The Molecular Heterogeneity of *MLL*-rearranged Pediatric AML

Eva Andrea Coenen

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J.E. Jurriaanse Stichting

The Molecular Heterogeneity of *MLL*-rearranged Pediatric AML

De moleculaire heterogeniteit van *MLL*-herschikte
acute myeloïde leukemie bij kinderen

Proefschrift

Ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus

prof.dr. H.G. Schmidt

en volgens besluit van het College voor Promoties.

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My candle burns at both ends;

It will not last the night;

But ah, my foes, and oh, my friends—

It gives a lovely light.

Edna St. Vincent Millay, “First Fig”, from “A few Figs from Thistles” (1892 –1950)

Onderzoek naar kinderkanker doe je met een goede reden.

Ter nagedachtenis aan alle kinderen met kanker bij wie het licht te vroeg doofde.

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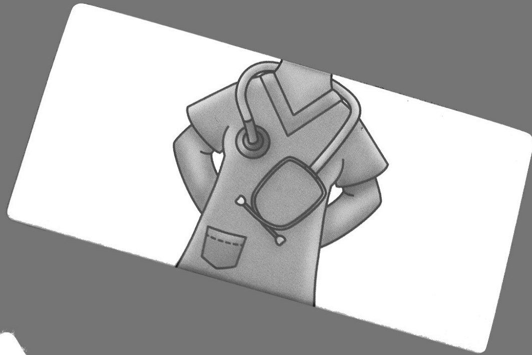
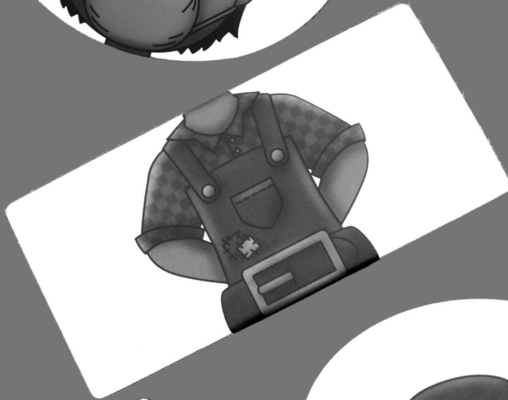
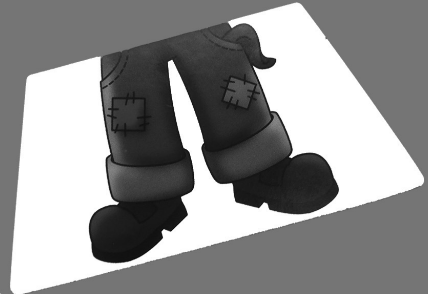
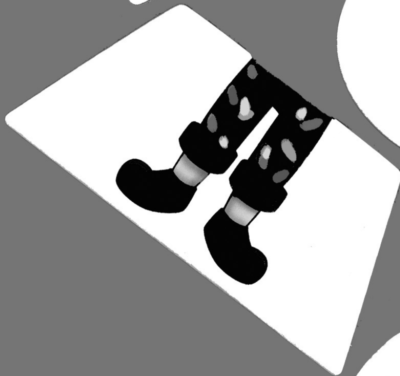
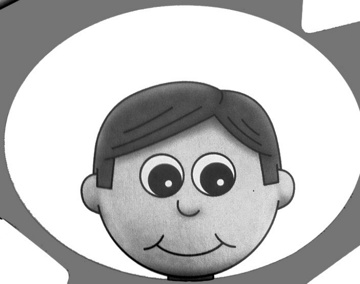
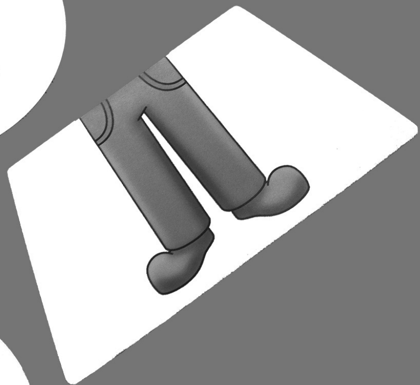
Introduction

1.1 Normal hematopoiesis

Continuous renewal of blood cells, so called hematopoiesis, is essential for human life. Starting from several months after birth the majority of blood cell production occurs in the bone marrow. Circulating blood cells are of diverse morphology and function and can be divided in red blood cells (erythrocytes), white blood cells (leukocytes), and platelets. The need for continuous production of blood cells is explained by the fact that the majority of mature blood cells have limited life span, i.e. erythrocytes live about 100-120 days and platelets only 5-9 days.[1]

All new blood cells arise from the self-renewing hematopoietic stem cell (HSC), which resides in the bone marrow. These HSCs produce daughter cells by cell division, thereby maintaining the stem cell population as well as creating offspring that can differentiate into more committed progenitors. Under strict regulation, these committed common lymphoid or myeloid progenitors, differentiate into mature blood cells (Figure 1) and are released in the peripheral blood. In the process of differentiation the blood cells gain specific functions for their mature life (such as the possibility to bind oxygen for the erythrocyte) but loose the capacity to proliferate. This shifting balance is essential for the body to keep control of the number of circulating blood cells.

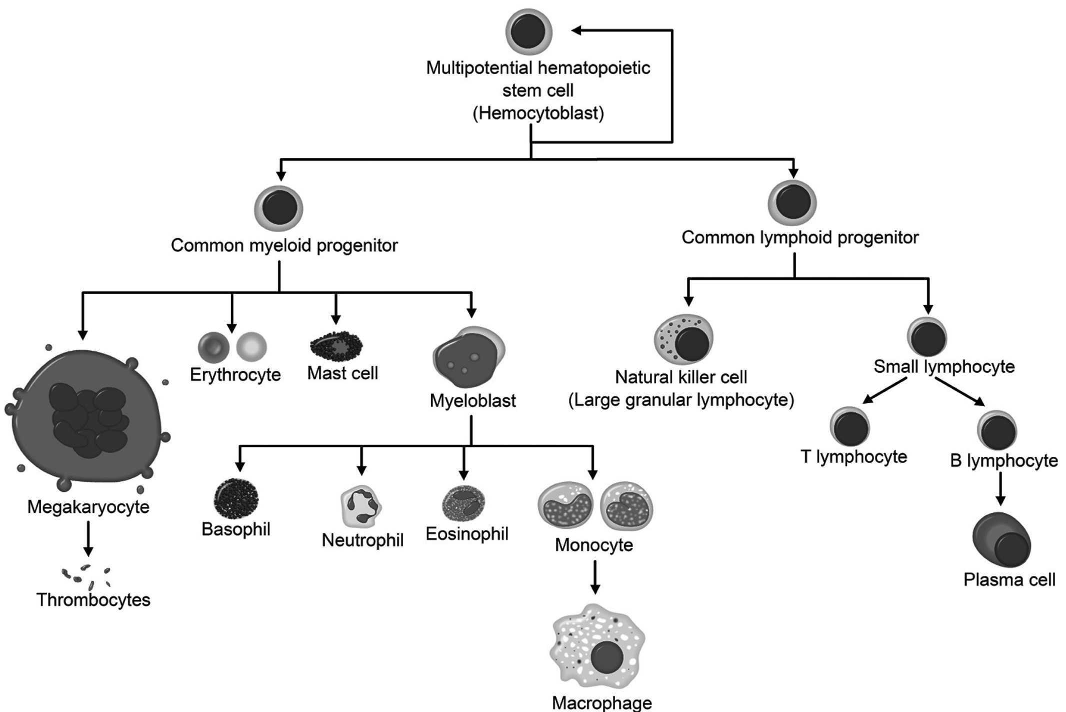


Figure 1 Schematic presentation of human hematopoiesis

1.2 Leukemia: disrupted hematopoiesis

Leukemia (Greek for 'white blood') is a disease of excessive growth of immature blood cells, so called blasts. Leukemias are divided by the lineage from which the blasts derived, myeloid or lymphoid lineage. Furthermore, leukemias are divided depending on the amount of proliferation potential that is retained by the cells. Fast proliferating cells with a complete maturation arrest result in acute leukemia, whereas cells with increased proliferation but retained maturation, cause a less aggressive chronic leukemia. This nomenclature defines four different types of leukemia: acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), chronic myeloid leukemia (CML) and chronic lymphoid leukemia (CLL).

1.3 Pediatric Acute Myeloid Leukemia

In children, leukemia is the most prevalent type of cancer and accounts for about 30% of all malignancies, which equals about 150 children newly diagnosed in the Netherlands per year. Acute lymphoblastic leukemia is the most frequently observed type of leukemia. Acute myeloid leukemia (AML) accounts for only about 15-20% of all pediatric leukemias.[2] Chronic leukemia is very rare in children.

Epidemiology

AML is diagnosed in about 20-25 children per year in the Netherlands (incidence less than 1 in 100.000), but AML incidence increases with age up to an incidence of over 20 in 100.000 in the elderly aged over 75 years. AML is slightly more frequent in males than in females, and furthermore geographic and ethnic variation exists, such as a higher frequency of the AML subtype acute promyelocytic leukemia in people from Hispanic or Mediterranean descent.[3-5]

Presenting symptoms and diagnosis

The clinical symptoms of leukemia patients at presentation are caused by the competition of expanding leukemic blasts with the normal bone marrow; fatigue, pallor, bruising or recurrent infections as a consequence of lack of healthy erythrocytes, platelets and leukocytes ('cytopenias') respectively.[1] Due to the infiltration and extramedullary hematopoiesis of leukemic cells in the liver or spleen these organs are often enlarged in AML, and in 5-15% of pediatric AML patients the central nervous system is infiltrated with blasts.[6,7] In certain AML subtypes, i.e. AML in infants, skin infiltrations or bone infiltrations can be present.

Currently the diagnostic work up of AML includes cytomorphology of the leukemic cells, immunophenotyping, karyotyping, and molecular genetics. AML is a heterogeneous group of disorders reflected by differences in clinical presentation, morphology, immunophenotype and molecular and cytogenetic aberrations. From the 1980's onwards, the French-American-British (FAB) morphology classification has been used to determine the lineage of origin and stage of maturation arrest, ranging from M0 (minimally differentiated AML) to M7 (acute megakaryoblastic leukemia) (Table 1).[8,9] As indicated in the table the type of maturation arrest can often be linked to the chromosomal aberrations involved in the development of AML (explained below).

FAB	Name	Cytogenetics	% of pediatric AML
M0	minimally differentiated acute myeloblastic leukemia		2-5
M1	acute myeloblastic leukemia, without maturation		10-15
M2	acute myeloblastic leukemia, with granulocytic maturation	t(8;21)(q22;q22), t(6;9)(p22;q34)	25-30
M3	promyelocytic, or acute promyelocytic leukemia (APL)	t(15;17)(q22;q12)	5-10
M4	acute myelomonocytic leukemia		15-25
M4eo	myelomonocytic together with bone marrow eosinophilia	inv(16)(p13;q22), t(16;16)(p13;q22)	10
M5	acute monoblastic leukemia (M5a) or acute monocytic leukemia (M5b)	*MLL*-rearrangement	15-25
M6	acute erythroid leukemias, including erythroleukemia (M6a) and very rare pure erythroid leukemia (M6b)		1-3
M7	acute megakaryoblastic leukemia	t(1;22)(p13;q13)	5-10

Table 1 French-American-British morphology classification of AML

Treatment and Prognosis

Over the last decades, the survival rate of pediatric AML has increased significantly.[10] About 40 years ago virtually no child with AML would survive, whereas treatment protocols of various collaborative study groups nowadays report 5-year overall survival rates of 60-70%.[5,11] Major improvements in remission rates were made by the introduction of intensive combination chemotherapy based on cytarabine combined with anthracyclines. In addition, treatment-related deaths were reduced by substantially improved supportive care. Current treatment protocols consist of 4-5 blocks of intensive combination chemotherapy with 3-4 weeks interval. The first blocks of chemotherapy)induction chemotherapy' are aimed to reach complete remission, the consecutive consolidation phase aims to maintain stable remission. The exact combination of drugs varies, but usually cytarabine-arabinoside (an anti-metabolite) and an anthracycline (drugs acting inhibition of DNA and RNA synthesis, topoisomerase inhibition and the production of free radicals) are included. Etoposide (a topoisomerase inhibitor) and/or 6-thioguanine (an anti-metabolite) may be included in induction therapy as a 3rd drug, although it is not clear whether this in fact increases complete remission rates or survival.

The majority of patients achieve complete remission after the first and/or second course of chemotherapy. Unfortunately, still approximately 30-40% of pediatric AML patients experience relapse (recurrence of the disease). At relapse, treatment protocols consist of re-induction chemotherapy, followed by allogenic stem cell transplantation. However, despite intensive treatment¸ still the prognosis of relapsed AML is poor; about 35% of these patients survive.[12] Especially the children with a high probability of relapse with current

therapy protocols, would benefit from new therapeutic options. Increasing the dose of currently used cytostatic drugs is not feasible, due to severe treatment-related morbidity and mortality. Therefore, novel therapeutic options have to arise from research towards the biologic background of the disease, as new molecular markers may identify potential specific treatment targets.

Biological background of pediatric AML: two-hit hypothesis

In 2002 Kelly and Gilliland hypothesized that the development of AML requires at least two types of molecular genetic events.[13,14] These aberrations result in maturation arrest (type II aberration) followed by hyperproliferation (type I aberration).

Type II aberrations in pediatric AML often involve chromosomal translocations (Figure 2) resulting in hybrid fusion genes. Classically, myeloid transcription factors are among the fusion genes, and as a consequence their gene expression is altered resulting in differentiation arrest. Several recurrent cytogenetic aberrations in pediatric AML classify as type II aberrations,[14] such as t(8;21)(q22;q22), inv(16)(p13;q22), t(16;16)(p13;q22), t(15;17)(q22;q12), t(6;9)(p22;q34) and translocations involving the *MLL* gene at chromosome 11q23. Not only translocations as identified by classical cytogenetic analysis but also cytogenetically cryptic aberrations, like the recently described NUP98/ NSD1 translo-

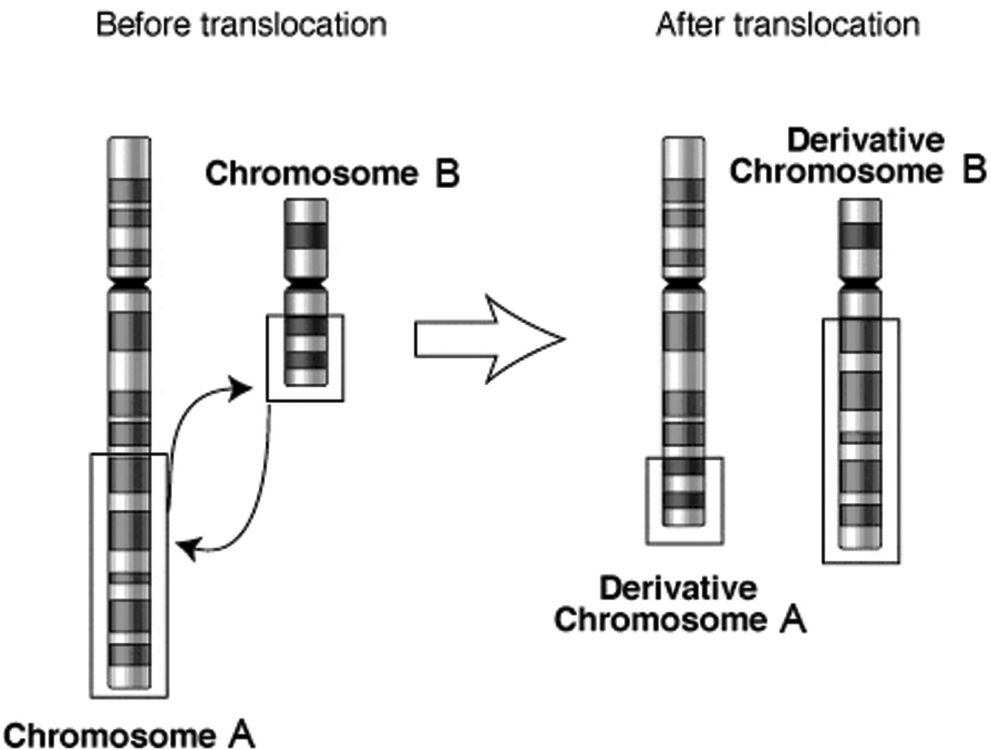


Figure 2 schematic presentation of a chromosomal translocation.

cation, can be considered as type II aberrations.[15] In addition, it was shown that also other genetic aberrations such as point-mutations in the NPM1 gene, *MLL*-partial tandem duplications and CEBPA double mutations should be classified as type II aberrations.[16-20]

The aberrations that cause increased proliferation potential, tend to be more subtle, as most of them are only detected by sequencing candidate genes for point mutations or small frame-shift mutations. Genes that were found to be involved in regulating proliferation are *N-RAS*, *K-RAS*, *FLT3*, *PTPN11* and *c-KIT*. These are grouped as type I mutations.[13] The recently described *CBL* mutations cause activation of the RAS-pathway,[21] and can therefore be judged as alternative type I mutations.

WHO classification (2008)		**% pediatric AML**
Acute myeloid leukemia with recurrent genetic abnormalities	AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1*	12-14%
	AML with inv(16)(p13.1q22) or t((16;16)(p13.1;q22); *CBFB-MYH11*	8%
	APL with t(15;17)(q22;q12); *PML-RARA*	6-10%
	AML with t(9;11)(p22;q23); *MLLT3-MLL*	7%
	AML with t(6;9)(p23;q34); *DEK-NUP214*	<2%
	AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1*	<1%
	AML (megakaryoblastic) with t(1;22)(p13;q13); *RBM15-MKL1*	Low, only infants
	Provisional entity: AML with mutated NPM1	*5-10%*
	Provisional entity: AML with mutated CEBPA	*5%*
Acute myeloid leukemia with myelodysplasia-related changes		Low
Therapy-related myeloid neoplasms		3.5%
Acute myeloid leukemia, not otherwise specified		~15% overall
	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		2-4%
Myeloid Proliferations related to Down Syndrome	Transient abnormal myelopoiesis	5% of DS newborn
	Myeloid leukemia associated with Down syndrome	~10%
Blastic Plasmacytoid Dendritic Cell Neoplasm		0%

Table 2 AML entities according to WHO and frequency in pediatric AML[10]

To include prognostically relevant aberrations in the classification of AML, the World Health Organization (WHO) has designed a classification that includes both cytogenetic aberrations and molecular aberrations (as provisional entities) and is applicable for both adult and pediatric AML (Table 2).[10,22] The classification contains most, but not all, pediatric cytogenetic subgroups, as t(7;12)(q36;p13) (*ETV6-HLXB9*) is exclusive for childhood AML and t(5;11)(q35;p15.5) (*NUP98-NSD1*) has only recently been described.[15]

1.4 *MLL*-rearranged pediatric AML

About 20% of the children with AML have an acquired chromosomal aberration involving the *mixed-lineage leukemia* (*MLL)* gene on chromosome 11q23, mostly a balanced translocation (Figure 2).[10,23] Within pediatric AML, patients with *MLL*-rearrangements are significantly younger. This is consistent with the theory that *MLL*-rearranged leukemias may already initiate in utero.[24] The proliferative capacity is illustrated by the frequent presence of high white blood cell counts at presentation.[5,16,25] *MLL*-rearranged pediatric AML is usually denominated by myelomonocytic and monoblastic morphology (FAB type M4 and M5).[25]

The *MLL*-gene can rearrange with more than 60 different translocation partner genes and new translocation partners are still being described, indicating that the full scope of

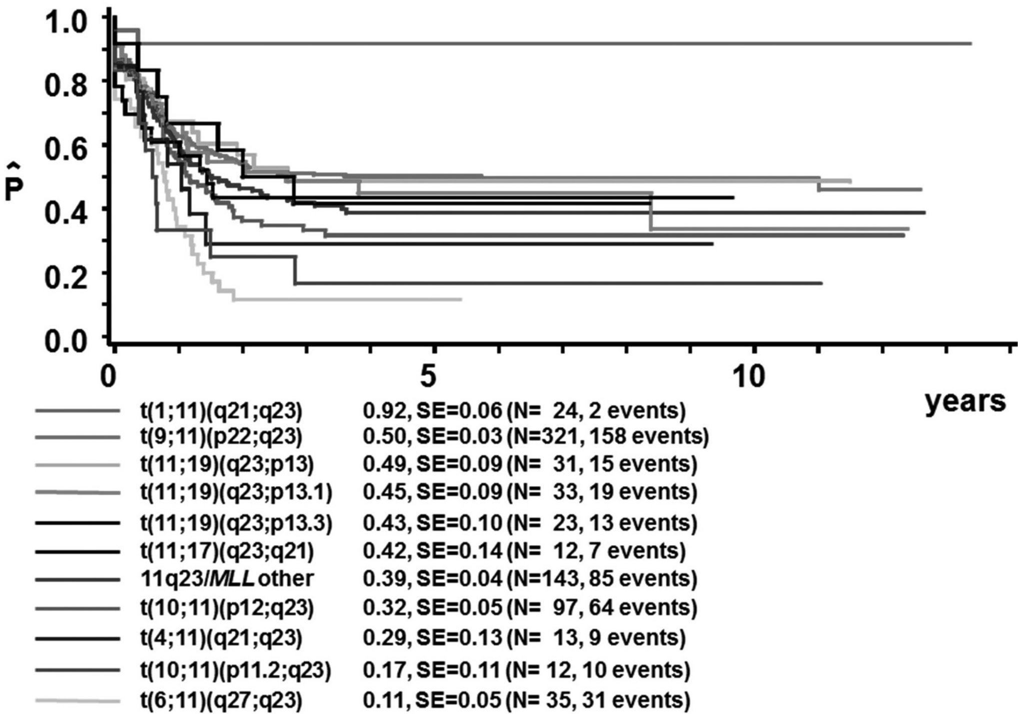


Figure 3 Event free survival of MLL-rearranged subgroups based on translocation partner.[25]
(A color version of this figure can be found in the appendices)

possible *MLL*-translocation partners has not been reached as yet.[26] Three partner genes account for about 60% of all *MLL*-rearranged pediatric AML patients: *MLLT3* (*AF9*) on chromosome 9p22 (~50%), *MLLT10* (*AF10*) on chromosome 10p12 (~10%) and *MLLT4* (*AF6*) on chromosome 6q27 (~5%).[5,26,27] AF9 is associated with intermediated outcome (event free survival (EFS) 50%), and AF10 and AF6 are associated with poor event free survival probabilities (EFS under 35%)(Figure 3). The biological background of these differences in outcome is currently unknown, but it is likely that the variety of target genes that are activated or repressed are responsible.

Other characteristics may also vary between specific translocation partner subgroups of *MLL*-rearranged pediatric AML, such as age at diagnosis and most prevalent FAB type.[25] This suggests that although *MLL*-rearranged AML patients have common characteristics, part of the phenotype is translocation partner specific. In addition, the presence of additional cytogenetic aberrations in *MLL*-rearranged pediatric AML may determine the biologic behavior and subsequent survival.[25]

The MLL-protein is an important epigenetic regulator and operates in a large multiprotein chromatin modification complex. Within this complex MLL has histone-methyltransferase and histone-acetyltransferase activity,[28] and although *MLL*-rearrangements are classically viewed as type II aberrations, interestingly mouse model studies have shown that an *MLL*-rearrangement can be sufficient to cause overt leukemia.[29-32] In pediatric *MLL*-rearranged AML patients however, non-random associations with type I mutations are found which in about half of the cases occur in genes involved in the RAS-pathway.[16] Over the last years many studies were conducted to identify *MLL*-driven target genes and collaborating proteins, which identified mainly *HOXA* and *MEIS1* gene overexpression. DOT1L, BRD4, EZH2 and Menin were identified as important epigenetic regulators within the MLL associated multiprotein complex.[29,30,33-35]

1.5 "*MLL*-like" pediatric AML subgroup with t(8;16)(p11;p13)

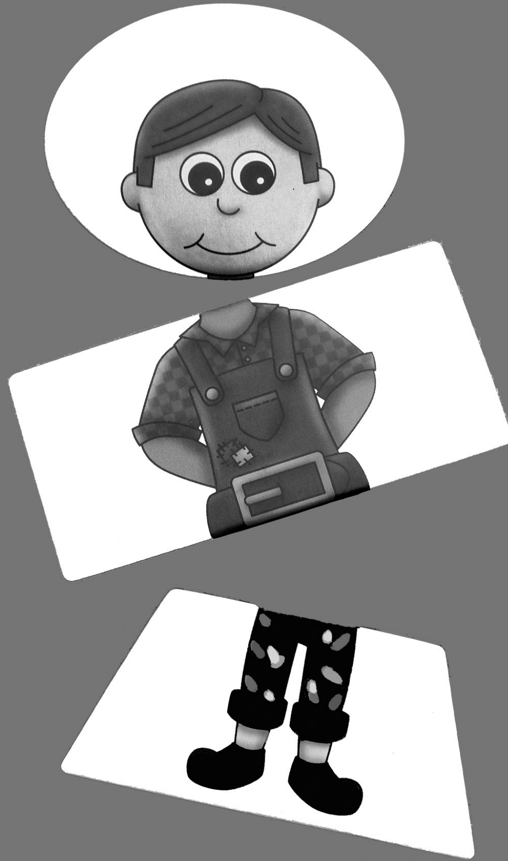
Several cytogenetic subgroups in pediatric AML are so rare that analysis of biological and clinical characteristics was stalled until now, due to low patient numbers. Among these are patients with t(8;16)(p11;p13) representing <1% of pediatric AML cases only.[27] This cytogenetic group is of interest because small series and case reports have suggested distinct clinical and prognostic profiles.[36,37] In addition, a recent study in adult AML suggested that this subtype of AML mimics *MLL*-rearranged AML, based on gene expression profiling, morphology and survival. To fully elucidate biological and prognostic features of this type of AML, we collaborated with international study groups.

1.6 Aims of the PhD project

The aim of this project was to further unravel the molecular and genetic signature of pediatric *MLL*-rearranged AML subgroups, exploring cytogenetics, molecular aberrations, gene expression signatures and methylation changes in gene promoters.

1.7 Outline of this thesis

As over 60 translocation partners have been described, *MLL* is considered a promiscuous gene. In collaboration with the Diagnostic Center of Acute Leukemia (DCAL) in Frankfurt, we identified two *novel* rare translocation partners of the *MLL* gene in pediatric AML, *KIAA1524* and *ABI2* (**Chapter 2** and **3**). **Chapter 4** describes the prognostic value of additional cytogenetic aberrations in *MLL*-rearranged AML in a large international retrospective study. In **Chapter 5** we report on the frequency of *CBL* mutations in pediatric AML, and the effect of *CBL* knock-down for activation of the RAS-pathway. Another interesting gene, *IGSF4*, was found to be highly upregulated in t(9;11)(p22;q23) pediatric AML by gene expression profiling, and it was shown to be associated with a favorable outcome (**Chapter 6**). **Chapter 7** describes a study on the gene expression signature of pediatric *MLL*-rearranged AML patients with t(6;11)(q27;q23), a subgroup that is associated with a very poor outcome. **Chapter 8** describes an international study on the clinical and biological characteristics of "*MLL*-like" pediatric AML patients with t(8;16)(p11;p13). The thesis ends with a summary and discussion of our findings.



Chapter 2

KIAA1524: a *novel MLL* translocation partner in acute myeloid leukemia

Eva A. Coenen[1], C. Michel Zwaan[1], Claus Meyer[2], Rolf Marschalek[2], Rob Pieters[1], Lars T. van der Veken[3], H. Berna Beverloo[3] and Marry M. van den Heuvel-Eibrink[1]

[1]Department of Pediatric Oncology and Hematology; [3]Department of Clinical Genetics; Erasmus MC, Sophia Children's Hospital, Rotterdam, The Netherlands

[2]Institute of Pharmaceutical Biology, ZAFES, Diagnostic Center of Acute Leukemias (DCAL), Frankfurt, Germany

Leukemia Research, 2011;35:133-5

Abstract

The Mixed Lineage Leukemia gene on chromosome 11q23 is a frequent site of recurrent translocations in acute leukemias. Its promiscuous character is reflected by the more than 60 different translocation partners described in literature. Prompted by karyotype and atypical FISH results, we identified a new translocation partner in infant acute myeloid leukemia, *KIAA1524* on 3q13.13, also known as `Cancerous Inhibitor of Protein phosphatase 2A (*CIP2A*)'. This gene was recently identified as a proto-oncogene stabilizing MYC protein in gastric carcinoma. *KIAA1524* has never been related to hematologic malignancies before, and the current AML case is the first case in which an *MLL-KIAA1524* fusion was described.

Introduction

MLL-rearrangements are frequently involved in both adult and childhood ALL and AML. In childhood they are especially frequent in infant leukemia.[25] The *MLL*-gene exhibits an 8-kb breakpoint cluster region in which virtually all rearrangements occur. So far more than 60 different fusion partners have been identified, some of them being observed in single cases only. In contrast, in 75% of the *MLL*-rearranged pediatric AML cases, 4 partners are involved (*AF9/MLLT3* on 9p21, *AF10/MLLT10* on 10p12, *ELL* on 19p13.1, and *AF6/MLLT4* on 6q27).[25] In infant AML, *AF9* and *AF10* are among the most frequent *MLL* fusion partners.[25,38] New translocation partners are still being reported to add to the diversity of *MLL*-rearranged leukemia.[39] Our case describes a new translocation partner, *KIAA1524*, which was indentified after an atypical finding using Fluorescent In Situ Hybridization (FISH) in a 4-months old girl with AML.

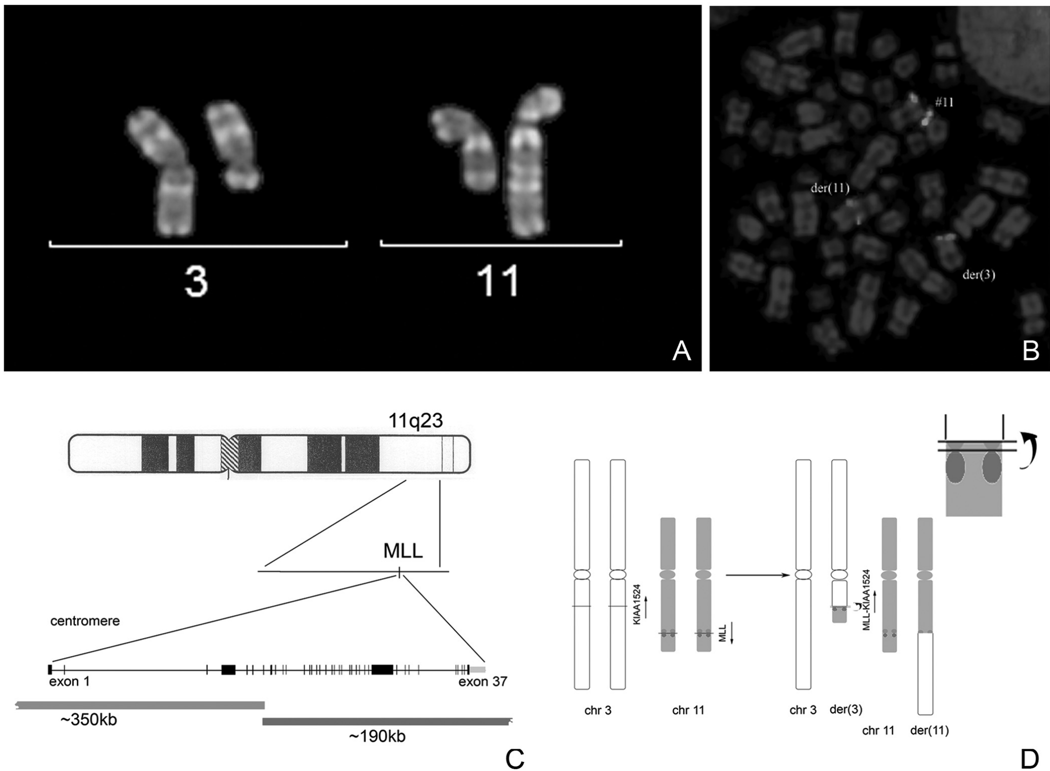
Case Report

A 4-months old Caucasian girl presented with AML-M5 and central nervous system involvement. The presenting white blood cell count was 394,0x10^9/l. The leukemic blasts showed the clonal immunophenotype CD34-, CD117-, CD133+, CD13-, CD33+, MPO+. The girl was treated according to the DCOG/MRC AML 15 protocol. Treatment, however, was complicated by a prolonged aplastic phase of 7 weeks following the first ADE-course. Despite rigorous supportive care she died in aplasia, approximately 9 weeks after diagnosis, without evidence for relapsing leukemia. A post-mortem liver biopsy was indicative of an aseptic toxic hepatitis.

Results and Discussion

RBA- and QFQ-banded karyotyping of the bone marrow were performed and showed 46,XX,t(3;11)(q12~13;q23)[20] (Figure 1A). Subsequently performed FISH with the Vysis LSI MLL Dual Color Break Apart Rearrangement Probe (Vysis/ Abbott, Des Plaines, IL, USA) showed unexpected results. Normally, in case of a translocation, a fused signal is observed on the normal chromosome 11, a green signal on the der(11) (containing the 5' part of *MLL*), and a red signal on the der(partner). In this case, a fused signal was observed on the normal 11 as well as on the der(3) (of which the green was weaker) and a separate green signal on the der(11) (Figure 1B). This suggested that the breakpoint on 11q23 was more upstream of the *MLL* breakpoint cluster region, or even upstream of *MLL*, since the green probe of this probe set extends towards the centromere and not only covers the 5’ part of *MLL* (Figure 1C). Subsequent FISH analysis with a different *MLL* dual color break apart probe set in which the 5’ probe extended less far centromeric than the Vysis probe[40], revealed fused signals on both the normal chromosome 11 and the der(3) and no signal on the der(11), suggesting that both 5' and 3' *MLL* were located on the der(3) (data not shown).

Figure 1 Karyotype and FISH results



A: Partial RBA-banded karyogram showing chromosomes 3 and 11. *The der(3) and the der(11) are shown on the right.* ***B: Metaphase showing FISH results using the LSI MLL dual color break apart probe set*** *(Vysis/ Abbott, Des Plaines, IL, USA)****.*** *The normal chromosome 11 shows fusion of the green and the red signal, the der(3) shows fusion with a weaker green signal whereas the der(11) shows a single green signal representing the 5'-part of MLL.* ***C: Genomic environment of Vysis MLL dual color break apart probe.*** *The 5' probe covers about 350kb and includes the first part of the MLL gene up to exon 6, the 3' probe, covering about 190 kb, overlaps with the 5' probe at exon 6 and covers the latter half of the gene.* ***D: Schematic display of proposed rearrangement.*** *FISH results can be explained by at least a third breakpoint. In this picture we propose this breakpoint to be just upstream of the MLL gene allowing inversion of the first part of MLL. After this inversion direction of transcription is identical to KIAA1524, from telomere to centromere. (A color version of this figure can be found in the appendices)*

Although a classical *MLL*-translocation seemed unlikely according to these FISH results, age of the patient and FAB classification prompted us to analyze the translocation in more detail. Since the possible translocation partner was not known, long distance inverse-PCR (LDI-PCR) was performed[41] and revealed an in frame fusion of *MLL* (intron 10) to *KIAA1524* (intron 16), a gene located on chromosome 3q13.13 (Figure 2). The *MLL-KIAA1524* transcript was confirmed with Reverse Transcription (RT)-PCR using an exon 8 specific *MLL* primer (5'-CCGCCAAGAAAAGAAGTT-3') combined with an exon 17 specific *KIAA1524* primer (5'-CTGCGCTCTTTCTGATTC-3') (Figure 2).

Figure 2 MLL-KIAA1524 fusion product

DNA sequence:

TTGTTTATGTTGGAAACATGTTTTTTAGATCTATTAATAAAATTTGTCATTTGCATTATTATCTGTTGCAA
ATGTGAAGGCAAATAGGGTGTGATTTTGTTCTATATTCATCTTTTGTCTCCTTAG**GAAAAACCACCTCC**
GGTCAATAAGCAGGAGAATGCAGGCACTTTGAACATCCTCAGCACTCTCTCCAATGGCAATAGTTCT
AAGCAAAAAATTCCAGCAGATGGAGTCCACAGGATCAGAGTGGACTTTAAGGTAAAGGTGTTCAGT
GATCATAAAGTATATTGAGTGTCAAAGACTTTAAATAAAGAAAATGCTACTACCAAAGGTGTTGAAAGA
GGAAATCAGCACCAACTGGGGGAATGAATAAGAACTCCCATTAGCAGGTGGGTTTAGCGCTGGGAGA
GCTTTGGTCAGTGTTGTTAGGTCACTGTTTGTGAACTGACTGCAGAACATACATAATGAAACATTCCTA
TCCATCCTGAGCAGTATCAGAGGAAGTAATTCCTTCACATGGAAAGTATCAAACCATGATGATTCCTTG
AGTCAGCAAAACTGTAAGAGAAATTCAATCCCAGTGTATTTTCGCAATATATTCAATATGAATTGAACAA
CTAGGTGAGCCTTTTAATAGTCCGTGTCTGA•GA•GATTGTTTTTATCAGATAATGTATTAAGTTAAAAAA
ATATAACAGTATCCAACTTATTAGAAGTAAAGTCCTAAGTATATGTGACTAATACAGCTTTGCATGACCC
AACAGATACCAGCTTATTAGATACTTAGTAAACTTTTAAATTGTAGGTATGTGTTGAGCATATGATATAG
ATGATAGTGGTAAAGTCTATCTTCTTTTTTTTTTTTGAG**GCACGGACACTTGCTAGTATGTTGAGAGAAG**
TTGAGAGAAAAAATGAAGAGCTTAGTGTGTTGCTGAAGGCGCAGCAAGTTGAATCAGAAAGAGCGC
AGAGTGATATTGAGCATCTCTTTCAACATAATAGGAAGTTAGAGTCTGTGGCTGAAGAACATGAAAT
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CCTAGAATGAACATGCAGCATTTCTTTTCTGAGACCCGAAAATCCTTTTGTAAGCATTTCATATATTCTG
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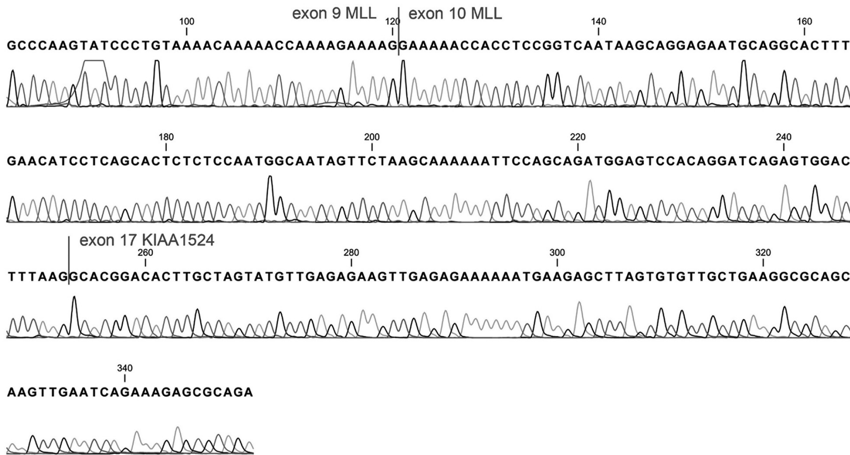
cDNA sequence:



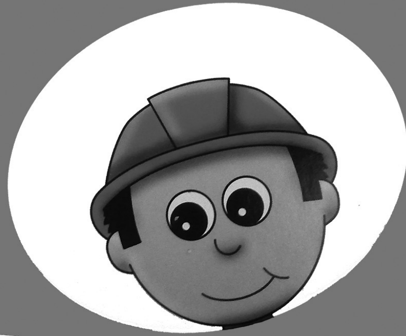
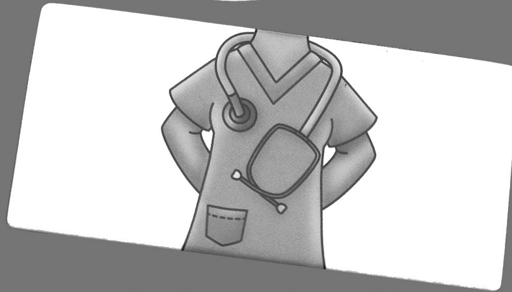
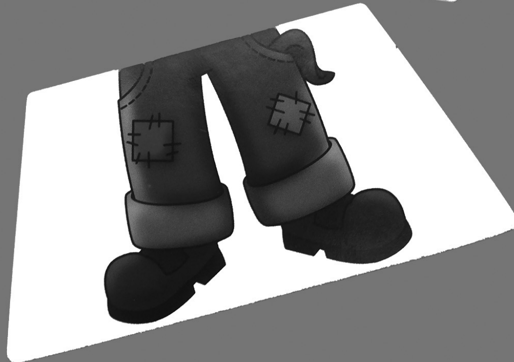
DNA sequence *as derived from a Long distance inverse-PCR experiment. Shown in black is the sequence from MLL intron 9, exon 10 (Bold) and intron 10 respectively, in blue two inserted nucleotides, in red the sequence from KIAA1524 intron 16, exon 17 (Bold) and intron 17 respectively.* ***cDNA sequence*** *is shown as derived from Reverse Transcription-PCR using an exon 8 specific MLL and an exon 17 specific KIAA1524 primer.* ***MLL-KIAA1524 fusion protein*** *is shown and its wildtype predecessors. AT: AT-hook, SNL1 and SNL2: subnuclear localization sites, MT: methyltransferase, PHD1-3 and PHD4: plant homology domains, BD: binding domain, FYRC and FYRN: domains involved in heterodimerisation, TASPASE1: taspase 1 cleavage sites, TAD: transcriptional activation domain, SET: Su(var)3-9, enchancer-of-zeste trithorax domain, CC: coiled coil domain, TMD: transmembrane domain, aa: amino acids. (A color version of this figure can be found in the appendices)*

In this case several factors, including the typical age, the phenotype (FAB M5) and a translocation involving 11q23, prompted us to search for an *MLL*-rearrangement, even though FISH results suggested otherwise. The LDI-PCR finding was unexpected in the light of FISH results, since intron 10 is within the common *MLL* breakpoint cluster region normally residing on the der(11). Furthermore, whereas *MLL* is transcribed from centromere to telomere, *KIAA1524* is transcribed in the opposite direction. Therefore at least one gene segment had to be inverted in order to produce a fusion transcript. This suggested mechanism bears great similarity to the *MLL-AF10* fusions, in which *AF10* is also normally transcribed in the opposite direction as *MLL* and for which the 5'part of *MLL* is also located on the der(partner). In these cases micro-inversions are known to occur.[42] The *MLL-KIAA1524* fusion thus is another fusion gene for which micro-inversions are needed to create the fusion gene (Figure 1 C-D). This indicates that at least 3 DNA-breaks are involved in such cases.[42]

The newly identified translocation partner, *KIAA1524*, recently renamed *CIP2A* (Cancerous Inhibitor of Protein phophatase 2A)[43], is a human proto-oncogene identified to be overexpressed in head and neck squamous cell carcinoma, colon cancer and gastric carcinoma.[43,44] The protein, encoded by 21 exons, harboring a transmembrane and a coiled coil domain, is involved in c-MYC signaling by inhibiting specific MYC-directed function of protein phosphatase 2A (PP2A). PP2A functions as a phosphatase destabilizing MYC, therefore overexpression of CIP2A results in increased stability of MYC.[43] MYC possesses a very broad scale of functions in cell cycle regulation, proliferation, differentiation and metabolism. KIAA1524 is required for sustained proliferation in gastric cancer and appears to regulate MYC in a positive feedback loop.[43,44] So far, *KIAA1524* has never been implicated in hematological malignancies, but MYC overexpression has been shown to cause AML in mice.[45]

Coiled coil domains are quite abundant structures, formed by about 3-5% of amino acids in proteins. They are also incorporated in more complex structures like leucine zippers and are involved in many biological actions, including transcriptional control.[46] A number of other *MLL* translocation partners also harbor a coiled coil domain, including *MLLT4* (*AF6*) and *MLLT10* (*AF10*).

