetic aberrations, which in the future may benefit to risk-stratification.

Thereafter, **part 2** describes mutations and translocations found in all

morphologic subtypes of pediatric AML. In the search for unknown aberrations, we used multiplex ligand probe amplification (MLPA) and identified *IKZF1* deletions in pediatric AML. In **chapter 6** we analyzed the frequency of *ETV6* alterations in pediatric AML using MLPA, sequencing and FISH. Using the same MLPA kit we identified deletions of *IKZF1*, which we describe in **chapter 7**. In **chapter 8** we describe the frequency of *BCOR* and *BCORL1* mutations in pediatric AML, and in **chapter 9** we focused on the frequency of *PHF6* mutations, and found that these are not specific for male patients, as seen in other types of leukemia. In **chapter 10** a new translocation fusing *ZMYND11* to *MBTD1* resulting in high *HOXA* expression is described. **Chapter 11** summarizes this thesis and **chapter 12** comprises the general discussion and future perspectives.

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PART ONE

Molecular insights in pediatric acute megakaryoblastic leukemia

Chapter 2

NUP98/JARID1A is a novel recurrent abnormality in pediatric acute megakaryoblastic leukemia with a distinct *HOX*-gene expression pattern

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Abstract

Cytogenetic abnormalities and early response to treatment are the main prognostic factors in acute myeloid leukemia (AML). Recently, *NUP98/NSD1*, a cytogenetically cryptic fusion, was described as recurrent event in AML, characterized by dismal prognosis and *HOXA/B*-gene overexpression. Using split-signal FISH, other *NUP98*-rearranged pediatric AML cases were identified, including several acute megakaryoblastic leukemia (AMKL) cases with a cytogenetically cryptic fusion of *NUP98* to *JARID1A* (t(11;12)(p15;q13)). In this study we screened 105 pediatric AMKL cases to analyse the frequency of *NUP98/JARID1A* and other recurrent genetic abnormalities. *NUP98/JARID1A* was identified in 11/105 patients (10.5%). Other abnormalities consisted of *RBM15/MKL1* (n=16), C*BFA2T3/GLIS2* (n=13), and *MLL*-rearrangements (n=13). Comparing *NUP98/JARID1A* positive with other pediatric AMKL patients, no significant differences in sex, age, and white blood cell count were found. *NUP98/JARID1A* was not an independent prognostic factor for 5-year overall (pOS) or event-free survival (pEFS), although the 5-year pOS for the entire AMKL cohort was poor (42±6%). Cases with *RBM15/MLK1* fared significantly better in terms of pOS and pEFS, although this was not independent from other risk factors in multivariate analysis. *NUP98/JARID1A* cases were characterized by *HOXA/B* gene overexpression, which is a potential druggable pathway. In conclusion, *NUP98/JARID1A* is a novel recurrent genetic abnormality in pediatric AMKL.

Introduction

Acute myeloid leukemia (AML) is a heterogeneous disease characterized by recurrent genetic aberrations.[1-3] At present, survival rates in pediatric AML are approximately 60-70%.[4, 5] The most important factors predicting clinical outcome are genetic and molecular aberrations, and early response to treatment. It is hypothesized that AML results from at least two different types of mutations which non-randomly collaborate in leukemogenesis.[6] The type-I aberrations confer a proliferative or survival advantage, and consist for instance of mutations in tyrosine kinases, such as *FLT3* or *RAS* mutations. Type-II mutations lead to impairment of the hematopoietic differentiation, and frequently consist of translocations, such as 11q23/*MLL*-rearrangements, *AML1/ETO* or *CBFB/MYH11*.[1, 6]

Genome wide studies have revealed many novel single gene mutations.[7, 8] Recently, we described a cytogenetically cryptic translocation fusing *nucleoporin 98 (NUP98)*, located on chromosome 11p15, to *nuclear receptor binding SET domain protein 1 (NSD1)*, located on chromosome 5q35, as a recurrent event in cytogenetically normal AML (CN-AML).[9, 10] In children with CN-AML the frequency was 16.1%, whereas in adult CN-AML the frequency was 2.3%.[9, 11] AML patients with *NUP98/NSD1* translocated leukemia had a dismal prognosis.[9] Of interest, *NUP98/NSD1*-rearranged cases were associated with overexpression of *HOXAB*-genes, suggesting an oncogenic mechanism similar to AML cases characterized by *MLL*-rearrangements or *NPM1*-gene mutations.[9, 12] Searching for other *NUP98*-driven leukemias, we screened a large cohort using split signal FISH to detect other *NUP98* breaks, as *NUP98* is known to have many different partner genes.[10]

In one patient another fusion partner was identified, i.e. *JARID1A*, which was previously described by Van Zutven *et al* in a single case from our institute.[13] This case was classified as acute megakaryoblastic leukemia (AMKL), which can either occur *de novo* or in the context of Down Syndrome associated myeloid leukemia with mutations in *GATA1.* Non-Down Syndrome AMKL is characterized by a limited number of specific genetic abnormalities, including the t(1;22)(p13;q13) translocation fusing *RBM15* to *MKL1*, formerly known as *OTT/MAL*.[14] In addition, recently Gruber *et al* described an inversion of chromosome 16 resulting in *CBFA2T3/GLIS2* gene fusion as a repetitive event in AMKL, with an overall frequency of 27%.[15] This frequency was confirmed by Thiollier *et al*.[16]

In this study, we show that *NUP98/JARID1A* is a novel recurrent cryptic translocation in pediatric AMKL. In addition, we characterized the genetic abnormalities of a larger series of pediatric AMKL patients, including the *RBM15/MKL1* translocation and the recently identified fusion of *CBFA2T3* to *GLIS2,* and investigated the prognostic significance of these aberrations.

Material and methods

Patient samples

The cohort of newly diagnosed pediatric AML patients with available peripheral blood or bone marrow samples taken at initial diagnosis included in this study was previously described by Hollink *et al* (n=293; age, 0-18 years).[9] Samples were provided by the

Dutch Childhood Oncology Group (DCOG, The Hague, The Netherlands; n=141), the AML–Berliner-Frankfurt-Münster (BFM) Study Group (Germany and Czech Republic; n=128), and the Saint-Louis Hospital (Paris, France; n=24) (Supplementary Table 2.1).[9] Morphological classification and karyotyping were centrally reviewed by each study group. Cytogenetic aberrations in this group were distributed representative for the frequencies seen in pediatric AML.[17, 18]

Based on the identification of *NUP98/JARID1A* in AMKL patients, 105 additional samples of newly diagnosed pediatric non-Down Syndrome AMKL patients were obtained. These were kindly provided by the aforementioned study groups (n=45), and the North-American Children's Oncology Group (COG, Arcadia, California, United States of America; n=60). In total 33 samples, consisting of viable frozen BM or PB were available, as well as 72 samples of which only RNA or cDNA was available.

To investigate the presence of *NUP98/JARID1A* in Down Syndrome AMKL patients, a series (n=16) was provided by the DCOG and the AML-BFM Study Group.

After thawing the samples, leukemic cells were purified as previously described.[19] Blast percentages were assessed morphologically using cytospins stained with a May-Grünwald-Giemsa staining. Isolation of genomic DNA and total cellular RNA from the samples was performed using Trizol reagent. All patients were treated with intensive collaborative group cytarabine-anthracycline based pediatric AML treatment protocols. Each collaborative study group provided us with centrally reviewed morphological and cytogenetic classification and clinical follow-up data of the AMKL patient cohort.

Institutional review board approval for these studies had been obtained in the participating centers.

Split signal FISH for NUP98

For detection of *NUP98* rearrangements, FISH experiments were performed on newly diagnosed pediatric AML cases, excluding cases where the driving oncogenic type-II abnormality was already known, such as *PML/RARA* or *MLL*-rearrangements. Dual-color FISH analysis for detection of *NUP98* breaks was performed on thawed cytospin slides using the 44-kb overlapping bacterial artificial chromosome probes RP11-120E20 and RP11-348A20 (BACPAC Resources Center, Oakland, USA), as described previously.[20]

Detection of the NUP98/JARID1A transcript

The presence of *NUP98/JARID1A* and the reciprocal *JARID1A/NUP98* were determined by RT-PCR (Supplementary Table 2.2a). Purified PCR products were directly sequenced on a PRISM 3100 genetic analyzer (Applied Biosystems) and analyzed using CLCWorkbench (Version 3.5.1; CLC Bio).

Cytogenetic and molecular characterization

Samples were screened for cytogenetic aberrations using standard chromosome banding analysis at initial diagnosis by the collaborative study groups. All cases with unknown karyotype were screened for *MLL*-rearrangements, and those with a reported *MLL*-rearrangement were confirmed, using split signal FISH for *MLL*. In case of a positive split signal as seen with FISH, partner gene identification was performed with translocation-partner specific PCR for the following translocations; *MLL/AF6 (*also known as *MLL/MLLT4), MLL/AF9* (also known as *MLL/MLLT3), MLL/AF10* (also known as

MLL/MLLT10), MLL-ENL (also known as *MLL/MLLT1),* and *MLL-ELL*.[21] Fusions of *RBM15/MKL1* and *CBFA2T3/GLIS2* were detected using conventional karyotyping, RT- PCR and sequencing.

To identify other aberrations, RT-PCR was performed on hotspot areas of the following genes; *N-RAS, K-RAS, NPM1, MLL-PTD, CEBPA, FLT3, PTPN11, KIT* and *WT1* as previously described by Balgobind *et al*.[1] Primers and probes used for analysing these molecular aberrations are shown in Supplementary Table 2.2a.

Gene expression profiling

Gene expression profiling (GEP) data of 274/293 pediatric AML patients, of which 8 were classified as AMKL cases, were available from earlier studies.[22] Checking RNA integrity, processing the Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA, USA), and normalizing and analyses of the data were performed as described previously.[9] Original data are available in the Gene Expression Omnibus repository (http://www.ncbi.nlm.nih.gov/geo;accession GSE17855).

Taqman validation of HOX-gene expression levels

GEP data were only available for a limited number of patients with AMKL, hence we performed specific real-time quantitative PCR (RT-qPCR) for various *HOXA* and *-B* genes to study *HOX* gene expression in AMKL patients. Results obtained with GEP were validated with RT-qPCR in a series of pediatric AML cases, and in addition pediatric AMKL cases were studied with RT-qPCR. RT-qPCR was performed in duplicate in cases with at least 70% blasts. Primer and probe sets are shown in Supplementary Table 2.2b. The average cycle threshold (CT) value was used to determine the expression levels of the different *HOX*-genes in comparison to the expression levels of the reference gene *GAPDH*, using the comparative cycle time method. Correlation between GEP data and RT-qPCR was examined.

Statistical analysis

Statistical analyses were performed with SPSS 17.0 (SPSS). Correlation coefficients were measured using Spearman's correlation. Categorical variables were compared using the chi-square or Fisher exact test and continuous variables using 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 tri-lineage hematopoiesis, plus absence of leukemic cells in the cerebrospinal fluid or elsewhere, probability of event-free survival (pEFS), defined as the time between diagnosis and first event including non-responders (calculated as an event on day 0), death of any cause and second malignancies, and probability of overall survival (pOS), defined as the time between diagnosis and death. Both pEFS and pOS were estimated by the Kaplan-Meier method and groups were compared with the log-rank test. The cumulative incidence of non-response or relapse (pCIR), defined as time between diagnosis and relapse and with non-responders included as an event on day 0, was analysed by the Kalbfleisch and Prentice method and groups were compared with the Gray's test. Multivariate Cox regression analysis was used to determine the independency of prognostic markers. Statistical significance was considered if p-values were below 0.05.

Results

Identification of the NUP98/JARID1A fusion

Of 293 well-characterized pediatric AML cases, 122 patients with available material and in which the driving oncogenic type-II abnormality had not been identified, were analyzed with split signal FISH for *NUP98*-abnormalities. In 26 patients a split signal was detected, suggesting a break in the *NUP98* gene. Using a candidate gene approach to identify potential cytogenetically cryptic fusion partners, we identified *JARID1A* to be fused to *NUP98.* Subsequently, the entire cohort (n=293) was screened for presence of the *NUP98/JARID1A* fusion, which revealed three additional cases, of whom no material for FISH was available. In all patients the fusion was cryptic. Three out of 4 *NUP98/JARID1A* positive cases were morphologically diagnosed with AMKL (FAB M7). One patient was classified as an acute monoblastic leukemia (FAB M5), and this patient was the only *NUP98/JARID1A* positive case out of 65 (1.5%) acute monoblastic leukemia patients (Supplementary Table 2.1).

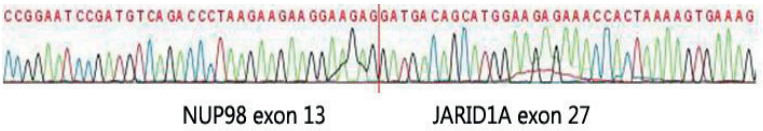


Figure 2.1. Sequence analysis of a *NUP98/JARID1A* positive case. RT-PCR analysis using *NUP98*- and *JARID1A*-specific primers revealed a *NUP98/JARID1A* fusion. Sequence analysis confirmed an in-frame fusion between *NUP98* exon 13 and *JARID1A* exon 27. The in-frame fusion was identical in 10 out of 11 cases.

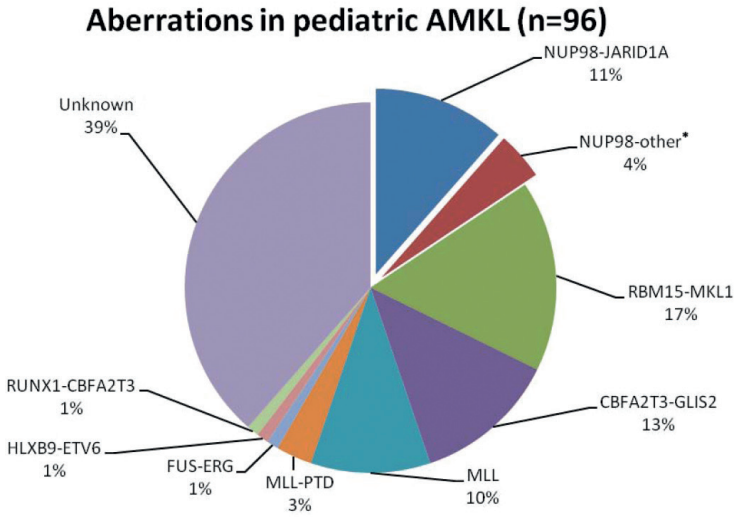


Figure 2.2. *NUP98/JARID1A* is a recurrent event in pediatric AMKL. Pie chart showing percentage of different type-II aberrations found in pediatric AMKL for the 96 patients of whom all data were available. All patients were tested with RT-PCR for *NUP98/JARID1A, RBM15/MKL1, CBFA2T3/GLIS2,* and tested with FISH on *MLL*-rearrangements, or in case of unavailable material for FISH tested with RT-PCR on *MLL-AF6, MLL-AF9, MLL-AF10,* and *MLL-ELL*. * Partner gene unknown, tested for fusion to *JARID1A, NSD1, NSD3, TOP1, DDX10* and *PHF23.*

NUP98-aberrations in AMKL

To identify the frequency of *NUP98/JARID1A* in *de novo* AMKL, we screened an extended pediatric AMKL cohort of 105 *de novo* non-Down Syndrome samples using RT-PCR. The *NUP98/JARID1A* fusion was detected in 11 out of 105 (10.5%) AMKL patients, and confirmed with direct sequencing. Sequence analysis showed an identical in-frame fusion of exon 13 of *NUP98* to exon 27 of *JARID1A* in 10 cases (Figure 2.1). In 1 case the breakpoint of *NUP98* was detected in exon 14, and part of exon 14 of *NUP98* was fused in-frame to exon 27 of *JARID1A*. The reciprocal product was found in 2 out of 8 cases only, three cases could not be tested due to lack of material.

Split signal FISH of *NUP98* could be performed in 25 AMKL cases, and revealed four additional cases with a break in the *NUP98* gene. In three patients the fusion partner has not been identified yet. These patients were negative for *NUP98* fusion to *PHF23, NSD1, TOP1, DDX10,* and *JARID1A* with breakpoints previously described.[9, 23, 24] One patient carried the *NUP98/NSD1* fusion. Among the 16 Down Syndrome AMKL patients, no *NUP98/JARID1A* fusions were found.

Extensive genetic characterization of the AMKL cohort

We further investigated the 105 pediatric AMKL cases for various type-II abnormalities, as depicted in Figure 2.2. The classical t(1;22)(p13;q13) fusing *RBM15 to MKL1* was found in 16 out of 105 patients (15.2%). In 13 out of 105 cases we found the recently reported *CBFA2T3/GLIS2* fusion, as described by Gruber *et al*.[15] *MLL*-rearrangements were identified in 10/96 cases with available material, i.e. *MLL-AF9* (n=6), *MLL-AF6* (n=1), *MLL-AF10* (n=1), *MLL-AF17* (n=1), and *MLL-ENL* (n=1). In addition, three *MLL-PTD* cases were identified. Other translocations consisted of one case with t(16;21)(p11;q22), resulting in the *FUS/ERG* fusion gene, and a t(7;12)(q36;p13), resulting in a fusion of *HLXB9* with *ETV6* in another patient. All aberrations were mutually exclusive.

In 39% of the AMKL cases, currently no type-II aberration could be detected using RT-PCR or FISH screening for the abnormalities mentioned above. However, in 36 cases cytogenetic data did reveal various complex and other random aberrations.

Forty-five out of 105 patients had material available for type-I mutation screening. Only 3 out of these 45 patients harboured a type-I mutation in one of the screened hot spot areas. All three consisted of a *RAS* mutation (Table 1.1a). No other mutations were found.

Characteristics of genetically distinct groups of pediatric AMKL

When we compared the clinical characteristics of the children with AMKL included in this study versus a population-based AMKL cohort included in the AML-BFM 93/98 studies no significant differences in sex (p=0.88), age (p=1.0) or white blood cell count (p=1.0) were seen. Concerning clinical outcome, there were no significant differences in event-free (p=0.44) or overall survival (p=0.56), nor in the cumulative incidence of relapse (p=0.93).

The median age at diagnosis of the 105 pediatric AMKL cases was 1.5 years (0.1-15.4 years). *NUP98/JARID1A* positive cases (Table 2.1b) did not differ significantly from the other pediatric AMKL cases concerning age at diagnosis (median age 1.8 years, range 0.9-4.9 years) compared to 1.5 years (range 0.1-15.1 years), respectively

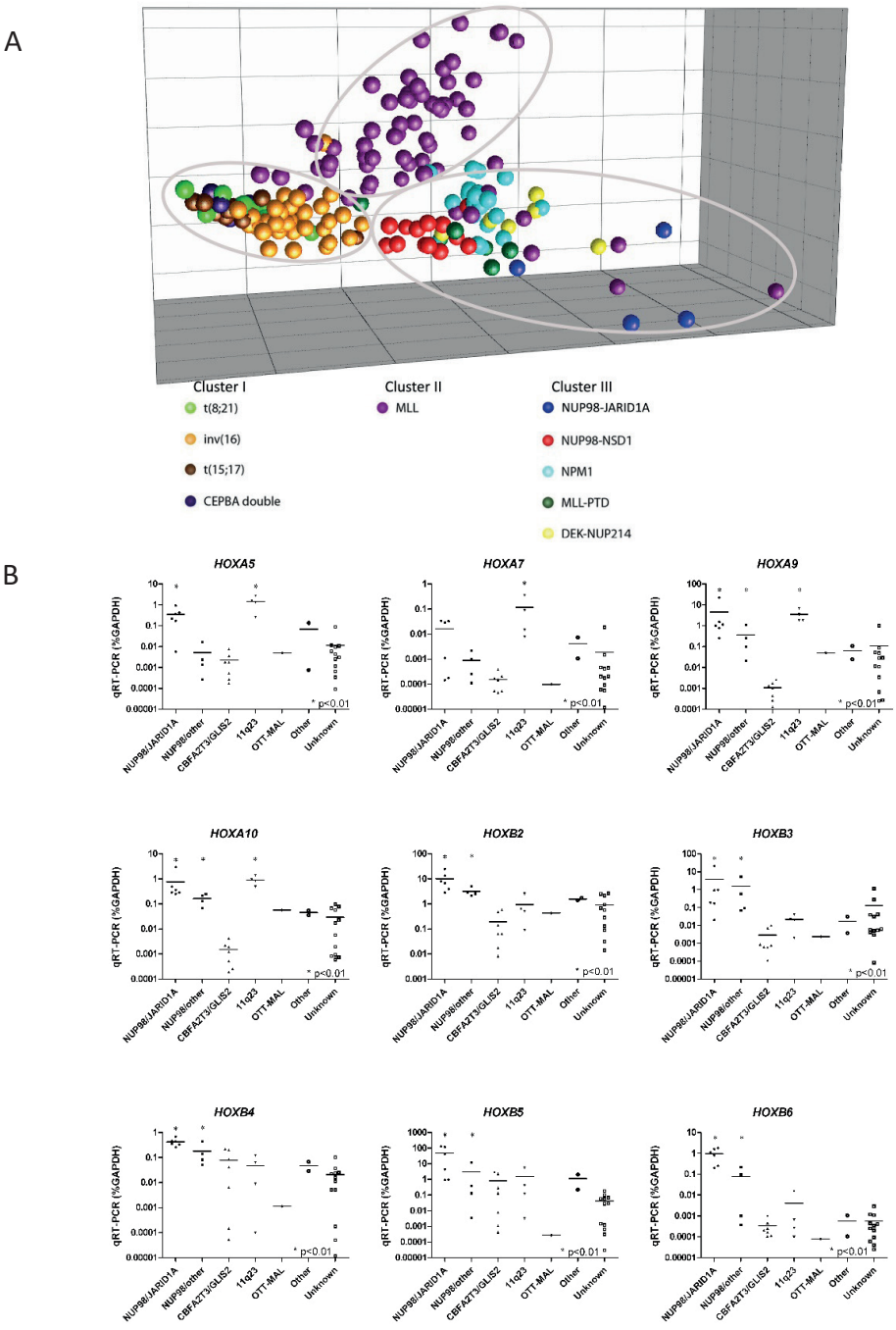


Figure 2.3. Distinct *HOX* expression pattern in *NUP98/JARID1A* positive cases. (A) Principal component analysis of pediatric AML subgroups characterized by specific type-II aberrations was carried out based on all *HOXA* and *–B* annotating probe sets present on the HGU133 Plus 2.0 microarray by Affymetrix. Each circle represent an individual case. Three clusters are observed as indicated by the grey circles. The cluster harbouring the *NUP98/JARID1A* positive cases is the cluster with both *HOXA* and *–B* expression. (B) RT-qPCR expression of *HOXA5, -A7, -A9, -A10, -B2, -B3, -B4, -B5* and *–B6* in percentage of *GAPDH* expression of pediatric AMKL cases (n=36) grouped by their specific type-II aberration. *NUP98/JARID1A* cases were characterized by high *HOXA5, -A9, -A10, -B2, -B3, -B4, -B5* and *–B6* expression.

(p=0.169); nor in sex (respectively 55% female vs. 53% female); nor in white blood cell count at diagnosis (median respectively 11.8 x10^9/L vs. 16.0 x10^9/L, p=0.141, Table 2.1a). Also in patients with the *CBFA2T3/GLIS2* fusion (n=13, Table 2.1c) age at diagnosis, sex and white blood cell count did not differ significantly in comparison to all other AMKL patients. Patients with AMKL characterized by t(1;22)(p13;q13) (Supplementary Table 2.3a) were significantly younger in comparison to all other AMKL cases (median 0.5 yrs vs. 1.6 yrs, p<0.01), but sex, and white blood cell count at diagnosis did not differ significantly (Table 2.1a). Moreover, the *MLL*-rearranged AMKL cases (Supplementary Table 2.3b) did not differ significantly from the other AMKL cases for the aforementioned clinical characteristics (Table 2.1a).

HOX-gene expression

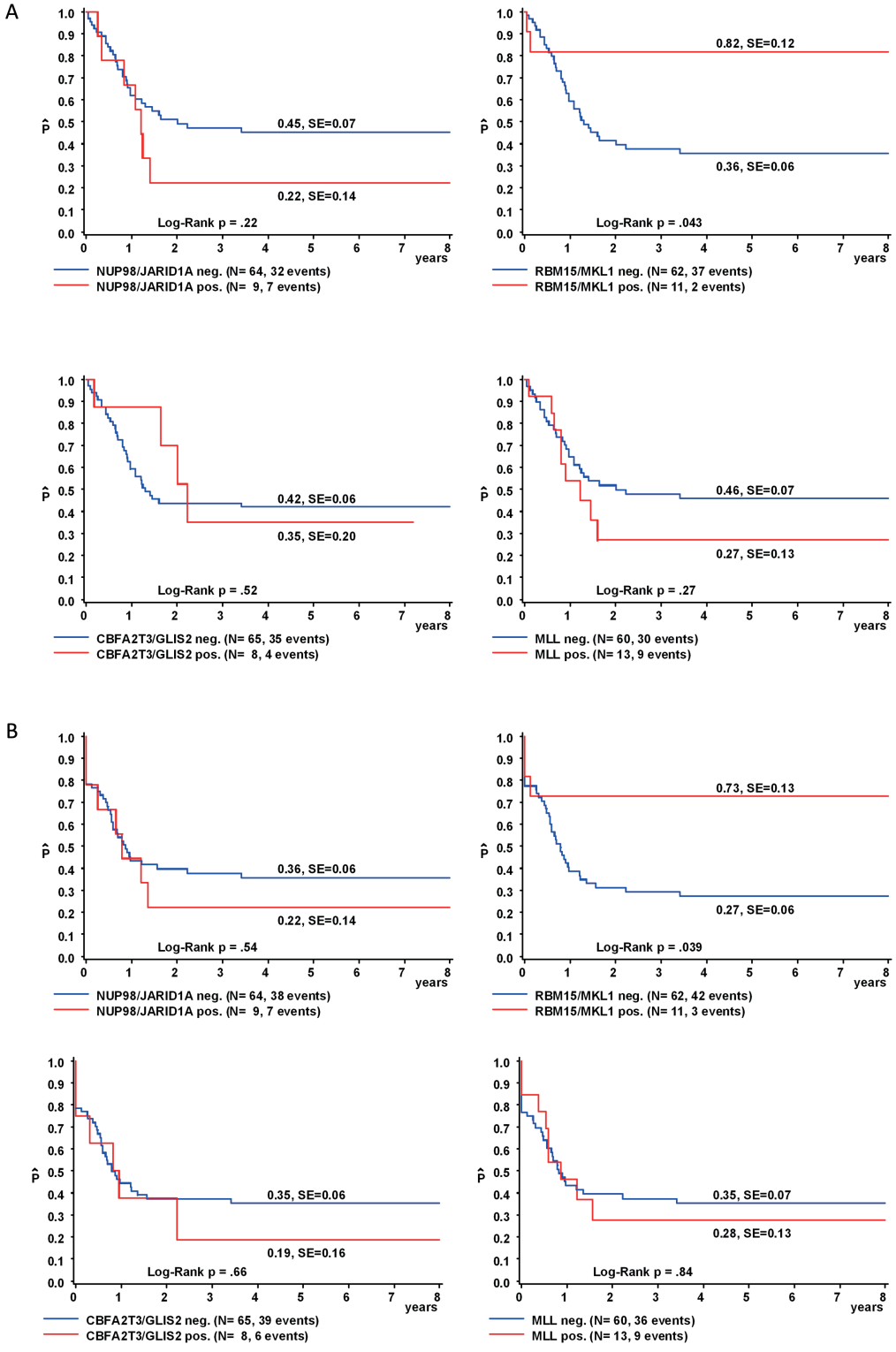
Principal component analysis based on *HOXA* and *-B* gene expression in the 274 patients with available gene expression profiles, as we reported previously, showed that AML can be subdivided in three clusters based on *HOXA* and *HOXB* expression.[9] All four *NUP98/JARID1A* fused patients of whom GEP data were available were categorized in the cluster with overexpression of both *HOXA* and *HOXB* genes, together with the *NUP98/NSD1* rearranged cases, *DEK/NUP214* rearranged cases and the *NPM1*-gene mutated cases (Figure 2.3a).
Strong correlations (r=0.6192-0.9184, p<0.05) between GEP data and RT-qPCR were found for *HOXA5, -A7, -A9, -A10, -B2, -B4, -B5,* and *–B6* (n=40, selection of well-characterized representative pediatric AML cases, Supplementary Figure 2.1).

We further studied *HOXA* and *–B* expression with RT-qPCR in 36 AMKL cases, of which 6 were positive for *NUP98/JARID1A*. The other 30 cases comprised of other *NUP98* rearrangements (n=4), *CBFA2T3/GLIS2* (n=7), *MLL*-rearrangements (n=4), *RBM15/MKL1* (n=1), *MLL-PTD* (n=1), t(7;12) (n=1) and 12 cases with unidentified type-II aberration. Significantly higher expression levels of *HOXA5, -A9, -A10,* and *HOXB2,-B3, -B4, -B5,* and *–B6* were found in the *NUP98/JARID1A* fused patients in comparison to the other pediatric AMKL patients (p<0.01) (Figure 2.3b). Of interest, *CBFA2T3/GLIS2* and *RBM15/MKL1* translocated patients did not have aberrant *HOX* gene expression. In the *MLL*-rearranged patients, as expected, we could confirm higher *HOXA5, -A7, -A9, and –A10* expression, but not *HOXB* over expression, in comparison to other AMKL patients.

Prognostic relevance of NUP98/JARID1A and other genetic subgroups in AMKL

Follow-up data for survival analysis was available in 73 cases. Follow-up data was not available for the AAML00531 study of the COG. Patient characteristics did not differ between the total cohort (n=105) and the cases used for prognostic markers (n=73), and was comparable to other studies earlier described. Overall 5-year pOS in pediatric AMKL was 42±6%, and pEFS was 34±6%. The pOS, pEFS and pCIR of the included cohort did not show significant differences compared to the pediatric non-DS AMKL patients included in the BFM93/98 protocols (p=0.56, p=0.44 and p=0.93 respectively).

The CR rate of *NUP98/JARID1A* positive cases was 78%, similar to the negative cases (78%). The 5-year pEFS was 22±14% in *NUP98/JARID1A* positive cases (n=9), and 36±6% in the negative cases (p=0.54). Although the 5-year pOS of *NUP98/JARID1A* positive cases was 22±14% compared to 45±7% for *NUP98/JARID1A* negative


A
0.45, SE=0.07
0.22, SE=0.14
Log-Rank p = .22
NUP98/JARID1A neg. (N= 64, 32 events)
NUP98/JARID1A pos. (N= 9, 7 events)
0.82, SE=0.12
0.36, SE=0.06
Log-Rank p = .043
RBM15/MKL1 neg. (N= 62, 37 events)
RBM15/MKL1 pos. (N= 11, 2 events)
0.42, SE=0.06
0.35, SE=0.20
Log-Rank p = .52
CBFA2T3/GLIS2 neg. (N= 65, 35 events)
CBFA2T3/GLIS2 pos. (N= 8, 4 events)
0.46, SE=0.07
0.27, SE=0.13
Log-Rank p = .27
MLL neg. (N= 60, 30 events)
MLL pos. (N= 13, 9 events)
B
0.36, SE=0.06
0.22, SE=0.14
Log-Rank p = .54
NUP98/JARID1A neg. (N= 64, 38 events)
NUP98/JARID1A pos. (N= 9, 7 events)
0.73, SE=0.13
0.27, SE=0.06
Log-Rank p = .039
RBM15/MKL1 neg. (N= 62, 42 events)
RBM15/MKL1 pos. (N= 11, 3 events)
0.35, SE=0.06
0.19, SE=0.16
Log-Rank p = .66
CBFA2T3/GLIS2 neg. (N= 65, 39 events)
CBFA2T3/GLIS2 pos. (N= 8, 6 events)
0.35, SE=0.07
0.28, SE=0.13
Log-Rank p = .84
MLL neg. (N= 60, 36 events)
MLL pos. (N= 13, 9 events)
years

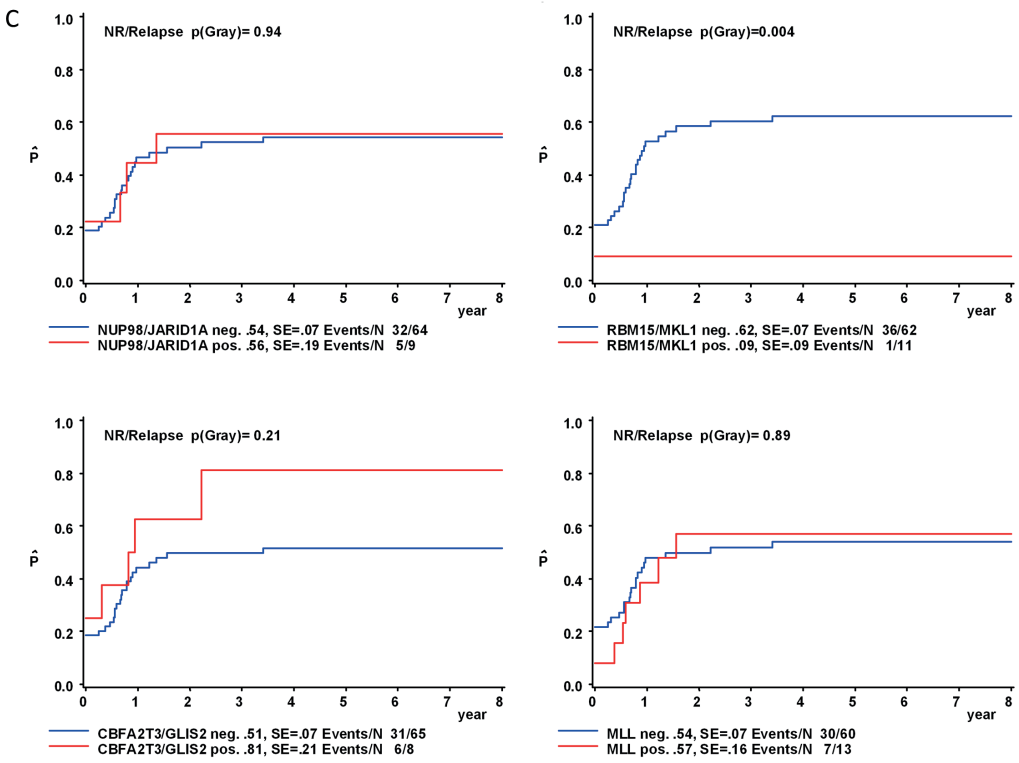


Figure 2.4. Survival estimates of specific type-II aberrations compared to other AMKL. (A) Kaplan-Meier estimates of the 5-year overall survival in specific type-II aberrations compared to other AMKL. *RBM15/MKL1* fusion has a significantly higher pOS compared to other AMKL. (B) Kaplan-Meier estimates of the 5-year event-free survival in specific type-II aberrations compared to other AMKL. Significantly higher pEFS is seen in *RBM15/MKL1* positive cases versus other AMKL. (C) Cumulative incidence of relapse according to the Kalbfleisch and Prentice method in specific type-II aberrations compared to other AMKL. Significantly lower relapse rate is seen in *RBM15/MKL1* positive patients as pCIR was also significantly better in this subgroup versus other AMKL.

cases, this difference was not statistically significant (p=0.22). In addition, there was no significant difference in pCIR; 56±19% in *NUP98/JARID1A* positive versus 54±7% in *NUP98/JARID1A* negative cases (p=0.94, Figure 2.4). Out of the 9 patients positive for *NUP98/JARID1A*, 2 were non-responders, 3 died after relapse and 2 patients died in complete remission due to treatment-related mortality. Cases with a positive split signal FISH on *NUP98* or other *NUP98*-aberration did not differ significantly from other cases with respect to pOS, pEFS and pCIR.

In *CBFA2T3/GLIS2* positive patients (n=8), the 5-year pEFS, pOS and pCIR did not differ significantly from all other AMKL cases; pEFS was 19±16% in positive cases and 35±6% in negative cases (p=0.66), 5-year pOS was 35±20% in positive cases compared to 42±6% in negative cases (p=0.52), pCIR was 81±21% versus 51±7% at 5-years respectively (p=0.21, Figure 2.4).

The 5-year pOS was significantly better for *RBM15/MKL1* positive patients (n=11, 82±12% versus 36±6% in negative cases, p=0.043), and 5-year pEFS was also significantly better with 73±13% in the positive cases versus 27±6% in other AMKL

(p=0.039). This was mainly caused by a lower relapse rate as pCIR was also significantly lower in this group (9±9% versus 62±7%, p=0.004, Figure 2.4).

As cases with *RBM15/MKL1* are associated with younger age, we performed multivariate analysis for *RBM15/MKL1* including age at diagnosis and white blood cell count as co-variable. We found that *RBM15/MKL1* was not an independent prognostic factor for pOS (HR 0.31 [95%CI 0.07-1.36], p=0.12) or pEFS (HR 0.40 [0.12-1.37], p=0.15), nor was age for pOS (HR 0.56 [0.24-1.32], p=0.19); and pEFS (HR 0.65 [0.30-1.41], p=0.28).

Discussion

In this study, we identified fusion of *NUP98* located on chromosome 11p15 to *JARID1A* located on chromosome 12p13 as a novel recurrent event in approximately 11% of patients with pediatric AMKL. The *NUP98/JARID1A* fusion is cryptic as *NUP98* is located 3Mb from the 11p telomere and *JARID1A* is located at the telomeric end of 12p13, and therefore undetectable with conventional karyotyping. This fusion gene was mutually exclusive with other type-II aberrations. This provides further evidence for the role of *NUP98* abnormalities in pediatric AML, in addition to the 16% of pediatric CN-AML cases which harbour a *NUP98/NSD1* fusion.[9]

The frequency of the *NUP98/JARID1A* fusion in AMKL is comparable to the *RBM15/MKL1* fusion (14% in our series), which is a well-known aberration in AMKL, and also comparable to the frequency of the recently described *CBFA2T3/GLIS2* fusion.[16] In our series the *CBFA2T3/GLIS2* fusion was detected in 12% of the AMKL cases, which is lower than reported by Gruber *et al* (27%) and Thiollier *et al* (31%).[15, 16] The latter studies included less patients (n=35 and n=22 respectively) and extensive information about selection bias concerning age at diagnosis in these studies is not available.

Altogether, with the identification of *NUP98/JARID1A* specific non-random type-II abnormalities can now be defined in roughly 60% of pediatric AMKL cases. *NUP98* breaks were seen in 3% of the cases, but the underlying aberration could not be identified.

Interestingly, the number of patients with a type-I aberration was very low (7%, n=45), as previously reported by Hama *et al*.[25] According to the Gilliland hypothesis, collaborating type-I and -II aberrations are needed in order to develop full-blown leukemia.[6] Recent evidence suggests that different types of *NUP98* fusions are frequently associated with *FLT3/ITD* (56-91%) and *WT1* (36-50%).[9, 26, 27] However, all AMKL patients were negative for *WT1* and *FLT3/ITD* mutations, including the *NUP98*-rearranged cases. Therefore, the collaborating type-I events in AMKL remain to be elucidated.

Recently, we described *NUP98/NSD1* as an independent variable for dismal outcome in pediatric AML, due to a high frequency of relapsed/refractory disease.[9] Although outcome of the *NUP98/JARID1A* cases was poor, there was no significant difference compared with other AMKL cases. However, outcome in AMKL patients in general was poor with a 5-year survival rate of only 41%, and a high incidence of refractory disease or relapse despite modern intensive therapy. Exceptions were patients with the *RBM15/MKL1* fusion, which had a clearly better outcome with approximately 80% overall survival, which was due to a low relapse rate. However, this fusion transcript could not be confirmed as an independent prognostic factor, probably due to the number of patients.

By unravelling the driving oncogenic events, the potential for targeted therapy needs to be taken into account which may ultimately increase survival rates. A prime example of the potential for targeted therapy in *HOX*-upregulated AML is the activity of DOT1L inhibitors in *MLL*-rearranged AML in vitro and in mouse-models.[28] Similar to *NUP98/NSD1* positive patients, *NPM1* mutated patients and in patients with a *DEK/NUP214* fusion, *NUP98/JARID1A* is associated with higher expression of several *HOXA* and *–B* genes, suggesting a common mechanism of leukemogenesis in these cases. This expression pattern is distinct from *MLL*-rearranged cases, which are characterized by overexpression of *HOXA* genes only. The leukemogenic role of activation of HOXB genes is however unknown to date.

NUP98, like *MLL*, has many different partner genes.[10] Almost all fusion genes contain the *NUP98* promoter and nearly all the *NUP98* FG/GLFG repeats. These GLFG-repeats result in recruitment of the co-activator CREBBP/p300, causing histone acetylation, resulting in transcriptional activation of different target genes including several *HOX*-genes as shown by he principal component analysis (Figure 2.2).[6, 29, 30] This may suggest that specific histone acetyltransferase inhibitors may be potential candidate drugs in *NUP98*-rearranged leukemias.

JARID1A contains three plant homeodomain (PHD) fingers of which the C-terminal PHD finger is fused to *NUP98* in the *NUP98/JARID1A* fusion. This is essential for leukemogenic transformation as demonstrated by Wang *et al*.[31] This PHD finger recognizes various histone 3 lysin 4 methylation (H3K4me) states.[32] Fusion of *NUP98* to this PHD finger of *JARID1A* leads to methylation of H3K4, which results in sustained upregulation of *HOX*-genes and other transcription factors, maintaining a stem-cell phenotype.[31, 33] This mechanism might function as second druggable target in *NUP98/JARID1A* positive patients. In absence of available cell-lines we are currently generating a model to study this further.

As AMKL is rare and frequently associated with myelofibrosis, it is often difficult to obtain sufficient material for additional laboratory studies. Therefore we had to obtain samples from various regions of Europe and from North America. A potential drawback of that approach is that patients were treated on different protocols over a longer period of time. However, all protocols consisted of intensive chemotherapy using an anthracycline and cytarabine backbone, including stem-cell transplantation in selected cases. Moreover, when comparing the clinical characteristics and outcome data of the patients presented in this paper versus a population-based cohort of the AML-BFM Study Group no significant differences were found in those parameters, suggesting that our cohort was representative for pediatric AMKL.

In conclusion, we report *NUP98/JARID1A* as a novel recurrent cryptic translocation which appears to be specific for pediatric AMKL. *NUP98/JARID1A* is associated with a high expression of *HOXA* and *HOXB* genes, indicative of a stem-cell phenotype, similar to what we reported for *NUP98/NSD1.* With exception of the *RBM15/MKL1* translocated patients, non-Down-Syndrome AMKL in general has a poor outcome, including the *NUP98/JARID1A* cases.

Taken together, with the identification of the *NUP98/JARID1A* fusion, a type-II aberration can now we found in approximately 60% of pediatric AMKL cases.

Table 2.1a. Patient characteristics of different subgroups identified in pediatric AMKL.

	NUP98-JARID1A positive	*RBM15/MKL1* positive	*CBFA2T3/GLIS2* positive	*MLL*-rearranged	Other
Total, n	11	16	13	13	52
Age, y					
Median	1.8	0.5 *	1.4	1.8	1.6
Range	0.9-4.8	0.1-2.7	0.6-3.4	0.7-12.0	0.1-15.1
Sex, %					
Female	55	67	77	20	48
Male	45	33	23	80	52
WBC, x10^9/L					
Median	11.8	15.6	19.1	11.2	14.8
Range	5.9-20.1	5.6-32.7	9.5-300.1	1.1-31.0	1.1-378.5
Karyotype, n (%)					
MLL	-	-	-	9 (90)	-
t(7;12)	-	-	-	-	1 (1.9)
CN-AML	-	2 (13.3)	1 (7.7)	-	5 (9.6)
Other	10	12 (80)	8 (61.5)	-	37 (71.2)
Unknown	1	1 (6.7)	4 (30.8)	1 (10)	9 (17.3)
Mutations, n (%)					
RAS	-	-	-	1 (7.7)	2 (3.8)
CEBPA	-	-	-	-	-
MLL-PTD	-	-	-	-	-
FLT3-ITD	-	-	-	-	-
FLT3-TKD	-	-	-	-	-
KIT	-	-	-	-	-
WT1	-	-	-	-	-
PTPN11	-	-	-	-	-
NPM1	-	-	-	-	-

* Significant younger age at diagnosis in *RBM15/MKL1* positive patients versus others ($p<0.01$).
Abrreviations: n indicates number of cases; y, years; WBC, white blood cell count.

Table 2.1b. Individual characteristics of the *NUP98/JARID1A*-positive (n=12) pediatric AML cases, including one FAB M5 case.

ID	Age (y)	Sex	WBC ($x10^9$/L)	FAB	Mutations*	Relapse	Death	Karyotype
1	1,3	M	8,4	M7	None	No	No	46,XYder(1)t(1;13)(p36;q1?4),add(11)(p15), der(13)t(1;13)(p36;q1?2),der(21)t(11;21)(p15;p13)
2	1,8	M	11,5	M7	None	No	Yes	44~51,XY,i(3)(q10)[15],+6[18],+8[17],-13,add(14)(q3?),-15[4],-16[3],add(17)(p1?)[18],+21,+mar1,+2~7mar[6][cp20]/46,XY[3]
3	1,4	F	19,0	M7	None	Yes	Yes	46,XX,del(11)(q13),der(12)t(11;12)(q13;p12),del(13)(?q13q22),tas(19;15;21)(q13;q26p13;p12)[17]/46,XX[3]
4	1,4	F	14,9	M7	None	No	No	46,XX,t(8;13)(q22;q1?4)[8]/48,idem,+6,+mar[4]/46,XX[8]
5	4,9	F	NA	M7	None	Yes	No	45,XX,-9,del(9)(p21),del(13)(q14q31),der(16)?t(9;16)(q34;q24)[15]/46,XX[3]
6	1,7	F	NA	M7	None	No	No	46,X,add(X)(q2?3),del(2)(q3?4),del(4)(q2?3),inc[7]/47,idem,+mar[4]/46,XX[9]
7	1,5	M	11,7	M7	None	No	Yes	46,XY,del(12)(p11p12)[3]
8	1,8	F	9,7	M7	NA	No	Yes	49,XX,add(3)(q21),t(3;12;7;4;13)(q25;q13;q22;q31;q22)or der(3)t(3;13)(q25;q22),+6,+8,+21[22]
9	3,2	M	5,9	M7	NA	NA	NA	47,XY,-13,add(17)(q21),+21,+21[1]/ 47,idem,t(3;18)(p21;q23)[6]/48,idem,+6,-21,+22[cp4]/46,XY[9]
10	3,3	F	13,1	M7	NA	NA	NA	49,XX,+3,+6,del(13)(q12q14),+21[11]/46,XX[9]
11	1,6	F	9,7	M7	NA	Yes	Yes	46,XX,dup(21)(q22q21)[5]/47,idem,+r[9]/46,XX[3]
12	5,9	M	156	M5	kRAS	Yes	Yes	50,XY,+X,+6,+7,+19[13]/46,XY[2]

* Mutations screened are hotspot areas in *NRAS, KRAS, KIT, WT1, CEBPA, FLT3,* and *PTPN11.* See materials and methods section for more details. Abbreviations: y indicates years; Sex M, male; Sex F, female; WBC, white blood cell count; FAB, French-American-British classification; NA, not available.

Table 2.1c. Individual characteristics of the *CBFA2T3/GLIS2*-positive (n=13) pediatric AMKL cases.

ID	Age (y)	Sex	WBC ($x10^9$/L)	FAB	Mutations*	Relapse	Death	Karyotype
13	1.4	F	12.4	M7	None	Yes	Yes	NA
14	0.6	F	10.5	M7	None	No	Yes	47,XX,+3[2]/46,XX[28]
15	1.4	M	19.1	M7	None	No	No	46,der(X)t(X;Y)(p2?3;q12)(WCPY+;WCPX+),?der(Y)t(Y;?)(q12;?)(WCPY+;?WCPY-)[10].nuc ish (MLLx2),(EVI1x2)[200]
16	1,2	M	155.5	M7	NA	No	Yes	NA
17	2.6	M	9.5	M7	NA	Yes	No	NA
18	3.1	F	21.9	M7	NA	Yes	Yes	NA
19	3.3	F	19.6	M7	NA	No	No	47,XX,+3[7]/46,XX[13]
20	0.8	F	300.1	M7	NA	NA	NA	46,XX,t(3;16)(p11.2;q24)[15]/46,idem,i(21)(q10)[4]/46,XX[1]
21	1.1	F	18.6	M7	NA	NA	NA	46,XX,add(22)(q13.3)[18]/46,XX[2]
22	2.1	F	31.1	M7	NA	NA	NA	46,XX,+21[15]/46,XX[5]
23	1.7	F	24.6	M7	NA	NA	NA	47,XX,+3[2]/46,XX[24]
24	0.8	F	11.7	M7	NA	NA	NA	46,XX[20]
25	3.4	F	16.0	M7	NA	No	No	46,XX,del(11)(q21q23)[19]/46,XX[1]

* Mutations screened are *NRAS, KRAS, KIT, WT1, CEBPA, FLT3,* and *PTPN11*. See materials and methods section for more details. Abbreviations: y indicates years; Sex M, male; Sex F, female; WBC, white blood cell count; FAB, French-American-British classification; NA, not available.

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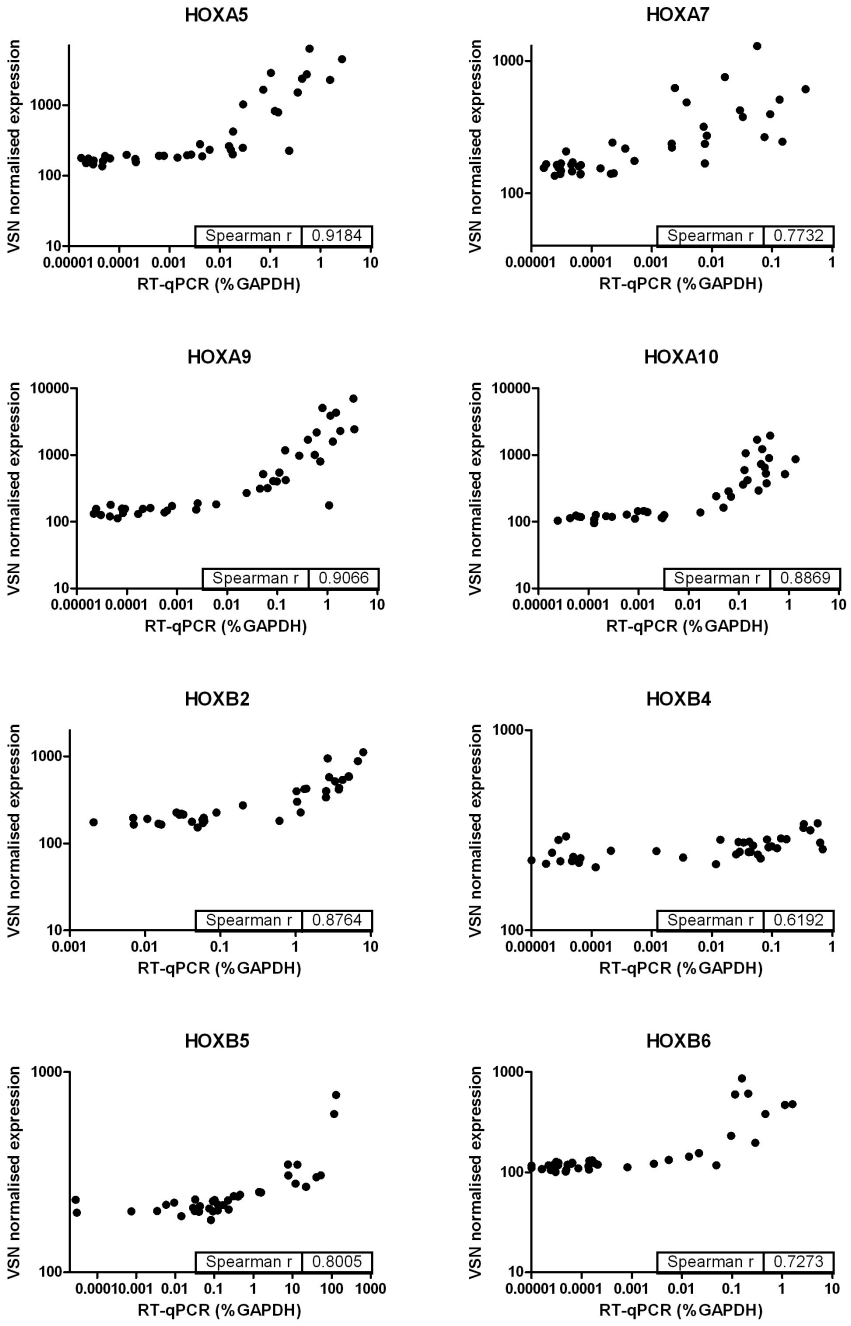
Supplementary data



Supplementary figure 2.1. Correlation of GEP-data and qRT-PCR in several *HOX* genes. Correlation between *HOXA5, -A7, -A9, -A10, -B2, -B4, -B5,* and *–B6* expression values as measured by Affymetrix Human Genome U133 Plus 2.0 Array and RT-qPCR, assessed with Spearman's rho correlation coefficient. Strong positive correlation was found for *HOXA5, -A7, -A9, -A10, -B2, -B4, -B5,* and *–B6*.

Supplementary table 2.1. Clinical and molecular characteristics of *NUP98/JARID1A* positive versus –negative cases in pediatric AML (n=293).

	***NUP98-JARID1A* positive patients (n=4)**	***NUP98-JARID1A* negative patients (n=289)**	**p**
Age, y			0.008
Median	1.6	9.5	
Range	1.2-5.9	0.1-18	
Sex, %			0.637
Female	40	42.7	
Male	60	57.3	
WBC, x10^9/L			0.253
Median	14.9	42.3	
Range	8.4-155.9	1.1-483.0	
FAB type, n (%)			
M0	-	13 (4.5)	
M1	-	38 (13.1)	
M2	-	53 (18.3)	
M3	-	20 (6.9)	
M4	-	80 (27.7)	
M5	1 (25.0)	64 (22.1)	
M6	-	4 (1.4)	
M7	3 (75.0)	6 (2.1)	
Mixed lineage	-	2 (0.7)	
Missing	-	9 (3.1)	
Cytogenetic aberrations			
MLL	-	66 (22.8)	
AML-ETO	-	25 (8.7)	
CBFB-MYH11	-	35 (12.1)	
t(15;17)	-	18 (6.2)	
CN-AML	-	62 (21.5)	
Other	4 (100.0)	64 (22.1)	
Unknown	-	19 (6.6)	
Mutations, n (%)			
RAS	1 (25.0)	56 (19.4)	
CEBPA	-	21 (7.3)	
MLL-PTD	-	6 (2.1)	
FLT3-ITD	-	60 (20.8)	
FLT3-TKD	-	10 (3.5)	
KIT	-	20 (6.9)	
WT1	-	29 (10.0)	
PTPN11	-	4 (1.4)	
NPM1	-	21 (7.3)	

Abbreviations: n indicates number of cases, y, years; WBC, white blood cell count; FAB, French-American-British classification.

Supplementary table 2.2A. Primers and PCR conditions molecular characterization.

Gene	Region	Direction	Primer-sequence	PCR condition
NUP98 *JARID1A*	Exon 12 Exon 27	Forward Reverse	5'-TGGACAGGCATCTTTGTT-3' 5'TCAGCTCCTTTGATTTGTCT 3'	10' 95°C, 40 x (15" 95°C, 1'60°C), 10' 72°C
JARID1A *NUP98*	Exon 26 Exon 14	Forward Reverse	5'CACCCCAAGGAAACAAC3' 5'GCCCATCAAAGAGATGTG 3'	10' 95°C, 40 x (15" 95°C, 1'60°C), 10' 72°C
RBM15 *MKL1*	Exon 1 Exon 6-7	Forward Reverse	5' TTCCCACCTTGTGAGTTCT 3' 5' TCAGCCGAGGTCTCTTC 3'	10' 95°C, 40x (15" 95°C, 1' 60°C), 10' 72°C
CBFA2T3 *GLIS2*	Exon 11 Exon 5	Forward Reverse	5' AGCGCACGAGCTCAT 3' 5' GAGGGGCAGGAAGAACT 3'	10' 95°C, 40x (15" 95°C, 1' 60°C), 10' 72°C
NUP98 *TOP1*	Exon 12 Exon 10	Forward Reverse	5' TGGACAGGCATCTTTGTT 3' 5' GTCATGAAGCTGAGCCC 3'	10' 95°C, 35x (15" 95°C, 1' 60°C), 10' 72°C
NUP98 *DDX10*	Exon 12 Exon 7	Forward Reverse	5' GTTTTGGCACAAATACCAG 3' 5' TGCAGCTCACAGACTATGTAG 3'	10' 95°C, 35x (15" 95°C, 1' 60°C), 10' 72°C
KRAS	Exon 2	Forward Reverse	5' CGTCGATGGAGGAGTTT 3' 5' AACCCAAGGTACATTTCAGA 3'	10' 95°C, 40x (15" 95°C, 1' 60°C), 10' 72°C
NRAS	Exon 2	Forward Reverse	5' GGGGGTTGCTAGAAAACTA 3' 5' ATCCGACAAGTGAGAGACA 3'	10' 95°C, 40x (15" 95°C, 1' 60°C), 10' 72°C
	Exon 3	Forward Reverse	5' CCCAGGATTCTTACAGAAAA 3' 5' TCCCCATAAAGATTCAGAAC 3'	10' 95°C, 40x (15" 95°C, 1' 60°C), 10' 72°C
CEBPA	Whole gene	Forward Reverse Forward Reverse	5' CGCCATGCCGGGAGAACTCT 3' 5' CTTGGCTTCATCCTCCTCGC 3' 5' CGGCCGCTGGTGATCAAG 3' 5' CCAGGGCGGTCCCACAGC 3'	10' 95°C, 35x (15" 95°C, 1' 60°C, 1' 72°C), 10' 72°C 10' 95°C, touchdown 20 cycles (15" 95°C, 1' 70-60°C, 1' 72°C), 20x (15" 95°C, 1' 60°C, 1' 72°C), 10' 72°C
KIT	Exon 8	Forward Reverse	5' CCGCCTCCTTGTACCTT 3' 5' TTCAGCAAACAAAATTAATGTCTA 3'	10' 95°C, touchdown 20 cycles (15" 95°C, 1' 66-56°C, 1' 72°C), 14x (15" 95°C, 1' 56°C, 1' 72°C), 10' 72°C
	Exon 17	Forward Reverse	5' TCCTCCAACCTAATAGTGTATTC 3' 5' CATTCCGAAATCAAACAGTT 3'	10' 95°C, touchdown 20 cycles (15" 95°C, 1' 66-56°C, 1' 72°C), 14x (15" 95°C, 1' 56°C, 1' 72°C), 10' 72°C
MLL	PTD's	Forward Reverse	5' AGGAGAGAGTTTACCTGCTC 3' 5' GGAAGTCAAGCAAGCAGGTC 3'	10' 95°C, 40x (15" 95°C, 1' 60°C), 10' 72°C
WT1	Exon 7	Forward Reverse	5' CATGGGGATCTGGAAGCAGGTC 3' 5' TGGGTCCTTAGCAGTGTGAGA 3'	10' 95°C, 40x (15" 95°C, 1' 60°C), 10' 72°C
	Exon 9	Forward Reverse	5' TAGGGCCGAGGCTAGACC 3' 5' TTCCAATCCCTCTCATCACAAT 3'	10' 95°C, 40x (15" 95°C, 1' 60°C), 10' 72°C
FLT3	Exon 14,15	Forward Reverse	5' GCAATTTAGGTATGAAAGCCAGC 3' 5' CTTTCAGCATTTTGACGGCAACC 3'	10' 95°C, 40x (15" 95°C, 1' 60°C), 10' 72°C
	Exon 20	Forward Reverse	5' TCACCGGTACCTCCTACTG 3' 5' AAATGCACCACAGTGAGTG 3'	10' 95°C, 40x (15" 95°C, 1' 60°C), 10' 72°C
PTPN11	Exon 3	Forward Reverse	5' TTGGGTTTCTTTCAACACTT 3' 5' GCCTTTGGAGTCAGAGAGT 3'	10' 95°C, 40x (15" 95°C, 1' 60°C), 10' 72°C
	Exon 13	Forward Reverse	5' TGGCTCTGCAGTTTCTCT 3' 5' CATTCCGAAATCAAACAGTT 3'	10' 95°C, 40x (15" 95°C, 1' 60°C), 10' 72°C
NPM1	Exon 12	Forward Reverse	5' CTGGTGGTAGAATGAAAAATAGAT 3' 5' GGCAGGGACATTCTCATAG 3'	10' 95°C, 40x (15" 95°C, 1' 60°C), 10' 72°C

Abbreviations: PCR indicates polymerase chain reaction; PTD, partial tandem duplication.

Supplementary table 2.2B. Primers and probes RT-qPCR *HOX* genes.

Gene	Direction	Primer-sequence	PCR condition
HOXB2	Forward Reverse Probe	5' CCTCCCGGTCCTTTC 3' 5' TGCCCAATATTTTAGAAGAAG 3' 5' AAATCGAGCCTCTCCCACCC 3'	10' 95°C, 40x (15" 95°C, 1' 60°C)
HOXB3	Forward Reverse	5' TGCTGCCTCCATCTCA 3' 5' TGCCTGGGACAGTTTG 3'	10' 95°C, 40x (15" 95°C, 1' 61°C)
HOXB4	Forward Reverse Probe	5' AGGGCCTTCCTCTGAAA 3' 5' GAGGGGCACATTTTATTTC 3' 5' AGTTCTGGGAGCTGGGCACTACT 3'	10' 95°C, 40x (15" 95°C, 1' 60°C)
HOXB5	Forward Reverse	5' AAGGGATGGTATTGAGAAGAG 3' 5' CCGCAAAGACAGATTTCA 3'	10' 95°C, 40x (15" 95°C, 1' 60°C)
HOXB6	Forward Reverse	5' CCCGCTGAGACATTACC 3' 5' CCCAAAGGAGGAACTGTT 3'	10' 95°C, 40x (15" 95°C, 1' 60°C)

Abbreviations: PCR indicates polymerase chain reaction.

Supplementary table 2.3A. Individual characteristics of the *RBM15/MKL1*-positive (n=16) pediatric AMKL cases.

ID	Age (y)	Sex	WBC ($x10^9$/L)	FAB	Mutations*	Relapse	Death	Karyotype
26	0.2	M	NA	M7	None	No	Yes	46,XY,t(1;2;22;2)(p1?3;q2?1;q13;p2?3)[26]/46,XY[4]
27	0.3	M	NA	M7	None	No	No	46,XY,t(1;22)(p13;q13)[8]/46,XY,[12]
28	0.2	F	NA	M7	None	No	No	46,XX,t(1;22)(p13;q13)
29	0.6	M	6.9	M7	None	No	No	46,XY[10]
30	0.6	F	23.4	M7	None	No	Yes	46,XX [15] / 46,XX,susp.t(1;22)(p13;q13) - low mitoses quality [8]
31	2.7	F	5.9	M7	None	No	No	53,XX,t(1;22)(p13;q13),+der(1;22),+8,+19,+20,+21,+22,+mar[cp2]/46,XX[78]
32	1.3	F	18.9	M7	NA	No	No	54,XX,1,+2,+4,+6,+7,+10,+19,der(?)t(?:1)(?:q21)x2, der(?)t(?:1)(?:p13)[5]/46,XX[14]
33	0.2	F	22.4	M7	NA	No	No	46,XX,t(1;22)(p13;q13)[7]/46,XX[22]
34	0.4	F	12.3	M7	NA	No	No	46,XX,t(1;22)(q13;q13)[6]/46,XX[14]
35	0.1	F	32.7	M7	NA	NA	NA	46,XX,t(1;22)(p13;q13.1)[15]/46,XX[5]
36	0.8	F	13.2	M7	NA	NA	NA	NA
37	0.6	F	11.7	M7	NA	NA	NA	46,XX,t(1;22)(p13;q13)[7]/46,XX[12]
38	0.1	M	27.6	M7	NA	NA	NA	46,XY,t(1;22)(p13;q13)[7]/46,XY[13]
39	1.9	F	16.4	M7	NA	NA	NA	46,XX,t(1;22)(p13;q13)[2]/46,XX[19]
40	0.4	M	14.8	M7	NA	No	No	46,XY,t(1:22)(p13:q13)[16]/46,XY[4]
41	2.6	F	5.6	M7	NA	No	No	46,XX[20]

* Mutations screened are NRAS, KRAS, KIT, WT1, CEBPA, FLT3, and PTPN11. See materials and methods section for more details. Abbreviations: y indicates years; Sex M, male; Sex F, female; WBC, white blood cell count; FAB, French-American-British classification; NA, not available.

Supplementary table 2.3B. Individual characteristics of the 11q23-mutated (n=13) pediatric AMKL cases.

ID	Age	Sex	WBC ($x10^9$/L)	FAB	Mutations*	Relapse	Death	Karyotype	11q23 aberration
42	1.2	M	23.0	M7	None	Yes	Yes	46, XY, t(9;11)(p22;q23)	*MLL/AF9*
43	7.4	M	1.1	M7	None	No	Yes	46,XY,del(11)(q23),del(12)(p12)	*MLL/AF6*
44	1.8	M	16.3	M7	None	Yes	Yes	47, XY, t(9;11)(p22;q23), +19 [27]	*MLL/AF9*
45	0.7	F	68.4	M7	KRAS pQ61R	Yes	Yes	45,XX,-7 [24]	*MLL* partial tandem duplication
46	1.8	F	NA	M7	None	Yes	Yes	47,X,i(X)(q10),t(9;11)(p22;q23),+19[1]/53,idem,+2,+6,+13,+13,+20,+21[1]/52,X,-X,+6,+9,t(9;11)(p22;q23),+19,+20,+21,+21,+22[11]	*MLL/AF9*
47	4.1	M	NA	M7	None	No	No	48,XY,+6,?ins(10;11)(p12?;q13q23),+21[15]	*MLL/AF10*
48	1.0	F	NA	M7	NA	No	No	47,XX,+21/46,XX	*MLL* partial tandem duplication
49	2.1	M	50.4	M7	None	No	No	49,XY,+16,del(17)(p11),+19,+21,+21,-22 [5]/46,XY [22]	*MLL* partial tandem duplication
50	0.7	M	31.0	M7	None	Yes	Yes	NA	*MLL/AF9*
51	4.0	F	5.6	M7	NA	NA	NA	89,XXXX,-3,-5,+6,add(7)(q22),+8,t(11;17)(q23;q21), add(11)(q23)x2,-15,-16,-17[15]/46,XX[5]	*MLL/AF17*
52	1.4	M	6.6	M7	NA	NA	NA	50,XY,+6,t(9;11)(p22;q23),+19,+21,+22[3]/46,XY[16]	*MLL/AF9*
53	1.4	M	15.8	M7	NA	NA	Yes	NA	*MLL/AF9*
54	12.0	M	1.1	M7	NA	NA	No	46,XY,t(11:19)(q23:p13.3)[20]	*MLL/ENL*

* Mutations screened are NRAS, KRAS, KIT, WT1, CEBPA, FLT3, and PTPN11. See materials and methods section for more details. Abbreviations: y indicates years; Sex M, male; Sex F, female; WBC, white blood cell count; FAB, French-American-British classification; NA, not available.

Chapter 3

RB1 loss as a unique and universally required additional hit in *NUP98/KDM5A* rearranged acute megakaryoblastic leukemia

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Abstract

Pediatric acute megakaryoblastic leukemia (AMKL) in non-Down Syndrome patients is a rare subtype of pediatric acute myeloid leukemia (AML), characterized by poor outcome. The fusion *NUP98/KDM5A*, which is present in ~10% of these cases, is hypothesized to result in a differentiation arrest. KDM5A, formerly known as retinoblastoma binding protein2 (RBP2), forms complexes with pRB that are detected in response to signals that affect cell-cycle exit and induction of differentiation. While studying *IKZF1* alterations in pediatric AML using MLPA, we were triggered to study the role of *RB1*, located on chromosome 13, since this method identified *RB1* loss exclusively in three patients with *NUP98/KDM5A* translocated AMKL. We screened a selected cohort of pediatric AMKL cases (n=31), non-AMKL *NUP98/KDM5A* translocated cases (n=2), and pediatric AML cases with a karyotypic chromosome 13 abnormality (n=5) for *RB1* deletions and mutations. All 8 *NUP98/KDM5A* translocated AMKL cases exclusively showed a heterozygous *RB1* deletion. Aditionally, one of these cases also showed a mutation in *RB1*, resulting in a stopcodon.

To analyze the effect of *NUP98/KDM5A* and *RB1* deletions on differentiation and proliferation, we transduced the murine 32D cell-line with *NUP98/KDM5A*, and performed shRNA knockdown of *Rb1*. Expression of *NUP98/KDM5A* alone resulted in a partial differentiation arrest, accompanied by overexpression of *HOXA* and *HOXB* genes, and a partial G1 cell cycle arrest. Knockdown of *Rb1* reverted the arrest to normal cell cycle progression.

We conclude that *RB1* deletions cooperate with the *NUP98/KDM5A* translocation in AMKL leukemogenesis to offset the cell cycle arrest induced by the fusion gene.

Introduction

Pediatric acute megakaryoblastic leukemia (AMKL) in non-Down Syndrome patients is a rare subtype of pediatric acute myeloid leukemia (AML) with a poor prognosis.[1-5] AML arises as a result of collaborative genetic aberrations.[6, 7] Recently, several novel abnormalities have been described in pediatric AMKL, such as *CBFA2T3*/*GLIS2*, and *NUP98/KDM5A,* in addition to well-known aberrations such as *KMT2A*-rearrangements and t(1;22)(p13;q13). [1, 2, 4] *NUP98/KDM5A* occurs in ~10% of pediatric AMKL, and thus far no collaborative genetic aberrations have been identified.[1, 2, 4, 8]

KDM5A is a histone lysine specific demethylase, originally identified as a retinoblastoma 1 (RB1) binding protein and previously named Retinoblastoma Binding Protein 2 (RBP2), although its role in the RB1 pathway has not been fully understood.[9-11]. RB1 is a tumor suppressor gene localized on chromosome band 13q14. RB proteins (pRB) play an important role in regulating the cell cycle checkpoint from the G1 to S phase, by repressing E2F. The effects of RB1 inactivation are cell type specific, and described in a wide variety of cancers.[12-14] Depletion of KDM5A has been shown to result in a block of proliferation and expression of differentiation markers in Saos-2 cells, a human osteosarcoma cell line, and similar effects were seen with overexpression of Rb1.[15]

Triggered by our MLPA results, that showed RB1 loss in three patients with *NUP98/KDM5A* translocated AMKL while studying IKZF1 alterations in pediatric AML[16], we further explored the role of RB1 in the leukemogenesis of pediatric AMKL with *NUP98/KDM5A* rearrangements, by screening a large representative cohort of pediatric AML cases (n=259) for *RB1* deletions, and an additional cohort (n=38), selected for AMKL, *NUP98/KDM5A*-rearrangements and cases with chromosome 13 aberrations in the karyotype, for deletions and mutations of *RB1*. We showed that in pediatric AML *RB1* deletions were exclusively found in all included *NUP98/KDM5A* positive AMKL. In a cell line model, *NUP98/KDM5A* alone resulted in a differentiation arrest, accompanied by a partial G1 arrest. *NUP98/KDM5A* in combination with a *RB1* deletions did not result in a G1 arrest. We conclude that *RB1* deletions cooperate with the *NUP98/KDM5A* translocation in AMKL leukemogenesis to offset the cell cycle arrest induced by the fusion gene.

Material and Methods

Patient samples and clinical characteristics

Patient samples, clinical characteristics and follow-up data were kindly provided by the Dutch Childhood Oncology Group (DCOG, The Netherlands), the AML–Berliner-Frankfurt-Münster Study Group (Germany and Czech Republic), the Saint-Louis Hospital (Paris, France), and the St Jude Children's Research Hospital (Memphis, TN, USA). Institutional review board approval for these studies had been obtained in the participating centers. Morphological and cytogenetic classification were centrally reviewed by each study group. Data on frequently seen mutations was available from earlier studies.[1, 16] Leukemic cells were purified as previously described.[17] Blast percentages were assessed morphologically using cytospins stained with a May-Grünwald-Giemsa staining. Isolation of genomic DNA and total cellular RNA from the samples was performed using Trizol reagent. Gene expression profiles using the HGU133 Plus 2.0 microarray platform (Affymetrix, Santa Clara, CA, USA) were available from earlier studies.[18] Original data

are available in the Gene Expression Omnibus repository (http://www.ncbi.nlm.nih.gov/geo; accession GSE17855).

Multiplex ligant-dependent probe amplification

Multiplex ligation-dependent probe amplification (MLPA) was performed on 250ng DNA using the SALSA MLPA-kit p335-B1;ALL-IKZF1 for analysis of *RB1* exon 6, 14, 19, 24 and 26 and the p047-C1/D1;RB1 kit for analysis of all *RB1* exons (MRC Holland, Amsterdam, The Netherlands, data available on http://www.mlpa.com). The data were analyzed with GeneMarker (version 1.85; SoftGenetics, State College, PN, USA). The data were normalized to reference probes and control samples. A deletion was defined as a peak ratio below 0.75, an amplification was defined as a peak ratio above 1.25.[19]

Mutational analysis of RB1

Using reverse transcriptase polymerase chain reaction (RT-PCR) and direct-sequencing, the complete coding region of *RB1* (exon 1 through exon 27) was screened for mutations (Primers; Supplementary Table 3.1). Purified PCR products were directly sequenced on a PRISM 3100 genetic analyzer (Applied Biosystems) and analyzed using CLCWorkbench (Version 3.5.1; CLC Bio, Aarhus, Denmark). Reference genome used was ENST00000267163.

Rb1 knockdown experiments in an inducible NUP98/KDM5A translocated cell line model

To create a cell line model the Zeocin resistant and doxycycline inducible pTripZ vector was used (Open Biosystems (GE Healthcare Dharmacon), Eindhoven, The Netherlands). The full length fusion of *NUP98/KDM5A* was PCR amplified using cDNA of a positive patient, and cloned into this vector, resulting in pTripZ-*NUP98/KDM5A* ($^{pT\text{-}NK}$). As a leukemic fusion control full length *NUP98/NSD1* was PCR amplified from cDNA of a positive patient, and cloned into the vector, resulting in pTripZ-*NUP98/NSD1* ($^{pT\text{-}NN}$). As a negative control, a pTripZ vector without the fusion gene was used ($^{pT\text{-}Empty}$). Lentiviral particles were produced in HEK293T cells and transduction occurred during a 45 minute 1800 rpm spin-oculation of $5x10^6$ 32D cells (DSMZ, Braunschweig, Germany, authentication using DNA fingerprinting, last tested December 2015), an IL3 growth factor dependent cell line established from murine bone marrow cells, altered with overexpression of the human granulocyte colony stimulating factor receptor (kindly provided by M. Jongen-Lavrencic, department of Hematology, Erasmus MC, Rotterdam, the Netherlands; 32D cell line purchased from DSMZ, Braunschweig, Germany). 36 hours after transduction, cells were positively selected for Zeocin resistance, with 750ug/mL Gibco Zeocin (Life Technologies, Bleiswijk, the Netherlands).

pLKO.1 mission short-hairpin RNA vector TRCN0000218099 ($^{/sh99}$) against murine *Rb1* with G418 (Geneticin) selection marker was purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands). Using restriction enzymes for removal of the *Rb1* targeting sequence of the pLKO.1 mission vector, an empty control ($^{/shEmpty}$) was created. Virus production and transduction was performed as above in $5x10^6$ cells of $32D^{pT\text{-}NK}$, $32D^{pT\text{-}NN}$, $32DpT^{-Empty}$ and in 32D non-infected ($32D^{NI}$) cells. After 36h cells were positively selected for G418 resistance, using 500ug/mL G418 (Life Technologies, Bleiswijk, the Netherlands), and a non-infected shRNA control ($^{/NI}$) was included for all aforementioned conditions as negative controls.

All cell cultures were divided in two, of which in one condition the vector was induced by adding 1.0 ug/mL doxycycline hyclate (Sigma-Aldrich, Zwijndrecht, the Netherlands) on day -2, 0, 3, 5, 7 and 10.

RNA was extracted using a Rneasy minikit (Qiagen, Venlo, the Netherlands) according to manufacturer's protocol. Expression levels of *Rb1*, the fusion genes *NUP98/KDM5A* and *NUP98/NSD1,* and several *Hoxa* and *Hoxb* genes were analysed using a SybrGreen based quantitative real-time PCR, with murine *Gapdh* as reference gene (primers; Supplementary Table 3.1).

DNA content analysis to assess cell-cycle distribution was done using Vybrant DyeCycle Violet (Life Technologies, Bleiswijk, the Netherlands) according manufacturers' protocol. Experiments were performed in quintuplet and repeated two times.

Differentiation assay in an inducible NUP98/KDM5A translocated cell line model

Differentiation analyses were performed in duplicate by depletion of IL3 from the cell culture medium followed by the addition of 100ng/mL recombinant human granulocyte colony stimulating factor (rh G-CSF, ImmunoTools, Friesoythe, Germany) for 11 days. Differentiation towards the granulocyte lineage was assessed by flow cytometry using the anti-mouse Ly-6G (Gr-1) antibody (BD Biosciences, Breda, The Netherlands) in addition to morphologic analysis of May-Grunwald-Giemsa stained cytospins. Cell cultures were analysed on days 0, 3, 4, 6, 7, 10 and 11.

Statistical analyses

Statistical analyses were performed with IBM SPSS 21 (IBM, Armonk, NY, USA). To compare the gain of percentages of cells in different cell cycle phases, the Mann-Whitney test was used to compare two groups, and the Kruskal-Wallis test to compare three or more groups. Statistical significance was considered if p-values were below 0.05.

Results

Deletions of RB1 are exclusively found in NUP98/KDM5A rearranged pediatric AMKL

In total, samples of 259 pediatric *de novo* AML cases, including 8 (3%) AMKL cases, were screened with the MLPA p335-B1;IKZF1 kit as previously described.[16] The median age of children included in this cohort was 9.6 years (range 0.1-18.0 years). Five cases were positive for *NUP98/KDM5A,* 3 of which were AMKL cases, and 2 with FAB-M5 morphology. Of the 259 pediatric cases, 3 cases showed a heterozygous *RB1* deletion of exon 6, 14, 19, 24 and 26. All 3 *RB1* deleted cases were *NUP98/KDM5A* rearranged AMKL cases (Table 3.1A). The non-AMKL *NUP98/KDM5A* cases both carried an amplification of *IKZF1*, as a result of a trisomy of chromosome 7, as evident from the karyotype (Table 3.1B).

To confirm the association between *RB1* deletional events and *NUP98/KDM5A,* an additional cohort, specifically selected for AMKL, *NUP98/KDM5A*-rearrangements and cases with cytogenetic aberrations of chromosome 13, was collected. In total, 38 cases, of which 11 were included in the aforementioned cohort, were analyzed for mutational screening and MLPA for *RB1*, including 8 samples of *NUP98/KDM5A* positive AMKL, 23 pediatric non-DS AMKL cases lacking *NUP98/KDM5A* rearrangements, 2 non-AMKL *NUP98/KDM5A* positive cases, and 5 cases with a chromosome 13 abnormality

in the karyotype (Table 3.1B, Supplementary Table 3.2). To analyze the frequency of partial or complete *RB1* deletions in this cohort, we used the MLPA p047-C1/D1;RB1 kit, containing an unique probe in every exon of *RB1*.

This analysis revealed 8/38 carried a heterozygous deletion of *RB1* (Figure 3.1). Remarkably, all 8 *RB1*-deleted cases were present in *NUP98/KDM5A* rearranged AMKL (Table 3.1B, Supplementary Table 3.2). Of the *RB1* deleted cases, three had germline or remission DNA material available, all of which carried two copies of *RB1*, suggesting that the deletions in *RB1* are somatic. None of the other samples in this selected cohort showed a deletion of *RB1*.

In one patient harboring a heterozygous *RB1* deletion, a point mutation resulting in a stop-codon on the remaining allele was found with mutational screening. No other patients were identified that carried a *RB1* mutation, however 13 of the samples had low blast counts (<50%) after purifying the cells, therefore we cannot exclude false-negative results in some patients. Both non-AMKL *NUP98/KDM5A* positive cases showed a trisomy 7 and therefore presented with an *IKZF1* amplification based on MLPA; in addition one of these cases was identified with a mutation in the RAS-pathway (Table 3.1B).

Gene expression profiles show low RB1 and high KDM5A expression in NUP98/KDM5A positive AMKL cases.

Gene expression data was available from 254 pediatric AML cases from previous studies, including 4 *NUP98/KDM5A* positive cases, of which 3 were AMKL combined with an *RB1* deletion, and one FAB-M5 case with two copies of *RB1*.[1, 18] Despite small numbers, analysis suggested a lower expression level of *RB1* in the *RB1* deleted cases (n=3 vs n=255, p=0.038, Supplementary Figure 3.1A), and a higher *KDM5A* expression in the *NUP98/KDM5A* positive cases (n=4 vs n=254, p=0.031, Supplementary Figure 3.1B). Expression levels obtained with micro-array were validated with qPCR, and expression was strongly correlated for *RB1* as well as for *KMD5A* (r=0.745 and r=0.719 respectively, Supplementary Figure 3.1C and 3.1D). These results indicate that heterozygous deletion of *RB1* leads to lower *RB1* expression, and the presence of the *NUP98/KDM5A* fusion gene leads to higher expression of *KDM5A*.

32D cells transduced with NUP98/KDM5A and NUP98/NSD1 show high Hoxa and Hoxb gene expression

32D cells ($32D^{NI}$) were transduced with constructs carrying either *NUP98/KDM5A* (pT-NK), *NUP98/NSD1* (pT-NN), or empty vector control (pT-Empty) and samples were analyzed for expression of the fusion genes by qPCR at 0, 3, 5, 7, and 10 days after transduction. $32D^{pT\text{-}NK}$ and $32D^{pT\text{-}NN}$ cells demonstrated low level expression of their fusion genes in the absence of doxycycline, and high expression levels upon induction with doxycycline (Supplementary Figure 3.2).

No expression of the fusion genes was found when not introduced to the cell, regardless of induction with doxycyline. Expression of the fusion gene was stable during the experiment, with measurements over 10 consecutive days. Expression of NUP98/KDM5A and NUP98/NSD1 protein of the pTripZ-vector with *NUP98/KDM5A* and *NUP98/NSD1* respectively, was confirmed by western blotting and immunofluorescence in HEK293 cells (Supplementary Figure 3.3).

Patients with the *NUP98/KDM5A* and *NUP98/NSD1* translocation are characterized with high *HOXA* and *HOXB* expression.[1, 18] In the doxycycline induced 32D$^{pT\text{-}NK}$ and 32D$^{pT\text{-}NN}$ cells we found similar elevations in *Hoxa* and *Hoxb* expression, whereas 32D$^{pT\text{-}Empty}$ showed sustained low level expression of *Hox* genes after induction with doxycycline (Supplementary Figure 3.4).

To evaluate the effect of Rb1 depletion on Hox locus upregulation, an *Rb1* specific shRNA (sh99) or a control shRNA lentiviral construct (shEmpty) was introduced into the cell lines. Knockdown of *Rb1* was confirmed by qPCR and showed more than 70% reduction in *Rb1* expression in the cells after selection with G418 (Supplementary Figure 3.2B). *Hoxa* and *Hoxb* expression was found to be independent of *Rb1* knockdown, suggesting that *RB1* deletion is not required for *Hox* upregulation by the fusion gene (Supplementary Figure 3.4).

NUP98/KDM5A positive cells show a differentiation arrest

To assess the effects of NUP98/KDM5A and NUP98/NSD1 fusion proteins on differentiation, 32D$^{pT\text{-}NK}$, 32D$^{pT\text{-}NN}$ and 32D$^{pT\text{-}Empty}$ were exposed to human G-CSF. The 32D cells containing an empty vector differentiated over time as demonstrated by increasing Gr1 expression with greater than 65% positivity by day 11, whereas *NUP98* fusion positive cells lacked this granulocyte differentiation phenotype with less than 30% Gr1 positivity at the same time point (Figure 3.2) , indicative of a differentiation arrest. This confirmed the results previously described.[20-22]. Analyses of the cells with *Rb1* knockdown showed that this differentiation arrest persists, suggesting the differentiation arrest to be driven by the fusion gene and not dependent on Rb1 depletion. May-Grünwald-Giemsa staining of the cells for morphology was consistent with flow cytometry results with a paucity of mature granulocytes in fusion containing cells (Figure 3.2).

NUP98/KDM5A positive cells show a G0-G1 arrest dependent of Rb1 expression

To evaluate cell cycle progression in our model, the transduced cells and controls were analyzed with DyeCycle Violet staining. A significant increase in G0-G1 arrested cells was found in 32D$^{pT\text{-}NK}$, but not in 32D$^{pT\text{-}NN}$, 32D^{NI}, and 32D$^{pT\text{-}Empty}$ as measured by a gain in G0-G1% upon doxycycline induction (mean increase of cells in G0-G1 phase 15.8%, versus -2.2%, -1.6% and 0.5%, respectively, $p<0.0001$, Figure 3.3A). This suggests that *NUP98/KDM5A* induces a portion of cells to undergo cell cycle arrest that may limit its ability to transform cells in the absence of a cooperating mutation.

Rb1 has a known role in cell cycle progression, therefore we examined the ability of *Rb1* knockdown to relieve *NUP98-KDM5A* carrying cells of the observed G0-G1 arrest. *Rb1* knockdown did not alter the number of cells in G0-G1 phase in cells lacking the *NUP98/KDM5A* fusion gene (data not shown). In contrast, a significantly lower number of cells were in the G0-G1 phase was found in *Rb1* depleted *NUP98/KDM5A* cells compared to *Rb1* expressing cells (mean 8.4%, versus 15.8% in 32D$^{pT\text{-}NK/NI}$ and 14.9% in 32D$^{pT\text{-}NK/shEmpty}$, $p<0.005$, Figure 3.3B). We conclude that deletion of *Rb1* in *NUP98-KDM5A* positive cells relieves the cell cycle arrest induced by the fusion.

Discussion

We recently described *NUP98/KDM5A* as a recurrent fusion gene in pediatric non-DS AMKL. This translocation is not fully exclusive for this morphological subtype, but very

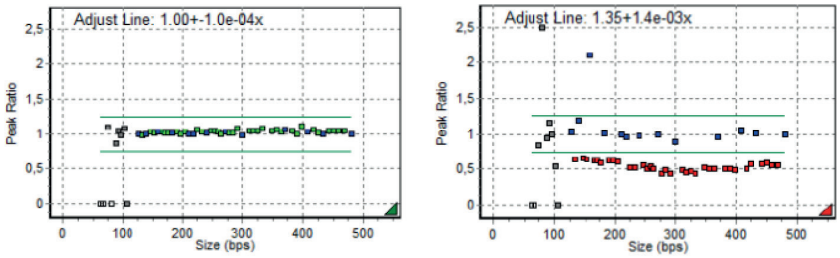

Figure 3.1. MLPA using the p047-RB1 kit shows a deletion of *RB1* in *NUP98/KDM5A* positive AMKL cells. Left panel shows a normal control, with a peak ratio of 0.75-1.25 of all RB1 probes included in the p047-RB1 kit indicated in green, and control probes in blue. Right panel shows a heterozygous deletion of the probes located in RB1 as indicated in red, and a normal peak ratio of the control probes, indicated in blue.

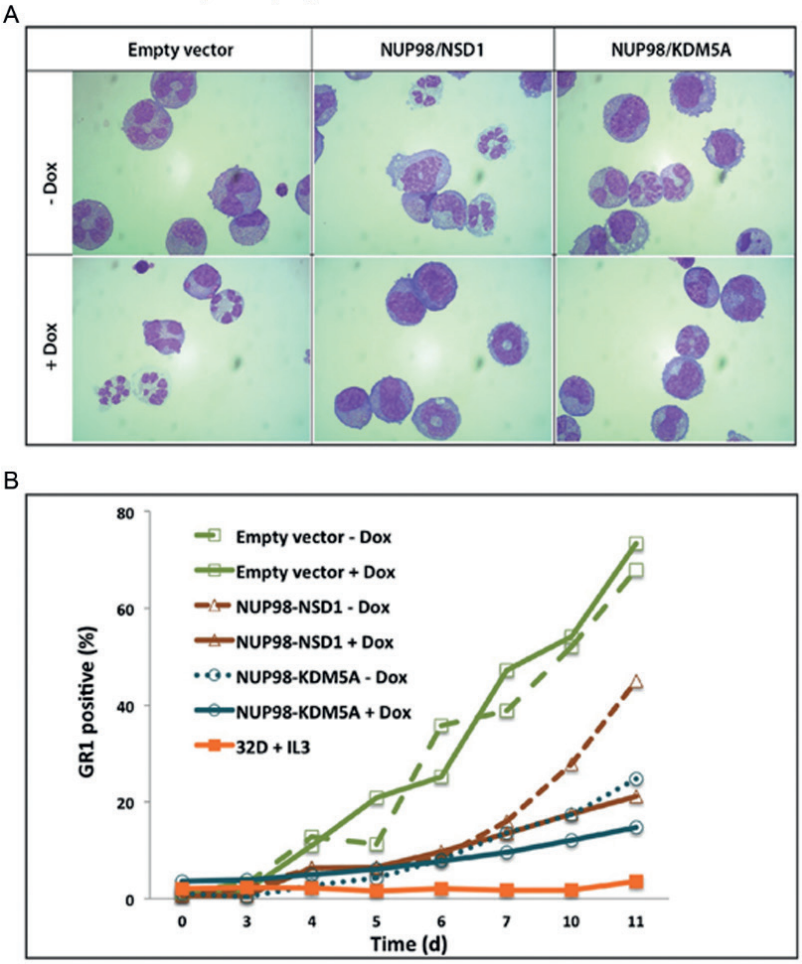

Figure 3.2. 32D cells expressing *NUP98/NSD1* and *NUP98/KDM5A* arrest differentiation of myeloid progenitors. Cells were untreated or treated with 1.0µg/mL of doxycycline. After depleting the cells from IL3, cells were induced for differentiation for 11 days with 100ng/ml of GCSF. (A) May-Grunewald-Giemsa staining of 32D cells expressing NUP98-NSD1, NUP98/KDM5A or control (empty vector). (B) Fluorescence-activated cell sorting analysis was carried out for GR1 positive cells.

rare in other morphologic subtypes of AML.[1] Myeloid leukemogenesis requires at least two different aberrations. [6, 7] RB1 is a well-known tumor suppressor gene which is linked to proliferation by its role in cell cycle checkpoints, but RB1 deletions have thus far not been reported in pediatric AML. Deletions of chromosomal band 13q14 have been detected by conventional karyotyping and array comparative genomic hybridization in pediatric AMKL, but the role of RB1 was not yet identified as a potential critical event in these cases.[1, 23] In this study, we found that *RB1* deletions exclusively occurred in all non-Down Syndrome AMKL cases with a *NUP98/KDM5A* rearrangement. Interestingly we did not find any *RB1* deletions in non-AMKL *NUP98/KDM5A* rearranged cases, nor in any other case of AML without *NUP98/KDM5A* rearrangement.

Non-random combinations of genetic aberrations are well-known in pediatric AML and are considered to collaborate in leukemogenesis.[1, 7, 24] For instance, the *NUP98/NSD1* translocation is often seen in combination with a *FLT3*-ITD, conferring a poor outcome.[18, 25, 26] The translocation *NUP98/NSD1* results in a differentiation arrest, but *FLT3*-ITD is required to induce leukemia in mouse models.[22] In the present study, we provide evidence that loss of RB1 offsets a *NUP98/KDM5A* induced cell cycle arrest, and the combination of the two aberrations results in both a differentiation arrest and proliferation advantage.

KDM5A was originally identified as a RB1-binding protein.[9, 27] The RB1 protein promotes senescence and differentiation, and these activities coincide with its ability to bind KDM5A.[11, 15, 28] Interestingly, Kdm5a deficient mouse embryo fibroblasts have a decreased proliferation rate, which could partially be rescued by depletion of Rb1.[11] These data demonstrate an intimate functional relationship between Kdm5a and Rb1. We have observed a decreased proliferation rate in *NUP98/KDM5A* expressing, early myeloid cells, accompanied with a G0-G1 arrest, which could be rescued by Rb1

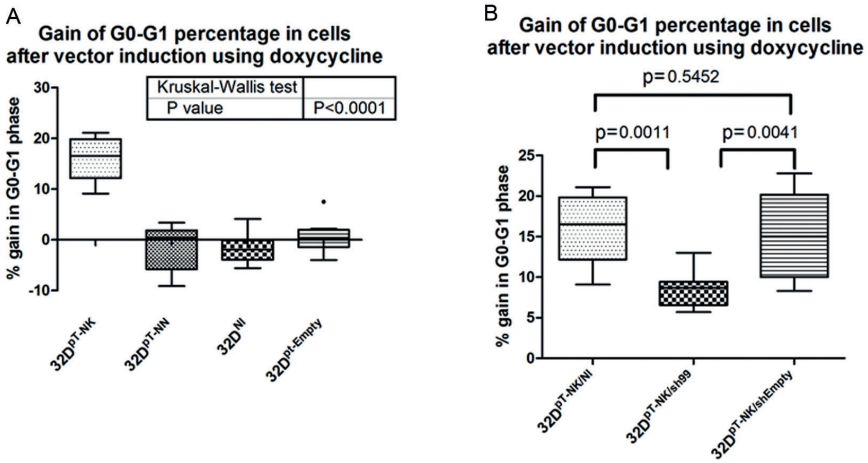

Figure 3.3. *NUP98/KDM5A* leads to a significant higher gain in G0-G1 percentage. *RB1* knockdown results in a significant decrease of G0-G1 percentage in *NUP98/KDM5A* positive cells. (A) Induction with doxycycline leads to a significantly higher gain in G0-G1 percentage in $32D^{pT\text{-}NK}$ compared to the other conditions ($p<0.0001$), indicating a G0-G1 arrest in *NUP98/KDM5A* expressing cells. The boxplot shows the values found in the experiment that was performed in quintuplet and, except for $32D^{pT\text{-}NN}$, repeated two times. (B) *Rb1* knockdown results in a significantly decrease of G0-G1 percentage in $32D^{pT\text{-}NK}$ ($p<0.01$). The boxplot shows the results of the experiment that was performed in quintuplet and repeated two times. Groups were compared using Mann Whitney-test.

depletion. This is consistent with the concept that the NUP98/KDM5A fusion protein constitutes a loss of KDM5A function in regulating RB1, leading to an anti-proliferative effect. While we show that the *NUP98/KDM5A* fusion also prevents differentiation through upregulation of *HOXA* and *HOXB* genes, creating a stem cell phenotype, this anti-proliferative effect may hinder the development of leukemia. We therefore hypothesize that loss of one RB1 allele compensates for this effect. We note that in the majority of cases the other RB1 allele was intact, contrary to the "classical" loss of RB1 function in other cancer types, which requires loss of both alleles.[29, 30] The differentiation arrest as seen in *NUP98/KDM5A* positive 32D cells suggests that the translocation of *NUP98/KDM5A* can indeed be classified as a type-2 aberration, as postulated by Kelly et al, and confirmed by Wang et al.[6, 20] The effect of the *Rb1* knockdown on proliferation, with a decreased percentage of cells in G0-G1 phase, suggests that the observed *Rb1* deletion in the *NUP98/KDM5A* positive AMKL cases cooperates to induce leukemia in *NUP98/KDM5A* positive AMKL by enhancing proliferation.

The pediatric AMKL cases with *NUP98/KDM5A* typically arise at very young age, but young age is a characteristic of most pediatric AMKL.[1-4, 8] In DS AMKL the somatic mutation in *GATA1* resulting in the leukemia is already present at birth, resulting in a transient myeloproliferative disease followed by spontaneous remission, and in approximately 20% followed by the development of AMKL.[31-34] Greaves *et al* showed that fusion genes can be detected in material derived from neonatal Guthrie cards, suggesting the presence of the fusion gene, although in small subclones, is present in utero.[35] In cases for which germline material was available, we confirmed the identified deletion of *RB1* in the leukemic cells to be somatic. Guthrie cards may show whether the most frequent identified somatic aberrations in non-DS AMKL, such as *NUP98/KDM5A*, might be present at birth as well, which later, in combination with a second hit, such as a *RB1* deletion, provides a proliferative advantage and the development of clinically overt leukemia.

In conclusion, we found heterozygous *RB1* deletions in pediatric AML were restricted to *NUP98/KDM5A* positive pediatric AMKL cases. We confirm that *NUP98/KDM5A* results in a differentiation arrest as has been previously shown, and *Rb1* knockdown in combination with this fusion results in a proliferation advantage. *RB1* deletions in *NUP98/KDM5A* positive cases are likely to be a compensatory aberration to the translocation, offsetting the anti-proliferative side effects of the differentiation inhibiting fusion gene.

Table 3.1A. Patient characteristics of the pediatric AML cohort (n=259).

		other pediatric AML cases (n=256)	*RB1* deleted cases (n=3)
Age at dx	Median (y)	9,6	1,4
	Range (y)	0,1-18,0	1,4-1,8
Sex	Female (%)	0,42	0,67
WBC	Median (x10^9/L)	47,9	14,9
	Range (x10^9/L)	1,2-438,0	11,5-19,0
FAB-type	M0	16	0
	M1	32	0
	M2	45	0
	M3	20	0
	M4	73	0
	M5	55	0
	M6	2	0
	M7	5	3
Type-2 Aberrations	*KMT2A*-rearrangement	62	0
	RUNX1/RUNX1T1	23	0
	inv(16)	33	0
	PML/RARA	18	0
	ETV6/MNX1	4	0
	DEK/NUP214	6	0
	KAT6A/CREBBP	6	0
	NUP98/NSD1	13	0
	NUP98/KDM5A	2	3
	*CEBPA*dm	13	0
	NPM1	17	0
	Other	3	0
	RBM15/MKL1	0	0
	CBFA2T3/GLIS2	0	0
	Unknown	56	0

Abbreviations: dx indicates diagnosis; WBC, white blood cell count; FAB, French-American-British classification; y, years.

Table 3.1B. Patient characteristics of the selected cohort for NUP98/KDM5A positive samples (n=10).

UPN	FAB	Result MLPA p047 RB1	Mutational screening of RB1	Age at dx (yrs)	Sex	Remission status	Event	SCT	Dead	COD	Karyotype sample	Result MLPA p330 ALL-IKZF1
1	M7	Deletion of RB1	wt	1,4	F	complete remission	no event	no	no		46,XX,t(8;13)(q22;q12)[8]/48,idem,+6,+mar[4]/46,XX[8]	Deletion of RB1
2	M7	Deletion of RB1	wt	1,3	M	complete remission	no event	no	no		46, XY, der(1)t(1;13)(p36;q12),add(11)(p15), der(13),t(1;13)(p36;q12), der(21)t(11;21(p15;p13)	NA
3	M7	Deletion of RB1	mutation	1,8	M	non-remitter	non-remitter	no	yes	leukemia	NA	Deletion of RB1
4	M7	Deletion of RB1	wt	8,5	M	complete remission	relapse	no	yes	leukemia	46,XY,del(12)(p11p12) [3]	NA
5	M7	Deletion of RB1	wt	1,8	M	NA	died	NA	yes	NA	46,XY,t(3;13)(p21;q14),t(7;12)(p13;p13),del(9)(p22),del(13)(q12q14),del(16)(p11.2[18]/46,XY[2]	NA
6	M7	Deletion of RB1	wt	2,7	M	NA	died	NA	yes	NA	46,XY,t(7;14)(q22;q32),-del(9)(p22),del(13)(q12q22)[19]/46,XY[1]	NA
7	M7	Deletion of RB1	wt	1,8	M	yes	no event	yes	no		46,XY,der(13)(q14),del(13)(q13q?)[5]/46,XY,?t(8;3)(p1?2;q1?4),del(13)(q14q?)[11]/46,XY[16]	NA
8	M7	Deletion of RB1	wt	4	M	yes	relapse	yes	yes	leukemia	46,XY,-13,der(17)t(13;17)(q14;q22) ,+21[18]46,XY[2]	NA
9	M5	no aberration	wt	5,9	M	complete remission	relapse	no	yes	NA	50,XY,+X,+6,+7,+19[13]/46,XY[2]	Amplification of IKZF1 and PAR1
10	M5	no aberration	wt	14,1	F	complete remission	relapse	no	yes	hemmor-rhage	49,XX,+6,+7,+8,?t(11;12)(p;p)[6]	Amplification of IKZF1

Abbreviations: UPN indicates unique patient number; FAB, French-American-British classification; wt, wildtype; dx, diagnosis; yrs, years; NA, not available; SCT, stem cell transplantation; COD, cause of death.

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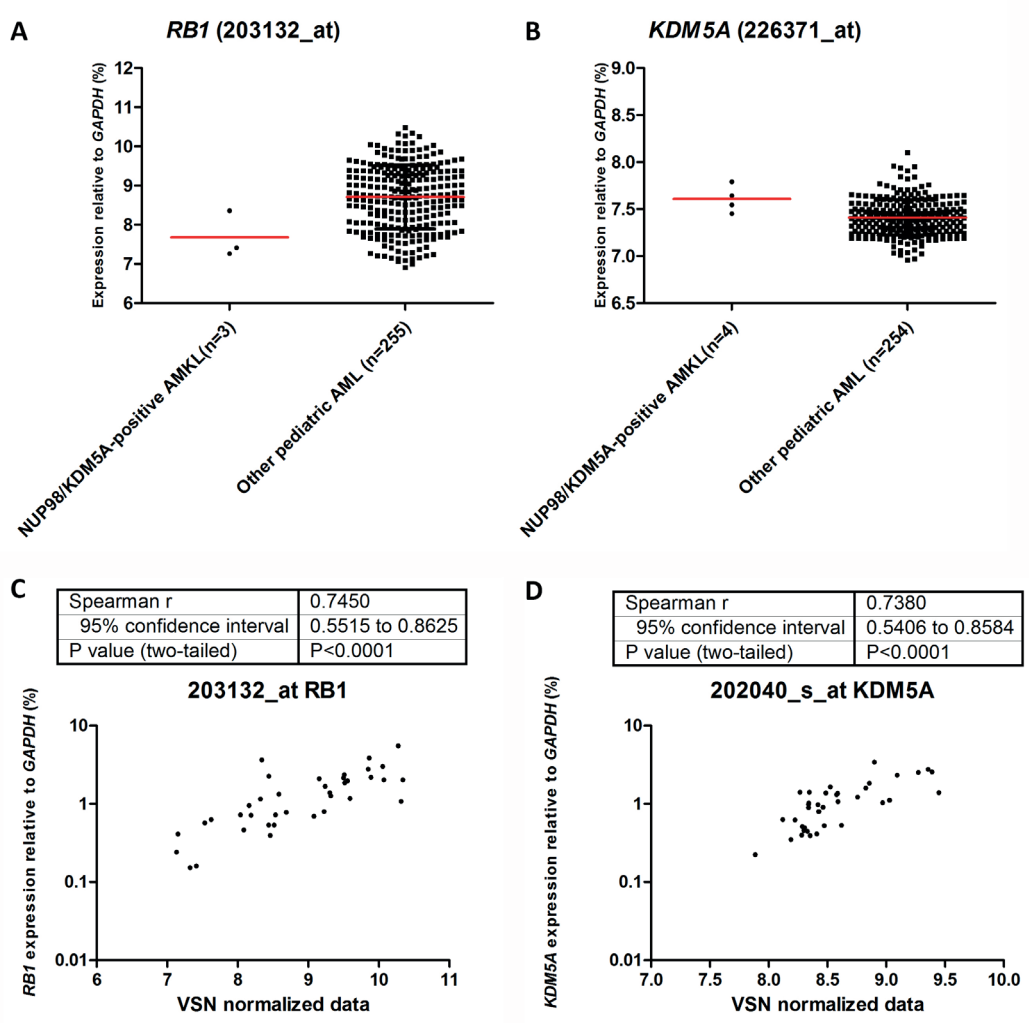
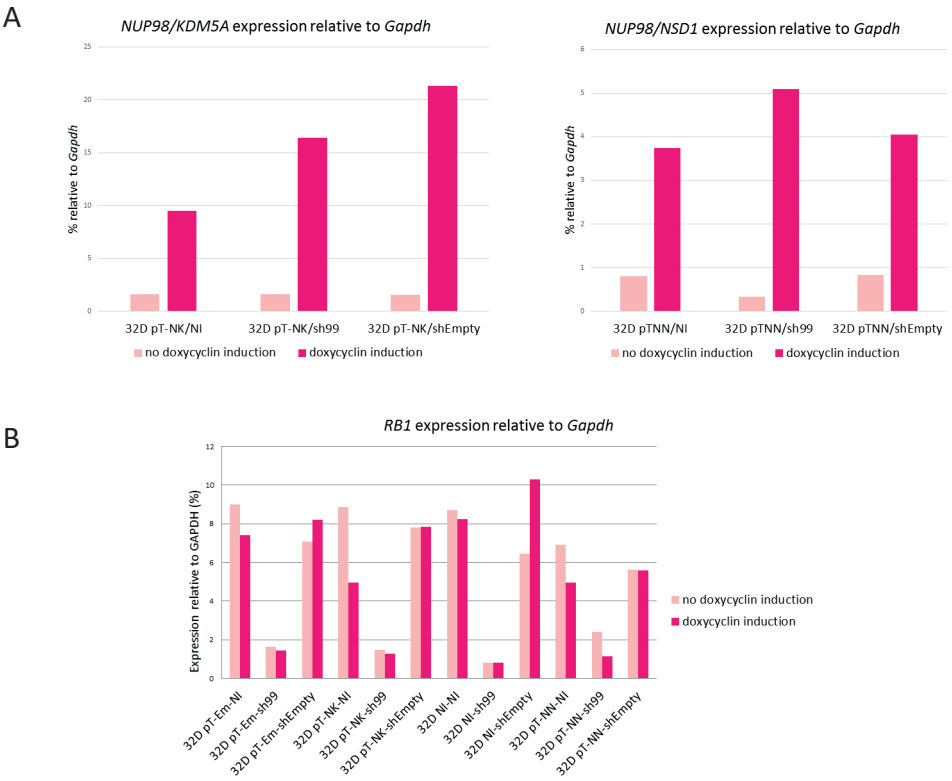
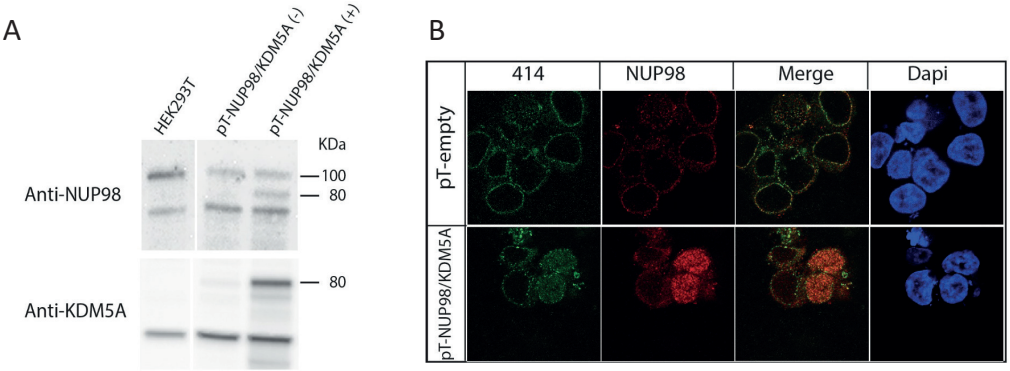
Supplementary data



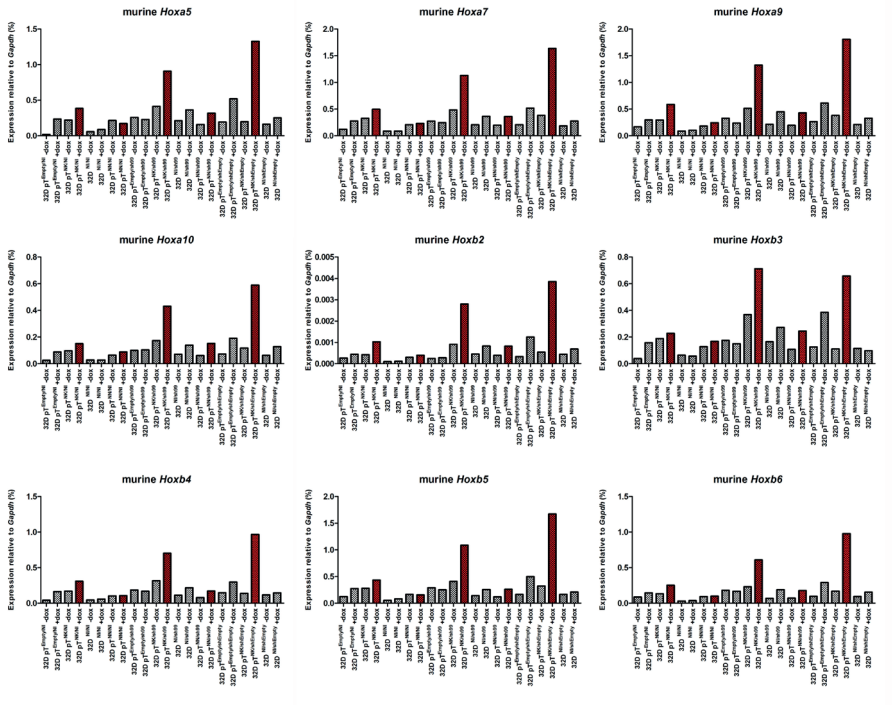
Supplementary Figure 3.1 (A) *RB1* expression seems lower in *RB1* deleted pediatric AML cases (n=3), compared to other pediatric AML (n=255; mean 7.68 compared to 8.69 of micro-array VSN normalized data, p=0.038). (B) *KDM5A* expression seems higher in *NUP98/KDM5A* positive pediatric AML (n=4) compared to other pediatric AML (n=254; mean 7.61 compared to 7.41 of micro-array VSN normalized data, p=0.031). (C) *RB1* expression as found with GEP validated with qPCR (n=38). *RB1* shows a strong correlation (r=0.745) comparing micro-array values with qPCR values. (D) *KDM5A* expression as found with GEP validated with qPCR (n=38). *KDM5A* shows a strong correlation (r=0.719) comparing micro-array values with qPCR values.