In summary we identified a new *MLL* translocation partner in AML, *KIAA1524,* which was recently recognized as a proto-oncogene with important function in MYC signaling in solid tumors.

Chapter 3

Abl-interactor 2 (ABI2): a *novel MLL* translocation partner in acute myeloid leukemia

Eva A. Coenen[1], C. Michel Zwaan[1], Claus Meyer[2], Rolf Marschalek[2], Ursula Creutzig[3], Rob Pieters[1], Jutta Bradtke[4], and Marry M. van den Heuvel-Eibrink[1]

[1] Department of Pediatric Oncology and Hematology, Erasmus MC, Sophia Children's Hospital, Rotterdam, The Netherlands;

[2] Institute of Pharmaceutical Biology, ZAFES, Diagnostic Center of Acute Leukemias (DCAL), Frankfurt, Germany;

[3] AML-BFM Study Group, Hannover, Germany;

[4] Department of Pediatric Hematology and Oncology, Justus-Liebig-University, Giessen, Germany.

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Introduction

Mixed-Lineage Leukemia (MLL)-rearrangements are frequently involved in both adult and childhood acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML). In childhood they are especially frequent in infant leukemia.[25] The *MLL*-gene exhibits an 8-kb breakpoint cluster region in which virtually all rearrangements occur. So far more than 60 different fusion partners have been identified, some of them being observed in single cases only. In contrast, in 75% of the *MLL*-rearranged pediatric AML cases, 4 partners are involved: *AF9/MLLT3* on chromosome 9p21, *AF10/MLLT10* on 10p12, *ELL* on 19p13.1, and *AF6/MLLT4* on 6q27.[25] In infant AML, *AF9* and *AF10* are among the most frequent *MLL* fusion partners.[25] New translocation partners are still being reported to add to the diversity of *MLL*-rearranged leukemia.[39,47] Here we describe a new translocation partner, *Abl-interactor 2* (*ABI2)*, which was identified in a 5-months old female with AML.

Case Report

A 5-months old Caucasian female presented with hepatosplenomegaly and a white blood cell count of 68,2x10^9/l. She was diagnosed with AML-M5 and treated according to the AML-BFM 2004 protocol (Clinical Trial NCT00111345). No remission was achieved after cytarabine, idarubicin and etoposide (AIE)-induction nor after the second induction course with high dose cytarabine and mitoxantrone (HAM). Treatment was continued according to international BFM relapse protocol including two courses of fludarabine, cytarabine, idarubicine and G-CSF (FLAG-IDA) resulting in complete remission. A matched unrelated donor (MUD) stem cell transplantation was performed after which she has been in continuous complete remission for 3 years now.

Results and Discussion

At diagnosis GTG-banded karyotyping of the bone marrow showed 46,XX,t(2;11)(q33;q23)[11]. FISH, using the Vysis LSI MLL Dual Color Break Apart Rearrangement Probe (Vysis/ Abbott, Des Plaines, IL, USA) showed one fused signal and deletion of the second 3' *MLL* signal (nuc ish 11q23(5'MLLx2)(3'MLLx1)(5'MLL con 3'MLLx1)) in 65% of nuclei (bone marrow blast percentage of 50%). Usually, in case of a translocation, a fused signal is observed on the normal chromosome 11, a green signal on the der(11) (containing the 5' part of *MLL*), and a red signal on the der(partner) (containing the 3' part of *MLL*). The configuration found in this case can be explained by loss of parts of telomeric MLL material that were undetectable by conventional karyotyping, similar to other translocations such as t(9;22)(q34;q11).[48]

As no recurrent translocation partners of *MLL* are known on the 2q33 locus, and RT-PCR on *MLL-AF4*, *MLL-AF9*, *MLL-ENL* and *MLL-AF10* were negative, long distance inverse-PCR (LDI-PCR) was performed to identify the partner gene.[41] This revealed an in-frame fusion of *MLL* (intron 9) to Abl-interactor 2 (*ABI2)* (intron 4), a gene located on chromosome 2q33.2.(Figure 1) The *MLL-ABI2* transcript was confirmed with Reverse Transcription (RT)-

PCR using an exon 8 specific *MLL* primer (5'-CGTCGAGGAAAAGAGTGA-3') combined with an exon 6 specific *ABI2* primer (5'-TTGGGCTAGGTACGTAATCATT-3'). With this primer pair three transcripts were detected. One transcript was the expected fusion of *MLL* exon 9 with *ABI2* exon 5 and two additional splice variants. In one splice variant *MLL* exon 8 is fused to *ABI2* exon 6 and in the other *MLL* exon 9 to *ABI2* exon 6 (Figure 1). The *MLL* exon 9- *ABI2* exon 5 results in an in-frame fusion, both other transcripts are out of frame and are not likely to result in functional protein.

ABI2 is a functional homologue of *ABI1*, a recurrent translocation partner of *MLL* located on 10p11.2. The *MLL-ABI1* fusion is associated with poor prognosis.[25,49] Abi1 and Abi2 can act as inhibitors as well as substrates of c-Abl.[50] c-Abl is an important non-receptor tyrosine kinase that has been implicated in cell growth, reorganization of the cytoskeleton, apoptosis and stress responses.[51] In chronic myeloid leukemia and acute lymphoblastic leukemia c-Abl is known for its chimeric fusion to the breakpoint cluster region (*BCR*) gene located on 22q11.2, resulting in a constitutively active Bcr-Abl protein.

Protein domains of both Abi proteins include an Abl-interactor homeodomain homologous region (HHR), SRC homology 3 (SH3) domains and proline rich stretches, with an overall homology of 69%.[50] Both the SH3 domain as well as the Abl-interactor HHR domain and adjacent proline rich stretches of Abi-proteins are required for inhibitory binding of c-Abl. The Abi2 protein lacking the Abl-interactor HHR domain and part of the proline rich stretches still binds c-Abl, but the inhibition of c-Abl is lost.[50] Instead c-Abl is converted into a transforming protein by the truncated Abi2 due to activation of its tyrosine kinase activity, which resembles the activity of BCR-ABL fusion proteins.[50] Overexpression of wild type *ABI1* on the other hand suppresses the transforming activity of v-Abl, the oncogene carried by the retrovirus Abelson murine leukemia virus. Moreover, the Bcr-Abl oncogenic fusion protein elicits the ubiquitin dependent degradation of Abi2, [52] and mutations in certain regions of Bcr-Abl abrogate the Abi2 interaction and prevent leukemogenesis.[53] Selective ubiquitylation of Abi2 is also exerted by Tripartite Motif Protein 32 (TRIM32), facilitating cell growth and migration in an epidermoid carcinoma cell line. These data indicate that Abl-interactor proteins are crucial tumour suppressors by inhibiting downstream signaling of c-Abl.

The translocation described in our case results in loss of the first 4 exons of *ABI2*, including part of its Abl-interactor HHR domain, and resulting in fusion to the N-terminal *MLL* gene segment. This loss resembles the deletion variants designed by Dai et al., but is not as extensive.[50] Therefore it is not clear whether this could have been sufficient in this patient to cause transforming potential of c-Abl.

In summary we identified *ABI2* on chromosome 2q33.2 as a new *MLL* translocation partner in an infant with AML-M5 leukemia refractory to standard induction chemotherapy. This important c-Abl regulator is a functional homologue of *ABI1*, a recurrent *MLL*-translocation partner located on chromosome 10p11.2 and is implicated as a tumour suppressor by its inhibitory function in c-Abl signaling.

Figure 1 MLL-ABI2 fusion product.

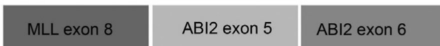
DNA sequence

TGAAGTCTTCAGTTCAAGAAAATCAGCTCTCTTTCTAACTATTATGTTTAATAATA
AAGAAACAGAAACAAAAAAAAACAGTTAAATTGGAGGTATTGTTTTAATTTCCTGT
TCGAAGCCTAGAGTTTAAATAGTTTTTTTTTTTTTTTTCTAATGGCCCTTTCTTCA
CAGGTAGCAGTCAGTACTAAAGTAGTCGTTGCCAGCATCTGACTGCAATTTATT
CTGAATTTTTTAG**GTCCAGAGCAGAGCAAACAGAAAAAAGTGGCTCCCCG**
CCCAAGTATCCCTGTAAAACAAAAACCAAAGAAAAGGTGAGGAGAGATT
TGTTTCTCTGCCATTTCTCAGGGATGTATTCTATTTTGTAGGGAAAAGCCTTATC
CTTGACTTCTATGTAGATGGCAGTGGAATTTCTTAAAATTAAGAAACTTCAAGTT
TAGGCTTTTAGCTGGGCACGGTGGCTCACGCTGGTAATCCCAACACTTAGTGA
GGCTGAGGTGGGAGGATTGCTTGAGGCCAGCAGTTCAAGACCAGCCTGGGCA
ACATAGCAAGACCCTGTCTTTATTTAAACAAAAAAAAAAAAAAAAGAAGAAGAAGA
AGAAGT●TA●CAGAAGATACTGCAATTTAAACAAAAACACCTTGCATACCAAAA
CTTAAAACAAATATAAGCTGGAAAGCTCTTTTCAAATATTTTAGCTATCCCATGT
ATAGAATAAAATTCTCTCTTAAATTCCTGCTCCTTTTCCTTCCTGTTTCTCAGCAA
ATTGATGTAGTTTTAGTGTTTGGTTCACTTCTCCCTCATCACTTAAAGTAACTTTG
GGTTTTATGTTGTTTAAGGTTGAGCTTAAAGGACTTTGGTTACCACTTTAAAAAA
ATCTTCTGGTTTCAGAAATTAAGATATGCAGTTTTAAAAGCTATTCATTATTTGTC
ATATTACTCCTAAAATTTTCCCCAAATGTGGATTGATTTTTTTAAAAAAACTTTTT
CTGGCATTTTCATTTTAAGATACAAGTAGTATACCCTCCGCTTATTAATGAGAGG
GAGAGCAAGGATAAGTTAATCTTTACTCTTACTTGATAATGTTTGTTTTTATTGTT
TAAAAGCAACAACAAAAGGATTACAGA

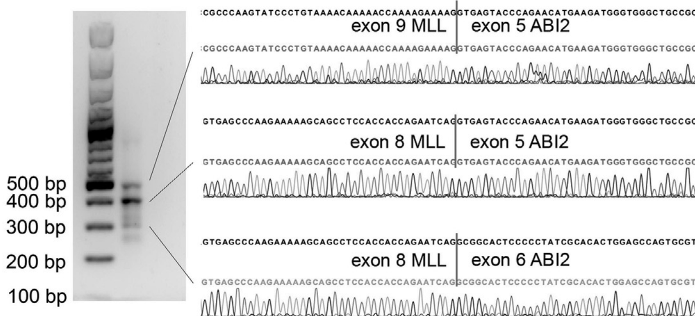
Schematic overview of transcripts

Expected transcript

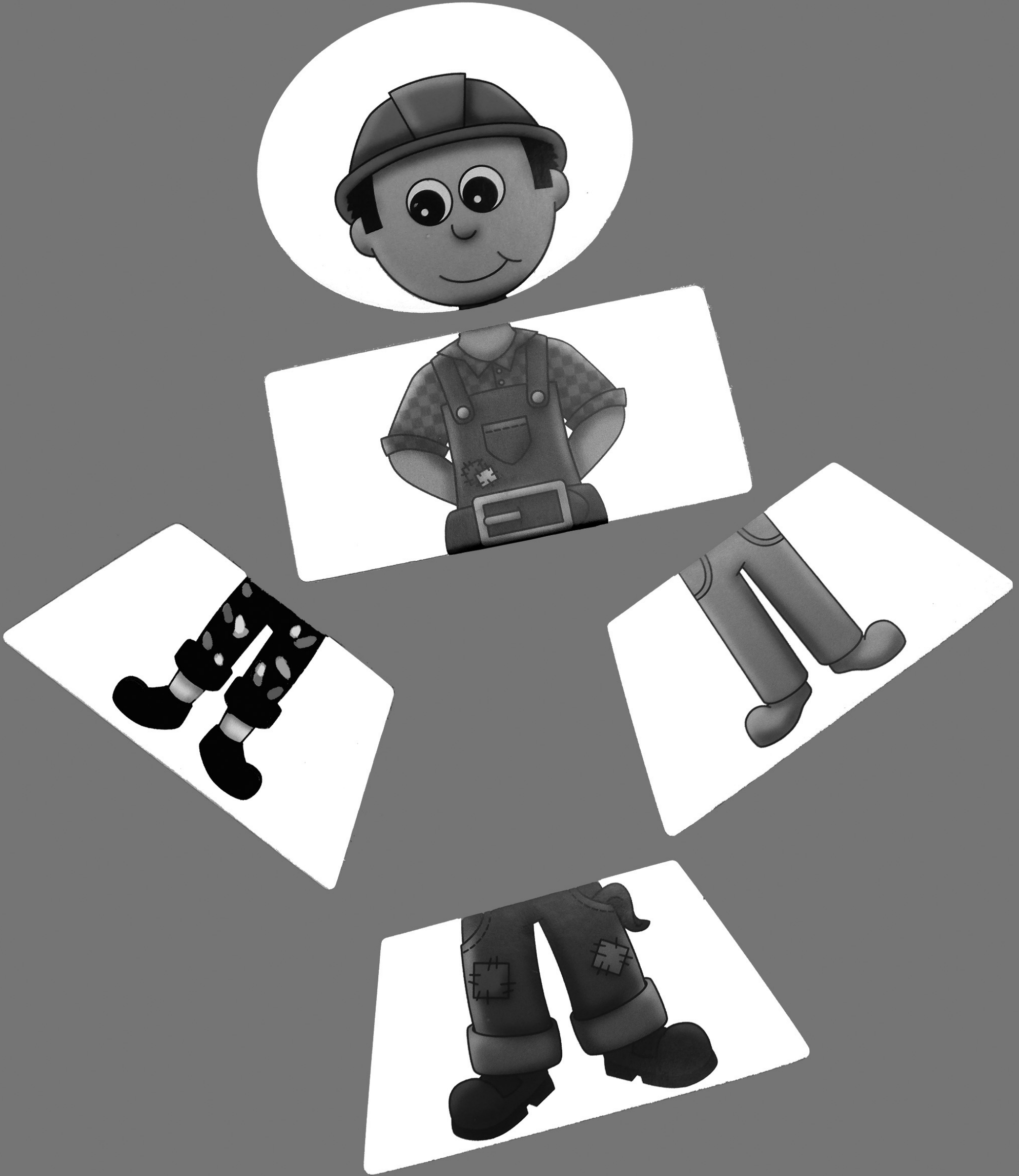
Other transcripts present:



cDNA sequence



DNA sequence is shown as derived from a Long distance inverse PCR experiment. Shown in black is the sequence from MLL intron 8, exon 9 (Bold) and intron 9 respectively, in blue two inserted nucleotides, in red the sequence from ABI2 intron 4. The schematic overview of transcripts shows the different splice variants that were present. cDNA sequence is shown as derived from Reverse Transcription-PCR using an exon 8 specific MLL and an exon 6 specific ABI2 primer. The gel picture shows multiple bands that were derived with these primers and were confirmed by cloning. (A color version of this figure can be found in the appendices)



Chapter 4

Prognostic significance of additional cytogenetic aberrations in 733 de novo pediatric 11q23/*MLL*-rearranged AML patients: results of an international study

Eva A. Coenen,[1] Susana C. Raimondi,[2,3] Jochen Harbott,[4] Martin Zimmermann,[5] Todd A. Alonzo,[3] Anne Auvrignon,[6] H. Berna Beverloo,[7,8] Myron Chang,[9] Ursula Creutzig,[10] Michael N. Dworzak,[11] Erik Forestier,[12] Brenda Gibson,[13] Henrik Hasle,[14] Christine J. Harrison,[15] Nyla A. Heerema,[3,16] Gertjan J. L. Kaspers,[17-19] Anna Leszl,[20] Nathalia Litvinko,[21] Luca Lo Nigro,[22] Akira Morimoto,[23,24] Christine Perot,[6] Dirk Reinhardt,[5] Jeffrey E. Rubnitz,[2] Franklin O. Smith,[3,25] Jan Stary,[26] Irina Stasevich,[21] Sabine Strehl,[11] Takashi Taga,[23,27] Daisuke Tomizawa,[23,28] David Webb,[18,29] Zuzana Zemanova,[30] Rob Pieters,[1] C. Michel Zwaan,[1,17] and Marry M. van den Heuvel-Eibrink[1,17]

[1]Department of Pediatric Oncology/Hematology, Erasmus MC–Sophia Children's Hospital, Rotterdam, The Netherlands; [2]St Jude Children's Research Hospital, Memphis, TN; [3]Children's Oncology Group, Arcadia, CA; [4]Acute Myeloid Leukemia-Berlin-Frankfurt-Münster Study Group, Department of Pediatric Hematology and Oncology, Justus-Liebig-University, Giessen, Germany; [5]Acute Myeloid Leukemia-Berlin-Frankfurt-Münster Study Group, Pediatric Hematology/Oncology, Medical School Hannover, Hannover, Germany; [6]French Leucémie Aique Myeloide Enfant, Hopital Trousseau, Paris, France; [7]Dutch Childhood Oncology Group, Dutch Working Group on Hemato-Oncologic Genome Diagnostics, The Hague, The Netherlands; [8]Department of Clinical Genetics, Erasmus MC, Rotterdam, The Netherlands; [9]Children's Oncology Group, Data Center, Gainesville, FL; [10]Acute Myeloid Leukemia-Berlin-Frankfurt-Münster Study Group, Pediatric Hematology/Oncology, University Hospital, Munster, Germany; [11]Children's Cancer Research Institute, Vienna, Austria; [12]Nordic Society for Pediatric Hematology and Oncology, Department of Clinical Science, Pediatrics, Umeå University, Umeå, Sweden; [13]Department of Pediatric Oncology/Hematology, Royal Hospital for Sick Children, Glasgow, United Kingdom; [14]Nordic Society for Pediatric Hematology and Oncology, Department of Pediatrics, Aarhus University Hospital Skejby, Aarhus, Denmark; [15]Northern Institute for Cancer Research, Newcastle University, Newcastle upon Tyne, United Kingdom; [16]Department of Pathology, Ohio State University, Columbus; [17]Dutch Childhood Oncology Group, The Hague, The Netherlands; [18]Acute Myeloid Leukemia Committee I-Berlin-Frankfurt-Münster Study Group; [19]Department of Pediatric Oncology/Hematology, VU University Medical Center, Amsterdam, The Netherlands; [20]Italian Association of Pediatric Hematology Oncology, Clinica Pediatrica, Università Padova, Padova, Italy; [21]Research Center for Pediatric Oncology and Hematology, Minsk, Belarus; [22]Italian Association of Pediatric Hematology Oncology, Clinica Pediatrica, Universita` Catania, Catania, Italy; [23]Japanese Pediatric Leukemia/Lymphoma Study Group, Nagoya, Japan; [24]Department of Pediatrics, Jichi Medical University School of Medicine, Tochigi, Japan; [25]Hematology/Oncology and Pediatrics, Cincinnati Children's Hospital Medical Center and the University of Cincinnati College of Medicine, OH; [26]Czech Pediatric Hematology/Oncology, University Hospital Motol and 2nd Medical School, Charles University, Prague, Czech Republic; [27]Department of Pediatrics, Shiga University of Medical Science, Shiga, Japan; [28]Department of Pediatrics and Developmental Biology, Tokyo Medical and Dental University, Tokyo, Japan; [29]Great Ormond Street Hospital for Children, London, United Kingdom; and [30]Center of Oncocytogenetics, General University Hospital and First Faculty of Medicine, Charles University, Prague, Czech Republic.

S.C.R. and J.H. contributed equally to this paper.
C.M.Z. and M.M.vdH.-E. contributed equally to this paper.

Blood, 2011;117:7102-11

Abstract

We previously showed that outcome of pediatric 11q23/*MLL*-rearranged AML depends on the translocation partner (TP). In this multicenter international study on 733 children with 11q23/*MLL*-rearranged AML, we further analyzed which additional cytogenetic aberrations (ACA) had prognostic significance. ACAs occurred in 344/733 (47%) and were associated with unfavorable outcome (5-year overall survival (OS) 47% *vs.* 62%, $P<0.001$). Trisomy 8, the most frequent specific ACA (n=130/344, 38%), independently predicted favorable outcome within the ACAs group (OS 61% *vs.* 39%, $P=0.003$; Cox model for OS Hazard Ratio (HR) 0.54, $P=0.03$), based on reduced relapse rate (26% *vs.* 49%, $P<0.001$). Trisomy 19 (n=37/344, 11%) independently predicted poor prognosis in ACAs cases, which was partly caused by refractory disease (remission rate 74% *vs.* 89%, $P=0.04$; OS 24% *vs.* 50%, $P<0.001$; HR 1.77, $P=0.01$). Structural ACAs had independent adverse prognostic value for event free survival (EFS) (HR 1.36, $P=0.01$). Complex karyotype, defined as ≥3 abnormalities, was present in 26% (n=192/733), and showed worse outcome than those without complex karyotype (OS 45% *vs.* 59%, $P=0.003$) in univariate analysis only. In conclusion, like TP, specific ACAs have independent prognostic significance in pediatric 11q23/*MLL*-rearranged AML, and the mechanism underlying these prognostic differences should be studied.

Introduction

Pediatric acute myeloid leukemia (AML) is a clinically and genetically heterogeneous disease. In addition to initial response to treatment, prognosis is largely determined by the presence of cytogenetic abnormalities and genetic lesions.[5,20,54-57] Several recurrent cytogenetic abnormalities, such as 11q23/*MLL*-rearrangements, predict outcome in myeloid neoplasms and acute leukemia.[22] So far, more than 60 different translocation partners (TP) have been identified, and new partners are still being reported to add to the diversity of *MLL*-rearranged leukemia.[39,47] A recent international study highlighted the heterogeneity of 11q23/*MLL*-rearranged pediatric AML by showing that outcome is dependent on TP.[25] This study also revealed that additional cytogenetic aberrations (ACAs) were an independent adverse prognostic factor,[25] but so far, it is unknown which additional aberration(s) determine this unfavorable outcome signature.

A recent large study in an adult AML cohort showed that additional cytogenetic abnormalities in t(9;11)(p22;q23) AML did not affect outcome.[23] However, the Berlin-Frankfurt-Münster (BFM) group showed that children with t(9;11)(p22;q23) with additional aberrations had lower rates of overall survival (OS) than those with other subgroups of AML.[5]

To date, no large studies have been undertaken to study the prognostic relevance of specific ACAs in pediatric *MLL*-rearranged AML. In this multicenter international study, we retrospectively analyzed data from a large cohort (n = 733) to determine which ACAs contribute to the prognostic effect in pediatric *MLL*-rearranged AML.

Patients and Methods

Patients

Patients' data collected in the retrospective international study by Balgobind *et al.*[25] were included in this study. In summary, data from 756 patients with 11q23/*MLL*-rearranged pediatric AML were collected from 11 collaborative study groups – the BFM Study Group (Germany and Austria); the Japanese Pediatric Leukemia/Lymphoma Study Group (Japan); the Leucémies Aiguës Myéloblastiques de l'Enfant Cooperative Group (France); the Czech Pediatric Hematology Working Group (Czech Republic); the St. Jude Children's Research Hospital (USA); the Associazione Italiana Ematologia Oncologia Pediatrica (Italy); Belarus; the Children's Oncology Group (USA); the Nordic Society for Pediatric Hematology and Oncology (Denmark, Finland, Iceland, Norway, and Sweden); the Dutch Children's Oncology Group (The Netherlands); and 2 centers of the Medical Research Council (United Kingdom). Patients were treated on national/collaborative group AML trials.[58-68] The treatment protocols were approved according to local law and guidelines, by the Institutional Review Boards of each participating center with informed consent obtained in accordance with the Declaration of Helsinki. Inclusion criteria for the current analyses were diagnosis between January 1, 1993, and January 1, 2005; age less than 18 years at diagnosis and involvement of 11q23 or *MLL* as determined by G-, Q- or R-banded karyotyping, fluorescence in situ hybridization (FISH) or reverse-transcribed polymerase chain reaction (RT-PCR). Exclusion criteria were secondary AML after congenital bone marrow

failure disorders, aplastic anemia, prior chemotherapy or radiotherapy for other diseases, and prior myelodysplastic syndrome (MDS). Patients with Down Syndrome were included if they met the other inclusion criteria.

All clinical data obtained at initial diagnosis, data on treatment [therapy protocol, including hematopoietic stem cell transplantation (HSCT)], and all events during follow-up were checked for consistency and completeness.[25]

Cytogenetic analysis

All karyotypes were centrally reviewed by 2 cytogeneticists (JH, SCR) and assigned to 11q23/*MLL*-rearranged groups on the basis of TP.[25] All karyotypes were designated according to the International System for Human Cytogenetic Nomenclature 2005, ISCN (2005).[69]

To analyze ACAs, data from all patients with incomplete karyotypes were excluded. For all cases included in the analysis, the number of aberrations was counted. Each aberration separated from the rest of the karyotype by a comma was counted as one abnormality (regardless of its complexity), counting every aberration only once (if present in multiple clones), and excluding constitutional aberrations. Triploidy and tetraploidy were counted as 1 aberration (1 event). In this cohort of 11q23/*MLL*-rearranged cases, ACAs cases were defined as having 2 or more aberrations, including the 11q23/*MLL*-rearrangement (n = 344). All cases with 3 or more aberrations were considered having a complex karyotype, consistent with previously used definitions.[70,71] Numerical aberrations were defined as loss or gain of a full chromosome. Balanced translocations were defined as translocations in which no material seemed to be gained or lost as determined by conventional karyotyping. Structural aberrations were defined as aberrations resulting from breakpoints within a chromosome. In all unbalanced translocations we described which material was lost and gained and also whether 11q23 was involved. The presence of a balanced overall karyotype was defined as a karyotype with two complete copies of all autosomes and complete copies of sex chromosomes without any additional material (2n). Definitions used for cytogenetic classification are summarized in Supplementary Table S1.

Statistical analyses

Complete remission (CR) was defined as less than 5% blasts in the bone marrow, with regeneration of trilineage hematopoiesis plus absence of extramedullary disease.[72] Early death was defined as any death within the first 6 weeks of treatment. Treatment of patients who did not obtain CR within the specified time-period in the protocol, was considered a failure on day 0. OS was measured from the date of diagnosis to the date of last follow-up or death from any cause. Event-free survival (EFS) was calculated from the date of diagnosis to the first event or to the date of last follow-up. Events included nonremittance, relapse, secondary malignancy, or death from any cause. Cumulative incidence of relapse (CIR) was calculated from the date of CR to the first relapse. Refractory disease was included in the EFS and CIR analyses by arbitrarily setting the event date on day 0. For OS, EFS, and CIR analyses, patients who did not experience an event were censored at the time of last follow-up. The Kaplan-Meier method was used to estimate the 5-year probabilities of OS and EFS, and survival estimates were compared by the log-rank test.

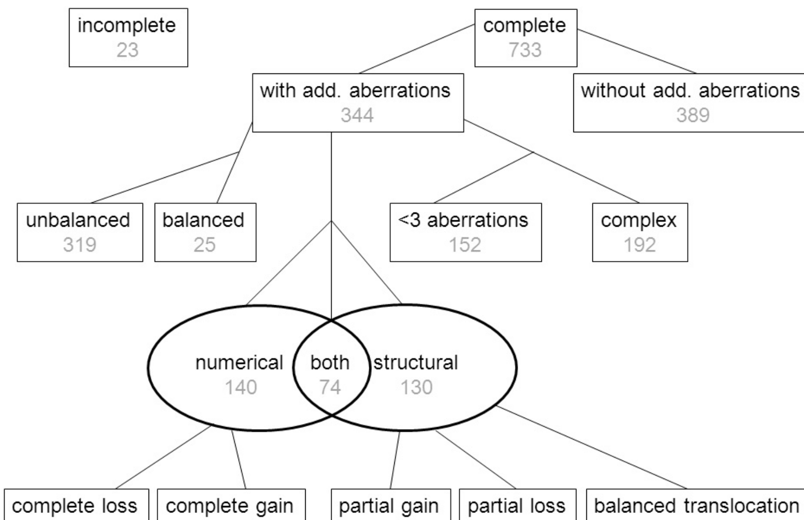
Gray's test for competing risks was used for CIR analysis. Multivariate analyses were performed with the Cox proportional hazards model. Continuous variables known to be of prognostic value in AML were categorized according to cutoff points [e.g. age more than 2 or 10 years, white blood cell (WBC) count less than 20×10^9/L or more than 100×10^9/L]. The χ^2 or Fisher's exact test was used to compare differences in proportions of variables among groups; the Mann-Whitney U test was used for continuous variables. All *P*-values are descriptive and explorative and were considered significant if less than or equal to 0.05. All statistical data were analyzed by using SAS-PC, Version 9.1 (SAS Institute, Inc., Cary, NC).

Results

Distribution of additional cytogenetic aberrations (ACAs)

Of the 756 patients, 733 (97%) had complete karyotypes and their data were included in the study (see flowchart in Figure 1). There were no significant differences in the patients included (n = 733) and not included (n = 23) in this study with respect to sex, age, WBC count, and TP group (data not shown). ACAs were found in 344 of 733 cases (47%) (Figure 1). The number of additional aberrations ranged from 0 to 15 (mean, 1.2 additional aberrations; Supplementary Figure S1).

Figure 1: Flow chart showing the presence and type of additional cytogenetic aberrations (ACAs) in 756 pediatric patients with 11q23/MLL-rearranged AML.



Complete karyotypes were not available for 23 patients and they were therefore excluded from analyses. Presence or absence of ACAs was determined for 733 patients for whom complete karyotypes were available. In the cohort having ACAs balanced karyotype was coded for 25 patients; the remaining had an unbalanced karyotype. The types of aberrations were coded as numerical, structural or both and the number of aberrations was also coded. Losses and gains are further coded in other figures.

Table 1: Distribution of additional cytogenetic aberrations (ACAs) by translocation partner and clinically relevant parameters.

			ACAs type		
	N	**ACAs (%)**	**Numerical (%)**	**Structural (%)**	**Both (%)**
TP group					
9p22	316	148 (47)	**84 (57)**	**40 (27)**	**24 (16)**
10p12	96	48 (50)	**13 (27)**	**26 (54)**	**9 (19)**
6q27	35	17 (49)	**8 (47)**	**7 (41)**	**2 (12)**
19p13	30	10 (33)	**6 (60)**	**1 (10)**	**3 (30)**
19p13.1	34	13 (38)	**5 (38)**	**4 (31)**	**4 (31)**
19p13.3	25	13 (52)	**5 (38)**	**4 (31)**	**4 (31)**
1q21	24	6 (25)	**2 (33)**	**3 (50)**	**1 (17)**
4q21	13	8 (62)	**2 (25)**	**4 (50)**	**2 (25)**
10p11.2	12	7 (58)	**0**	**5 (71)**	**2 (29)**
17q21	12	3 (25)	**1 (33)**	**1 (33)**	**1 (33)**
other	136	71 (52)	**14 (20)**	**35 (49)**	**22 (31)**
	733				
Sex					
Male	358	171 (48)	78 (46)	63 (37)	30 (18)
Female	375	173 (46)	62 (36)	67 (39)	44 (25)
	733				
Age dx					
<2	344	143 (42)	*45 (31)*	*68 (48)*	*30 (21)*
2-9	219	115 (53)	*57 (50)*	*35 (30)*	*23 (20)*
≥10	170	86 (51)	*38 (44)*	*27 (31)*	*21 (24)*
	733				
WBC					
<20	339	175 (52)	*84 (49)*	*51 (29)*	*40 (23)*
20-99	203	87 (43)	*28 (32)*	*40 (46)*	*19 (22)*
≥100	171	73 (43)	*25 (34)*	*35 (48)*	*13 (18)*
	713				
FAB					
M0	23	12 (52)	6 (50)	3 (25)	3 (25)
M1	39	20 (51)	9 (45)	7 (35)	4 (20)
M2	32	12 (38)	7 (58)	4 (33)	1 (8)
M4	134	49 (37)	21 (43)	21 (43)	7 (14)
M5	446	217 (49)	88 (41)	83 (38)	46 (21)
M7	19	15 (79)	7 (47)	2 (13)	6 (4)
n.d.	7	5 (71)	0	2 (40)	3 (60)
	700				
Overall		344 (47)	140 (41)	130 (38)	74 (22)

Note: *Values significantly different at the $P<0.05$ level (χ^2) are given in italics and those significantly different at the $P<0.01$ level (χ^2) are given in bold.* ***Abbreviations:*** *ACAs (%) indicates number of cases with additional aberrations and percentage within this group; Total, total number of cases within this group; Balanced karyotype (%), number of cases with balanced karyotype and percentage of specific group (row); Numerical (%), number of cases with numerical additional aberrations and percentage of specific group (row); Structural (%), number of cases with structural additional aberrations and percentage of specific group (row); TP group, site of translocation on partner chromosome; Age dx, age in years at diagnosis; WBC, white blood cell count x 10^9/L at diagnosis; FAB, French American British morphology classification subtype; n.d., not determined.*

There were 3 or more aberrations (including the 11q23/*MLL*-rearrangement) in 192 of 733 (26%) cases, which were therefore defined as complex karyotypes. Of the 344 cases with ACAs, 140 (41%) had numerical ACAs only, 130 (38%) had structural ACAs only, and 74 (22%) had both numerical and structural ACAs (Figure 1). There were 25 (7%) cases of ACA that had only balanced structural abnormalities in their karyotypes (Figure 1).

Distribution of ACAs in clinically relevant groups

Tables 1 and 2 show the distribution of ACAs by translocation partner (TP) group and clinically relevant parameters [sex, age, WBC count, and FAB (French-American-British) subtype]. TP groups 9p22 and 19p13 were characterized by a relatively high frequency of numerical ACAs, whereas groups 10p12, 10p11.2, and 4q21 showed higher prevalence of structural ACAs ($P < 0.001$) (Table 1). Also, there were significant differences in the number of aberrations among TP groups: the 6q27 group had a relatively high number of ACAs ($P = 0.002$) whereas groups 9p22, 19p13, and 1q21 had a lower number of ACAs (Table 2).

ACAs were less likely to occur in young children (<2 years old) than in children 2–9 years old or 10 years or older (42% *vs.* 53% *vs.* 51%, $P = 0.02$, Table 1). However, structural ACAs were more frequent children <2 years than in children 2–9 years old or 10-18 years (48% *vs.* 30% *vs.* 31%, $P < 0.01$) (Table 1). There was a higher prevalence of highly complex karyotypes (>5 aberrations) in children 10-18 years old than those younger than 2 years or 2–9 years old (11% *vs.* 4% *vs.* 5%, $P = 0.02$, Table 2).

Although the number of patients with FAB M7 was small, ACAs were more likely to occur in patients with AML FAB M7 as compared to those with other FAB types (79% *vs.* 46%, $P = 0.008$), whereas patients with AML FAB M2 and M4 had the lowest occurrence of ACAs (Table 1). Also, patients with AML FAB M7 seem to have a higher number of aberrations than those with other FAB morphologies ($P = 0.003$) (Table 2).

Specific recurrent aberrations

Trisomy 8 was the most frequently occurring numerical abnormality (130/733, 18% of all cases and 38% of ACA cases, Figure 2A). In addition, trisomy 4, 6, 13, 19 and 21 were recurrent ACAs (at least 15 cases each). Two cases with Down's syndrome were included in this study. However, since constitutional aberrations were not included in the additional aberrations, they were not included in the trisomy 21 group. Only 11 patients had losses of full chromosomes, collectively accounting for 25 monosomies (Figure 2A).

Figure 2B shows the collective analysis of structural ACAs per chromosome arm, but does not include breakpoints involved in balanced 11q23/*MLL*-translocations. However, the figure includes unbalanced 11q23/*MLL*-translocations in which chromosomal material was lost or gained. Chromosomes 1 and 11 were most frequently involved in structural ACAs. Analysis of specific breakpoints showed that 11q23 was the only breakpoint found more than 10 times (data not shown).

Table 2: Number of aberrations by 11q23 translocation partner and clinically relevant parameters.