Supplementary Figure 3.2 (A) Left panel shows expression levels of *NUP98/KDM5A* and *NUP98/NSD1* measured with qPCR. Doxycycline induction gives a >9-fold higher expression of *NUP98/KDM5A* in the pTripZ-*NUP98/KDM5A* induced cells. Right panel shows expression levels of *NUP98/NSD1* measured with qPCR. Doxycycline induction gives a 4-fold higher expression of *NUP98/NSD1* in the pTripZ-*NUP98/NSD1* induced cells. (B) *Rb1* expression as measured with qPCR. Knockdown with sh99 results in >60% reduction of *Rb1* compared to cells without knockdown.



Supplementary Figure 3.3 (A) NUP98/KDM5A protein is expressed after induction with doxycycline. Western blot analysis of NUP98 and KDM5A in total lysates from HEK293T cells transfected with pT^{NK}, before and after induction with doxycycline (1ug/ml) for 24hours. Equal amounts of total protein was loaded. (B) NUP98/KDM5A localizes to nucleus after induction with doxycycline. The 414 antibody was used as a general nucleoporin staining antibody, and localization of NUP98 was performed after induction with doxycycline (1ug/ml) for 24 hours in HEK293T cells transfected with pT^{NK} or pT^{empty}.



Supplementary Figure 3.4. Increased *Hoxa* and *Hoxb* gene expression was found in *NUP98/KDM5A* transduced 32D cells. *NUP98/KDM5A* and *NUP98/NSD1* transduced 32D cells with doxycycline induction are indicated in the red bars.

Supplemental Table 3.1. Overview of primers.

Gene		Location	Direction	Sequence 5' > 3'	Amplification conditions
RB1	mutational screening	Exon 1	Forward	CTCCCCGGCGCTCCTCCACAGC	10'94, 40x(30"94, 1'60,30"68),10'68
			Reverse	GGGCGCCCTTCCCGCGTGAG	
RB1	mutational screening	Exon 2	Forward	**TGTAAAACGACGGCCAGT**AACAAGTATGTACTGAATCAATTTG	2' 94, 40X(1'94, 35"57,40"72), 4'72
			Reverse	**CAGGAAACAGCTATGACC**CTATCTTTCAATTTTTGTATAGTGA	
RB1	mutational screening	Exon 3	Forward	**TGTAAAACGACGGCCAGT**GACTGACCCCTAAAGTTTCCACA	10' 95, 40X(15"95, 1'60, 30"72), 10'72
			Reverse	**CAGGAAACAGCTATGACC**CGTTTCCTTTTATGGCAGAGGCTT	
RB1	mutational screening	Exon 4	Forward	ACAAATTTTTAAGGTTACTGATTTAC	2' 94, 40X(1'94, 35"52,40"72), 4'72
			Reverse	CCAGAATCTAATTGTGAACAATGAC	
RB1	mutational screening	Exon 5	Forward	AACTACTATGACTTCTAAATTACG	2' 94, 40X(1'94, 35"52,40"72), 4'72
			Reverse	CTTAATTTATGAAGTAGCCTGCTA	
RB1	mutational screening	Exon 6	Forward	**TGTAAAACGACGGCCAGT**CTGGAAAACTTTCTTTCAGTGATA	2' 94, 40X(1'94, 35"52,40"72), 4'72
			Reverse	**CAGGAAACAGCTATGACC**GGAATTTAGTCCAAAGGAATGCC	
RB1	mutational screening	Exon 7	Forward	**TGTAAAACGACGGCCAGT**CTCTACCCTGCGATTTTCTCTC	2' 94, 40X(1'94, 35"52,40"72), 4'72
			Reverse	**CAGGAAACAGCTATGACC**CATCCTGTCAGCCTTAGAACCA	
RB1	mutational screening	Exon 8	Forward	**TGTAAAACGACGGCCAGT**AAACAGCTGTTATACCCATT	2' 94, 40X(1'94, 35"52,40"72), 4'72
			Reverse	**CAGGAAACAGCTATGACC**TCATCTATATTACATTCAT	
RB1	mutational screening	Exon 9	Forward	**TGTAAAACGACGGCCAGT**GTTCAAGAGTCAAGAGATTAGATT	2' 94, 40X(1'94, 35"52,40"72), 4'72
			Reverse	**CAGGAAACAGCTATGACC**CAATTATCCTCCCTCCACAGTCTCA	
RB1	mutational screening	Exon 10	Forward	**TGTAAAACGACGGCCAGT**GCCTCTGTGTGCTGAGAGATGTA	2' 94, 40X(1'94, 35"57,40"72), 4'72
			Reverse	**CAGGAAACAGCTATGACC**AATGATATCTAAAGGTCACTAAGC	
RB1	mutational screening	Exon 11	Forward	**TGTAAAACGACGGCCAGT**GATTTTATGAGACAACAGAAGCA	2' 94, 40X(1'94, 35"52,40"72), 4'72
			Reverse	**CAGGAAACAGCTATGACC**ATCTGAAACACTATAAAGCCATG	
RB1	mutational screening	Exon 12	Forward	**TGTAAAACGACGGCCAGT**AGAGACAAGTGGGAGGCAGTG	2' 94, 40X(1'94, 35"52,40"72), 4'72
			Reverse	**CAGGAAACAGCTATGACC**GATAACTACATGTTAGATAGGAG	
RB1	mutational screening	Exon 13	Forward	**TGTAAAACGACGGCCAGT**CCTAAAGAACTGCACAGTGAATCC	2' 94, 40X(1'94, 35"52,40"72), 4'72
			Reverse	**CAGGAAACAGCTATGACC**AATGGGGTGGGAGGTAGTT	
RB1	mutational screening	Exon 14	Forward	**TGTAAAACGACGGCCAGT**ATTGTGATTTTCTAAAATAGCAGG	2' 94, 40X(1'94, 35"52,40"72), 4'72
			Reverse	**CAGGAAACAGCTATGACC**CAGGATGATCTTGATGCCTTG	
RB1	mutational screening	Exon 15	Forward	**TGTAAAACGACGGCCAGT**CAATGCTGACACAAATAAGGTT	2' 94, 40X(1'94, 35"52,40"72), 4'72
		Exon 16	Reverse	**CAGGAAACAGCTATGACC**AAGAAACACACCACATTTTAACT	
RB1	mutational screening	Exon 17	Forward	**TGTAAAACGACGGCCAGT**AGCTCAAGGGTTAATATTTCATAA	2' 94, 40X(1'94, 35"52,40"72), 4'72
			Reverse	**CAGGAAACAGCTATGACC**AATTTGTTAGCCATATGCACATG	
RB1	mutational screening	Exon 18	Forward	**TGTAAAACGACGGCCAGT**ATGTACCTGGGAAAATTATGCTT	2' 94, 40X(1'94, 35"52,40"72), 4'72
			Reverse	**CAGGAAACAGCTATGACC**CTTTATAGAATGTTACATTGCAC	
RB1	mutational screening	Exon 19	Forward	**TGTAAAACGACGGCCAGT**ATCTGTGATTCTTAGCCAACTTG	2' 94, 40X(1'94, 35"52,40"72), 4'72
			Reverse	**CAGGAAACAGCTATGACC**AGTCAGCCTAGTTTCAGAGTC	
RB1	mutational screening	Exon 20	Forward	**TGTAAAACGACGGCCAGT**CTGGGGGAAAGAAAAGAGTGG	2' 94, 40X(1'94, 35"52,40"72), 4'72
			Reverse	**CAGGAAACAGCTATGACC**GAGGAGAGAAGGTGAAGTGCT	
RB1	mutational screening	Exon 21	Forward	**TGTAAAACGACGGCCAGT**GAACAAAACCATGTAATAAAATTCT	2' 94, 40X(1'94, 35"52,40"72), 4'72
			Reverse	**CAGGAAACAGCTATGACC**ACCTATGTTATGTTATGGATATGG	
RB1	mutational screening	Exon 22	Forward	**TGTAAAACGACGGCCAGT**ACTGTTCTTCCTCAGACATTCA	2' 94, 40X(1'94, 35"52,40"72), 4'72
			Reverse	**CAGGAAACAGCTATGACC**TTGGTGGACCCATTACATTAGA	
RB1	mutational screening	Exon 23	Forward	**TGTAAAACGACGGCCAGT**CTAATGTAATGGGTCCACCAAA	2' 94, 40X(1'94, 35"52,40"72), 4'72
			Reverse	**CAGGAAACAGCTATGACC**TCCCCCTCTCATTCTTTACTAC	
RB1	mutational screening	Exon 24	Forward	**TGTAAAACGACGGCCAGT**TTCATCTCTGCAAAATTGTATATGG	2' 94, 40X(1'94, 35"52,40"72), 4'72
			Reverse	**CAGGAAACAGCTATGACC**TATGCAATATGCCTGGATGAGG	

Gene		Location	Direction	Sequence 5' > 3'	Amplification conditions
RB1	mutational	Exon 25	Forward	TTGCTAACTATGAAACACTGGC	10’ 95, 40X(15”95, 1’60, 30”72), 10’72
	screening		Reverse	ATGACCATCTCAGGCTACTGGA	
RB1	mutational	Exon 26	Forward	**TGTAAAACGACGGCCAGT**TCGAAAGCATCATAGTTACTGG	2’ 94, 40X(1’94, 35”52,40”72), 4’72
	screening		Reverse	**CAGGAAACAGCTATGACC**ATGCATAAACAAACCTGCCAACT	
RB1	mutational	Exon 27	Forward	**TGTAAAACGACGGCCAGT**TGCAAGGTCCTGAGCGCCAT	2’ 94, 40X(1’94, 35”52,40”72), 4’72
	screening		Reverse	**CAGGAAACAGCTATGACC**GAGAGACAATGAATCCAGAGGTG	
RB1	qPCR		Forward	TGGCAGAAATGACTTCTACTC	10'95, 40x(15"95, 1'60)
			Reverse	CTGGGAAAGTTATACAGTCACA	
KDM5A	qPCR		Forward	CTGCAAGGACAAGGTAGACT	10'95, 40x(15"95, 1'60)
			Reverse	TCCTCCATTGGTAGTTTGTAG	
murine	qPCR		Forward	TGGCCTGTGCTCTTGA	10'95, 40x(15"95, 1'60)
Rb1			Reverse	TTGGCTTCCACTTTGATAA	
murine	qPCR		Forward	CTCCTGCGACTTCAACAG	10'95, 40x(15"95, 1'60)
Gapdh			Reverse	TCTGGGATGGAAATTGTG	
murine	qPCR		Forward	ATGGCATGGATCTCAGC	10'95, 40x(15"95, 1'60)
Hoxa5			Reverse	TTGCCCAGGGAGTTTT	
murine	qPCR		Forward	GCCGGACAACAAATCAC	10'95, 40x(15"95, 1'60)
Hoxa7			Reverse	TCGGCATTTTGGAAGAG	
murine	qPCR		Forward	CCTGCTGCAGTGTATCATC	10'95, 40x(15"95, 1'60)
Hoxa9			Reverse	GCTGGGTTGTTTTTCTCTATC	
murine	qPCR		Forward	GAGCGAGTCCTAGACTCCA	10'95, 40x(15"95, 1'60)
Hoxa10			Reverse	CGGCGAAGCTTTACTGT	
murine	qPCR		Forward	TCCCCTGGATGAAAGAG	10'95, 40x(15"95, 1'60)
Hoxb2			Reverse	GGCCATCTGATGGTGAT	
murine	qPCR		Forward	CCGCACCTACCAGTACC	10'95, 40x(15"95, 1'60)
Hoxb3			Reverse	TGCCTCGACTCTTTCATC	
murine	qPCR		Forward	CCGGAGGGTGGAGAT	10'95, 40x(15"95, 1'60)
Hoxb4			Reverse	TGGGCAACTTGTGGTC	
murine	qPCR		Forward	GCCCGGACTATCAGTTG	10'95, 40x(15"95, 1'60)
Hoxb5			Reverse	TCCATCCCATTGTAATTGTAG	
murine	qPCR		Forward	CGGCGTCCTCCTATTAC	10'95, 40x(15"95, 1'60)
Hoxb6			Reverse	GGCGTGGAGCACTTC	

Supplemental Table 3.2. Patient characteristics of *NUP98/KDM5A* negative samples screened for *RB1* deletions.

UPN	Aberrations	FAB-type	Result MLPA p047 RB1	Mutational screening of *RB1*	Age at dx (yrs)	Sex	Remission status	Event	SCT	Dead	COD	Karyotype sample	Result MLPA p330 ALL-IKZF1
11	*KMT2A/MLLT3*	M7	no aberration	wt	1,8	Male	complete remission	relapse	no	yes	leukemia	47, XY, t(9;11)(p22;q23), +19 [27]	NA
12	*KMT2A/MLLT3*	M7	no aberration	wt	1,2	Male	complete remission	relapse	no	yes	leukemia	46, XY, t(9;11)(p22;q23)	NA
13	*KMT2A/MLLT3*, *FLT3* mutation	M5	no aberration	wt	2,0	Male	early death	early death	no	yes		46,XY,t(9;13)(p21;q14) [11]	NA
14	*KMT2A/MLLT3*	M7	no aberration	wt	0,7	Male	complete remission	relapse	no	yes		NA	NA
15	*KMT2A/MLLT10*	M0	Amplification of RB1	wt	9,5	Female	complete remission	relapse	yes	yes	leukemia	51~53,XX,+2,+4,+6,+10,+add(11)(p?15),+13,-18,+21,+22,inc[cp10]	Amplification of RB1
16	*KMT2A/MLLT10*	M5	no aberration	wt	1,6	Female	complete remission	death in CR	no	yes	toxicity	46,XX,der(3)t(3;11)(3pter->3q23::11q23->11q?13::?),der(10)?t(10;11)(p12;q23),add(11)(q13),der(13)	no aberration
17	*KMT2A/MLLT10*	M7	no aberration	wt	2,4	Male	complete remission	relapse	no	yes		NA	NA
18	*CBFA2T3/GUS2*	M7	no aberration	wt	1,4	Female	complete remission	relapse	no	yes	NA	NA	NA
19	*RBM15/MKL1*	M7	no aberration	wt	0,2	Male	complete remission	death in CR	no	yes	NA	46,XY,t(1;2;22;2)(p13;q2?1;q13;p2?3)[26]/46,XY[4]	NA
20	*RBM15/MKL1*	M7	no aberration	wt	0,1	Female	complete remission	relapse	no	yes	leukemia	46, XX, t(1;2)(p21;p21) [21], 46,XX [2]	NA
21	*MNX1/ETV6*, *RAS* mutation	M7	no aberration	wt	1,1	Female	complete remission	toxic event	no	yes	toxicity	NA	NA
22	*BCR/ABL1*	M7	no aberration	wt	5,1	Male	non-remitter	non-remitter	yes	no		NA	NA
23	*NPM1* , *CEBPA and PTPN11* mutation	M7	no aberration	wt	NA	Male	complete remission	relapse	no	no		NA	NA
24	-	M7	no aberration	wt	1,5	Male	complete remission	no event	no	no		50~55,XY,t(2;7)(p11;p11),+der(2)?t(2;7)(p11;p11),+6,+der(7)t(7;14)(p12;q11)[2],+8,+8[3],+13[9],-14[2],+der(10)t(10;19)(q21;q13.4)[8],+der(19)t(10;19)(q21;q13.4),+21[4][cp13]/ 46,XY [7]	NA
25	*RAS* mutation	M7	no aberration	wt	2,0	Female	complete remission	no event	no	no		NA	no aberration
26	*RUNX1* mutation	M7	no aberration	wt	2,1	Male	complete remission	no event	no	no		49,XY,+16,del(17)(p11),+19,+21,-22 [5]/46,XY [22]	no aberration

UPN	Aberrations	FAB-type	Result MLPA p047 RB1	Mutational screening of *RB1*	Age at dx (yrs)	Sex	Remission status	Event	SCT	Dead	COD	Karyotype sample	Result MLPA p330 ALL-IKZF1
27	*WT1* mutation	M7	no aberration	wt	14,0	Male	non-remitter	non-remitter	yes	yes	leukemia	NA	NA
28	-	M1	no aberration	wt	7,3	Male	complete remission	no event	yes	no		46,XY,1qh+,t(2;13)(q33;q34),inc[7]/49,XY,idem,+21,+21,+21,inc[11]	no aberration
29	-	M7	no aberration	wt	2,1	Female	NA	NA	NA	NA		47,XX,+8.t(10;11)(p14;q14)[1]/48,idem,+19[14]/46,XX[5]	NA
30	-	M7	no aberration	wt	0,1	Male	complete remission	relapse	NA	NA		46,XY[28]	NA
31	-	M1	no aberration	wt	8,4	Female	NA	NA	NA	NA		47,XX,t(8;12;13)(q1?2;p21;q3?),+10[21]/46,XX [2]	NA
32	-	M7	no aberration	wt	2,1	Female	complete remission	relapse	no	yes	leukemia	42-44,XX,-2,add(5p),add(6q),+8,-16,-17,-19,+mar1,+mar2[cp10]/ 46,XX [14]	Deletion of CDKN2A/B
33	-	M7	no aberration	wt	1,4	Male	complete remission	no event	no	no		48,XY,+8,+21[3]/ 46,XY[40]	NA
34	-	M7	no aberration	wt	1,5	Female	complete remission	death in CR	no	yes	NA	38~47,XX,-7,-8,+8,+19[cp14]/46,XX[7]	NA
35	*GATA1* mutation	M7	no aberration	wt	1,6	Male	complete remission	no	no	no		48,XY,+19,+21	NA
36	-	M7	no aberration	wt	1,8	Female	complete remission	no	no	no		48,X,t(X;7)(q22?;p15),+10,-16,+der(16)t(2;16)(q12;p13),+der(1q)t(19;?)(p133;?)	NA
37	-	M7	no aberration	wt	0,6	Male	complete remission	no event	yes	no		Hyperdiploidy at 55~71 chromosomes	no aberration
38	-	M7	no aberration	wt	0,9	Male	complete remission	no event	no	no		NA	NA

Chapter 4

Pediatric non-Down syndrome acute megakaryoblastic leukemia is characterized by distinct genomic subsets with varying outcomes

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Submitted

Introductory paragraph

Acute Megakaryoblastic Leukemia (AMKL) is a subtype of acute myeloid leukemia (AML) that morphologically resembles abnormal megakaryoblasts. While extremely rare in adults, pediatric cases comprise 4-15% of newly diagnosed AML patients.[1-3] A significant proportion of pediatric cases occur in children with Down syndrome (DS). These patients have excellent outcomes and are characterized at the genomic level by a founding *GATA1* mutation.[4-6] In contrast, AMKL in patients without DS (non-DS-AMKL) have poor outcomes. Previous efforts have identified chimeric oncogenes in a significant number of cases, including *RBM15-MKL1*, *CBFA2T3-GLIS2, KMT2A* gene rearrangements and *NUP98-KDM5A*[7-9]. The etiology of 30-40% of cases, however, remains unknown. To better understand the genomic landscape of non-DS-AMKL, we performed RNA and exome sequencing on specimens from 99 patients. We demonstrate that pediatric non-DS-AMKL is a heterogeneous malignancy that can be divided into seven subgroups with varying outcomes. These subgroups are characterized by chimeric oncogenes with cooperating mutations in epigenetic and kinase signaling genes. In contrast, epigenetic mutations predominate in adults. These data shed light on the etiology of AMKL.

Letter to the editor

The earliest insight into the genomic alterations that drive non-DS-AMKL occurred with the recognition of a recurrent t(1;22) found in infants.[10] Ten years after this initial report, the *RBM15* and *MKL1* genes involved in this translocation event were characterized.[7, 11] Subsequent to this, a high resolution study of DNA copy number abnormalities (CNAs) and loss of heterozygosity (LOH) on pediatric *de novo* AML identified a significant number of alterations in non-DS-AMKL cases, suggesting that additional gene rearrangements may be present in this population.[12] To define the functional consequences of these structural variations, diagnostic leukemia specimens from 14 pediatric patients have previously undergone RNA and exome sequencing.[8] This effort identified chimeric transcripts encoding fusion proteins in 12 of 14 cases, including the novel recurrent *CBFA2T3-GLIS2* fusion which renders a poor prognosis. In parallel, a separate non-DS-AMKL cohort was evaluated by PCR and split-signal fluorescence in situ hybridization (FISH) for fusion events associated with myeloid malignancies including *NUP98* and *KMT2A* gene rearrangements (*KMT2A*r).[9] In this cohort, *NUP98* and *KMT2Ar* were identified in 15% and 10% of cases respectively. To gain a more comprehensive understanding of the genomic alterations that lead to non-DS-AMKL, specimens from 99 patients (75 pediatric and 24 adult cases) were subjected to RNA and/or exome sequencing. Combined with the 14 cases previously described, the pediatric cohort described in this manuscript yields 89 cases, the largest of this rare malignancy to undergo next generation sequencing to date.

Of the 93 patients for whom sufficient RNA was available, 5.5% (1/17) adult and 72.4% (55/76) pediatric cases carried a structural variation (SV) predicted to lead to a fusion product by RNAseq. Ten additional fusion events in patients that lacked RNA for sequencing could be recognized by RT-PCR, florescent in situ hybridization (FISH) or southern blotting (Figure 4.1). In the pediatric cohort, the most frequent fusion events include *CBFA2T3-GLIS2* (18.6%; 16/86), *KMT2A*r (17.4%; 15/86), *NUP98-KDM5A* (11.6%; 10/86), and *RBM15-MKL1* (10.5%; 9/86). Previously described low frequency non-DS-AMKL fusions identified in this expanded cohort, include a case of *NIPBL-HOXB9* and a novel, but functionally analogous *NIPBL-HOXA9* fusion.[8] Similarly, a case carrying *GATA2-HOXA10* was identified, which is functionally equivalent to the *GATA2-HOXA9* fusion that has been reported in a single case.[8] Chimeric transcripts not previously described include several fusions involving genes within the HOX cluster (*EWSR1-HOXB8*, *PLEK-HOXA11-AS*, *BMP2K-HOXD10, and EP300-HOXA7*). Collectively, fusions involving a HOX cluster gene (*HOX*r) occurred in 14% (12/86) of this cohort (Table 4.1). Many of the *HOX*r are predicted to lead to an in-frame functional fusion protein. As proof of principal, we evaluated three of these fusion events and found all three to enhance self-renewal as determined by an *in vitro* colony replating assay (Supplementary Figure 4.1). Several fusions, however, involve non-coding RNA species and are predicted to result in a loss of function of these regulatory transcripts. 3.5% (3/86) of the cases carried non-recurrent fusion proteins involving hematopoietic transcription factors and epigenetic regulators such as *MN1-FLI1*, *BCR-ABL1*, and *MAP2K2-AF10*. 3.5% (3/86) of cases were found to have chimeric transcripts involving the cohesin gene *STAG* which were all predicted to lead to a truncation in the protein. In 21% (18/86) of cases, no potentially oncogenic fusion event could be detected.

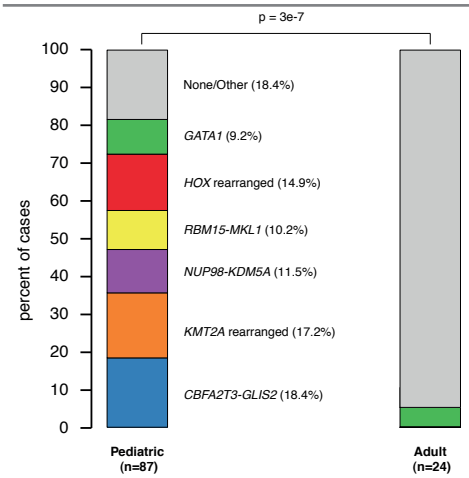

Figure 4.1. Pediatric and adult non-DS-AMKL are genomically distinct. Distributions of recurrent chromosome translocations and *GATA1* mutations in pediatric and adult non-DS-AMKL. p value according to Pearson's Chi squared test.

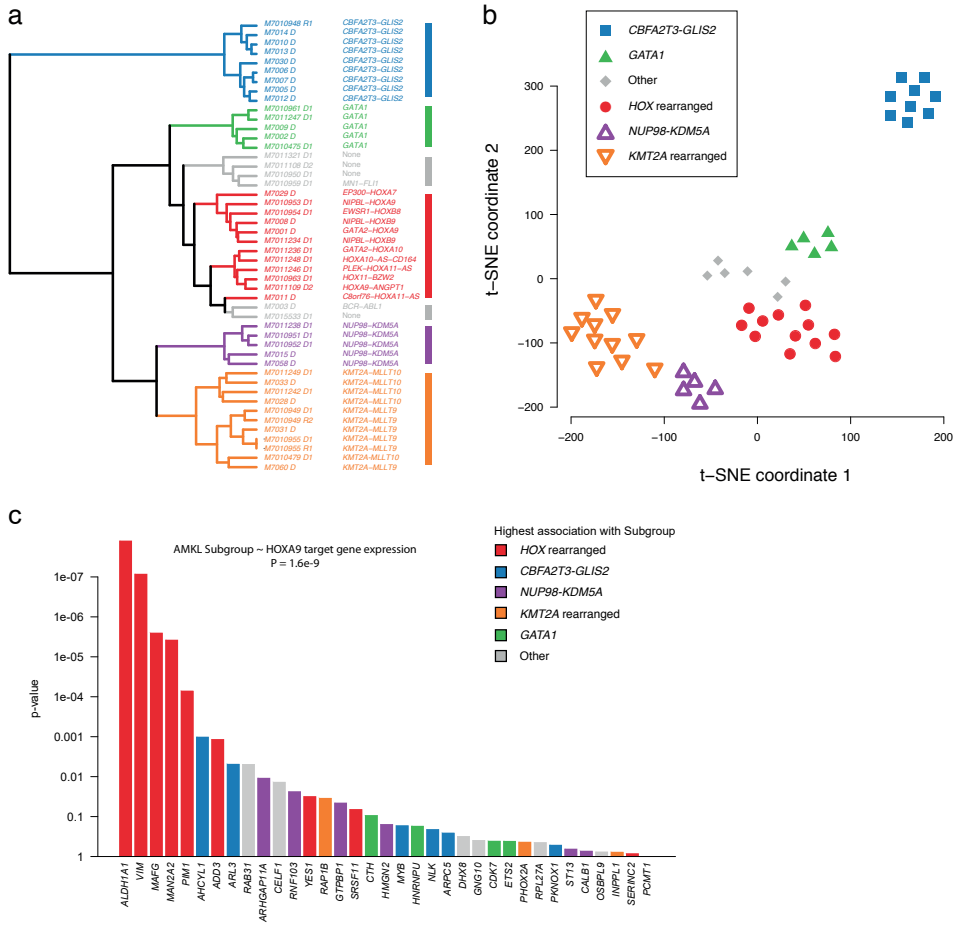

Figure 4.2. **Gene expression analysis confirms genomic subgroups.** (a) Unsupervised hierachical clustering of the gene expression correlation matrix of 100 most variant RNAs. Colors indicate patient samples with similar genomic aberrations. (b) t-SNE visualization. (c) Expression of myeloid *HOXA9* target genes most highly associates with gene expression in *HOX*-rearranged AMKL. Global association between AMKL subgroup and *HOXA9* target gene expression was estimated using a global test.[23] Contributions of gene to the overall association p value are shown ranked according to covariate p value and colored according to most highly associating subgroup.

However, in 50% of these cases, a truncating mutation in exon 2 or 3 of *GATA1* was found, amounting to 10.1% (9/89) of the pediatric cohort overall (further discussed below, Figure 4.1).

To determine if these fusion events contribute significantly to gene expression patterns, samples with greater than 60% purity were subjected to unsupervised clustering using the top 100 most variant genes by standard deviation (Figure 4.2A and B). Confirming the strength of the fusions in determining gene expression signatures, samples clustered according to fusion status, specifically *KMT2A*r, *HOX*r, *NUP98-KDM5A*, and *CBFA2T3-GLIS2* cases formed distinct clusters. When analyzing expression of the *HOX* gene cluster, we found upregulation of the *HOX* gene involved in the fusion construct (Supplementary Figure 4.2), often accompanied by upregulation of adjacent *HOX* genes. To determine whether this upregulation had downstream effects on HOX targets, we evaluated expression of a gene set defined by *HOXA9* overexpression in hematopoietic cells.[13] This demonstrated the highest association for the majority of target genes with the *HOX*r subgroup, providing further evidence of a *HOX* signature (Figure 4.2 and Supplementary Figure 4.3). Combined with *KMT2A*r and *NUP98-KDM5A*, chimeric oncogenes known to upregulate *HOX* cluster genes, roughly half of pediatric non-DS-AMKL patients carry a *HOX* gene expression program. These cases were distinct from those carrying the cryptic *CBFA2T3-GLIS2* inversion which clustered away from all other non-DS-AMKL as has been previously shown[8].

In addition to RNAseq data, 68 pediatric patients had DNA available for whole exome sequencing (WES), of which 30 had paired germline material. To identify single nucleotide variants and insertion/deletion events (SNV/Indels) in cases lacking WES, RNAseq data was also interrogated for these mutational events. To identify high confidence somatic calls, unpaired samples underwent a vigorous filtering process as described in the online methods. Of 83 pediatric cases at diagnosis for which SNV/Indel analysis was available, the most highly recurrent mutations occurred in *GATA1* (13.3%; 11/83), *JAK* kinase or *STAT* genes (16.9%; 14/83), Cohesin/*CTCF* genes (18.1%; 15/83), and RAS pathway genes (15.7%; 13/83, Figure 4.3). Additionally, 18.1% (15/83) of patients carried mutations in a cytokine receptor gene, the most frequent of which was the MPL receptor (n=10) that plays a role in normal megakaryoblast growth and survival. In contrast, in the adult cohort (n=24), the most highly recurrent mutations were in *TP53* (20.8%; 5/24), cohesin genes (16.7%; 4/24), splicing factor genes (16.7%; 4/24), *ASXL* genes (16.7%; 4/24) and *DNMT3A* (12.5%; 3/24). Paired exome specimens and single nucleotide polymorphism (SNP) arrays were available in 29 specimens for copy number alterations analysis to identify additional cooperating mutations (Figure 4.3). The tumor suppressor RB1 was found to be recurrently targeted with focal deletional events (Supplementary Table 4.1). Combined with SNV/Indels and structural variations, *RB1* mutational events occurred in 14.3% (12/84) of the pediatric cohort and 8.3% (2/24) of the adult cohort. Confirming previous reports, gains in chromosome 19 and 21 were also recurrent in the pediatric cohort as determined by WES and/or cytogenetics in 24% (19/79) and 39.2% (31/79) of cases respectively.[14,15]

Of the ten cases carrying *GATA1* truncating mutations in the pediatric cohort, none had physical stigmata consistent with DS (Supplementary Table 4.2). Karyotypes were available for all patients and found to be negative for constitutional trisomy 21. Four pediatric cases had matched germline available with an average coverage of 110X.

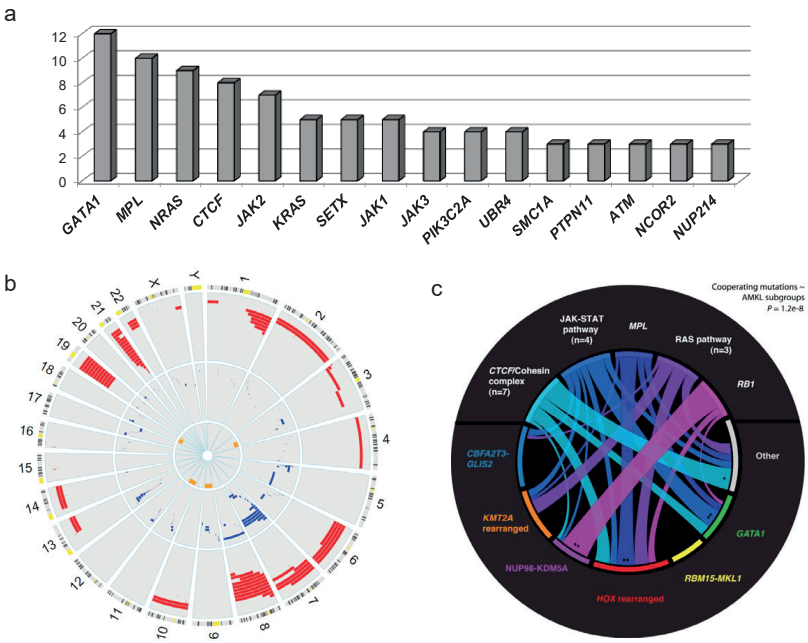

Figure 4.3. Cooperating mutations in pediatric non-DS-AMKL. (a) Recurrent genes in diagnostic and relapsed specimens targeted by SNV/Indel mutations. Genes for which four or more cases carried a lesion are shown. (b) Frequency of copy number alterations for cases with a paired germline specimen. The outer track indicates the chromosomal location. Amplifications are shown in red, deletions in blue, and loss of heterozygosity is shown in orange. (c) Non-random associations between genomic AMKL subgroup and cooperating mutation. Circos plot showing co-occurence in patients between grouped (n) cooperating mutations (top) and AMKL subgroup (bottom). CTCF/Cohesin: *CTCF, STAG2, STAG3, SMC1A, NIPBL, SMC3, RAD21*; JAK/STAT: *JAK1, JAK2, JAK3, STAT5B*; RAS: *NRAS, KRAS, PTPN11*. Global association *p* value of $1.3x10^{-8}$ is estimated according to global test using a multinomal regression model.[23]

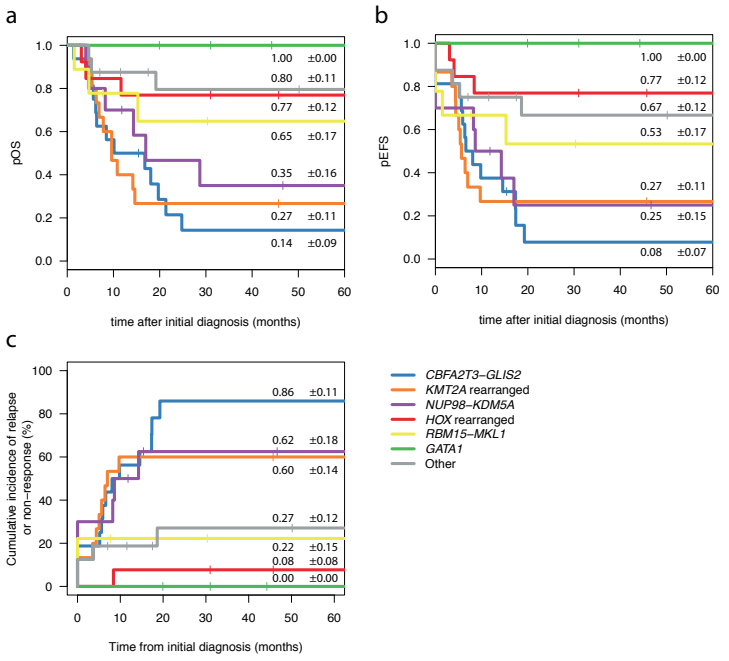

Figure 4.4. Clinical outcomes in pediatric non-DS-AMKL. (A) Overall survival of pediatric non-DS-AMKL patients according to subgroup. Medium follow up of survivors: 89 months. (B) Event free survival of pediatric non-DS-AMKL patients according to subgroup. (C) Cummulative incidence of relapse or non-response.

With the exception of one case that had evidence of low level tumor contamination in the germline specimen, *GATA1* mutant calls were absent from these samples, including a case with 160X coverage, arguing against low level mosaicism. The strong association of *GATA1* truncations in DS patients suggests cooperativity between amplification of the Down syndrome critical region (DSCR) on chromosome 21 and the *GATA1* mutant. We therefore evaluated cases for amplification of the DSCR using SNP arrays and exome read depth in paired samples. Nine of ten *GATA1* mutant cases had amplifications in the DSCR (Supplementary Table 4.2). Across the entire cohort, amplifications of chromosome 21 were found to be one of the most highly recurrent copy number alterations (Figure 4.3). Candidate genes in this region that play a role in megakaryopoiesis include *ERG*, a member of the *ETS* transcription gene family.[16] Furthermore, over-expression of ERG in hematopoietic progenitor cells by retroviral transduction and subsequent transplantation into mice results in megakaryoblastic leukemia, consistent with a role for this oncogene in promoting megakaryocytic disease.[17] *GATA*1 mutant cases comprised a distinct subset at the gene expression level (Figure 4.2), and this signature was strongly correlated with that found in DS-AMKL. Confirming cooperativity with chromosome 21, the *GATA1s* subset significantly overexpressed chromosome 21 genes, even in comparison to other samples carrying extra copies of this chromosome, including UBASH3A and LINC00478/MONC, genes previously implicated in AML and AMKL respectively (Supplementary Figure 4.4).[18, 19] Combined with RNA-seq data, this led us to divide our cohort into seven subsets based on genomic lesions for further analysis: *CBFA2T3-GLIS2*, *RBM15-MKL1*, *NUP98-KDM5A*, *KMT2A*r, *HOX*r, *GATA*1s, and "Other" which is comprised of cases not falling into any of the aforementioned subsets (Figure 4.1).

Cooperating mutations as identified by exome sequencing revealed a significant association between subgroups and recurrent mutations (p = 1.3e-8, global test, Figure 4.3). *NUP98-KDM5A* cases carried mutations in *RB1* almost without exception; as has been previously described *KMT2A*r often associated with RAS pathway lesions (p = 0.09 for enrichment, Fisher exact test) and *JAK* kinase or *STAT5B* mutations were commonly identified in *GATA1* mutant cases (p = 0.003).[5, 20] *HOX*r cases were found to be significantly enriched in activating *MPL* mutations (p = 0.007) and the "Other" category was enriched for mutations in cohesin and *CTCF* (p = 0.04).

Clinical outcomes for DS-AMKL are uniformly excellent, whereas studies on non-DS-AMKL are more variable with the majority reporting inferior survival rates compared to other AML subtypes.[2,3,6,21,22] Furthermore, the recommendation for stem cell transplant (SCT) in first remission for non-DS-AMKL patients is not uniform among pediatric cooperative groups. To further understand the association between genomic subgroups and outcome, we first utilized the global test to determine if subgroups correlated with survival and found a statistically significant association, with *CBFA2T3-GLIS2* carrying the strongest negative association and *GATA1* mutant cases carrying the strongest positive association (p=1.7x10^{-5} for overall survival, p=3.4x10^{-5} for event free survival)[23]. Kaplan-Meier visualizations of event free survival (EFS), overall survival (OS) and cumulative incidence of relapse or non-response (CIR, p[Gray] = 1.4x10^{-4}) calculations confirmed this trend (Figure 4.4). Specifically, *CBFA2T3-GLIS2*, *KMT2A*r were found to have statistically significant inferior EFS and OS in addition to higher CIR. *NUP98-KDM5A* cases had a trend towards inferior outcomes, however this did not reach

statistical significance. Conversely, *GATA1* and *HOX*r subgroups carried significantly superior outcomes. Of note, all *GATA1* mutant cases that lacked a fusion gene were cured, mimicking the excellent outcomes seen in DS-AMKL.[6] Thus these patients are not only biologically but clinically similar as well, suggesting they may benefit from the less intensive chemotherapy regimens given to DS-AMKL patients.[24, 25] Based on these results, we recommend all pediatric non-DS-AMKL patients be tested for the presence of *NUP98-KDM5A*, *CBFA2T3-GLIS2* and *KMT2A*r. Patients carrying one of these fusion genes have inferior outcomes and may benefit from transplant in first remission. In contrast, patients lacking these lesions have outcomes on par with or superior to other subtypes of pediatric AML and transplant in first remission should be reserved for those that have a poor response to therapy.

In summary, pediatric non-DS-AMKL is a heterogenous malignancy comprised of distinct subsets as defined by next generation sequencing with varying outcomes. Chimeric oncogenes that define two subsets, *CBFA2T3-GLIS2* and *NUP98-KDM5A* are missed by conventional karyotyping and therefore require split-signal FISH or RT-PCR for detection. The presence of *CBFA2T3-GLIS2* and *KMT2A*r should be determined on all newly diagnosed pediatric non-DS-AMKL patients as they may benefit from transplant in first remission.

Methods

Patient samples

Specimens were compiled from multiple institutions. All samples were obtained with patient or parent/guardian provided informed consent under protocols approved by the Institutional Review Board at each institution. Samples were deidentified prior to nucleic acid extraction and analysis.

Next Generation Sequencing

RNA and DNA library construction for RNA and whole exome DNA sequencing were done as per manufacturer's instructions using the Illumina True-seq RNA sample preparation V2 and Nextera rapid capture exome kits, respectively. Sequencing was completed on the Illumina HiSeq 2000 as per manufacturer's instructions. Analysis of RNA and whole-exome sequencing data which includes mapping, coverage and quality assessment, SNV/Indel detection, tier annotation for sequence mutations, and prediction of deleterious effects of missense mutations have been described previously.[8,26] Open reading frames predictions of fusion transcripts detected by RNAseq were validated by RT-PCR followed by Sanger sequencing of the purified PCR products.

Exome filtering

To identify high confidence somatic calls, unpaired samples underwent a vigorous filtering process including the removal of low quality calls and known polymorphisms. Rare variants (defined as a mutant allele frequence of <0.1% in the non-cancer NHLBI ESP cohort) were retained for further analysis. Known recurrent somatic variants present in the catalogue of somatic mutations in cancer database (COSMIC) were designated as high confidence lesions. Remaining calls were evaluated by damage-prediction algorithms. Those with mutations occurring in a conserved domain of a cancer consensus gene and predicted to be damaging were designated as intermediate

confidence lesions.

Gene expression analysis

Transcript expression levels were estimated as Fragments Per Kilobase of transcript per Million mapped reads (FPKM); gene FPKMs were computed by summing the transcript FPKMs for each gene using Cuffdiff2.[27, 28] A gene was considered "expressed" if the FPKM value >= 0.5 based on the distribution of FPKM gene expression levels. Genes that were not expressed in any sample group were excluded from the final data matrix for downstream analysis. For hierarchical clustering and t-SNE the top 100 most variant RNAs by standard deviation were used, excluding those encoded by sex specific genes, sno and miRNAs and mRNAs correlating with inflammatory response.

Associations of AMKL subgroups with cooperating mutations

High confidence SNVs/Indels from initial diagnostic samples excluding mutations in GATA1 were combined with structural alterations excluding those identifying genomic subgroups were subjected to mutational frequency analysis. One hypermutated sample was excluded from this analysis. Genes mutated in >4 cases were identified as *RB1, MPL, CTCF, JAK2* and *NRAS*. Based on these 5 genes, five non-overlapping proximal gene sets were constructed from mutated genes, covering 44% of identified cooperating mutations. Global association with AMKL subgroup was calculated using multinomal regression with the global test.[23] Enrichment of gene set mutations in AMKL subgroups was determined by one-sided Fisher exact tests.

Colony forming assay

All experiments involving mice were reviewed and approved by the Institutional Animal Care and Use Committee. Bone marrow from 4-6 week old female C57BL/6 mice was harvested, lineage depleted, and cultured in the presence of recombinant murine SCF (rmSCF, Peprotech, 50ng/ml), IL-3 (rmIL3, Peprotech, 50ng/ml), and IL-6 (rmIL6, Peprotech, 50ng/ml) for 24 hours prior to transduction on RetroNectin (Takara Bio Inc.) coated plates. Cultured supernatants containing ecotropic envelope pseudotyped retroviral vectors were produced by transient transfection of 293T cells as previously described.[29] Murine bone marrow cells were harvested 48 hours following transduction, sorted for vector-encoded mCherry or GFP expression, and plated on methylcellulose containing IL-3, IL-6, SCF and erythropoietin (EPO) (Stem Cell technologies, Vancouver, BC) as per manufacturer's instructions. Colonies were counted after 7 days of growth at 37°C, harvested and serially replated.

Affymetrix SNP arrays

Affymetrix SNP 6.0 array genotyping was performed for 14 of 15 AMKL cases in the discovery cohort, and array normalization and DNA copy number alterations identified as previously described.[30-33] To differentiate inherited copy number alterations from somatic events in leukaemia blasts from patient's lacking matched normal DNA, identified putative variants were filtered using public copy number polymorphism databases and a St. Jude database of SNP array data from several hundred samples.[34, 35]

Outcome analysis

Kaplan-Meier curves for overall survival (OS), event free survival (EFS) and cumulative incidence of relapse or non-response (CIR) were calculated and plotted using the survival package in R based on clinical survival data. Gray's test statistic and p value for CIR were calculated using the cuminc() function in the cmprsk package in R. Events in EFS calculations included relapses, deaths in remission by any cause and non-responses, which were included as events at date of diagnosis. For CIR, only relapses and non-responses were included. No significant differences were present in cumulative incidences of competing risk (p=0.7). For asssociation of disease outcome with AMKL subgroup, EFS and OS survival were formatted using the Survival package and used as response variables in the global test in R in a Cox regression model with group label as covariate.

Table 4.1. *HOX* cluster gene rearrangments identified in pediatric AMKL patients.

Chimeric Transcript	Junction	Breakpoint	Predicted Product
GATA2-HOXA9	exon 4 - exon 2	TTCAG/ATAAC	Protein coding
GATA2-HOXA10	exon 4 - exon 2	TTCAG/GCAAT	Protein coding
NIPBL-HOXA9	exon 6 - exon 1	ACAAG/TTGAT	Protein coding
NIPBL-HOXB9	exon 6 - exon 2	ACAAG/CCAAC	Protein coding
EP300-HOXA7	exon 31 - 5'UTR	TGGGA/TTCAA	Protein coding
EWSR1-HOXB8	exon 12 - 5'UTR	TTGAT/CCCCCA	Protein coding
BMP2K-HOXD10	exon 15 - exon 2	TTCAG/AGGAA	Protein coding
C8orf76-HOXA11AS	exon 1 - exon 1	ATCAG/GAGGT	Non-coding RNA
PLEK-HOXA11AS	exon 1 - 5'UTR	AGAAG/GAGGT	Non-coding RNA
HOXA9-ANGPT1	3'UTR - intergenic	AGGGT/GGAAA	Unknown
HOXA10AS-CD164	5'UTR - exon 3	TCCAG/ATGAG	Non-coding RNA

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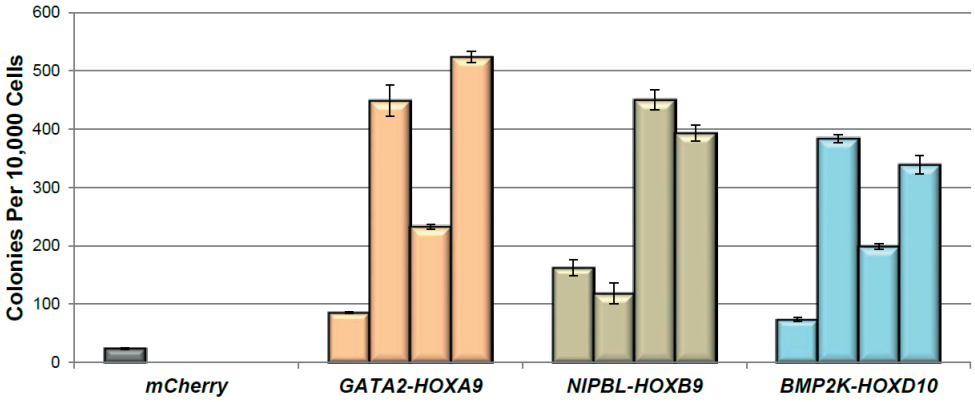
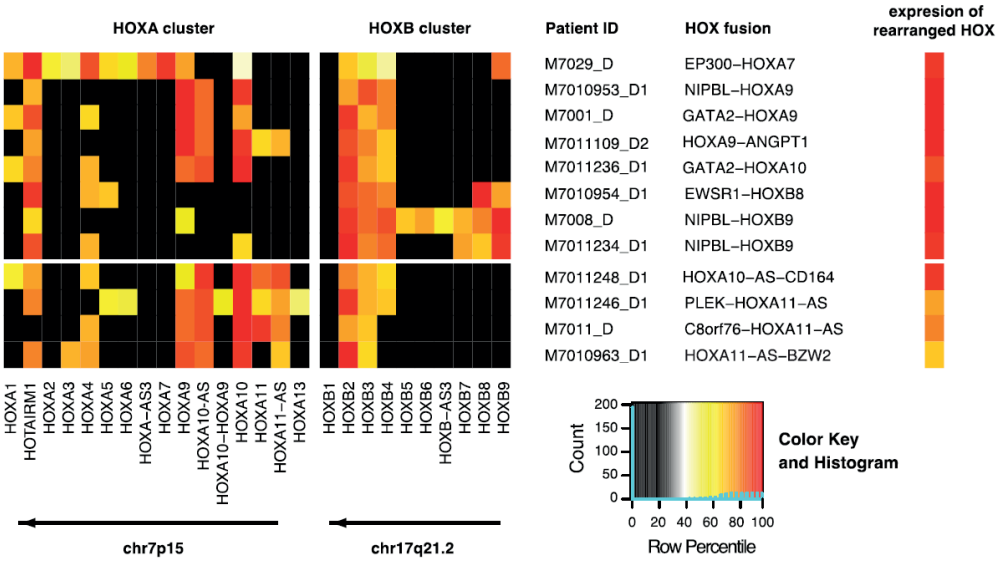
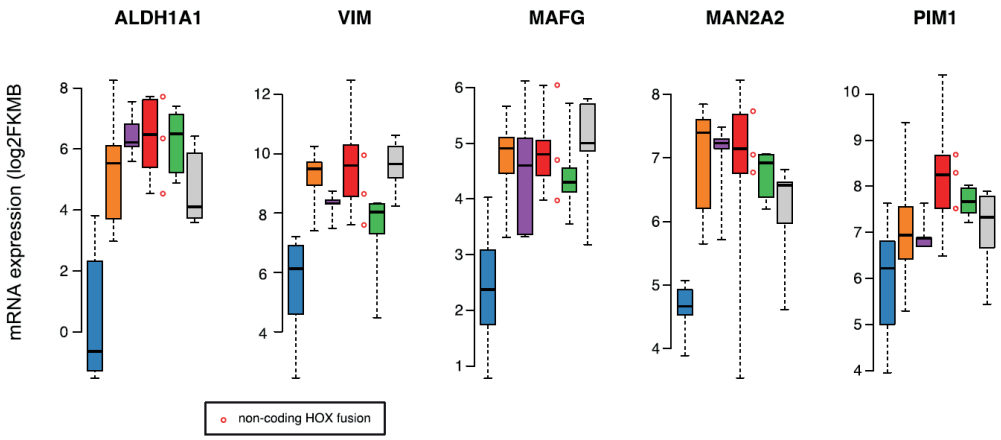
Supplementary data



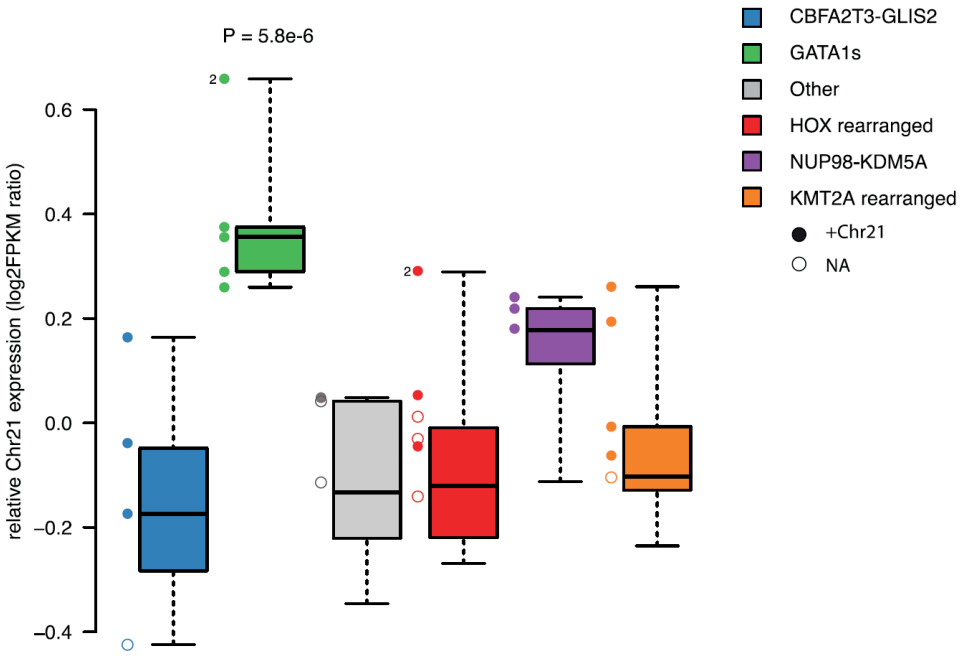
Supplementary Figure 4.1. *HOX* fusions enhance self-renewal capacity. Murine bone marrow was transduced with a retrovirus carrying one of three *HOX* fusion genes or the empty mCherry reporter construct as described in online methods. mCherry positive cells were flow sorted and grown on methylcellulose supplemented with cytokines. At one week intervals colonies were counted and cells were replated for a total of four platings. Each bar donates colony counts for a replating round. Error bars indicate standard error of the mean.



Supplementary Figure 4.2. *HOX* fusion partner genes are upregulated in *HOX* rearranged cases. Relative *HOX* gene expression values (row percentiles) are indicated by heat color (color key). Genes are ordered by chromosomal position. Arrows indicate direction of transcription of protein coding HOX genes. Fusion genes are divided by coding (top) and non-coding *HOX* fusions and ordered by *HOX* fusion partner. Left column summarized expression value of rearranged *HOX* gene.



Supplementary Figure 4.3. *HOXA9* target genes are upregulated in the *HOX* rearranged subgroup**.** Distribution of expression of genes within the *HOXA9* target upregulated gene set showing the highest association with AMKL subgroup4. Expression is highest in the *HOX* rearranged cases for both protein coding and non-protein coding *HOX* fusions (red circles).



Supplementary Figure 4.4. Chromosome 21 expression levels. Relative expression of genes on chromosome 21 in the AMKL subgroups is shown. Genomic amplification status for each sample is indicated by the circles. Filled circles represent amplified chromosome 21; open circles represent diploid chromosome 21.

Supplementary Table 4.1. Overview of *RB1* mutations.

UPN	Cohort	Group Assignment	Event 1 Genomic Alteration	Result	Event 2 Genomic Alteration	Result
1	Adult	NA	CNA	Deletion		
2	Adult	NA	SNV	V833E		
3	Pediatric	NUP98-KDM5A	CNA	Deletion	Structural Variation	RB1-FNDC3A Fusion Protein
4	Pediatric	NUP98-KDM5A	CNA	Deletion		
5	Pediatric	NUP98-KDM5A	CNA	Deletion		
6	Pediatric	NUP98-KDM5A	CNA	Deletion	SNV	Q354*
7	Pediatric	HOXr	CNA	Deletion		
8	Pediatric	NUP98-KDM5A	CNA	Deletion		
9	Pediatric	GATA1s	CNA	Deletion		
10	Pediatric	HOXr	CNA	Deletion		
11	Pediatric	NUP98-KDM5A	CNA	Deletion		
12	Pediatric	NUP98-KDM5A	CNA	Deletion		
13	Pediatric	NUP98-KDM5A	CNA	Deletion	Structural Variation	RB1 fused to a non-coding region of the genome
14	Pediatric	NUP98-KDM5A	CNA	Deletion	SNV	R445*

Abbreviations: UPN indicates unique patient number; NA, not available; CNA, copy number alteration; SNV, single nucleotide variant.

Supplementary Table 4.2. Overview of *GATA1s* mutations in diagnostic material.

UPN	Cohort	Group Assignment	Result	Cytogenetics	Other mutated genes	Chr21 Status
9	Peds	*GATA1s*	Q17*	50,XY,del(7)(p13),+8,+19,+21,+21[10]	*CTCF, JAK1, PIK3R1, RB1*	Somatic Amplification
12	Peds	*NUP98-KDM5A*	F173fs	46,XY,-13,der(17)t(13;17)(q14;q22) ,+21[18]46,XY[2]	*NUP98-KDM5A, RB1, PIK3CA*	Somatic Amplification
15	Peds	*GATA1s*	D65fs	47,XY,add(5)(p15.3),i(7)(q10),add(11)(p11.2),+21[20]	*JAK2, MPL, CTCF*	Somatic Amplification
16	Peds	*GATA1s*	Y69_P73fs	47,XY,del(5)(p14),der(16)t(1;16) (q21;p13.1),+21[3]/47,idem,del(7) (p15)[11]	*JAK1*	Somatic Amplification
17	Adult	NA	Y78*	46,XY[20]	*TET2, RAD21*	None
18	Peds	*GATA1s*	E13fs	47,XY,+21[17]/46,XY[3]	*GATA1s*	Somatic Amplification
19	Peds	*GATA1s*	c.220+1G>A (exon 2 skipped)	47,XY,del(6)(q?16q23),add(7)(p22),+21[12]/46,XY[8]	*MPL, BCOR*	Somatic Amplification
20	Peds	*GATA1s*	G31fs	48,XY,+19,+21	*JAK3, EP300 ITD*	Germline Mosaic 21
21	Peds	*GATA1s*	F33fs	46,XY[15]	*RAD21, SMC1A*	Somatic Amplification
22	Peds	*GATA1s*	V32fs	46,XY[15]	*RAD21, SMC1A*	Somatic Amplification
23	Peds	*GATA1s*	Y63fs	46,XY, i (21) (q10) / 46, XY	*GATA1s*	None
24	Peds	*GATA1s*	p.R243L	46,XY, i (21) (q10) / 46, XY	*GATA1s*	None
25	Peds	*KMT2A*	P50fs	50,XX,+6,der(6)(6pter->6q10::10p10->10p12::11q23->11qter),t(10;11;7)(p12;q23,p15)inv(11)(q13q23),add(16)(16q24), +19,+21,+22[20]50, idem, +add(1)(p13), -add(16),-19[4]	*KMT2A-MLLT10, GATA1s*	Somatic Amplification

Abbreviations: UPN indicates unique patient number; Peds, pediatric cohort; NA, not available; Chr, chromosome.

Chapter 5

Recurrent genetic abnormalities can be used for risk-group stratification in pediatric AMKL: results of a retrospective intergroup study.

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Abstract

Genetic abnormalities and early treatment response are the main prognostic factors in acute myeloid leukemia (AML). Acute megakaryoblastic leukemia (AMKL) is a rare subtype of AML. Recently, deep sequencing has identified *CBFA2T3/GLIS2* and *NUP98/KDM5A* as recurrent aberrations, occurring in similar frequencies as *RBM15/MKL1* and *KMT2A*-rearrangements. We studied whether these cytogenetic aberrations can be used for risk-group stratification.

To assess frequencies and outcome parameters of recurrent cytogenetic aberrations in AMKL, samples and clinical data of patients treated between 1998 and 2014 by the AIEOP, BFM-SG, COG, DCOG Centers and the Saint Louis Hopital were collected, enabling us to screen 153 newly diagnosed pediatric AMKL cases for the aforementioned aberrations, and to study their clinical characteristics and outcome.

CBFA2T3/GLIS2 was identified in 16% of the cases, *RBM15/MKL1* in 12%, *NUP98/KDM5A* and *KMT2A*-rearrangements in 9% each. These aberrations were mutually exclusive, and none of these aberrations were detected in the remaining 54% of patients. *RBM15/MKL1*-rearranged patients were significantly younger. No significant differences in sex, and white blood cell count were found between these subgroups. *NUP98/KDM5A, CBFA2T3/GLIS2* and *KMT2A*-rearranged (NCK) lesions predicted a poor outcome; compared to *RBM15/MKL1*-rearranged patients and to those with AMKL not carrying these molecular lesions, NCK-patients (n=52) showed a 4-yr pOS of 37±7% vs 66±5% in the *RBM15/MKL1*-other groups (n=101, p=0.001), and 4-yr pEFS of 34±7% vs 59±5% (p=0.011), the 4-yr cumulative incidence of relapse being 43±7% and 21±4% (p=0.006), respectively.

We conclude that these genetic aberrations may be used for risk-group stratification of pediatric AMKL and for treatment tailoring.

Introduction

Pediatric acute myeloid leukemia (AML) is a heterogeneous disease characterized by recurrent genetic aberrations. The most important factors predicting clinical outcome are presence of (molecular) genetic aberrations and early response to treatment.[1, 2] Acute megakaryoblastic leukemia (AMKL) is a rare subtype of AML and is seen mostly in Myeloid Leukemia of Down Syndrome (ML-DS) patients, where it is associated with mutations in the transcription factor *GATA1*.[3] In pediatric non-Down syndrome AML, AMKL accounts for approximately 10% of the cases, and has been reported to be associated with poor outcome.[4-7] Most study groups consider non-Down syndrome AMKL as high-risk AML, and some consider it to have the indication for allogeneic hematopoietic stem cell transplantation (HSCT) in first complete remission (CR1). [4, 8-10] However, risk group stratification and treatment protocols are not yet optimized for this subtype of pediatric AML.[11]

Translocation t(1;22)(p13;q13), resulting in a chimeric fusion of *RBM15* and *MKL1,* formerly known as *OTT/MAL,* until recently was the only recurrent aberration described in pediatric AMKL, occurring in ~10% of the patients.[12-14] Conflicting results on the prognostic relevance of *RBM15/MKL1* have been reported, as some series suggested the outcome to be poor and others favorable.[5, 6, 13, 15] Recently, the cytogenetically cryptic translocation t(11;12)(p15;p13), resulting in the chimeric fusion gene *NUP98/KDM5A,* was identified as recurrent aberration, found in ~10% of AMKL cases.[5, 7, 16] Moreover, another cytogenetically cryptic event, inv(16)(p13q24), resulting in a fusion between *CBFA2T3* and *GLIS2,* and accounting for ~15% of the cases, was identified as a recurrent aberration in this specific subtype of AML.[5, 7, 16, 17]. Both *NUP98/KDM5A* and *CBFA2T3/GLIS2* fusion transcripts were reported to confer poor outcome, but numbers of patients analysed to support this conclusion were small. Moreover, *CBFA2T3/GLIS2* was also reported to behave as an aggressive leukemia in mouse models.[7, 16] Another recurrent abnormality consists of *KMT2A*-rearrangements, which are seen in ~10% of the pediatric non-Down syndrome AMKL cases.[5] The clinical outcome of *KMT2A*-rearranged leukemia is dependent on the fusion-partner gene.[18, 19] *NUP98/KDM5A, CBFA2T3/GLIS2* and *RBM15/MKL1* are rarely or never seen in other pediatric AML subtypes, with *CBFA2T3/GLIS2* being the least specific for AMKL. [5, 17, 20]

In this study, we present the clinical, cell-biological and genetic characteristics of 153 pediatric patients with non-Down syndrome AMKL patients from the Associazione Italiana Ematologia Oncologia Pediatrica (AIEOP, Italian Association of Pediatric Hematology and Oncology, Italy), the Berlin-Frankfurt-Munster-Study Group (BFM-SG, Essen, Germany), the Dutch Childhood Oncology group (DCOG, The Hague, The Netherlands), the Saint Louis Hospital (SLH, Paris, France) and the Children's Oncology Group (COG, Philadelphia, United States of America), which were fully characterized for the aforementioned genetic aberrations; we also investigated the frequencies and prognostic relevance of these recurrent aberrations.

Material and methods

Patient inclusion

We collected data on clinical characteristics and outcome of newly diagnosed pediatric (younger than 18 years at disease onset) patients with non-Down syndrome AMKL.

These patients were diagnosed between 1998 and 2014, and must have available RNA or cDNA for molecular studies and a blast percentage over 20%; they were from 3 consortia: 1) AIEOP, 2) BFM-SG, DCOG and SLH, and 3) COG. Approval for this study had been obtained from the Institutional review board of the participating Centers. Morphological classification, karyotyping and the presence of *KMT2A*-rearrangements were centrally reviewed by each study group.

Screening for recurrent translocations

Screening for molecular aberrations was performed in the Erasmus MC-Sophia Children's Hospital for samples provided by the AML BFM-SG, COG, DCOG and the SLH, while samples provided by the AIEOP group were screened at the University of Padova and Bologna. All patients were screened for *NUP98/KDM5A, CBFA2T3/GLIS2* and *RBM15/MKL1* with RT-PCR, using specific primers for each fusion gene, as previously described (Primers; Supplementary Table 5.1). [5, 17] In case of a positive sample, purified PCR product was directly sequenced on a PRISM 3100 genetic analyzer (Applied Biosystems) and analyzed using CLCWorkbench (Version 3.5.1; CLC Bio) to confirm the translocation. A higher incidence of AMKL is seen in Down syndrome patients, and these children are usually characterized by a *GATA1*s mutation.[21-23] To analyze the presence of *NUP98/KDM5A, CBFA2T3/GLIS2* and *RBM15/MKL1* also in Down syndrome AMKL, a cohort of 16 cases was provided by DCOG and AML BFM-SG. All positive *KMT2A*-rearranged cases (diagnosis being based on either *KMT2A* split signal FISH or cytogenetic analysis as performed by each study group) were screened for partner genes using specific primers for *KMT2A/MLLT3, KMT2A/MLLT10, KMT2A/MLLT4(MLL/AF6), KMT2A/MLLT1(MLL/ENL), KMT2A/ELL,* and *KMT2A/MLLT6(MLL/AF17),* and the purified PCR product was directly sequenced (Primers; Supplementary Table 5.1).[18, 19]

Statistical analyses

Statistical analyses were performed with SPSS Statistics 20 (IBM, Armonk, NY, United States of America). To assess outcome, the following parameters were used; i) probability of achieving CR1; ii) probability of event-free survival (pEFS); iii) probability of overall survival (pOS); and iv) cumulative incidence of relapse or induction failure (pCIR). CR was defined as less than 5% blasts in the bone marrow, with regeneration of tri-lineage hematopoiesis, and absence of leukemic cells elsewhere. pEFS was defined as the time between diagnosis and first event, including relapse, death due to any cause, and second malignancy, whichever occurred first. For calculation of EFS, lack of achieving CR was considered an event occurring on day 0. pOS was defined as the time between diagnosis and death. Both pEFS and pOS were estimated by the Kaplan-Meier method, and groups were compared with the log-rank test. pCIR was defined as time between diagnosis and relapse, with non-responders being attributed an event on day 0, and was analysed by the Kalbfleisch and Prentice method taking into account death and second malignancy as competing event; groups were compared with the Gray's test. A Cox regression analysis was performed for EFS, OS and for the probability of relapse-free survival (pRFS) considering the following co-variables: cytogenetic subgroup, age, sex, white blood cell count (WBC) at diagnosis, and HSCT (as time-dependent variable), as well as the previously described risk factor monosomy 7.[11,24,25] Furthermore, we

included a hyperdiploid karyotype in the Cox regression, which was reported to result in a better outcome.[26,27] Statistical significance was considered if p-values were <0.05.

Results

Clinical characteristics

The AIEOP group included 47 patients, the BFM-SG, DCOG and SLH consortium included 45 patients, and COG provided 61 patients. All 153 patients were eligible for screening of the four aberrations. Median age was 1.6 years (range 0.1-17.1), median WBC at diagnosis was 13.7x109/L (range 1.1-378.5x109/L), and 46% of the included patients were males (Table 1). Patients were treated with different treatment protocols according to their respective study group. All protocols consisted of intensive chemotherapy using an anthracycline and cytarabine backbone for both induction and consolidation; in addition, 41% of patients received HSCT in CR1. HSCT was performed in 66% of the patients reported by the AIEOP group, in 44% of the COG cases, and in 11% of the cases provided by DCOG, BFM and SLH. Patient characteristics are shown in Table 1.

Twenty-four (16%) samples were positive for *CBFA2T3/GLIS2*, eighteen (12%) samples harbored the *RBM15/MKL1* fusion gene, *NUP98/KDM5A* was present in 14 (9%) of cases, and fourteen samples were positive for a *KMT2A*-rearrangement, all diagnosed with FISH. Three *KMT2A*-positive cases had no karyotype available, in the other 11 the *KMT2A* rearrangement was also detected through karyotype analysis. Different fusion partners were identified; 9/14 were positive for *KMT2A/MLLT3*, three were identified with fusion *KMT2A/MLLT10*, one case each harbored either *KMT2A/MLLT1* or *KMT2A/MLLT6* (Supplementary Table 2). Age at diagnosis was significantly lower in the RBM15/MKL1 positive group, compared to the other pediatric AMKL cases (0.7 years, range 0.1-2.7, p=0.035). Eighty-three patients were characterized as 'other', as they were negative for any of the above mentioned genetic aberrations. Other karyotypes identified were t(1:22)(p13;q13), but this case was negative for *RBM15/MKL1* using RT-PCR, and t(16;21)(q24;q22.1), which was positive for *RUNX1/CBFA2T3* (Supplemental Table 3). The 'other' subgroup had a median age of 1.6 years (range 0.1-17.1), and a median WBC of 13.8 x109/L (range 1.1-378.5 x109/L).

Detailed data on karyotype was lacking in 34/153 cases. Twenty cases were cytogenetically normal. Cytogenetics showed 30 cases with an acquired trisomy 21, another 8 cases were identified to carry monosomy 7, 33 cases were hyperdiploid with 49-84 chromosomes, and 31 showed a complex karyotype (defined as 3 or more aberrations excluding trisomy's). Some of these cases belonged to more than one of these cytogenetic subgroups (see also Supplementary Table 4 for details).

Survival analyses

The median follow-up of surviving patients was 67 months (range 2.7-259.0). The 4-year pOS of the entire pediatric non-Down syndrome AMKL cohort was 56±4%, the 4-year pEFS was 51±4% and the 4-year pCIR 29±4%. To test whether our cohort (n=146, excluding the 7 cases present in both this study and the study published by Inaba et al[11]) had a selection bias, our patients were compared for outcome with all non-Down syndrome AMKL cases in the BFM cohort recently published by Inaba et al[11] (n=97), and

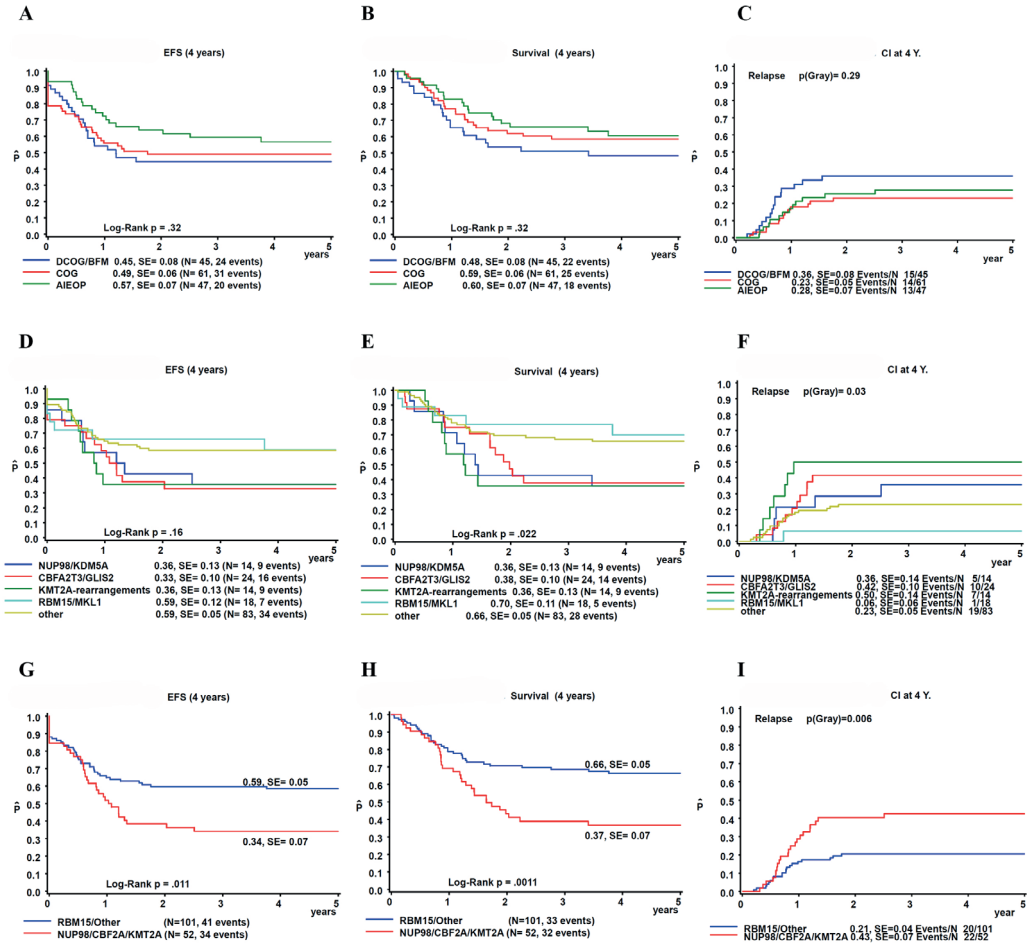

Figure 5.1. Survival curves of pediatric non-Down Syndrome AMKL patients. (A-C) 4-year pOS, pEFS and pCIR comparing the outcome of patients of the different enrolled study groups. There is no significant difference in outcome between the study groups. (D-F) 4-year pOS, pEFS and pCIR comparing the described cytogenetic subgroups as identified in pediatric non-Down syndrome AMKL. *RBM15/MKL1* positive cases and other pediatric non-Down syndrome AMKL have a favorable outcome compared to *NUP98/KDM5A, CBFA2T3/GLIS* and *KMT2A*-rearranged cases. (G-I) 4-year pEFS, pOS and pCIR of the *NUP98/KDM5A, CBFA2T3/GLIS2* and *KMT2A*-rearranged cases compared to the *RBM15/MKL1* and other pediatric non-Down syndrome AMKL cases. Harboring *NUP98/KDM5A, CBFA2T3/GLIS2* or *KMT2A*-rearrangements confers to poor outcome.

outcome was comparable for pOS (56±4% vs 60±5%, p=0.51), pEFS (51±4% vs 47±5%, p=0.62) and pCIR (29±4% vs 32±5%, p=0.62).

Outcome did not differ significantly between the different collaborative groups. The 4-year pEFS of all non-Down syndrome AMKL cases was 57±7%, 45±8%, and 49±6% for the AIEOP, BFM/DCOG/SLH, and COG cohorts, respectively (p=0.32, Figure 5.1A). The 4-year pOS was 60±7%, 48±8%, and 59±6% (p=0.32, Figure 5.1B), and 4-year pCIR was 28±7%, 36±8%, and 23±5% (p=0.29, Figure 5.1C).

When we analyzed the outcome of patients according to the specific recurrent molecular lesions, we found that *RBM15/MKL1* and other pediatric AMKL showed

a favorable prognosis with 4-year pOS of 70±11% and 66±5%, respectively, versus a poorer outcome in *NUP98/KDM5A, CBFA2T3/GLIS2* and *KMT2A*-rearranged (collectively referred to as 'NCK-positive') patients (4-year pOS 36±13%, 38±10% and 36±13%, respectively; p=0.022), with a favorable but not statistically significant different pEFS (p=0.16, Figure 5.1D-E). The difference in outcome was mainly due to the presence of relapsed and refractory cases, as 22 and 9 of the 52 NCK patients either relapsed or did not achieve CR, respectively. The pCIR of *RBM15/MKL1* translocated cases was 6±6%, compared with 36±14%, 42±10% and 50±14% for the cases positive for *NUP98/KDM5A, CBFA2T3/GLIS2* and the *KMT2A* rearrangements respectively (p=0.03, Figure 5.1F, Supplementary Table 5.2). The pCIR differed significantly between the NCK-positive patients and the remaining patients (4-yr pCIR of NCK-cases being 43±7% vs 21±4% for *RBM15/MKL1* and other cases, p=0.006). This lower pCIR translated into a worse EFS (34±7% for NCK-cases vs 59±5% for *RBM15/MKL1* and other cases, p=0.011), and a lower probability of OS (37±7% vs 66±5%, p=0.001, Figure 5.1G-I).

Apart from relapsed and refractory cases, other events leading to mortality among the patients with one of the identified fusions consisted of infections (n=3, all *CBFA2T3/GLIS2* positive), toxicity (n=2, in a *NUP98/KDM5A*-positive and a *RBM15/MKL1*-positive case), graft-versus-host disease (GVHD) after HSCT (1 *KMT2A*-rearranged and 1 *RBM15/MKL1*-positive case), hemorrhage in a *RBM15/MKL1* translocated case, and acute respiratory distress syndrome (ARDS) in a *KMT2A*-rearranged case (Supplementary Table 5.2).

Multivariate analysis

Harboring *NUP98/KDM5A, CBFA2T3/GLIS2* or a *KMT2A*-rearrangement was the an independent risk factor for RFS and OS (HR 2.13, 95% CI;1.08-4.23, p=0.030; and HR 2.17, 95% CI;1.26-3.77 p=0.006, respectively, Table 2), when other risk factors such as sex, age at diagnosis, WBC, HSCT, monosomy 7 or hyperdiploid karyotype were included in the model. Harboring a monosomy 7 was found to be an independent risk factor for overall survival, but not for EFS or RFS, and a hyperdiploid karyotype resulted in a favorable outcome for EFS and OS (Table 2).

In the NCK groups, 35% of the patients received HSCT, compared with 28% in the *RBM15/MKL1* and other cases. HSCT did not influence the RFS (HR 0.93, 95% CI;0.46-1.84, p=0.0.824, Table 2). Moreover, the study group reporting cases did not influence the outcome in multivariate analysis.

Discussion

We report the results of an international collaborative study on the prognostic value of the recently identified, recurrent (cyto-) genetic aberrations found in non-Down syndrome pediatric AMKL. Pediatric AMKL is a rare entity, which comprises 5-10% of the pediatric non-Down syndrome AML cases and is considered to be characterized by poor outcome. The high incidence of myelofibrosis may complicate cytogenetic diagnosis based on conventional karyotyping, and robust prognostic markers are still to be identified.[1, 4-6, 8, 11] In our cohort, the translocations *CBFA2T3/GLIS2, NUP98/KDM5A, RBM15/MKL1* and rearrangements of *KMT2A* accounted for 46% of the pediatric AML cases, and harboring translocation *CBFA2T3/GLIS2, NUP98/KDM5A,* or a *KMT2A*-rearrangement appeared to be an independent prognostic factor for poor

outcome. This poor outcome was mainly due to a high incidence of non-response and relapse in these patients, as seen in 43% of these cases in our cohort. Pediatric non-Down syndrome AMKL arises in very young patients, the median age at diagnosis being below 2 years. Our study shows that neither age, nor , sex, or WBC at diagnosis are independent prognostic factor in pediatric non-Down syndrome AMKL. In addition, there were no differences in presenting characteristics between the various genetic groups, with the exception of *RBM15/MKL1* positive cases, which had a significant younger age at diagnosis.

Our data confirm the poor prognosis for overall survival of monosomy 7 cases, which was previously described in pediatric AML.[11,24,25,28] Previous studies showed the association between a hyperdiploid karyotype and AMKL, and it was suggested that hyperdiploidy is associated with better outcome, a finding confirmed in this study for EFS and OS.[26,27] The outcome of *RBM15/MKL1* positive cases is not uniform in different studies due to the nature of the events. The studies of Inaba *et al* and Schweitzer *et al* describe an intermediate outcome, with low EFS. However, Inaba *et al* reported that most events were early deaths. Schweitzer *et al* also described an intermediate outcome, but low EFS (event described in 6/8), and a high non-remitter rate was reported in this group. In our study, only 1/18 patients harboring *RBM15/MKL1* suffered from early death, while two cases die due to treatment-related mortality, and two cases died due to leukemia progression. This better outcome may be the result of differences in supportive care, which is especially in patients of very young age.

An important difference between the treatment protocols of the various collaborative groups in this study was the inclusion of HSCT in first CR in case of an available donor. However, in multivariate analysis, HSCT did not influence the pRFS. The added value of HSCT for pediatric AML in general is under discussion. There is clear evidence that allogeneic HSCT has a greater anti-leukemic potential than chemotherapy as post-remissional treatment, but this favorable effect may be blunted by a higher risk of treatment-related mortality. [9,10,29] Nevertheless, HSCT in first remission is recommended in many AML treatment protocols for high-risk cases as defined by presence of genetic aberrations or early response to treatment (i.e. high levels of minimal residual disease, MRD, after 1 or 2 courses of induction chemotherapy). Based on our results, a benefit of HSCT could be demonstrated in this retrospective analysis of pediatric AMKL.

Pediatric AMKL is seen in >70% of ML-DS patients.[6,11,30] ML-DS AMKL cases are characterized by mutations in the hematopoietic transcription factor *GATA1,* and these patients have a good prognosis when treated with reduced-intensity chemotherapy due to the unique sensitivity of leukemia cells to chemotherapeutic agents.[21,22,30-34] *GATA1* mutations have been described in non-Down syndrome AMKL, often seen with an acquired trisomy 21, and it was suggested that these cases fare well in terms of prognosis, although numbers were small.[6,23,35-37] We did not identify the fusion genes *CBFA2T3/GLIS2, NUP98/KDM5A* or *RBM15/MKL1* in the group of Down syndrome patients we screened. Cases of non-Down syndrome AMKL that we could not classify based on the four studied translocations may harbor a *GATA1* mutation, since we were lacking DNA material of a large number of patients in this cohort. Next generation sequencing techniques may be instrumental to unravel other recurrent aberrations in the near future in this relatively large group of AMKL cases, negative for any of the

aforementioned aberrations.

Recently, Inaba *et al* described AMKL risk groups based on cytogenetic data obtained with conventional karyotyping, proposing three groups: good risk including patients carrying 7p abnormalities; poor risk including cases with monosomy 7, or 9p abnormalities, including *KMT2A/MLLT3,* -13/13q- and -15, and intermediate risk which encompasses all other AKML patients.[11] This risk classification may be difficult to use, since AMKL is frequently associated with myelofibrosis, which contributes to difficulties in obtaining sufficient material for cytogenetic analysis. In our study, 34/153 (22%) patients lacked data on karyotype, and could therefore not be included in any of the risk groups as proposed by Inaba *et al*. The percentage of missing karyotypes in our cohort is comparable to the findings of Inaba *et al*[11], although this is higher than that reported in pediatric AML including all morphology subtypes (7-13%).[25] Moreover, conventional karyotyping will not reveal cryptic translocations, such as *CBFA2T3/GLIS2* or *NUP98/KDM5A.* Through screening by RT-PCR, we were able to classify 38 cases with these cryptic events, of which 12 did not have an available karyotype, and the remaining had an analysis not showing the occurrence of these events. Therefore, screening for the most frequent recurrent aberrations will provide additional information to a risk stratification based on conventional karyotyping, revealing additional groups with poor outcome.

With international collaborations novel prognostic subgroups may be identified, which may lead to improved risk-group stratification. Although the overall survival is poor for non-Down syndrome AMKL in general, with an 4-yr pOS of 56% in this study, *NUP98/KDM5A, CBFA2T3/GLIS2* and *KMT2A*-rearranged cases fare worse. We suggest that *NUP98/KDM5A, CBFA2T3/GLIS2* and *KMT2A*-rearrangements in pediatric non-Down syndrome AMKL identify as high-risk patient subgroup, while children belonging to either *RBM15/MKL1* or other pediatric non-Down syndrome AMKL should be considered as a standard risk subgroup.

Altogether, our results indicate that non-Down syndrome AMKL is a heterogeneous disease, and that presence of *NUP98/KDM5A, CBFA2T3/GLIS2* or *KMT2A*-rearrangements confers poor clinical outcome. Screening for these translocations combined with conventional karyotyping in pediatric non-AMKL is advisable in future studies for improving risk-group stratification and tailoring treatment intensity according to the biological characteristics of the leukemic clone.

Table 5.1. Presenting clinical characteristics of pediatric non-Down syndrome AMKL patients (n=153).

		CBFA2T3/GLIS2 (n=24)	*NUP98/KDM5A* (n=14)	*KMT2A*-r (n=14)	*RBM15/MKL1* (n=18)	Other (n=83)	All (n=153)
Median age (range)		1.5 y (0.5-4.0)	1.9 y (0.8-8.5)	1.9 y (0.7-12.0)	0.7 y* (0.1-2.7)	1.6 y (0.1-17.1)	1.6 y (0.1-17.1)
Median WBC (range)		17.3 x10^9/L (7.5-300.1)	14.0 x10^9/L (5.8-188.0)	7.4 x10^9/L (1.1-31.0)	13.8 x10^9/L (5.6-32.7)	13.8 x10^9/L (1.1-378.5)	13.7 x10^9/L (1.1-378.5)
Male		25 %	43 %	71 %	33 %	51 %	46 %
Origin	AIEOP	42 %	21 %	36 %	17 %	31 %	31 %
	DCOG/BFM/SLH	17 %	50 %	36 %	28 %	29 %	29 %
	COG	42 %	29 %	29 %	56 %	40 %	40 %
HSCT		11 (46%)	4 (29%)	9 (64%)	9 (50%)	30 (36%)	63 (41%)
4-y pEFS		33±10%	36±13%	36±13%	59±12%	59±5%	
4-y pOS		38±10%	36±13%	36±13%	70±11%	66±5%	
4-y pCIR		42±10%	36±14%	50±14%	6±6%	23±5%	

* significant lower age at diagnosis (p=0.038).
Abbreviations: *KMT2A*-r indicates *KMT2A*-rearrangement; y, years; WBC, white blood cell count.

Table 5.2. Hazard ratios of cytogenetic and clinical markers for event-free, relapse-free and overall survival in multivariate analysis.

		HR	95%CI	p-value (χ^2)
NCK-positive	EFS	1.61	0.98-2.66	0.061
	RFS	2.13	1.08-4.23	0.030
	OS	2.17	1.26-3.77	0.006
Monosomy 7	EFS	2.44	0.99-6.01	0.052
	RFS	2.91	0.81-10.52	0.103
	OS	3.58	1.41-9.11	0.007
Hyperdiploidy	EFS	0.43	0.21-0.89	0.023
	RFS	0.42	0.16-1.12	0.084
	OS	0.43	0.19-0.96	0.040
Sex (male)	EFS	1.33	0.82-2.14	0.248
	RFS	1.11	0.59-2.10	0.748
	OS	1.37	0.81-2.31	0.237
Age <2 years	EFS	0.71	0.44-1.15	0.167
	RFS	0.58	0.30-1.10	0.097
	OS	0.83	0.49-1.39	0.471
WBC > 20.0x 10^9/L	EFS	1.30	0.78-2.16	0.312
	RFS	1.67	0.86-3.25	0.132
	OS	1.57	0.92-2.69	0.098
HSCT	EFS	1.11	0.60-2.05	0.731
	RFS	0.93	0.46-1.84	0.824
	OS	1.15	0.66-2.01	0.620

Abbreviations; HR indicates Hazard Ratio; CI, confidence interval; NCK, *NUP98/KDM5A, CBFA2T3/GLIS2* or *KMT2A*-rearranged; EFS, event free survival; RFS, relapse free survival; WBC, white blood cell count; HSCT, hematopoietic stem cell transplantation.

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Supplementary data

Supplementary Table 5.1. Primer design and PCR program for identification of *NUP98/KDM5A, RBM15/MKL1, CBFA2T3/GLIS2* and specific *KMT2A*-rearrangements.

Gene	Region	Direction	Primer-sequence	PCR condition
NUP98 *KDM5A*	Exon 12 Exon 27	Forward Reverse	5'-TGGACAGGCATCTTTGTT-3' 5'TCAGCTCCTTTGATTTGTCT3'	10' 95°C, 40 x (15" 95°C, 1'60°C) cc
RBM15 *MKL1*	Exon 1 Exon 6-7	Forward Reverse	5'TTCCCACCTTGTGAGTTCT3' 5'TCAGCCGAGGTCTCTTC3'	10' 95°C, 40x (15" 95°C, 1' 60°C) cc
CBFA2T3 *GLIS2*	Exon 11 Exon 5	Forward Reverse	5'AGCGCACGAGCTCAT3' 5'GAGGGGCAGGAAGAACT3'	10' 95°C, 40x (15" 95°C, 1' 60°C) cc
CBFA2T3 *GLIS2*	Exon 9 Exon 4	Forward Reverse	5'-CGCCGAGGACACAAAG-3' 5'-AGGGGCAGGAAGAACTG-3'	10' 95°C, 40x (15" 95°C, 1' 60°C) Cc
*KMT2A**	Exon 7	Forward	5'-CGTCGAGGAAAAGAGTGA-3'	
MLLT1 (ENL)	Exon 3	Reverse	5'-TACCCCGACTCCTCTACTT-3'	10' 95°C, 40x (15" 95°C, 1' 60°C)
MLLT3 (AF9)	Exon 11	Reverse	5'-ATGTTTCCAGGTAACTCTGTAGT-3'	10' 95°C, 45x (15" 95°C, 1' 60°C, 30" 72°C), 10' 72°C
MLLT4 (AF6)	Exon 3	Reverse	5'-TCCCGATCATCTTTGTTC-3'	10' 95°C, 45x (15" 95°C, 1' 59°C)
MLLT10 (AF10)	Exon 10	Reverse	5'-CTGGAAATTTGCATTTGTAA-3'	10' 95°C, 40x (15" 95°C, 1' 60°C)
ELL	Exon 3-4	Reverse	5'-CCCATGACTGGAGACATACT-3'	10' 95°C, 40x (15" 95°C, 1' 60°C)
MLLT6 (AF17)	Exon 11	Reverse	5'- TTCTGCTTCTATCTCCACCA-3'	10' 95°C, 45x (15" 95°C, 1' 60°C, 30" 72°C), 10' 72°C

* The KMT2A forward primer is used for every KMT2A-rearrangement with a specific reverse primer.

Supplementary Table 5.2. Clinical outcome of patients positive for *CBFA2T3/GLIS2, NUP98/KDM5A, RBM15/MKL1* and *KMT2A*-rearrangements.

PIN	Patient origin	Aberration	Sex	Age at diagnosis	WBC at diagnosis	CR achieved	Relapse	SCT	Event	Death	COD
1	AIEOP	CBFA2T3/GLIS2	male	0,7	12,79	yes	no	yes	no event	no	
2	AIEOP	CBFA2T3/GLIS2	female	0,5	22,8	no	no	no	non-remitter	yes	infection
3	AIEOP	CBFA2T3/GLIS2	female	2,0	9,6	yes	no	yes	no event	no	
4	AIEOP	CBFA2T3/GLIS2	female	1,4	65,08	no	no	no	non-remitter	yes	infection
5	AIEOP	CBFA2T3/GLIS2	female	3,2	13,63	yes	yes	yes	relapse	yes	leukemia
6	AIEOP	CBFA2T3/GLIS2	female	3,0	13,25	yes	no	yes	no event	no	
7	AIEOP	CBFA2T3/GLIS2	male	4,0	7,52	no	no	yes	no event	no	
8	AIEOP	CBFA2T3/GLIS2	female	0,7	55,48	yes	yes	yes	relapse	yes	leukemia
9	AIEOP	CBFA2T3/GLIS2	female	1,6	13,6	yes	no	NA	died in CR	yes	infection
10	AIEOP	CBFA2T3/GLIS2	female	1,9	24,22	yes	yes	yes	relapse	yes	
11	COG	CBFA2T3/GLIS2	male	1,2	155,5	no	no	no	non-remitter	yes	leukemia
12	COG	CBFA2T3/GLIS2	male	2,6	9,5	yes	yes	yes	relapse	no	
13	COG	CBFA2T3/GLIS2	female	3,1	21,9	yes	yes	yes	relapse	yes	leukemia
14	COG	CBFA2T3/GLIS2	female	3,3	19,6	no	no	yes	non-remitter	no	
15	COG	CBFA2T3/GLIS2	female	0,8	300,1	no	no	no	non-remitter	yes	leukemia
16	COG	CBFA2T3/GLIS2	female	1,1	18,6	yes	yes	no	relapse	yes	unknown
17	COG	CBFA2T3/GLIS2	female	2,1	31,1	yes	yes	yes	relapse	yes	leukemia
18	COG	CBFA2T3/GLIS2	female	1,7	24,6	yes	no	no	no event	no	
19	COG	CBFA2T3/GLIS2	female	0,8	11,7	yes	no	no	no event	no	
20	COG	CBFA2T3/GLIS2	female	3,4	16	yes	no	no	no event	no	
21	DCOG/BFM	CBFA2T3/GLIS2	male	1,2	9,5	yes	yes	no	relapse	yes	leukemia
22	DCOG/BFM	CBFA2T3/GLIS2	female	1,4	12,4	yes	yes	no	relapse	yes	leukemia
23	DCOG/BFM	CBFA2T3/GLIS2	female	0,6	10,5	yes	yes	NA	relapse	yes	leukemia
24	DCOG/BFM	CBFA2T3/GLIS2	male	1,5	19,1	yes	no	NA	no event	no	
25	AIEOP	NUP98/KDM5A	male	0,8	188	yes	yes	yes	relapse	yes	leukemia
26	AIEOP	NUP98/KDM5A	female	2,1	5,83	yes	no	yes	no event	no	
27	AIEOP	NUP98/KDM5A	female	2,3	28,4	yes	yes	NA	relapse	yes	leukemia
28	COG	NUP98/KDM5A	female	1,8	9,7	no	no	yes	non-remitter	yes	leukemia
29	COG	NUP98/KDM5A	male	3,2	5,9	yes	no	no	no event	no	
30	COG	NUP98/KDM5A	female	3,3	13,1	yes	no	no	no event	no	
31	COG	NUP98/KDM5A	male	2,5	20,1	yes	yes	yes	relapse	yes	leukemia
32	DCOG/BFM	NUP98/KDM5A	male	1,3	8,4	yes	no	no	no event	no	
33	DCOG/BFM	NUP98/KDM5A	female	1,4	14,9	yes	no	no	no event	no	
34	DCOG/BFM	NUP98/KDM5A	male	1,8	11,5	no	no	no	non-remitter	yes	leukemia
35	DCOG/BFM	NUP98/KDM5A	female	1,4	19	yes	yes	no	relapse	yes	leukemia
36	DCOG/BFM	NUP98/KDM5A	male	8,5	11,7	yes	no	no	died in CR	yes	toxicity
37	DCOG/BFM	NUP98/KDM5A	female	4,9	NA	yes	yes	no	relapse	yes	leukemia
38	DCOG/BFM	NUP98/KDM5A	female	1,7	NA	yes	no	no	died in CR	yes	died in CR
39	COG	KMT2A/MLLT1	male	12,0	1,1	yes	no	yes	no event	no	
40	AIEOP	KMT2A/MLLT10	male	2,1	6,8	yes	yes	yes	relapse	yes	leukemia
41	DCOG/BFM	KMT2A/MLLT10	male	4,1	NA	yes	no	no	no event	no	
42	DCOG/BFM	KMT2A/MLLT10	male	7,6	2,3	yes	yes	no	relapse	yes	leukemia
43	AIEOP	KMT2A/MLLT3	female	4,0	6,3	yes	no	yes	no event	no	GVHD
44	AIEOP	KMT2A/MLLT3	male	2,7	7,4	yes	no	yes	no event	no	
45	AIEOP	KMT2A/MLLT3	female	1,1	14,8	yes	yes	yes	relapse	yes	leukemia
46	AIEOP	KMT2A/MLLT3	male	1,0	12,5	yes	no	yes	no event	no	
47	COG	KMT2A/MLLT3	male	1,4	6,6	yes	yes	yes	relapse	yes	leukemia
48	COG	KMT2A/MLLT3	male	1,4	15,8	no	no	yes	non-remitter	yes	leukemia
49	DCOG/BFM	KMT2A/MLLT3	male	1,8	16,3	yes	yes	no	relapse	yes	leukemia
50	DCOG/BFM	KMT2A/MLLT3	female	1,8	NA	yes	yes	no	relapse	yes	leukemia
51	DCOG/BFM	KMT2A/MLLT3	male	0,7	31	yes	yes	no	relapse	yes	leukemia
52	COG	KMT2A/MLLT6	female	4,0	5,6	yes	no	yes	ARDS	yes	ARDS
53	AIEOP	RBM15/MKL1	female	1,5	10,69	yes	no	yes	no event	no	
54	AIEOP	RBM15/MKL1	male	1,1	9,8	yes	no	yes	GVHD	yes	GVHD
55	AIEOP	RBM15/MKL1	male	1,0	12,9	yes	no	yes	no event	no	
56	COG	RBM15/MKL1	female	1,3	18,9	yes	no	yes	no event	no	
57	COG	RBM15/MKL1	female	0,2	22,4	yes	no	no	no event	no	
58	COG	RBM15/MKL1	female	0,9	13,8	yes	yes	yes	relapse	yes	leukemia
59	COG	RBM15/MKL1	female	0,1	32,7	yes	no	yes	no event	no	
60	COG	RBM15/MKL1	female	0,8	13,2	no	no	no	non-remitter	yes	leukemia
61	COG	RBM15/MKL1	female	0,6	11,7	yes	no	no	no event	no	
62	COG	RBM15/MKL1	male	0,1	27,6	yes	no	yes	no event	no	
63	COG	RBM15/MKL1	female	2,0	16,4	yes	no	no	no event	no	
64	COG	RBM15/MKL1	male	0,4	14,8	no	no	yes	non-remitter	no	
65	COG	RBM15/MKL1	female	2,6	5,6	yes	no	no	no event	no	
66	DCOG/BFM	RBM15/MKL1	male	0,2	NA	yes	no	no	died in CR	yes	toxicity
67	DCOG/BFM	RBM15/MKL1	female	0,2	NA	yes	no	no	no event	no	
68	DCOG/BFM	RBM15/MKL1	male	0,6	6,9	yes	no	yes	no event	no	
69	DCOG/BFM	RBM15/MKL1	female	0,6	23,4	no	no	no	early death	yes	hemorrhage
70	DCOG/BFM	RBM15/MKL1	female	2,7	5,9	yes	no	NA	no event	no	

Supplementary Table 5.3. Patient characteristics and karyotype data for patients negative for *CBFA2T3/GLIS2, NUP98/KDM5A, RBM15/MKL1*, and *KMT2A*-rearrangements. (part 1/2)

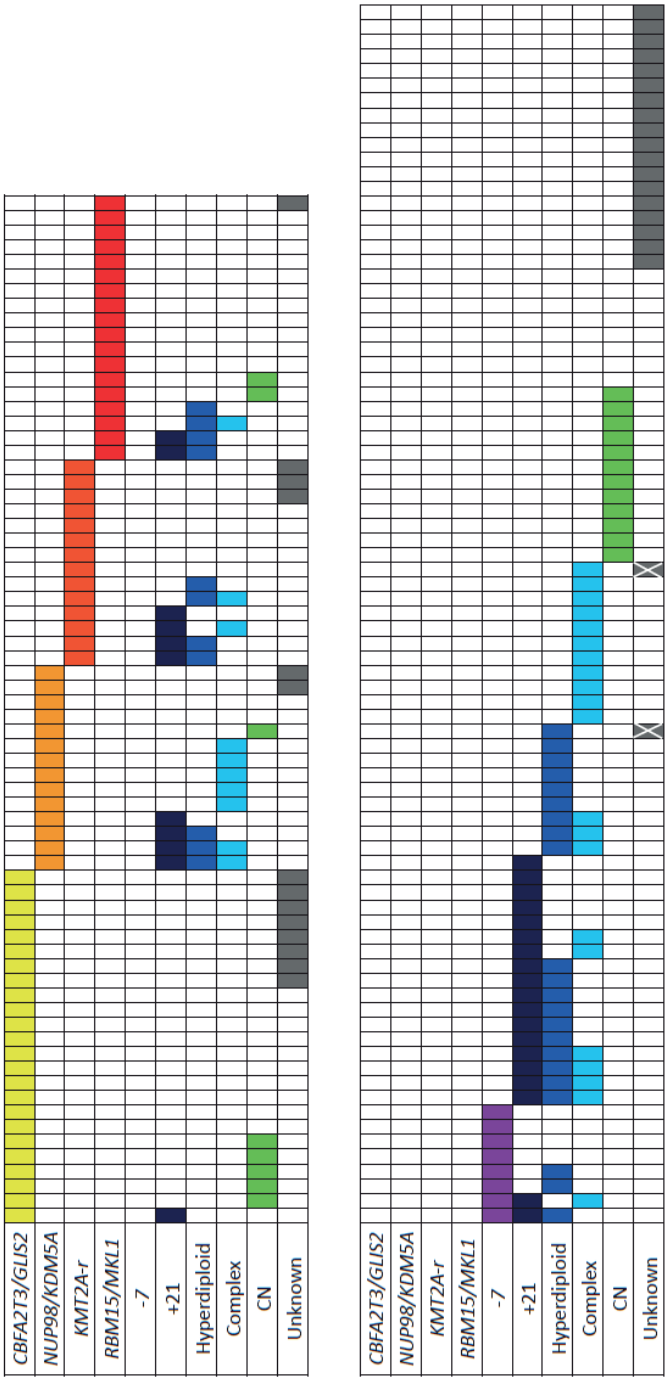
PIN	Patient origin	Karyotype	Sex	Age at diagnosis (y)	WBC at diagnosis
71	AIEOP	46,XX	Female	0,8	8,4
72	AIEOP	46,XX	Female	1,5	13,4
73	AIEOP	46,XX	Female	2,2	8,6
74	AIEOP	46,XY	Male	2,2	10,7
75	AIEOP	46,XY	Male	1,4	22,4
76	AIEOP	46,XX	Female	7,3	59,0
77	AIEOP	46,XX	Female	2,4	2,5
78	AIEOP	46,XY	Male	1,8	12,9
79	COG	46,XX[20]	Female	15,1	1,1
80	COG	46,XX[25]	Female	1,3	15,5
81	COG	46,XY[30]	Male	2,2	11,9
82	DCOG/BFM	46,XY[16]	Male	0,7	16,1
83	DCOG/BFM	46,XY[21]	Male	1,1	23,3
84	COG	45,XX,-7[15]	Female	17,1	16,8
85	COG	45,XX,-7[6]/45,XY,-7,del(11)(p11.2p15)[14]	Female	11,7	90,2
86	DCOG/BFM	38~47,XX,-7,-8,+8,+19[cp14]/46,XX[7]	Female	1,5	12,2
87	DCOG/BFM	45,XX,-7[24]	Female	0,7	68,4
88	DCOG/BFM	50,XY,del(7)(p13),+8,+19,+21,+21[10]	male	1,2	66,0
89	DCOG/BFM	46,XX,-6,-7,del(12)(p12),-19,add(21)(p11),+mar1,+mar2,+r[9]/46,XX[1]	Female	1,1	NA
90	AIEOP	49,XY+2,del(6)(q?),-7,-9,?17,+?19,+,mar,1 dmin(cp5)/46,XY(16)	Male	0,5	8,7
91	AIEOP	46,XY,i(21)(q10)[16]/46,XY[8]	Male	1,7	7,8
92	AIEOP	47,XY,+21[2]/46,XY[21]	Male	2,7	14,9
93	AIEOP	48,XX,+21,+21[15]/49,idem,+14[5]/47,XX,+21[1]	Female	1,4	19,3
94	COG	47,XY,+21[6]/47,idem,inv(6)(p24q21)[13]/46,XY[1]	Male	2,0	22,8
95	DCOG/BFM	47,XX,+21/46,XX	Female	1,0	NA
96	DCOG/BFM	48,XY,+19,+21	Male	1,6	14,5
97	DCOG/BFM	49,XY,+16,del(17)(p11),+19,+21,+21,-22[5]/46,XY[22]	Male	2,0	52,2
98	DCOG/BFM	48,XY,+19,+21,+21,-22[5]/46,XY[22]	Male	2,2	48,6
99	AIEOP	45,XX,t(3;6)(q27;q25)inv(3)(p13q27),-7[22]	Female	15,0	8,8
100	AIEOP	48,XY,der(1)t(1;13)(p35.1;q3?2),der(7)t(7;17)(p21;?).der(17),dup(17)(q21q23),+21,+21[cp2]/46,X,-Y,der(7)t(7;17),inv(13)(p12-13q14), -17,dup(17),+21,+21[20]	Male	1,6	6,3
101	AIEOP	51,XX,+2,add(2)(q3??),del(6)(q2?),del(7)(p1?4),+8,+9,add(9)(q?),+?10,+19[2]/46,XX[18]	Female	2,2	14,9
102	COG	45,XX,add(7)(p22),-9,add(10)(q26),add(13)(q34),add(15)(p11.1)[cp7]/46,idem,+8,add(22)(p11.1)[2]/46,XX[11]	Female	1,6	9,7
103	COG	45,XY,add(4)(p14),add(6)(p21.3),del(7)(p15),psudic(19;12)(p13.3;p13)[6]/46,XY[17]	Male	2,3	2,5
104	COG	46,X,der(X)t(X;22)(p22.1;q13),add(6)(q21),add(22)(q13)[22]	Female	0,1	378,5
105	COG	46,XY,+1,der(1:19)(q10:p10),add(4)(q21),-7,add(7)(p13),add(8)(q22),del(11)(q13q23),-12,-13,add(14)(q32),+2-7mar[cp13]/ 56,idem,+add(4)(q21),+7,+10,+11,+13,+14,+21,+22,+2mar[cp4]/46,XY[4]	Male	0,9	23,7
106	COG	46,XY,-1,-2,t(16:21)(q24:q22.1),+der(1)t(1:?)(p36.3:?),+der(2)t(2:?)(q37:?)[3]/48,XY,-1,-2,+19,t(16:21)(q24:q22.1), +der(1)t(1:?)(p36.3:?),+der(2)t(2:?)(q37:?),+der(21)t(16:21)(q24:q22.1)[4]/46,XY,-1,-2,-4,t(16:21)(q24:q22.1),+der(1),t(1:?)(p36.3:?), +der(2)t(?:2)(2:?)(?:p25q37:?),+der(4)t(1:4)(q32:q35)[6]/46,XY,-3,-20,t(16:21)(q24:q22.1),+der(3)t(3:?)(p2?6:?),+der(20) t(?:20)(20:?)(?:p13q13.3:?)[6]/46,XY[1]/46,XY,add(1)(p36.3),add(2)(q37),t(16:21)(q24:q22.1)[3]/48,idem,+19, +der(21) t(16:21)(q24:q22.1)[4]/46,idem,add(2)(q37),+der(2)add(2)(p25)add(2)(q27),der(4)t(1:4)(q32:q35)[6]/46,idem,+1,-add(1)(p36.3),+2, -add(2)(q37),add(3)(p2?6),der(20)add(20)(p13)add(20)(q13.3)[6]/46,XY[1]	Male	5,7	6,7
107	COG	46,XY,t(3;21)(q25;q22.3),del(5)(q31),r(7)(::p22->q36::?p15->?p22::),der(11)(11pter->11q13::11q24->11q22::7p15->7pter)	Male	2,5	18,2
108	COG	46,Y,inv(X)(p11.2q26),add(2)(q21),t(6;8)(q13;q13),t(15;20)(q22;q13.3),add(17)(p13)[12]/46,XY[10]	Male	1,6	26,2
109	COG	47,XX,add(3)(q21),add(5)(p14),add(10)(q26),der(11)add(11)(p13)del(11)(q13q22),+21[21]	Female	1,7	43,7
110	COG	47,XX,der(5)t(5;17)(p13;q21)t(1;5)(q21;q31),der(17)t(5;17)(p13;q21),+19[7]/56,idem,+2,+4,+5,+6,+10,+13,+20,+21, +22[cp13]/46,XX[1]	Female	1,2	90,8
111	COG	47,XY,der(2)del(2)(p11.2)t(2;7)(q11.2;p14),der(7)t(2;7)(q11.2;p14),t(11;16)(q14;q22),del(15)(q11.2q15),add(17)(q25), +mar[cp4]/46,XY[16]	Male	1,3	56,3
112	COG	48,XX,t(2;19)(q14.3;p13.3),der(3)inv(3)(p13q26.3)t(3;4)(q26.3;q21),der(4)t(4;7)(q21;p15),der(7)t(3;7)(q26.3;p15),del(9)(q13q22), +10,+der(19)t(2;19)[12]/46,XX[10]	Female	1,1	13,0
113	COG	49,Y,t(X;1)(q24;q22.1),+6,+8,+mar[15]/46,XY[5]	Male	1,6	18,6
114	COG	50,XX,+2,+4,add(7)(p12),del(7)(p15p21),+17,add(17)(p11.2),+mar[cp2]/46,XX[21]	Female	2,1	13,2
115	COG	50,XX,+7,+8,+10,der(17)t(2;17)(q21;p13),+19[14]/46,XX[11]	Female	2,0	19,1
116	COG	50,XY,+6,+7,t(7;10)(p15;q11.2),+8,+19[6]/46,XY[13]	Male	1,2	54,7
117	COG	51,XX,+8,+12,+19,+20,+21[15]/46,XX[5]	Female	0,1	4,0
118	COG	58,X,t(X:7:1)(q22:p15:q21),+5,+6,+8,+8,+8,+der(9)t(9:14)(q22:q32),t(9:14)(q22:q32),+t(9:14),+10,+15,+19,+22[16]/46,XY[4]	Female	1,5	10,1
119	COG	61,XX,+2,+6,+6,+7,+8,+8,+10,+13,+13,+17,+19,+19,der(19)t(1:19)(q11:q13.4),+20,+21,+21[cp10]/122,idemx2[cp4]/46,XX[6]	Female	2,3	8,7
120	COG	63,XY,+Y,+der(1:12)(p10:p10),+2,add(3)(q25),+4,der(7)t(3:7)(p22:q36),+der(7)t(3:7)(p22:q36),+8,+9,-10, +14,+15,+18,+19,+19,+20,+21,+22,+3mar[16]/46,XY[22]	Male	1,1	14,4
121	COG	84,XXX,-X,-1,-2,-4,-5,add(5)(q33)x2,-7,-9,+10,-11,-14,-16,-18,+20,+21,+21,-22,add(22)(q11.2)[13]/46,XX[7]	Female	3,0	9,8
122	DCOG/BFM	42-44,XX,-2,add(5p),add(6q),+8,-16,-17,-19,+mar1,+mar2[cp10]/46,XX[14]	Female	2,1	47,6
123	DCOG/BFM	47,XY,+3,der(20)t(1;20)(q12;q2?1)[14]/47,X,der(Y)t(1,Y)(q12,q12),+3[7]	Male	0,5	8,5
124	DCOG/BFM	48,X,t(X;7)(q22?;p15),+10,-16,+der(16)t(2;16)(q12;p13),+der(1q)t(19;?)(p133;?)	Female	1,8	16,8
125	DCOG/BFM	50~55,XY,t(2;7)(p11;p11),+der(2)?t(2;7)(p11;p11),+6,+der(7)t(7;14)(p12;q11)[2],+8,+8[3],+13[9],-14[2], +der(10)t(10;19)(q21;q13.4)[8],+der(19)t(10;19)(q21;q13.4),+21[4][cp13]/46,XY[7]	Male	1,5	13,7

Supplementary Table 5.3. Patient characteristics and karyotype data for patients negative for *CBFA2T3/GLIS2, NUP98/KDM5A, RBM15/MKL1*, and *KMT2A*-rearrangements. (part 2/2)

PIN	Patient origin	Karyotype	Sex	Age at diagnosis (y)	WBC at diagnosis
126	DCOG/BFM	52chromosomes,XY,+8,+14,+16,+21,+21,+21[16]	Male	2,1	8,5
127	DCOG/BFM	52,XY,del(1)(q41),+4,+6,t(7;22)(q31;q13),+del(8)(q24),+9,+19+21,inc[7]/46,XY[5]	Male	0,8	NA
128	DCOG/BFM	76~80,XX,+X,+X,+1,+2,+3,+3,+4,+5,+5,-6,+del(6(q16),+7,+8,+8,+der(9)t(1;9)(q2?2;q34)x2, +10,+11,+12,+13,+14,+15,+17,+18,+18,+19,+19,+der(21)t(16;21;?)x2,+22,+22,+1~4mar[cp24]/46,XX[2]	Female	12,5	8,7
129	AIEOP	47,Y,t(X;16)(q26;q23),+21[19]/47,XY,+21[5]	Male	0,5	8,3
130	COG	46,XX,t(1;22)(q13;q13)[6]/46,XX[14]	Female	0,4	12,3
131	COG	46,XY,del(5)(q31q34)[10]/46,XY,del(9)(q12q32)[2]/46,XY[8]	Male	13,2	1,1
132	DCOG/BFM	46,XY,t(1;22)(p13;q13)[8]/46,XY,[12]	Male	0,3	NA
133	DCOG/BFM	Hyperdiploidyat55~71chromosomes	Male	0,6	31,6
134	DCOG/BFM	Reported as complex karyotype, no specific data available.	Male	0,9	5,6
135	AIEOP	Not available	Male	2,0	26,2
136	AIEOP	Not available	Female	1,0	14,4
137	AIEOP	Not available	Female	3,5	10,9
138	AIEOP	Not available	Female	1,4	8,5
139	AIEOP	Not available	Male	1,5	8,6
140	AIEOP	Not available	Female	0,6	18,7
141	AIEOP	Not available	Male	1,7	52,3
142	AIEOP	Not available	Male	1,9	5,7
143	AIEOP	Not available	Female	1,1	15,7
144	AIEOP	Not available	Male	0,9	5,4
145	COG	Not available	Male	0,9	16,9
146	COG	Not available	Male	6,0	19,9
147	COG	Not available	Female	2,3	11,0
148	COG	Not available	Female	2,6	5,5
149	COG	Not available	Female	2,0	7,7
150	DCOG/BFM	Not available	Male	5,1	14,8
151	DCOG/BFM	Not available	Male	2,4	16,8
152	DCOG/BFM	Not available	Female	2,1	49,1
153	DCOG/BFM	Not available	NA	1,5	NA

Abbreviations: PIN indicates patient identification number; WBC, white blood cell count x109/L; CR, complete remission; SCT, stem cell transplantation; COD, cause of death; NA, not available.