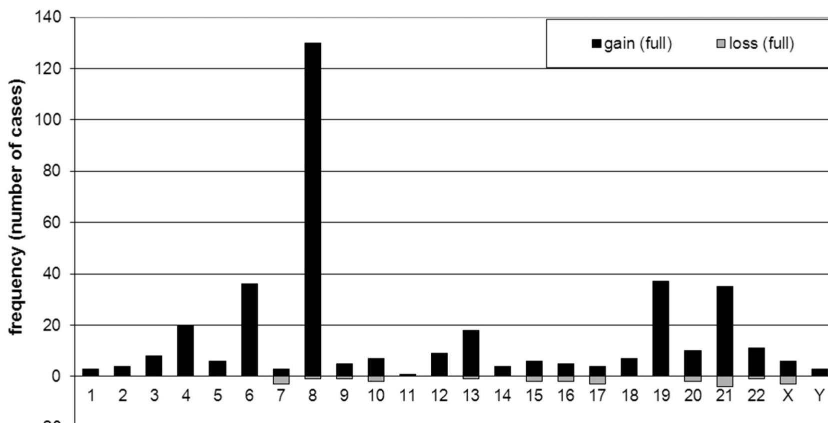
	Number of aberrations							
	0	**1**	**2**	**3**	**4**	**5**	**>5**	**All**
TP group								
9p22		**168 (44)**	**75 (49)**	**33 (41)**	**19 (43)**	**7 (28)**	**14 (33)**	316 (43)
10p12	**1 (14)**	**47 (12)**	**19 (13)**	**12 (15)**	**7 (16)**	**5 (20)**	**5 (12)**	96 (13)
6q27	**1 (14)**	**17 (4)**	**7 (5)**	**1 (1)**			**9 (21)**	35 (5)
19p13		**20 (5)**	**3 (2)**	**4 (5)**	**1 (2)**	**1 (4)**	**1 (2)**	30(4)
19p13.1	**1 (14)**	**20 (5)**	**7 (5)**	**2 (3)**	**1 (2)**	**3 (12)**		34 (5)
19p13.3		**12 (3)**	**7 (5)**		**3 (7)**	**2 (8)**	**1 (2)**	25 (3)
1q21		**18 (5)**	**3 (2)**	**1 (1)**	**1 (2)**		**1 (2)**	24 (3)
4q21		**5 (1)**	**2 (1)**	**4 (5)**	**1 (2)**	**1 (4)**		13 (2)
10p11.2		**5 (1)**	**2 (1)**	**3 (4)**		**2 (8)**		12 (2)
17q21		**9 (2)**	**1 (1)**	**1 (1)**			**1 (2)**	12 (2)
other	**4 (57)**	**61 (16)**	**26 (17)**	**19 (24)**	**11 (25)**	**4 (16)**	**11 (26)**	136 (19)
								733
Sex								
Male		*187 (49)*	*89 (59)*	*35 (44)*	*13 (30)*	*11 (44)*	*23 (53)*	358 (49)
Female	*7 (100)*	*195 (51)*	*63 (41)*	*45 (56)*	*31 (70)*	*14 (56)*	*20 (47)*	375 (51)
								733
Age dx								
<2	**4 (57)**	**197 (52)**	**61 (40)**	**39 (49)**	**12 (27)**	**16 (64)**	**15 (35)**	344 (47)
2-9		**104 (27)**	**49 (32)**	**29 (36)**	**22 (50)**	**5 (20)**	**10 (23)**	219 (30)
≥10	**3 (43)**	**81 (21)**	**42 (28)**	**12 (15)**	**10 (23)**	**4 (16)**	**18 (42)**	170 (23)
								733
WBC								
<20	5 (71)	159 (42)	76 (50)	39 (49)	23 (52)	16 (64)	21 (49)	339 (46)
20-99	1 (14)	115 (30)	38 (25)	20 (25)	14 (32)	5 (20)	10 (23)	203 (28)
≥100	1 (14)	97 (25)	34 (22)	19 (24)	7 (16)	2 (8)	11 (26)	171 (23)
								713 (97)
FAB								
M0		**11 (3)**	**4 (3)**	**2 (3)**	**3 (7)**	**1 (4)**	**2 (5)**	23 (3)
M1		**19 (5)**	**12 (8)**	**4 (5)**	**1 (2)**		**3 (7)**	39 (5)
M2	**1 (14)**	**19 (5)**	**7 (5)**	**2 (3)**	**3 (7)**			32 (4)
M4	**2 (29)**	**83 (22)**	**25 (16)**	**12 (15)**	**5 (11)**	**2 (6)**	**5 (12)**	134 (18)
M5	**4 (57)**	**225 (59)**	**97 (64)**	**54 (68)**	**24 (55)**	**17 (68)**	**25 (58)**	446 (61)
M7		**4 (1)**	**3 (2)**	**2 (3)**	**4 (9)**		**6 (14)**	19 (3)
n.d.		**2 (1)**	**1 (1)**	**1 (1)**	**1 (2)**	**1 (4)**	**1 (2)**	7 (1)
								700 (95)
Overall	7 (1)	382 (52)	152 (21)	80 (11)	44 (6)	25 (3)	43 (6)	733

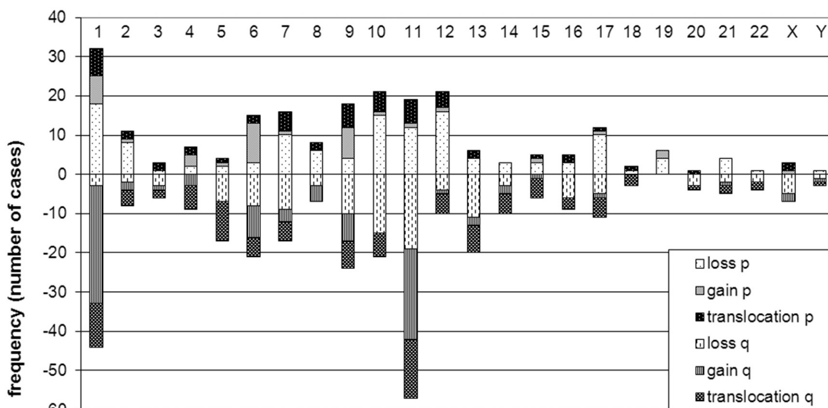
Note: *Values significantly different at the P<0.05 level (χ^2) are given in italics and those significantly different at the P<0.01 level (χ^2) are given in bold.* ***Abbreviations:*** *Number of aberrations indicates total number of aberrations in the karyotype, including 11q23/MLL-rearrangement, percentages per group shown in parentheses (per column); TP group, site of translocation on partner chromosome; Age dx, age in years at diagnosis; WBC, white blood cell count x 10^9/L at diagnosis; FAB, French American British morphology classification subtype; n.d., not determined.*

Univariate analysis of the prognostic impact of ACAs on survival

Table 3 summarizes results of the univariate analysis of survival parameters. The EFS and OS estimates of patients with ACAs were significantly lower than those without ACAs (EFS 38% *vs.* 48%, $P = 0.002$; OS 47% *vs.* 62%, $P < 0.001$) (Figure 3). CIR estimates of patients with ACAs were significantly higher than for those without ACAs (52% *vs.* 38%, $P < 0.001$; Figure 3).

Figure 2: Frequency (number of cases) of numerical ACAs (A) and structural ACAs (B).





Note: *In figure 2A gains are shown on the positive Y-axis, losses are shown on the negative Y-axis. Chromosomes are on the X-axis; figure 2B, the short arms (p) of the chromosomes are shown on the positive Y-axis, the long arms (q) on the negative Y-axis. Lightest shades are used for losses, medium shaded colors are used for gains, darkest shaded colors for translocation breakpoints. Chromosomes are on the X-axis. Balanced 11q23 translocations are not included in the figure.*

Patients with complex karyotypes had significantly worse outcomes than those without complex karyotypes (EFS 37% *vs.* 46%, $P = 0.02$; OS 45% *vs.* 59%, $P = 0.003$; CIR 53% *vs.* 42%, $P < 0.001$; Figure 3).

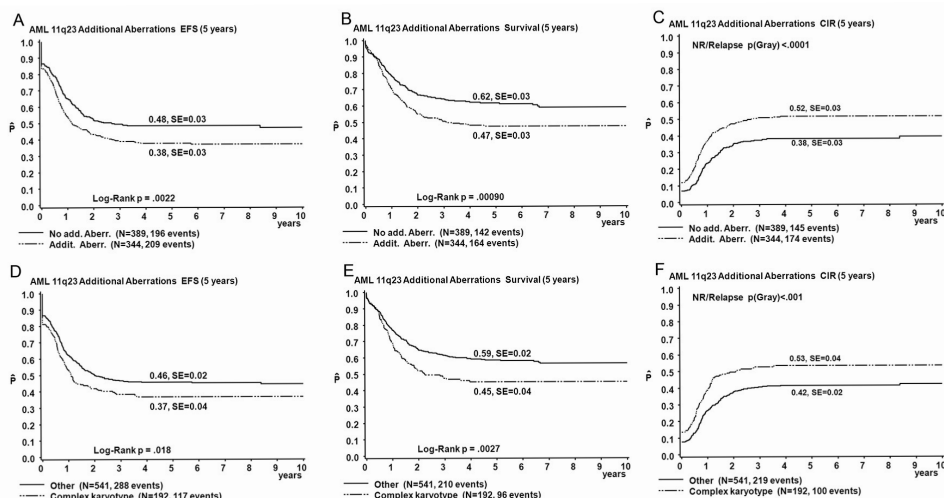
The presence of trisomy 8 (n = 130) was a favorable prognostic factor (EFS 53% *vs.* 29% for patients without trisomy 8, $P < 0.001$; OS 61% *vs.* 39% for patients without trisomy 8, $P = 0.003$; CIR 35% *vs.* 62% for patients without trisomy 8, $P < 0.001$) (Figure 4). Survival differences are mainly explained by reduced relapse rate in trisomy 8 patients (relapse rate 26% *vs.* 49% for patients without trisomy 8, $P<0.001$) (Figure 4).

Table 3: Univariate survival analysis of the complete cohort (n=733)

	Complete cohort						
	N	**EFS**	***P*(log-rank)**	**OS**	***P*(log-rank)**	**CIR**	***P*(Gray)**
Additional aberrations			0.002†		<.001†		<.001†
Absent	389	0.48		0.62		0.38	
Present	344	0.38		0.47		0.52	
No. of aberrations			<.001†		<.001†		0.001†
2	152	0.39		0.50		0.50	
3	80	0.45		0.53		0.48	
≥3	192	0.37	0.018*	0.45	0.003†	0.53	<.001†
4	44	0.40		0.50		0.53	
5	25	0.36		0.43		0.60	
>5	43	0.18	<.001†	0.25		0.61	
Type			0.001†		0.003†		<.001†
Numerical	140	0.47		0.56		0.41	
Structural	130	0.32		0.43		0.59	
Both	74	0.31		0.40		0.59	
Trisomy							
4	20	0.43	0.72	0.52	0.87	0.52	0.93
6	36	0.35	0.43	0.35	0.029*	0.54	0.65
8	130	0.53	<.001†	0.61	0.003†	0.35	<.001†
13	18	0.49	0.52	0.64	0.41	0.40	0.37
19	37	0.17	0.003†	0.24	<.001†	0.54	0.88
21	35	0.19	0.007†	0.28	0.015*	0.69	0.014*

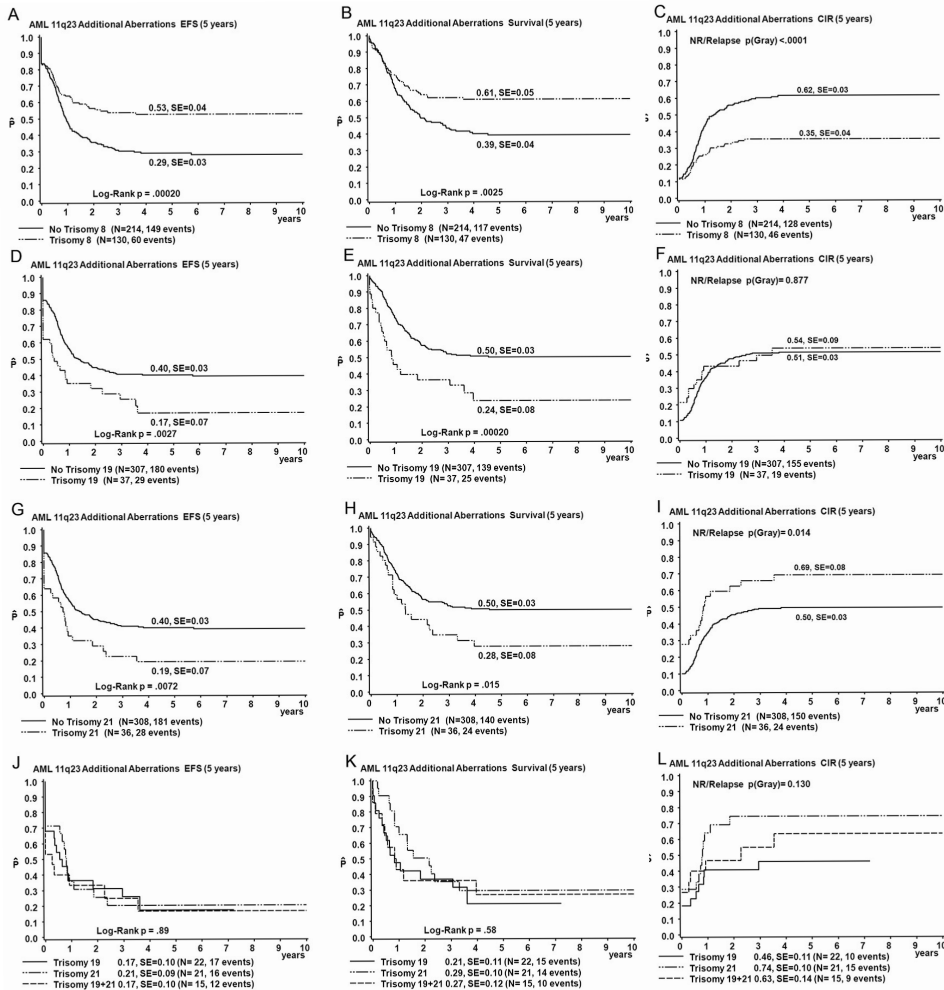
Abbreviations: *N indicates number of patients; EFS, 5-year event-free survival estimates; P(log-rank), P-value from log-rank test; OS, 5-year overall survival estimate; CIR, 5-year cumulative incidence of relapse; P(Gray), P-value from the Gray's test. * values significant at P<0.05 level. † values significant at P<0.01 level.*

Figure 3: Survival curves obtained from univariate analysis comparing patients with ACAs to patients without ACAs (A, B, C) and comparing patients with complex karyotype to all patients with less than 3 aberrations (D, E, F).



Event-free survival (EFS) (A, D), overall survival (Survival) (B, E), and cumulative incidence of relapse (CIR) (C, F).

Figure 4: Comparison of survival curves obtained from univariate analysis for patients with trisomy 8 (A, B, C), trisomy 19 (D, E, F) and those with trisomy 21 (G, H, I) and defined by strata of occurrence of trisomy 19 and trisomy 21 (J, K, L).



For curves A-I patients with a specific trisomy are compared to patients with other ACAs. Event-free survival (EFS) (A, D, G, J), overall survival (Survival) (B, E, H, K), and cumulative incidence of relapse (CIR) (C, F, I, L).

The presence of trisomy 19 (n = 37) and trisomy 21 (n = 36) was an unfavorable prognostic factor (EFS 17% *vs.* 40% for patients without trisomy 19, $P = 0.003$; OS 24% *vs.* 50% for patients without trisomy 19, $P < 0.001$; CIR 54% *vs.* 51% for patients without trisomy 19, $P = 0.88$; and EFS 19% *vs.* 40% for patients without trisomy 21, $P = 0.007$; OS 28% *vs.* 50% for patients without trisomy 21, $P = 0.02$; CIR 69% *vs.* 50% for patients without trisomy 21, $P = 0.01$) (Figure 4). Both trisomies 19 and 21 were present in 15 patients. Survival curves for patients with either trisomy 19 or 21 were not different from those for patients

with both trisomies 19 and 21 (Figure 4). Combined trisomy 19 and trisomy 8 was present in 23 patients. These patients showed a survival curve intermediate to that of trisomy 8 and trisomy 19 cases (EFS 30%, data not shown). The survival disadvantage of patients with trisomy 19 seems to be determined by refractory disease (probability of CR 74% for patients with trisomy 19 *vs.* 89% for patients with other ACAs, as calculated over the fraction of patients that survive beyond the first 6 weeks after diagnosis, $P = 0.04$) rather than relapse. In addition, patients with trisomy 19 had a significantly higher incidence of early death (16% *vs.* 3.3% in other ACA cases, $P = 0.004$), which could not be explained by adverse clinical prognostic factors such as high WBC or age.

Structural aberrations were diverse and randomly distributed among translocation partner groups, and survival analysis of patients with specific breakpoints was not feasible because none of the breakpoints was involved more than 10 times.

Multivariate analyses of the prognostic impact of ACAs on survival

Table 4 summarizes results of the multivariate survival analysis. Cox proportional hazards model for EFS, OS, and relapse incidence of the full cohort ($n = 733$) showed that trisomy 8 and trisomy 19 were independent prognostic factors at $P < 0.05$ for EFS (HR 0.57, $P = 0.02$ and HR 1.77, $P = 0.01$) and OS (HR 0.54, $P = 0.03$ and HR 2.11, $P = 0.002$) (Table 4). Structural aberrations as a general finding predicted EFS (HR 1.39, $P = 0.01$) (Table 4).

Table 4: Multivariate survival analysis of the complete cohort by using the Cox proportional hazards model

	Cox proportional hazards model								
	EFS			OS			Relapse incidence		
	HR	**CI**	***P***	**HR**	**CI**	***P***	**HR**	**CI**	***P***
TP									
9p22	1	reference		1	reference		1	reference	
other	1.15	(0.87-1.51)	0.328	1.13	(0.82-1.57)	0.461	1.17	(0.92-1.47)	0.195
10p12	1.36	(1.01-1.83)	0.042*	1.62	(1.16-2.27)	0.005†	1.76	(1.36-2.29)	0.000†
6q27	2.29	(1.54-3.39)	0.000†	2.72	(1.77-4.19)	0.000†	2.79	(1.80-4.33)	0.000†
19p13	1.06	(0.62-1.80)	0.832	1.44	(0.82-2.51)	0.204	0.88	(0.57-1.37)	0.579
19p13.1	1.11	(0.69-1.79)	0.667	0.97	(0.53-1.77)	0.931	1.04	(0.71-1.53)	0.841
19p13.3	1.06	(0.60-1.88)	0.832	1.64	(0.90-3.00)	0.105	1.18	(0.71-1.94)	0.522
1q21	0.12	(0.03-0.49)	0.003†	0.00			0.68	(0.44-1.05)	0.080
4q21	1.46	(0.74-2.88)	0.276	2.04	(1.02-4.09)	0.043*	1.84	(0.99-3.43)	0.054
10p11.2	2.12	(1.10-4.06)	0.024*	2.56	(1.24-5.32)	0.011*	1.37	(0.67-2.78)	0.384
17q21	1.14	(0.53-2.43)	0.743	1.15	(0.47-2.82)	0.763	1.28	(0.68-2.42)	0.446
Trisomy									
no trisomy	1	reference		1	reference		1	reference	
8	0.57	(0.36-0.92)	0.022*	0.54	(0.32-0.94)	0.028*	0.79	(0.56-1.12)	0.188
19	1.77	(1.13-2.78)	0.012*	2.11	(1.31-3.42)	0.002†	1.15	(0.68-1.94)	0.596
21	1.35	(0.85-2.13)	0.198	1.25	(0.76-2.03)	0.377	0.98	(0.60-1.60)	0.926
Type									
no ACAs	1	reference		1	reference		1	reference	
numerical	1.16	(0.83-1.63)	0.376	1.17	(0.84-1.62)	0.353	1.09	(0.81-1.47)	0.588
structural	1.39	(1.07-1.80)	0.013*	1.27	(0.98-1.63)	0.068	1.13	(0.90-1.43)	0.288

***Note:** Results of 3 independent analyses. **Abbreviations:** EFS indicates event-free survival; OS, overall survival HR, hazard ratio; CI, 95% confidence interval. * Values significant at P<0.05 level. † Values significant at P<0.01 level.*

The translocation partners identified by Balgobind *et al.*[25] (10p12, 6q27, 1q21 and 10p11.2) remained significant independent prognostic factors in these models. Trisomy 8, 19, and 21 were not significant factors in the model for the prediction of relapse incidence. Complexity of the karyotype, tested by different cut-off values (2 or more aberrations, 3 or more aberrations, and more than 5 aberrations), was not a significant factor for outcome in all models and was therefore excluded from the final model. A separate analysis of t(9;11)(p22;q23) cases showed that they did not differ considerably from the complete cohort (Supplementary Figure S2, Supplementary Table S2).

Discussion

The heterogeneity of pediatric AML is mainly determined by specific karyotypes and molecular aberrations which have become important prognosticators.[5,17,19,20,23,27,39,54,56,57,73-76] In addition, within distinct groups such as 11q23/*MLL*-rearranged AML, we have reported that additional cytogenetic aberrations are of prognostic relevance.[25] In the present exploratory study, we identified trisomy 8, trisomy 19, and trisomy 21 to be recurrent ACAs of prognostic significance in pediatric 11q23/*MLL*-rearranged AML. Multivariate analysis showed that only trisomy 8 and trisomy 19 as additional aberrations were of independent prognostic value. Notably, the adverse outcome for 11q23/MLL-rearranged AML patients harboring trisomy 19 was due to refractory disease and early death rather than an increased rate of relapse. Complex karyotype was a frequent finding (26%) and a negative prognostic factor in univariate analysis only.

Trisomy 19 in AML is an aberration that is rarely found as the sole aberration.[77] In infants with AML it is associated with t(7;12)(q36;p13) and t(7;12)(q32;p13).[78] In most of such cases it can seem to be the sole aberration due to the cryptic t(7;12).[78] Trisomy 19 has been described as an additional aberration with adverse prognostic significance in adult 11q23/*MLL*-rearranged AML.[23] It has been postulated that a gene dosage effect of the DNA methyltransferase 1 (DNMT1) located on 19p13.2 contributes to the hypermethylation seen in patients with MDS and thereby to prognosis.[79] Future studies may reveal whether this mechanism also contributes to aberrant methylation found in pediatric 11q23/*MLL*-rearranged AML.[80]

In our study, trisomy 8 was found to be an independent favorable prognostic factor. Kok *et al.* identified a gene expression signature with high *HOX*A gene expression in adult AML patients with AML with trisomy 8 as the sole abnormality, which clustered together with patents with *MLL*-rearranged AML.[81] This may suggest similarities in the biology of these diseases. In contrast, in pediatric MDS, trisomy 8 is recognized as a positive prognostic factor, possibly because of differences in apoptosis regulation between cells with trisomy 8 and cells with other abnormalities.[82,83] To date, it is not clear how trisomy 8 influences the biology of *MLL*-rearranged AML.

Interestingly, in our study, although 26% of all cases of 11q23/*MLL*-rearranged had complex karyotypes, this ACA was not an independent prognostic factor. Although the usage of definitions on complex karyotypes is not uniform, the occurrence of complex karyotypes in pediatric AML cohorts has been reported to range from 7% to 15%. [5,55,60,84] A Cancer and Leukemia Group B study on adult *de novo* AML showed that patients with increased num-

ber of aberrations had significantly worse outcome than those with normal karyotypes.[85] Recently, Göhring *et al.* used a new definition of "structural complex karyotype", defined as a karyotype with ≥3 chromosomal aberrations including at least one structural aberration.[86] This specific karyotype independently predicted very poor survival in a cohort of 192 children with advanced MDS.[86] Although all the cases of complex karyotype in our study fit their definition, we did not find the presence of such karyotype to be associated with the poor prognosis that was reported in pediatric advanced MDS.[86] Only some studies have specifically shown a correlation between complexity of the karyotype and outcome in pediatric AML.[5,27,55,60,87] EFS rates for patients with complex karyotype have ranged from 29% to 42% in these studies, which is comparable to the EFS obtained our study. Alternatively, a strong negative association between monosomal karyotype, defined as a karyotype with at least 2 monosomies or 1 monosomy combined with at least 1 structural aberration, and outcome was described in adult AML.[88] This monosomal karyotype was only present in 1.5% (n = 11) of our cases and therefore it was not possible to evaluate the predictive value in our pediatric 11q23/*MLL*-rearranged AML cohort.

Although this study is adding additional prognostic factors, the multivariate models still point out that previously determined risk factors (among which the translocation partners) retain their independent prognostic significance irrespective of ACAs status.

A limitation of our study is the variety of treatment regimens, although all protocols had a backbone including intensive chemotherapy with cytarabine/ anthracycline. Unfortunately numbers were too small to do specific analyses for different protocols, or to draw any meaningful conclusion regarding provided treatment and outcome.

In separate analysis of t(9;11)(p22;q23) cases, we confirmed most of the findings from the complete cohort, regarding frequent recurrent aberrations and predictive factors. In addition, FAB M5 morphology was still recognized as independent favorable prognostic factor in this group of patients.

In conclusion, in this exploratory study we have identified specific ACAs as novel independent prognostic variables in pediatric 11q23/*MLL*-rearranged AML, which can be identified by conventional karyotyping. Future studies should be aimed to test the associations found in this study in different patient cohorts. Our findings may also guide further studies that unravel the biological differences that determine outcome differences in 11q23/MLL-rearranged AML as well as future treatment stratification.

Acknowledgements

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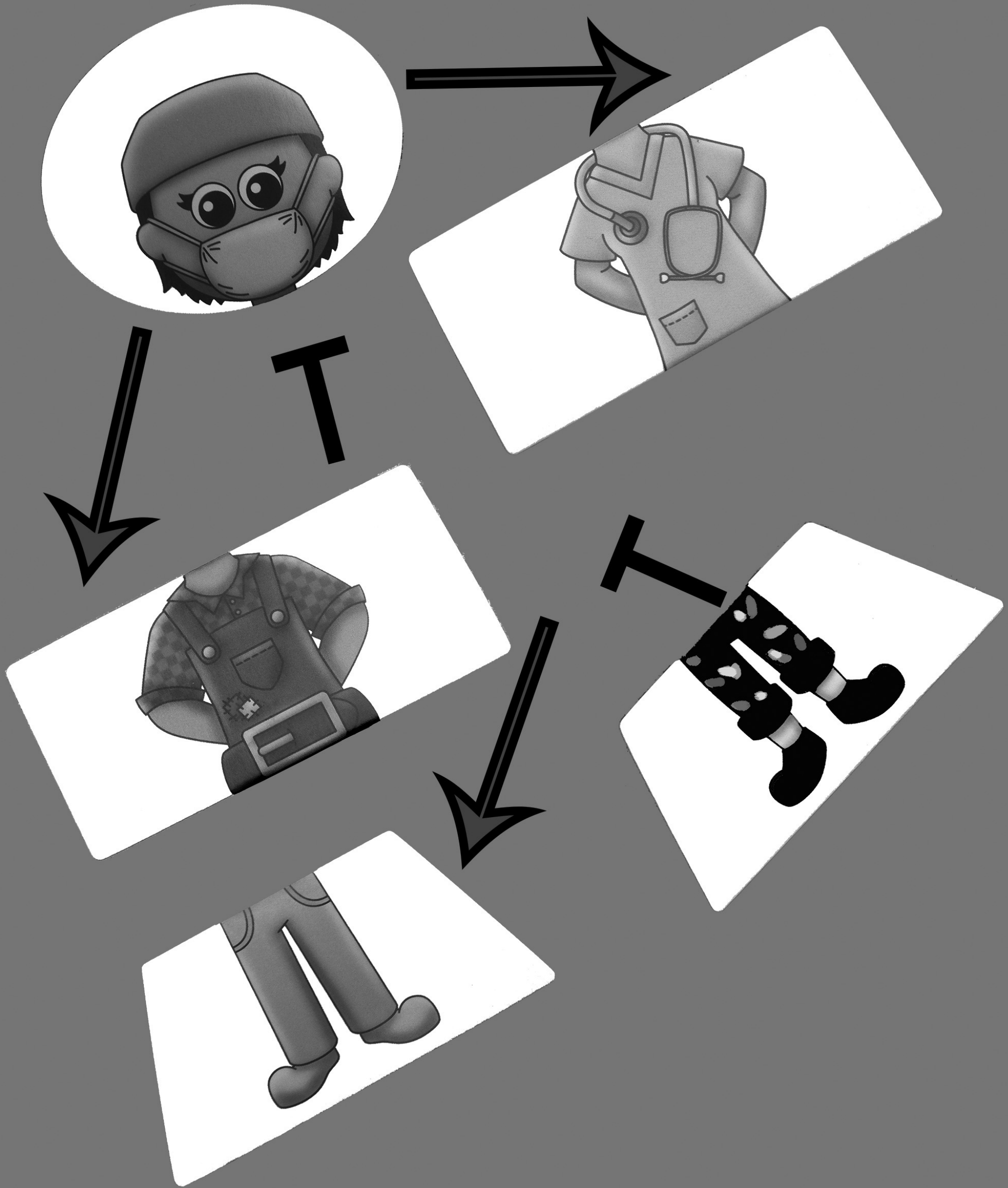

Chapter 5

CBL mutations do not frequently occur in paediatric acute myeloid leukaemia

Eva A. Coenen[1*], Emma M.C. Driessen[1*], C. Michel Zwaan[1], Jan Stary[2], Andre Baruchel[3], Valerie de Haas[4],Eveline S.J.M. de Bont[5], Dirk Reinhardt[6], Gertjan J.L. Kaspers[7], Susan T.C.J.M. Arentsen-Peters[1], Claus Meyer[8], Rolf Marschalek[8], Rob Pieters[1,4], Ronald W. Stam[1] and Marry M. van den Heuvel-Eibrink[1]

[*)] these authors contributed equally to this manuscript

[1]Pediatric Oncology/Haematology, Erasmus MC/Sophia Children's Hospital, Rotterdam, The Netherlands; [2]Pediatric Haematology/Oncology, 2nd Medical School, Charles University, Prague, Czech Republic; [3]Hematology, St. Louis Hospital, Paris, France; [4]Dutch Childhood Oncology Group (DCOG), The Hague, The Netherlands; [5]Pediatric Oncology/Haematology, Beatrix Children's Hospital, University Medical Centre Groningen, Groningen, The Netherlands; [6]AML-BFM Study Group, Hannover, Germany; [7]Pediatric Oncology/Haematology, VU University Medical Centre, Amsterdam, The Netherlands; [8]Institute of Pharmaceutical Biology, ZAFES, Diagnostic Centre of Acute Leukaemia's (DCAL), Frankfurt, Germany

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Summary

RAS-pathway mutations, causing a proliferative advantage, occur in acute myeloid leukaemia (AML) and *MLL*-rearranged leukaemia. Recently, mutations in the *Casitas B lineage lymphoma* (*CBL*) gene were reported to be involved in RAS-pathway activation in various myeloid malignancies, but their role in paediatric AML is still unknown.

We performed mutation analysis of 283 initial and 33 relapse paediatric AML and revealed only two mutant cases (0.7%) in initial paediatric AML samples, of which one *MLL*-rearranged case. Both mutant cases showed *CBL* mRNA expression in the range of the non-mutated cases. Phosphorylated extracellular signal-regulated kinase (pERK) was not correlated with CBL protein expression (n = 11).

In conclusion, we report a very low *CBL* mutation frequency in paediatric AML, which together with the lack of difference in protein and mRNA expression illustrates the limited role of CBL in paediatric AML.

Introduction

Enhanced proliferation and disrupted differentiation are known to constitute collaborative key events leading to the onset of leukaemogenesis. In acute myeloid leukaemia(AML), mutations in several members of the RAS-regulated signalling pathway result in proliferative advantage [14,89]. Recently, *Casitas B lineage lymphoma*(*CBL*), a protein involved upstream in this pathway, was found to be mutated at high frequency (15%) in juvenile myelomonocytic leukaemia(JMML) mutually exclusive from other RAS-pathway mutations [90]. The proto-oncogene *CBL* encodes an E3 ubiquitin ligase that negatively regulates receptor tyrosine kinases (like FLT3 and EGFR) and associated proteins including Grb2 and SOS, which are involved in RAS deactivation [91]. Mutations in the linker region and RING finger domain of *CBL* reduces its ubiquitin ligase potential, impairing the ability of Grb2 and SOS to suppress RAS signalling, and thereby over-activating downstream RAS targets [21].

CBL mutations have also been observed in 1-2% of adult AML, 8% of atypical chronic myeloid leukaemia, and 13% of chronic myelomonocytic leukaemia [92-94]. In paediatric AML, RAS-pathway-associated mutations are found in about 20% of cases, which mostly occur in specific subgroups, such as cytogenetically normal cases with *NPM1* mutations or *MLL*-partial tandem duplications, t(8;21), inv(16), and *MLL*-rearranged patients under the age of 2 years [16,35,95]. Furthermore, type I mutations in *MLL*-rearranged AML cases were almost always related with the RAS-pathway [16]. The collaboration of RAS-pathway mutations and *MLL*-rearrangements at young age is also reported in paediatric *MLL*-rearranged acute lymphoblastic leukaemia(ALL) cases (50%) [96]. The role of RAS-pathway mutations in leukaemogenesis of *MLL*-rearranged ALL is further stressed by studies in MLL/ AF4+ Kras mutation+ transgenic mice, which develop B-cell lymphoma and/ or leukaemia with a relatively short latency (6 months), in contrast to transgenic mice models without *RAS* mutations [97-99]. In one recent study the co-existence of *CBL* mutations and *MLL*-rearrangements was reported in ~5% of infant ALL patients. [100]

So far the frequency and clinical value of *CBL* mutations in paediatric AML is unknown. Therefore, we studied the frequency of *CBL* mutations and expression in a large cohort of paediatric AML.

Methods

Patient Samples

Viably frozen diagnostic bone marrow or peripheral blood from 319 AML samples, including 277 *de novo,* 9 secondary and 33 relapsed paediatric AML samples, were provided by the Dutch Childhood Oncology Group(DCOG), the AML 'Berlin-Frankfurt-Münster' Study Group (AML-BFM-SG), the Czech Paediatric Haematology Group (CPH), and the St. Louis Hospital in Paris, France. Patient characteristics of the 277 *de novo* cases are shown in Table I. In order to elucidate the role of CBL related to MLL in general, we used a control group of an *MLL*-rearranged enriched cohort (18 infant ALL patients, 100% t(4;11)(q21;q23), 7 males).

Informed consent was obtained from all patients after Institutional Review Board approval according to national law and regulations.

Table I. Patient characteristics de novo AML cohort consisting of 277 patients.

	No. of samples (%)
Sex	
Male	159 (57)
Female	115 (42)
Unknown	3 (1)
Age (median, range, years)	9,4 (0-18,5)
WBC x 10^9/l at Dx (median, range, n=247)	43,6 (1,2-483)
FAB	
M0	14 (5)
M1	34 (12)
M2	52 (19)
M3	21 (8)
M4	77 (28)
M5	61 (22)
M6	4 (1)
M7	7 (3)
uknown	7 (3)
Cytogenetics	
***MLL*-rearrangements**	64 (23)
t(8;21)	25 (9)
inv(16)	35 (13)
t(7;12)	5 (2)
t(15;17)	18 (6)
Cytogenetically Normal (CN)-AML	53 (19)
AML-other	61 (22)
Unknown	16 (6)
Molecular abnormalities (n samples)	
***KIT* (261)**	21 (8)
***KRAS* or *NRAS* (261)**	54 (21)
***FLT3*-ITD (269)**	54 (20)
***FLT3*-D835/6 (43)**	2 (5)
***PTPN11* (261)**	3 (1)
***CEBPA* (238)**	17 (7)
***NPM1* (252)**	18 (7)
***MLL*-PTD (232)**	5 (2)
***WT1* (259)**	22 (12)
***TET2* (38)**	1 (3)
***DNMT3A* (142)**	3 (2)
***IDH1/2* (199)**	12 (6)
***NUP98/ NSD1* (261)**	12 (5)
***CBL* (277)**	2 (1)

Patient characteristics of the de novo acute myeloid leukaemia cohort. Numbers indicate frequency (%) unless specified otherwise. AML: acute myeloid leukaemia, WBC: white blood cell count, Dx: diagnosis, FAB: French American British morphology classification.

Materials

Leukemic cells were isolated and enriched as previously described[101-103]. All resulting samples contained 80% or more leukemic cells, as determined morphologically by May-Grünwald-Giemsa (Merck, Darmstadt, Germany)–stained cytospins. A minimum of 5 x 10^6 leukemic cells was lysed in Trizol reagent (Gibco BRL/Life Technologies, Breda, The Netherlands) and stored at -80°C. Isolation of genomic DNA and total cellular RNA was performed as described before.[104]

AML samples were routinely investigated for *MLL*-rearrangements as previously described [57]. The other common cytogenetic abnormalities in AML (such as t(8;21), inv(16), t(15;17)) were confirmed by conventional karyotyping, FISH or RT-PCR. For all infant ALL samples karyotyping and analysis of possible *MLL*-rearrangements was performed with split-signal FISH, PCR or both (Primers are described in Table S1).

CBL mutation analysis

PCR analysis for *CBL* mutations was performed on genomic DNA. A 2720 Thermal cycler (Applied Biosystems, Foster City, CA, USA) was used. Primers and conditions used for *CBL* screening are identical to those used by Loh et al [90]. Briefly, these primers target exon 8 and exon 9 including the intron-exon boundaries of the *CBL* gene. For reference primer sequences are listed in Table S1. Sequencing was done using BigDye terminator v 1.1 Cycle Sequencing kit (PE Applied Biosystems, Foster City, CA, USA) on an Applied Biosystems 3130x/ Genetic Analyzer. Sequence analysis was done with CLC Workbench software (CLCbio, Aarhus, Denmark) with reference sequence ENST00000264033 (www.ensembl.org, release 59).

Of the mutated cases, germline material was obtained to investigate the *CBL* mutation status in healthy cells. We obtained DNA isolated from a cytospin at remission state from one case. From the other case lymphocytes were isolated by autoMACS sorting (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's guidelines.

Gene expression profiling and RT-qPCR

Gene expression profiling(GEP) (Affymetrix HU133plus2.0, Affymetrix, Santa Clara, CA) was performed on 272/ 277 of *de novo* paediatric AML patients as a subset of the recently published study [74]. GEP data were deposited in the GEO database under accession number GSE17855. To validate mRNA expression levels, Quantitative real-time PCR(RT-qPCR) was performed on cDNA of 12 AML cell lines and 11 paediatric AML patient samples, selected on availability of cDNA, produced as previously described [105]. An ABI PRISM 7900HT sequence detector (Applied Biosystems, Foster City, CA, USA) was used to validate the GEP results. Primers used for *CBL* are described in Table S1. The average cycle threshold(Ct) value was used to calculate mRNA expression levels of *CBL* relative to the expression level of *GAPDH* using the comparative Cycle threshold(ΔCt) method [106]. For analysis of the *CBL* mutant transcripts we designed primers listed in Table S1 targeting exon 6 to 11 of *CBL*.

Western Blot

For protein expression analysis 11 AML samples were selected based on *CBL* mutational status. Analysis was performed as previously described [57]. Cell lysates containing 20 ug of protein were separated on 10% polyacrylamide gels and transferred onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Western Blots were probed with rabbit polyclonal IgG CBL (#2179, Cell Signaling, Danvers, MA, USA), rabbit polyclonal IgG phospho ERK1/2 (#4377, Cell Signaling) and mouse anti-beta-actin (ab6276, Abcam, Cambridge, MA, USA) antisera. Subsequently, the blots were probed with IRDye 800CW Goat-anti-Rabbit antibody (#926-32211, LI-COR, Lincoln, NE, USA) and IRDye 680 Goat-anti-Mouse antibody (#926-32220, LI-COR). Fluorescence was detected by the LI-COR Odyssey system (LI-COR).