Supplementary Table 5.4. Overview of aberrations found in the karyotypes of pediatric AMKL (n=153). Each column represents a single case. A cell colored means positive for the aberration corresponding with that particular row.



X; karyotype is not available, only information given is hyperdiploid, or complex karyotype.
Abbreviations: KMT2A-r indicates KMT2A-rearrangement; +21, trisomy 21; CN, cytogenetically normal; -7, monosomy 7.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PART TWO

Aberrations and translocations in pediatric acute myeloid leukemia

Chapter 6

ETV6 aberrations are recurrent in pediatric acute myeloid leukemia

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Submitted

Abstract

ETV6 encodes a transcription factor that acts as tumor suppressor and is required for proper hematopoiesis of all lineages. *ETV6*-aberrations strongly associate with leukemia. In pediatric AML, the *MNX1/ETV6* fusion is found in infants, and characterizes by a poor clinical outcome. However, the role of alterations of the *ETV6* gene in pediatric AML have not been fully addressed. We aimed to determine whether *ETV6* aberrations are recurrent by screening for mutations with direct sequencing, for deletions by MLPA, and for translocations using split-signal FISH and RNA-sequencing, and analyzed the clinical relevance by studying outcome parameters. In a cohort of *de novo* pediatric AML patients (n=275) we identified 6 patients with mutations in *ETV6*, 4 patients with an *ETV6* deletion and 9 patients with an *ETV6* translocation. In patients with an *ETV6* mutation or deletion, several genes were significantly up-regulated, including *CLDN5, DPEP1* and *BIRC7,* as reported earlier for T-cell ALL with functional ETV6 silencing. Six patients were identified harboring *MNX1/ETV*6. RNA-sequencing revealed three additional translocations; *ETV6/ARNT1*, *ETV6/HOXCas2* and *ETV6/HOXA11as*. Poorer survival parameters for *ETV6* aberrated cases were found for overall survival. We conclude that *ETV6* mutations, deletions and translocations are rare but recurrent in pediatric AML.

Introduction

The overall survival (OS) of pediatric acute myeloid leukemia (AML) is ~70% and the 5 year event-free survival (EFS) is ~50-60%.[1-3] Treatment stratification is based on early treatment response and the presence of prognostically relevant genomic aberrations.[4-6] Although many recurrent pathologic mutations in pediatric AML have been defined, in ~20% of the patients the oncogenic event(s) driving the malignancy remain unidentified.[1, 7]

The ETS-Variant gene 6 (*ETV6*, also known as Translocation Ets Leukemia (*TEL*)) located on chromosome 12p13 encodes a transcription factor that is required for homing and survival of all hematopoietic lineages in the bone marrow niche.[8-10] ETV6 operates as a transcriptional repressor using its C-terminal ETS domain for binding ETS binding sites and its N-terminal helix-loop-helix (HLH) domain for homodimerizaton. [11-17] ETV6 was originally identified due to its involvement in translocation t(5;12)(q33;p13) found in chronic myelomonocytic leukemia (CMML).[17] This translocation generates the fusion protein *ETV6*/platelet derived growth factor receptor, beta polypeptide (*ETV6/PDGFRB*).

Over 30 fusion partners of ETV6 have been described, and these gene rearrangements show strong association with leukemia.[18] In pediatric acute lymphoblastic leukemia (ALL), t(12;21)(p13;q22) (*ETV6/RUNX1*, also known as *TEL/AML1* or *TEL/CBFA2*) is the most common alteration with a prevalence of ~25%.[19] It is often accompanied by a complete or partial deletion of the non-translocated *ETV6* allele, leading to absent expression of *ETV6*. [20-22] However, in some cases it has been shown that the expression of *ETV6* is absent, although the second *ETV6* allele is present, suggesting that other mechanisms, such as hyper-methylation or point mutations, may silence the *ETV6* gene.[22]

In early immature adult T-cell acute lymphoblastic leukemia (T-ALL), mutations affecting the predicted amino-acid sequence of *ETV6* were found in 24%.[23] These mutations result in the expression of N-terminally of C-terminally truncated ETV6 proteins unable to repress gene expression, leading to a gene expression profile dominated by up-regulation of target genes. In adult AML, silencing somatic heterozygous *ETV6* mutations and deletions have been found. [24, 25]

In pediatric AML, translocations involving *ETV6* (t(7;12)(q36;p13) (*MNX1/ETV6* or *HLXB9/ETV6*) and t(7;12)(q32;p13)) have only been described under the age of 18 months. These translocations associate with a poor clinical outcome, and are characterized by over-expression of *MNX1*.[26, 27] Mutations of *ETV6* have been identified in pediatric AML using whole genome sequencing.[28] However, the role of alterations of the *ETV6* gene in pediatric AML have not been fully addressed. Therefore, we screened a cohort of pediatric AML patients for mutations, deletions and translocations of the *ETV6* gene.[29] We identified repetitive abnormalities in a small number of cases including heterozygous mutations affecting the predicted amino-acid sequence of *ETV6,* partial or complete heterozygous deletions of *ETV6*, and translocations of *ETV6*, with *MNX1*, *ARNT1* and novel fusion partners. We also assessed the clinical outcome of *ETV6* gene alterations, we determined outcome parameters in patients with *ETV6* gene alterated cases.

Material and methods

Patient samples

Samples were provided by the Dutch Childhood Oncology Group (DCOG, The Hague, The Netherlands), the AML–Berliner-Frankfurt-Münster (BFM) Study Group (Germany and Czech Republic), and the Saint-Louis Hospital (Paris, France). The cohort of newly diagnosed pediatric AML patients with available peripheral blood or bone marrow samples taken at initial diagnosis included in this study was previously described by us (n=293; age, 0-19 years).[29] In case of positivity for an *ETV6* gene alteration, available peripheral blood or bone marrow samples drawn at remission, and relapse material in case of a relapse, were obtained from the cell-banks. Clinical characteristics, morphological classification and karyotyping were centrally reviewed by each study group.

After thawing the samples, leukemic cells were purified as previously described.[30] Blast percentages were assessed morphologically using cytospins stained with a May-Grünwald-Giemsa staining. Isolation of genomic DNA and total cellular RNA from the samples was performed using Trizol reagent. All patients were treated with intensive collaborative group cytarabine-anthracycline based pediatric AML treatment protocols. Each collaborative study group provided us with centrally reviewed morphological and cytogenetic classification and clinical follow-up data. Institutional review board approval for these studies had been obtained in the participating centers.

Detection of ETV6 mutations

Using reverse transcriptase polymerase chain reaction (RT-PCR) and direct-sequencing *ETV6* exon 2 through exon 8 were screened for mutational status. Exon specific primers were used (supplementary table 1). Purified PCR products were directly sequenced on a PRISM 3100 genetic analyzer (Applied Biosystems) and analyzed using CLCWorkbench (Version 3.5.1; CLC Bio, Aarhus, Denmark). Reference genome used was ENST00000396373.

Multiplex ligation-dependent probe ampliciation (MLPA)

MLPA was performed on DNA using the SALSA MLPA kit p335-B1 ALL-IKZF1 (MRC Holland, Amsterdam, The Netherlands), which contains 6 probes covering *ETV6*: 2 probes are located in exon 1, the other probes are located in exon 2, 3, 5 and 8. Additionally there are 4 probes covering the *BTG1* gene, which is located on 12q22.33, for confirmation of the presence of chromosome 12 (data available on http://www.mlpa.com). The data were analyzed with GeneMarker (version 1.85; SoftGenetics, State College, Pennsylvania, USA). The data were normalized to reference probes and healthy control samples. A deletion was defined as a peak ratio below 0.75, an amplification was defined as a peak ratio above 1.25 in two independent MLPA experiments. A succeeded MLPA was considered if all reference probes had a peak ratio between 0.75 and 1.25, excluding cases where the karyotype showed a monosomy of the chromosome of the correlating reference probe.[31]

Array comparative genome hybridization (Array-CGH)

Array-CGH was performed on 1µg DNA using the human genome CGH Microarray

105K (Agilent Technologies, Santa Clara, California, USA) according to manufacturer's protocol, and data were analysed with Genomic Workbench (version 5.0.14; Agilent Technologies, Santa Clara, California, USA).

Gene expression profiling

Gene expression profiling (GEP) data of 293 pediatric AML patients were available from earlier studies.[29] Checking RNA integrity, processing the Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, California, USA), and normalizing and analyses of the data were performed as previously described.[29] Original data are available in the Gene Expression Omnibus repository (http://www.ncbi.nlm.nih.gov/geo;accession GSE17855).

Identification of translocations involving ETV6

Split signal FISH experiments were performed for detection of *ETV6* rearrangements, and to confirm deletions as identified with MLPA. Split signal FISH was performed on all cases, excluding the cases where already another driving oncogenic type-II event had been identified. Dual-color FISH experiments were performed using the Y5400 probe (Dako, Glostrup, Denmark) with a texas red-labelled DNA-probe covering the upstream *ETV6*-part including exon 1-3, and a fluorescein-labelled DNA-probe covering a downstream segment of 12p13, on thawed cytospin slides. At least 100 cells were counted. A negative split signal was defined as 2 fusions in ≥80% of the cells. A positive split signal was defined as >20% split signal cells and one fusion present in one sample. A deletion was defined as one fusion in >20% with the expected second fusion absent. All positive samples for split signal were confirmed in a second experiment. The presence of the fusions *MNX1/ETV6* and *ETV6/PDGRFB* was analysed by RT-PCR (primers in Supplementary Table 6.1). Paired-end transcriptome sequencing was performed on diagnostic leukemic cells on the pediatric AML cases with a positive split signal FISH and available high-quality RNA using the Illumina platform as previously described by Zhang et al.[32]

Cytogenetic and molecular characterization

Samples were screened for cytogenetic aberrations using standard chromosome banding analysis at initial diagnosis by the collaborative study groups. To identify other aberrations, RT-PCR was performed on hotspot areas of the following genes; *NRAS, KRAS, NPM1, MLL-PTD, CEBPA, FLT3, PTPN11, KIT* and *WT1,* as previously described by us.[33]

Validation of expression levels

We performed specific real-time quantitative PCR (RT-qPCR) to validate *HOXC9* and *HOXC10* gene expression levels. RT-qPCR was performed in duplicate in cases with at least 80% blasts only. Primer and probe sets are shown in Supplementary Table 6.1. The average cycle threshold (CT) value was used to determine the expression levels of *ETV6* in comparison to the expression levels of the reference gene *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH)*, using the comparative cycle time method. Correlation between GEP data and RT-qPCR was examined.

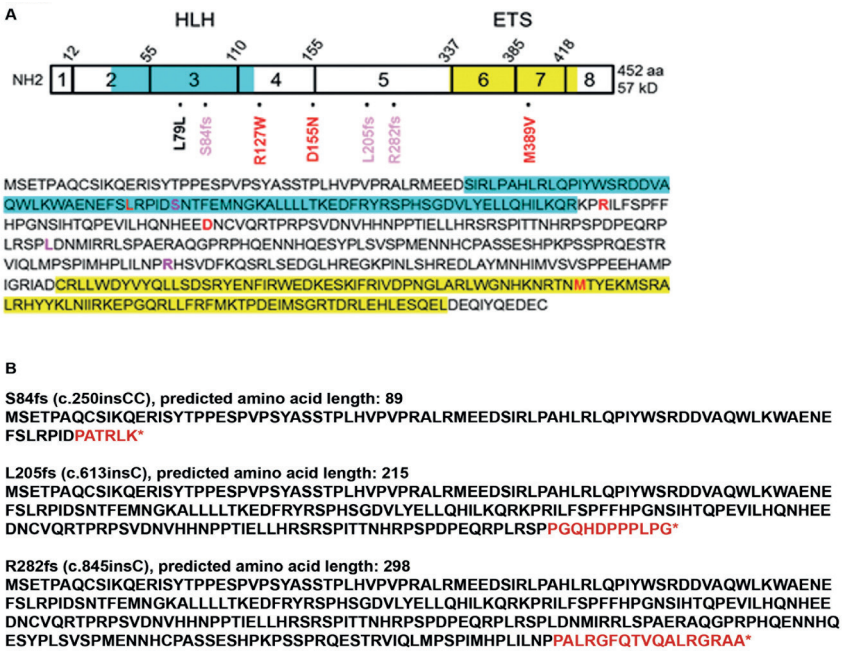

Figure 6.1. Six patients demonstrated a mutation affecting the predicted amino-acid sequence of *ETV6*. (A) Schematic representation of the protein structure of ETV6. The boxes with numbers represent exons 1 through 8 of the ETV6 gene.The in blue highlighted region represents the HLH-domain, the in yellow highlighted region represents the ETS-domain. The letters in green are internal translation initiation sites.[24] The letters in red are the identified point mutations. The letters in pink indicate the amino acids were the identified frame shifts start. (B) The in green highlighted regions represent changed amino acid sequence due to the identified frame shifts.

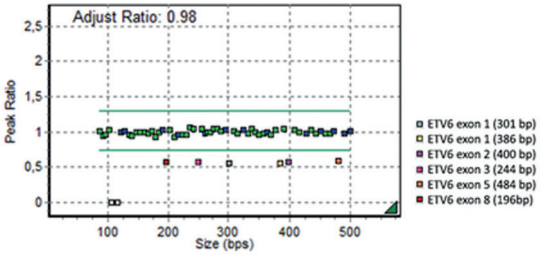

Figure 6.2. Heterozygous *ETV6* deletions in AML. Multiplex ligation-dependent probe amplification results of a patient with heterozygous deletion of exons 1-8 of *ETV6*.

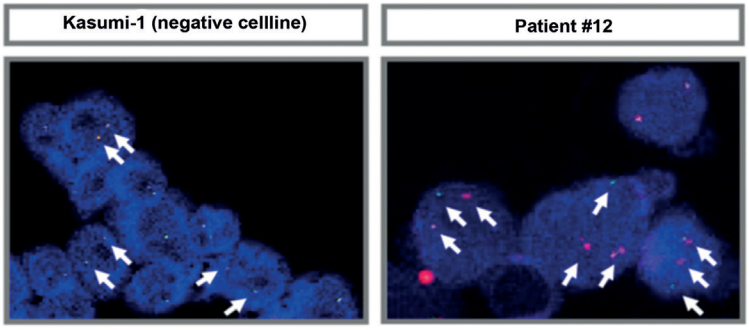

Figure 6.3. Dual-color split-signal FISH analysis of *ETV6*. Split-signal FISH of a (A) negative cell line and (B) positive patient, using the Y5400 probe (Dako, Glostrup, Denmark) with a texas red-labelled DNA-probe covering the upstream *ETV6*-part including exon 1-3, and a fluorescein-labelled DNA-probe covering a downstream segment of 12p13, on thawed cytospin slides.

Statistical analysis

Statistical analyses were performed with IBM SPSS 21 (IBM, Armonk, New York, USA) and SAS for Windows, version 9.3 (SAS, Cary, North Carolina, USA). Categorical variables were compared using the chi-square or Fisher exact test and continuous variables using Mann-Whitney U test. Correlation coefficients were measured using Spearman's correlation. To assess outcome the following parameters were used; complete remission (CR), defined as less than 5% leukemic blasts in the bone marrow, with regeneration of tri-lineage hematopoiesis, and absence of leukemic cells in the cerebrospinal fluid or elsewhere; probability of event-free survival (pEFS), defined as the time between diagnosis and first event including non-responders calculated as an event on day 0, death of any cause and second malignancies; and probability of overall survival (pOS), defined as the time between diagnosis and death. Both pEFS and pOS were estimated by using the Kaplan-Meier method and groups were compared using the log-rank test. The cumulative incidence of relapse (pCIR), defined as time between diagnosis and relapse, was analysed by the Kalbfleisch and Prentice method and groups were compared with the Gray's test. Statistical significance was considered if p-values were below 0.05.

Results

Mutations affecting the predicted amino acid sequence of ETV6 are recurrent in pediatric AML.

We screened exon 2 through 8 of *ETV6* by direct sequencing on 20ng genomic DNA of 275 pediatric AML patients. In one patient (patient #1) we found a silent point mutation (TTA>TTG, p.L79L) that has not been previously described (Table 6.1, Figure 6.1A). In 6 patients, we identified heterozygous mutations affecting the predicted amino-acid sequence of *ETV6* (Table 6.1, Figure 6.1A). Three of these 6 patients demonstrated a heterozygous point mutation, leading to the change of one amino acid. Two of those mutations (Patient #2: p.R127W and Patient #3: p.D155N) were located in exon 4, upstream of the HLH-domain (Figure 6.1A). The mutation found in patient #4 (p.M389V) was located in exon 7 and resulted in an amino acid change in the ETS-domain of the *ETV6* gene (Figure 6.1B). SIFT analyses (http://sift.jcvi.org/) predicted that the point mutations found in patient #2 (p.R127W) and #4 (p.M389V) are damaging to the protein function (Table 6.1). Patients #5, #6 and #7 presented with a heterozygous insertion (c.250insCC, c.613insC and c.845insC, respectively) resulting in a frame shift and a shorter predicted amino acid length of 89, 215 and 298 amino acids, respectively (Figure 6.1C). Patient #4, #5, and #6 had remission material available, which were negative for mutations in *ETV6*. Patient #4 and #7 had relapse material available, which showed in both cases the mutation retained at relapse. Together these data demonstrate recurrent mutations affecting the predicted amino acid sequence of *ETV6* at a low frequency (6/275=2.2%) in pediatric AML patients.

ETV6 deletions are recurrent in pediatric AML patients.

To determine deletions of the *ETV6* gene we performed MLPA analyses in 259 cases with available material. We specifically analyzed the probe sets located on chromosome 12, for *ETV6* and the B-cell translocation gene 1 (*BTG1).* We analyzed 14 healthy control

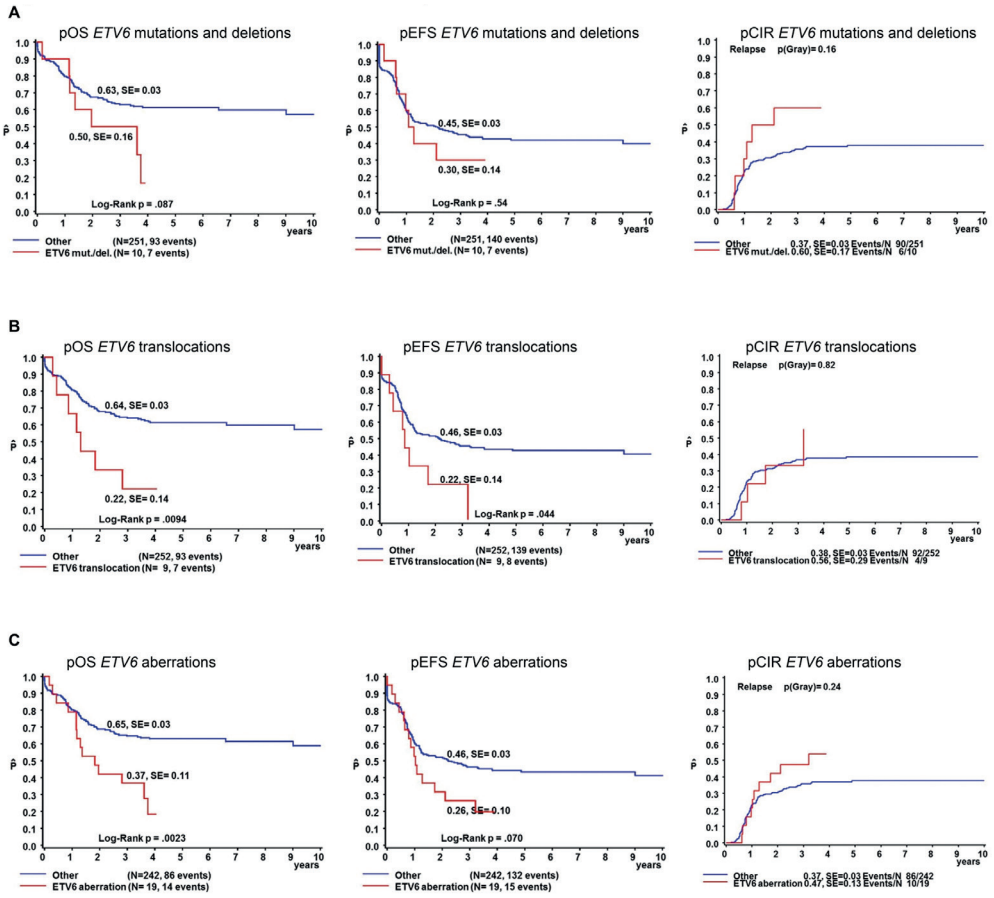

Figure 6.4. Survival estimates of *ETV6*-aberrations compared to other AML. (A) Kaplan-Meier estimates of the 3-year overall survival, event-free survival and cumulative incidence of relapseaccording to the Kalbfleisch and Prentice method of *ETV6*-mutated or deleted cases versus other pediatric AML. (B) Kaplan-Meier estimates of the 3-year overall survival, event-free survival and cumulative incidence of relapse according to the Kalbfleisch and Prentice method of *ETV6*-translocated cases versus other pediatric AML. (C) Kaplan-Meier estimates of the 3-year overall survival, event-free survival and cumulative incidence of relapse according to the Kalbfleisch and Prentice method of *ETV6*-aberrated cases versus other pediatric AML.

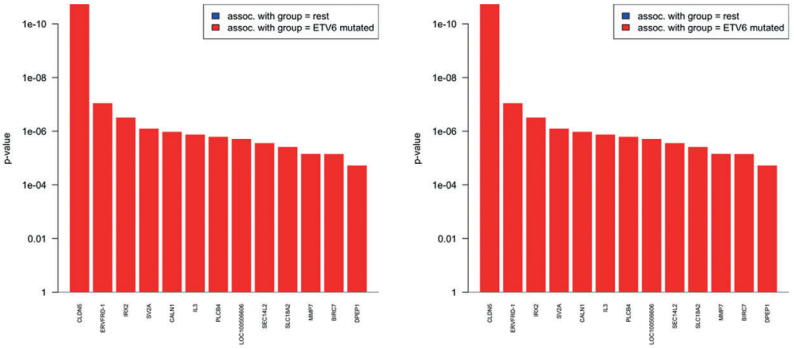

Figure 6.5. *ETV6*-inactivating mutations and deletions associate with an up-regulation of gene expression. Left panel shows plot of the 13 significantly up-regulated genes, ordered by p-value, based on gene expression profiles of *ETV6*-mutated cases versus non-mutated cases. Right panel shows plot of the 38 significantly up-regulated genes of *ETV6*-deleted cases versus non-deleted cases.

samples as negative controls, and the Reh cell line, a B-cell precursor leukemia cell line harboring the *ETV6/RUNX1* fusion and a heterozygous deletion of *ETV6,* as positive control. In three patients, we detected a heterozygous deletion of all analyzed *ETV6* exons (Figure 6.2). In none of these cases the *BTG1* gene was deleted, indicating the event was focal. Array CGH analyses confirmed that the chromosome 12p deletions included the *ETV6* gene locus (Table 6.1). In one patient, we detected a partial *ETV6* deletion, covering exon 1 through 5 and amplification of exon 8 of the *ETV6* gene and the *BTG1* gene by MLPA. Array CGH analyses of this patient confirmed deletion of chromosome 12p13.2, including the telomeric part of the *ETV6* gene (Table 6.1), and demonstrated amplification of chromosome 12q (including the *BTG1* gene) and the centromeric part of 12p, including *ETV6* exon 8. Together, these results show that *ETV6* deletions are recurrent in pediatric AML patients at a low frequency (4/259=1.6%).

ETV6 gene translocations are recurrent in pediatric AML patients.

To identify *ETV6* translocations, we only screened the patients without a known type II mutation (n=65), assuming these type II mutations are mutually exclusive. We used an *ETV6* split signal FISH approach on cytospins to identify translocations involving *ETV6,* including two patients previously identified with the *MNX1/ETV6* translocation as confirmation and positive control (patients #13 and #17, Table 6.2). Three patients were identified with a positive *ETV6* split signal as defined as >20% of the cells containing a split signal and one fusion, were negative for *MNX1/ETV6* as screened with RT-PCR (Figure 3, patients #18-20, Table 6.2).

To determine the translocation partner of *ETV6* in these patients, we performed paired-end RNA sequencing. This resulted in the identification of three different translocations. We identified one case with translocation *ETV6/ARNT*, a fusion previously described in pediatric AML and T-ALL.[34, 35] We found 2 *HOX*-antisense genes as novel *ETV6* translocation partners in pediatric AML; *ETV6/HOXC-AS2,* and *ETV6/HOXA11-AS*. The antisense molecules are not protein coding, and these fusions are most likely to result in a loss of function of *ETV6*, as well as a loss of the specific *HOX*-antisense.

All fusions were confirmed using RT-PCR and direct sequencing (primers in Supplemental Table 6.1). Together these data demonstrate *ETV6* translocations are recurrent in pediatric AML.

Clinical characteristics of the pediatric AML cohort

The median age at diagnosis of the pediatric AML cohort was 9.6 years (range 0.1-18.5yrs), 43% of the cases were female, and the median white blood cell count (WBC) at diagnosis was $45.2x10^9$/L (range 1.2-481.8). Cytogenetic aberrations in this group showed a representative distribution for the frequencies seen in pediatric AML in general (Supplementary Table 6.2).[1, 5] Age at diagnosis (<2 years of age) or treatment including a hematopoietic stem cell transplantation did not influence outcome (HR:0.65; 95%CI0.38-1.11, p=0.113 and HR:0.99; 95%CI0.95-1.95, p=0.97, respectively), and standard risk cytogenetics conferred to a favourable prognostic outcome as expected (HR:0.27, 95%CI0.15-0.50, p<0.01).

The median age of patients with an *ETV6*-mutation or deletion (n=10, excluding the silent mutation) was 11.3 years (range 4.0-15.3 years) and 40% was

female. Median WBC was significantly lower ($15.1x10^9$/L, range 2.8-196.0, p<0.01) in comparison to all other pediatric AML cases. Co-occuring cytogenetic aberrations found in the *ETV6*-mutated or deleted cases were *RUNX1/RUNX1T1* (n=3), *PML/RARA* (n=1), *MLL/AF6* (n=1) and one *NPM1*-mutated case (Table 2). Other co-occuring aberrations in the translocated group were a *KIT* mutation, and the fusion of *BCR* and *ABL1*, both considered to increase cell proliferation, and therefore classified as type-1 mutation.[36, 37]All *ETV6*-mutated or deleted cases achieved complete remission, versus 80% of the other AML cases. Six out of ten patients encountered a relapse and one patient died in complete remission, suggesting poor clinical outcome (Table 6.2) although this could not be proven statistically, as the 3-yr pOS was 50±16% in the *ETV6*-mutated or deleted group (n=10) versus 63±3% in the other AML cases (n=251, p=0.09), the pEFS was 30±14% vs 45±3% (p=0.54), and the pCIR was 60±17% vs 37±3% (p=0.16) (Figure 6.4). Four out of 9 translocated cases encountered a relapse, one patient did not achieve remission and these 5 died due to the leukemia, another two patients died due to treatment-related toxicity.

The 3-yr pOS was 22±14% in the *ETV6*-translocated (n=9) versus 64±3% in the other AML cases (n=252, p<0.01), the pEFS was 22±14% vs 46±3% (p=0.04), and the pCIR was 56±29% vs 38±3% (p=0.82, Figure 6.4).

The age at diagnosis for patients with an *ETV6*-translocation (n=9) was significantly lower compared to all other pediatric AML cases (median 1.1 years, range 0.2-10.4, p=0.0006), median WBC was $21.8x10^9$/L (range 2.4-226.8 $x10^9$/L) vs $45.7x10^9$/L (p=0.52), and 44% was female (table 2).The 3-yr pOS for all combined *ETV6*-aberrant cases (n=19) was 37±11% vs 65±3% for the other pediatric AML cases (n=242, p=0.002), 3-yr pEFS was 26±10% vs 46±3% (p=0.07), and 3-yr pCIR 47±13% vs 37±3% (p=0.24). Harboring an *ETV6* mutation or deletion was an independent factor for prognosis with an increased risk for poor outcome concerning OS (Hazard Ratio (HR):2.45; 95%CI1.11-5.43, p=0.027), but not EFS (HR:1.42; 95%CI0.66-3.08, p=0.373), which was also true for harbouring any *ETV6* aberration (HR:2.27; 95%CI1.27-4.07, p=0.006 and HR:1.62; 95%CI0.94-2.79, p=0.082), also when other well-known risk factors such as sex, age at diagnosis, WBC, treatment including a hematopoietic stem cell transplantation, and standard risk cytogentics such as CBF-AML and *PML/RARA* were included in the model.

ETV6-inactivating mutations and deletions associate with an up-regulation of gene expression.

To assess the effect of *ETV6* inactivation on gene expression, we compared the gene expression profiles of patients with an *ETV6* mutation (n=6) or deletion (n=4) to those of pediatric AML patients without an *ETV6* gene alteration (n=260). In patients with an *ETV6* mutation (n=6) or deletion (n=4) 13 and 38 genes, respectively, were significantly up-regulated (Figure 5) and no genes were significantly down-regulated, consistent with the role of ETV6 as a transcriptional repressor.[11-13]. Interestingly, Claudin 5 (*CLDN5*), the gene that is at the top of the list of differently expressed genes in patients with *ETV6* mutations, is the only gene that overlaps between the top lists of patients with *ETV6* mutations and of patients with *ETV6* deletions. Other genes that are up-regulated in the six patients with *ETV6*-inactivating mutations (Figure 5), are baculoviral IAP repeat containing 7 (*BIRC7*) and dipeptidase 1 (*DPEP1*).

ETV6-HOX translocations associate with an up-regulation of specific HOX genes

RNA antisense has been implicated to control RNA sense expression by sense-antisense pairing. [38, 39] The antisense genes may be a silencer of the assigned *HOX* gene.[40, 41] When comparing gene expression profiles of the *ETV6-HOX* fusions (n=2), we identified up-regulation of specific *HOX* genes. *ETV6/HOXC-AS2* showed a high upregulation of two *HOX* genes; *HOXC9* and *HOXC10*, respectively, which was confirmed with qPCR (data not shown), and *ETV6/HOXA11-AS* showed high expression of *HOXA11* (gene expression previously validated with qPCR[42]).

Discussion

In this study we analyzed the occurrence and frequencies of *ETV6* aberrations in pediatric AML. We identified mutations, deletions and translocations in the *ETV6* gene as recurrent events in pediatric AML, although at low frequency (7%). *ETV6* mutations were found in 2.2% of the cases, which is comparable to the frequency found in adult AML.[24] Additionally we found *ETV6* deletions in 1.6% of patients in our cohort. This is at a lower frequency then identified by Helton *et al*[28] (6%), but they selected for patients that did not have any other cytogenetic or molecular risk features. Helton *et al* found deletions of *ETV6* predominantly in patients with core-binding factor (CBF-)AML. We found an *ETV6* translocation in 9/275 (3.3%) cases, only screening the patients without any known type II mutation, assuming that type II aberrations are mutually exclusive. This is a much lower frequency in comparison to the frequency of *ETV6* translocations found in pediatric ALL.[23] Among the pediatric AML cases identified with an *ETV6* mutation, deletion or translocation, various other non-repetetive genetic aberrations were detected. Our data underscore the heterogeneity in underlying genetic abnormalities seen in AML.[6, 33]

Comparing gene expression patterns of mutated and deleted cases versus other pediatric AML patients, *CLDN5* is the only differentially expressed gene in both categories. This gene has been described as one of the up-regulated transcripts after functional *ETV6*-silencing in T-ALL.[23] This suggests that the mutations and deletions in our series also result in impaired *ETV6* function. *BIRC7* and *DPEP1* are upregulated in the *ETV6* mutated cases. These genes have been associated with leukemia[43, 44] and are also up-regulated transcripts after functional *ETV6*-silencing in T-ALL[23]. No genes were significantly down-regulated in our patients with *ETV6* mutations or deletions, consistent with the role of ETV6 as a transcriptional repressor.[11-13] Together, these findings indicate that, consistently with the role of ETV6 as a transcriptional repressor, *ETV6*-inactivating mutations and deletions result in an up-regulated expression of several genes, amongst which are genes that are associated with leukemia.

ETV6 is known to have many different translocation partners. We identified 4 different, of which two novel, partner genes for *ETV6* in this cohort. The median age of all translocated cases was significantly lower compared to the other pediatric AML cases. This lower age seems specific for *MNX1/ETV6* and *ETV6/ARNT*, since the *ETV6-HOX*-antisense translocated cases were older, 9 and 10 years of age at diagnosis respectively. The novel identified partner genes were *HOX* antisense genes. We previously identified *HOX* gene upregulation in a large subset of pediatric AML.[29, 42] In the two identified translocations, the translocation resulted in an upregulation of the

assigned *HOX* gene(s). The function of the *HOX*-antisense genes is not fully understood. They may be a silencer of the assigned *HOX* gene.[40, 41] Assuming this, the fusion may lead to a loss-of-function of this silencing, resulting in an up-regulation of the *HOX* gene(s).

Although the number of patients with *ETV6* aberrations is low, the survival analyses suggests that *ETV6* aberrations associate with a poor clinical outcome. We could not prove a statistically higher relapse rate perhaps due to small numbers., The poorer outcome in this series was due to both leukemic events and treatment-related mortality.

Altogether, we identified *ETV6* aberrations as rare but recurrent event in pediatric AML, occurring in 7% of the cases, associated with poor overall survival.

Table 6.1. *ETV6* mutations and deletions identified in pediatric AML patients.

ID	base pair change	protein change	Deleted ETV6 probes as seen with MLPA	Deleted coordinates based on a-CGH	predicted amino acid length	SIFT analyses	Relapse material	Remission material
1	TTA>TTG	p.L79L	-	-	452	-	-	NA
2	CGG>TGG	p.R127W	-	-	452	damaging	NA	NA
3	GAT>AAT	p.D155N	-	-	452	tolerated	NA	NA
4	ATG>GTG	p.M389V	-	-	452	damaging	M389V	Negative for mutation
5	250ins.CC	p.S84fs	-	-	89	-	NA	Negative for mutation
6	613ins.C	p.L205fs	-	-	215	-	NA	Negative for mutation
7	845ins.C	p.R282fs	-	-	298	-	845ins.C	NA
8	-	-	1,2,3,5,8	33904 – 21363053		-		NA
9	-	-	1,2,3,5,8	11419189 - 12851240		-		NA
10	-	-	1,2,3,5,8	64510 – 31359743		-	-	NA
11	-	-	1,2,3,5	10289523 - 11923630		-	-	NA

Abbreviations: ID indicates identification number; MLPA, multiplex-ligand probe amplification; a-CGH, array-comparative genome hybridization; SIFT, sorting intolerant from tolerant; fs, frameshift; NA, not available.

Table 6.2. Patient characteristics of *ETV6*-aberrated cases.

ID	Age (y)	Sex	WBC ($x10^9$/L)	FAB	Type-1 aberrations	Type-2 aberrations	CR	Relapse	Death	SCT	COD	Karyotype
1	15.8	M	180.0	M4	*RAS*	inv(16)	Yes	No	No	No	-	46, XY, inv(16)(p13;q22)
2	7.1	M	24.4	M3		*PML/RARA*	Yes	No	No	No	-	46,XY,t(15;17)(q22;q21)
3	10.6	M	22.0	M2	*KIT*	*RUNX1/RUNX1T1*	Yes	No	No	No	-	46, XY
4	6.3	M	196.0	M0	*RAS, WT1*		Yes	Yes	Yes	No	Leukemia	46,XY,del(17)(p12,p13) or add(17)(p11)[2]
5	14.4	M	33.8	M2	*FLT3*-ITD	*RUNX1/RUNX1T1*	Yes	Yes	Yes	Yes	Leukemia	46,XY,t(1;21;8)(p32?5;q22;q22)[18]\47,idem,+8[2]
6	8.5	F	2.9	M4			Yes	Yes	Yes	No	Leukemia	46,XX,del(7)?(p13p21)[1]/46,idem,t(3;7)?(p25;p15)[11]/46,idem,t(3;7)?(p25;p15),del(11)?(p11p14)[13]
7	12.3	F	22.0	M2		*NPM1*	Yes	Yes	Yes	No	Leukemia	46,XX
8	11.9	M	7.7	M0			Yes	Yes	Yes	Yes	Leukemia	46, XY [30]
9	14.1	F	4.4	M4	*RAS*		Yes	Yes	Yes	Yes	Neurologic syndrome	46,XX,-7[21]
10	4.0	F	8.1	M0		*MLL/AF6*	Yes	No	Yes	No	Death in CR	46,XX,t(11;12)(q2?3;p13)[15]/46,XX,6?q,t(11;12)(q2?3;p13)[6]
11	15.3	M	2.8	M2		*RUNX1/RUNX1T1*	Yes	No	No	No	-	90~91,XY,t(8;21)(q22;q22),inc[cp3]/46,XY[3]
12	0.2	F	217.9	M2	*KIT*	*MNX1/ETV6*	Yes	Yes	Yes	No	Leukemia	47,XX,t(7;12)(q36;p13),+19[32]
13	1.5	M	226.8	M3		*MNX1/ETV6*	Yes	Yes	Yes	Yes	Leukemia	47,XY,del(12)(p12p13).ish t(7;12)(q3?;p13),+19[20]
14	0.3	F	88.8	M0		*MNX1/ETV6*	Yes	Yes	Yes	Yes	Leukemia	47,XX,+19[8]/46,XX[2]
15	0.3	F	21.1	M0		*MNX1/ETV6*	Yes	No	Yes	Yes	Toxic	46,XX,t(7;12)(q32;p13)[2],47,XX,t(7;12)(q32;p13),+19[18]
16	0.3	M	14.0	M1		*MNX1/ETV6*	Yes	No	Yes	No	Death in CR	47,XY,del(12)(p13).ish t(7;12)(q3?;p13),+19[20]
17	1.1	F	21.8	M7		*MNX1/ETV6*	Yes	No	Yes	No	Toxic	NA
18	10.4	M	10.0	M2	*BCR/ABL1*	*ETV6/HOXA11as*	No	No	Yes	Yes	Leukemia	46, XY,t(9;22)(q34;q11)
19	9.7	M	2.4	M0		*ETV6/HOXCas2*	Yes	Yes	No	No	-	47,XY,inv(12)(p13q1?3),+19[6]/46,XY[14]
20	2.3	M	29.0	M4		*ETV6/ARNT*	Yes	No	No	Yes	-	46, XY, t(6;11)(q27;q23)[15]

Abbreviations: ID indicates identification number; y, years; WBC, white blood cell count; L, liter; FAB, French-American-British classification; CR, complete remission; SCT, hematopoietic stem cell transplantation; COD, cause of death; M, male; F, female.

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Supplementary Data

Supplementary Table 6.1. Primers and PCR conditions used for the identification of mutations and translocations.

Gene		Direction	Primer-sequence	PCR condition
ETV6 *Mutational screening*	Exon 2	Forward	5'-CCCCGAGATGGTCTCAT-3'	10' 95°C, 40x (15" 95°C, 1' 60°C)
		Reverse	5'-TGGCGAAGTCCTGTGAA-3'	
	Exon 3	Forward	5'-AGGGCTCTTGAGATGTGG-3'	
		Reverse	5'-ATCCCTTCCTTGTGATGACT-3'	
	Exon 4	Forward	5'-GGTGCGCTCCAATTGTA-3'	
		Reverse	5'-CTTCAGGGAACCAAGAGTGT-3'	
	Exon 5	Forward	5'-CACGCTCCTCCATTTACC-3'	
		Reverse	5'-GCTGGCTGCAAAGATCA-3'	
	Exon 6	Forward	5'-TCCCGGTAAAACAAGGAA-3'	
		Reverse	5'-TGCCCAGCAAGGAATAA-3'	
	Exon 7	Forward	5'-CAGGCAGCAGCTGAAGA-3'	
		Reverse	5'-CCCCGTTATTTAAAGAAAACAG-3'	
	Exon 8	Forward	5'-CGGGGTTCAGTAGCTCTC-3'	
		Reverse	5'-TCGCTTCCTTTTCCACTC-3'	
MNX1	Exon 1	Forward	5'-GCATGATCCTGCCTAAGA-3'	10' 95°C, 40x (15" 95°C, 1' 60°C, 30" 72°C), 10' 72°C
ETV6	Exon 3	Reverse 1	5'-GCCATTCATTTCAAACGT-3'	
ETV6	Exon 4	Reverse 2	5'-AATCCGAGGTTTCCTCTG-3'	
ETV6	Exon 2	Forward	5'-TCAGGATGGAGGAAGACTCG-3'	10' 95°C, 40x (15" 95°C, 1' 60°C, 30" 72°C), 10' 72°C
PDGFRB	Exon 14	Reverse	5'-CCCCAACAGGTTGACCACGTTCAG-3'	
ETV6	Exon 4	Forward	5'-AGCCGGAGGTCATACTG-3'	
PDGFRB	Exon 11	Reverse	5'-TGCCAAAGCATGATGAG-3'	
ETV6	Exon 4	Forward	5'-AGCCGGAGGTCATACTG-3'	
ARNT	Exon 6	Reverse	5'-CCCGCAAGGACTTCA-3'	
ETV6	Exon 1	Forward	5'-GCCGGGAGAGATGCT-3'	
HOXA11-AS	Exon 5	Reverse	5'-CTGCCTGGACCTTGAAG-3'	
ETV6	intronic 3-4	Forward	5'-CAGTGCTTCCTTTAAACACAT-3'	
HOXC-AS2	antisense	Reverse	5'-GCAGGCAGCTCAAGAGT-3'	
HOXC9 *qPCR*	Exon 1	Forward	5'-ACCCGGACTACATGTACG-3'	10' 95°C, 40x (15" 95°C, 1' 60°C)
	Exon 2	Reverse	5'-GCTCGGTGAGATTGAGAA-3'	
HOXC10 *qPCR*	Exon 1	Forward	5'-GCCCCAATGAAATCAAG-3'	10' 95°C, 40x (15" 95°C, 1' 60°C)
	Exon 2	Reverse	5'-CTCGCGTCAAATACATATTG-3'	

Abbreviations: PCR indicates poly chain reaction; qPCR, quantitative PCR.

Supplementary Table 6.2. Clinical characteristics of patients with *ETV6*-inactivating mutations or deletions.

		pediatric AML cohort (n=267)	ETV6 deleted or mutated cases (n=10)	ETV6 translocated cases (n=9)	other pediatric AML cases (n=248)
Age at dx	*Median (y)*	9.6	11.3	1.1 (p<0.01)	9.6
	Range (y)	0.1-18.5	4.0-15.3	0.2-10.4	0.1-18.5
Sex	*Female (%)*	43	40	44	43
WBC	*Median ($x10^9$/L)*	46.7	11.3 (p<0.01)	21.8	48.2
	Range ($x10^9$/L)	1.2-483.0	2.8-193.2	2.4-226.8	1.2-481.8
CR	*Achieved CR (%)*	80	100	89	79
FAB-type	*M0*	14	3	3	8
	M1	32	0	1	31
	M2	49	4	2	43
	M3	19	0	1	18
	M4	76	2	1	73
	M5	59	0	0	59
	M6	2	0	0	2
	M7	7	0	1	6
	Unknown	9	1	0	8
Type 2 aberrations	*MLL-rearrangement*	69	1	0	68
	RUNX1/RUNX1T1	25	3	0	22
	inv(16)	35	0	0	35
	PML/RARA	18	1	0	17
	DEK/NUP214	6	0	0	6
	KAT6A/CREBBP	6	0	0	6
	NUP98-rearrangement	18	0	0	18
	CEBPAdm	14	0	0	14
	NPM1	19	1	0	18
	Other/Unknown	56	4	9	43

Abbreviations: dx indicates diagnosis; y, years; L, liter; CR, complete remission; FAB, French-American-British classification; M, morphology type; dm, double mutation.

Chapter 7

Recurrent deletions of *IKZF1* in pediatric acute myeloid leukemia

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Abstract

IKAROS family zinc finger 1/IKZF1 is a transcription factor important in lymphoid differentiation, and a known tumor suppressor in acute lymphoid leukemia. Recent studies suggest that IKZF1 is also involved in myeloid differentiation. To investigate whether *IKZF1* deletions also play a role in pediatric acute myeloid leukemia, we screened a panel of pediatric acute myeloid leukemia samples for deletions of the *IKZF1* locus using multiplex ligation-dependent probe amplification and for mutations using direct-sequencing. Three patients were identified with a single amino acid variant without change of IKZF1 length. No frame-shift mutations were found. Out of 11 patients with an *IKZF1* deletion, eight samples revealed a complete loss of chromosome 7, and three cases a focal deletion of 0.1-0.9Mb. These deletions included the complete *IKZF1* gene (n=2) or exons 1-4 (n=1), both leading to a loss of *IKZF1*-function. Interestingly, differentially expressed genes in monosomy 7 cases (n=8) when comparing to non-deleted samples (n=247) significantly correlated with gene expression changes in focal *IKZF1* deleted cases (n=3). Genes with increased expression included genes involved in myeloid cell self-renewal and cell cycle, and a significant portion of GATA target genes and *GATA* factors. Together, these results suggest that loss of *IKZF1* is recurrent in pediatric acyte myeloid leukemia and might be a determinant of oncogenesis in AML patients with monosomy 7 and focal *IKZF1* deletions.

Introduction

Acute myeloid leukemia (AML) is a heterogeneous disease characterized by dysregulated hematopoiesis initiated by recurrent non-random genetic aberrations.[1, 2] Despite major progress in improving treatment over the past decades, AML remains a life-threatening malignancy in children. The 5 year event-free survival (EFS) rates of pediatric AML are currently ~50-60% and the overall survival (OS) rate ~70%.[3, 4] Treatment stratification is mainly based on early response to treatment and prognostic relevant genetic abnormalities. Although many driving recurrent genetic aberrations causing AML have been defined, in approximately 20% of the pediatric AML patients the driving genetic events remain unidentified.[3]

The DNA-binding protein IKAROS family zinc finger 1 (IKZF1) is a zinc finger containing transcription factor that is encoded in 8 exons on chromosome 7 (7p12.2). IKZF1 functions upon homo-dimerization and acts as a chromatin remodeling protein involved in several chromatin remodeling complexes, such as nucleosome-remodeling and histone deacetylation (NuRD).[5] Alternative splicing of *IKZF1* pre-mRNA gives rise to several isoforms.[6] The functional differences among the various isoforms of IKZF1 are mostly due to alterations of zinc finger structures required for DNA binding (4 zinc fingers in the N-terminal domain) or protein-protein interactions (2 zinc fingers in the C-terminal domain).[6, 7] Some of these splice variants lack the ability to properly bind DNA and act as dominant-negative repressors when dimerized with functional isoforms.

Loss of Ikzf1 function in mice homozygous for an *Ikzf1* null allele or homozygous for a dominant-negative isoform of Ikzf1 results in an early arrest of lymphoid differentiation with lack of T- and B-lymphocytes and NK cells, as well as their earliest identifiable progenitors.[8, 9] In heterozygous *Ikzf1* mutant mice, lymphocytes appear normal right after birth, but proliferation increases and differentiation arrest occurs rapidly, leading to the development of leukemia and lymphoma within three months after birth.[10] In humans, heterozygous loss of *IKZF1* is found in 15% of B-cell precursor ALL (BCP-ALL) patients; in 70-80% of *BCR/ABL1* (t(9;22)(q34;q11)) positive BCP-ALL patients and in 33% of *BCR/ABL1* negative BCP-ALL patients. [11-15] In *BCR/ABL1* ALL patients, heterozygous loss of *IKZF1* associates with a poor clinical outcome. In T-ALL heterozygous loss of *IKZF1* is found in only 4%, but it remains unclear if this has any prognostic value.[11, 12]

While the role of IKZF1 in lymphoid differentiation and ALL is firmly established, the role of IKZF1 in myeloid differentiation is less clear. However, there are some strong indications that IKZF1 is also involved in myeloid differentiation. In early myeloid progenitors, loss of IKZF1 function prolongs cell survival.[16] During erythropoiesis, IKZF1 supports survival of the erythroid lineage, and promotes erythrocyte differentiation at the expense of granulocyte and monocyte differentiation.[17-19] In early megakaryopoiesis IKZF1 represses differentiation by inhibiting genes associated with the NOTCH pathway and later on in differentiation towards megakaryocytes by controlling transcriptional regulators, such as GATA1 and RUNX1.[18, 20] Although IKZF1 is not required for the initial differentiation towards granulocytes and monocytes, IKZF1 represses differentiation of the basophilic granulocyte lineage and promotes early maturation and survival of the neutrophil granulocyte lineage.[17, 21, 22]

In pediatric AML, monosomy 7 is found in only 4-5% of the patients. The 5-year

OS and EFS of pediatric AML patients with monosomy 7 with or without additional cytogenetic or chromosomal aberrations are poor.[23] In adult AML, monosomy 7 is the most frequent single monosomy and has a similar poor 4-year overall survival as compared to other single autosomal monosomies.[24] Deletions of the short arm of chromosome 7, 7p (del7p) were recurrently found in adult *de novo* AML and secondary AML developed from myelodysplastic syndrome (MDS) or myeloproliferative neoplasms (MPN).[25-27] Furthermore, 7p deletions were mapped to the *IKZF1* locus and it was shown that loss of *IKZF1* is acquired during the progression of MPN to secondairy AML, indicating that loss of *IKZF1* contributes to the transformation from MPN to AML.[25]

Together, these studies demonstrate that IKZF1 may play a role in myeloid differentiation and that loss of *IKZF1* may contribute to myeloid oncogenesis. We therefore hypothesized that in pediatric AML patients with monosomy 7, IKZF1 may be an important player. In this study we analyzed the frequency of *IKZF1* deletions in a pediatric AML cohort, and studied the difference in gene expression of cases with focal *IKZF1* deletions, and cases with monosomy 7.

Methods

Patient samples

Samples were provided by the Dutch Childhood Oncology Group (DCOG, The Netherlands), the AML–Berliner-Frankfurt-Münster Study Group (Germany and Czech Republic), the Saint-Louis Hospital (France), and the Royal Hospital for Sick Children (UK). Isolation of genomic DNA and total cellular RNA was performed using Trizol reagent. Each study group provided morphological and cytogenetic classification and clinical data. Institutional review board approval for these studies had been obtained in the participating centers.

Detection of deletions and mutations of IKZF1

Multiplex ligation-dependent probe amplification **(**MLPA) was performed using the p335-B1;ALL-IKZF1 kit (MRC Holland, Amsterdam, The Netherlands, data available on http://www.mlpa.com). The data were analyzed with GeneMarker (version 1.85; SoftGenetics, State College, Pennsylvania, USA). The data were normalized to reference probes and control samples. A deletion was defined as a peak ratio below 0.75, an amplification was defined as a peak ratio above 1.25.

Array comparative genomic hybridization (Array-CGH) was performed using the human genome CGH Microarray 105K (Agilent Technologies, Santa Clara, California, USA) according to manufacturer's protocol, data were analysed with Genomic Workbench (version 5.0.14; Agilent Technologies, Santa Clara, California, USA).

Direct sequencing was used to analyze mutations or frameshifts in exon 4, 5 and 8, which are found to be hotspot areas of mutations in pediatric BCP-ALL (primers Supplementary Table 7.1).[13, 28]

Cytogenetic and molecular characterization

Reverse-transcription-PCR was performed on hotspot areas as previously described by Balgobind *et al*[29] and to analyse the frequency of the *BCR/ABL1* translocation (primers Supplementary Table 7.1).

Gene expression profiling

GEP data were available from earlier studies.[30] Quantitative reverse-transcription-PCR (qRT-PCR) analysis was performed on a subset of patients to analyze the correlation between GEP data and qRT-PCR on relevant genes in patients of different cytogenetic subgroups and a variety of expression measured with GEP. The mRNA expression was determined using the average cycle threshold (Ct) in comparison to expression levels of *GAPDH*, using the comparative cycle threshold method.

Statistical analysis

Statistical analyses for disease outcome and correlation were performed with IBM SPSS 21 (IBM, Armonk, New York, USA). To assess clinical outcome, complete remission (CR, defined as less than 5% blasts in the bone marrow, with regeneration of tri-lineage hematopoiesis, plus absence of leukemic cells), probability of event-free survival (pEFS, defined as the time between diagnosis and first event including non-responders calculated as an event on day 0), and probability of overall survival (pOS, defined as the time between diagnosis and death) were analyzed. Both pEFS and pOS were estimated by the Kaplan-Meier method and differences compared using logrank-tests. The cumulative incidence of non-response or relapse (pCIR, defined as time between diagnosis and relapse and with non-responders included as an event on day 0) was analysed by the Kalbfleisch and Prentice method and compared with the Gray's test. Statistical significance was considered if p-values were below 0.05.

Differential expression analyses between groups were conducted using the R-package ShirnkBayes[31] at the probe level. In contrast to commonly used two-sample tests, this test is proven to be powerful for small sample sizes. Bayesian false discovery rate (BFDR)<0.05 was considered statistically significant.

Results

Identification of IKZF1 deletions in pediatric AML

The cohort of newly diagnosed pediatric AML patients with available peripheral blood or bone marrow samples taken at initial diagnosis included in this study was previously described by Hollink *et al* (n=293; age, 0-18 years).[30] Of 293 well-characterized pediatric AML cases, 258 with available high quality DNA were analyzed with MLPA using 250ng of DNA. Cytogenetic aberrations in this group were representative for the frequencies seen in pediatric AML.[3, 32] In addition, 14 healthy control samples as negative controls, and one pediatric ALL sample known to harbor an *IKZF1* deletion as positive control were analyzed. A succeeded MLPA was considered if all reference probes had a peak ratio between 0.75 and 1.25, except where a deletion or amplification of a reference probe could be explained by an aberration visible in the karyotype. The P335-B1;ALL-IKZF1 MLPA kit contains a probe in each of the 8 exons of *IKZF1*. All probes on chromosome 7 were analyzed individually, including reference probe 15370-L13762 located on 7q11.23 in *P450 cytochrome oxidorectase* (*POR*). Additional high-resolution array-CGH was performed on patients with an *IKZF1* deletion detected by MLPA, but without knowledge of the existence of monosomy 7, and one additional case of monosomy 7 from which no material was available for MLPA analysis. Mutational screening of exon 4, 5 and 8 of *IKZF1* was performed on the same cohort of 258 patients. This revealed

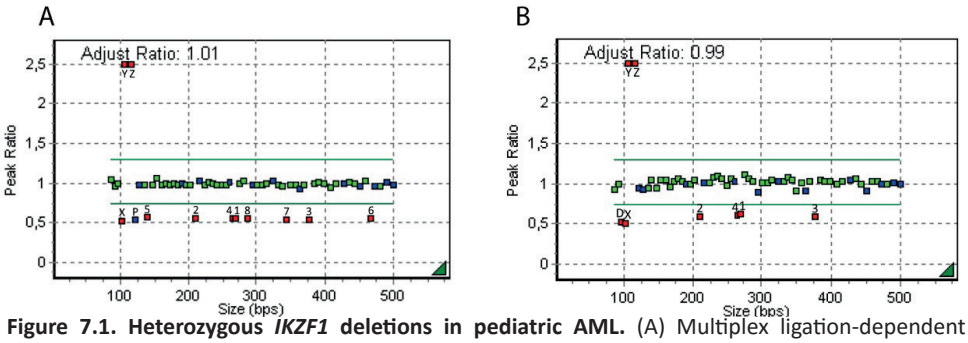

Figure 7.1. Heterozygous *IKZF1* deletions in pediatric AML. (A) Multiplex ligation-dependent probe amplification results of a patient with heterozygous deletion of exons 1-8 of *IKZF1* including the reference probe in *POR* located on 7q11.23. (B) MLPA results of a patient with heterozygous deletion of exons 1 through 4 of *IKZF1*. 1-8 indicates *IKZF1* exons 1-8; D, reference D-fragment 96, a control probe for denaturation during the procedure; P, *POR* (7q11.23); X, X-chromosome specific fragment, a reference probe for gender; Y, Y-chromosome specific fragment, a reference probe for gender; Z, *ZFY* (Yp11.31), a reference probe for gender.

no patient harboring a frameshift mutation, as described to be damaging in BCP-ALL. We did identify three patients with a heterozygous point mutation, p.V382M, which was predicted as tolerated by SIFT analysis and as benign by PoyPhen analysis, and p.G158S and p.L188P, which were predicted as deleterious by SIFT analysis and probably damaging by PolyPhen analysis, but not resulting in a truncated protein. 77/258 patients presented with synonymous SNP rs61731355, 17/258 with synonymous SNP rs61731356, and 1/258 with non-synonymous SNP rs376657964 (p.T333A), which is in line with the presence of this SNP's in the normal population (21%, 4% and 1% respectively).[33]

In total, 10 patients carried a heterozygous *IKZF1* deletion as identified by MLPA, and one additional case as identified by array-CGH (Table 7.1). From 258 patients, as determined by MLPA, 9 cases showed an *IKZF1* deletion in all 8 exons, and 1 case showed a deletion of exon 1-4 with a normal peak ratio in the remaining exons (Figure 7.1). Four of these 10 cases showed a monosomy 7 with conventional karyotyping (Table 7.1). Using array CGH on 7 patients, we identified 2 cases with monosomy 7 in patients with an unknown karyotype, 2 patients with monosomy 7 of whom the karyotype was (wrongly) defined as cytogenetically normal, and 3 cases with a focal 0.1-0.9Mb deletion of 7p12.2, where the *IKZF1* gene is located (Figure 7.2, Table 7.1).

Combining the data gathered from MLPA, karyotype and array CGH, we defined 11 patients with an *IKZF1* deletion, 8 of which showed complete loss of one copy of chromosome 7 (monosomy 7), and 3 of which showed a focal *IKZF1* deletion. As shown in Figure 7.2, patient #10 demonstrated a focal deletion (~0.1Mb) including exon 1-4 of *IKZF1*. The focal deletion in patient #2 included the *IKZF1* gene and part of the C7orf72 gene (~0.3Mb), whereas patient #3 showed a focal deletion (~0.9Mb) including the *IKZF1* gene and the genes for *VWC2*, *ZPBP*, *C7orf72* and *DDC*. Together, these results map the deletions in these patients to the *IKZF1* gene locus as the common deleted region.

Characteristics of IKZF1 deleted cases in pediatric AML

IKZF1 deleted cases did not differ significantly from the other pediatric AML cases concerning age at diagnosis (median age 9.1 years, range 0.7-14.1, compared to 9.5

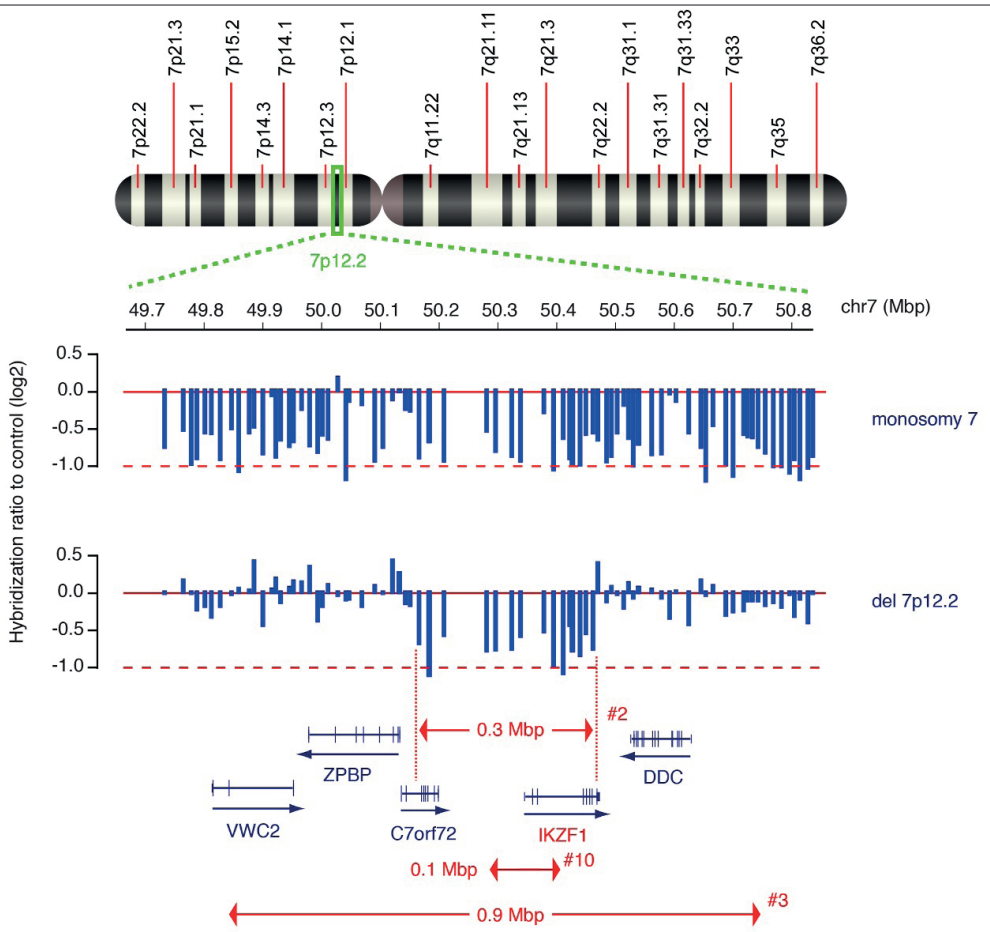

Figure 7.2. Array CGH analysis of monosomy 7 and focal *IKZF1* deletions. DNA isolated from pediatric AML samples taken at initial diagnosis and containing *IKZF1* deletion based on MLPA results were analyzed using array CGH. Representative samples for monosomy 7 (patient #4, upper panel) and *IKZF1* focal del7p12.2 deletions (patient #2, #3 and #10, lower panel) are shown.

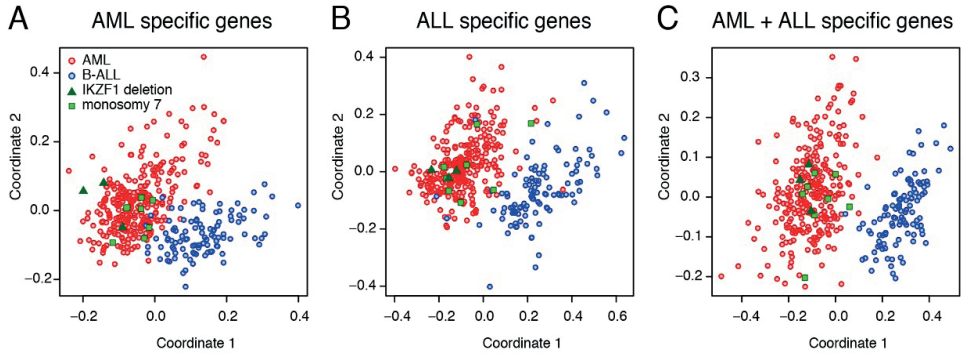

Figure 7.3. *IKZF1* haploinsufficient AML cells display an AML-specific gene expression signature. AML (n=297, red) and B-ALL (n=107, blue) patients were projected onto the first two principal coordinates, obtained via multidimensional scaling of expression values of AML specific genes (A), ALL specific (B) genes and the combination of these (C) (Thomas et al., 2001). Focally deleted *IKZF1* (dark green triangles) and monosomy 7 (light green squares) AMLs were located among other AMLs and away from B-ALLs.

years, range 0.1-18.5, respectively, p=0.41); nor in gender (55% female versus 42% female, p=0.41); nor in white blood cell count at diagnosis (median 30.2 $x10^9$/L versus 47.5 $x10^9$/L, p=0.24, Supplementary Table 7.2). All AML patients were treated with intensive collaborative group cytarabine-anthracycline based pediatric AML treatment protocols. Clinical outcomes were not significantly different between *IKZF1* focal deleted, monosomy 7 and other AML cases. The 3-year pOS in *IKZF1* deleted cases (n=11) was 70±14% versus 63±3% (p=0.82) in cases with 2 *IKZF1* alleles (n=231). The 3-year pEFS was 36±15% versus 46±3% (p=0.87), and 3-year pCIR was 64±16% versus 36±3% (p=0.09) respectively (Supplementary Figure 7.1).

No specific morphology subtypes based on the French–American–British (FAB) classification were related to *IKZF1* deletions. *IKZF1* deleted samples showed none or various additional somatic mutations, most frequently activating the RAS pathway with mutations in *NRAS* or *PTPN11* (n=4, Table 7.1). As in BCP-ALL, *IKZF1* deletions are associated with *BCR-ABL1* fusions, we additionally screened the *IKZF1* deleted cases for the *BCR/ABL1* fusion and all were negative.[12]

IKZF1 haploinsufficient AML cells display an AML-specific gene expression signature

Original gene expression data are available in the Gene Expression Omnibus repository (http://www.ncbi.nlm.nih.gov/geo;accession GSE17855). Focal *IKZF1* deletions are also found in mixed phenotype acute leukemia.[34] To ensure the included cases were AML patients and not patients with a mixed ALL and AML phenotype we used a previously defined AML vs ALL classifier.[35] Patients with focal *IKZF1* deletions and patients with monosomy 7 clustered amongst the other AML patients and away from the ALL patients, based on the expression signatures of the AML-specific genes, the ALL-specific genes and the combined AML/ALL- specific genes (Figure 7.3). This indicates that the *IKZF1* haploinsufficient AML cells of included patients indeed displayed pure AML cell gene expression profiles. Only of one of the three patients with a focal *IKZF1* deletion immunophenotypical data was available (patient #2). This patient was classified as *PML/RARA*-negative AML FAB M3V (TdT-/MPO+/cyCD79a-/CD34+/HLADR+/CD19-/CD10-/CD7+/CD13+/CD33+).

Similarity between gene expression profiles of monosomy 7 and focal IKZF1 deleted cases

Loss of IKZF1 function, a well-known tumor inducing event in ALL, is conceivably one of the reasons for the recurrent heterozygous loss of chromosome 7, as this would include losing the *IKZF1* locus on 7p12.2. We hypothesized that, if loss of *IKZF1* would be one of the driving leukemic events in monosomy 7, the gene expression signature of monosomy 7 AML cells may resemble those that only have a focal *IKZF1* deletion. However, we anticipated that this similarity might be quite weak because of the heterogeneity across the samples in our cohort, both derived from somatic and germ line genetic variation and the small sample size of monosomy 7 and *IKZF1* deleted cases. Therefore, we performed genome wide differential gene expression analyses using a robust Bayesian method implemented in the R-package ShrinkBayes.[31] Probe sets were limited to those targeting a unique genes or having a selectivity score of >0.8 (www.geneannot.com). Further statistical analyses were performed in R, version 3.0.0. R scripts are available upon request. We first compared gene expression profiles of monosomy 7 samples

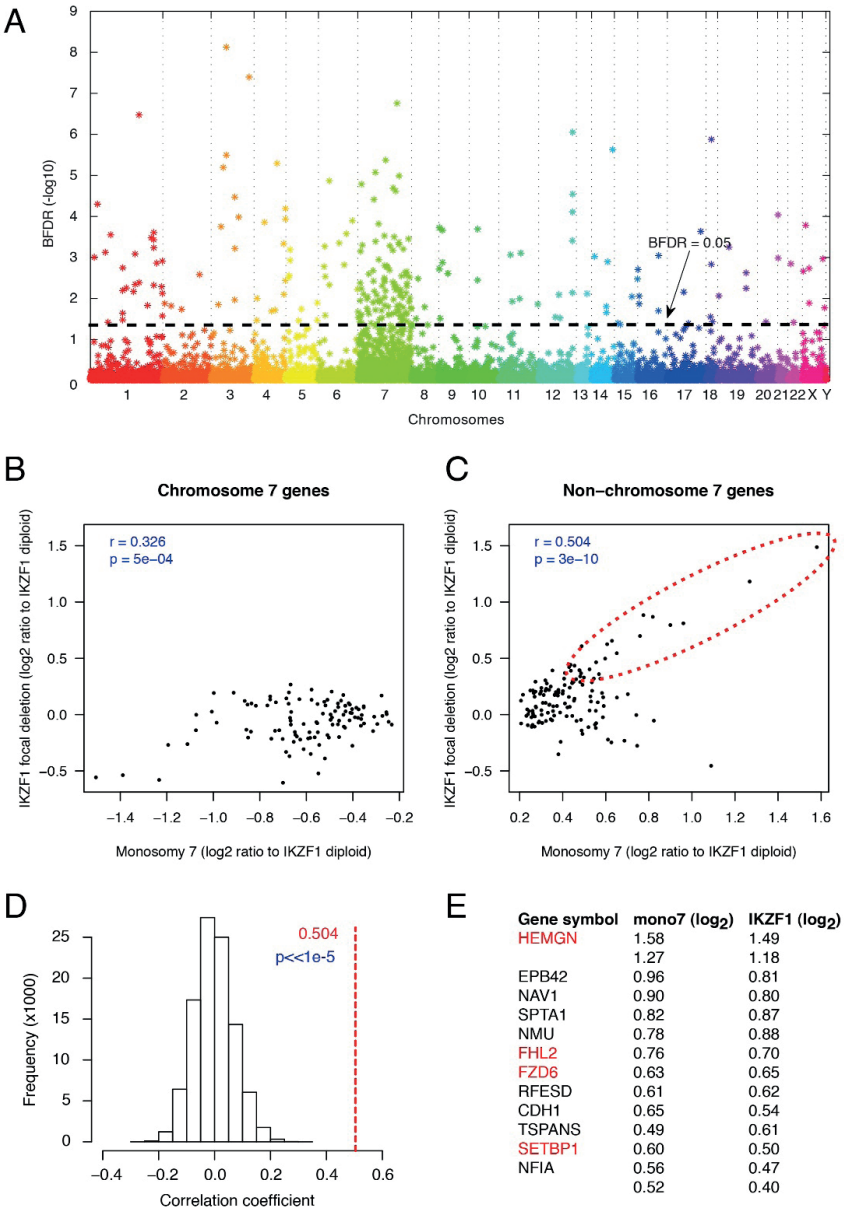

Figure 7.4. Monosomy 7 and focal *IKZF1* deletion associate with gene expression profiles with significant similarity. (A) Analysis of differences in gene expression between monosomy 7 samples (n=8) and *IKZF1*-non-deleted samples (n=247). Genes are ranked on Bayesian false discovery rate (BFDR) (y-axis) and binned in their chromosome of origin (x-axis). Using a BFDR of 5% (dashed line), a total of 198 genes are detected differentially expressed, of which 111 are not encoded on chromosome 7. (B,C) Mean differences per gene between monosomy 7 and non-*IKZF1*-deleted samples versus those of *IKZF1* focal deletions and non-*IKZF1*-deleted samples for chromosome 7 encoded genes (B) and non-chromosome 7 encoded genes (C). Pearson's correlation coefficients (r) are indicated in the plot, together with accompanying p values. (D) Bootstrap analyses of the correlation between monosomy 7 cases (n=8) and 3 randomly picked *IKZF1*-non-deleted samples (n=247). The red dashed line indicates correlation coefficient from the analyses displayed in C. (E) Top correlating genes (dashed elipse in (C)) between monosomy 7 and focal *IKZF1* deletions. Highlighted in red are those reported to be positively acting on leukemic cell self-renewal or cell cycle.

(n=8) to those of *IKZF1*-non-deleted samples (n=247). We detected 244 probe sets representing 198 genes differently expressed (BFDR<0.05) between these two groups (Figure 7.4A). As might be expected, a large proportion of these genes (44%) is located on chromosome 7, and is down-regulated (Figure 7.4B).

Next, we compared the mean differences in expression of these 198 genes in monosomy 7 samples (n=8) to those in samples with *IKZF1* focal deletions (n=3) (Figure 7.4C). For non-chromosome 7 genes, highly significant positive correlation was observed in gene expression differences (r=0.50, p=3e-10). A less significant positive correlation (r=0.33, p=5e-4) was observed for chromosome 7 genes (Figure 7.4B). To further test that the correlation of gene expression changes between monosomy 7 and focal *IKZF1* deletions is not based on chance, we performed a bootstrap analyses taking 3 random patients and comparing them to the 8 patients with monosomy 7. As shown in Figure 7.4D, none of 100,000 comparisons displays a correlation approaching a value of 0.5 found for the patients with focal *IKZF1* deletions.

We next asked which non-chromosome 7 genes were responsible for the similarity between monosomy 7 and focal *IKZF1* deletion gene expression profiles. Genes that substantially contribute to the correlation between monosomy 7 and *IKZF1*-only deleted gene expression differences are up-regulated in both groups (Figure 7.4C). Interestingly, the gene most clearly up-regulated in both monosomy 7 and *IKFZ1*-focal deleted samples was Hemogen (*HEMGN*), of which over-expression has been described to induce expansion of myeloid progenitor cells in a murine model.[36] Other genes with known pro-leukemic functions in the top-correlating list are Four and a half LIM domains 2 (*FHL2*), Frizzled 6 (*FZD6*) and SET binding protein 1 (*SETBP1*) (Figure 7.4E). Very strong correlations (r=0.9117-0.9562, p<0.0001) were found between the GEP data and qRT-PCR analyses for *HEMGN* and *FHL2*, and strong correlations (r=0.7913-0.7962, p<0.0001) were found for *SETBP1* and *FZD6* (n=30, Supplementary Figure 7.2).

As up-regulation of *HEMGN* in *IKZF1* hemizygous cells is relative to a fairly heterogeneous group, we stratified the control group in various more homogenous cytogenetic AML subgroups (Figure 7.5A). Median expression of *HEMGN* in each of those subgroups is clearly lower than in the *IKZF1* deleted group. The same is true for *FHL2*, *FZD6* and *SETBP1* (Figure 7.5B-D), although it should be noted that *FZD6* and *SETBP1* are also highly expressed in patients with *MNX1-ETV6* (t(7;12)). When excluding the patient harboring both an *IKZF1* deletions and *MNX1-ETV6* (t(7;12)), *FZD6* and *SETBP1* remain significantly higher expressed in the *IKZF1*-deleted cases compared to the other pediatric AML subgroups (Figure 7.5E-F). These data indicate that up-regulation of these genes in *IKZF1* deleted samples is not confounded by specific cytogenetic aberrations in pediatric AML.

Gene expression profiles of the IKZF1-deleted samples demonstrate enrichment in up-regulated GATA targets.

HEMGN, the gene most clearly up-regulated in both monosomy 7 and *IKFZ1*-focal deleted samples, is transcriptionally activated by GATA1 in the myeloid cell line K562.[37] We therefore investigated whether other genes that demonstrated a correlation between monosomy 7 and focally deleted *IKFZ1* samples are also GATA1 targets in K562. For this purpose, we used a GATA1 ChIP seq dataset in K562 cells.[38] From the 23 genes significantly up-regulated in the *IKZF1*-deleted samples, 11 genes had a GATA1 peak

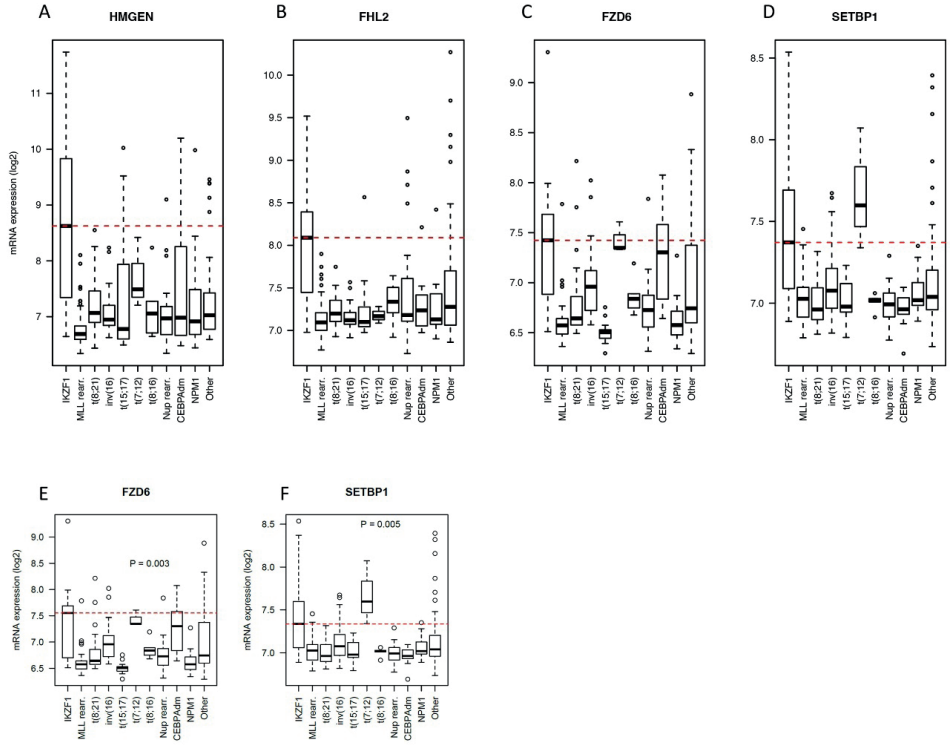

Figure 7.5. *HEMGN*, *FHL2*, *FZD6* and *SETBP1* expression in *IKZF1*-deleted patients as compared to other cytogenetically classified AML groups. Comparison of mRNA abundances in *IKZF1*-deleted samples to major cytogenetic AML subgroups revealed that the median expression of *HEMGN*, *FHL2*, *FZD6* and *SETBP1* in the *IKZF1* deleted group is clearly higher than in each of the subgroups, except for *FZD6* and *SETBP1* in comparison to t(7;12)/*MNX1-ETV6*. When excluding the patient harboring both the *IKZF1* deletion and t(7;12)/*MNX1-ETV6*, *FZD6* and *SETBP1* remain significantly higher expressed in the *IKFZ1*-deleted cases compared to the other pediatric AML subgroups (p=0.003 and p=0.005 respectively).

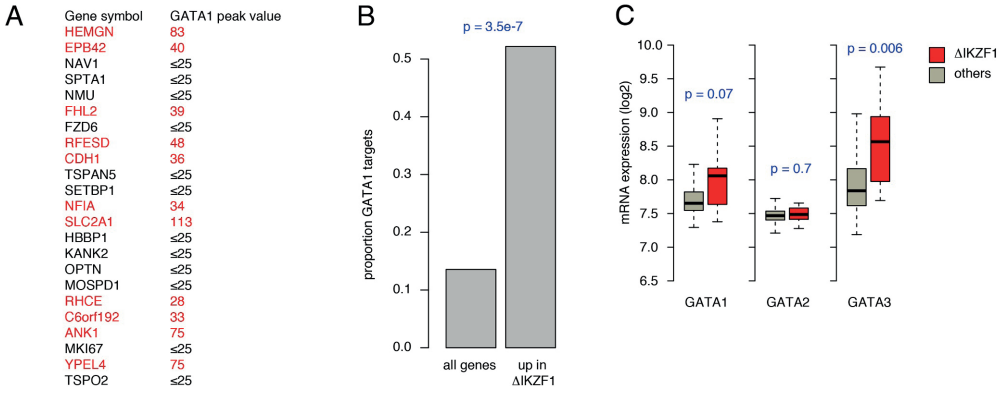

Figure 7.6. Genes commonly upregulated in *IKZF1* focally deleted and monosomy 7 cases are enriched in myeloid GATA1 targets. (A) GATA1 chromatin interaction values (GATA1 peak value) in K562 cells of commonly upregulated genes (n=23) in monosomy 7 and focal *IKZF1* deletion (Δ*IKZF1*). (B) Proportion of all matching genes (n=22564) or Δ*IKZF1* upregulated (n=23) displaying a GATA1 chromatin interaction in K562 cells. p value from Fisher's exact test. (C) mRNA expression of *GATA1, GATA2* and *GATA3* in *IKZF1* deleted samples (red) versus *IKZF1* WT samples (grey). p values according to Mann-Whitney tests.

ratio larger than 25, indicating the gene being a GATA1 target (Figure 7.6A). Further analysis showed that whereas 13% of genes on our expression platform is a GATA1 target, 55% of genes that are up-regulated in *IKZF1* deleted cases are GATA1 targets (p=3.5 e-7, Figure 7.6B). While GATA transcription factors exhibit diverse functions in cellular proliferation, differentiation and gene regulation, only GATA1, GATA2, and GATA3 are expressed in developing blood cells and are critical for hematopoiesis.[39,40] As shown in Figure 7.6C, *GATA1* (p=0.07) and *GATA3* (p=0.006) were up-regulated in the *IKZF1*-deleted samples (n=11) as compared to the rest (n=247). Direct interaction of IKZF1 with GATA proteins was previously demonstrated.[41] Together, these data emphasize the similarity in gene expression between monosomy 7 and focal *IKZF1* deleted pediatric AML patients.

Discussion

In the last ten years, *IKZF1* has been widely studied in the context of B-cell differentiation and acute lymphoblastic leukemia, both in adults and children. Accumulating evidence indicates that IKZF1 also plays a role in various stages of myeloid differentiation. So far, the best indication that loss of *IKZF1* may contribute to myeloid leukemogenesis are deletions of the short arm of chromosome 7 associated with MPN-preceded secondary AML in adults, where the commonly deleted region is mapped to the *IKZF1* locus.[25] Our finding of recurrent focal deletions that map to the *IKZF1* gene locus in pediatric AML patients cells now provides additional support for a role for IKZF1 in myeloid leukemia.

Reduced *IKZF1* gene function is a well-known recurrent event in BCP-ALL. In pediatric BCP-ALL, the overall frequency of focal *IKZF1* deletions is around 15%, but is greatly enriched in *BCR/ABL1* positive BCP-ALL at 70-80%.[15, 42] Here we report that focal *IKZF1* deletions are also a recurrent event in pediatric AML, although with much lower frequency. In a cohort of 258 patients, representing all cytogenetic AML subgroups, we found 3 cases of a focal inactivating deletion of this gene (1.2%). In addition, three patients were identified with a single nucleotide variation leading to amino acid changes. In contrast to mutations found in BCP-ALL, no frame shift mutations were detected, and the functional status of these SNV containing alleles remain to be determined.

In adult and pediatric BCP-ALL, in addition to focal *IKZF1* deletions, monosomy 7 is a recurrent chromosomal aberration. In *BCR/ABL1* positive BCP-ALLs, 16% (adult) and 13% (pediatric) of the cases of *IKZF1* deletion can be attributed to monosomy 7.[14, 42] In pediatric AML, monosomy 7 is a recurrent chromosome loss, in particular in myeloproliverative diseases, such as juvenile myelomonocytic leukemia, progressed towards AML (40% of cases), but also in primary AML (4-5% of cases).[23, 43] In our pediatric *de novo* AML cohort, we found 8 cases (3.1%) of monosomy 7, consistent with earlier estimates. Across these monosomy 7 AML cases, we find various additional somatic mutations, most prominently *PTPN11* (n=2) and *NRAS* mutations (n=2), adding up to half of cases presenting with an activated RAS pathway.

We hypothesized that in pediatric AML, as presumed in BCP-ALL, an important biological determinant for leukemogenesis in monosomy 7 patients is loss of the *IKZF1* gene.[44] Evidence for this hypothesis was obtained by comparing gene expression profiles of monosomy 7 and focal *IKZF1* deletions. We found a statistically significant correlation on non-chromosome 7 genes. We attribute the changes in chromosome 7 genes to bystander effects of deletion of the entire chromosome 7, leading to a widespread

reduction in chromosome 7 encoded genes, many of which can be assumed to be without much consequence to the leukemogenic process. In contrast, the effects on non-chromosome 7 genes can be expected to be more enriched for leukemia related changes in the genetic program due to loss of one or more chromosome 7 encoded driving oncogenic mutations. The similarity of gene expression signatures between AML cases with monosomy 7 and those with a focal *IKZF1* deletion therefore suggests that IKZF1 is one of the driving oncogenic events in monosomy 7 pediatric AML. Further data supporting this hypothesis is the observation that pediatric AML patients harboring monosomy 7 have a worse disease outcome compared to those harboring 7q deletions.[23] Our findings may also explain the prevalence of monosomy 7 and focal *IKZF1* deletions in mixed phenotype acute leukemia, as loss of *IKZF1* now appears to be able to contribute to both acute lymphoblastic and myeloid leukemogenesis.[34]

Several genes up-regulated in both monosomy 7 and focal *IKZF1* deleted AML cases are previously implicated in leukemogenesis. In a large cohort in adult BCP-ALL, comparison between focal *IKZF1* deletions and *IKZF1* wild-type cases revealed up-regulation of genes involved in cell cycle, JAK-STAT signaling and stem cell self-renewal.[45] We find up-regulation of *HEMGN*, a transcription factor implicated in myeloid self-renewal[36], *FHL2*, enhanced expression of which leads to enhanced cell cycle entry, *FZD6*, that has shown to be involved in the pathologically reactivation of the Wnt/Fzd-mediated self-renewal signals that are enlisted during B-cell development and may be pathologically reactivated in the neoplastic transformation of mature B cells, and *SETBP1*, of which over-expression promotes the self-renewal of myeloid progenitors.[46,47,48] It is interesting that similar cellular processes are affected in the *IKZF1* deleted cases, while none of the genes deregulated in *IKZF1* deleted adult BCP-ALL are found deregulated in this pediatric *IKZF1* deleted AML cohort.[45]

Of note, we find a strong enrichment of myeloid GATA1 targets among genes commonly up-regulated in focal *IKZF1* deleted AML cases and monosomy 7 AML cases. This could be the confirmation that the genes commonly up-regulated in *IKZF1* focal deletions and monosomy 7 did not present themselves by chance. However, it is tempting to speculate that *IKZF1* haploinsufficiency in pediatric AML is influencing GATA transcription factor function. Supporting this idea, *IKZF1* over-expression in transgenic mice represses GATA1 expression, implying that loss of IKZF1 function may increase GATA1 function.[21]

Together, we find evidence for *IKZF1* as a tumor suppressor gene in pediatric acute myeloid leukemia and suggest that *IKZF1* deletion might be one of the driving events of monosomy 7 in various myeloid diseases.

Table 7.1. Patient characteristics of *IKZF1* deleted pediatric AML cases (n=11).

id	*IKZF1* status		Karyotype provided	Age (y)	Sex	WBC x10^9/L	FAB	Aberrations*	Therapy protocol	CR	Relapse	Death	SCT	COD
	MLPA	array-CGH												
1	del ex1-8	NA	45, XX, -7, t(8;16)(p11;p13) [21]	7.4	F	58.1	M5	*KAT6A/CREBBP, FLT3-ITD*	MRC12	yes	no	no	no	-
2	del ex1-8	del 7p12.2	47,XY,del(12)(p12p13),ish t(7;12)(q3;p13),+19 [20]	1.5	M	226.8	M3	*MNX1-ETV6*	DCOG94	yes	yes	yes	yes	leukemia
3	del ex1-8	del 7p12.2	NA	11.3	F	NA	M5	*WT1*	MRC12	yes	yes	no	no	-
4	del ex1-8	del 7	NA	4.9	F	31.4	M6	None	BFM04	yes	yes	no	yes	-
5	del ex1-8	NA	45, XX, inv(2)(p24q14), -7	10.9	F	54.9	M4	None	DCOG94	yes	yes	yes	no	infection
6	del ex1-8	del 7	46,XY	0.7	M	8.2	M5	*PTPN11*	BFM87	yes	yes	yes	yes	leukemia
7	del ex1-8	NA	45,XY,-7	9.6	M	66.0	M2	*CEBPAdm, NRAS, IDH2*	BFM98	yes	no	no	no	-
8	del ex1-8	NA	45,XX,-7[21]	14.1	F	4.4	M4	*NRAS*	ELAM 2001	yes	yes	yes	yes	toxicity
9	del ex1-8	del 7	46, XY	10.6	M	22.0	M2	*RUNX1-RUNX1T, CKIT*	BFM98	yes	no	no	no	-
10	del ex1-4	del 7p12.2	46, XY, t(6;11)(q27;q23)[15]	2.3	M	29.0	M4	None	BFM04	yes	no	no	yes	-
11	NA	del 7	NA	9.1	F	4.4	M5	*PTPN11*	BFM98	yes	yes	yes	yes	

Abbreviations: del indicates deletion; WBC, white blood cell count; FAB, French-American-British classification; CR, complete remission; SCT, stem-cell transplantation; COD, cause of death; NA, not available. *Aberrations screened are AML associated fusions and hotspot areas in *NRAS, KRAS, KIT, WT1, CEBPA, FLT3,* and *PTPN11.*

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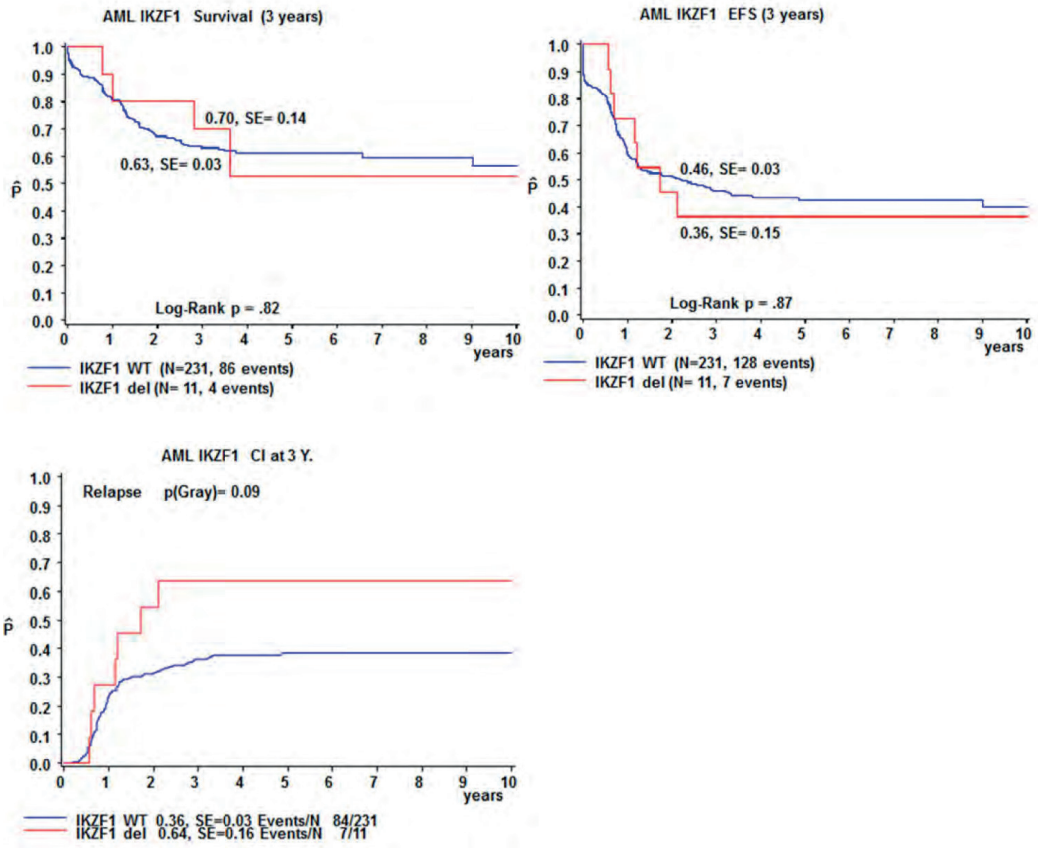
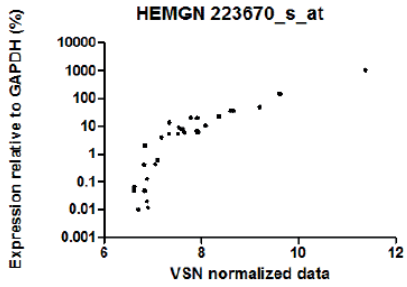
Supplementary data

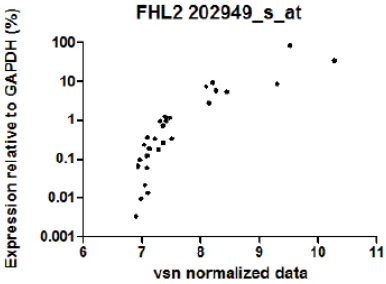


Supplementary Figure 7.1. Survival estimates of IKZF1-deleted patients compared to other AML. Kaplan-Meier estimates of the 3-year overall survival, event-free survival and cumulative incidence of relapse according to the Kalbfleisch and Prentice method of *IKZF1*-deleted patients versus other pediatric AML.

Spearman r	0.9246
95% confidence interval	0.8409 to 0.9651
P value (two-tailed)	P<0.0001

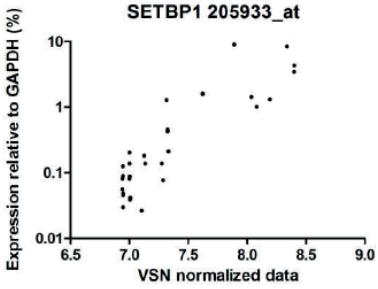
Spearman r	0.9177
95% confidence interval	0.8292 to 0.9613
P value (two-tailed)	P<0.0001

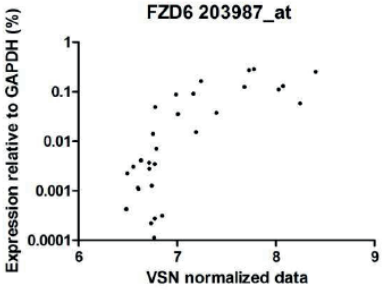




Spearman r	0.7962
95% confidence interval	0.6042 to 0.9008
P value (two-tailed)	P<0.0001

Spearman r	0.7913
95% confidence interval	0.5957 to 0.8983
P value (two-tailed)	P<0.0001





Supplementary Figure 7.2. Comparison of microarray values with qRT-PCR values. Four of the genes most highly upregulated in monosomy 7 and focal *IKZF1* deletions based on microarray analysis were selected for qRT-PCR validation using RNA from 30 primary AML cases. *GAPDH* qRT-PCR values were used for normalization. *HEMGN* and *FHL2* showed very strong correlations (r>0.9), *SETBP1* and *FZD6* showed strong correlations (r>0.7) comparing micro-array values with qRT-PCR values.

Supplementary Table 7.1. Primer sequences used for RT-PCR and qRT-PCR.

Gene	Location	Direction	Primers
RT-PCR primers			
IKZF1	Exon 4	Forward Reverse	5'-GCTGCTGTGTTGTTTTG-3' 5'-TGCTTTCCTCCTTCAAACCC-3'
IKZF1	Exon 5	Forward Reverse	5'-CGTGGGAAACAACTTTCTCG-3' 5'-CAGAGTGGAGGAATCCCG-3'
IKZF1	Exon 8	Forward Reverse	5'-GACCTGACCGGTTCC-3' 5'-CAGTCTATGCTGCTGGCG-3'
BCR *ABL1*	Exon 13 Exon 3	Forward Reverse	5'-TTCCGCTGACCATCA-3' 5'-CGCTGCTCAGCAGATACT-3'
qRT-PCR primers			
HEMGN	Exon 3 Exon 4	Forward Reverse	5'-CCTGGAGTTTGAGAAACAGA-3' 5'-AGGCTCCACCTTCAATTC-3'
FHL2	Exon 5 Exon 6	Forward Reverse	5'-CGCAGTGCAGAAACTCA-3' 5'-CTGCCCTTGTACTCCATCT-3'
SETBP1	Exon 2 Exon 2	Forward Reverse	5'-CCGGAAGACTGTAGAGATTG-3' 5'-CCGCTTTGTGGTCTGA-3'
FZD6	Exon 4 Exon 5	Forward Reverse	5'-CCGGAACCAAGAAAAACTA-3' 5'-ATGCCAACAATTAATGTCATC-3'

Supplementary Table 7.2. Patient characteristics of pediatric AML cohort (n=258).

		IKZF1 deleted cases (n=3)	Monosomy 7 cases (n=8)	Other pediatric AML (n=247)
Age at diagnosis	Median (y)	2.3	9.4	9.5
	Range	1.5-11.3	0.7-14.1	0.1-18.5
Sex	Female (%)	33	63	42
WBC	Median (x10^9/L)	127.9	26.7	47.3
	Range	29.0; 226.8	4.4-66.0	1.2-483.0
FAB-type	M0	0	0	16
	M1	0	0	32
	M2	0	2	43
	M3	1	0	19
	M4	1	2	70
	M5	1	3	51
	M6	0	1	1
	M7	0	0	8
Type 2 aberration	*MLL*-rearrangement	0	0	62
	RUNX1/RUNX1T1	0	1	22
	inv(16)	0	0	33
	PML/RARA	0	0	18
	ETV6/MNX1	1	0	3
	DEK/NUP214	0	0	6
	KAT6A/CREBBP	0	1	5
	NUP98-rearrangement	0	0	18
	*CEBPA*dm	0	1	12
	NPM1	0	0	17
	Other	0	0	2
	Unknown	2	5	49

Chapter 8

BCOR and *BCORL1* mutations in pediatric acute myeloid leukemia

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Letter to the editor

Acute myeloid leukemia (AML) is a heterogeneous disease characterized by the presence of different collaborating cytogenetic and molecular aberrations that are associated with treatment response.[1-3] Approximately 20% of pediatric AML patients are classified as cytogenetically normal AML (CN-AML).[4] In the past decade, new prognostic relevant aberrations have been identified in CNAML, such as *NPM1, WT1, FLT3-ITD* and *CEBPA* double mutations (*CEBPA*dm), which may improve future riskgroup stratification.[5-8] In addition to these mutations, we recently described recurrent cryptic *NUP98/NSD1* and *NUP98/KDM5A* translocations in pediatric CN-AML.[9, 10] Still, some CN-AML samples have not been fully characterized. Recently, Grossmann *et al*. discovered a somatic mutation in the *BCL6* co-repressor (*BCOR*) gene in an adult CNAML patient using whole-exome sequencing.[11] BCOR mutations were subsequently identified in 10 of 262 (approx. 4%) adult CN-AML patients, unselected for cytogenetic abnormalities, and for whom a poor outcome had been predicted. In addition, Li *et al.* discovered mutations in the *BCL6 corepressor-like 1* (*BCORL1*) gene in 2 of 8 patients with secondary adult AML.[12] Further exploration identified somatic *BCORL1* mutations in 10 of 173 (6%) of adult AML patients, of which 5 cases were diagnosed with secondary or treatment-related AML. In 8 of 10 patients these included nonsense, splice site, and frame-shift mutations that were predicted to result in truncation of the protein, suggesting that *BCORL1* is a tumor suppressor gene that may be inactivated by mutations.[13] In pediatric AML, the role of *BCOR* and *BCORL1* is unknown. Therefore, we explored the frequency and impact of *BCOR* and *BCORL1* mutations in pediatric AML in a molecularly well documented cohort of 230 pediatric AML patients.

Genomic DNA was PCR amplified using the primers described in Supplementary Table 8.1. Purified PCR products were directly sequenced. Sequence data were analyzed using CLCWorkbench (CLC Bio, Aarhus, Denmark). *BCOR* variations were determined in comparison to the coding DNA sequence ENST00000342274, and *BCORL1* variations to ENST00000540052. A mutation was defined as a nucleotide change not reported in the dbSNP database. SNPs were defined as a nucleotide change as described in the dbSNP database. Characteristics of the 230 pediatric AML patients included in this mutational screening are listed in Table 8.1.

The complete coding sequence of *BCOR* and *BCORL1* was screened in a nested cohort of 83 *de novo* pediatric AML patients, and 17 AML cell lines. This cohort represented all relevant cytogenetic subgroups in pediatric AML, with an enrichment for CN-AML (n=48). All CNAML patients were screened for *NPM1, CEBPAdm, FLT3- ITD, IDH1/2, WT1, cKIT, N/K-RAS, DNMT3A*, *ASXL1* and *RUNX1* mutations, and *MLL*-PTD, *NUP98/NSD1*, *NUP98/KDM5A*, and MLL-rearrangements (Table 8.3). None of the cell lines showed a mutation. In 4 of 83 patients a *BCOR* mutation (detailed in Table 8.2) was identified; 3 in exon 4 and 1 in exon 12. Three of these cases were CN-AML (3 of 48, 6.3%), and the fourth mutation was seen in an *MNX1/ETV6* translocated AML. In only one of 83 patients a *BCORL1* mutation was identified which resulted in a stop codon (Table 8.2). This CN-AML patient had an underlying xeroderma pigmentosum. An additional 147 pediatric AML patients were screened for mutations in *BCOR* and *BCORL1* exon 4, based on the occurrence of mutations in this exon; 136 cases were *de novo* AML, 11 cases were secondary AML, of which 6 cases were MDS which progressed

to AML and 5 were secondary to earlier therapy because of another malignancy. None of the additional cases were CN-AML. No additional mutations were found. In 2 of 230 cases a missense SNP was found in *BCOR* (p.V679I, rs144722432) and in 35 of 230 (15%) a missense SNP in *BCORL1* (p.G209S, rs5932715). Both SNPs are predicted as tolerated by SIFT analysis, and in line with the predicted prevalence in the normal population.14 In one case missense SNP rs139887979 (p.D94N) was identified in *BCORL1*, predicted as damaging by SIFT analysis. We were unable to confirm this in germline or remission because such material was not available for these patients.