Functional analysis of CBL in AML

To study the influence of *CBL* expression on RAS pathway activation in paediatric AML cells we used the Kasumi-1 cell line (DSMZ GmbH), originally derived from a paediatric AML patient, as a model. Cells were cultured in RPMI-1640 medium supplemented with 20% fetal calf serum (FCS, Integro, Zaandam, the Netherlands) and penicillin 100 U/mL, streptomycin 100 μg/mL, and fungizone 0.125 μg/mL (PSF; Invitrogen, Breda, the Netherlands) and grown as suspension cultures at 37°C in humidified air containing 5% CO2.

Cells (10 x 10^6) were transfected by electroporation in 400 μL of RPMI 1640 with L-alanyl-L-glutamine (Invitrogen) containing 250nM of either a mix of equal amounts of *CBL* siRNAs (Dharmacon ON-TARGETplus L-003003; Thermo Fisher Scientific, Etten-Leur, the Netherlands) or Non-targeting siRNA (Dharmacon ON-TARGETplus D-001810-01-05; Thermo Fisher Scientific), in 4-mm electroporation cuvettes (Bio-Rad, Veenendaal, the Netherlands; target sequences are described in Table S1). Electroporation was performed by the use of a BioRad Genepulser MXcell (Biorad) by applying a rectangle pulse of 400 V for 10 milliseconds. To compensate for the amount of cell death induced merely as a consequence of the electroporation procedure, control cells were electroporated in the absence of siRNA. After incubating for 15 minutes at room temperature, the cells were diluted in 15 mL RPMI 1640 supplemented with 10% FCS and PSF and incubated at 37°C and 5% CO2. They were maintained in culture for 168 hours. Cell counts were determined daily (t=6 hours, t=24 hours, t=48 hours, t=72 hours, t=96 hours, and t=168 hours). Cell samples of both experimental and control conditions were collected from the medium at every time point. They were washed with phosphate-buffered saline and stored as dry pellet or lysed in Trizol reagent and stored at -80°C. RT-qPCR and Western Blot were used as described above to validate CBL knockdown and consecutive phosphorylated extracellular signal-regulated kinase(pERK) up-regulation as a marker for RAS-pathway activity. Results from three repetitive experiments were analyzed together.

Statistical Analysis

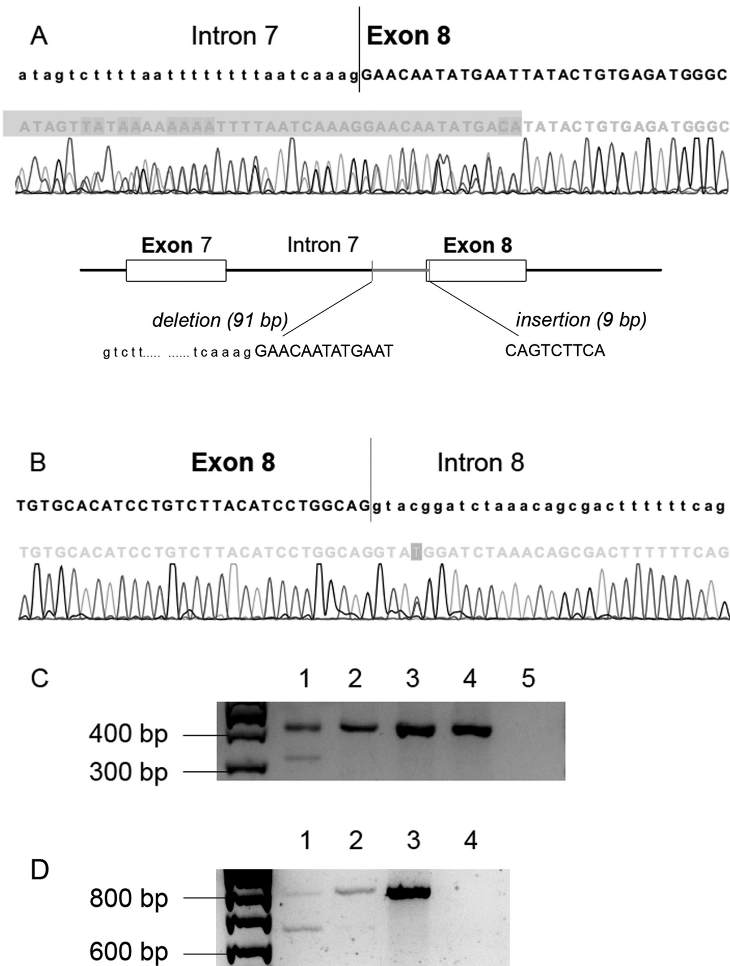
Statistical analysis of GEP data was performed as previously reported [74,107]. For comparison of *CBL* expression in different cytogenetic AML groups the Kruskal-Wallis test was used. For paired analyses of non-parametric variables, the Wilcoxon Signed Ranks Test was used. All analyses were performed with SPSS Statistics version 17.0 (SPSS Inc. Chicago, IL, USA). All tests were two-tailed and a p-value <0.05 was considered significant.

Results

Mutation screening

CBL mutation screening was performed in 277 newly diagnosed *de novo* AML samples, 9 newly diagnosed secondary AML samples and 33 AML samples at relapse, including 22 paired diagnosis-relapse samples, and in the enriched MLL/ AF4+ ALL cohort (n = 18).

Figure 1. Mutation analysis CBL exon 8 of mutant cases.



Case 1 has a large deletion at the intron 7-exon 8 boundary, shown in pink (Figure 1A). A schematic overview is shown as well (Figure 1A). Case 2 has a point mutation at the exon 8-intron 8 splice site, exon 8 +4C>T (Figure 1B). Figure 1C shows a PCR electrophoresis on genomic DNA. Lane 1 corresponds to case1, lane 2 corresponds to case 2, lane 3 and 4 correspond to wild-type controls, and lane 5 corresponds to a negative control. Figure 1D shows a PCR electrophoresis on cDNA. Lane 1 corresponds to case 1, lane 2 corresponds to case 2, lane 3 corresponds to a wild-type control, and lane 4 corresponds to a negative control. (A color version of this figure can be found in the appendices).

In 2/277 of the *de novo* AML patients a heterozygous mutation was found (0.7%, confidence interval 0.2-2.6%); case 1 had a 91 base pair deletion combined with a 9 base pair insertion of the intron 7-exon 8 boundary; case 2 had a single nucleotide exchange of the exon 8-intron 8 splice site (Fig 1). Patient characteristics of both mutants are listed in Table II. Interestingly, the 2 patients with *CBL* mutations did not carry any other AML related molecular aberrations (*NPM1, WT1, NRAS, KRAS, CEBPA, PTPN11, KIT, FLT3, IDH1/2, DNMT3A, NUP98/NSD1*). All other AML cases, including all relapses and secondary AML cases, were homozygous for the wild type allele. No *CBL* mutations were found in the *MLL*-rearranged infant ALL samples.

Germline material of both mutated cases was analysed. DNA derived from remission material of case 1 was wild type for *CBL* (data not shown), while the sorted lymphocytes of case 2 showed the same heterozygous point mutation in the exon 8-intron 8 splice site as the matched diagnosis sample (Figure S1).

Table II. Patient characteristics CBL mutant cases.

	case 1	**case 2**
Sex	female	male
Age at diagnosis (years)	0.8	15.0
WBC at diagnosis (x 10^9/L)	475	1.4
FAB	M4	M6
Cytogenetic subgroup	*MLL* (*FNBP1,* 9q34.11)	CN
Molecular abnormalities	None	None
Follow-up data	CCR, 27 months	LFU, day 0

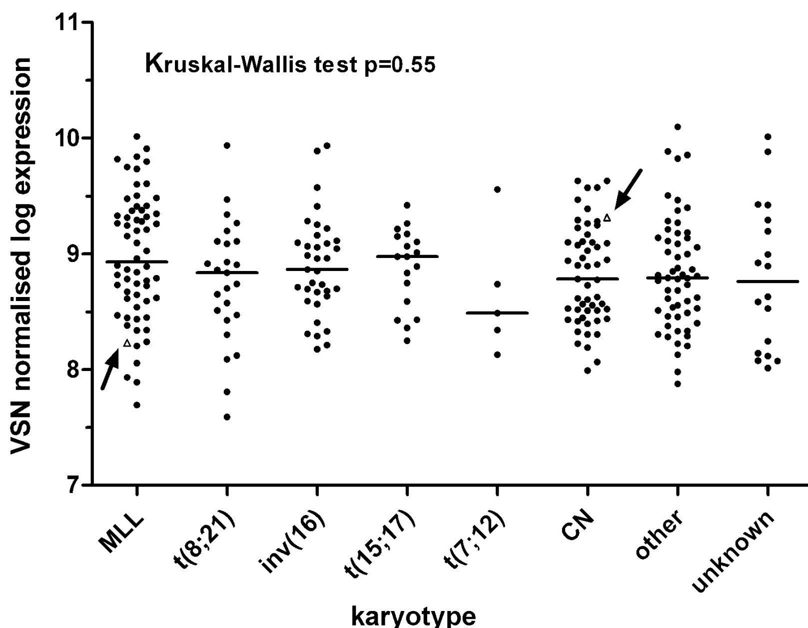
Patient characteristics of both mutant cases. WBC: white blood cell count, FAB: French American British morphology classification, CN: cytogenetically normal, CCR: continuous complete remission, LFU: loss to follow-up.

CBL mRNA expression

Figure 2 shows *CBL* mRNA expression of paediatric AML patients as measured by gene expression array (probe 225231_at). RT-qPCR correlated moderately with the results obtained from GEP (Spearman r=0.41) (Figure S2). *CBL* mRNA expression was not different between the cytogenetic groups in paediatric AML (Kruskal-Wallis test, p=0.55) (Fig 2). The two mutated cases expressed *CBL* mRNA levels within the range of all other cases.

Since alternative splicing can occur for splice site mutations of *CBL* we analyzed the transcripts of case 1 and case 2. Case 1 expressed alternative splicing with a full length transcript and one lacking exon 8 (Fig 1D). Case 2 only expressed the full length transcript (Fig 1D).

Figure 2. CBL mRNA expression of 272 initial paediatric AML patients.



Graph showing the expression of probe set 225231_at, representing the CBL gene, after log transformation. Bars represent the median expression in each group. The arrows point to the mutant cases, given as a triangle rather than a dot. VSN: Variance Stabilization and Normalization, CN: cytogenetically normal.

Western Blotting

Western blotting showed no significant differences in protein expression of the two *CBL* mutants versus nine *CBL* wild type AML cases (p=0.8) (Fig 3). We did not find evidence of truncated protein in both mutant cases (Fig 3). We could not detect pERK over expression in patients with low CBL protein expression (Fig 3). Correlation of CBL protein expression with *CBL* mRNA expression on gene expression array was very poor (Spearman r=0.0) (Fig S2).

CBL knock-down by siRNA transfection

To study whether *CBL* mRNA down-regulation affects RAS-pathway activation in paediatric AML, *CBL* mRNA knock-down experiments were performed in a paediatric AML cell line. A reduction of *CBL* mRNA and CBL protein expression of 50-60% did not significantly affect cell counts and proliferation compared to the non-targeting siRNA control condition (Fig 4), whereas CBL protein knock-down did result in pERK up-regulation compared to the non-targeting siRNA control (p=0.03), with a maximum of 3-fold at t=48 hours (Fig 4).

Figure 3. Western Blot analysis of CBL from 11 AML patients.

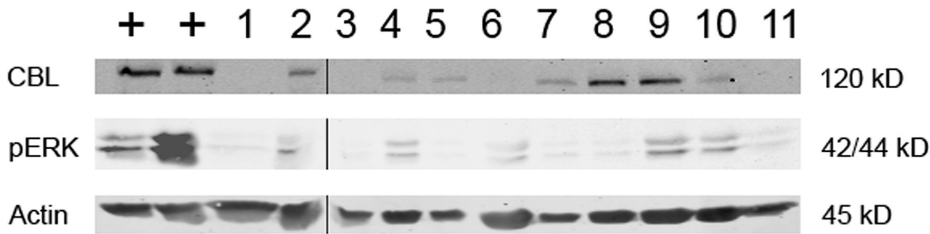


Figure displaying three western blot sections. The 120 kD product represents CBL protein, the bands on 42/44 kD represent pERK. Actin was used as loading control (45 kD). Both lanes marked as + represent positive controls (cell lines HL60 and ME1 respectively), lanes 1 to 9 correspond to protein lysates of patients from diverse cytogenetic subgroups of paediatric AML, lane 10 corresponds to mutant case 1, lane 11 corresponds to mutant case 2. At the thin line one lane was spliced out. Differences in the protein levels of CBL do not correspond with CBL mutation status. There is no strict correlation between CBL protein levels and pERK protein levels in paediatric AML samples.

Figure 4. CBL knockdown by siRNA transfection.

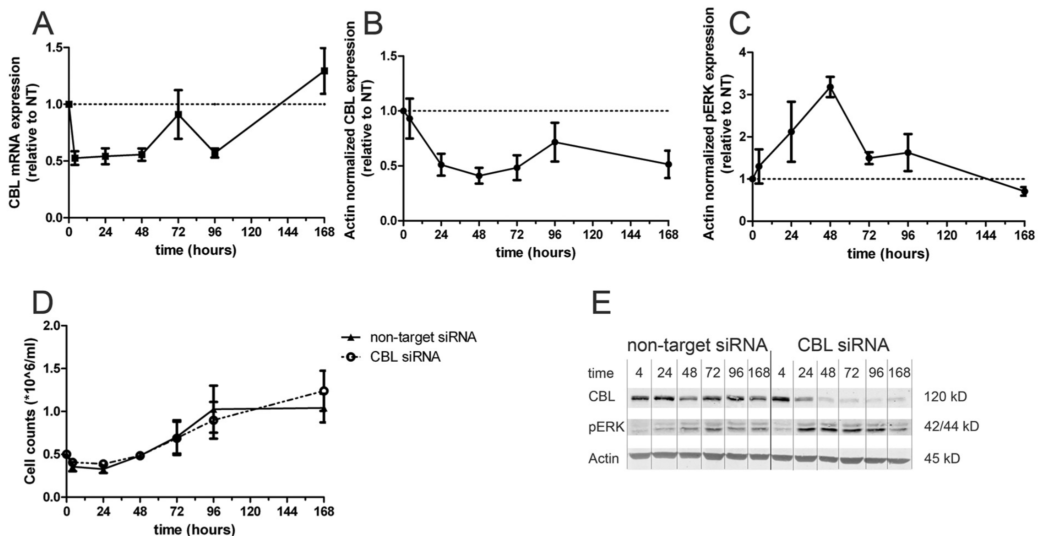


Figure showing results from CBL knockdown by siRNA transfection by electroporation in Kasumi cell line. CBL mRNA expression is shown relative to the non-target siRNA over time (A); mean and standard error of 3 consecutive experiments are shown. CBL protein expression normalized to the loading control actin is shown relative to non-target siRNA (B); mean and standard error of 3 consecutive experiments are shown. pERK protein expression normalized to the loading control actin is shown relative to non-target siRNA (C); mean and standard error of 3 consecutive experiments are shown. Cell counts during culture of both the experimental (CBL siRNA) and control (non-target siRNA) condition (D); mean and standard error of 3 consecutive experiments are shown. Western blot of both control (non-target siRNA) and experimental (CBL siRNA) condition (E); CBL, pERK and actin levels were determined. Time is shown in hours after electroporation in all panels. NT: non-target siRNA.

Discussion

In this study we aimed to elucidate the role of *CBL* in paediatric AML, triggered by reports showing high frequencies of inactivating *CBL* mutations in various myeloid neoplasms [21,90,92-94,108,109]. Our results show that in paediatric AML *CBL* mutations occur only in a very low frequency (0.7%) in our well-documented and representative cohort (n = 319) [16]. This report shows similar low frequencies of *CBL* mutations as in adult AML (1-2%) [92,93]. In adults *CBL* mutations were associated with core-binding factor leukaemia [92], which we could not confirm in this paediatric AML series, i.e. one of the mutated patients carried an *MLL*-rearrangement and the karyotype of the second mutated patient was normal. Like in the adult AML cohorts, in our two patients the identified mutations were heterozygous, which is in contrast to findings reported in JMML and CMML [90,92,93,110-114].

In the design of the study, since *CBL* mutations occur in high frequency in the RAS-pathway mediated disease JMML, we hypothesized that *CBL* mutations may reveal a new, more upstream mechanism for RAS-pathway activation in paediatric AML. For that reason, we anticipated that *CBL* mutations could be related to *MLL*-rearrangements, given the previously observed association between *MLL*-rearrangements and RAS-pathway mutations in paediatric acute leukaemias [16,95,96]. Surprisingly, only 1/64 (1.6%) of our *MLL*-rearranged AML samples was mutated and no mutations were found in the *MLL*-rearranged enriched cohort of infant ALL samples. Thus, we could not confirm the previously suggested relation between *MLL*-rearrangements and *CBL* mutations [100].

The splice site *CBL* mutation of case 2 was previously reported in JMML and CMML [90,94]. RT-PCR confirms that this specific mutation does not affect the *CBL* splicing, which was already reported in a recent study on adult myeloproliferative neoplasms [94]. Analysis of germline material showed that the point mutation was also present in sorted lymphocytes. The patients in the previous reports, which harboured this specific nucleotide change, were not analyzed for the presence of the mutation in their germline material [90,94]. Therefore, the relevance of this specific germline mutation for leukaemogenesis is not clear.

Even though several studies have reported on *CBL* mutations in myeloid neoplasms, we are the first to study CBL mRNA and protein expression concomitantly in diagnostic patient AML material. We found that *CBL* mRNA expression of the two mutated cases was not different from other paediatric AML cases, that there was no differential expression between the cytogenetic subgroups, and that mRNA levels were highly variable. The CBL protein expression of *CBL* mutants **was low, and the** expression in non mutants showed a wide range. No evidence of truncated protein was found, and protein expression did not correlate with mRNA expression, suggesting posttranscriptional mechanisms for CBL protein regulation.

Nevertheless, in JMML where mRNA and protein levels were not compared, *CBL* mutated cases clearly demonstrated aberrant RAS-pathway activation [90]. Others have demonstrated interaction between CBL and FLT3 or KIT, and have shown cytokine independent growth for co-expression of these RTKs with mutant CBL in murine myeloid cells [21,115]. Co-expression of wild-type CBL, which is the case in our patients, reduced this potential significantly [116]. So far, it has not been reported whether CBL protein inactivation in paediatric

AML leads to RAS-pathway activation like in JMML. We found that CBL protein knock-down results in RAS-activation by means of pERK protein up-regulation in an AML cell line Kasumi-1. This may suggest that low levels of CBL protein or dysfunctional CBL protein may activate the RAS-pathway in AML. This is important, as RAS-pathway activation is known to play an important proliferative role in paediatric AML, which can be targeted by already available therapy, such as MEK-inhibitors [16,95,117-119]. Nevertheless, pERK activation was not uniformly apparent in our patient samples, nor well correlated to CBL protein levels.

In conclusion, this study shows that *CBL* mutations are very rare in paediatric AML. Nevertheless, we show that decreased CBL protein expression may be a mechanism for RAS-pathway activation in paediatric AML, like in JMML [90]. Therefore other factors, besides *CBL* mutations such as post-translational processing or enhanced degradation may be involved in this process in at least of subset of paediatric AML patients.

Acknowledgments

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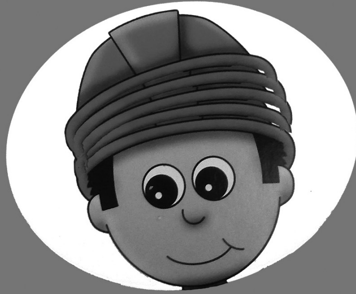
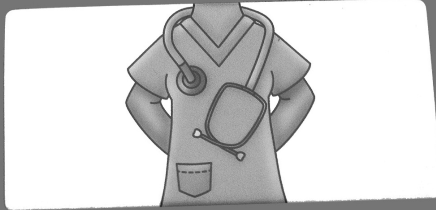
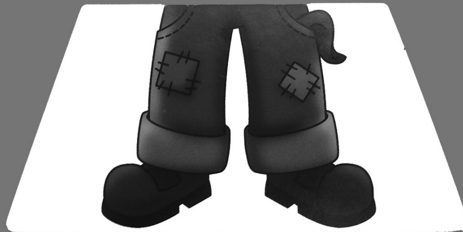

Chapter 6

High *IGSF4* expression in pediatric M5 acute myeloid leukemia with t(9;11)(p22;q23)

Jenny E. Kuipers[1*], Eva A. Coenen[1*], Brian V. Balgobind[1], Jan Stary[2], Andre Baruchel[3], Valerie de Haas[4], Eveline S.J.M. de Bont[5], Dirk Reinhardt[6], Gertjan J.L. Kaspers[7], Jacqueline Cloos[7], Astrid A. Danen- van Oorschot[1], Monique L. den Boer[1], Rolf Marschalek[8], Claus Meyer[8], Rob Pieters[1], C. Michel Zwaan[1] and Marry M. van den Heuvel-Eibrink[1]

[*)] these authors contributed equally to this manuscript

[1] Pediatric Oncology/Hematology, Erasmus MC/Sophia Children's Hospital, Rotterdam, The Netherlands;

[2] Pediatric Hematology/Oncology, 2nd Medical School, Charles University, Prague, Czech Republic;

[3] Hematology, St. Louis Hospital, Paris, France;

[4] Dutch Childhood Oncology Group (DCOG), The Hague, The Netherlands;

[5] Pediatric Oncology/Hematology, Beatrix Children's Hospital, University Medical Center Groningen, Groningen, The Netherlands;

[6] AML-BFM Study Group, Hannover, Germany;

[7] Pediatric Oncology/Hematology, VU University Medical Center, Amsterdam, The Netherlands;

[8] Institute of Pharmaceutical Biology, ZAFES, Diagnostic Center of Acute Leukemias (DCAL), Goethe-University of Frankfurt, Frankfurt, Germany

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Abstract

Pediatric *MLL*-rearranged acute monoblastic leukemia with t(9;11)(p22;q23) has favorable outcome compared to other *MLL*-rearranged AML. The biological background for this difference remains unknown. Therefore, we compared gene expression profiles (GEP, Affymetrix HGU133+ 2.0) of 26 t(9;11)(p22;q23) patients with 42 other *MLL*-rearranged AML patients to identify differentially expressed genes. *IGSF4*, a cell-cell adhesion molecule, was found to be highly expressed in t(9;11)(p22;q23) patients, which was confirmed by RT-qPCR and Western Blot. *IGSF4* expression within t(9;11)(p22;q23) patients was 4.9 times higher in FAB-M5 versus other FAB-types (p=0.001). Methylation status investigation showed that high *IGSF4* expressing t(9;11)(p22;q23) patients with FAB-M5 have no promoter hypermethylation, whereas all other cases do. Cell line incubation with demethylating agent decitabine resulted in promoter demethylation and increased expression of *IGSF4*. Downregulation of *IGSF4* by siRNA did not affect proliferation nor drug sensitivity. In a cohort of 79 *MLL*-rearranged AML cases we show significant better overall survival (OS) for cases with high *IGSF4* expression (5-yr OS 0.70 *vs* 0.37, p=0.03) In conclusion, we identified *IGSF4* overexpression to be discriminative for t(9;11)(p22;q23) patients with FAB-M5, regulated partially by promoter methylation and resulting in survival benefit.

Introduction

Pediatric acute myeloid leukemia (AML) is a heterogeneous disease. Currently, apart from response to treatment, the most important prognostic factor is cytogenetic aberrations. Well known cytogenetic abnormalities that predict differences in survival are t(15;17)(q22;q21) (*PML-RARα*), t(8;21)(q22;q22) (*RUNX1-RUNX1T1*), inv(16)(p13q22) (*CBF-MYH11*) and Mixed Lineage Leukemia *(MLL)*-rearranged AML.[27,120,121] Intensive chemotherapy has improved survival rate over the past decades (5 year event free survival (EFS) 60%). Future therapeutic strategies should be directed towards outcome as well as towards limitation of short and long term toxicity.[122] It is anticipated that such strategies can be based on molecular targeting of abnormally expressed genes in specific genetic types of pediatric AML.[123]

In recent years, more than 60 different translocation partners of the *MLL*-gene have been described.[26] In pediatric *MLL*-rearranged AML the most common translocations are t(9;11)(p21;q23) (*MLL-AF9*) (approximately 50% of patients), t(10;11)(p12;q23) (*MLL-AF10*), t(6;11)(q27;q23) (*MLL-AF6*) and t(11;19)(q23;p13.3) (*MLL-ENL*).[27,120] Of interest is that t(9;11) has been linked with favorable outcome.[63,124,125] Recently, we identified that superior prognosis in the t(9;11) cases was restricted to those with French-American British morphology classification (FAB) M5 phenotype.[25]

So far the underlying biological factors that determine the differences in clinical outcome of *MLL*-rearranged AML cases based on translocation partner are not known as scarce information is available on the molecular aberrations. Therefore, the aim of this study was to investigate the biological background of t(9;11)(p22;q23) AML with and without FAB M5 compared to AML with other *MLL*-translocation partners.

Materials and methods

Patients

Viably frozen diagnostic bone marrow or peripheral blood samples from 269 de novo and 8 secondary pediatric AML cases were provided by the Dutch Childhood Oncology Group (DCOG), the AML 'Berlin-Frankfurt-Münster' Study Group (AML-BFM-SG), the Czech Pediatric Hematology Group (CPH), and the St. Louis Hospital in Paris, France. Samples were chosen to represent all common cytogenetic groups and were selected based on availability of high-quality RNA. Each study group performed central morphological reviews according to the FAB classification. Informed consent was obtained from all patients, after Institutional Review Board approval according to national law and regulations.

The samples included 33 pediatric *MLL*-rearranged cases with t(9;11)(p22;q23) and 52 with other *MLL*-rearrangements, the other 192 samples represented all other common AML cytogenetic groups (Table 1). Among the 8 secondary AML cases, 3 harbored a t(9;11)(p22;q23). These 3 cases were all classified as FAB-M5. The 5 other secondary AML cases did not harbor an *MLL*-rearrangement.

Table 1: Clinical characteristics of GEP cohort of initial pediatric AML samples.

	Original GEP cohort (n=245)	Additional cases (n=32)	Total (n=277)
Sex			
male	137 (56)	13 (45)	150
female	108 (44)	16 (55)	124
Age (median, range, years)	9,8 (0-18,8)	3.0 (0,4-17,3)	
WBC x 10^9/l (median, range)	41,3 (0,0-483)	117,5 (1,8-475)	
FAB			
M0	14 (6)	3 (9)	17 (6)
M1	25 (10)	1 (3)	26 (9)
M2	55 (22)	1 (3)	56 (20)
M3	20 (8)		20 (7)
M4	56 (23)	8 (25)	64 (23)
M5	53 (22)	18 (56)	71 (26)
M6	3 (1)		3 (1)
M7	8 (3)		8 (3)
unknown	11 (4)	1 (3)	12 (4)
Cytogenetics			
***MLL*-rearrangements**	53 (23)	32 (100)	85 (31)
t(9;11)(p22;q23)	*21 (9)*	*12 (38)*	*33 (12)*
other MLL-rearrangements	*32 (13)*	*20 (63)*	*52 (19)*
t(8;21) (q22;q22)	28 (11)		28 (10)
inv(16)(q13q22)	27 (11)		27 (10)
t(15;17)(q22;q21)	18 (7)		18 (6)
Cytogenetically Normal (CN)-AML	41 (17)		41 (15)
AML-other/unknown	78 (32)		78 (28)
Successful GEP	245	14	259
Successful RT-qPCR	76	19	95

GEP: gene expression profiling array, WBC: white blood cell count, FAB: French American British morphology classification, RT-qPCR: Quantitative real-time PCR. Numbers reflect number of cases (%) unless otherwise specified.

Materials

Leukemic cells were isolated and enriched as previously described.[101,126] All resulting samples contained 80% or more leukemic cells, as determined morphologically by May-Grünwald-Giemsa (Merck, Darmstadt, Germany)–stained cytospins. A minimum of $5x10^6$ leukemic cells was lysed in Trizol reagent (Gibco BRL/Life Technologies, Breda, The Netherlands) and stored at -80°C. Isolation of genomic DNA and total cellular RNA was performed as described before.[104] Leukemic samples were routinely investigated for *MLL*-rearrangements by standard chromosome-banding analysis and/or FISH. If translocation with one of the common partners (*MLL-AF9, MLL-AF10, MLL-AF6, MLL-ENL* and *MLL-*

ELL) was suspected, Reverse Transcriptase PCR (RT-PCR) was performed (Primers are described in Supplementary Table S1). Of the 85 *MLL*-rearranged cases, 33 harbored a t(9;11)(p22;q23), 19 a t(10;11)(p12;q23) and 15 a t(6;11)(q27;q23). The remaining 18 cases were confirmed with Long Distance Inverse PCR (LDI-PCR) as *MLL*-other.

Gene expression profiling

Gene expression profiling (GEP) was performed on the RNA of a cohort of 237 de novo and 8 secondary pediatric AML samples. We included 14 additional cases of *MLL*-rearranged AML (5 of which carried a t(9:11)) for GEP to increase group size. Integrity of total RNA was checked using the Agilent 2100 Bio-analyzer (Agilent, Santa Clara, CA, USA). cDNA and biotinylated cRNA was synthesized, hybridized and processed on the Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's guidelines. Data-acquisition was performed using Expresso (Bioconductor package Affy) and probe-set intensities were normalized using the variance stabilization normalization (Bioconductor package VSN) in the statistical data analysis environment R, version 2.7.0.[127,128] Expression levels were log-transformed during this normalization. An empirical Bayes linear regression model was used to compare the signatures for the t(9;11) cases to all other *MLL*-rearranged AML cases.[129] Moderated T-statistics p-values were corrected for multiple testing using the False Discovery Rate (FDR) method defined by Benjamini and Hochberg.[130] *IGSF4* was identified from a top-50 differentially expressed gene list. For the expression analysis of *IGSF4* probe set 209031_at was used.

Quantitative real-time PCR

Quantitative real-time PCR (RT-qPCR) was performed on cDNA of 95 pediatric AML patient samples, selected on availability of remaining cDNA, produced as previously described.[105]. Within this group, 57 were classified as *MLL*-rearranged leukemia of which 24 harbored a t(9;11) (Table 1). An ABI PRISM 7900HT sequence detector (Applied Biosystems, Foster City, CA, USA) was used to validate the GEP results. Primers used for *IGSF4* are described in Supplementary Table S1. For expression analysis of *IGSF4* SYBRgreen was used. The expression of the genes was compared to *GAPDH*, with primers and probe as previously described (sequences are shown in Supplementary Table S1).[105] The average cycle threshold (Ct) value was used to calculate mRNA expression levels of *IGSF4* relative to the expression level of the reference gene (*GAPDH*) using the comparative Cycle time (ΔCt) method.[106]

Western Blot

For Western Blot 10 leukemia samples were selected based on availability of material, of which 3 harbored a t(9;11), 3 harbored another *MLL*-translocation and 4 had a karyotype other than *MLL* (AML-other, containing a case with t(8;21), one with inv(16), one with t(15;17) and one with a normal karyotype). Cell pellets stored at –80°C were quickly thawed and resuspended in 100 μL lysis buffer composed of 25 mM Tris (tris(hydroxymethyl)aminomethane) buffer, 150 mM NaCl, 5 mM EDTA (ethylenediaminetetraacetic acid), 10% glycerol, 1% Triton X-100, 10 mM sodium pyrophosphate, 1 mM sodium or-

thovanadate, 10 mM glycerolphosphate, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% aprotinin (Sigma-Aldrich, St Louis, MO, USA), 10 mM sodium fluoride, and 20 μL freshly prepared sodium pervanadate. Subsequently, cell lysis was allowed for 30 minutes on ice. Cell lysates were cleared by centrifugation for 15 minutes at 10 000 g (13 000 rpm) and 4°C. Protein concentration was determined using the bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Rockford, IL, USA) with different concentrations of bovine serum albumin (BSA) as standards. Cell lysates containing 20 μg of protein were separated on 10% polyacrylamide gels and transferred onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Western Blots were probed with goat polyclonal IgG anti-TSLC1 (synonym of IGSF4, sc-25077, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse anti-beta-actin (ab6276, Abcam, Cambridge, MA, USA) antisera. Subsequently, the blots were labeled with peroxidase-conjugated anti-goat antibody (sc-2020, Santa Cruz Biotechnology) or anti-mouse antibody (DAKO, Glostrup, Denmark). Chemiluminescence (SuperSignal West Femto Maximum Sensitivity Substrate; Pierce Biotechnology) was used to detect luminescence using the Syngene chemigenius (Syngene, Cambridge, United Kingdom).

Methylation Specific PCR

To investigate the methylation status of *IGSF4* Methylation Specific PCR (MS-PCR) was used. Fourteen leukemia samples were selected, 5 samples with a t(9;11) based on their high *IGSF4* expression with GEP and RT-qPCR. These samples with t(9;11) were compared to 5 *MLL*-rearranged samples with other translocation partners and 4 other AML samples. The primers as described by Overmeer et al. were used, using three different areas of the promoter (designated 1 M/U, 5 M/U and 9 M/U) (sequences are shown in Supplementary Table S1).[131] Unmodified genomic DNA was used to test the specificity of the primers for bisulfate converted DNA. One DNA sample was first treated with DNA methylase SSS1 and methyl donor SAM (M0226S New England Biolabs, Ipswich, MA, USA) and then bisulfate converted, creating a hypermethylated sample (M) as a control for the methylation specific primers. As a control for the unmethylated specific primers bisulfate converted DNA of healthy, adult male donors was used (U).

The specificity of the methylation (M) and unmethylation (U) specific primers was tested on a dilution range with a mix of M and U DNA (Supplementary Figure 1). The dilution ranges indicated that the combination of 9M and 9U is the most specific.

Demethylating agents

Cell lines ML-2 (*MLL* t(6;11)), HL-60 (AML other) and MONO-MAC-1 (*MLL* t(9;11)) (DSMZ GmbH, Braunschweig, Germany) were cultured with and without demethylating agent decitabine. ML-2 and HL-60 were selected for their low expression of *IGSF4* on RT-qPCR, MONO-MAC-1 was used as a control, since it shows high *IGSF4* expression. Decitabine concentration was chosen after an in vitro drug assay with decitabine concentrations ranging from 0.125- 4,0 µM and was determined for each cell line to be the approximate 50% lethal concentration (LC50). ML-2 was cultured with a concentration of decitabine of 2 µM, HL-60 and MONO-MAC-1 with a concentration of 4 µM. Decitabine and culturing medium (RPMI 1640 with L-Alanyl-L-Glutamine (Invitrogen, Breda, The Netherlands), 10% Fetal

Calf Serum (FCS) (Integro, Zaandam, The Netherlands), penicillin 100 U/ml, streptomycin 100 µg/ml and fungizone 0.125 µg/ml (PSF) (Invitrogen)) were refreshed daily. The experimental condition started with 100x10^6 cells. Cell counts were determined on a daily basis and cells were maintained in culture at a concentration of 0.5x10^6 cells/ml. Cell samples of both test and control conditions were taken from the medium every other day for the first 6 days and daily thereafter. They were washed with Phosphate Buffered Saline (PBS) and samples for protein studies were frozen at -80°C as dry cell pellets, for DNA and RNA extraction cells were lysed in Trizol reagent and stored at -80°C. The experiment ended as soon as all remaining experimental cells were apoptotic.

siRNA transfection

The MONO-MAC-1 (t(9;11)) and NOMO-1 (t(9;11)) cell line (DSMZ, Braunschweig, Germany) were cultured in RPMI-1640 medium supplemented with 10% FCS and PSF, and grown as suspension cultures at 37°C in humidified air containing 5% CO2.

Cells from both cell lines (10x10^6) were transfected by electroporation in 400 µl RPMI 1640 with L-Alanyl-L-Glutamine (Invitrogen) containing 250nM of either a mix of equal amounts of *IGSF4* siRNAs (Thermo Fisher Scientific Dharmacon ON-TARGETplus LQ-016565, Lafayette, CO, USA) or Non-targeting siRNA (Thermo Fisher Scientific Dharmacon ON-TARGETplus D-001810-01-05, Lafayette, CO, USA), in 4 mm electroporation cuvettes (BioRad, Hercules, CA, USA) (sequences are described in Supplementary Table S1). Electroporation was performed using an EPI 2500 gene pulser (Fischer, Heidelberg, Germany) applying a rectangle pulse of 300 V for 10 ms. To compensate for the amount of cell death induced merely as a consequence of the electroporation procedure, control cells were electroporated in the absence of siRNA. After incubating for 15 min at room temperature, the cells were diluted in 10 ml RPMI 1640 supplemented with 10% FCS and PSF and incubated at 37°C and 5% CO2. They were maintained in culture for 72 hours. Cell counts were determined daily (t= 6 hours, t= 24 hours, t= 48 hours, t= 72 hours). Cell samples of both test and control conditions were taken from the medium at every time point. They were washed with Phosphate Buffered Saline (PBS) and lysed in Trizol reagent and stored at -80°C.

DNA content and cell cycle phase were assessed using PI staining and measured by flow cytometry using a FACSCalibur (Becton Dickinson, San Jose, CA, USA).

In vitro drug resistance

After transfection *in vitro* drug resistance for daunorubicin (DNR; Cerubidine, Sanofi Aventis, Gouda, The Netherlands), cytosine arabinoside (ARA-C; Cytosar, Pharmacia, Woerden, The Netherlands), Cladribine (2-CDA; Leustatin, Janssen-Cilag, Tilburg, The Netherlands), and Etoposide (VP16; Toposin, Pharmachemie, Haarlem, The Netherlands) and as a control also Vincristine (VCR; Pharmachemie), L-Asparaginase (ASP; Paronal, Nycomed Christiaens, Breda, The Netherlands), Prednisolone (PRED; Bufa Pharmaceutical Products, Uitgeest, The Netherlands) and Dexamethason (DXM; Erasmus MC, Rotterdam, The Netherlands) was determined using the 2-, 3- and 4-day 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously.[132] Six con-

centrations of each drug were tested in duplicate. The ranges of the final concentrations of these drugs were as follows: DNR: 0.002 - 2.0 μg/ml; ARA-C: 0.01 – 10 μg/ml; 2-CDA: 0.0004 - 4 μg/ml; VP16: 0.05 – 50 μg/ml; VCR: 0.05 - 50 μg/ml; ASP: 0.003 - 10 IU/ml; PRED: 0.008 - 250 μg/ml and DXM: 0.0002 – 6 μg/ml.

Statistical Analyses

Statistical workup of GEP data is described under 'gene expression profiling'. For comparison of the gene expression in different groups the Mann-Whitney test was used. For assessment of correlation of the results from gene expression profiling and RT-qPCR, Spearman's Correlation coefficient was used. All MLL-rearranged de novo AML cases with available follow-up data were included for survival analysis. Probabilities of overall survival, event-free survival (events: non remitter, relapse, secondary malignancy, death from any cause) and cumulative incidence of relapse (events: non remitter, relapse) were estimated by the method of Kaplan and Meier and compared by the Log-rank Test. Median *IGSF4* expression in the t(9;11) group was used to split all MLL cases in high and low *IGSF4* expression. The Cox Proportional hazards model analysis was applied to determine the association of *IGSF4* overexpression with overall and event free survival adjusted for prognostic factors as described for pediatric AML (white blood cell count (WBC), age and karyotype). All analyses were performed with SPSS Statistics version 16.0 (SPSS Inc. Chicago, IL, USA). All used tests were two-tailed and a p-value of less than 0.05 was considered significant.