Altogether we identified a *BCOR* mutation in 4 of 230 (1.7%) cases. The frequency of mutations in pediatric CNAML patients (6.3%; 95%CI: 2.1-16.8) was comparable to that in adult CN-AML (3.8%; 95%CI: 2.1-6.9; *P*=ns). The frequency of *BCORL1* mutations (1 of 83, 1.2%; 95%CI: 0.2-6.5) was significantly lower than in adults (10 of 173, 5.8%; 95%CI: 3.2-10.3; *P*<0.05) in patients of whom we screened the complete exon, and also lower when only analyzing exon 4 of *BCORL1* [1 of 230, 0.4% (0.1-2.4) *vs.* 7 of 173, 4.0% (2.0-8.1); *P*<0.05]. This may be due to the enrichment for secondary AML and treatment-related AML in the adult cohort, 73 of 173 (42%) in the cohort of Li *et al*. versus 12 of 230 (5%) in this cohort.[11, 13, 14]

Apart from mutation analyses, we studied *BCOR* and *BCORL1* gene expression levels using RT-qPCR in 65 patients of the initial cohort, including all mutated cases. Expression levels were determined using a SYBRgreen (Finnzymes Inc, Woburn, MA, USA) Taqman assay and average cycle threshold (CT) values were compared to the reference gene *GAPDH*, using the comparative cycle time method. Expression levels of 2 *BCOR*-mutated cases seemed lower than the other pediatric AML cases, but the 4 mutated cases did not significantly deviate from the nonmutated pediatric AML patients (Supplementary Figure S8.1). This is in accordance with findings in adult AML patients where both decreased and normal BCOR levels were observed in cases with *BCOR* mutations.[11] In adult AML, *BCORL1* mutations did not affect mRNA levels, similar to the finding of our single case [0.297% expression relative to *GAPDH*, mean in group (n=65) 0.275%, range 0.049-1.358].[13, 14] In the adult CN-AML cohort, the presence of *BCOR* mutations conferred a poor outcome. In our cohort, the low frequency of mutations did not allow survival analysis to be performed. Clinical outcome of the mutated cases is shown in Table 8.2. The presence of the non-synonymous SNP in *BCORL1*, rs5932715, did not influence clinical outcome (5-year probability of overall survival 57±9% *vs.* 64±4%, *P*=0.4; 5-year probability of event-free survival 49±9% *vs.* 45±4, *P*=0.9).

In conclusion, in pediatric AML, *BCOR* and *BCORL1* mutations rarely occur. Consequently, the clinical relevance is difficult to determine.

Table 8.1. Clinical characteristics of pediatric acute myeloid leukemia cohort.

	***BCOR* and *BCORL1* complete coding sequence**	***BCOR* and *BCORL1* exon 4**
Number	83	147
Median age (y) range	9.8 0.2-18.0	8.2 0.1-18.0
Female, n (%)	36 (43)	58 (42)
Median WBC x10^9/L range	94.3 1.2-377.6	45.2 2.4-475.0
Karyotype, n (%)		
11q23	5 (6)	46 (31)
t(8;21)	5 (6)	13 (9)
inv(16)	6 (7)	28 (19)
t(15;17)	7 (8)	9 (6)
t(7;12)	5 (6)	-
CN	48 (58)	-
Other	7 (8)	40 (27)
Unknown	-	11 (8)
FAB-type, n (%)		
M0	8 (10)	6 (4)
M1	17 (21)	10 (7)
M2	19 (23)	19 (13)
M3	9 (11)	9 (6)
M4	22 (27)	49 (33)
M5	4 (5)	42 (29)
M6	-	2 (1)
M7	1 (1)	7 (5)
Unknown	3 (4)	3 (2)
NUP98/NSD1, n (%)	10 (12)	2 (1)
NPM1, n (%)	14 (17)	4 (3)
*CEBPA*dm, n (%)	7 (8)	3 (2)

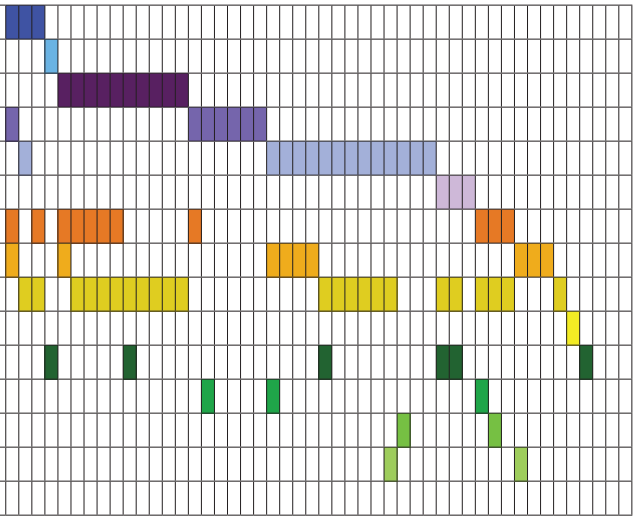
Abbreviations: y indicates years; n, number; CN, cytogenetically normal; FAB, French-American-British classification.

Table 8.2. Characteristics of *BCOR* and *BCORL1* aberrations detected in pediatric cytogenetically normal AML patients.

UPN	Age (y)	Sex	WBC x10^9/L	FAB	Other mutations	Relapse	Death	Karyotype	*BCOR* mutation	*BCORL1* mutation
1	4	M	351.0	M1	*CEBPAdm, WT1, NRAS*	No	No	46,XY[20]	p.A854T	
2	11	F	48.2	M4	*FLT3-itd, NPM1*	No	No	46,XX	p.T60M	
3	12	F	140.6	M5	*FLT3-itd, WT1*	No	No	46,XX[20]	p.D1302E	
4	0	F	217.9	M2	*MNX1/ETV6, KIT*	Yes	Yes	47,XX,t(7;12) (q36;p13),+19[32]	p.G86E	
5	17	M	9.8	M1	*RUNX1*	No	Yes	46,XX[26]		p.G158*

Abbreviations: UPN indicates unique patient number; y, years; M, male; F, female; WBC, white blood cell count; L, liter.

Table 8.3. Characteristics of pediatric CN-AML cases (n=48) screened for *BCOR* and *BCORL1* mutations. Each column represent a single case. A colored field means this case is positive for a mutation or translocation in the corresponding gene(s). A white field indicates the case was tested negative for a mutation or translocation in the corresponding gene(s).

BCOR and *BCORL1* positive cases	*BCOR*	
	BCORL1	
Type 2 mutation	*NUP98/NSD1*	
	*CEBPA*dm	
	NPM1	
	MLL-PTD	
Type 1 mutation	*WT1*	
	N/K-RAS	
	FLT3-ITD	
	PTPN11	
Epigenetic regulator gene mutations	*IDH1/2*	
	DNMT3A	
	RUNX1	
	TET2	
	ASXL1	

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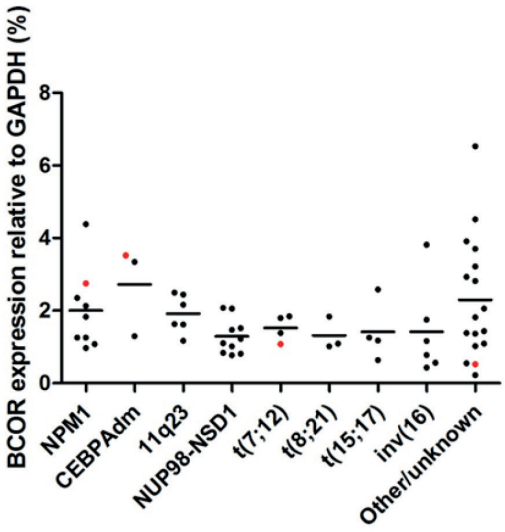
Supplementary data



Supplementary figure 8.1. *BCOR* expression levels determined by RT-qPCR per cytogenetic subgroup. Graphs showing expression levels of *BCOR* for a selected cohort of pediatric AML patients (n=65, initial cohort). Each dot represents a patient and horizontal bars represent median expression for every cytogenetic subgroup. Black indicates wild-type for *BCOR*, red indicates mutated for *BCOR*. 11q23 indicates *MLL*-rearranged or *MLL*-ptd; t(8;21), the *RUNX1/RUNX1T1* translocation; t(15;17), *PML/RARA*; inv(16), *CBFB/MYH11*. Other or unknown indicates patient group without the other mentioned aberrations.

Supplementary Table 8.1A. Overview of *BCOR* primers.

Exon	Direction	Primer
2	Forward Reverse	5'-GGCTCCCAGACCACTGT-3' 5'-GCGGGAAGCTCTTTCTCT-3'
3	Forward Reverse	5'-GCGGAGGGTTAAGGACA-3' 5'-GGGGCCTTGTCCTCTC-3'
4.1	Forward Reverse	5'-GCCGGAAGCCTGTCTT-3' 5'-GGCAGCCGCAGATAAC-3'
4.2	Forward Reverse	5'-GCCAGCGACAAACAGAG-3' 5'-GGGGGCAACAGGAGA-3'
4.3	Forward Reverse	5'-CCACGCCTATCCTCACA-3' 5'-AGCTTTGGAAGCATCTACATC-3'
4.4	Forward Reverse	5'-AGCGGTTCAAGACAGAAAA-3' 5'-GCCCACGTGCTGAATAA-3'
4.5	Forward Reverse	5'-CACCGATGCTGTCATCAC-3' 5'-GTGCCCAGGAAACAGACT-3'
4.6	Forward Reverse	5'-GAGGGCATTGCTGTAAGTC-3' 5'-TCGCTTTTGACAACAGTCTT-3'
4.7	Forward Reverse	5'-TCCCCACCGACAAGAA-3' 5'-GGGGGTCACATCCACA-3'
4.8	Forward Reverse	5'-TGGCCCTGCTGTAACTTT-3' 5'-CCCCAATCCTGTTTACACA-3'
5	Forward Reverse	5'-AAAGGTTGCTTAAAGGGATAGA-3' 5'-ACCATGGCCCACAAACT-3'
6	Forward Reverse	5'-TTTGGGCACTTTTCTTTGA-3' 5'-GGTTGCCACCATTCATAAG-3'
7	Forward Reverse	5'-CCCTCCCTCTGGAAAGTT-3' 5'-CCGCACATCCACATCTC-3'
8	Forward Reverse	5'-CCCCCACCCCATTAGT-3' 5'-CCCGCATACCTTGTTCA-3'
9	Forward Reverse	5'-CGCCCGCTTCTTCTC-3' 5'-AAGCCGGGGTCAAGAG-3'
10	Forward Reverse	5'-CTCTCCACGCAGGTTTG-3' 5'-CTCGCCCACCACAGTC-3'
11	Forward Reverse	5'-GCCCGAGAGGTTCTCAG-3' 5'-ATTGGGAGCTTTACATCTACATT-3'
12	Forward Reverse	5'-TGGCGGTGACTGTGC-3' 5'-TCGGCTGCTCTCCTAAAA-3'
13	Forward Reverse	5'-CTTCCAGCCTGTCATGAAT-3' 5'-GCCCACCATCCACTTTC-3'
14	Forward Reverse	5'-CTGGGAAAGAATCTTGTTATTTA-3' 5'-CCCCCCACCACACTG-3'
15	Forward Reverse	5'-GGCGCACTTTTCATTTTAC-3' 5'-CCAGCTTGCTCACCAGTAG-3'
RT-qPCR	Forward Reverse	5'-ACCGATTCAAATGTGTCACT-3' 5'-GGCCACCTTCTCTTTCTT-3'

PCR cycle: 10'95 ^{o}C, 40x(15"95^{o}C, 1'60^{o}C, 30"72^{o}C), 10'72^{o}C
qRT-PCR cycle: 10'95^{o}C, 40x(15"95^{o}C, 1'60^{o}C)

Supplementary Table 8.1B. Overview of *BCORL1* primers.

Exon	Direction	Primer
2	Forward Reverse	5'-CTTCCCGGTTTAGACCTTAAT-3' 5'-CCCAGGCCCTATTGTATG-3'
3	Forward Reverse	5'-CCCCCTCACAAGTTACTACAG-3' 5'-AGCCAGCCAATTTGTGTC-3'
4.1	Forward Reverse	5'-CGGGCCTCAGGACAC-3' 5'-GGGCTGGCAGAGGACT-3'
4.2	Forward Reverse	5'-AGGCCAGCAACAGCAG-3' 5'-AGGGGGTTCGAGTCAGA-3'
4.3	Forward Reverse	5'-TGTTCCAGTCCAAGTTGCCACTTC-3' 5'-ATGGGTGTAGGGGCTGGAGTAAA-3'
4.4	Forward Reverse	5'-TGCCCACGCTCATCTC-3' 5'-TGGAGGCGGGATATATACC-3'
4.5	Forward Reverse	5'-CCGGCCTCCTTCAGTT-3' 5'-CAGGGGAGCCTGTTCA-3'
4.6	Forward Reverse	5'-CCCGAGCTCCGTTCTT-3' 5'-CAGGCAGGAGGTGACATT-3'
4.7	Forward Reverse	5'-CCCCCATGCCTGTGT-3' 5'-GTGGGTTTCCACAAAGAGA-3'
4.8	Forward Reverse	5'-AGCCCCATCTCCATCATTG-3' 5'-AGGCCCCTGATTCATTC-3'
4.9	Forward Reverse	5'-CTGCCAAGCCTTATGAAG-3' 5'-GCCCAACAGGACTGTGT-3'
5	Forward Reverse	5'-ACCCCTGGAGAGCTTTCT-3' 5'-CTGCCCTCACCACTGTG-3'
6	Forward Reverse	5'-TGGGCTTTCTGGTCTTTAAG-3' 5'-GTGGGCAATATTTGAGGACT-3'
7	Forward Reverse	5'-GTCCAGGGCATTCACTTC-3' 5'-AGCACCGGGTTTCTCTG-3'
8	Forward Reverse	5'-GGGCTGGGAGCTGTCT-3' 5'-CGCCACACACACCTTCTA-3'
9	Forward Reverse	5'-GTGGCTCAGGACTGATGTT-3' 5'-GGCAAGGTCTTTTGAGTGA-3'
10	Forward Reverse	5'-TTCCCCTGATGCAGTAGC-3' 5'-GCCCCAAGGACAACAGT-3'
11	Forward Reverse	5'-TGGCAGTCTCTGACTGAGAA-3' 5'-TGAGGGATGGCTGTGTC-3'
12	Forward Reverse	5'-GTGCCCTCTGAAGGATGT-3' 5'-AGCCTTTGTCATGTGTGATG-3'
13	Forward Reverse	5'-TGGGCTTTGCAGAGTGT-3' 5'-GGTCGGGGAAAGACAAG-3'
RT-qPCR	Forward Reverse	5'-TGCCTCCAGAAAGACAGT-3' 5'-CCACCGCATCATGAAC-3'

PCR cycle: 10'95 ^{o}C, 40x(15"95^{o}C, 1'60^{o}C, 30"72^{o}C), 10'72^{o}C
qRT-PCR cycle: 10'95^{o}C, 40x(15"95^{o}C, 1'60^{o}C)

Chapter 9

PHF6 mutations in pediatric acute myeloid leukemia

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Letter to the editor

Pediatric acute myeloid leukemia (AML) is a heterogeneous disease characterized by the presence of different collaborating cytogenetic aberrations which are associated with outcome [1, 2]. In the past decade, new prognostically relevant genetics aberrations have been identified, such as mutations in *NPM1* and *FLT3*, which further improve risk-group stratification. In ~20% of the pediatric AML cases the underlying genetic abnormalities are not fully understood [1, 2]. Unravelling the underlying aberrations involved in leukemogenesis may improve risk-group stratification.

PHF6 is an X-linked tumor suppressor gene, with a role in transcriptional regulation, and was identified as germline mutation causing Borjeson-Forssman-Lehmann syndrome [3, 4]. Mutations of *PHF6* are reported in adult T-ALL, and somatic mutations in *PHF6* were identified in 3% of adult AML, predominantly in males, in cases classified as French-American-British (FAB) M0, M1 and M2 [5-7]. Using whole genome sequencing, *PHF6* mutations were identified in 2% of a pediatric AML series strongly enriched for core-binding factor AML [8]. However, a large pediatric AML cohort has never been examined. In this study, we screened a large pediatric *de novo* AML cohort including all FAB-types, enriched for FAB-M0, M1 and M2, for *PHF6* mutations.

Samples were provided by the Dutch Childhood Oncology Group (DCOG, The Netherlands), the AML–Berlin-Frankfurt-Münster Study Group, and the Saint-Louis Hospital (France). Each study group provided centrally reviewed morphological and cytogenetic classification, and clinical data, such as age at diagnosis, white blood cell count (WBC) and outcome. Institutional review board approval had been obtained in the participating centres. Genomic DNA was PCR amplified. Purified PCR products were directly sequenced, and analyzed using CLC Workbench (CLC Bio, Aarhus, Denmark, primers: Supplementary Table 9.1). In case of an aberration, RNA and germline or remission DNA was analyzed, if available. Reverse-transcription-PCR was performed on known hotspot areas and for the presence of fusion genes as previously described [9].

318 *de novo* paediatric AML cases were eligible for mutational screening, including 20 FAB-M0, 47 FAB-M1 and 76 FAB-M2 cases (Table 9.1A). 175 males and 141 females were screened (2 patients unknown). Direct sequencing analysis revealed the presence of *PHF6* mutations in 6/318 (2%;95%CI:0.9-4.1%) AML cases, representing 6/143 (4%;95%CI:1.9-8.9%) pediatric FAB M0, M1 and M2 cases. Most mutations were predicted to result in a loss-of-function with four truncating frameshift mutations, and one point mutation in an intron resulting in loss of exon 4 on RNA-level (Supplementary Figure 9.1A). In the latter case germline material derived from fibroblasts did not harbor this aberration.

Additionally, one missense variation (p.S145N, Supplementary Figure 9.1B) was identified, which was also present in material obtained at complete remission and relapse, and predicted as damaging in SIFT and PolyPhen analysis. No SNPs are known at this position (dbSNP, dbVar, accessed 2015/02/17). However, the variation localized outside the most conserved PHD-finger domains. Based on the material obtained at complete remission this may be considered a germline variation.

Median age at diagnosis was 12.6 years in the *PHF6*-mutated group, compared to 9.5 years in other pediatric AML (p=0.11). Median WBC was $3.8x10^9$/L and $43.0x10^9$/L respectively (p=0.03, Table 9.1A). After successful induction, 4/6 mutated cases had

a relapse, and 1 patient did not experience an event. One other patient was a non-remitter. Two patients are alive in complete remission, and four died from the disease.

All *PHF6*-mutated cases were morphology subtype FAB M0, M1 or M2. Other genetic abnormalities found in these cases were translocations *RUNX1/RUNX1T1* and *NUP98/KDM5A*, and mutations in *WT1, RAS, ETV6, TET2, IDH1* and *BCORL1* (Table 9.1B). None of these aberrations were recurrent in the *PHF6*-mutated group. Wang et al reported that in T-cell ALL *PHF6* mutations were associated in combination with the *SET/NUP214* translocation [10]. This translocation was present in 11 cases in our series, but none harboured a *PHF6* mutation.

In all reported series of *PHF6* mutations in T-ALL and adult AML mutations were almost exclusively found in males [5, 7]. In our data, 4/6 of the patients with a mutation were female. Distribution of sex among the mutated cases did not differ significantly (Fisher's exact test; p=0.1), in contrast to the findings in adult AML [7]. One female *PHF6*-mutated case showed loss of a sex chromosome in the leukemic cells based on karyotyping. PHF6 is hypothesized to be inactivated on one copy of the X-chromosome in females due to lyonization. Quantitative real-time PCR (qRT-PCR) analysis of mRNA expression of *XIST* was performed using the average cycle threshold (Ct) in comparison to expression levels of *GAPDH*, using the comparative Ct method. *XIST* is highly expressed (>5% compared to *GAPDH*) if at least two X-chromosomes are present. The patient with known loss of an X-chromosome indeed showed low expression (<1% compared to GAPDH) of *XIST.* The other females with a *PHF6* mutation showed high expression of *XIST,* indicating both X-chromosomes were present in the leukemic blasts. Whereas on DNA the frameshift mutations were heterozygous in female, analysis of the frameshifts on RNA level showed mono-allelic presence of the mutated gene, indicating inactivation of the wild type *PHF6* gene (Supplementary Figure 9.1C).

qRT-PCR analysis of *PHF6* was performed on patients with available RNA (n=240). FAB-types M0, M1 and M2 did not show lower mRNA expression compared to other FAB-types (Figure 9.1A). There was no difference in *PHF6* expression between males and females (p=0.7, Figure 9.1B). *PHF6* was significantly lower expressed in patients with a frameshift mutation compared to patients with normal predicted amino-acid length, suggesting the frameshifts result in a loss-of-function (p=0.038, Figure 9.1C). When comparing mutated cases to patients with FAB-M0, M1 or M2 paediatric AML without *PHF6* mutation, again a significant difference was found (p=0.026, Figure 9.1D). Hairpin-mediated suppression of Phf6 promoted AML in vivo.[4] Accordingly, the presence of loss-of-function mutations in *PHF6* in pediatric AML suggests a role for *PHF6* in leukemogenesis.

Overall, our data show that *PHF6* mutations occur in a low frequency in pediatric AML, exclusively in FAB-M0, M1 and M2, with a significantly lower mRNA expression of *PHF6*. In contrast to T-ALL and adult AML, we found mutations of *PHF6* in both female and male patients in pediatric AML. The potential prognostic impact of *PHF6* mutations needs to be determined in a larger series.

Table 9.1A. Clinical characteristics of *PHF6* mutated and *PHF6* wild type pediatric AML patients.

		***PHF6* mutated cases (n=6)**	***PHF6* wild type cases (n=312)**	**p-value**
Age at diagnosis	Median (y)	12.6	9.5	p=0.11
	Range (y)	6.3-17.0	0.1-18.0	
Sex	Female	67%	44%	p=0.06
WBC	Median (x10^9/L)	3.8	43.0	p=0.03
	Range (x10^9/L)	2.2-196.0	1.0-534.6	
FAB-type	M0	2	18	
	M1	2	45	
	M2	2	74	
	M3	0	20	
	M4	0	75	
	M5	0	62	
	M6	0	2	
	M7	0	9	
	Not available	0	7	
Type-II aberrations	*RUNX1/RUNX1T1*	1	32	
	NUP98-rearrangements	1	20	
	Inv(16)	0	35	
	KMT2A-rearrangements	0	70	
	PML/RARA	0	18	
	DEK/NUP214	0	11	
	Other/Unknown	4	126	

Abbreviations: y indicates years; WBC, white blood cell count; FAB-type, French-American-British morphology type.

Table 9.1B. Patient characteristics of pediatric AML with a *PHF6* mutation.

UPN	PHF6 mutation (location)	Predicted amino acid length	Age (yrs)	Sex	WBC ($x10^9$/L)	FAB	Mutations	Rem	Rel	Death	SCT	Karyotype	*XIST* expression
1	pS145N (exon 6)	-	6.3	M	196.0	M0	*WT1, RAS, ETV6*	CR	Y	Y	N	46,XY,del(17)(p12,p13) or add(17)(p11)[2]	-
2	c.224delAA (exon 3)	94	10.3	F	NA	M0	-	CR	N	N	N	46,XX,t(3;4)(q11-12;p15-16)[6]/47,XX, t(3;4)(q11-12;p15-16),+mar [12]/46,XX[2]	High (13.9%)
3	c.834-839delGTTTTA insTCAGTTC (exon 6)	146	14.3	M	2.2	M2	*TET2, RAS*	PR	N	N	Y	46,XY	-
4	c.28insA (exon 2)	21	10.9	F	3.8	M2	*RUNX1/ RUNX1T1*	CR	Y	Y	Y	46,X,-X,+8,t(8;21)(q22;q22)[15]	Low (0.84%)
5	c.904delC (exon 9)	350	14.6	F	2.2	M1	*NUP98/ KDM5A*	CR	Y	Y	N	Not available	High (10.0%)
6	Deletion of exon 4	92	17.0	F	9.4	M1	*IDH1, BCORL1*	NR	-	Y	N	46,XX[26]	High (6.6%)

Abbreviations: UPN indicates unique patient number; yrs, years; WBC, white blood cell count; FAB, French-American-British morphology subtype; SCT, stem cell transplantation; M, male; F, female; Y, yes; N, no.

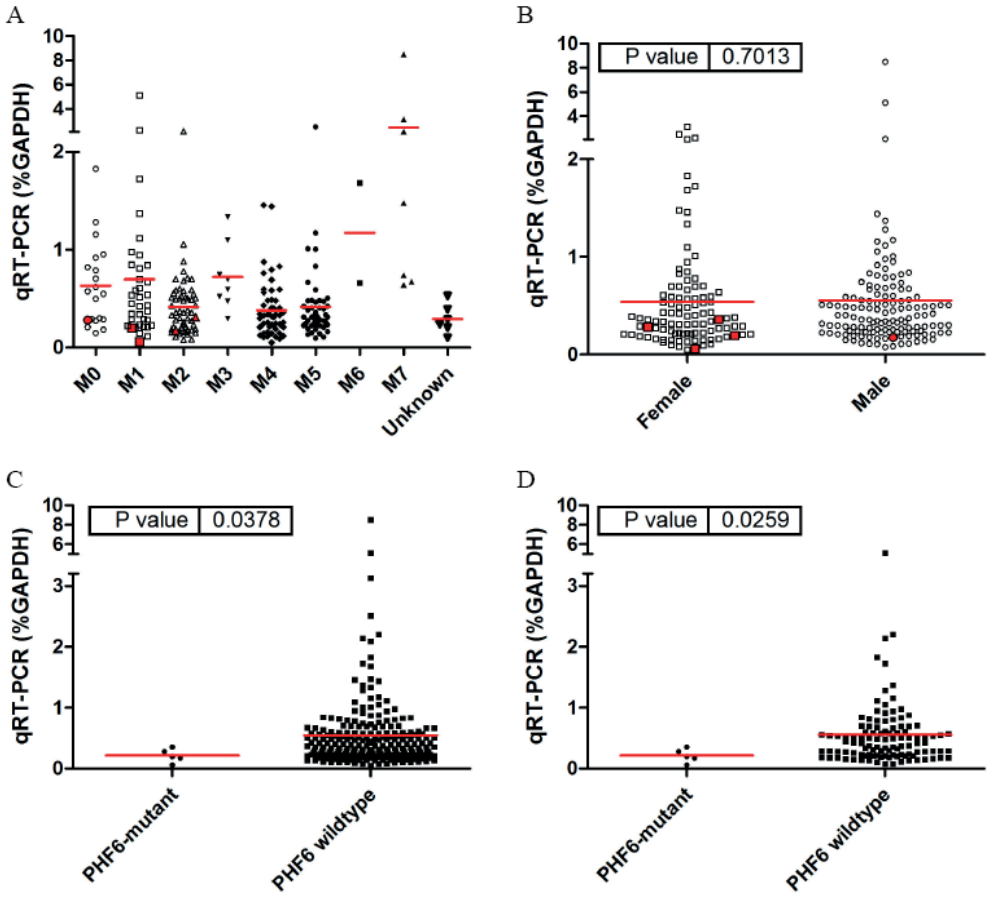

Figure 9.1. *PHF6* mRNA expression as measured with qPCR. Mean expression is indicated with the red line. (A) Patients divided per FAB-type. Patients with a frameshift mutation are found in FAB-M0, M1 and M2, and indicated in red. (B) Patients divided per sex. Patients with a frameshift mutation are indicated in red. There is no significant difference in mRNA expression of *PHF6* between male and female pediatric AML patients (Mann-Whitney test, p=0.7). (C) Comparing mRNA expression levels of pediatric AML patients with a frameshift mutation to other pediatric AML, patients with a frameshift have a significant lower mRNA expression compared to wild type *PHF6* pediatric AML patients (Mann-Whitney test, p=0.0378). (D) Comparing mRNA expression levels of pediatric AML patients with FAB M0, M1 and M2, patients with a frameshift mutation have a significant lower mRNA expression compared to wild type *PHF6* pediatric AML patients of the selected FAB-types (Mann-Whitney test, p=0.0259).

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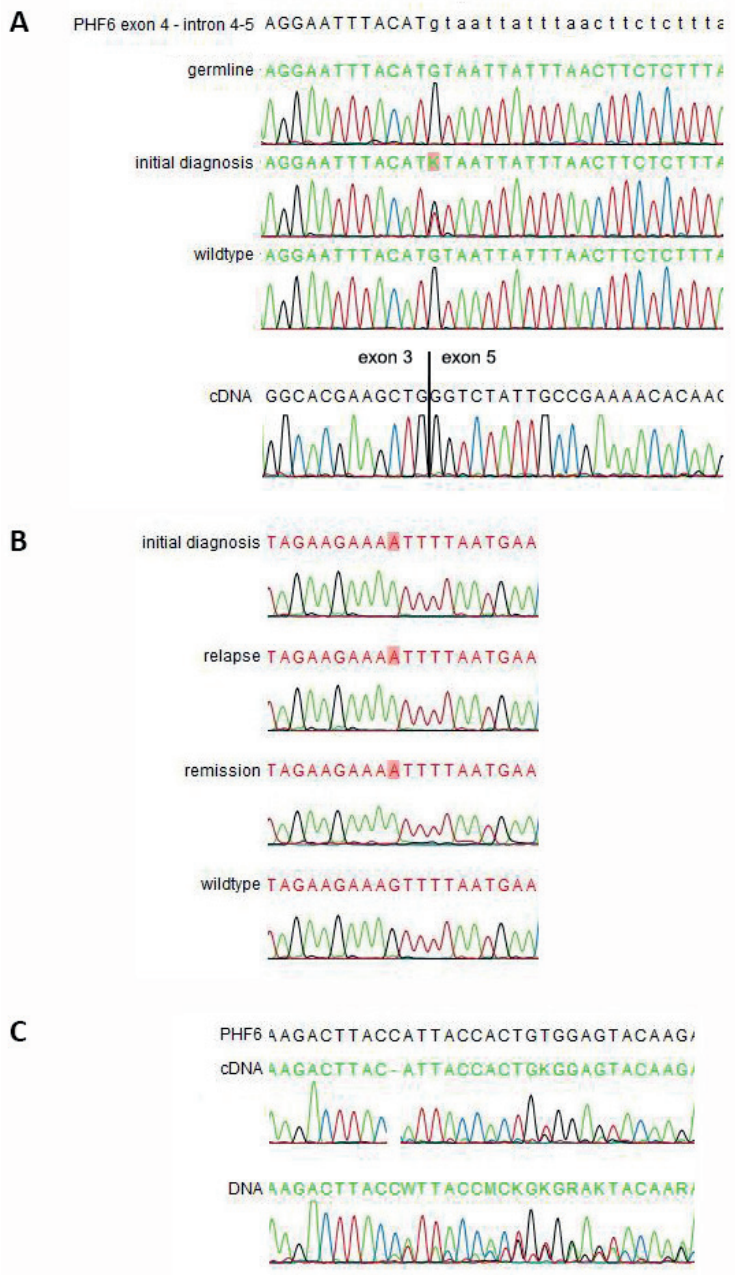
Supplementary data



Supplementary Figure 1. *PHF6* mutations in pediatric AML cases. (A) A point mutation (G>A) in intron 4-5 resulting in deletion of exon 4 in a girl. The mutation, indicated with orange background, is present in the initial diagnosis sample, but not in germline material of this patient. On cDNA the sequence of exon 3-5, missing exon 4. The uppercase letters indicate the sequence of exons 3, 4 and 5, the lowercase letters indicate the sequence of intron 4-5. (B) A point mutation (G>A) resulting in p.S145N in a boy. The mutation, indicated with orange background, is present in samples of initial diagnosis, remission and relapse. A wild type sequence of a different patient is added as reference. (C) A deletion of C resulting in a frameshift in exon 9 in a girl with high XIST expression. On DNA this deletion is seen heterozygously, while the sequence on cDNA shows solely the mutated allele, indicating silencing of the X-chromosome with wild type PHF6.

Supplementary Table 9.1. Primers used for mutational screening and qRT-PCR.

Exon	Direction	Primer	PCR cycle
2	Forward Reverse	5'-TTTCTTGGGGCTTAGAGTG-3' 5'-AAATGGCATAGCATTAGTGA-3'	10'95°C, 40x(15''95°C, 1'60°C, 15''72°C), 10'72°C
3	Forward Reverse	5'-GCTATGCCATTTTTACTAGAAA-3' 5'-GCTGGCTCAGAGAAAAAAAA-3'	10'95°C, 40x(15''95°C, 1'60°C)
4-5	Forward Reverse Forward*	5'-CCCCAGAAGAATTTTATTCC-3' 5'-AAACGTGGCTAAATGATGTA-3' 5'-AAAGGGTGTTTTTGATAAGA-3'	10'95°C, 40x(15''95°C, 1'60°C, 20''72°C), 10'72°C
6-7	Forward Reverse	5'-AGGCCACATGTTGCTAGT-3' 5'-GGCTTTAGATCACAGGGGAAAC-3'	10'95°C, 40x(15''95°C, 1'60°C, 45''72°C), 10'72°C
8	Forward Reverse	5'-CTCCACACTGGCAGTCCTCATTTT-3' 5'-GGCAGCCCTGTGTATGTGTTCTTT-3'	10'95°C, 40x(15''95°C, 1'60°C, 45''72°C), 10'72°C
9	Forward Reverse	5'-GAGGGCTTATCAAAGTATGG-3' 5'-AGGAAAATGCCAATTGTAGT-3'	10'95°C, 40x(15''95°C, 1'60°C)
10	Forward Reverse	5'-TGGGCACTAGCCTCATCCACTAAT-3' 5'-GCAAGCCTACAAAATCCTCAGCA-3'	10'95°C, 40x(15''95°C, 1'60°C)
qPCR	Forward Reverse	5'-AGGGGAGGAAGAAAATGA-3' 5'-GTGCCAGAAGAAAACAACA-3'	10'95°C, 40x(15''95°C, 1'60°C)

* This primer is only used for direct sequencing of exon 5.

Chapter 10

Recurrent translocation t(10;17)(p15;q21) in minimally differentiated acute myeloid leukemia results in *ZMYND11/MBTD1* fusion

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Abstract

Pediatric acute myeloid leukemia (AML) is a heterogeneous disease, characterized by different collaborating karyotypic and molecular abnormalities, which are used in risk group stratification. In ~20% of the pediatric AML cases a specific genetic aberration is still unknown. Minimally differentiated myeloid leukemia or FAB-type M0 is a rare morphological subtype of AML. The translocation t(10;17)(p15;q21) is described to be recurrent in minimally differentiated AML, but the involved genes and location of the breakpoints have so far not been identified.

In this study we show that this translocation results in an in-frame translocation fusing exon 12 of the tumor suppressor gene *ZMYND11* to exon 2 of the chromatin protein *MBTD1,* encoding a protein of 1054 amino acids, while the reciprocal fusion product is predicted to lack a productive start codon. Gene expression profiling of the leukemic cells showed high *HOXA* expression. *ZMYND11,* also known as *BS69*, is a tumor suppressor that specifically recognizes H3K36me3, which is linked to aberrant *HOXA* expression in leukemogenesis. Aberrant expression of the genes involved in this fusion may thus contribute to the *HOXA*-phenotype observed with gene expression profiling.

Introduction

Pediatric acute myeloid leukemia (AML) is a heterogeneous disease, characterized by different collaborating gene aberrations, such as translocations and mutations. These abnormalities, together with early treatment response, predict outcome.[1-3] In ~20% of the pediatric AML cases no specific genetic aberration has been identified.[4] Based on morphology, minimally differentiated AML (French-American-British (FAB) type M0) is defined by expression of markers such as CD13, CD33 and CD117 in the absence of lymphoid differentiation and myeloperoxidase (MPO) positivity. Minimally differentiated AML is seen in <5% of all AML cases, being more common in adult AML patients compared to children.[5] The translocation t(10;17)(p15;q21) is recurrent in minimally differentiated AML, but the translocation partners in this fusion remain unknown to date.[6-8] In total 6 cases of AML harboring this karyotypic aberration have been described in the literature in both pediatric and adult AML, and one case was described in a pediatric precursor B-cell acute lymphoblastic leukemia (Supplementary Table 10.1). Based on FISH analyses it has been suggested that this translocation in AML most likely results in fusion of *ZMYND11* on chromosome band 10p15 and *MBTD1* on 17q21.[7] In this study we confirm this by identifying the translocation at the molecular level in a not previously described patient, and suggest the translocation results in high *HOXA* expression.

Material and methods

Patient Material

Blood and bone marrow samples taken at initial diagnosis and at relapse of the index patient were provided by the Dutch Childhood Oncology Group (DCOG). Leukemic cells were purified as described before.[9] Isolation of genomic DNA and total cellular RNA was performed using Trizol reagent. Conventional karyotyping was performed at the center of diagnosis and centrally reviewed.

Molecular Screening

Molecular work-up included mutational analysis on hot-spot areas in *CEBPA, NPM1, WT1, KIT, NRAS, KRAS, FLT3* (including *FLT3*-ITD), *PTPN11, IDH1/2, DNMT3A, ETV6,* and *RUNX1,* and recurrent translocations were screened for, such as *RUNX1/RUNX1T1, KMT2A*(formerly known as *MLL)*-rearrangements and *NUP98*-rearrangements.

To investigate whether the t(10;17)(p15;q21) resulted in the *ZMYND11/MBTD1* fusion as previously suggested[7], reverse transcriptase polymerase chain reaction (RT-PCR) primers were designed in exon 8, 10, 11, 12, 13 and 15 of *ZMYND11,* and exon 1 and 3 of *MBTD1* (Supplementary Table 10.2) and compared to the coding sequences ENST00000397962 for *ZMYND11* and ENST00000586178 for *MBTD1*.

Gene expression profiling (GEP) of 274 *de novo* pediatric AML samples and confirmation of mRNA expression results by quantitative reverse-transcription-PCR (qRT-PCR) analysis for *HOXA5, HOXA7, HOXA9* and *HOXA10* was available from earlier studies.[10] Original data are available in the Gene Expression Omnibus repository (http://www.ncbi.nlm.nih.gov/geo; accession GSE17855). A centered principal component analysis, based on singular value decomposition, was performed using all *HOX*-gene probesets available. K-means clustering optimizing squared Euclidean distances was

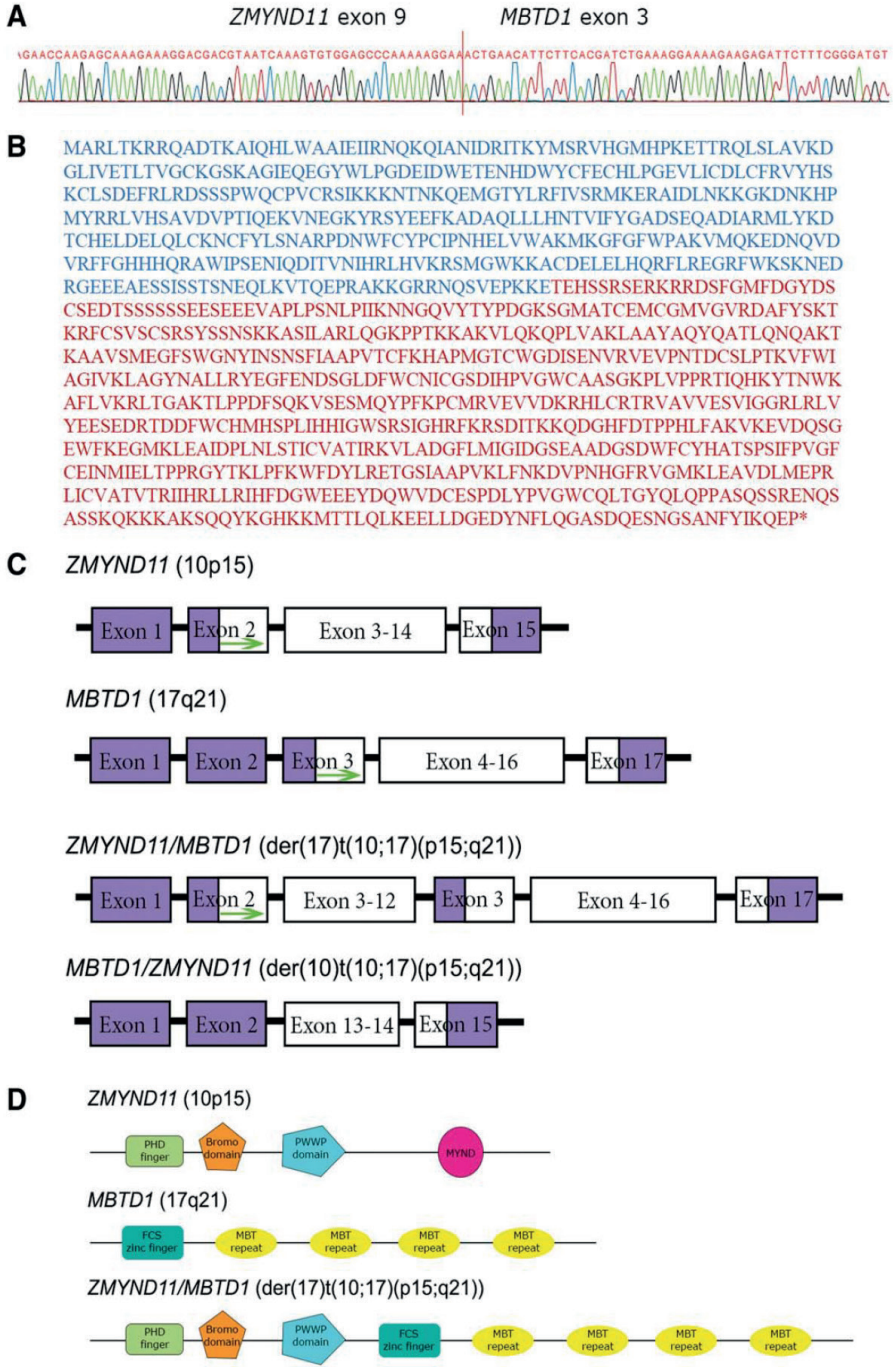

Figure 10.1. Fusion of *ZMYND11* and *MBTD1*. (A) Sequence of *ZMYND11* exon 12 fused to *MBTD1* exon 3. In red the sequence found in the sample obtained at diagnosis. In green the sequence found in the sample at time of relapse. (B) Fusion protein of ZMYND11/MBTD1. In blue the protein originating from ZMYND11, in red the protein originating from MBTD1, * indicates the stopcodon. (C) Schematic overview of the fused exons in *ZMYND11/MBTD1* and *MBTD1/ZMYND11*. The purple rectangles indicate untranslated region of the exon. A green arrow indicates a start-codon. The white rectangles indicate translated region of the exon. (D) Schematic overview of the different domains of *ZMYND11, MBTD1* and *ZMYND11/MBTD1*.

performed to create three clusters.

Results and discussion

The index patient was a 13 year old boy, who was diagnosed with pediatric AML with FAB type-M0. The white blood cell count at diagnosis was $6.5x10^9$/L. Immunophenotyping showed the leukemic blasts to be positive for CD7, CD33, CD34, CD45, CD56, CD117, and negative for MPO and HLA-DR. Conventional karyotyping showed 46,XY,t(10;17)(p15;q21)[9]/46,XY[1]. The boy was treated according to the DCOG ANLL97/MRC AML 12 protocol. He achieved complete remission 4 weeks after start treatment, but relapsed 11 months after diagnosis. Conventional karyotyping at this time showed 47,XY,t(10;17)(p15;q21),+13[1]/48,idem,+7[11]/46,XY[8]. He was treated with reinduction chemotherapy according to the Relapsed AML2001/01 protocol and stem cell transplantation with a matched unrelated donor. The post-transplantation period was complicated due to severe graft versus host disease (GVHD) of the skin, intestines and liver, followed by pulmonary chronic GVHD. He was treated with high dose immune-suppressive therapy. Unfortunately he died 30 months after initial diagnosis due to a combination of infection and hemorrhage.

Screening for all aforementioned mutational hot-spot areas and other known AML gene rearrangements was negative. Samples of this patient from diagnosis and relapse were screened for the *ZMYND11/MBTD1* fusion and *MBTD1/ZMYND11* fusion with the designed primer sets. Direct sequencing was performed for obtained bands present on agarose gel. This led to the identification of the fusion product *ZMYND11/MBTD1* and its reciprocal product in both bone marrow and peripheral blood samples taken at initial diagnosis and relapse. Sequencing showed that the translocation resulted in an in-frame fusion fusing exon 12 of *ZMYND11* to exon 3 of *MBTD1*, encoding a 1054 amino acid long protein (Figure 10.1A and 10.1B) on the der(17). The reciprocal product *MBTD1/ZMYND11* resulted in fusion of exon 2 of *MBTD1* to exon 13 of *ZMYND11*. Because exon 1, 2 and a minor part of exon 3 of *MBTD1* encode part of the 5' untranslated region, no productive start codon is encoded in this fusion transcript (Figure 10.1C) on the der(10).

Gene expression profiling was available for the initial diagnosis sample, but not for the relapse sample. *HOX* genes have various roles in hematopoiesis, and dysregulation may lead as a common pathway to leukemogenesis induced by various underlying genetic events.[11] Different *HOX* expression patterns are described and the distinct *HOX* expression pattern is most likely the result of the underlying translocation or mutation activating different *HOX*-genes.[11-13] In previous studies we could identify three distinct clusters of pediatric AML cases based on *HOX* expression.[10, 14, 15] Although overexpression of several *HOX* genes is described to correlate with poor outcome, other studies describe outcome to be correlated with the underlying translocation or mutation resulting in the aberrant *HOX* expression.[14-17] Understanding the underlying mechanism causing *HOX* gene dysregulation, may lead to the development of targeted treatment options for the *HOX* overexpressing cases. The development of DOT1L inhibitors for *KMT2A*-rerranged leukemias is a prime example of this.[18]

To investigate the *HOX* expression in this case we used the first three principal components, together 85% component variance, to form the three requested clusters based on k-means clustering using (Figure 10.2A). This method identified the previously

described *HOX* clusters.[10, 14, 15] Performing a principal component analysis on all these *HOX* genes, the index-patient clustered in the group with high *HOXA* expressing cases, showing an expression profile similar to the expression observed in *KMT2A*-rearranged cases (Figure 10.2B).

ZMYND11, also known as *BS69*, is a tumor suppressor, specifically repressing active genes through binding of H3.3K36me3.[19-21] *ZMYND11* contains a PHD finger, bromodomain, PWWP domain and a MYND domain (Figure 10.1D). In the fusion product *ZMYND11/MBTD1*, the MYND domain is replaced by the full length MBTD1 protein (Figure 10.1D). Wen *et al* showed that deletion of the MYND domain did not affect the binding of H3K36me3.[19] *MBTD1* is a poorly characterized putative Polycomb Group (PcG) protein, which functions as transcriptional repressor of genes affecting differentiation and tumor suppression.[22-24] *MBTD1* contains four malignant brain tumor (MBT) repeats, which specifically recognize H4K20me1/2.[22, 25] In another family member of MBTD1*,* the dSfmbt protein, the MBT repeats are described to specifically bind mono- or dimethylated states of the H3 and H4 histones, thereby targeting *HOX* gene Polycomb response elements, required for silencing *HOX* gene expression.[26] Together, the genes involved in this fusion are likely to play a role in gene repression by epigenetic mechanisms, and may conceivably be responsible for the observed high *HOXA* expression.

In conclusion, we identified in this pediatric AML case the genes involved in the t(10;17)(p15;q21), resulting in fusion of *ZMYND11* and *MBTD1* on the der(17)t(10;17). This fusion might result in *HOXA* overexpression, which is a well-known leukemogenic pathway in AML.

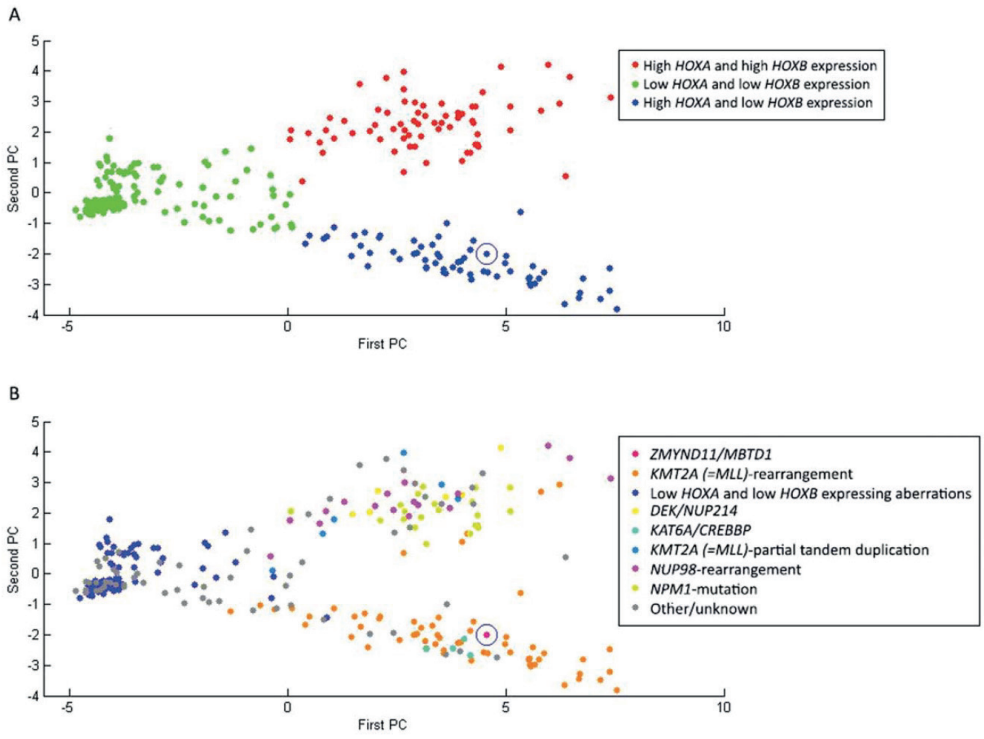

Figure 10.2. *HOX* expression in pediatric AML. (A) Three clusters were created using k-means clustering using all available *HOX*-gene probesets. The index patient is indicated with a halo. (B) Principal component analysis based on *HOX* genes. Indicated in red with halo is the index case harboring the *ZMYND11/MBTD1* fusion.

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Supplementary Data

Supplementary Table 10.1. Overview of cases of t(10;17)(p15;q21) described in literature.

id	karyotype	Type	Age at diagnosis	reference
1	46,XY,del(6)(q14q16),t(10;17)(p15;-q21),?der(11)	AML-M1	NA (range 15.2-76.8 y)	Barjesteh van Waalwijk van Doorn-Khosrovani S et al., 2003
2	46,XY,t(10;17)(p15;q21)/47,idem,+13	AML-M1	13 y	Tempescul A et al., 2007
3	46,XX,t(10;17)(p15;q21)/46,idem,-der(11)del(11)(q13q23)t(11;13)(q14;-q34),der (13)t(11;13)(q23;q34)	AML-M1	40 y	Tempescul A et al., 2007
4	46,XX,t(10;17)(p15;q21)/47,idem,+7	AML-M0	NA (range 20-86 y)	Dicker F et al., 2007
5	46,XY,t(10;17)(p15;q21)/46,idem,-del(6)(q15q23),del(9)(p21p22),-del(14) (q21q24)	AML-M0	16 y	Pollak C et al., 1987
6	46,XY, i(7)(q10),t(10;17)(p15;q21)	AML-M1	11 y	Shah D et al., 2001
7	46,XX,t(10;17)(p15;q21)[24]/46,XX[3]	Pre B-ALL	6-y	Silva ML et al., 2002

Abbreviations; id indicates identification number; AML-M, AML FAB-type; y, years.

Supplementary Table 10.2. Primers used for identification of involved genes and the breakpoint of *ZMYND11/MBTD1.*

Primer id	Location	Direction	Used together with	Primer sequence
1	*ZMYND11* exon 8	Fw	5	5'-TTGCGAGGATGCTATATAAAG-3'
2	*ZMYND11* exon 10	Fw	5	5'-GGCCAGCCAAAGTCAT-3'
3	*ZMYND11* exon 12-13	Fw	5	5'-GAGCCCAAAAAGGAAGAA-3'
4	*ZMYND11* exon 13	Fw	5	5'-AAGCGGGAGACAGAGC-3'
5	*MBTD1* exon 3	Rv	1-4	5'-TGCCCATTGTTTTTGATAA-3'
6	*MBTD1* exon 1	Fw	7-9	5'-AGGCCCTGATGGAAAA-3'
7	*ZMYND11* exon 11	Rv	6	5'-CCTCGGTCCTCATTCTTAG-3'
8	*ZMYND11* exon 13	Rv	6	5'-ATCCGGTCTTTGAAGTCAT-3'
9	*ZMYND11* exon 15	Rv	6	5'-ACCGCTGTGTCTGAAAAG-3'

Abbreviations; id indicates identification number; Fw, forward; Rv, reverse.

Chapter 11

Summary
Samenvatting

Summary

In studies presented in this thesis, novel recurrent aberrations were identified in pediatric AML, with a specific focus on pediatric non-Down syndrome AMKL. With the identification of *NUP98/KDM5A, HOX*-rearrangements and *GATA1s* mutations in pediatric AMKL, a standard risk and high risk group could be identified in patients diagnosed with this rare subtype of AML.