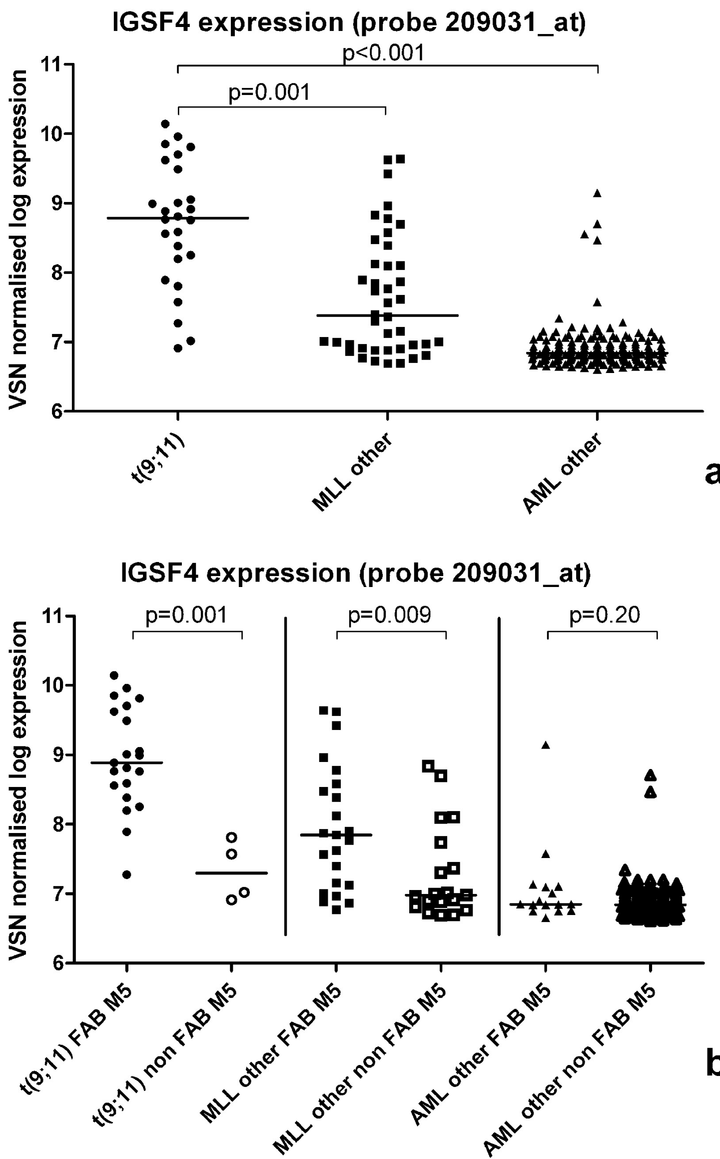
Results

Gene expression profiling

In a comparison of t(9;11)(*MLL-AF9*) AML with other *MLL*-rearranged AML cases, *IGSF4* was among the highly differentially expressed genes. Recent literature described *IGSF4* as a tumor suppressor gene in solid tumors, but so far no data are available about the function of *IGSF4* in leukemia.[133-135] As this gene was never described to be expressed in pediatric AML, we decided to further study *IGSF4* specifically.

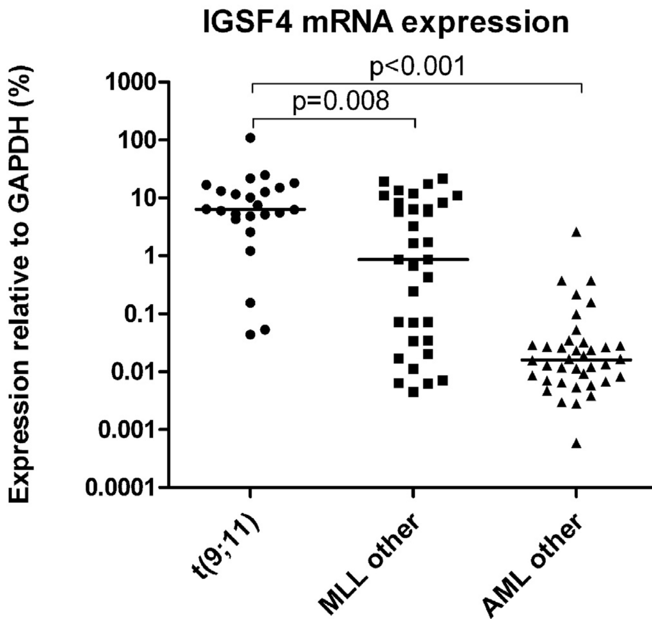
We chose probe set 209031_at, which revealed the most significant differences, to compare differential expression in AML subgroups. Patients with t(9;11) (n=26) had a 4.1 fold higher median *IGSF4* mRNA expression as compared to patients with other *MLL*-rearrangements (n=42) (8.8 Arbitrary Units (AU) *vs* 7.4 AU, p<0.001) (Figure 1a). *IGSF4* expression was also significantly higher in t(9;11) when compared to non *MLL*-rearranged AML cases with other karyotypes (n=192) (fold change 7.0, median expression of 8.8 *vs* 6.8 AU, p<0.001) (Figure 1a). Within the t(9;11) group, expression of *IGSF4* was 4.9 fold higher in FAB-M5 (n=21) versus other FAB-types (n=4) (median: 8.9 AU *vs* 7.3 AU, p=0.001) (Figure 1b). This difference, associated with FAB-classification, was also present in *MLL*-rearranged AML patients with other translocation partners (n=23 *vs* n=19) (median 7.8 *vs* 7.0, fold change 2.4, p=0.009), but not in the AML-other group (n=16 *vs* n=166) (median 6.8 *vs* 6.8, p=0.20) (Figure 1b). All cases with unknown FAB-type were excluded from these analyses (t(9:11) n=1, AML-other n=10).

Figure 1: IGSF4 gene expression in pediatric AML as determined by gene expression arrays.



Graphs showing the expression of probe set 209031_at, representing the IGSF4 gene, after log transformation. Bars represent the median expression in each group. ***a****: Significant differences are shown between patients with a t(9;11) (n= 26) and patients with another MLL-rearrangement (MLL other, n=42) (8.8 vs 7.4, p<0.001) or AML patients without an MLL-rearrangement (AML other, n=192) (8.8 vs 6.8, p<0.001).* ***b****: Expression of probe set 209031_at with all groups divided based on morphology, i.e. FAB M5 vs other FAB-types (non FAB M5). All cases with unknown FAB-type were excluded from this analysis (t(9;11) n=1, AML-other n=10). We detected a significant difference for median expression within the patients with a t(9;11) (n= 21 vs n=4) (8.9 vs 7.3, p=0.001) and the patients with other MLL-rearrangements (n= 23 vs n=19) (7.8 vs 7.0 p=0.009). This difference was not detected in the remaining patients without an MLL-rearrangement (AML other, n=16 vs n=166) (6.8 vs 6.8, p=0.20).*

Figure 2: IGSF4 gene expression in pediatric AML as determined by RT-qPCR.



Graph showing the expression of IGSF4 on mRNA level measured with RT-qPCR. Bars represent the median expression in each group. Significant differences are observed between patients with a t(9;11) (n=24) and patients with another MLL-rearrangement (MLL other) (n=33) (6.4 vs 0.9, p= 0.008) or AML patients without a MLL-rearrangement (AML other) (n=38) (6.4 vs 0.02, p<0.001).

Real-time quantitative PCR

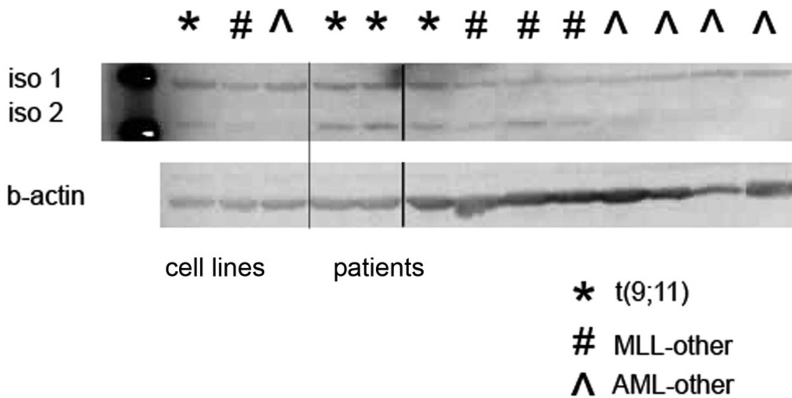
Gene expression results were confirmed with RT-qPCR in 78 cases. An additional 17 cases of which no GEP-data were available were used to expand the number of cases. The median relative expression of *IGSF4* in patients with t(9;11) was 7.4 fold higher compared to *MLL*-rearranged patients with another translocation (6.4% *vs* 0.9%, p= 0.008) (Figure 2). Relative mRNA expression of *IGSF4* in other AML patients was,396 fold lower than in t(9;11) patients (0.02% *vs* 6.4%, p<0.001).

A correlation coefficient was calculated comparing the expression of *IGSF4* by GEP and RT-qPCR. Because of the use of SYBRgreen in the RT-qPCR reaction Ct-values >32 can be considered noise. The remaining 56 pairs resulted in a highly correlated Spearman R=0.839 (p=0.01) (Supplementary Figure 2).

Western Blot

The IGSF4 antibody specifically identified two different isoforms that have previously been described.[136] We did not find a difference in isoform 1 expression, but the expression of isoform 2 was higher in t(9;11) positive patients than in the other patients (Figure 3).

Figure 3: Protein expression analysis of IGSF4 with Western Blot.



Sections of Western Blot showing data from 3 cell lines and 10 patient samples. Two described isoforms (iso 1 and iso 2) are shown at 48 kDa and 45 kDa respectively. The lower panel shows loading control with beta-actin. The protein expression of IGSF4 isoform 2 in patients with a MLL t(9;11) is higher than protein expression of IGSF4 isoform 2 in the other groups. The first lane shows the ladder, the other lanes contain cell lysates from cell lines and patients (separated by the thin line). At the thick line one lane was spliced out.

Methylation Specific PCR

In the selected *MLL*-rearranged AML cases with t(9;11), the *IGSF4* promoter was unmethylated. In contrast, in other *MLL*-rearranged cases and cases without an *MLL*-rearrangement the *IGSF4* promoter was methylated (Figure 4). This difference between cytogenetic groups was also found in the cell lines (Figure 4).

Treatment with demethylating agent

To study the role of promoter methylation in the regulation of *IGSF4* expression, AML cell lines were cultured with and without decitabine. RT-qPCR showed an increase of *IGSF4* RNA expression, up to at least 1000-fold on day 8-9 for the treated hypermethylated cell lines ML-2 (t(6;11)(q27;q23)) and HL-60 (AML-other) when compared to their non treated counterparts (Figure 5a). Bisulfate treated DNA tested on MS-PCR showed methylation status changes, most significantly with selected primers 9M and 9U (Figure 5c). The control cell line MONO-MAC-1 (t(9;11)) that has a high *IGSF4* mRNA expression and moderate methylation, showed a 10-fold increase of expression during treatment with decitabine (Figure 5b). In this cell line MS-PCR showed moderate methylation at the start of treatment which was lost during treatment (Figure 5c).

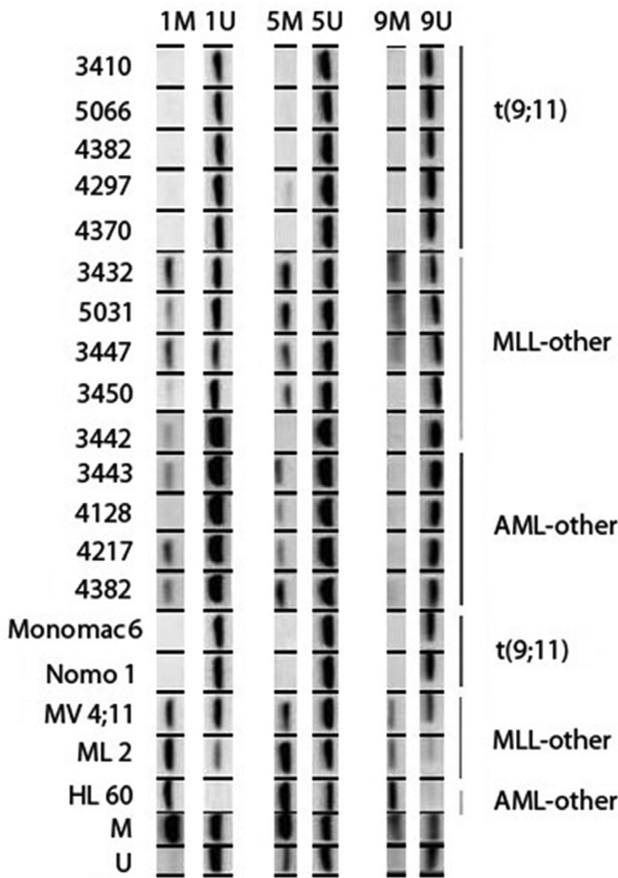


Figure 4: Methylation status of IGSF4 tested by MS-PCR.

Figure showing results of methylation specific PCR in AML patients and cell lines. Three separate regions of the promoter were investigated; region 1, 5 and 9. Each column shows results for a specific primer pair (M: methylated, U: unmethylated). The upper part of the figure shows the IGSF4 methylation status of several patients (indicated by number), the lower part shows methylation status of cell lines and methylated and unmethylated control DNA. On the left the identity of each sample is indicated, on the right the cytogenetic group each patient or cell line belongs to is shown. In patients with t(9;11) (n=5) no bands are seen with the methylated specific primers and heavy bands are seen with the unmethylated specific primers. In contrast, the other MLL patients (n=5) and other AML patients (n=5) do show a band with the methylated primer. This difference is also seen in the cell lines.

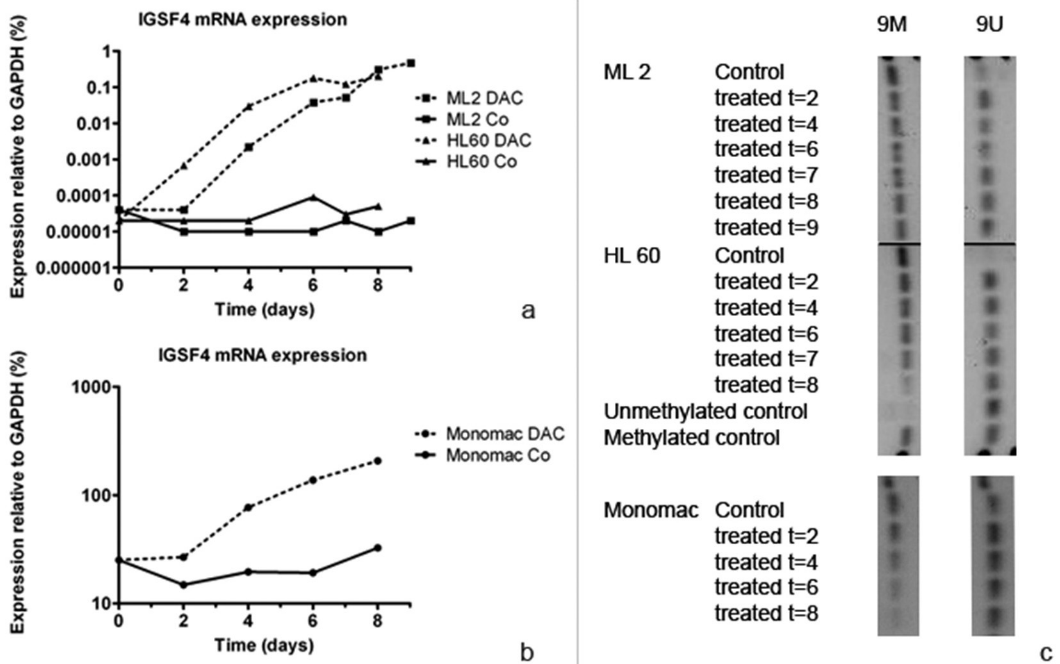
Transfection

Transfection of siRNAs targeting *IGSF4* by electroporation in the cell line MONO-MAC-1 resulted in 50-70% silencing of *IGSF4* mRNA in repeated experiments (Figure 6). NOMO-1 was more difficult to transfect than MONO-MAC-1, and therefore was not used in further experiments. No significant differences were found between transfected and control conditions in MONO-MAC-1 cells, neither in apoptosis or cell cycle arrest (Supplementary Figure 3) nor in cell proliferation (data not shown).

In vitro drug resistance

No significant differences in drug toxicity were found after 2, 3 or 4 days of consecutive culturing of transfected MONO-MAC-1 cells with the most commonly used cytostatic drugs in AML and ALL (Supplementary Figure 4).

Figure 5: relative expression and promoter methylation of IGSF4 in cell lines after culture with decitabine.

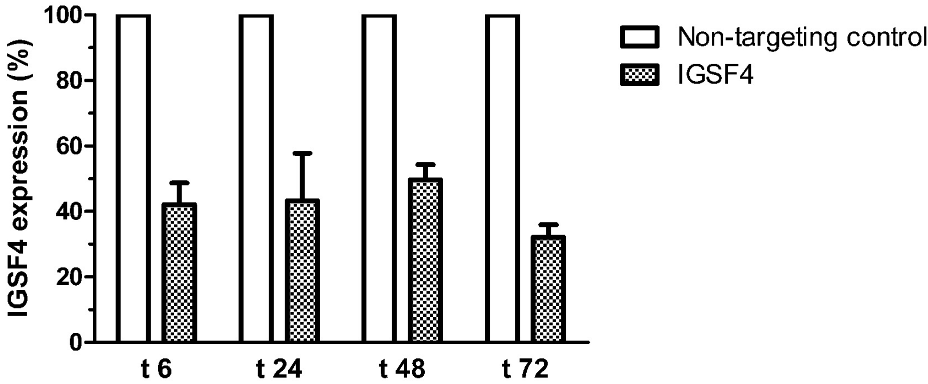


Graph showing IGSF4 mRNA expression in cell lines ML-2, HL-60 and MONO-MAC-1 at different time points during culture with demethylating agent decitabine (DAC). ***a****: Solid lines correspond to untreated conditions of cell lines ML-2 (t(6;11)) and HL-60 (AML-other), dotted lines reflect values from treated conditions of the same cell lines. Shown is >1000-fold upregulation of IGSF4 expression during culture with decitabine, whereas in control conditions expression remained stable.* ***b****: Treatment of cell line MONO-MAC-1 with decitabine. The solid line reflects values from untreated condition, the dotted line from corresponding treated samples. A 10-fold upregulation was found over a treatment period of 8 days.* ***c****: Figure showing results for methylation specific PCR in cell lines cultured with demethylating agent decitabine for 2-9 days. Left panel provides results for 9M primers, the right panel for 9U primers. Under control conditions ML-2 and HL-60 mainly show a methylated promoter region. Shortly after start of treatment unmethylated bands are visible and methylated bands decrease in intensity. MONO-MAC-1 shows both bands at the start of the experiment, and the methylated band clearly weakens during treatment.*

Outcome

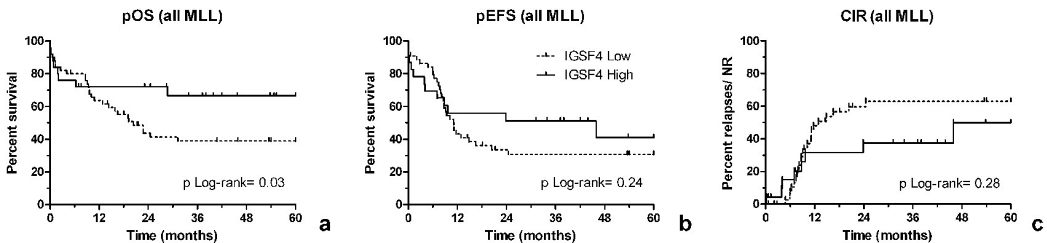
Five year overall survival of *MLL*-rearranged patients with high IGSF4 expression is 70%, which is significantly better than an overall survival of 37% in *MLL*-rearranged patients with low IGSF4 expression (n= 79, p= 0.03) (Figure 7a-c). This group included 28 patients with t(9;11). When analyzed separately this group proved to be too small to show significant survival differences (data not shown). Using the Cox proportional hazards model, no correlation with outcome could be shown after adjustment for known prognostic factors (WBC, age) (data not shown).

Figure 6: IGSF4 knock-down in MONO-MAC-1 after transfection.



The figure shows IGSF4 expression levels after transfection with IGSF4 siRNAs measured by RT-qPCR relative to levels measured in corresponding samples transfected with non-targeting siRNA. Time points are given in hours after transfection. Shown are the means of 4 experiments. Error bars represent standard error of the mean.

Figure 7: Survival plots for patients with high and low IGSF4 expression.



Plots showing overall survival (pOS) (panel a), event free survival (pEFS) (panel b) and cumulative incidence of relapse (CIR) (panel c) for all MLL-rearranged patients (n=26 (IGSF4 high) vs n=53 (IGSF4 low)). Median expression in the t(9;11) group was chosen as a cut-off for the division in high and low IGSF4 expression. The solid line represents patients with high IGSF4 expression, the dotted line represents patients with low IGSF4 expression. NR: non remitter.

Discussion

In pediatric *MLL*-rearranged AML, t(9;11) is the most common genetic aberration. Recently we showed that prognosis of this patient group largely depends on the morphologic FAB classification, i.e. patients with t(9;11) with FAB M5 had a significantly better prognosis than patients with other FAB types.[25] However, so far the biological background for this survival difference is largely unknown. In order to study differentially expressed genes in this specific group, we performed gene expression profiling and identified *IGSF4* as a discriminative gene.

In non-malignant cells, IGSF4 is known to play a role in cell-cell adhesion, cell polarity and as a signaling molecule for NK- and T-cell cytotoxicity.[134,137] Recently, Kawano *et al.* showed that IGSF4 participates in the ErbB2/ErbB3 pathway as a competitive antagonist of ErbB2 in complex formation with ErbB3.[138] Loss of *IGSF4* expression resulted in AKT pathway stimulation which resulted in improved cell movement and survival.[138] We could not confirm similar pathway activation in our pediatric AML dataset using microarray analysis (data not shown). In non small cell lung carcinoma (NSCLC), *IGSF4* was found to be located in an area with a common loss of heterozygosity. Transferring this gene in A549 cells (NSCLC cell line) inhibited tumor formation in nude mice.[133] In neuroblastoma and cervical carcinoma, aberrant promoter methylation of *IGSF4* influenced tumor growth.[131,135]

We found a high *IGSF4* mRNA expression in *MLL*-rearranged pediatric patients with t(9;11) which was associated with increased protein expression, in combination with a hypomethylated promoter region of *IGSF4.* This indicates that indeed epigenetic regulation plays a major role in the expression of *IGSF4* in pediatric AML as was further illustrated by cell line studies with demethylating agents. The expected effect of *IGSF4* on proliferation could not be shown in the siRNA experiments in a t(9;11) cell line. Our *in vitro* studies however do not represent the normal cell environment. Future studies using a design including environmental factors (like homing assays) are more potent to show proliferative advantage caused by differential expression of this cell surface protein. We are not the first group to report gene silencing by methylation in pediatric AML. CCAAT/enhancer binding protein (CEBPA) is a well known gene that is linked to mutations as well as methylation differences and whose expression predicts survival in AML.[19,139] In this context, we might consider designing clinical studies to assess whether the outcome of patients with epigenetic silencing can be improved by adding demethylating agents.

So far only one study in AML cell lines reported on IGSF4, showing hypermethylation of its promoter region in MLL-rearranged AML cell lines versus cell lines without an MLL-rearrangement.[140] In adult T-cell leukemia IGSF4 overexpression resulted in a proliferation advantage.[141] However, the precise role of IGSF4 in hematopoiesis and leukemogenesis is currently unknown. It remains to be determined whether cell-cell adhesion plays a role in IGSF4+ leukemia like in solid tumors. The finding by Boles et al. that expression of IGSF4 protein on the cell surface is a trigger for NK- and CD8+ T-cell mediated cytotoxicity, might support our finding of overexpression of IGSF4 in a group with a more favorable outcome.[137] Normally, circulating leukocytes do not express high levels of IGSF4. If Boles' hypothesis proves to be true in pediatric AML, we would expect blasts with high IGSF4 expression to be more easily recognized by the immune system. Blasts with low IGSF4 ex-

pression are able to evade this mechanism. As low expression is often derived from promoter hypermethylation, these patients might benefit from demethylating agents. In conclusion, we hypothesize that silencing of IGSF4 could be considered as a secondary event, causing the leukemic blasts to be immunologically silent and thereby allowing longer survival.

The ErbB-RAC-AKT pathway, influenced by IGSF4 interaction with ErbB3, could also be of interest for leukemias, since this pathway is linked to proliferation and apoptosis.[138]

We found that *IGSF4* was mainly and most apparently expressed in monoblastic t(9;11) rearranged patients. As this subgroup of *MLL*-rearranged pediatric AML has recently been identified as important prognostic group[25], the role of *IGSF4* deserves further attention. Interestingly, the *IGSF4* expression also seems to be determined by the cell type (M5) in which the maturation arrest occurs. This reflects a unique *novel* collaboration of a specific (epi-)genetic aberration and type II mutations (i.e. MLL) together with maturation status in pediatric AML.

In this retrospective study, which included AML samples from differently treated pediatric patients, we show there was no significant difference for EFS between cases with high and low IGSF4 expression. However, there was a significant difference in overall survival favoring patients with high IGSF4 expression, due to a better salvage rate following relapse in these patients. Further studies in larger prospective cohorts will be necessary to determine the full role of *IGSF4* in pediatric AML.

In conclusion, we found *IGSF4* mRNA and protein to be differentially expressed in *MLL* rearranged pediatric monoblastic AML patients with the highest expression in t(9;11) M5 AML. This expression seems to be largely regulated by promoter hypermethylation. Further studies are needed to be able to determine the biological role and prognostic relevance of *IGSF4* expression in pediatric AML.

Financial support

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Chapter 7

Unique *BHLHB3* overexpression in pediatric acute myeloid leukemia with t(6;11)(q27;q23)

Eva A. Coenen[1], C. Michel Zwaan[1,4], Jan Stary[2], Andre Baruchel[3], Valerie de Haas[4], Ronald W. Stam[1], Dirk Reinhardt[5], Gertjan J.L. Kaspers[6], Susan T.C.J.M. Arentsen-Peters[1], Claus Meyer[7], Rolf Marschalek[7], Luca Lo Nigro[8], Michael Dworzak[9], Rob Pieters[1,4], and Marry M. van den Heuvel-Eibrink[1,4]

[1]Pediatric Oncology/Hematology, Erasmus MC/Sophia Children's Hospital, Rotterdam, The Netherlands;

[2]Pediatric Hematology/Oncology, Charles University and University Hospital Motol, Prague, Czech Republic;

[3]Pediatric Hematology, Hôpital Robert Debré AP-HP and University ParisDiderot, Paris, France;

[4]Dutch Childhood Oncology Group (DCOG), The Hague, The Netherlands;

[5]AML-BFM Study Group, Hannover, Germany;

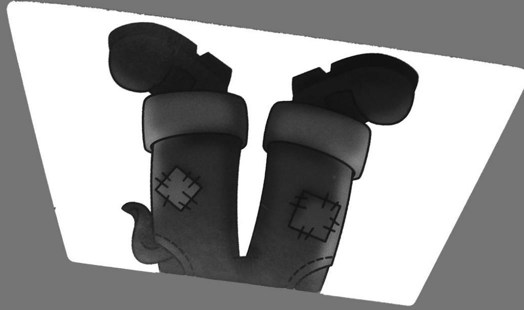
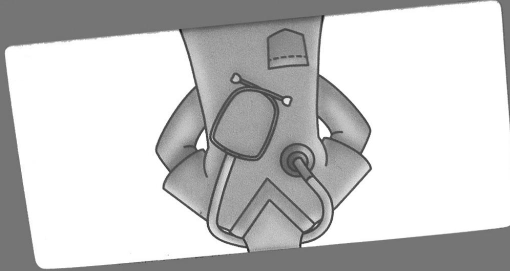
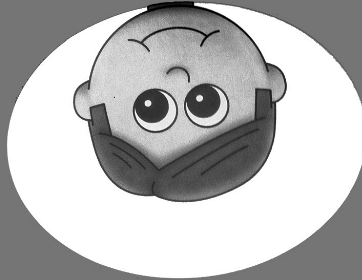
[6]Pediatric Oncology/Hematology, VU University Medical Center, Amsterdam, The Netherlands;

[7]Institute of Pharmaceutical Biology, ZAFES, Diagnostic Center of Acute Leukemia (DCAL), Frankfurt, Germany;

[8]Center of Pediatric Hematology Oncology, Azienda Policlinico, Catania, Italy;

[9]St. Anna Children's Hospital and Children's Cancer Research Institute, Department of Pediatrics, Medical University of Vienna, Austria

Submitted

Chapter 8

Pediatric Acute Myeloid Leukemia with t(8;16)(p11;p13): a distinct clinical and biological entity.

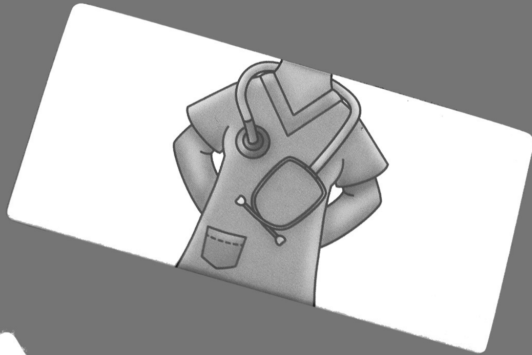
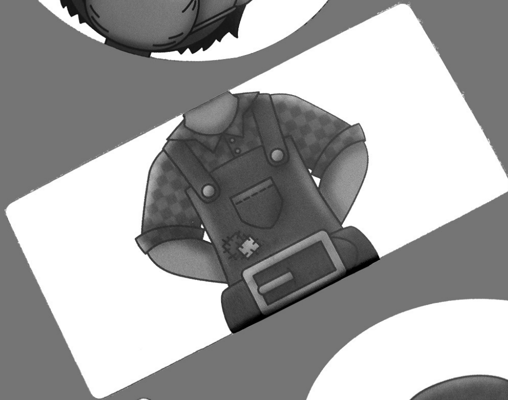
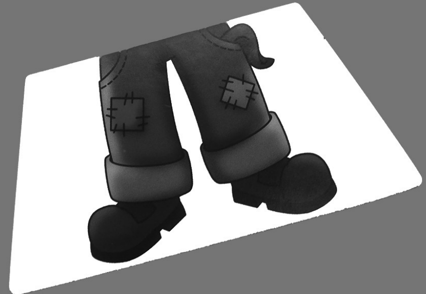
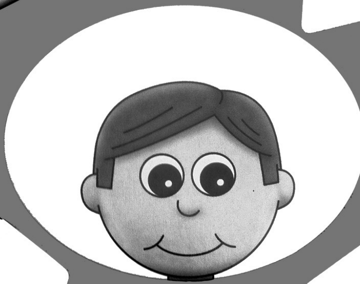
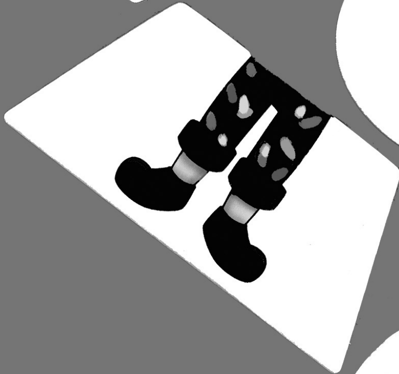
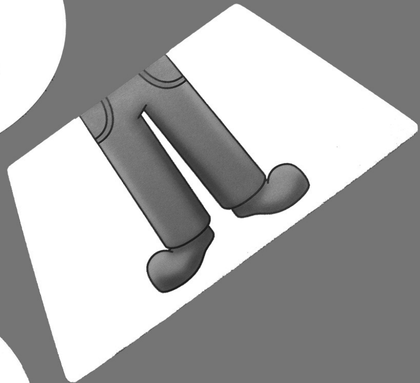
A collaborative study by the International-Berlin-Frankfurt-Münster AML-study group.

Eva A. Coenen[1], C. Michel Zwaan[1,2], Dirk Reinhardt[3], Christine J. Harrison[4], Oskar A. Haas[5], Valerie de Haas[2], Vladimir Mihál[6], Barbara De Moerloose[7], Marta Jeison[8], Jeffrey E. Rubnitz[9], Daisuke Tomizawa[10], Donna Johnston[11,12], Todd A. Alonzo[11,13], Henrik Hasle[14], Anne Auvrignon[15], Michael Dworzak[5], Andrea Pession[16], Vincent H.J. van der Velden[17], John Swansbury[18], Kit-fai Wong[19], Kiminori Terui[20], Sureyya Savasan[21], Mark Winstanley[22], Goda Vaitkeviciene[23], Martin Zimmermann[3], Rob Pieters[1], Marry M. van den Heuvel-Eibrink[1,2]

[1] Department of Pediatric Oncology/ Hematology, Erasmus MC- Sophia Children's Hospital, Rotterdam, The Netherlands; [2] Dutch Childhood Oncology Group, the Hague, The Netherlands; [3] Acute Myeloid Leukemia-Berlin-Frankfurt-Münster Study Group, Pediatric Hematology/Oncology, Medical School Hannover, Hannover, Germany; [4] Northern Institute of Cancer Research, Newcastle University, Newcastle upon Tyne, United Kingdom; [5] St. Anna Children's Hospital and Children's Cancer Research Institute, Department of Pediatrics, Medical University of Vienna, Vienna, Austria; [6] Czech Pediatric Hematology/Oncology, Department of Pediatrics, University Hospital, Palacky´s University, Olomouc, Czech Republic; [7] Belgian Society of Paediatric Haematology Oncology, Ghent University Hospital, Department of Pediatric Hematology/Oncology, Ghent, Belgium; [8] Cancer Cytogenetic Laboratory, Pediatric Hematology Oncology Department, Schneider Children's Medical Center of Israel, Petach Tikvah, Israel; [9] St Jude Children's Research Hospital, Memphis, TN, USA; [10] Japanese Pediatric Leukemia/Lymphoma Study Group, Department of Pediatrics, Tokyo Medical and Dental University, Tokyo, Japan; [11] Children's Oncology Group, Arcadia, CA, USA; [12] Children's Hospital of Eastern Ontario, Ottawa, ON, Canada; [13] Department of Preventive Medicine, University of Southern California, Los Angeles, CA, USA; [14] Nordic Society for Pediatric Hematology and Oncology, Department of Pediatrics, Aarhus University Hospital Skejby, Aarhus, Denmark; [15] French Leucémie Aique Myeloide Enfant, Hopital Trousseau, Paris, France; [16] Italian Association of Pediatric Hematology Oncology, Clinica Pediatrica, Università Padova, Padova, Italy; [17] Department of Immunology, Erasmus MC, Rotterdam, The Netherlands; [18] Department of Clinical Cytogenetics, The Royal Marsden Hospital & The Institute of Cancer Research, Sutton, United Kingdom; [19] Department of pathology, Queen Elizabeth Hospital, Hong Kong, China; [20] Department of pediatrics, Hirosaki University Graduate School of Medicine, Hirosaki, Japan; [21] Division of Hematology/Oncology, Children's Hospital of Michigan, Detroit, MI, USA; [22] Department of Pediatric Oncology, Starship Children's Hospital, Auckland, New Zealand; [23] Clinic for Children's Diseases, Centre for Oncology/Haematology, Vilnius University, Vilnius, Lithuania.

Submitted

Summary and Discussion

Summary

Currently, intensive treatment schedules of pediatric AML have resulted in a survival of about 70%. The intensive chemotherapy poses considerable risks for serious acute as well as long term side effects and therefore does not allow further intensification. For an optimal trade off in efficiency and minimal side effects, new therapeutic options need to be obtained from insights in biological processes that drive leukemogenesis. This will allow development of targeted therapies which ideally kill leukemic cells while leaving the healthy cells unharmed.

In this thesis we focused on *MLL*-rearranged pediatric AML, comprising about 20% of pediatric AML cases. Until recently this group was considered to represent an adverse prognostic group, but recent studies showed marked heterogeneity in outcome depending on clinical characteristics as well as translocation partner. We aimed to elucidate part of this heterogeneity by studying the biological features of *MLL*-rearranged AML patients and cell lines.

MLL is a promiscuous gene, for which over 60 translocation partners have been described, and it is likely that in the future still more partners will be identified. In **Chapter 2** and **Chapter 3** we describe two *novel* translocation partners, *KIAA1524* and *ABI2*, in two patients harboring an *MLL*-translocation. The newly described fusion genes add to the growing list of *MLL* fusion genes, and elucidate pieces of the pathways that are preferably disrupted by *MLL*-rearranged AML. Both patients were very young children under the age of one year, which is a classical age-group for *MLL*-rearrangements.

In **Chapter 4** we show in a large international retrospective *MLL*-rearranged cohort that not only the translocation partner but also specific additional cytogenetic aberrations contribute independently to outcome in pediatric 11q23/*MLL*-rearranged AML. These were mainly numerical cytogenetic aberrations, such as trisomy 8 and trisomy 19 specifically, that can be readily identified by conventional karyotyping, and can be translated to prognosis. We determined that *MLL*-rearrangements frequently occurred as part of a complex karyotype, but showed that complex karyotype in *MLL*-rearranged AML is not an independent prognostic factor, in contrast to what is known from complex karyotype in other AML types.

In **Chapter 5** we show that *CBL* mutations are very rare in pediatric AML and are not correlated with *MLL*-rearranged pediatric AML Nevertheless, forced knockdown of CBL protein expression by siRNAs, activated the RAS-pathway in a pediatric AML cell line. So apart from KRAS and NRAS mutations, we hypothesized that factors influencing CBL protein status, such as post-translational processing or enhanced protein degradation, may be involved in RAS-pathway activation.

In **Chapter 6**, comparing gene expression profiles of pediatric AML patients with t(9;11)(p22;q23) with pediatric AML patients with a different *MLL*-translocation, we found *IGSF4* mRNA and protein to be overexpressed in *MLL*-rearranged pediatric monoblastic AML patients with t(9;11)(p22;q23). This expression seemed to be largely regulated by promoter hypomethylation in this specific subgroup. Within *MLL*-rearranged pediatric AML, we showed a better overall survival for cases with high *IGSF4* expression.

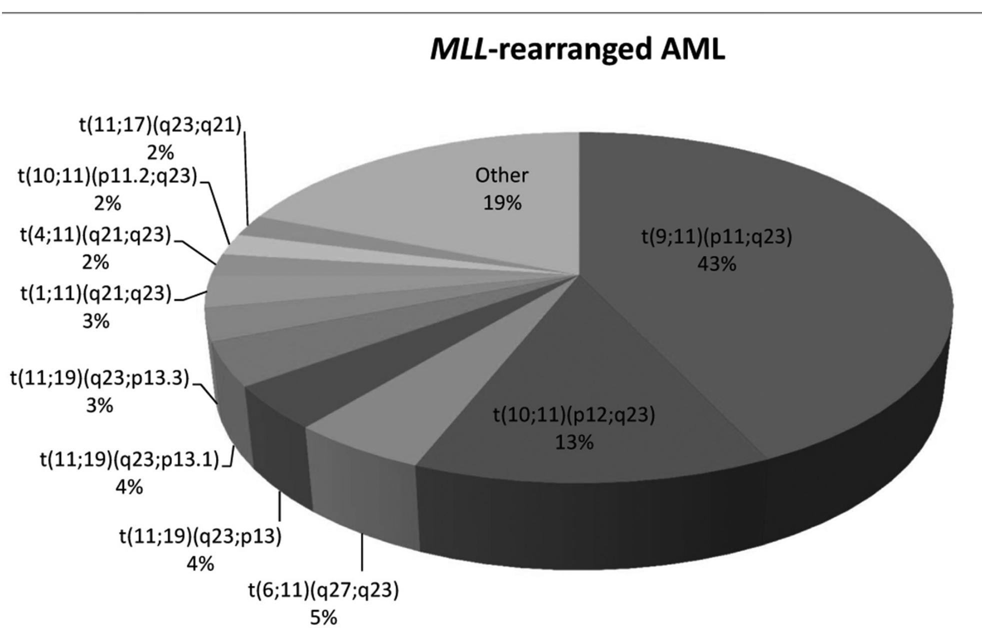
Chapter 7 focuses on a cohort of t(6;11)(q27;q23) pediatric AML patients. Using gene expression profiling, we identified the unique overexpression of *BHLHB3* in this t(6;11)(q27;q23) pediatric AML subgroup. From functional experiments however, we could not provide evidence for *BHLHB3* as a driving oncogene in this patient group, which urges further research in other directions on this previously identified poor prognostic AML subgroup, in order to identify targets for therapy.

In **Chapter 8,** we describe an international retrospective study combining data from 62 pediatric AML patients, collected from registries and literature reports, to elucidate the clinical and biological characteristics of pediatric AML with t(8;16)(p11;p13). AML patients with t(8;16)(p11;p13) were significantly younger, had a predominant M4-M5 FAB type, and frequent occurrence of erythrophagocytosis, leukemia cutis and disseminated intravascular coagulation. Strikingly, spontaneous remissions occurred in some neonates with t(8;16)(p11;p13) AML. The 5-year overall survival of patients that received intensive chemotherapy was not different from other AML types. Gene expression profiles of t(8;16)(p11;p13) pediatric AML cases resemble *MLL*-rearranged AML and identified high expression of the *RET*, *PERP* and *GGA2* genes as discriminative and specific for t(8;16)(p11;p13).

General discussion and future perspectives

In this thesis we studied biological features of *MLL*-rearranged pediatric AML aiming to identify novel molecular and genetic aberrations that determine prognosis and could serve as new treatment targets. *MLL*-rearranged pediatric AML patients are generally rather young patients that frequently present with myelomonocytic or monoblastic phenotype and high white blood cell counts. In half of the cases t(9;11)(p22;q23) is identified and an additional 25% of the cases shows t(10;11)(p12;q23), t(11;19)(q23;p13) and t(6;11)(q27;q23) (Figure 1).[35] Previous studies from our group have shown substantial heterogeneity in outcome estimates, based on translocation partner.[25]

Figure 1: The distribution of MLL translocation partners in pediatric AML.[35]



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Within the landscape of AML, *MLL* is a unique genetic abnormality. Whereas the majority of recurring cytogenetic aberrations in pediatric AML are characterized by the disruption of hematopoietic transcription factors, and translocations typically occur fusing transcription factors to fixed partner genes, such as *AML1-ETO*, and *CBFB-MYH11*, the *MLL*-gene is a chromatin modifying gene with a promiscuous selection of partner genes and involved in the leukemogenesis of both ALL and AML. This indicates a unique potential of *MLL*-translocations to disrupt hematologic development in both lymphoid and myeloid lineage, and partly independent of the gene it is fused to.[212-215] Among the frequently recurring genes in both AML and ALL however, there is a strong preference for genes coding for transcriptional elongation proteins. Many of these proteins collaborate with the wild type MLL in a

large multi-protein chromatin modifying complex, facilitating gene expression in a tissue specific manner.[28,216-221] In *MLL*-rearranged AML however, both sporadic and recurrent fusion proteins from many other functional classes have been described as well, such as cell surface proteins, cell adhesion proteins, and proteins involved in endocytosis, signaling, cytoskeleton organization, metabolism, nucleic acid binding, RNA decay metabolism, histone acetylation, and transcription.[26,222] Although the majority of pediatric *MLL*-translocated AML samples share important characteristics and can be identified by gene expression profiling as a distinct group,[74] the translocation partner also contributes specific gene expression characteristics and is an independent prognostic factor in pediatric *MLL*-rearranged AML.[25,54,57]

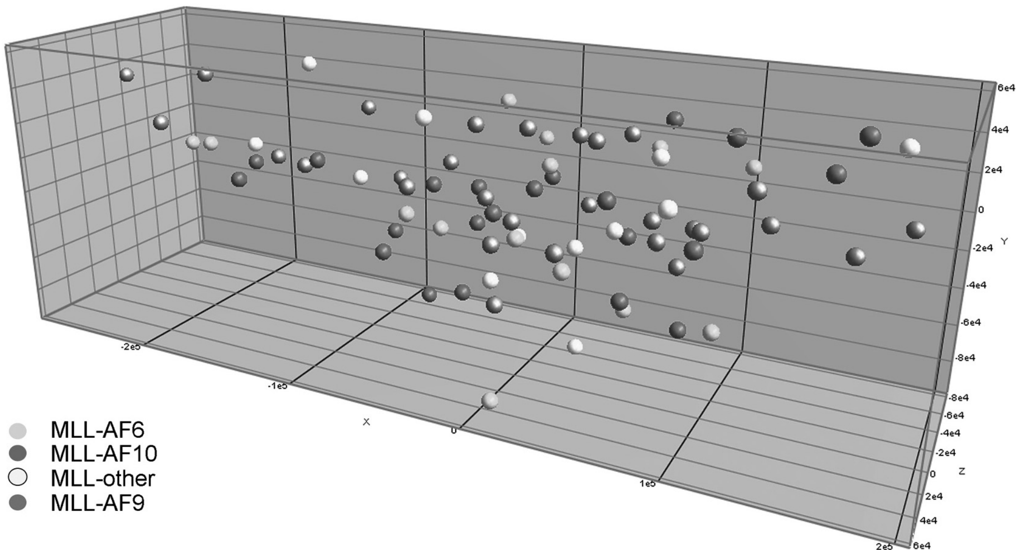
For this reason the determination of the involved translocation partner in *MLL*-rearranged leukemias is of great importance. In this thesis we described two *MLL* fusion partners that had not been previously described, *ABI2*, and *KIAA1524*. *ABI2* is a gene regulating the c-Abl pathway, and is closely related to *ABI1*, which is a known recurrent *MLL*-fusion partner in pediatric AML. *KIAA1524*, also known as *cancerous inhibitor of PP2A* (*CIP2A*), is a regulator of PP2A and involved in the c-Myc pathway. Genes regulating this pathway were not previously described to be involved in *MLL*-translocations, but overexpression of c-Myc has been shown to cause AML in mice.[45] In the two cases, both younger than 1 year and of FAB M5 morphology, by cytogenetic analysis involvement of the *MLL* locus on 11q23 was suspected, but the locus of the potential fusion partner was not a common locus for recurrent *MLL*-translocations. If no fusion genes are known on the involved locus, screening with RT-PCR can not identify the translocation partner, and long distance inverse PCR (LDI-PCR) is a useful additional diagnostic tool.[41] Also in case of unsuccessful cytogenetic analysis and positive FISH result, or if the RNA required for RT-PCR is difficult to obtain, but DNA is available, LDI-PCR can be used to determine the translocation partner. Knowledge of the translocation partner can be translated into a more accurate estimate of prognosis and could be used for more targeted treatment options in future. However, at this moment, no translocation partner directed therapeutic options are available, partly due to the lack of our understanding of the biology driving these leukemic cells.

From previous work we knew that the presence of additional cytogenetic aberrations in general is a poor prognostic factor in pediatric *MLL*-rearranged AML.[25] We found that structural aberrations, which were highly diverse, and +19 independently predict adverse outcome and +8 independently predicts favorable outcome. In addition, it was recently reported that trisomy 4 is of unfavorable prognostic value in t(8;21)(q22;q22) pediatric AML.[223] This indicates that additional aberrations may aid in treatment stratification of pediatric AML. In pediatric *MLL*-rearranged AML, it appears that gene dosage rather than structural gene disruption plays a role in the prognostic value, since numerical aberrations were highly recurrent but specific structural breakpoint were not. Trisomy 8 is a common cytogenetic abnormality in AML and MDS, both as sole abnormality and in addition to other cytogenetic abnormalities such as inv(16), t(7;12) and *MLL*-rearrangements, and seems to be associated with intermediate outcome.[5,55,224] The fact that individuals with constitutional trisomy 8 mosaicism are at increased risk of myeloid malignancy, also points to an additive effect of trisomy 8 in leukemogenesis.[225-229] Until now however, studies failed to show that trisomy 8 AML has a specific gene expression profile, and no candidate gene on chromosome 8 was convincingly shown to contribute to leukemogenesis.[224,230] At the

moment this leaves us with a significant association of cytogenetic findings and outcome, but lacking a biological substrate which could guide us to alternative therapeutic options. In general, in pediatric AML complex karyotype is recognized as a poor prognostic finding.[5,27,55,60,87] In pediatric *MLL*-rearranged AML complex karyotype occurs frequently (26%), but was only a significant prognostic factor in univariate analysis. Overall survival of pediatric *MLL*-rearranged AML patients with complex karyotype (45%) however, is within the range reported for pediatric AML patients with complex karyotype in general (44-46%).[5,55] This suggests that also in *MLL*-rearranged pediatric AML patients, complex karyotype can be used in treatment stratification.

Factors contributing to *MLL*-rearranged leukemogenesis include aberrations that enhance proliferation (type I aberrations).[14] Although the *MLL*-rearrangement alone may be sufficient to induce leukemogenesis in some patients,[24] about half of the pediatric *MLL*-rearranged AML patients carry additional molecular genetic aberrations, consisting mainly of RAS-pathway aberrations.[16] In our laboratory, RAS mutations were identified in ¼ of t(4;11)(q21;q23) *MLL*-rearranged infant ALL.[155] This is of interest because *MLL*-rearranged infant ALL in general shows a very low number of aberrations. Mouse studies have shown the importance of RAS mutations for leukemogenesis in *MLL*-translocated hematopoietic stem cells,[99] but 50% of *MLL*-rearranged pediatric AML patients did not harbor type I mutations.

Figure 2: principal component analysis of all MLL-rearranged pediatric AML patients clustered on all probe sets from gene expression array (Affymetrix Human Genome U133 plus 2).



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Hypothesizing that *MLL*-rearranged leukemias benefit from RAS-pathway mutations, we studied the frequency of mutations in *CBL*, an E3 ubiquitin ligase that negatively regulates receptor tyrosine kinases and therefore can also regulate the RAS-signaling pathway. It appeared that *CBL*-mutations occurred in only 1% of pediatric AML, and in 1.6% of *MLL*-rearranged cases. This is in contrast to findings in juvenile myelomonocytic leukemia (JMML) and adult chronic myelomonocytic leukemia (CMML), diseases with high mutation frequency in both *RAS* and *CBL*.[90,110] Until now it is not known whether additional molecular aberrations are required for leukemogenesis in the *MLL*-rearranged AML patients that do not harbor known type I mutations. Genome wide techniques such as exome sequencing or RNA-sequencing may be used to search for such *MLL*-collaborating genetic aberrations, and have proven to be fruitful in other AML types, identifying DNMT3A and IDH1 mutations as recurrent aberrations in adult cytogenetically normal AML.[231-233]

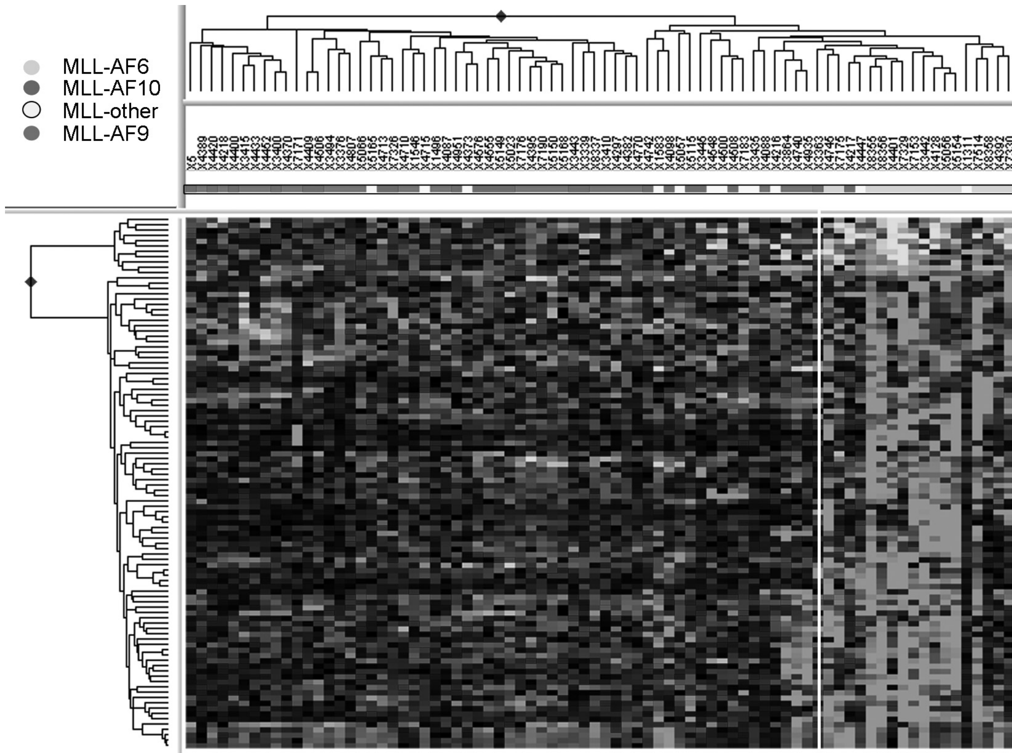
We studied *MLL*-related gene expression signatures in pediatric AML by gene expression profiling (GEP), that previously showed its value for classification of some cytogenetic AML subgroups, including t(8;21)(q22;q22), inv(16)(p13q22), t(15;17)(q21;q22) and *MLL*-rearranged AML.[15,74]

Within this thesis we focused on gene expression differences within the *MLL*-rearranged subgroups, aiming to find biological markers that explain the survival differences. In our analyses MLL-subgroups were mainly divided by translocation partner. Unsupervised analysis of the gene expression profiles of *MLL*-rearranged pediatric AML, results in poor discrimination of translocation partner subgroups (Figure 2). By supervised analysis however, comparing subgroups of *MLL*-rearranged pediatric AML to other *MLL*-rearranged AML patients, we could identify specific gene expression profiles (Figure 3) differentially expressed genes, such as *BRE* and *IGSF4* in t(9;11)(p22;q23) patients.[54,57] We further investigated highly expressed genes in t(9;11)(p22;q23) and t(6;11)(q27;q23) and validated unique upregulation of genes in these specific patient groups. Using siRNA and shRNA methods resulting in efficient knockdown of *IGSF4* and *BHLHB3*, we were unable to detect changes in proliferation, drug sensitivity and apoptosis. The limited effects could be caused by limitations from the used methods of in vitro culturing,

but it may also be that these genes are not the leukemia driving genes. Approaches to more readily identify leukemia driving genes include knockdown of the *MLL*-translocation transcript followed by gene expression profiling.[172] Within our laboratory this technique has successfully been used in t(4;11)(q21;q23) (*MLL-AF4*) and t(11;19)(q23;13) (*MLL-ENL*) rearranged ALL cell lines (MH van der Linden *et al*, manuscript in preparation), but was unsuccessful in t(6;11)(q27;q23) (*MLL-AF6*) AML. The failure in t(6;11)(q27;q23) positive cell lines is possibly caused by the expression of alternative splice variants of the *MLL*-fusion transcript, or the limited susceptibility for transfection by electroporation of these cell lines. Other approaches were recently shown to be of great value to determine real target genes in *MLL*-rearranged leukemias, such as the analysis of changes in histone marks in gene promoters after interference with the *MLL*-fusion transcript.[33,165]

We also studied the clinical and biological characteristics of the largest cohort of pediatric t(8;16)(p11;p13) AML so far. This study illustrates again that rare AML types, can only be studied in the framework of international collaboration such as the International Berlin-

Figure 3: heatmap showing the top-100 differentially expressed probe sets of t(6;11)(q27;q23) (MLL-AF6) vs other-MLL comparison (LIMMA) from gene expression array (Affymetrix Human Genome U133 plus 2). t(6;11)(q27;q23) cases are labeled green, t(10;11)(p12;q23) (MLL-AF10) cases red, t(9;11)(p22;q23) (MLL-AF9) purple and MLL-other cases yellow. The cluster of t(6;11)(q27;q23) cases is located in the white box on the right side of the figure.



(A color version of this figure can be found in the appendices)

Frankfurt-Münster (IBFM) pediatric AML Study Group (chair Prof.dr. D. Reinhardt, Hannover, Germany).[25,121,173,223,234] Interestingly, congenital cases of t(8;16)(p11;p13) pediatric AML showed spontaneous remission. During follow-up however, about half of them were diagnosed with disease recurrence. The knowledge that some congenital AML patients carrying specific cytogenetic aberrations may show a transient leukemia, similar to transient myeloproliferative disease of Down syndrome, is of clinical relevance as at least in some of these neonates toxic chemotherapy can be avoided. Until now we have not been able to explain the curious phenomenon of spontaneous remission, and with the rarity of the disease and the lack of good models it is unlikely that answers will come easily. Future research could start from comparing paired samples from the neonatal phase of self-limiting leukemia to the AML that developed later in life, using genome-wide screening techniques such as exome sequencing and gene expression profiling to search for differences.[235] It would also be interesting to explore the properties of the t(8;16)(p11;p13) transcript *MYST3-CREBBP* in hematopoietic stem cells.

Both t(8;16)(p11;p13) pediatric AML and *MLL*-rearranged AML, share overexpression of HOXA genes and have no overexpression of other HOX genes. Several other pediatric AML cytogenetic subgroups, such as t(6;9), *NUP98/NSD1* and *NPM1* mutated cases overexpress both *HOXA* and *HOXB* genes.[15] Especially *HOXA9* gene overexpression, present in about 50% of AML patients, possesses important transforming capacities, but due to its role in normal hematopoiesis it seems less likely to be a potential target for therapy.[236]

The studies presented in this thesis were performed in an era in which genome wide platforms became widely available. Using gene expression profiling we were able to show strong correlations between cytogenetic subgroups and gene expression profiles. On the other hand, this thesis also demonstrates the importance of 'old techniques' for the detection of prognostic variables in *MLL*-rearranged pediatric AML, such as karyotyping, FISH and PCR. At this time, no single technique is available that detects all prognostic variables. Alternative strategies that could still elucidate more of the driving processes in *MLL*-rearranged pediatric AML include integrative analyses of different genome wide platforms, such as miRNA screenings, DNA methylation arrays, and gene expression profiling.[237-239]

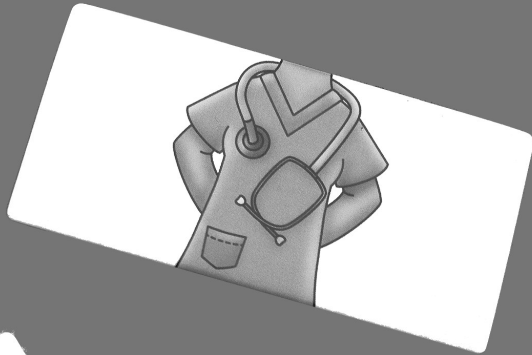
Significant progress in the treatment of pediatric AML has been made over the last decades, using standard chemotherapeutics. However, still about 35% of the patients suffer from relapse.[5] At relapse, AML blasts have frequently gained or lost aberrations, indicating clonal evolution of the blast population, possibly due to therapy resistance of subclones.[240,241] For future research and improvement of AML outcome the detection of small subclones at initial diagnosis is of great importance. Studying these subclones with sequencing techniques that acquire greater depth, such as exome, RNA or DNA capture sequencing seem to hold great promises.[242]

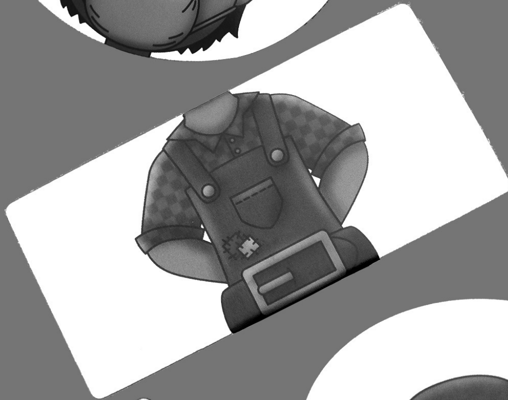
Especially gene silencing caused by promoter hypermethylation could serve as a possible treatment target, because drugs targeting hypermethylation have shown to be well tolerated and successful in some hematologic diseases, although they have not yet been widely tested in children.[243,244] For successful analysis of such cross-platform datasets, the involvement of bio-informaticians is required.

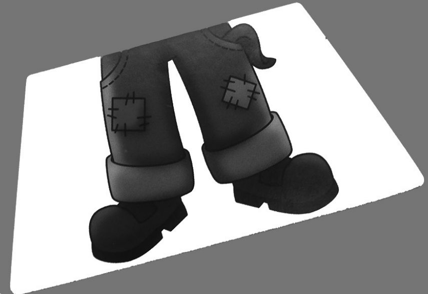
The treatment of *MLL*-rearranged AML may improve by drugs targeting specific epigenetic regulators that collaborate with the *MLL*-rearrangement. Histone methyltransferase DOT1L has proven to be a very potent target in mouse models, because DOT1L is responsible for promoter H3K79 trimethylation in *MLL*-rearranged leukemia, providing an active gene expression histone mark.[33,165] The first DOT1L-inhibitor is currently tested in a phase I clinical trial in adult leukemia and MDS patients (NCT01684150). Recent data have indicated that also t(6;11)(q27;q23) AML patients might benefit from these drugs, although previous studies suggested that *MLL-AF6* does not associate with DOT1L.[245] More studies are needed on the mechanisms by which DOT1L is recruited to *MLL*-target genes by the *MLL-AF6* fusion.

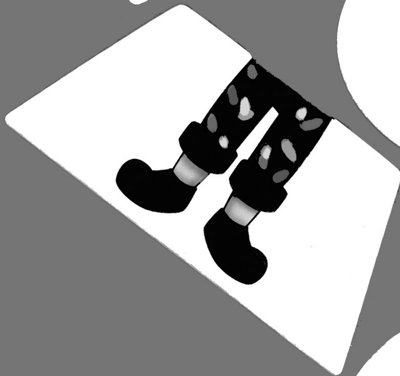
In conclusion, the heterogeneity of *MLL*-rearranged pediatric AML has become more apparent over the last years, and demands for a more detailed understanding of the biologic processes in the leukemic cells. The studies described in this thesis have contributed to this understanding, but have not yet been able to point to novel therapeutic options. Future research should continue to focus on the *MLL*-rearranged AML types with the poorest outcome, such as t(6;11)(q27;q23), t(10;11)(p11;q23) and t(10;11)(p12;q23), and risk group stratification and risk adapted therapy.

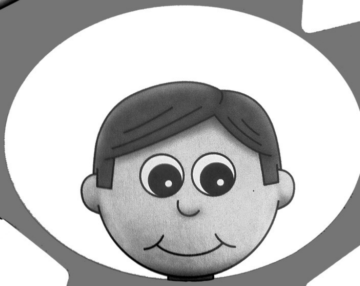
Nederlandse samenvatting

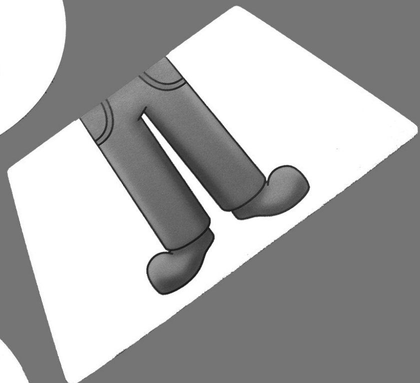












Nederlandse samenvatting

Op dit moment hebben kinderen met AML een overlevingskans van ongeveer 70%. De overleving is de laatste decennia fors toegenomen dankzij de inzet van intensieve behandeling met chemotherapie en betere supportive care. De intensieve chemotherapie levert echter ook aanzienlijke risico's voor zowel acute als lange termijn bijwerkingen, en daarom is verdere intensivering niet mogelijk. Voor een combinatie van optimaal behandeleffect met minimale bijwerkingen, moeten nieuwe therapeutische opties worden bedacht. Inzicht in de biologische processen die leukemische cellen van gezonde cellen onderscheiden is hiervoor essentieel. Het is de opzet dat deze kennis zal leiden tot de ontwikkeling van gerichte therapieën die idealiter de leukemische cellen doden, terwijl de gezonde cellen ongemoeid gelaten worden.

In dit proefschrift hebben we ons gericht op *MLL*-herschikte AML bij kinderen, een groep die ongeveer 20% van de gevallen van kinder-AML beslaat. Tot voor kort werd deze groep beschouwd als een prognostisch ongunstige groep, maar recente studies toonden grote verschillen in uitkomst afhankelijk van klinische kenmerken, en vooral de translocatie partner. Het doel van onze studies was deze heterogeniteit te verklaren door het bestuderen van de biologische kenmerken van *MLL*-herschikte AML en cellijnen.

MLL is een promiscue gen, waarvan meer dan 60 translocatie partners zijn beschreven, en het is waarschijnlijk dat in de toekomst nog meer partners worden vastgesteld. In **hoofdstuk 2** en **hoofdstuk 3** beschrijven we twee nieuwe translocatie partners, respectievelijk *KIAA1524* en *ABI2*, in twee patiënten met een niet-typische *MLL*-translocatie. Beide patiënten waren bij diagnose nog geen jaar oud, hetgeen een klassieke leeftijdsgroep is met een relatief frequent vóórkomen van *MLL*-herschikte AML. De nieuw beschreven fusie-genen zijn toegevoegd aan de groeiende lijst van *MLL* fusie-genen, en verhelderen delen van de processen die bij voorkeur worden verstoord door *MLL*-herschikte AML.

In **hoofdstuk 4** tonen we in een groot internationaal retrospectief *MLL*-herschikt cohort aan dat niet alleen translocatie partners belangrijk zijn voor de prognose van deze ziekte, maar ook specifieke bijkomende cytogenetische afwijkingen. Dit zijn voornamelijk numerieke cytogenetische afwijkingen, zoals trisomie 8 en trisomie 19, die gemakkelijk kunnen worden geïdentificeerd door conventionele karyotypering. Ook bleek dat patiënten met *MLL*-herschikkingen vaak een complex karyotype hebben, maar complex karyotype op zich bleek in *MLL*-herschikte AML geen onafhankelijke prognostische factor te zijn. In andere subgroepen van AML bij kinderen wordt complex karyotype vaak geassocieerd met zeer ongunstige prognose.

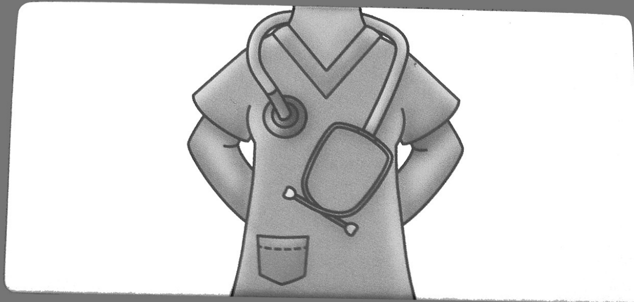
In **hoofdstuk 5** laten we zien dat *CBL* mutaties zeer zeldzaam zijn bij kinderen met AML en niet gecorreleerd zijn aan *MLL*-herschikkingen. Uitschakelen van CBL eiwit expressie door siRNA's, activeerde echter wel de RAS-route in een pediatrische AML cellijn. Wij veronderstellen daarom dat factoren die leiden tot een verlaging van CBL eiwitexpressie, zoals post-translationele processen of versnelde eiwitafbraak, net als mutaties in KRAS en NRAS kunnen leiden tot RAS-activatie.

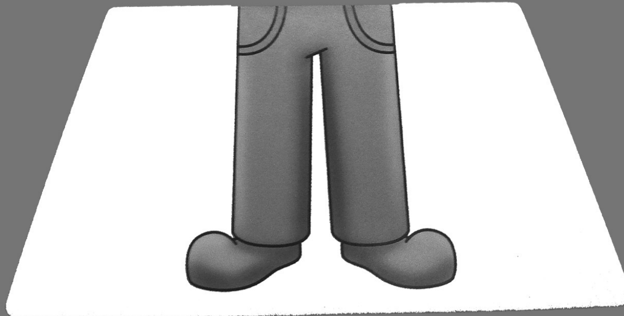
In **hoofdstuk 6** vergelijken we genexpressie profielen van kinderen met t(9;11)(p22;q23) positieve AML met kinderen met AML met een andere *MLL*-translocatie. We tonen aan dat *IGSF4* mRNA en eiwit tot overexpressie komt in *MLL*-herschikte monoblastaire AML patiënten met t(9;11)(p22;q23). Deze overexpressie leek grotendeels gereguleerd door promoter hypomethylatie in deze specifieke subgroep. Binnen de groep van kinderen met *MLL*-herschikte AML toonden we bovendien een betere overleving aan voor patiënten met een hoge *IGSF4* expressie.

Hoofdstuk 7 richt zich op een cohort van kinderen met t(6;11)(q27;q23) positieve AML. Met behulp van genexpressie profilering identificeerden we de unieke overexpressie van *BHLHB3* in deze t(6;11)(q27;q23) positieve AML. Met functionele experimenten konden we echter niet bewijzen dat *BHLHB3* een belangrijk aansturend oncogen is in deze groep patiënten. Verder onderzoek is dan ook vereist in deze subgroep van kinderen met *MLL*-herschikte AML, omdat deze kinderen in de huidige behandelprotocollen een zeer slechte prognose hebben.

In **hoofdstuk 8** rapporteren we een internationale retrospectieve studie in 62 kinderen met AML met t(8;16)(p11;p13). Klinische en biologische gegevens over deze patiënten werden verzameld uit databanken van nationale studiegroepen en uit publicaties in de literatuur. AML patiënten met t(8;16)(p11;p13) waren significant jonger, hadden frequent FAB M4-M5 (myelomonocytaire of monoblastaire) morfologie en daarnaast frequent voorkomen van erythrofagocytose, cutane leukemie en diffuse intravasale stolling. Opvallend is bovendien dat spontane remissies gemeld worden in een aantal neonaten met t(8;16)(p11;p13) AML. De 5-jaars overleving van patiënten met t(8;16)(p11;p13) was vergelijkbaar met andere AML types. Genexpressie profielen van kinderen met t(8;16)(p11;p13) AML lijken in bepaalde opzichten op die van *MLL*-herschikte AML, maar tonen ook unieke kenmerken, zoals hoge expressie van de *RET*, *PERP* en *GGA2* genen.

About the author





List of publications

(Authored and co-authored by Eva Coenen)

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Coenen EA, Zwaan CM, Meyer C, Marschalek R, Pieters R, van der Veken LT, Beverloo HB, van den Heuvel-Eibrink MM, *KIAA1524*: a novel *MLL* translocation partner in acute myeloid leukemia. Leuk Res 2011;35:133-5

Kuipers JE, **Coenen EA**, Balgobind BV, Stary J, Baruchel A, de Haas V, de Bont ES, Reinhardt D, Kaspers GJ, Cloos J, Danen-van Oorschot AA, den Boer ML, Marschalek R, Meyer C, Pieters R, Zwaan CM, van den Heuvel-Eibrink MM, High *IGSF4* expression in pediatric M5 acute myeloid leukemia with t(9;11)(p22;q23), Blood 2011;117:928-35

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Coenen EA, Harbott J, Zwaan CM, Raimondi SC, van den Heuvel-Eibrink MM, 11q23 rearrangements in de novo childhood acute myeloid leukemia. Atlas Genet Cytogenet Oncol Haematol. March 2012. URL:
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Curriculum vitae

Eva Coenen werd op 5 september 1982 geboren te Utrecht en woonde de eerste 18 jaar in De Bilt. Ze doorliep de middelbare school aan het Utrechts Stedelijk Gymnasium van 1994 tot 2000 en verkoos voor haar studie geneeskunde de stad Maastricht. Tijdens haar studie werkte Eva enkele jaren als student-assistent bij de vakgroep Anatomie-Embryologie. Na keuzestage's in de kinderoncologie en primary health (Akosombo, Ghana) koos ze voor een keuzeonderzoek naar de effecten van gesuperviseerde looptherapie bij perifeer arterieel vaatlijden (Atrium Medisch Centrum, Heerlen, supervisor dr. J.A.W. Teijink). Na het afronden van haar studie geneeskunde (cum laude) koos Eva voor een aanstelling als arts-assistent interne geneeskunde in het Diakonessenhuis Zeist, waar haar brede interesse voor het artsenvak goed tot zijn recht kwam. In 2007 verhuisde zij naar Rotterdam om daar als ANIOS kindergeneeskunde bij het Sint Franciscus Gasthuis te beginnen. De wetenschap bleef echter roepen en in 2009 startte ze als arts-onderzoeker in het Erasmus Medisch Centrum Rotterdam. Binnen de vakgroep kinderoncologie/hematologie (hoofd prof.dr. Rob Pieters) deed zij tot eind 2012, onder supervisie van dr. Marry van den Heuvel-Eibrink en dr. Michel Zwaan, onderzoek naar moleculaire afwijkingen van leukemiecellen van kinderen met acute myeloide leukemie. In het bijzonder was haar onderzoek gericht op patiënten met een *MLL*-herschikking. Dit onderzoek wordt beschreven in dit proefschrift. Momenteel is Eva werkzaam als ANIOS kindergeneeskunde op de intensive care van het Sophia Kinderziekenhuis in Rotterdam. Eva is getrouwd met Seppe Koopman.

PhD Portfolio

Summary of PhD training and research
Name PhD student: Eva A. Coenen
ErasmusMC Department: Pediatric Oncology
Research School: Molecular Medicine (MM)
PhD period: 1 January 2009 – 1 January 2013
Promotor: Prof. Dr. R. Pieters
Co-promotors: Dr. M.M. van den Heuvel-Eibrink, Dr. C.M. Zwaan

1. PhD training	Year	Workload (ECTS)
General courses		
Classical Methods for Data Analysis (CC02) (NIHES)	2009	5.7
Biomedical English Writing and Communication	2010-2011	4.0
Basic R course (MM)	2010	1.4
Specific courses		
Microarray Data Analysis using R & Bioconductor Intensive Course (CMSB)	2009	1.4
Molecular Diagnostics (MM)	2009	1.0
Biomedical Research Techniques (MM)	2009	1.5
Photoshop CS3 (MM)	2010	0.3
Browsing Genes and Genomes in Ensembl (MM)	2010	0.6
Indesign workshop (MM)	2012	0.15
Seminars and workshops		
COST workshop on bioinformatics (European Genomics and Epigenomics Study on MDS and AML, EuGESMA), Modena, Italy	2011	1.2
Molecular diagnositics in haematologic malignancies (MODHEM) meeting (oral presentation)	2011	1.2
4th AML-BFM Research Symposium (oral presentation)	2012	1.2
KIKA promovendi dag (oral presentation)	2013	1.2
Annual Molecular Medicine day, ErasmusMC	2009-2012	0.6
Presentations		
See 'Seminars and workshops' and '(Inter)national conferences'		
7 oral presentations at the weekly Pediatric Research Meetings and Pediatric Oncology Research Meetings	2009-2012	4.0
(Inter)national conferences		
51st ASH Annual Meeting, New Orleans, USA	2009	1
7th Bi-annual I-BFM Leukemia Symposium, Antalya, Turkey (oral presentation)	2010	1.6
52nd ASH Annual Meeting, Orlando, USA (oral presentation)	2010	2.0
43rd SIOP Annual Meeting, Auckland, New Zealand (oral and poster presentation)	2011	2.0
54th ASH Annual Meeting, Atlanta, USA (poster presentation)	2012	2.0
17th European Hematology Association (EHA), Amsterdam (poster presentation)	2012	1.6
7th Dutch Hematology Congress (oral presentation)	2013	1.2
Other		
2nd prize publication award Molecular Medicine Day, ErasmusMC	2011	0.1
Travel grant for the 52nd and 54th ASH Annual meeting	2010, 2012	0
Travel grants Awarded by the ErasmusMC Trustfonds	2010-2012	0.2
Writing grant application for KIKA (grant assigned of €100.000)	2011	4.0
2. Teaching	**Year**	**Workload (ECTS)**
Teaching		
Practical and seminar 'Biology of pediatric AML', 3rd year medical students (ErasmusMC, Rotterdam)	2011	2.0
Supervising		
Supervising Derya Ay, student Applied Science (Fontys, Eindhoven)	2011	3.0
Supervising Anne Bras, 4th year medical student (ErasmusMC, Rotterdam)	2012	2.0
Supervising Danique Jacobsen, 1st year medical student (ErasmusMC, Rotterdam)	2012	2.0

Total		**50.15**

Dankwoord / acknowledgements

Vier jaar zijn voorbij, en de tijd is voorbij gevlogen. Dit is mede te danken aan de mensen die ik om me heen had de afgelopen 4 jaar. Er was hulp en steun tijdens het praktisch werk op het lab, aanmoediging en deadlines wanneer het even niet zo makkelijk ging, gezelligheid en plezier in de vrije uurtjes rondom het werk. Mijn puzzel met de *MLL*-herschikkingen in de cellen van kinderen met AML, maakte mij van dokter tot half bioloog. Dit, in combinatie met de bijzondere eigenschap van *MLL*-herschikkingen, namelijk dat het eerste deel van het fusiegen (het hoofd of het bovenlijf) steeds hetzelfde is, maar het tweede deel van het fusiegen (de voeten) varieert tussen de verschillende patiënten, was de inspiratiebron voor de opmaak van de kaft. Inmiddels sta ik al weer voor nieuwe uitdagingen in de kliniek, maar een terugblik op de afgelopen jaren is hier op zijn plaats.

Om te beginnen was dit onderzoek niet mogelijk geweest zonder de medewerking van kinderen met AML en hun ouders. Op het moment dat zij deze levensbedreigende diagnose te horen kregen, gaven ze toestemming voor deelname aan wetenschappelijk onderzoek. In het onderzoek dat beschreven is in dit proefschrift werd gebruik gemaakt van gegevens en materiaal van kinderen met AML uit 20 landen over de hele wereld.

Prof. Dr. Pieters, beste Rob; zonder jou en de goede onderzoeksgroep op het lab was ik hier nooit gekomen. Ik herinner me onze kennismaking nog en de belofte van jouw kant: promoveren is hard werken, we eisen je volle inzet. Deze instelling heeft me in mijn werk veel voldoening gebracht. Jouw tomoloze inzet de afgelopen jaren voor het Prinses Máxima Centrum voor Kinderoncologie geeft aan dat alleen het beste goed genoeg is. Bedankt voor het overbrengen van deze boodschap, waar ik volledig achter sta.

Mijn co-promoter Dr. van den Heuvel-Eibrink, beste Marry; dankzij jou stapte ik in een rijdende trein. Mijn voorganger Brian Balgobind en ik hebben ruim een half jaar samen op de afdeling naast elkaar kunnen werken. Deze dakpanconstructie heeft mij een vliegende start gegeven waarvoor ik je zeer dankbaar ben. Jouw resultaat gerichte blik en enorm hoog werkethos hielden altijd de vaart erin. De laatste twee jaar moest ik mijn manuscripten weliswaar op een groter lettertype afdrukken, maar dat kwam omdat je het anders ’s avonds op de bank (!) niet meer kon lezen. En als ik in zeldzame gevallen meer dan drie dagen moest wachten totdat je stukken had nagekeken, dan kwam dat bijna altijd omdat er iets mis was gegaan bij het printen of verzenden van de mail. Dankzij jou heb ik ook menig internationaal contact gemaakt, waarvan ik hoop nog lang plezier te kunnen hebben. Bedankt!

Mijn tweede co-promoter Dr. Zwaan, beste Michel; het feit dat jij en Marry de AML groep samen leiden, is voor mij altijd een uitkomst geweest. Meer dan eens maakten we, wanneer Marry op vakantie was, vlak voor de deadline een abstract in orde om naar een internationaal congres te sturen. Bovendien was jij dikwijls de eerste ‘reviewer’ van mijn stukken, met een heldere en kritische blik. Ik denk met veel plezier terug aan het SIOP congres van 2011in Auckland, waarvoor we samen binnen een week de wereld rondvlogen, en dat konden voelen in de jetlag die daarbij hoorde.