Chapter 2 describes the identification of the fusion of *NUP98* to *JARID1A* (later referred to as *KDM5A*) as a novel recurrent event in approximately 11% of patients with pediatric AMKL. This is a cryptic fusion, because the telomeric location of both genes, and therefore it can easily be missed with conventional karyotyping. The frequency of the *NUP98/KDM5A* fusion in AMKL was comparable to the *RBM15/MKL1* fusion (~10%). Patients with this abnormality showed poor outcome compared to the latter group, although this did not reach statistical significance, most likely due to small numbers. Murine models previously showed the capacity of *NUP98/KDM5A* to result in a differentiation arrest with a short latency time to develop leukemia. This suggested that the translocation of *NUP98/KDM5A* can indeed be classified as a type-2 aberration, based on the model of genetic events in AML as postulated by Kelly et al.[1, 2] AML patients carrying the *CBFA2T3/GLIS2* fusion, another cryptic event, had a similar poor outcome. These three fusions are more or less specific for pediatric AMKL, as they are rarely or never seen in other morphological subtypes of pediatric AML. The fourth group identified in this study consisted of the *MLL*-rearranged (later referred to as *KMT2A*) cases, which are not specific for AMKL, but are also found in other morphologic leukemia subtypes. Fusions of *KMT2A* with the partner-genes *MLLT3* and *MLLT10* were most frequently found in this study.[3, 4] Altogether, we identified a driving leukemic aberration in roughly 60% of the pediatric AMKL cases.

Recent evidence showed that *NUP98* aberrations, specifically *NUP98/NSD1*, are frequently co-expressed with *FLT3*/ITD (in 56–91%) and *WT1* mutations (in 36–50%). In the samples positive for a *NUP98/KDM5A*-aberration in this study, all were negative for an aberration in one of these genes, suggesting the presence of another specific leukemic mechanism in *NUP98/KDM5A* rearranged AMKL.[1, 5] In **chapter 3**, *RB1,* a tumor suppressor which is linked to proliferation by its role in cell cycle checkpoints, and one of the most frequently described cancer genes, was identified to be deleted in all *NUP98/KDM5A* positive non-Down syndrome AMKL. Interestingly, *RB1* deletions were not found in non-AMKL *NUP98/KDM5A* rearranged cases, or any other subtype of pediatric AML, underlining its specificity for cooperation with translocation *NUP98/KDM5A* in pediatric AMKL. Furthermore, this chapter describes the evidence that loss of *RB1* is required to offset a *NUP98/KDM5A* induced cell cycle arrest, and the combination results in both a differentiation arrest and proliferation advantage. A decreased proliferation rate was observed in *NUP98/KDM5A* expressing, early myeloid cells, accompanied with a G0-G1 cell cycle arrest, which could be rescued by *RB1* depletion. This is consistent with the idea that the NUP98/KDM5A fusion protein constitutes a loss of KDM5A function in regulating RB1, leading to an anti-proliferative effect. While it was shown that the *NUP98/KDM5A* fusion also prevents differentiation through up-regulation of *HOXA* and *HOXB* genes, creating a stem cell phenotype, this anti-proliferative effect may prevent the development of a full blown leukemia with two *RB1* copies present. Therefore was

hypothesized that loss of one RB1 allele is required to compensate for this effect. In the majority of cases the other *RB1* allele was intact, contrary to the "classical" loss of RB1 function in for instance retinoblastoma, which requires loss of both alleles.[6, 7]

The use of next-generation sequencing, including RNA-sequencing and whole exome sequencing, on pediatric non-Down Syndrome AMKL samples, led to the identification of more distinct cytogenetic groups within pediatric non-Down syndrome AMKL, as described in **chapter 4**. Using samples derived from an international collaboration, two additional groups next to the in chapter 2 described aberrations, were identified. In 9% of the cases, a truncating *GATA1* mutation was found. This is the driving leukemic aberration found in the majority of Down syndrome AMKL cases. The patients in whom a *GATA1* mutation was identified were however not diagnosed with Down syndrome, but the *GATA1* mutations were frequently seen in combination with an acquired trisomy of chromosome 21. Another group that was identified were *HOX*-rearrangements. We analyzed gene expression signatures to confirm the unique character of all cytogenetic groups identified in this study, and samples clustered according to specific group and were distinct from any of the other cytogenetic groups identified. Similar to *KMT2A*-rearrangements and *NUP98-KDM5A* positive cases, the *HOX*-rearrangements were characterized by a '*HOX* signature', but in these cases the *HOX* gene showing upregulation was the specific *HOX* gene involved in the translocation. *HOX* genes play a significant role in normal hematopoietic development and deregulated expression has a central role in the etiology of several subtypes of acute leukemia, in part through the acquisition of enhanced self-renewal.[8] In this study the leukemic potential was further studied using murine colony formation assays to assess the ability to serially replate *HOX*-rearrangements, which showed differences in replating-capacity for the different *HOX*-fusions.

In the study described in **chapter 5**, the aim was to further refine the current risk group stratification within pediatric non-Down syndrome AMKL. We reported an international collaborative study on the prognostic value of the recently identified recurrent cytogenetic aberrations found in non-Down syndrome pediatric AMKL, with a special focus on cases positive for *NUP98/KDM5A, CBFA2T3/GLIS2, RBM15/MKL1* and *KMT2A*-rearrangements. Cases positive for these translocations represented 46% of the pediatric AML cases in this study, and harboring translocation *CBFA2T3/GLIS2, NUP98/KDM5A,* or a *KMT2A*-rearrangement appeared to be an independent prognostic factor for poor outcome. This poor outcome was mainly due to a high incidence of relapse in these patients. This study showed, similar to the study described in chapter 2, that age was not an independent prognostic factor in pediatric non-DS AMKL, nor was sex, nor WBC, nor treatment with a SCT. In addition, there were no differences in presenting characteristics between the various genetic groups with the exception of *RBM15/MKL1* positive cases, which presented with a significantly lower age at diagnosis (median age 0.7 years, range 0.1-2.7). When looking at treatment, we could not provide an answer whether children benefitted from SCT. In general, the value of SCT for pediatric AML is under discussion: there is clear evidence that SCT has anti-leukemic potential, but this may be off-set by transplant-related mortality.[9-11] Nevertheless, SCT in first remission is recommended for high risk cases in many AML treatment protocols, as defined by specific high-risk genetic aberrations or poor early response to treatment (high MRD

after 1 or 2 courses of chemotherapy). Based on our results, no definitive answer could be provided on the potential benefit of SCT in pediatric AMKL, but we suggest that children with good-risk AMKL may be spared from this intensive treatment.

In part 2 of this thesis, we analyzed the presence of different aberrations in a pediatric AML cohort, including all morphologic subtypes. In **chapter 6**, *ETV6*, a well-known gene to be aberrant in other types of leukemia, was studied, based on a finding by the COG groups of *ETV6* abnormalities in AML as presented at the ASH 2012.[12] Using MLPA, direct sequencing and FISH, we identified deletions, mutations and translocations in the *ETV6* gene as recurrent events in pediatric AML in 7% of the cases. *ETV6* mutations were found in 2.2% of the cases included in this cohort, which is comparable to the frequency found in adult AML.[13] Additionally we found *ETV6* deletions in 1.6% of patients in our cohort, and *ETV6* translocation in 9/275 (3.3%) cases, however, we only screened the patients without any known type II mutation, assuming that type II aberrations are mutually exclusive. This was a much lower frequency in comparison to the frequency of *ETV6* translocations found in pediatric ALL.[14] Comparing gene expression patterns of mutated and deleted cases versus other pediatric AML patients, several genes were upregulated, such as *CLDN5, BIRC7* and *DPEP1*. These three genes have been described as up-regulated in *ETV6*-silenced in T-ALL.[15-17] This suggested that the mutations and deletions in our series also result in impaired *ETV6* function. In addition, we identified 4 different *ETV6* translocations in this cohort. Next to *ETV6/MN1*, identified in 6 cases, we identified single cases of *ETV6/ARNT*, *ETV6/HOXCas2* and *ETV6/HOXA11as*. The translocations involving *HOX* antisense genes both showed high *HOX* expression specific for the *HOX* gene involved in this fusion, confirming the *HOX* gene upregulation in the *HOX*-rearrangements found in **chapter 4**. Although the number of patients with *ETV6* aberrations was low, the survival analyses suggested that *ETV6* aberrations associated with a poor clinical outcome.

To investigate the presence of *ETV6* aberrations in pediatric AML, we used the MLPA probe mix, which also contained probes for the analysis of *IKZF1*. **Chapter 7** describes the identification of *IKZF1* deletions as recurrent event in pediatric AML. *IKZF1,* localized on chromosome *7,* has been widely studied in the context of B-cell differentiation and ALL, both in adults and children. Reduced *IKZF1* gene function is a well-known recurrent event in B-Cell Precursor (BCP-)ALL, and caused by focal *IKZF1* deletions in approximately 15%. The frequency however is greatly enriched in *BCR/ABL1* positive BCP-ALL at 70%-80%.[18, 19] In this study, focal *IKZF1* deletions were shown to be a recurrent event in pediatric AML, although in a low frequency, and not in combination with *BCR/ABL1*. In contrast, mutations of *IKFZ1*, also found in BCP-ALL patients, were not detected in this AML cohort. In pediatric *de novo* AML, monosomy 7 is a recurrent cytogenetic abnormality seen in ~4% of the cases, and monosomy 7 is also seen in myelodysplastic syndrome (MDS) and juvenile myelomonocytic leukemia (JMML) which could progressed towards AML.[20, 21] In this pediatric *de novo* AML cohort, 8 cases (3.1%) of monosomy 7 were identified. We found a statistically significant correlation in gene expression profile between focally *IKZF1* deleted cases (n=3) and monosomy 7 cases, when analyzing the expression of non-chromosome 7 genes. The similarity of gene expression signatures between AML cases with monosomy 7 and those with a focal *IKZF1* deletion suggested that *IKZF1* may be one of the driving oncogenic events

in monosomy 7 pediatric AML.

In **chapter 8 and 9**, mutations described in adult AML were analyzed for their frequency in pediatric AML. Mutations in *BCOR* and *BCORL1* were identified in adult AML using whole exome sequencing, and given their frequency in adult cytogenetically normal AML we studied a pediatric cohort for their frequency.[22, 23] *BCOR* mutations were identified in 6.3% of the pediatric cytogenetically normal AML cases, which was comparable to the frequency in adult CN-AML. The frequency of *BCORL1* mutations was significantly lower than in adults. This may be due to the enrichment for secondary AML and treatment-related AML in the adult cohort: 73 of 173 (42%) in the adult cohort of Li *et al*., versus 12 of 230 (5%) in this cohort.[22-24] Expression levels of *BCOR*-mutated cases did not significantly deviate from the non-mutated pediatric AML patients, which was in accordance with findings in adult AML patients. In adult AML, *BCORL1* mutations did not affect mRNA levels, similar to the finding of our single case. In the adult CN-AML cohort, the presence of *BCOR* mutations conferred a poor outcome. In our cohort, the low frequency of mutations did not allow survival analysis to be performed, and is yet to be determined in a larger cohort.

Mutations in *PHF6,* an X-linked tumor suppressor gene, with a role in transcriptional regulation, were previously identified as germline mutation causing Borjeson-Forssman-Lehmann syndrome.[25, 26] Mutations of *PHF6* are reported in adult T-ALL, and somatic mutations in *PHF6* were identified in 3% of adult AML, predominantly in males, in cases classified as French-American-British (FAB) M0, M1 and M2. [27-29] Given the presence in adult AML, we studied a pediatric cohort to reduce the number of cases with unknown aberrations. In **chapter 9**, we identified mutations in *PHF6* in 6/318 (2%; 95% CI: 0.9-4.1%) AML cases. They were exclusively found in FAB-M0, M1 and M2, representing 6/143 (4%; 95%CI: 1.9-8.9%) pediatric FAB M0, M1 and M2 cases. Presenting with a significantly lower mRNA expression of *PHF6,* mutations were predicted to result in a loss of function. In contrast to T-ALL and adult AML, we found mutations of *PHF6* in both female and male patients in pediatric AML. The potential prognostic impact of *PHF6* mutations needs to be determined in a larger series.

In **chapter 10**, a translocation resulting in high *HOX* expression was described. The recurrent described t(10;17)(p15;q21) was analyzed for its breakpoints using reverse transcriptase PCR in a single case found in a pediatric *de novo* AML cohort. This resulted in the identification of translocation of *ZMYND11* and *MBTD1.* The case harboring this fusion was identified with a high *HOXA* expression, suggesting the fusion to result in a well-known leukemogenic pathway in AML.

Samenvatting

In dit proefschrift worden nieuwe afwijkingen beschreven die voorkomen bij AML op de kinderleeftijd, met specifieke aandacht voor acute megakaryoblastaire leukemia (AMKL) bij kinderen zonder Downsyndroom. Met de ontdekking van *NUP98/KDM5A, HOX*-herschikkingen en *GATA1s* mutaties in de leukemische cellen van deze kinderen, is er een groep met een slechte prognose, en een groep met een gemiddelde prognose geïdentificeerd, met een direct gevolg voor risicostratificatie en behandeling voor kinderen met deze zeldzame groep binnen de AML.

In **hoofdstuk 2** wordt de fusie van *NUP98* met *JARID1A* (later hernoemd tot *KDM5A)* beschreven. Deze afwijking is gevonden in ongeveer 11% van de kinderen met AMKL. Het is een cryptische translocatie die vaak wordt gemist met conventionele karyotypering, gezien de telomerische locatie van de genen die getransloceerd zijn. Het voorkomen van deze translocatie is vergelijkbaar met de in AMKL veel beschreven fusie van *RBM15* en *MKL1*, maar geeft een infauste prognose in vergelijking met deze fusie. *In vivo* studies hebben aangetoond dat *NUP98/KDM5A* leidt tot een differentiatiestop, deze fusie wordt daarom gezien als type-2-afwijking zoals beschreven door Kelly *et al.* [1, 2] In deze studie werd ook de translocatie *CBFA2T3/GLIS2* gevonden, een andere cryptische afwijking, met eveneens een slechte prognose. Als vierde groep binnen de AMKL-patiënten op de kinderleeftijd werden de *KMT2A*-herschikkingen gevonden, en dan met name *KMT2A/MLLT3* en *KMT2A/MLLT10*. [3, 4] Deze laatste groep wordt bij alle morfologische subtypes van AML gevonden, terwijl de andere groepen maar zeer zelden voorkomen bij andere morfologische subtypes en voornamelijk worden gezien bij AMKL. In deze studie is de onderliggende afwijking in 60% van de patiënten gevonden.

Naast de type-2-afwijkingen zoals beschreven door Kelly *et al*, is er ook gezocht naar additionele afwijkingen binnen de groep van *NUP98/KDM5A* getransloceerde patiënten. In **hoofdstuk 3** wordt de deletie van *RB1* besproken, een tumorsuppressorgen, met een belangrijke rol in de proliferatie van de cel, met name vanwege zijn rol in het checkpoint van de celcyclus. In alle *NUP98/KDM5A* positieve AMKL-patiënten werd een deletie van *RB1* gevonden, en bij geen enkele andere AML-patiënt. Deze deleties waren nog niet eerder beschreven in AML op de kinderleeftijd, maar deleties van chromosoomband 13q14, de locatie van *RB1*, waren al wel geobserveerd in AMKL-cellen. [3, 30] In een cellijn model werd gevonden dat *NUP98/KDM5A* leidde tot hoge *Hoxa* en *Hoxb* expressie, zoals ook gezien werd in patiënten, en dat de fusie een differentiatiestop gaf, maar eveneens een stop gaf van de G0- naar de G1-fase in de celcyclus. De deletie van *RB1* zorgde er in dit model voor dat deze stop in de celcyclus afzwakte, en de combinatie van *NUP98/KDM5A* en een *RB1* deletie resulteerde in een differentiatiestop met daarbij een proliferatie voordeel.

Door het gebruik van nieuwe, meer sensitieve, technieken, zoals RNA-sequencing en whole exome sequencing, werden nog twee nieuwe groepen geïdentificeerd binnen de AMKL op de kinderleeftijd. In **hoofdstuk 4** is de identificatie van *GATA1* mutaties, voorkomend in 9% van de kinderen met AMKL zonder Downsyndroom, en de groep van *HOX*-herschikkingen beschreven. De *GATA1*-mutaties zijn de meest voorkomende afwijking gevonden in AMKL bij kinderen met het Downsyndroom. In de patiënten zonder Downsyndroom in deze studie worden in de meerderheid van de patiënten in

de leukemiecel ook een additioneel chromosoom 21 gevonden. De *HOX*-herschikkingen bestaan uit fusies met een *HOX*-gen. Hiervan werden verschillende varianten gevonden. Met behulp van genexpressieprofielen werd gevonden dat de samples met een *HOX*-herschikking een gelijkend genexpressieprofiel hadden. Ook alle andere groepen gevonden in deze studie lieten een uniek genexpressieprofiel zien, waarbij de samples van patiënten met dezelfde afwijking bij elkaar groepeerden en de verschillende subgroepen niet bij elkaar. Daarnaast werd een specifieke *HOX*-expressie gezien bij de samples met een *HOX*-herschikking, naast de bekende *HOX*-profielen bij samples positief voor *NUP98/KDM5A* of *KMT2A*-herschikkingen. *HOX*-genen spelen een belangrijke rol in de normale hematopoëse en verschillende studies hebben aangetoond dat een gedereguleerde *HOX*-expressie een rol kan spelen in het ontstaan van leukemie. In deze studie hebben we het leukemisch effect van de *HOX*-herschikkingen bekeken door de capaciteit te analyseren om de cellen positief voor een *HOX*-herschikking in serie uit te platen. Deze methode liet zien dat verschillende *HOX*-herschikkingen een matige tot grote capaciteit hebben om in serie uitgeplaat te worden.

In **hoofdstuk 5** is gekeken naar de prognostische waarde van de meest voorkomende cytogenetische subgroepen binnen de AMKL op de kinderleeftijd van kinderen zonder Downsyndroom. Er werd een internationale samenwerking gevormd, waarbij er 153 patiënten werden geanalyseerd voor de aanwezigheid van *NUP98/KDM5A, CBFA2T3/GLIS2, RBM15/MKL1* of *KMT2A*-herschikkingen en er werd gekeken naar uitkomstparameters. Tot nu toe werden alle kinderen met AMKL zonder Downsyndroom geclassificeerd als hoogrisico. Indien de leukemiecel cel positief was voor *CBFA2T3/GLIS2, NUP98/KDM5A,* of een *KMT2A*-herschikking, resulteerde dit in een ongunstige overlevingskans. Deze slechte overlevingskans was met name het gevolg van de hoge incidentie van recidieven bij deze patiënten. Wanneer de leukemiecel negatief was voor een van deze translocaties was de overlevingskans groter, overeenkomend met de standaardrisicogroep. AMKL bij kinderen zonder Downsyndroom ontstaat meestal op zeer jonge leeftijd, met een gemiddelde diagnose leeftijd onder de twee jaar. Onze studie toonde aan dat leeftijd geen onafhankelijke factor is voor prognose binnen deze ziekte, evenals geslacht en hoeveelheid aanwezige witte bloedcellen. Er waren geen verschillen in patiëntkarakteristieken tussen de verschillende groepen, met uitzondering van de leeftijd van diagnose van kinderen met *RBM15/MKL1*-positieve AMKL; zij waren bij diagnose significant jonger dan de andere kinderen met AMKL. Een belangrijk verschil in de therapieprotocollen van de verschillende samenwerkende groepen binnen deze studie, is het toepassen van een hematopoietische stamcel transplantatie (hSCT) in het geval van een beschikbare donor. In een multivariaatanalyse bleek het behandelen met een hSCT niet van invloed op de overlevingskans. Over de waarde van een hSCT binnen de behandeling van AML op de kinderleeftijd wordt gediscussieerd; een succesvolle hSCT heeft een antileukemisch effect, maar er is een hoge kans op transplantatiegerelateerde mortaliteit. [9-11] Desalniettemin wordt in vele therapieprotocollen een hSCT in eerste remissie aangeraden voor hoogrisicogroepen binnen de AML. Hoogrisicogroepen worden gedefinieerd door specifieke hoogrisico genetische afwijkingen of aan de hand van de slechte vroege respons op chemotherapeutische behandeling (hoge minimale residuale ziekte na het eerste of tweede blok van de chemotherapie). Onze resultaten toonden geen meerwaarde aan van een hSCT als behandeling van AMKL op

de kinderleeftijd. Kinderen vallend in de standaardrisicogroep zouden mogelijk niet in aanmerking komen voor deze intensieve behandeling. Kinderen zonder Downsyndroom met AMKL die niet tot een van de vier groepen in deze studie behoorden, zouden mogelijk een *GATA1* mutatie of *HOX*-herschikking kunnen hebben. We hadden echter niet de mogelijkheid om dit te testen bij gebrek aan voldoende of kwalitatief goed patiëntmateriaal.

In **deel 2** van dit proefschrift hebben we de aanwezigheid van afwijkingen van verschillende genen bestudeerd in een cohort met alle morfologische subtypes van AML op de kinderleeftijd. In **hoofdstuk 6** zijn afwijkingen van *ETV6*, een gen bekend door de afwijkingen in andere types van leukemie, bestudeerd, door de vondst van afwijkingen van dit gen binnen AML door de Children's Oncology Group (Noord-Amerika), zoals gepresenteerd op de ASH 2012. [12] Door MLPA, direct sequencing en FISH te gebruiken, identificeerden we deleties, mutaties en translocaties van dit gen, voorkomend bij 7% van de onderzochte patiënten met AML op de kinderleeftijd. *ETV6*-mutaties zijn gevonden bij 2.2% van de geteste patiënten, wat overeenkomt met de beschreven frequentie van AML bij volwassenen. [13] Daarnaast werden *ETV6*-deleties gevonden bij 1.6%, en *ETV6*-translocaties bij 3.3% van de kinderen in dit cohort. De translocaties komen veel minder vaak voor dan *ETV6*-translocaties bij kinderen met acute lymfatische leukemie (ALL). [14] Bij de vergelijking tussen genexpressieprofielen van kinderen met *ETV6*-mutaties of -deleties versus de profielen van alle andere kinderen met AML in deze studie, vonden we verschillende genen met overexpressie, onder andere *CLDN5, BIRC7* en *DPEP1.* De overexpressie van deze drie genen is ook beschreven bij T-ALL met *ETV6*-afwijkingen resulterend in een verminderde functie. [15-17] Dit suggereert dat de mutaties en deleties die we in deze studie hebben gevonden, ook resulteren in een dergelijke *ETV6*-afwijking. Hiernaast hebben we in deze studie vier verschillende *ETV6*-translocaties geïdentificeerd. Naast *ETV6/MNX1*, een bekende *ETV6*-translocatie bij jonge kinderen met AML die we in zes gevallen vonden, identificeerden we drie unieke casus met *ETV6/ARNT*, *ETV6/HOXCas2* en *ETV6/HOXA11as*. De translocaties met een *HOX*-antisense gen gaven hoge *HOX*-expressie specifiek voor het betrokken *HOX*-gen. Dit bevestigt de resultaten die we eerder bespraken in **hoofdstuk 4**. Hoewel de hoeveelheid gevonden *ETV6*-afwijkingen relatief laag waren voor een goede statistische analyse van de overlevingskans, suggereerde de analyse een slechte overlevingskans voor kinderen met AML met een *ETV6*-afwijking.

Voor de studie naar *ETV6*-afwijkingen bij AML op de kinderleeftijd, hebben we MLPA gebruikt met een probe mix, welke onder andere ook probes bevatte voor het gen *IKZF1.* In **hoofdstuk 7** beschrijven we de vondst van *IKZF1*-deleties binnen AML. *IKZF1* is een gen dat gelokaliseerd is op chromosoom 7. Dit gen is uitgebreid bestudeerd met betrekking tot de B-cel differentiatie en binnen de ALL zowel op de kinderleeftijd als bij volwassenen. Verminderde *IKZF1*-genfunctie is een veelvoorkomende afwijking bij B-cel precursor (BCP-)ALL en wordt in 15% van deze gevallen veroorzaakt door een focale *IKZF1*-deletie. Dit komt voornamelijk voor bij *BCR/ABL1* positieve BCP-ALL, waarbij *IKZF1*-deleties voorkomen in 70-80% van de gevallen. [18, 19] In onze studie vonden we focale *IKZF1*-deleties, maar nooit in combinatie met *BCR/ABL1.* Ook konden we geen mutaties van *IKZF1* vinden, zoals wel beschreven bij BCP-ALL. Bij AML op de kinderleeftijd werd eerder al het voorkomen van een monosomie van chromosoom

7 beschreven, dit komt voor bij ongeveer 4% van alle AML op de kinderleeftijd. Ook wordt monosomie 7 gezien bij het myelodysplastisch syndroom (MDS) en bij juveniele myelomonocytaire leukemie (JMML), die beiden kunnen uitgroeien tot AML. [20, 21] Ook in ons cohort vonden we in 3.1% van de casus een monosomie 7. We vonden een statistisch significante correlatie in genexpressieprofielen tussen de focale *IKZF1*-gedeleteerde casus (n=3) en de casus met monosomie 7 (n=8), van de genen die zich niet bevonden op chromosoom 7. Deze overeenkomst in genexpressieprofielen suggereert dat *IKZF1* een belangrijk gen leidend tot leukemie is bij de patiënten met een monosomie 7.

In **hoofdstuk 8 en 9** wordt de frequentie van mutaties in genen beschreven, die eerder werden gevonden bij AML van volwassenen. Mutaties in *BCOR* en *BCORL1* werden bij volwassenen met AML gevonden door middel van whole exome sequencing. Gezien de hoge frequentie met name bij cytogenetisch normale patiënten, onderzochten wij een cohort van AML van de kinderleeftijd.[22, 23] Wij vonden *BCOR* mutaties in 6.3% van ons cytogenetisch normale cohort, vergelijkbaar met het voorkomen bij volwassenen. We vonden een lagere frequentie van *BCORL1*-mutaties in vergelijking met volwassenen. Een oorzaak hiervan kan zijn dat het volwassencohort was verrijkt met secundaire- en therapie-gerelateerde AML; 73/173 (42%) in het volwassencohort van Li *et al.*, versus 12/230 (5%) in ons kindercohort. [22-24] *BCOR*-genexpressie was niet significant verschillend tussen de *BCOR*-gemuteerde en niet-gemuteerde patiënten, een vergelijkbaar resultaat met de bevindingen bij volwassen patienten. Ook de *BCORL1*-mutatie leidde niet tot veranderde genexpressie in het volwassencohort, dezelfde bevinding als bij de *BCORL1*-gemuteerde casus uit ons cohort. In het volwassencohort leidde een *BCOR*-mutatie tot een lagere overlevingskans. In ons cohort vonden wij te weinig casus voor een betrouwbare analyse met betrekking tot overlevingskans. Dit zal in een groter cohort plaats moeten vinden.

Mutaties van *PHF6*, een tumorsuppressorgen op het X-chromosoom, met een rol in transcriptionele regulatie, zijn gevonden als germline mutatie leidend tot het Borjeson-Forssman- Lehmannsyndroom. [25, 26] Mutaties in dit gen zijn ook geïdentificeerd in T-ALL en AML bij volwassenen. De mutaties werden voornamelijk gevonden bij mannen, en alleen in het geval van AML bij de morfologische classificaties French-American-British (FAB) M0, M1 en M2. [27-29] Gezien het voorkomen bij mutaties in dit gen bij AML bij volwassenen, hebben we een kindercohort geanalyseerd voor mutaties van *PHF6*. In **hoofdstuk 9** wordt beschreven dat mutaties voorkomen bij 6/318 (2%; 95% CI: 0.9-4.1%) kinderen met AML. Ook bij kinderen zijn ze louter gevonden bij FAB M0, M1 en M2. Gezien de significant lagere mRNA-expressie van *PHF6*, wordt voorspeld dat deze *PHF6*-mutaties leiden tot een verlies van de functie van het gen. In tegenstelling tot de bevindingen bij T-ALL en AML bij volwassenen, werden de mutaties bij zowel vrouwen als mannen gevonden. De prognostische waarde van deze mutaties moet worden beoordeeld in een groter cohort.

In **hoofdstuk 10** wordt een translocatie beschreven welke resulteert in hoge *HOX*-expressie. Het breekpunt van de vaker beschreven t(10;17)(p15;q21) werd, geanalyseerd met behulp van reverse transcriptase PCR, in een unieke casus gevonden in een cohort van AML ontstaan op de kinderleeftijd. Dit resulteerde in de identificatie van de fusie van *ZMYND11* en *MBTD1*. Deze casus resulteerde in hoge *HOXA*-expressie, dat frequent wordt gezien als oorzaak van het ontstaan van leukemie, zoals AML.

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Chapter 12

General discussion

International collaboration is needed to study rare diseases such as pediatric AMKL

Pediatric AML is a heterogeneous disease. By unraveling the underlying aberrations resulting in pediatric AML, potential disease-driving mutations may be identified, which may be important for risk stratification and targeted therapy, with the final aim to improve prognosis. To study rare diseases such as pediatric non-Down syndrome AMKL, international collaboration was set-up to be able to not only identify aberrations, but also analyze their prognostic impact. In the studies discussed in this thesis, samples from various regions of Europe, Asia and North America were included. A potential drawback of that approach is that patients were treated on different protocols over a longer period of time, which is not ideal to study prognostic factors. However, all AML treatment protocols consisted of intensive chemotherapy using an anthracycline and cytarabine backbone, including stem cell transplantation in selected cases. Moreover, when comparing the clinical characteristics and outcome data of the AMKL patients in chapter 5 versus a population-based cohort of the AML-BFM Study Group, no significant differences were found, suggesting that this cohort was a representative sample for pediatric AMKL. This thesis shows, that with intensive international collaboration, new risk groups can be identified.

Next generation sequencing contributes to the unravelling of aberrations which can be used for risk-group stratification

Inaba *et al* described AMKL risk groups based on cytogenetic analysis obtained with conventional karyotyping, resulting in three groups; good risk in patients with AMKL carrying 7p abnormalities; poor risk in cases with monosomy 7, or 9p abnormalities including the *KMT2A/MLLT3,* -13/13q- and -15; and intermediate risk in all others.[1] This risk classification may be difficult, since AMKL is frequently associated with myelofibrosis, which renders it difficult to obtain sufficient material for cytogenetic analysis. In this thesis, we showed in chapter 5 that 32/153 (21%) patients lacked data on karyotype, comparable to the findings of Inaba *et al*[1] in pediatric AMKL, but a much higher percentage than reported on missing karyotypes in pediatric AML including all morphology subtypes (7-13%).[2] Moreover, conventional karyotyping will not reveal cryptic translocations, such as *CBFA2T3/GLIS2* or *NUP98/KDM5A*. Using RT-PCR as screening method, we were able to classify 12/32 cases without an available karyotype, not only including the aforementioned cryptic translocations, but patients harboring *KMT2A*-rearrangements and *RBM15/MKL1* as well. Therefore, screening of the most frequent recurrent aberrations with PCR will add value to risk stratification next to cytogenetic analysis based on conventional karyotyping, revealing additional groups with poor outcome. Although the overall survival is poor for non-DS AMKL in general, with a 4-year probability of overall survival of ~56% in this study, *NUP98/KDM5A, CBFA2T3/GLIS2* and *KMT2A*-rearranged cases fared worse. Using RNA-sequencing, we were able to identify two additional genetic groups within pediatric AMKL; the *GATA1* mutated cases, and cases with *HOX*-rearrangements, interestingly both predicting for better survival. Altogether we identified an aberration in over 80% of the pediatric AMKL cases (chapter 4 and Figure 12.1).

The group of *HOX*-rearrangements has a wide variety of different *HOX* genes and partner genes involved, and with that, targeted screening for any of these fusions is

hardly feasible. This emphasizes the need for next generation sequencing as screening tool. Screening for these translocations combined with conventional karyotyping in pediatric non-AMKL is advisable for accurate risk-group stratification. Based on our data we suggest that within pediatric non-Down syndrome AMKL *NUP98/KDM5A, CBFA2T3/GLIS2* and *KMT2A*-rearrangements are stratified as high risk patients. Moreover, we propose that *RBM15/MKL1, GATA1s* mutated cases and *HOX*-rearrangements are stratified as standard risk, with a 5-year pOS of 65±17%, 100%, and 77%±12%, respectively.[3] For proper risk group stratification conventional karyotyping combined with screening for *NUP98/KDM5A, CBFA2T3/GLIS2, KMT2A*-rearrangements, *RBM15/MKL1* and mutational screening of *GATA1* may be used if next generation sequencing is not feasible.

Fusions seen in pediatric non-Down Syndrome AMKL might be present at birth before the development of frank leukemia

Pediatric non-Down Syndrome AMKL arises at a median age below two years. [1,3-5] Greaves *et al* showed that AML fusion genes can be detected in circulating bone marrow cells derived from neonatal Guthrie cards, suggesting the fusion gene, although in small subclones, to be present in utero.[6] In Down syndrome AMKL, the *GATA1s* mutation induces transient myeloproliferative disease originating in the fetal liver, followed by spontaneous remission in ~80% of cases, and in approximately 20% followed by the development of AMKL before the age of 4-5 years.[7-10] Guthrie cards may show whether the most frequent identified somatic aberrations in non-Down syndrome AMKL, such as *NUP98/KDM5A, RBM15/MKL1,* and *CBFA2T3/GLIS2*, all seen in leukemias which present themselves at a very young age, might be present at birth as well, which later, in combination with a second hit, such as a *RB1* deletion, may result in frank leukemia. Analyzing the presence of the fusion genes specific for pediatric AMKL may reveal new insights in the development of frank leukemia, and may be an explanation of the specific age pattern for these types of leukemia. Since the incidence of pediatric AML is 0.4-1.6 per 100.000 individuals, screening in non-Down syndrome newborns will not contribute to early detection. In Down syndrome screening by Guthrie cards may be useful, since ~20% of the cases with a transient myeloid leukemia will develop AML.

Non-Down syndrome GATA1 mutated AMKL cases may potentially benefit from less intensive chemotherapy

AMKL in children with Down syndrome is treated with a reduced chemotherapy treatment protocol compared to non-Down syndrome patients. Given the favorable prognostic outcome of *GATA1* mutated cases in pediatric non-Down Syndrome AMKL not harboring any other fusion event (in our cohort 0/7 patients had an event), it may be interesting to see whether patients with this specific genotype of pediatric AMKL could be treated with a reduced chemotherapy treatment protocol. The *GATA1* mutation is most likely resulting in a reduced expression of cytidine deaminase.[11-14] Cytidine deaminase is an enzyme that converts cytarabine in its inactive metabolite.[12,13] The decreased expression of cytidine deaminase results in a higher intracellular concentration, and therefore a lower dose of cytarabine may have a higher potential in anti-leukemic effect in these patients.[11,14] When comparing the drug sensitivity of Down syndrome AML versus non-Down syndrome AML, Down syndrome AML cells were not

only more sensitive for cytarabine, but also for daunorubicin, idarubicin, mitoxantrone, etoposide, 6-thioguanine, amsacrine, vincristine, and busulfan.[15] This effect was not observed in Down syndrome ALL, suggesting that the *GATA1* mutation is responsible for this effect. Further research should be performed to investigate whether non-Down syndrome *GATA1s* mutated AMKL cells show a similar sensitivity for chemotherapy as seen in Down syndrome AMKL, to optimize treatment protocols for this specific subgroup of non-Down syndrome AML. In case chemotherapy can be reduced without compromising anti-leukemic efficacy this will also diminish treatment-related morbidity and mortality, further improving outcome. Nevertheless, it is important to acknowledge that *GATA1* was not mutually exclusive in our patient cohort. The combination with *NUP98/KDM5A,* as seen in our cohort in one patient, may lead to a poorer outcome.

Future strategies to treat pediatric AML: newly identified drugable targets and immunotherapy may lead to improved outcome of pediatric AML

One option to improve outcome of pediatric AML is through targeted therapy, since intensification of the current treatment protocols is not feasible due to the high incidence of treatment related morbidity and mortality. Targeted therapy could focus on disrupting the complexes formed by fusion genes. In the *KMT2A*-rearranged cases, DOT1L inhibitors are currently under investigation. DOT1L is a protein that is recruited in the KMT2A-complex, resulting in the *HOXA9* and *MEIS*-overexpression that drive the leukemogenesis.[16-18] Where in vitro and in vivo models show a great potential for DOT1L inhibitors, clinical studies so far have not shown the promising results anticipated by preclinical models.[19]

Another interesting player in the field of immunotherapy is the category of bispecific T-cell engager (BiTE) antibodies. These anti-cancer drugs use the ability to bind T-cells via the CD3 receptor, and tumor cells via a tumor specific molecule; for instance CD19 for ALL, or CD33 for AML cells. This causes T-cells to exert cytotoxic activity, resulting in apoptosis of the leukemic cells.[20-22] In other types of cancer, such as Non-Hodkin's lymphomas and ALL, clinical studies showed a therapeutic potential for BiTE-antibodies.[23-26] A drawback for this therapy is that CD33 is not specific for the leukemic cell; as CD33 is also expressed on healthy myeloid cell progenitors. Therefore this therapy may result in severe neutropenia, precluding its use to for instance induce remission in a pre-SCT window. Clinical studies in adults are initiated and should provide insight in how to best use these drugs.

Chimeric antigen receptor expressing T (CART) cells already showed great potential in the treatment of CD19-positive ALL and lymphoma.[27-29] The difficulty in using CART cells in AML is the lack of antibodies only expressed at the leukemic cells, similar to BITES. Targeting a specific antibody on an AML cell may therefore also attack healthy cells in the myeloid lineage and may therefore lead to neutropenia.[30] Specific antibodies or combinations of antibodies that will not attack non-malignant cells need to be developed both for BITES and CART technology.[31] Further optimization and extension of this concept of anti-cancer therapy may well lead to striking clinical benefits, without harming the healthy myeloid progenitors.

Perspective

In this thesis, specific novel translocations resulting in *HOX* upregulation are described,

which can be classified according to the *HOX* clusters as previosuly reported by us (Figure 12.2).[32] These cases may benefit from targeted therapy assigned to disrupt this *HOX* upregulation in the future. For instance, we identified *ZMYND11/MBTD1, NUP98/KDM5A,* and *HOX*-rearrangements as new groups resulting in *HOX* overexpression (Figure 12.2). Future research should be performed to investigate the interaction between the *HOX* genes and the fusion proteins causing *HOX*-upregulation. This may reveal potential targets for therapy. To find drugable targets in *HOX*-overexpressed cases such as *NUP98/KDM5A*, methods unraveling protein-protein interaction, such as Chip-sequencing, can be used to determine molecules involved in the protein interaction of the fusion resulting in the *HOX*-overexpression. Finding key proteins involved in the fusion-gene induced protein complexes resulting in leukemia will give lead towards targeted therapy. Since *HOX*-upregulation is not specific for pediatric AML, finding targets resulting in *HOX*-upregulation may be of interest for other leukemias as well.

Furthermore, screening of neonatal Guthrie cards may reveal the presence of fusion genes in utero in non-Down syndrome patients. Analyzing the presence of the fusion genes specific for pediatric AMKL may reveal new insights in the development of leukemia, and may explain the early onset of the disease.

To investigate the potential of reduced chemotherapy as treatment for non-Down syndrome AMKL with a *GATA1s* mutation, the ex-vivo drug sensitivity of non-Down syndrome *GATA1s* mutated leukemic cells needs to be determined.

In conclusion, there is clear medical need for new therapeutic options in pediatric AML, since survival is currently ~70%, and intensification of the current chemotherapeutic protocols is not feasible due to high rates of treatment related morbidity and mortality. We have identified multiple recurrent (cyto)genetic aberrations in pediatric AML, which gives leads to new therapeutic options and better risk-group stratification.

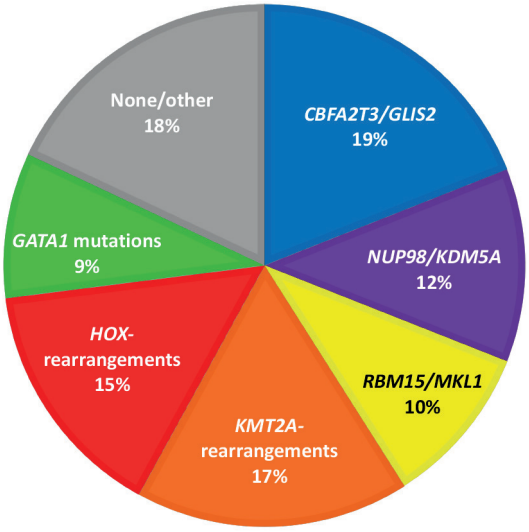

Figure 12.1. Schematic overview of different aberrations found in pediatric non-Down Syndrome AMKL using RNA-sequencing.

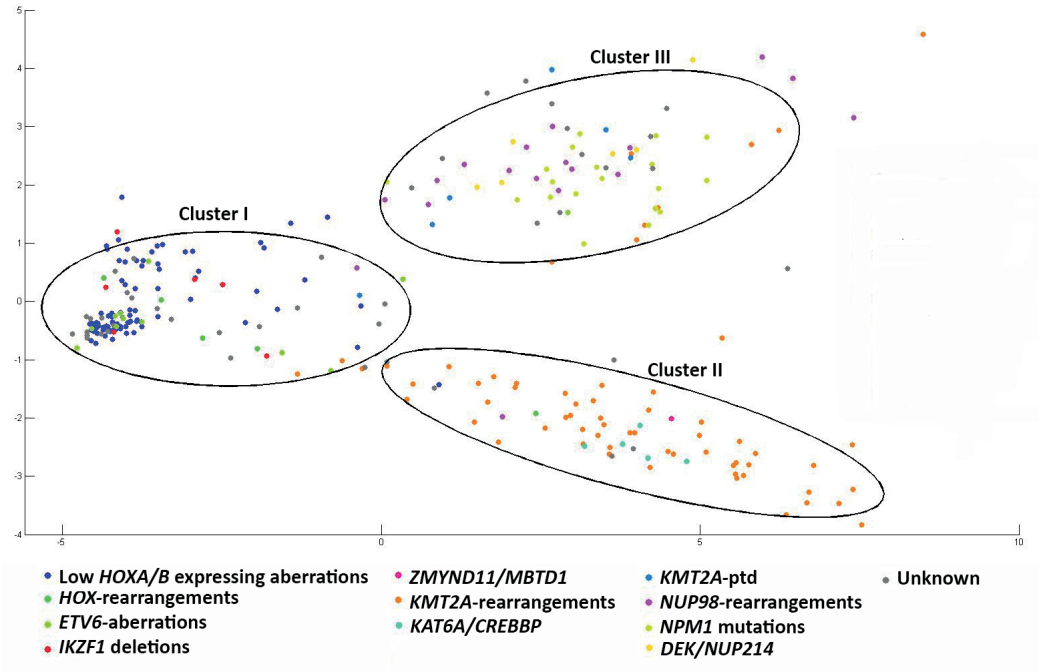

Figure 12.2. Principal component analysis based on HOX genes. Three clusters were formed, cluster I contains samples with low *HOXA* and *HOXB* expression, cluster II contains samples with high *HOXA* overexpression, such as *KMT2A*-rearrangements and *ZMYND11/MBTD1*, cluster III contains samples resulting in high *HOXA* and *HOXB* overexpression, including *NUP98*-rearrangments.

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Chapter 13

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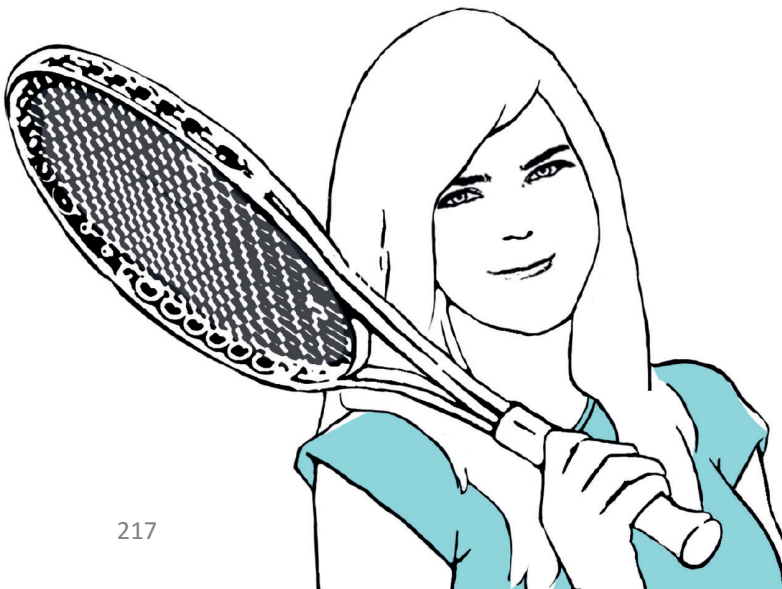

Curriculum vitae

Jasmijn Diantha Eliane de Rooij werd op 18 oktober 1986 geboren te Leiden. Tijdens haar middelbare school volgde zij de FaFa dansacademie in Den Haag. Na het behalen van haar VWO-diploma aan het Segbroek college te Den Haag in 2004, startte zij met de Bachelor Medicine, en aansluitend in 2007 met de Master Medicine aan de Faculty of Health, Medicine and Life Science van de Universiteit Maastricht. Zij volgde een wetenschappelijke stage op de afdeling Pathologie in het Ospedali Riuniti di Bergamo in Italië begeleid door Dr. A. Sonzogni, gevolgd door een keuze-coschap op de afdeling Stamceltransplantatie in het LUMC Willem-Alexander kinderziekenhuis te Leiden onder leiding van Prof. dr. M. Egeler, en een semi-artsstage kindergeneeskunde in het Maxima Medisch Centrum te Veldhoven gesuperviseerd door dr. L. Bok, waarna zij in 2010 haar studie afsloot. Hierna werkte zij als arts-assistent op de afdeling kindergeneeskunde en neonatale intensive care unit in het Maxima Medisch Centrum in Veldhoven onder supervisie van dr. P. Andriessen. Vanwege haar wetenschappelijke interesse voor myeloïde leukemie op de kinderleeftijd startte zij in 2011 als arts-onderzoeker op het promotie-onderzoek “Molecular genetic aberrations and their prognostic relevance in pediatric acute myeloid leukemia” op de afdeling kinderoncologie/hematologie van het Erasmus MC-Sophia kinderziekenhuis te Rotterdam. Het resultaat van dit onderzoek is beschreven in dit proefschrift. Momenteel werkt zij als arts-assistent in het Haga Juliana kinderziekenhuis te Den Haag, onder supervisie van dr. F. Brus.

List of publications

de Rooij JDE, Branstetter C, Ma J, Li Y, Walsh MP, Cheng J, Obulkasim A, Easton J, Verboon LJ, Mulder HL, Dang J, Koss C, Gupta P, Edmonson M, Rusch M, Lim J, Reinhardt K, Pigazzi M, Song G, Yeoh A, Shih LY, Liang DC, Halene S, Krause DS, Zhang J, Downing JR, Locatelli F, Reinhardt D, van den Heuvel-Eibrink MM, Zwaan CM, Fornerod M, Gruber TA. Pediatric non-Down syndrome acute megakaryoblastic leukemia is characterized by distinct genomic subsets with varying outcomes. *Submitted.*

Beuling E, **de Rooij JDE**, van den Heuvel-Eibrink MM, Verboon LJ, Obulkasim A, Baruchel A, Trka J, Reinhardt D, Sonneveld E, Zimmermann M, Pieters R, Gruber TA, Fornerod M, Zwaan CM. ETV6 aberrations are recurrent in pediatric acute myeloid leukemia. *Submitted.*

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de Rooij JDE, van den Heuvel-Eibrink MM, Hermkens MCH, Verboon LJ, Fornerod M, Baruchel A, Stary J, Reinhardt D, de Haas V, Pieters R, Zwaan CM. BCOR and BCORL1 mutations in pediatric Acute Myeloid Leukemia. *Haematologica.* 2015;100(5):194-195.

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den Hoed MA, Lopez-Lopez E, te Winkel ML, Tissing W, **de Rooij JDE**, Gutierrez-Camino A, Garcia-Orad A, den Boer E, Pieters R, Pluijm SM, de Jonge R, van den Heuvel-Eibrink MM. Genetic and metabolic determinants of methotrexate-induced mucositis in pediatric acute lymphoblastic leukemia. *Pharmacogenomics J.* 2015;15(3):248-254.

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Been JV, Vanterpool SF, **de Rooij JDE**, Rours GI, Kornelisse RF, van Dongen MC, van Gool CJ, de Krijger RR, Andriessen P, Zimmermann LJ, Kramer BW. A clinical prediction rule for histological chorioamnionitis in preterm newborns. *PLoS One*. 2012;7(10):e46217.

PhD portfolio

Summary of PhD training and teaching	
Name PhD student:	Jasmijn D.E. de Rooij
Erasmus MC department:	Pediatric Oncology
Research school:	MolMed
PhD period:	1st June 2011-31st May 2015
Promotors:	Prof. dr. R. Pieters Prof. dr. CM Zwaan

1. PhD training		
General Courses	**year**	**Workload**
Biostatistical Methods I: Basic Principles (CC02, Nihes)	2012	5.7 ECTS
Biomedical Scientific Writing (short, MolMed)	2013	2.0 ECTS
Specific courses		
Molecular Diagnostics VI (MolMed)	2011	1.0 ECTS
Biomedical Research Techniques X (MolMed)	2011	1.6 ECTS
Basic Course on 'R' (MolMed)	2011	1.0 ECTS
The Introductory Course on Statistics & Survival Analysis for Research master/ PhD students and MDs (MolMed)	2011/2012	0.5 ECTS
Analysis of micro array gene expression data using R/BioC and web tools (MolMed)	2012	2.0 ECTS
Seminars and workshops		
Ensembl Workshop (MolMed)	2011	0.5 ECTS
ESH-EHA Scientific Workshop AML "Molecular", Mandelieu la Napoule, France	2011	1.0 ECTS
RNA-seq technology, mapping and statistical analysis of RNA-seq data, EuGESMA Bioinformatics school, Modena, Italy	2012	1.0 ECTS
Indesign CS6 Workshop for PhD-students and other researchers (MolMed)	2014	0.15 ECTS
Presentations		
Six 30 minute presentations at weekly meetings of the department of Pediatric Oncology	2011-2015	4.0 ECTS
(Inter)national conferences		
16th Molecular Medicine Day, Rotterdam, the Netherlands (poster presentation, poster award winner)	2012	1.0 ECTS
54th ASH annual meeting, Atlanta, United States of America (oral presentation, ASH abstract achievement award winner)	2012	1.5 ECTS
HOVON dagen, Arnhem, the Netherlands (oral presentation)	2013	0.5 ECTS
17th Molecular Medicine Day, Rotterdam, the Netherlands (poster presentation)	2013	0.5 ECTS
Acute Leukemias XIV - Biology and Treatment Strategies, Munich, Germany (poster presentation)	2013	1.0 ECTS

1. PhD training		
24th Annual Meeting of the International BFM Study Group, Kiel, Germany (oral presentation)	2013	1.0 ECTS
45th Congress of the International Society of Paediatric Oncology, Hong Kong, China (oral presentation)	2013	1.0 ECTS
55th ASH Annual Meeting, New Orleans, United States of America (poster presentation)	2013	1.5 ECTS
25th Annual Meeting of the International BFM Study Group, Prague, Czech Republic (oral presentation)	2014	1.0 ECTS
46th Congress of the International Society of Paediatric Oncology, Toronto, Canada (two oral presentations)	2014	1.0 ECTS
56th ASH annual meeting, San Francisco, United States of America (two poster presentations, ASH abstract achievement award winner)	2014	1.5 ECTS
19th Molecular Medicine Day, Rotterdam, the Netherlands (poster presentation)	2015	0.5 ECTS
26th Annual Meeting of the International BFM Study Group, Budapest, Hungary (two oral presentations)	2015	1.0 ECTS
47th Congress of the International Society of Paediatric Oncology, Cape Town, South Africa (oral presentation)	2015	1.0 ECTS
57th ASH annual meeting, Orlando, United States of America (oral and poster presentation, ASH abstract achievement award winner)	2015	1.5 ECTS

2. Teaching		
Practical during Medicine course 'pediatric oncology' (minor)	2011-2014	1.0 ECTS
Supervising research project of medical student: Catherine Owusuaa	2011-2012	1.5 ECTS
Supervising international PhD student: Elixabet Lopez-Lopez	2012-2013	4.5 ECTS
Supervising research project of medical student: Nina van de Rijdt	2014-2015	2.5 ECTS

Dankwoord

Allereerst gaat mijn dank uit naar alle patiënten en hun ouders voor deelname aan het onderzoek door het beschikbaar stellen van samples.

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Rust zacht, Jocefros ♥ 29 mei 1987 - 10 december 2015 ♥

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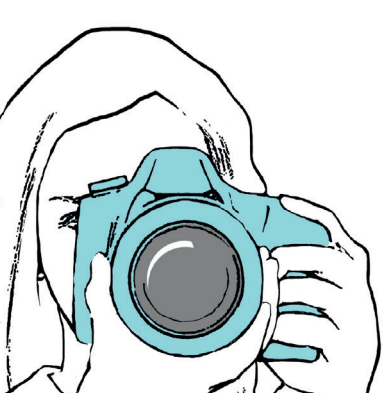
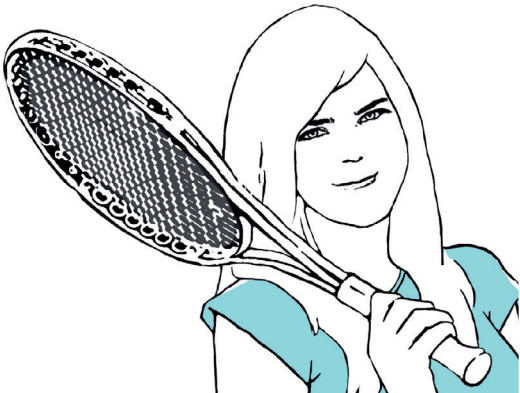
Jasmijn



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