Als dokter op een lab gaan werken, is vragen om problemen. Nee 'uitdagingen', dat is het juiste woord. Het enige wat hetzelfde blijft is de witte jas! Ik dwaalde het eerste jaar niet alleen figuurlijk door het lab, maar ook letterlijk: ik heb vier werkplekken gehad. In het begin benoemde ik menige vraag als die van een 'domme dokter', want zo voelde het ook. Maar de leercurve was stijl en de handvaardigheid groeide rap. Dat is vooral te danken aan geduldige collega's, Susan in het bijzonder, die het aandurfden mij in te werken. Het is voor mij dan ook niet meer dan logisch geweest om jou als paranymf te vragen. Bedankt! Ook de andere leden van de AML groep waren een grote hulp; Brian voor zijn voorwerk en kennis in het project van *MLL*-herschikte AML, Iris voor al haar kennis in de breedte van kinderAML, en Jenny met wie ik, terwijl zij nog student was, mijn eerste project startte. Ook Nicola, Lonneke, Trudy, Marjolein, Andrica, Anna, Astrid, Malou, Eva B, Jasmijn ("louder") en Daria ("holy mackers") hebben allemaal hun steentje bijgedragen aan dit boekje. Jasmijn is de bedenker van het thema voor de kaft. Ik zal jullie de komende tijd als collega's moeten missen, maar hoop dat de vriendschap blijvend is. Dominique, jij was de schakel die mij bij het lab heeft gebracht. Jij werkte er al even en kende Seppe nog van de geneeskunde studie. Via jou kwam ik in contact met Marry, en werden we collega's. Je bent een voorbeeld geweest in diverse opzichten: je kon vol overgave research bedrijven, geniaal presenteren, en succesvol promoveren. Ook je doorzettingsvermogen bij tegenslag hoop ik een beetje van je te hebben kunnen afkijken. Floor, ook jij was een speciaal persoon voor mij op het lab. Ook jou kende ik reeds eerder en je ging me voor in het zoeken naar de balans tussen het klinisch en biologisch denken. Van de afgelopen promoties, herinner ik me die van jou als één van de sterkste. Ik ben overtuigd dat ook jij spoedig een baan zal vinden waarin je gelukkig wordt. Anne, Danique en Maite; ook een klein woordje aan jullie. Het is oprecht dapper om als geneeskunde student een project op een laboratorium op te pakken, maar jullie hebben dat gedaan. Ik vond het erg leuk om een deel van de begeleiding daarin te mogen doen en zie jullie als krachtige toekomstige collega's.

Het laboratorium kindergeneeskunde is groot, en iedereen is behulpzaam naar elkaar; of je nu voor de 'onco', de 'neo', de 'gastro' of de 'infectie' werkt. Bedankt daarvoor! In het bijzonder ook de andere werkgroepleiders van de 'onco', Jules, Ronald en Monique; bedankt voor jullie kennis en ondersteuning en heel veel succes gewenst met de nog lopende projecten.

Het succes van de afdeling kinderoncologie van het SKZ, wordt mede bepaald door de integratie van de kliniek en het onderzoek. Niet alleen het translationele onderzoek vanuit het lab, maar ook dat van (para)medici naar late effecten is daarbij van belang. De wekelijkse woensdagochtend bespreking bracht ons samen en maakte me wijzer. Bedankt!

Julie, it is not without reason that you are standing next to me at this thesis defense. Thanks to the work of both our supervisors, we became colleagues and friends. Your brave step to include a biological part in your international retrospective study on t(6;9) pediatric AML, brought you to Rotterdam for three weeks. I still remember these days very well, and next to the practical work in the lab, we had a lot of fun. Thank you for the joy you brought in our work ('for some reason I have been smiling the past minutes when I wrote this'), thank you for being my paranymph.

Due to the rarity of the disease, working in the field of pediatric AML requires international collaboration. None of my projects would have been possible without the collaborators from the DCOG, BFM, CPH and LAME and all other collaborators participating in the I-BFM AML study group. I would especially like to thank Prof. Dr. Hasle from Denmark for participating in the opposition at my thesis defense.

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Lieve pap en mam. 'Wat Eva in d'r kop heeft, heeft ze nergens anders', zou mamma zeggen. Ik geloof niet dat mijn eigenwijsheid altijd makkelijk was in de opvoeding, maar ik wil jullie bedanken voor alle wijze lessen die jullie me leerden. 'Nee heb je, ja kun je krijgen', bijvoorbeeld, heeft zich vertaald in hard werken en resultaat. Ik hoop dat jullie na het lezen van dit proefschrift nog wat beter begrijpen wat ik de afgelopen jaren heb gedaan. Bedankt voor alle steun!

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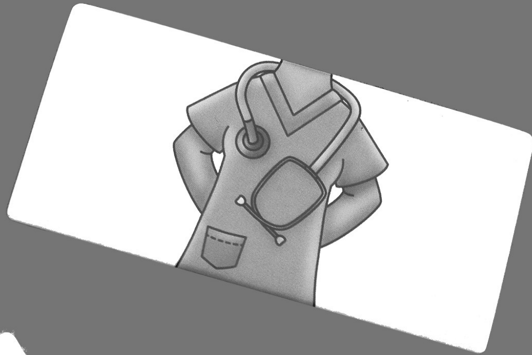
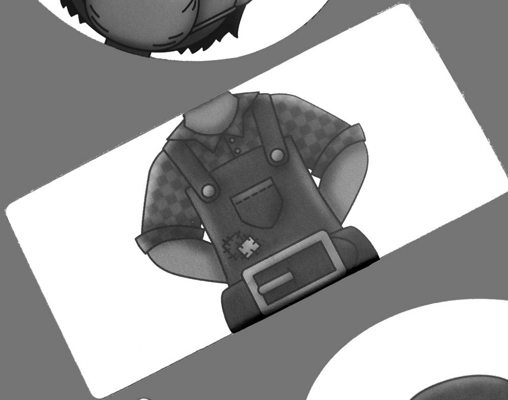
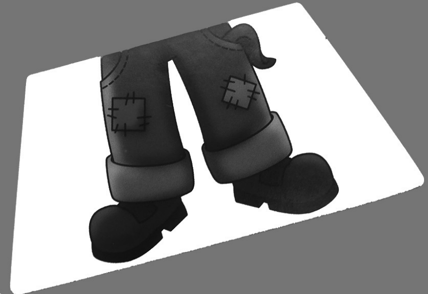
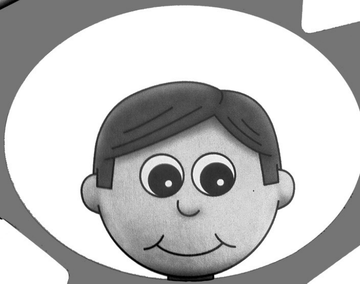
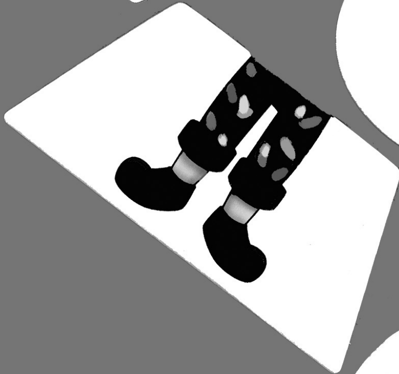
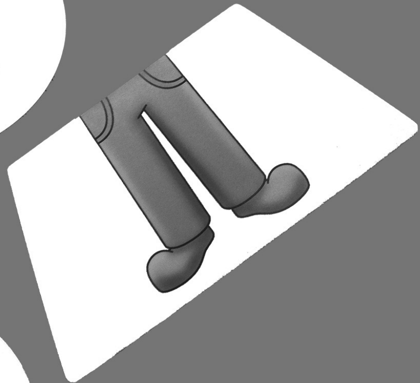
Appendices

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Affiliations co-authors

T.A. Alonzo	Children's Oncology Group (COG), Arcadia, CA, USA
T.C.J.M. Arentsen-Peters	Department of Pediatric Oncology/Hematology, Erasmus MC–Sophia Children's Hospital, Rotterdam
A. Auvrignon	French Leucémie Aiquë Myéloblastique Enfant (LAME), Hopital Trousseau, Paris, France
A. Baruchel	French Leucémie Aiquë Myéloblastique Enfant (LAME), Hopital Trousseau, Paris, France
B.V. Balgobind	Department of Pediatric Oncology/Hematology, Erasmus MC–Sophia Children's Hospital, Rotterdam
H.B. Beverloo	Department of Clinical Genetics, Erasmus MC, Rotterdam
M.L. den Boer	Department of Pediatric Oncology/Hematology, Erasmus MC–Sophia Children's Hospital, Rotterdam
E.S.J.M. de Bont	Pediatric Oncology/Hematology, Beatrix Children's Hospital, University Medical Center Groningen, Groningen
J. Bradtke	Department of Pediatric Hematology and Oncology, Justus-Liebig-University, Giessen, Germany
M. Chang	Children's Oncology Group (COG), Data Center, Gainesville, FL, USA
J. Cloos	Pediatric Oncology/Hematology, VU University Medical Center, Amsterdam
U. Creutzig	Acute Myeloid Leukemia-Berlin-Frankfurt-Münster Study Group (AML-BFM), Pediatric Hematology/Oncology, Medical School Hannover, Hannover, Germany
A.A. Danen-van Oorschot	Department of Pediatric Oncology/Hematology, Erasmus MC–Sophia Children's Hospital, Rotterdam
E.M.C. Driessen	Department of Pediatric Oncology/Hematology, Erasmus MC–Sophia Children's Hospital, Rotterdam
M.N. Dworzak	St. Anna Children's Hospital and Children's Cancer Research Institute, Department of Pediatrics, Medical University of Vienna, Vienna, Austria

E. Forestier — Nordic Society for Pediatric Hematology and Oncology (NOPHO), Department of Clinical Science, Pediatrics, Umeå University, Umeå, Sweden

B. Gibson — Department of Pediatric Oncology/Hematology, Royal Hospital for Sick Children, Glasgow, United Kingdom

O.A. Haas — St. Anna Children's Hospital and Children's Cancer Research Institute, Department of Pediatrics, Medical University of Vienna, Vienna, Austria

V. de Haas — Dutch Childhood Oncology Group (DCOG), The Hague

J. Harbott — Acute Myeloid Leukemia-Berlin-Frankfurt-Münster Study Group (AML-BFM), Department of Pediatric Hematology and Oncology, Justus-Liebig-University, Giessen, Germany

C.J. Harrison — Northern Institute of Cancer Research, Newcastle University, Newcastle upon Tyne, United Kingdom

H. Hasle — Nordic Society for Pediatric Hematology and Oncology (NOPHO), Department of Pediatrics, Aarhus University Hospital Skejby, Aarhus, Denmark

N.A. Heerema — Children's Oncology Group (COG), Department of Pathology, The Ohio State University, Columbus, OH, USA

M. Jeison — Cancer Cytogenetic Laboratory, Pediatric Hematology Oncology Department, Schneider Children's Medical Center of Israel, Petach Tikvah, Israel

D. Johnston — Children's Oncology Group (COG), Arcadia, CA, USA; Children's Hospital of Eastern Ontario, Ottawa, ON, Canada

M.M. van den Heuvel-Eibrink — Department of Pediatric Oncology/Hematology, Erasmus MC–Sophia Children's Hospital, Rotterdam

G.J.L. Kaspers — Pediatric Oncology/Hematology, VU University Medical Center, Amsterdam

J.E. Kuipers — Department of Pediatric Oncology/Hematology, Erasmus MC–Sophia Children's Hospital, Rotterdam

A. Leszl — Italian Association of Pediatric Hematology Oncology (AIEOP), Clinica Pediatrica, Università Padova, Padova, Italy

N. Litvinko — Research Center for Pediatric Oncology and Hematology, Minsk, Belarus

R. Marschalek — Institute of Pharmaceutical Biology, ZAFES, Diagnostic Center of Acute Leukemia (DCAL), Frankfurt, Germany

C. Meyer — Institute of Pharmaceutical Biology, ZAFES, Diagnostic Center of Acute Leukemia (DCAL), Frankfurt, Germany

V. Mihál — Czech Pediatric Hematology/Oncology (CPH), Department of Pediatrics, University Hospital, Palacky´s University, Olomouc, Czech Republic

B. De Moerloose — Belgian Society of Paediatric Haematology Oncology (BSPHO), Ghent University Hospital, Department of Pediatric Hematology/Oncology, Ghent, Belgium

A. Morimoto — Japanese Pediatric Leukemia/Lymphoma Study Group (JPLSG), Department of Pediatrics, Jichi Medical University School of Medicine, Tochigi, Japan

L. Lo Nigro — Italian Association of Pediatric Hematology Oncology (AIEOP), Center of Pediatric Hematology Oncology, Azienda Policlinico, Catania, Italy

C. Perot — French Leucémie Aiquë Myéloblastique Enfant (LAME), Hopital Trousseau, Paris, France

A. Pession — Italian Association of Pediatric Hematology Oncology (AIEOP), Clinica Pediatrica, Università Padova, Padova, Italy

R. Pieters — Department of Pediatric Oncology/Hematology, Erasmus MC–Sophia Children's Hospital, Rotterdam

S.C. Raimondi — St Jude Children's Research Hospital, Memphis, TN, USA

D. Reinhardt — Acute Myeloid Leukemia-Berlin-Frankfurt-Münster Study Group (AML-BFM), Pediatric Hematology/Oncology, Medical School Hannover, Hannover, Germany

J.E. Rubnitz — St Jude Children's Research Hospital, Memphis, TN, USA

S. Savasan	Division of Hematology/Oncology, Children's Hospital of Michigan, Detroit, MI, USA
F.O. Smith	Children's Oncology Group (COG), Hematology/Oncology and Pediatrics, Cincinnati Children's Hospital Medical Center and the University of Cincinnati College of Medicine, Cincinnati, OH, USA
R.W. Stam	Department of Pediatric Oncology/Hematology, Erasmus MC–Sophia Children's Hospital, Rotterdam
J. Stary	Czech Pediatric Hematology/Oncology (CPH), Pediatric Hematology/Oncology, Charles University and University Hospital Motol, Prague, Czech Republic
I. Stasevich	Research Center for Pediatric Oncology and Hematology, Minsk, Belarus
S. Strehl	St. Anna Children's Hospital and Children's Cancer Research Institute, Department of Pediatrics, Medical University of Vienna, Vienna, Austria
J. Swansbury	Department of Clinical Cytogenetics, The Royal Marsden Hospital & The Institute of Cancer Research, Sutton, United Kingdom
T. Taga	Japanese Pediatric Leukemia/Lymphoma Study Group (JPLSG), Department of Pediatrics, Shiga University of Medical Science, Shiga, Japan
K. Terui	Department of pediatrics, Hirosaki University Graduate School of Medicine, Hirosaki, Japan
D. Tomizawa	Japanese Pediatric Leukemia/Lymphoma Study Group (JPLSG), Department of Pediatrics, Tokyo Medical and Dental University, Tokyo, Japan
G. Vaitkeviciene	Clinic for Children's Diseases, Centre for Oncology/Haematology, Vilnius University, Vilnius, Lithuania
L.T. van der Veken	Department of Clinical Genetics, Erasmus MC, Rotterdam
V.H.J. van der Velden	Department of Immunology, Erasmus MC, Rotterdam

D. Webb	Acute Myeloid Leukemia Committee International-Berlin-Frankfurt-Münster Study Group (I-BFM), Great Ormond Street Hospital for Children, London, United Kingdom
M. Winstanley	Department of Pediatric Oncology, Starship Children's Hospital, Auckland, New Zealand
K.F. Wong	Department of pathology, Queen Elizabeth Hospital, Hong Kong, China
Z. Zemanova	Czech Pediatric Hematology/Oncology (CPH), Center of Oncocytogenetics, General University Hospital and First Faculty of Medicine, Charles University, Prague, Czech Republic
M. Zimmermann	Acute Myeloid Leukemia-Berlin-Frankfurt-Münster Study Group (AML-BFM), Pediatric Hematology/Oncology, Medical School Hannover, Hannover, Germany
C.M. Zwaan	Department of Pediatric Oncology/Hematology, Erasmus MC–Sophia Children's Hospital, Rotterdam

Abbreviations

ABI1	abl-interactor 1 (gene)
ABI2	abl-interactor 2 (gene)
ABL1	c-abl oncogene 1, non-receptor tyrosine kinase (gene)
ACA	additional cytogenetic aberrations
AF10	ALL1-fused gene from chromosome 10 protein, also known as MLLT10 (gene)
AF6	ALL1-fused gene from chromosome 6 protein, also known as MLLT4 (gene)
AF9	ALL1-fused gene from chromosome 9 protein, also known as MLLT3 (gene)
AIE	chemotherapy combination given in pediatric AML protocols: cytarabine arabinoside (Ara-C), idarubicin, etoposide
AIEOP	Associazione Italiana Ematologia Oncologia Pediatrica, Italian pediatric oncology study group
AKT	v-akt murine thymoma viral oncogene homolog 1 (gene)
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
AML-BFM-SG	acute myeloid leukemia, Berlin Frankfurt Münster study group
APL	acute promyelocytic leukemia
AU	arbitrary units
BCR	breakpoint cluster region gene (gene)
BHLHB2	basic helix-loop-helix family, member B2, also known as BHLHE40, DEC1, SHARP2 (gene)
BHLHB3	basic helix-loop-helix family, member B3, also known as BHLHE41, DEC2, SHARP1 (gene)
BM	bone marrow
BRD4	bromodomain containing 4 (gene)
BSPHO	Belgian Society of Paedatric Haematology Oncology

c-Abl	c-abl oncogene 1, non-receptor tyrosine kinase, also known as ABL1 (gene)
CBFB	core binding factor beta (gene)
CBL	Cbl proto-oncogene, E3 ubiquitin protein ligase, also known as casitas B-lineage lymphoma proto-oncogene (gene)
CCR	continuous complete remission
cDNA	complementary DNA
CEBPA	CCAAT/enhancer binding protein (C/EBP), alpha (gene)
CIP2A	cancerous inhibitor of PP2A, also known as KIAA1524 (gene)
CIR	cumulative incidence of relapse
CLL	chronic lymphoblastic leukemia
CML	chronic myeloid leukemia
CMML	chronic myelomonocytic leukemia
CN-AML	cytogenetic normal acute myeloid leukemia
CNS	central nervous system
COG	Children's Oncology Group
CPH	Czech Pediatric Hematology/Oncology
CR	complete remission
CREBBP	cAMP responsive element binding protein 1, binding protein (gene)
cRNA	complimentary RNA
Ct	cycle threshold
DCAL	Diagnostic Center of Acute Leukemia
DCOG	Dutch Childhood Oncology Group
DEK	DEK oncogene (gene)
DIC	disseminated intravascular coagulation
DNA	Deoxyribonucleic acid
DNMT3A	DNA (cytosine-5-)-methyltransferase 3 alpha (gene)

DOT1L	DOT1-like, histone H3 methyltransferase (S. cerevisiae) (gene)
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
ED	early death
EDTA	ethylenediaminetetraacetic acid
EFS	event free survival
EGFR	epidermal growth factor receptor (gene)
ELL	elongation factor RNA polymerase II (gene)
EMD	extra medullary disease
ENL	eleven nineteen leukemia, also known as myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 1 (gene)
EP300	E1A binding protein p300 (gene)
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian) (gene)
ERBB3	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian) (gene)
ERK	extracellular regulated MAP kinase, also known as mitogen-activated protein kinase 1 (MAPK1) (gene)
ETV6	ets variant 6 (gene)
EVI1	ecotropic viral integration site 1 (gene)
EZH2	enhancer of zeste homolog 2 (Drosophila) (gene)
FAB	French American British morphology classification
FCS	fetal calf serum
FDR	false discovery rate
FISH	fluorescence in situ hybridization
FLAG	chemotherapy combination given in pedatric AML protocols: fludarabine, high-dose cytarabine, G-CSF
FLAG-IDA	chemotherapy combination given in pedatric AML protocols: fludarabine, high-dose cytarabine, G-CSF and idarubicin

FLT3	fms-related tyrosine kinase 3 (gene)
FLT3-ITD	fms-related tyrosine kinase 3 internal tandem duplication
FLT3-TKD	fms-related tyrosine kinase 3 tyrosine kinase domain mutation
FNBP1	formin binding protein 1 (gene)
GAPDH	glyceraldehyde-3-phosphate dehydrogenase (gene)
G-CSF	Granulocyte colony-stimulating factor
GEP	gene expression profiling
GGA2	golgi-associated, gamma adaptin ear containing, ARF binding protein 2 (gene)
GRB2	growth factor receptor-bound protein 2 (gene)
GTPase	guanosine triphosphate hydrolase
HAM	chemotherapy combination given in pedatric AML protocols: high-dose cytarabine and mitoxantrone
HL60	AML cell line
HLXB9	homeobox gene HB9, also known as MNX1(motor neuron and pancreas homeobox 1) (gene)
HOXA	homeobox A cluster
HOXA10	homeobox A10 (gene)
HOXA11	homeobox A11 (gene)
HOXB	homeobox B cluster
HR	hazard ratio
HSC	hematopoietic stem cell
HSCT	hematopoietic stem cell transplantation
I-BFM	international Berlin-Frankfurt-Münster study group
IDH1	isocitrate dehydrogenase 1 (NADP+), soluble (gene)
IDH2	isocitrate dehydrogenase 2 (NADP+), mitochondrial (gene)
IGSF4	immunoglobulin superfamily member 4, also known as CADM1 and TSLC1 (gene)

INTERFANT	International study group for treatment of infants younger than 1 year with acute lymphoblastic leukaemia
ISCN	International System for Human Cytogenetic Nomenclature
JARID1A	Jumonji, AT rich interactive domain 1A (RBBP2-like), also known as KDM5A (gene)
JMML	juvenile myelomonocytic leukemia
JPLSG	Japanese Pediatric Leukemia/Lymphoma Study Group
kDa	kilo Dalton, measurement of atomic mass commonly used to indicate protein mass
KIAA1524	KIAA1524 gene, also known as CIP2A (gene)
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (gene)
KOCR	Kinderoncologisch centrum Rotterdam, Stichting KOCR is a funding organization for pediatric oncology research and training
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (gene)
LAME	Leucémie Aiguë Myéloblastique Enfant, French pediatric AML study group
LDI-PCR	long distance inverse PCR
LFU	loss to follow-up
MAF	minor allele frequency
MDS	myelodysplastic syndrome
ME1	AML cell line
MEIS1	Meis homeobox 1 (gene)
miR	microRNA, small non-coding RNA molecule
miRNA	microRNA, small non-coding RNA molecule
MKL1	megakaryoblastic leukemia (translocation) 1, also known as MAL (gene)
ML-2	AML cell line
MLH1	mutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli) (gene)
MLL	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila) (gene)

MLL-PTD	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila) partial tandem duplication
MLLT10	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 10, also known as AF10 (gene)
MLLT3	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 3, also known as AF9 (gene)
MLLT4	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 4, also known as AF6 (gene)
MONO-MAC-1	AML cell line
MRC	Medical Research Council
mRNA	messenger RNA
MS-PCR	methylation specific PCR
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MUD	matched unrelated donor
MYC	v-myc myelocytomatosis viral oncogene homolog (avian) (gene)
MYH11	myosin, heavy chain 11, smooth muscle (gene)
MYST3	MYST histone acetyltransferase (monocytic leukemia) 3, also known as KAT6A (gene)
MYST4	MYST histone acetyltransferase (monocytic leukemia) 4, also known as KAT6B (gene)
NBM	normal bone marrow
NOMO-1	AML cell line
NOPHO	Nordic Society of Pediatric Haematology and Oncology, pediatric hematology/oncology study group of the scandinavian coutries (Sweden, Norway, Denmark, Iceland, Finland)
NPM1	nucleophosmin (nucleolar phosphoprotein B23, numatrin) (gene)
NR	non response
NRAS	neuroblastoma RAS viral (v-ras) oncogene homolog (gene)
NSC	non silencing control

NSD1	NSD1 nuclear receptor binding SET domain protein 1 (gene)
NUP214	nucleoporin 214kDa (gene)
NUP98	nucleoporin 98kDa (gene)
OCI-AML-3	AML cell line
OS	overall survival
P53	tumor protein p53 (gene)
PCR	polymerase chain reaction
pERK	phophorylated extracellular regulated MAP kinase protein
PERP	TP53 apoptosis effector Related to PMP22 (gene)
PI	propidium iodide
PML	promyelocytic leukemia
PP2A	protein phosphatase 2A activator, regulatory subunit 4 (gene)
PR	partial response
PSF	penicillin, streptomycin, fungizone
PTPN11	protein tyrosine phosphatase, non-receptor type 11 (gene)
Rap1A	RAP1A, member of RAS oncogene family (gene)
RARA	retinoic acid receptor, alpha (gene)
RBM15	RNA binding motif protein 15 (gene)
RET	ret proto-oncogene (gene)
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute medium, cell culture medium
RPN1	ribophorin I (gene)
RT-PCR	Reverse transcription PCR
RT-qPCR	quantitative real time PCR
RUNX1	runt-related transcription factor 1 (gene)
RUNX1T1	runt-related transcription factor 1; translocated to, 1 (gene)

SCT	stem cell transplantation
SHI1	AML cell line
shRNA	short hairpin RNA
siRNA	small interfering RNA
SJCRH	St. Jude Children's Research Hospital
SNP	Single-nucleotide polymorphism
SOS1	son of sevenless homolog 1 (Drosophila) (gene)
TET2	tet methylcytosine dioxygenase 2 (gene)
THP1	AML cell line
TMD	transient myeloproliferative disorder
TP	translocation partner
TRIM32	tripartite motif containing 32 (gene)
VSN	variance stabilization normalization
WBC	white blood cell count
WHO	world health organization
WT1	Wilms tumor 1 (gene)

Supplementary data

Chapter 4 supplementary data

Supplementary Table S1: Definitions used in ACAs classification

Aberration	• Count 1 for each aberration separated from the rest of the karyotype by a comma, regardless of the complexity • Count each aberration only once (in multiple clones) • Constitutional abnormality: count 0, if uncertain count 1 • Clonal tetraploidy or triploidy: count 1
ACAs cases	all cases harboring 2 or more aberrations
Complex karyotype	all cases harboring 3 or more aberrations
Numerical ACAs	loss or gain of a complete chromosome. Cases also harboring a marker chromosome were excluded for numerical losses
Structural ACAs	all aberrations that include breakpoints within the chromosomes
Partial chromosomal loss or gain	all unbalanced structural aberrations that result in loss or gain of chromosomal material, not being a complete chromosome
Balanced translocation	exchange of material from one chromosome to another and vice versa without loss of material (as observed by conventional karyotyping)
Balanced karyotype	karyotype of a patient which contains two complete copies of all autosomes and complete copies of sex chromosomes, without additional material (2n)

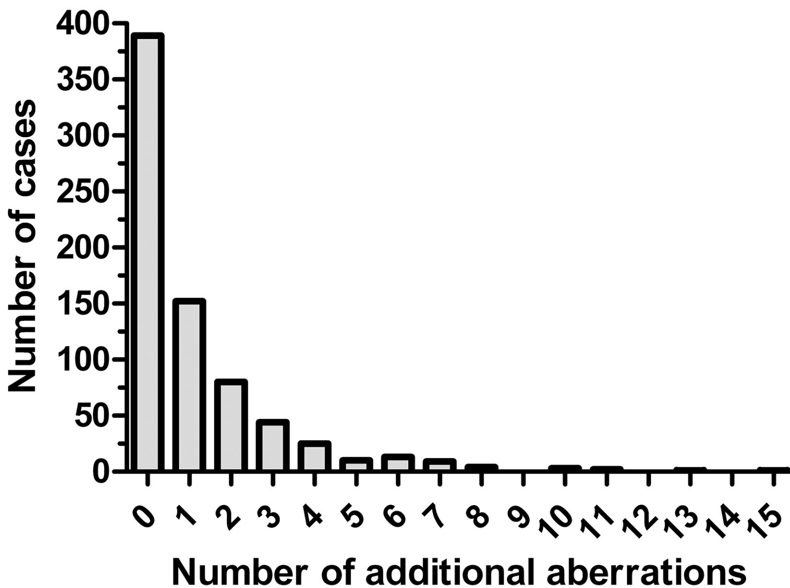
Abbreviations: *ACAs indicates additional cytogenetic aberrations.*

Supplementary Table S2: Multivariate survival analysis of the t(9;11)(p22;q23) subgroup by using the Cox proportional hazards model

	Cox proportional hazards model								
	EFS			OS			Relapse incidence		
	HR	**CI**	***P***	**HR**	**CI**	***P***	**HR**	**CI**	***P***
Trisomy									
no trisomy	1	reference		1	reference		1	reference	
8	0.68	(0.37-1.25)	0.214	0.75	(0.37-1.51)	0.425	0.56	(0.32-0.99)	0.046*
19	2.12	(1.08-4.18)	0.029*	3.02	(1.44-6.34)	0.003†	2.24	(1.08-4.64)	0.029*
21	0.91	(0.44-1.89)	0.794	0.90	(0.40-1.99)	0.785	0.68	(0.32-1.44)	0.314
Type									
no ACAs	1	reference		1	reference		1	reference	
numerical	1.32	(0.78-2.23)	0.292	1.38	(0.74-2.57)	0.310	1.67	(1.01-2.76)	0.045*
structural	1.39	(0.94-2.06)	0.102	1.41	(0.88-2.26)	0.148	1.08	(0.77-1.51)	0.650
FAB									
non **M5**	1	reference		1	reference		1	reference	
M5	0.47	(0.33-0.69)	0.000†	0.48	(0.31-0.75)	0.001†	0.75	(0.53-1.06)	0.106
Age									
2-9 yr	1	reference		1	reference		1	reference	
<2 yr	1.68	(1.13-2.50)	0.011*	1.92	(1.18-3.12)	0.008†	1.21	(0.89-1.64)	0.220
≥10 yr	1.45	(0.92-2.29)	0.110	1.61	(0.93-2.81)	0.090	1.22	(0.86-1.73)	0.272

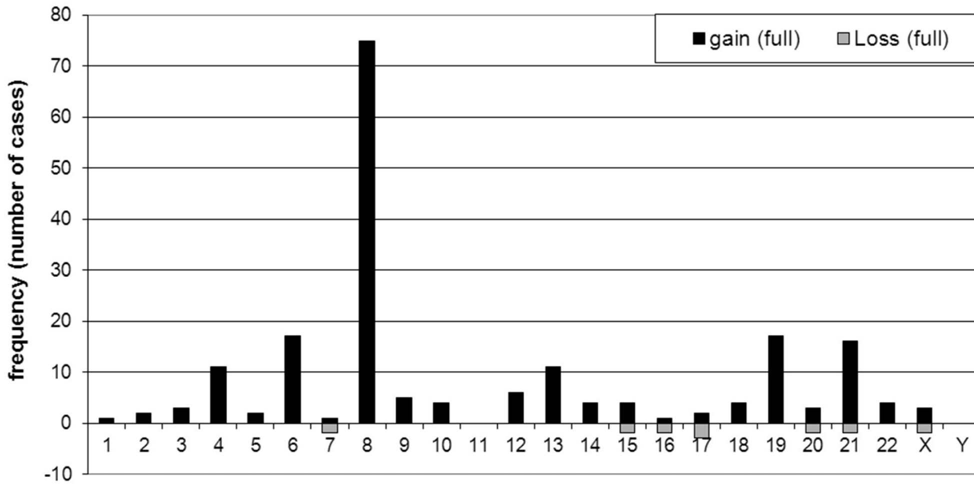
Note: *Results of 3 independent analyses.* ***Abbreviations:*** *EFS indicates event-free survival; OS, overall survival HR, hazard ratio; CI, 95% confidence interval. * Values significant at P<0.05 level. † Values significant at P<0.01 level.*

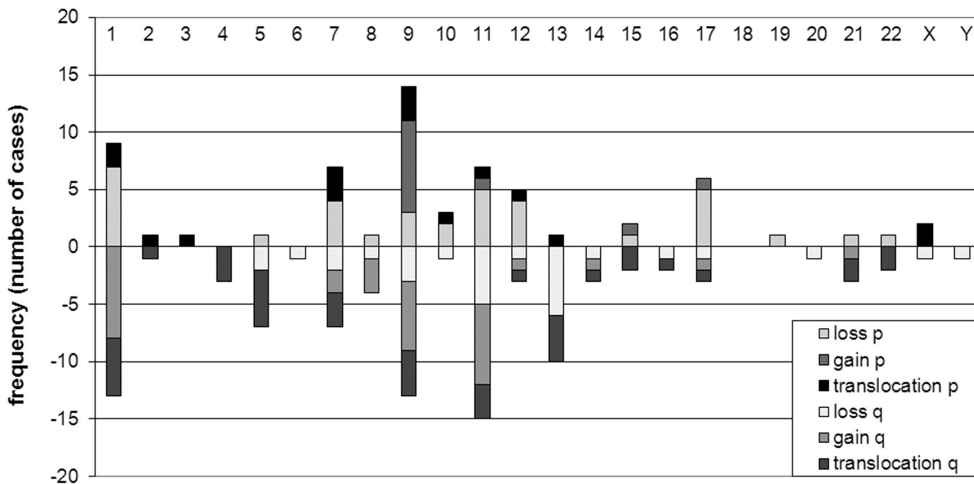
Supplementary Figure S1: Histogram showing number of additional aberrations found in 733 cases



Note: *Number of additional aberrations are on the X-axis, number of cases are on the Y-axis. All aberrations additional to a balanced 11q23/MLL-rearrangement are included in the total number of additional aberrations.*

Supplementary Figure S2: Frequency (number of cases) of numerical ACAs (A) and *structural ACAs (B) in the t(9;11)(p22;q23) subgroup.*





Note: *In figure 3A gains are shown on the positive Y-axis, losses are shown on the negative Y-axis. Chromosomes are on the X-axis; figure 3B, the short arms (p) of the chromosomes are shown on the positive Y-axis, the long arms (q) on the negative Y-axis. Lightest shades are used for losses, medium shaded colors are used for gains, darkest shaded colors for translocation breakpoints. Chromosomes are on the X-axis. Balanced 11q23 translocations are not included in the figure.*

Chapter 5 supplementary data

Supplementary Table S1 Sequences of primers and probes.

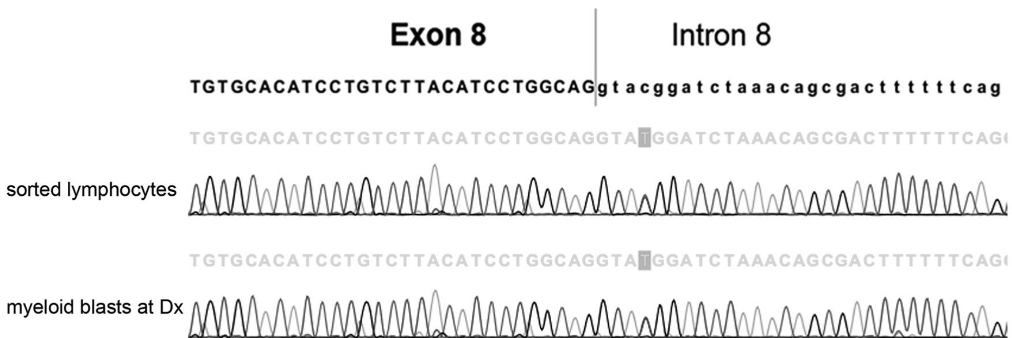
Primer/Probe	Sequence (5'-3')
MLL Forward	CGT CGA GGA AAA GAG TGA
AF6 Reverse	TCC CGA TCA TCT TTG TTC
AF10 Reverse	CTG GAA ATT TGC ATT TGT AA
AF9 Reverse	ATG TTT CCA GGT AAC TCT GTA GT
ENL Reverse	TAC CCC GAC TCC TCT ACT T
ELL Reverse	CCC ATG ACT GGA GAC ATA CT
AF4-Reverse	CTG-GGG-TTT-GTT-CAC
CBL-8F	ACC CAG ACT AGA TGC TTT CTG
CBL-8R	AGG CCA CCC CTT GTA TCA GT
CBL-9F	TTC AGA TGC ATC TGT TAC TAT CT
CBL-9R	AGT GTT TTA CGG CTT TAG AAG ACA
CBL-FW (RT-qPCR)	GCC GCC TTC TCC ATT CT
CBL-RV (RT-qPCR)	CAG GGG GCA GTT TGT CTC
GAPDH Forward	GTC GGA GTC AAC GGA TT
GAPDH Reverse	AAG CTT CCC GTT CTC AG
GAPDH Probe	(FAM)-RCA ACT ACA TGG TTT ACA TGT TCC AA (TAMRA)
CBL siRNA J-003003-09	AAUCAACUCUGAACGGAAA
CBL siRNA J-003003-10	GACAAUCCCUCACAAUAAA
CBL siRNA J-003003-11	UAGCCCACCUUAUAUCUUA
CBL siRNA J-003003-12	GGAGACACAUUUCGGAUUA

Note: for siRNAs described sequences are target sequences.

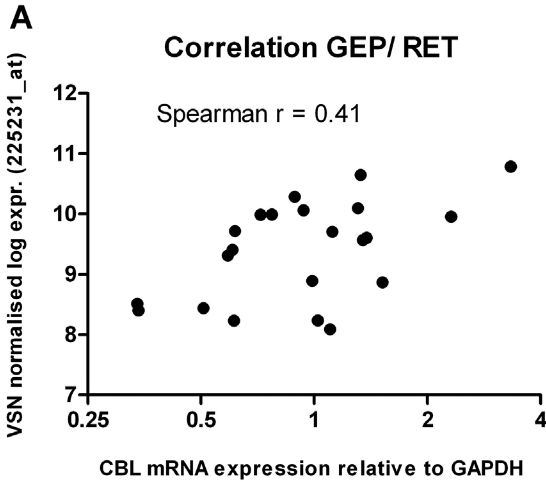
CBL PCR conditions: 95ºC for 2 minutes, 35 cycles of 94ºC for 40 seconds, 58ºC for 30 seconds, and 72ºC for 40 seconds, one cycle of 72ºC for 5 minutes.

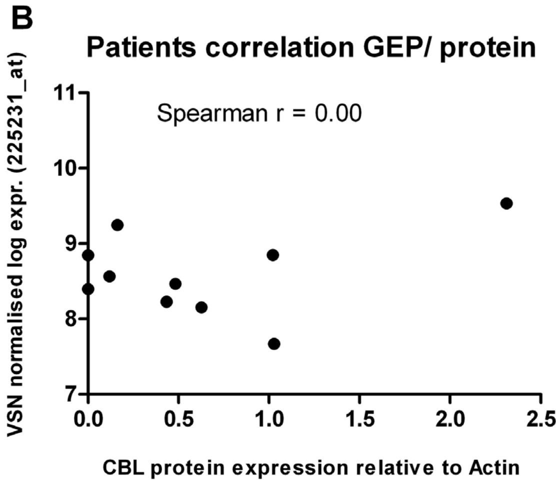
CBL RT-q-PCR conditions: 50 ºC for 2 minutes, 95ºC for 10 minutes, 40 cycles of 95ºC for 15 seconds, 60ºC for 60 seconds.

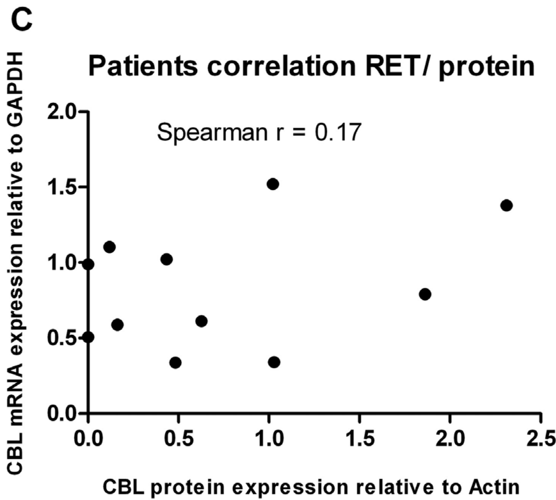
Supplementary Figure S1 Mutation analysis of sorted lymphocytes and myeloid blasts at diagnosis of case 2.



Note: *Both cell fractions have the same point mutation at the exon 8-intron 8 splice site, exon 8 +4C>T.* ***Abbreviations:*** *Dx, diagnosis. (A color version of this figure can be found in the appendices)*







Supplementary Figure S2 Correlation of protein and mRNA expression.

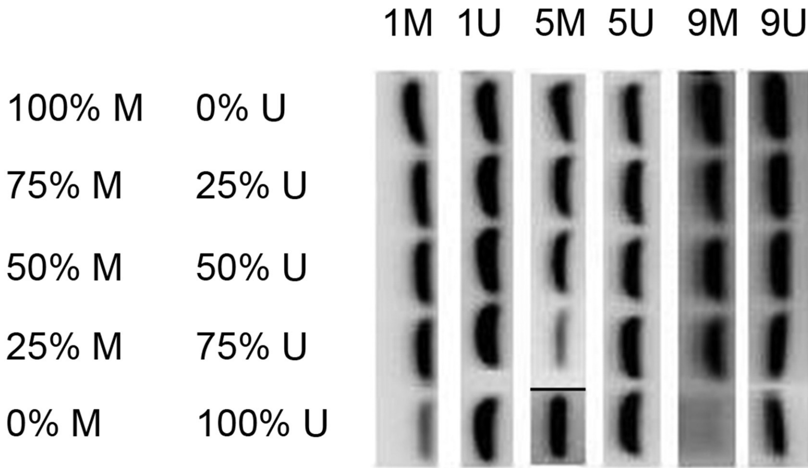
Note: *graph showing CBL mRNA expression from GEP (probe set 225231_at) in correlation with mRNA expression measured by RT-qPCR (A); CBL mRNA expression from GEP (probe set 225231_at) in correlation with protein expression from western blot (B); and CBL mRNA expression measured by RT-qPCR in correlation with protein expression from western blot (C). Panel A shows data from 10 patient samples and 12 cell lines, panel B shows data from 10 patient samples and panel C from 11 patient samples.* ***Abbreviations:*** *GEP, gene expression profiling; RET, relative expression calculation from RT-qPCR relative to GAPDH levels.*

Chapter 6 supplementary data

Supplementary Table 1: Primer and Probe sequences.

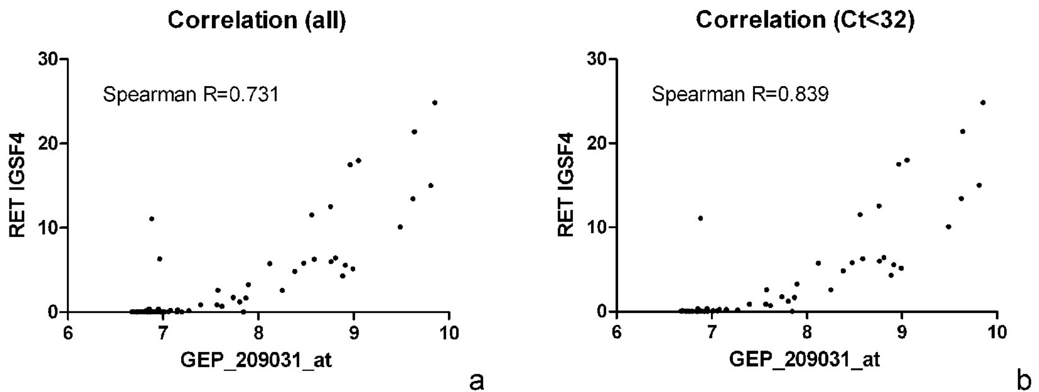
Primer/Probe	Sequence (5'-3')
MLL Forward	CGT CGA GGA AAA GAG TGA
AF6 Reverse	TCC CGA TCA TCT TTG TTC
AF10 Reverse	CTG GAA ATT TGC ATT TGT AA
AF9 Reverse	ATG TTT CCA GGT AAC TCT GTA GT
ENL Reverse	TAC CCC GAC TCC TCT ACT T
ELL Reverse	CCC ATG ACT GGA GAC ATA CT
IGSF4 Forward	TGA TGG GCA GAA TCT GTT
IGSF4 Reverse	GAG CTG GCA AAA GTA TCT TC
MSP region 1 *IGSF4* methylated forward	GAA AAT TTT AGA ATT CGA TTT TAC G
MSP region 1 *IGSF4* methylated reverse	AAA ATA CAT ACG TAC TTT ACA CG
MSP region 1 *IGSF4* unmethylated forward	GAA AAT TTT AGA ATT TGA TTT TAT G
MSP region 1 *IGSF4* unmethylated reverse	AAA AAA ATA CAT ACA TAC TTT ACA CA
MSP region 5 *IGSF4* methylated forward	AAG GGA GAT TTT TTA GTC GTC
MSP region 5 *IGSF4* methylated reverse	CGA ATT TTA CTT TCC CCG AA
MSP region 5 *IGSF4* unmethylated forward	AAG GGA GAT TTT TTA GTT GTT G
MSP region 5 *IGSF4* unmethylated reverse	AAT TCA AAT TTT ACT TTC CCC AAA
MSP region 9 *IGSF4* methylated forward	TTA GTT GTT CGT TCG GGT TTC GG
MSP region 9 *IGSF4* methylated reverse	CGC ACA CTA AAA TCC GCT CGA
MSP region 9 *IGSF4* unmethylated forward	TTA GTT GTT TGT TTG GGT TTT GGA GG
MSP region 9 *IGSF4* unmethylated reverse	CAC CAC ACA CTA AAA TCC ACT CAA
GAPDH Forward	GTC GGA GTC AAC GGA TT
GAPDH Reverse	AAG CTT CCC GTT CTC AG
GAPDH Probe	(FAM)-RCA ACT ACA TGG TTT ACA TGT TCC AA (TAMRA)
	`Target sequence
IGSF4 siRNA J-016565-05	CGAAAGACGUGACAGUGAU
IGSF4 siRNA J-016565-06	GUAAUCUGAUGAUCGAUAU
IGSF4 siRNA J-016565-07	AAAGCUCACUCGGAUUAUA
IGSF4 siRNA J-016565-058	GCGCUUGAGUUAACAUGUG

Supplementary Figure 1: MS-PCR primer test IGSF4.



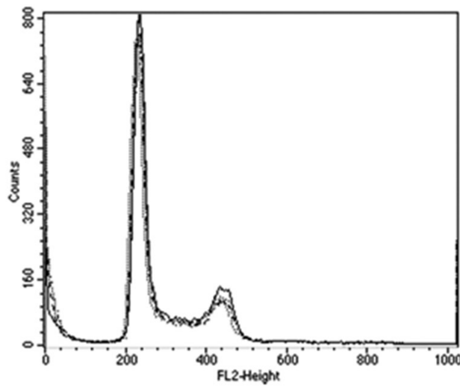
The MS-PCR primers targeting IGSF4 were tested for specificity with a dilution range of a mix with methylated (M) and unmethylated (U) bisulfate converted DNA. On the left the composition of the used DNA mix, on the top the different primer pairs.

Supplementary Figure 2: Correlation of IGSF4 expression measured by GEP and RT-qPCR.



These figures show the correlation of IGSF4 expression measurements by GEP and RT-qPCR. On the X-axis VSN normalized and log-transformed expression as measured by gene expression profiling on micro array (probe 209031_at) is shown. On the Y-axis IGSF4 expression relative to GAPDH (Relative Expression (RET) in percentage) measured by RT-qPCR is shown. When all acquired data were used, 78 data pairs could be analyzed, resulting in a Spearman R of 0.731 with p=0.01(panel a). When the pairs that had raw ct-values >32 were excluded, 56 pairs could be analyzed, resulting in Spearman R=0.839 with p=0.01 (panel b).

Supplementary Figure 3: PI-staining showing cell cycle phase and apoptosis 48 hours after transfection.



	No	Electroporation	NT	IGSF4
Apoptosis	8,85	17,47	17,18	14,21
G0/1	52,91	50,55	48,9	52,09
S	13,96	11,39	12,75	12,16
G2/M	18,66	14,81	15,09	15

Percentage of cells in specific cell cycle phase

Figure showing PI staining in cell line MONO-MAC-1 48 hours after transfection with siRNA. Four lines are shown: untreated control (No, solid line), electroporation only (Electroporation, dotted line), transfection with non-targeting siRNA (NT, dashed line) and transfection with IGSF4 siRNAs (IGSF$, mixed dashed/ dotted line). The profile of all conditions did not show significant differences. Fractions of the cells that were in sub-G0-phase (apoptosis), G0/1-phase, S-phase or G2/M phase are shown in the table on the right.

Supplementary Figure 4: Drug response profile after transfection: AML drug panel.

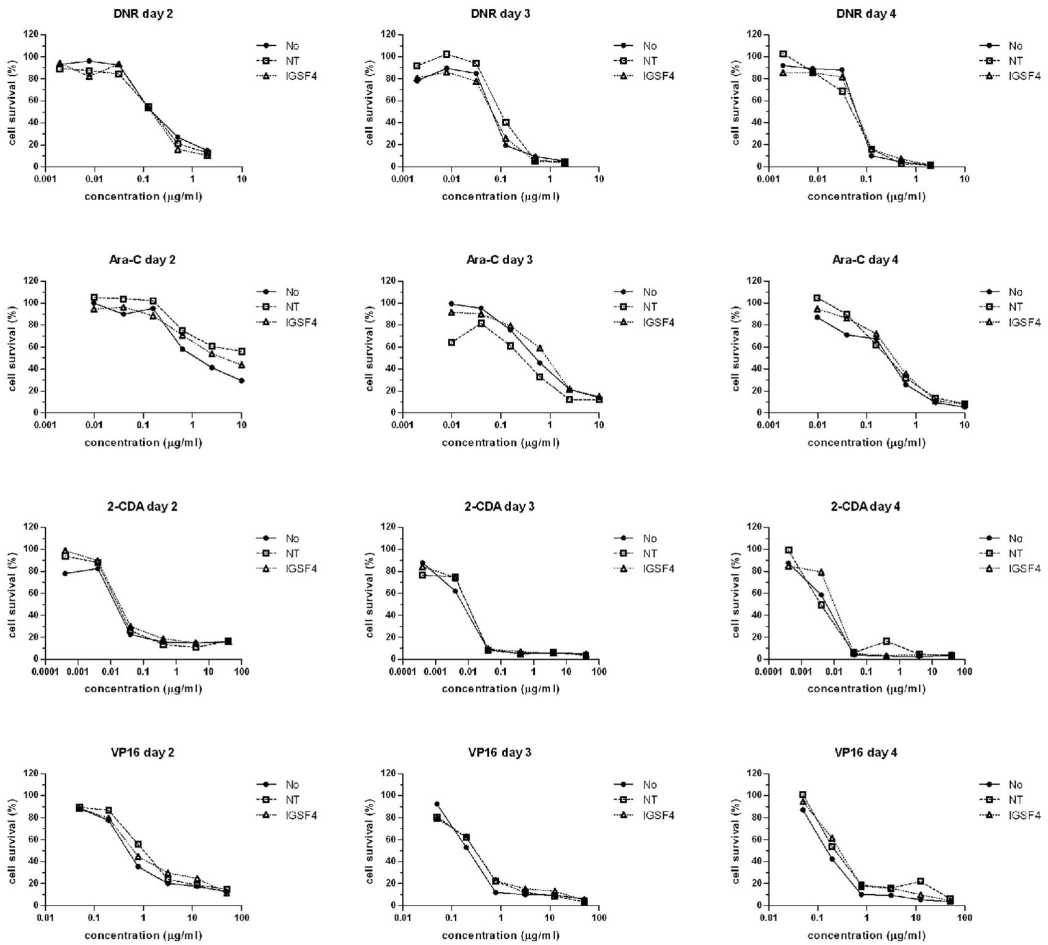


Figure showing results of drug resistance test for cell line MONO-MAC-1 after transfection with siRNA. No: control condition, NT: transfection with non-targeting control siRNA, IGSF4: transfection with IGSF4 siRNAs. Cells were incubated for 2, 3 and 4 days respectively. DNR: daunorubicine, Ara-C: cytosine arabinoside, 2-CDA: cladribine, VP16: etoposide. We did not find significant differences for any of the drugs at any time point.

Supplementary Figure 5: Drug response profile after transfection: ALL drug panel.

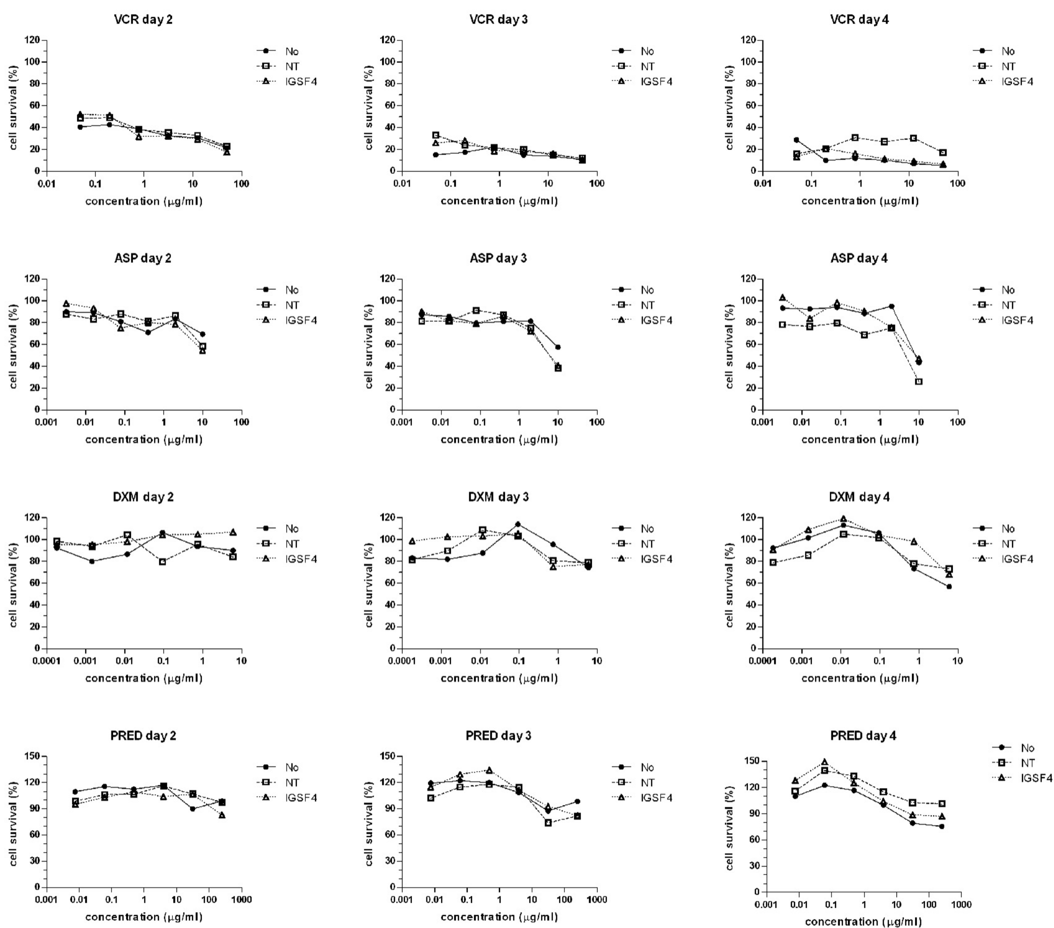


Figure showing results of drug resistance test for cell line MONO-MAC-1 after transfection with siRNA. No reflects control condition, NT transfection with non-targeting control siRNA and IGSF4 transfection with IGSF4 siRNAs. Cells were incubated for 2, 3 and 4 days respectively. VCR: vincristine, ASP: L-Asparaginase, PRED: prednisolone, DXM: dexamethason. We did not find significant differences for any of the drugs at any time point.

Chapter 7 supplementary data

Supplementary Table S1: Sequences of primers and shRNA constructs.

Primer/Probe	Sequence (5'-3')
BHLHB3_ex1_FW	GGG GGG GGA CCA AC
BHLHB3_ex1_RV	TCG CCA GCT CCA TAC C
BHLHB3_ex2_FW	CCC TTG CGA ATA TCT GTC TA
BHLHB3_ex2_RV	TTG CGG CGA GAG CT
BHLHB3_ex3_FW	CTT CCC CAA CTG TGA ATT TT
BHLHB3_ex3_RV	AAC ACG CCC TTG GAG AG
BHLHB3_ex4_FW	TCC CGC AAA CTG GAG TA
BHLHB3_ex4_RV	GGG CTC GTT CCA ATG A
BHLHB3_ex5.1_FW	CCC CGC TTC TCT CGT
BHLHB3_ex5.1_RV	GAC GGG CAC GCA GTA
BHLHB3_ex5.2_FW	CCC CCT GCC TGG AG
BHLHB3_ex5.2_RV	CCG CCG GGT ACA GAT A
BHLHB3_ex5.3_FW	AGC CCT TCC TGG ACA AG
BHLHB3_ex5.3_RV	GCG GGA TGA GCA AAA C
BHLHB3_FW	AGG CGT TCC ACA AAC AG
BHLHB3_RV	TGG CCT TCT CAT CCA TC
P53_ex5_FW	CCG TCT TCC AGT TGC TTT
P53_ex5_RV	CAA CCA GCC CTG TCG T
P53_ex6_FW	GCA CAT GAC GGA GGT TG
P53_ex6_RV	AGG AGA AAG CCC CCC TA
P53_ex7_FW	CGG TGG AGC TTG CAG T
P53_ex7_RV	CCG GGG ATG TGA TGA G
P53_ex8_FW	GGT TTT TTA AAT GGG ACA GG
P53_ex8_RV	TAG GAA AGA GGC AAG GAA AG
	Target sequence
shNSC	SHC002 MISSION Non-Target shRNA Control Vector
sh5101	CCT ACA AAT TAC CGC ACA GAT
sh5104	GCA TCA GAA GAT AAT TGC TTT

Supplementary Table S2: Characteristics P53 mutated samples.

Patient ID	Sample time	P53 mutation	Karyotype	Type I mutation	Type II mutation	Age at Dx.	Clinical course
3460	initial	exon 5, C176Y heterozygous	50,XY,+X,+6,+7,+19[13]/46, XY[2]	KRAS	NUP98-JARID1A	5.9	CR, relapse, death, OS 16 months
4499	initial	exon 5, R181H heterozygous	46,XY,t(15;17)(q22;q21)	-	PML-RARα	7.1	CR, no events, OS 42+ months
8358	second relapse	exon 8, R273C heterozygous	47,XY,+6	-	MLL-AF6	12.9	CR, relapse, SCT, 2nd relapse, death, OS 9 months

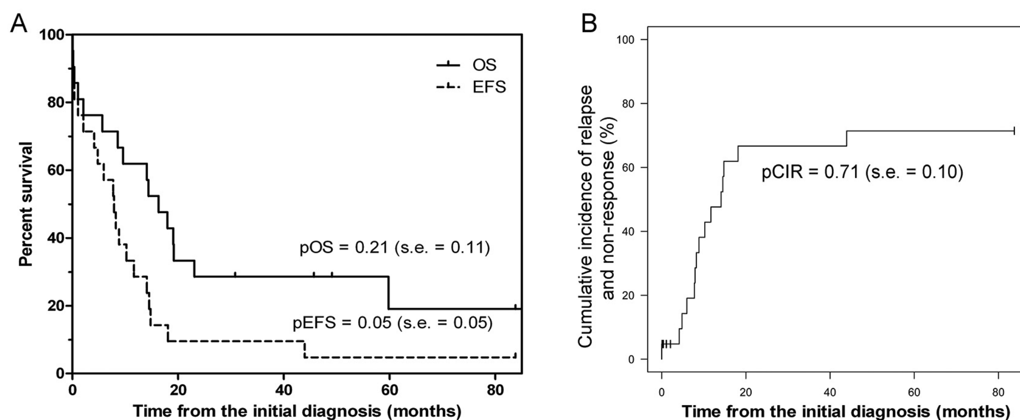
Note: *in the column of P53 mutation the exon number and protein changes are given, age at diagnosis is given in years.* ***Abbreviations:*** *Dx indicates diagnosis; CR, complete remission; SCT, stem cell transplantation; OS, overall survival; + in clinical course indicates the patient was alive at last follow-up.*

Supplementary Table S3: Allele frequency of rs11048413 in pediatric AML and control samples.

	t(6;11)	MLL	t(8;21)	inv(16)	t(15;17)	t(7;12)	CN	Other	Unknown	Remission	CD34+	NBM/ NPB	Overall AML
Samples	20	15	9	6	5	4	13	9	2	18	3	17	83
Minor allele	22	15	4	11	6	6	17	4	2	18	1	10	87
Major allele	18	15	14	1	4	2	9	14	2	18	5	24	79
MAF	0.55	0.50	0.22	0.92	0.60	0.75	0.65	0.22	0.50	0.50	0.17	0.29	0.52

Results from mutation screening on rs11048413 is shown per cytogenetic subgroup. Samples indicates the number of samples per group; Minor allele, the frequency of the minor allele; Major allele, the frequency of the major allele; and MAF, the minor allele frequency as a proportion of all alleles. MLL reflects the results of samples of MLL-rearranged patients with a translocation other than t(6;11)(q27;q23), CN indicates cytogenetically normal AML; other, AML samples with miscellaneous cytogenetics; unknown, AML samples with unknown cytogenetics; CD34+, healthy CD34+ bone marrow cells; NMB/NPB, normal bone marrow and normal peripheral blood.

Supplementary Figure S1: Survival of pediatric t(6;11)(q27;q23) AML patients.



*In **panel A** overall survival (OS) and event free survival (EFS) curves are shown for 21 pediatric t(6;11)(q27;q23) AML patients, in **panel B** cumulative incidence of relapse and non-response (CIR) is shown for 21 pediatric t(6;11)(q27;q23) AML patients.*

Chapter 8 supplementary data

Supplementary Table S1: Primers and Probes sequences.

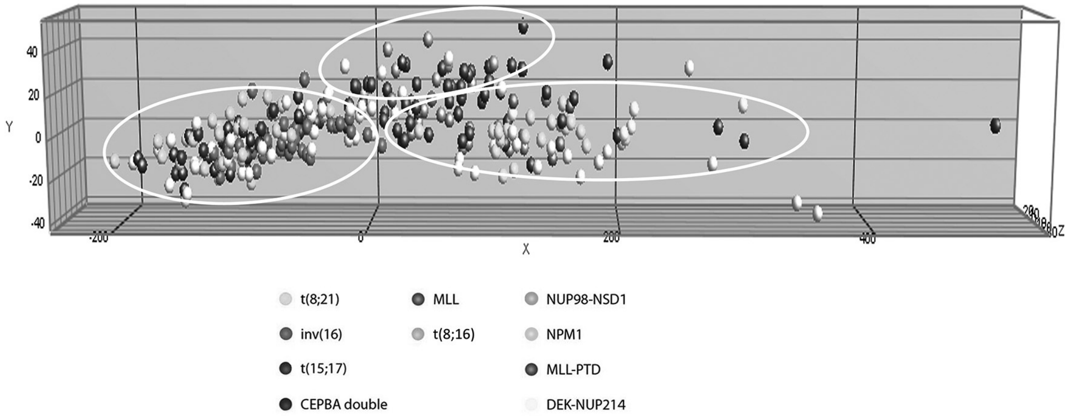
Primer/Probe	**Sequence (5'-3')**
GGA2 Forward	GTG-CGG-TGG-AGG-AAG-TG
GGA2 Reverse	TTC-CGC-GAG-TGC-ATC-AT
PERP Forward	CGG-CTG-CCA-TGC-TCT-T
PERP Reverse	TCA-GCA-GCA-GCG-ATT-TTC
GAPDH Forward	GTC-GGA-GTC-AAC-GGA-TT
GAPDH Reverse	AAG-CTT-CCC-GTT-CTC-AG
GAPDH Probe	(FAM)-CA-ACT-ACA-TGG-TTT-ACA-TGT-TCC-AA-(TAMRA)
shRNA 7107 (PERP)	target sequence: GCTTTCCTTTAAGTGTGAAAT
shRNA 7108 (PERP)	target sequence: CGTGAAGTACACCCAGACCTT
shRNA 7109 (PERP)	target sequence: CCAGATCATCTCCCTGGTAAT
shRNA 7112 (GGA2)	target sequence: GCTTCTGACAAGGCTTCTAAA
shRNA 7113 (GGA2)	target sequence: GCTTATCAGATGCTGAAGAAA
shRNA 7114 (GGA2)	target sequence: GCATGGTTTCTGGTCAGAATT
shRNA 7115 (GGA2)	target sequence: CCGTGCCCTCTGAATTATGTT
shRNA 7116 (GGA2)	target sequence: CTTACGGTACAAGCTGACATT

Supplementary Table S2: Top-50 most discriminative probe sets from a linear regression model.

Probe	Name	adj.P.Val
238107_at	--	2,43E-50
217540_at	--	1,18E-29
243606_at	--	6,6E-28
202834_at	angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	1,86E-25
205445_at	Prolactin	7,15E-25
239641_at	Friend leukemia virus integration 1	1,28E-24
222392_x_at	PERP, TP53 apoptosis effector	8,11E-24
203722_at	aldehyde dehydrogenase 4 family, member A1	4,65E-21
213153_at	SET domain containing 1B	8,1E-21
208914_at	golgi associated, gamma adaptin ear containing, ARF binding protein 2	3,84E-19
204381_at	low density lipoprotein receptor-related protein 3	4,41E-19
229257_at	trinucleotide repeat containing 18	1E-18
219872_at	chromosome 4 open reading frame 18	6,38E-18
33197_at	myosin VIIA	3,97E-16
213436_at	cannabinoid receptor 1 (brain)	2,06E-15
213772_s_at	golgi associated, gamma adaptin ear containing, ARF binding protein 2	5,8E-14
226796_at	hypothetical protein LOC116236	6,63E-13
222799_at	WD repeat domain 91	1,07E-13
217744_s_at	PERP, TP53 apoptosis effector	1,49E-12
206277_at	purinergic receptor P2Y, G-protein coupled, 2	1,6E-12
205879_x_at	ret proto-oncogene	6,4E-12
210658_s_at	golgi associated, gamma adaptin ear containing, ARF binding protein 2	1,4E-11
223204_at	chromosome 4 open reading frame 18	1,51E-11
235147_at	SATB homeobox 2	1,6E-11
207156_at	histone cluster 1, H2ag	8,75E-11
1555124_at	hypothetical protein MGC40574	2,01E-10
230888_at	WD repeat domain 91	2,07E-10
219937_at	thyrotropin-releasing hormone degrading enzyme	2,54E-10
227002_at	family with sequence similarity 78, member A	4,27E-10
243011_at	family with sequence similarity 55, member C	4,74E-10
208913_at	golgi associated, gamma adaptin ear containing, ARF binding protein 2	5,85E-10
235030_at	family with sequence similarity 55, member C	1,21E-09
206079_at	choroideremia-like (Rab escort protein 2)	1,51E-09
213823_at	homeobox A11	1,75E-09
205573_s_at	sorting nexin 7	2,61E-09
208915_s_at	golgi associated, gamma adaptin ear containing, ARF binding protein 2	4,8E-09
224393_s_at	cat eye syndrome chromosome region, candidate 6	4,8E-09
219288_at	chromosome 3 open reading frame 14	1,47E-08
210436_at	chaperonin containing TCP1, subunit 8 (theta)	1,82E-08
216699_s_at	kallikrein 1	1,82E-08
1559049_a_at	BAH domain and coiled-coil containing 1	1,99E-08
239934_x_at	homeo box A11, antisense	5E-08
223658_at	potassium channel, subfamily K, member 6	7,09E-08
244387_at	--	1,16E-07
204458_at	lysophospholipase 3 (lysosomal phospholipase A2)	1,35E-07
219126_at	PHD finger protein 10	3,54E-07
213147_at	homeobox A10	4,81E-07
65718_at	G protein-coupled receptor 124	5,14E-07
220230_s_at	cytochrome b5 reductase 2	5,51E-07
222101_s_at	dachsous 1 (Drosophila)	6,99E-07

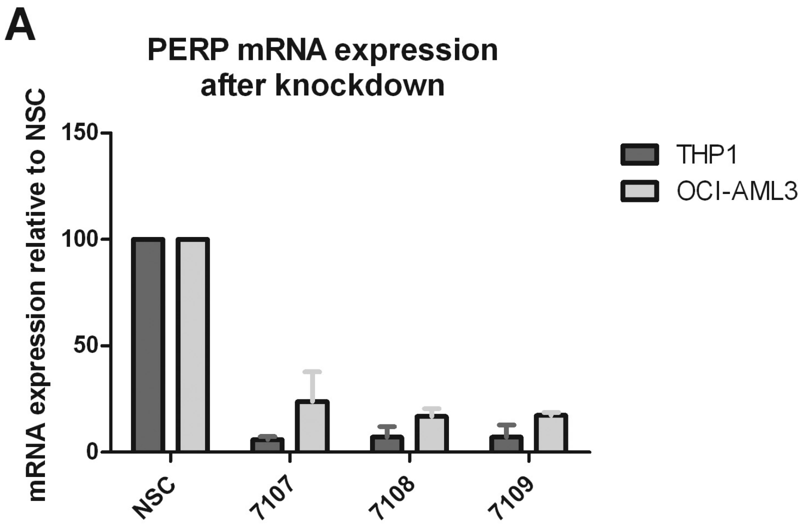
adj.P.Val. indicates FDR-adjusted P-value. Probe-sets representing PERP are shaded light grey, the sets representing GGA2 are shaded dark grey.

Supplementary Figure S1: Principal component analysis of pediatric AML samples on HOXA and HOXB genes



Principal component analysis of pediatric AML patients with a specific type II aberration (N = 278) was carried out based on all HOXA and HOXB annotating probe sets present on the HGU133 Plus 2.0 microarray (Affymetrix). Each color-coded sphere represents an individual AML case from the cytogenetic and molecular groups explained in the figure. White spheres represent patient samples that have no know type II aberration. Three groups are observed in the principal component analysis, which are indicated by the white circles; on the left samples with t(8;21), inv(16), t(15;17) and CEBPA double mutations that do not have HOXA of HOXB expression, in the middle MLL-rearranged samples and t(8;16) samples characterized by HOXA expression only, and on the lower right NUP98-NSD1, NPM1, MLL-PTD and DEK-NUP214 samples characterized by both HOXA and HOXB expression.

Supplementary Figure S2: Results after shRNA mediated PERP knockdown



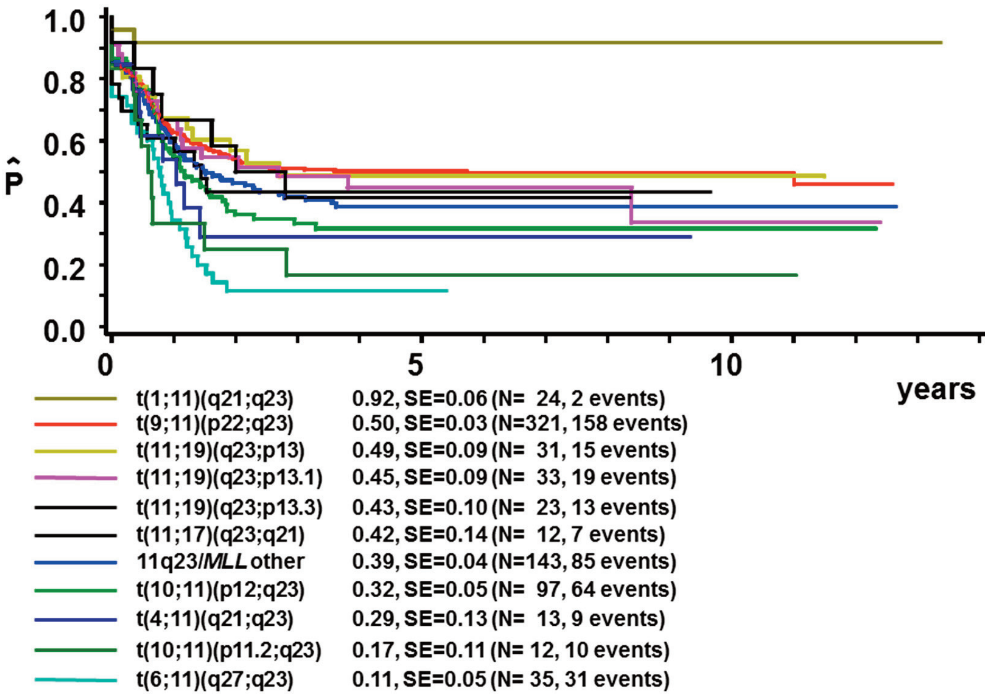
Panel A *shows the PERP mRNA knockdown by 3 different shRNA constructs targeting PERP (7107, 7108, 7109) as a percentage of mRNA expression in the none silencing control (NSC) in both cell lines (THP1 and OCI-AML3) as a median of 3 repetitive experiments. Efficient knockdown was achieved.*

Panel B *shows cell growth in 3 different shRNA infected conditions of both THP1 and OCI-AML3 cell line relative to cell growth of the NSC-condition of the respective cell lines as a median of 3 repetitive experiments. In OCI-AML3 the treated conditions grew faster than NSC, but with large error-bars. In THP1 no difference in cell growth was detected.*

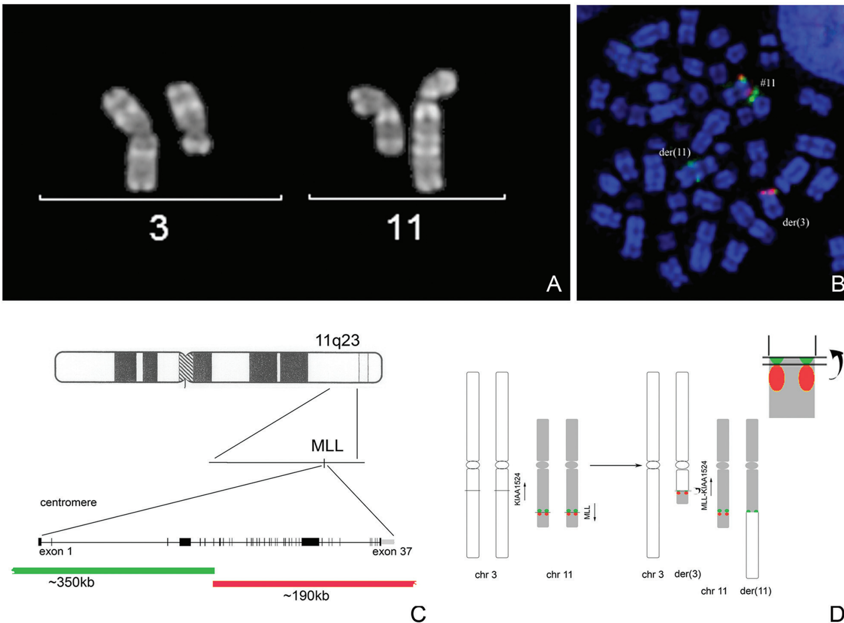
Panel C *shown results from annexin/PI staining with the median cell proportion gated in alive, apoptotic and dead gates of 2 independent experiments. No consistent changes of the 3 different shRNA conditions were seen when comparing to the NSC condition.*

Color figures

Chapter 1 Figure 3



Chapter 2 Figure 1



Chapter 2 Figure 2

DNA sequence:

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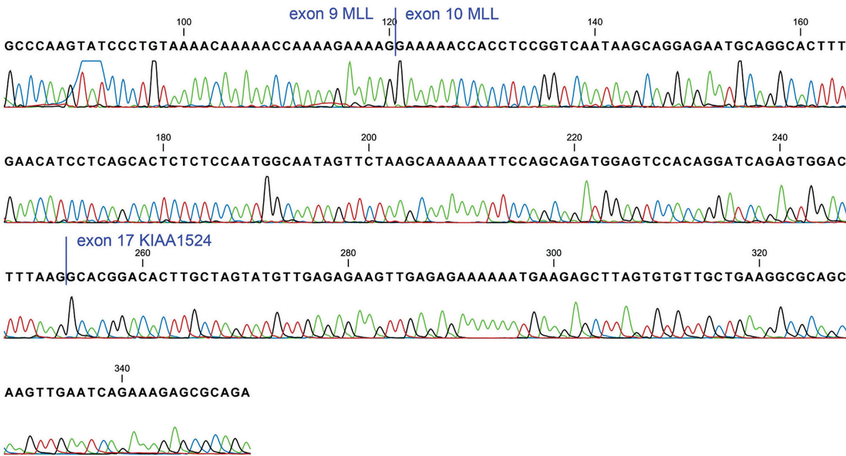
cDNA sequence:



Chapter 3 Figure 1

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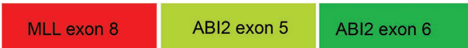
Schematic overview of transcripts

Expected transcript

Other transcripts present:



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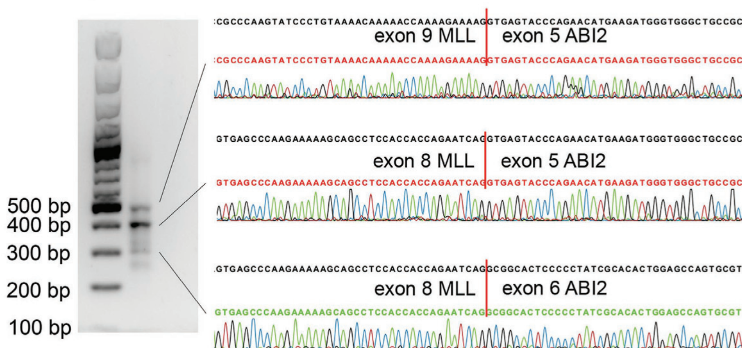
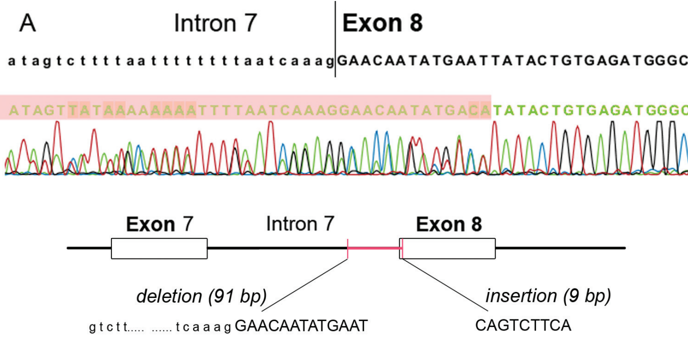
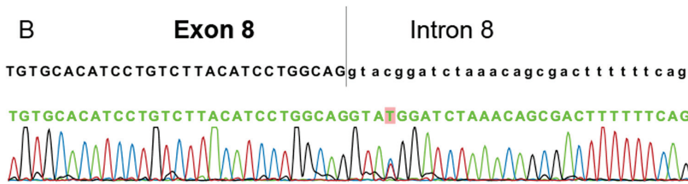
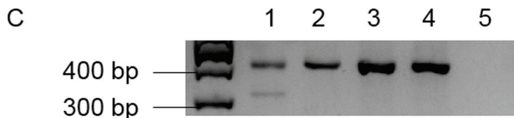
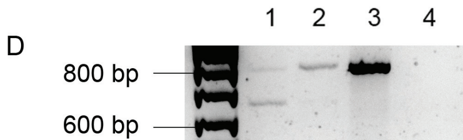


Chapter 5 Figure 1



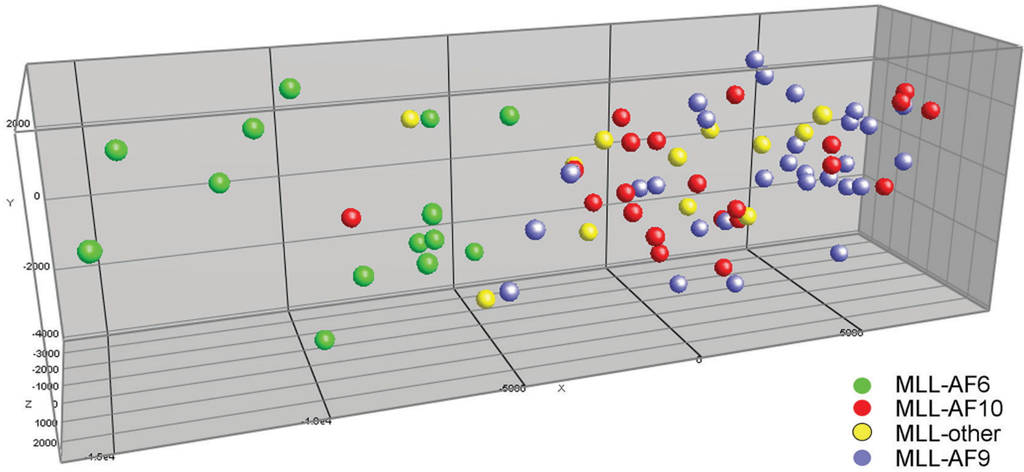






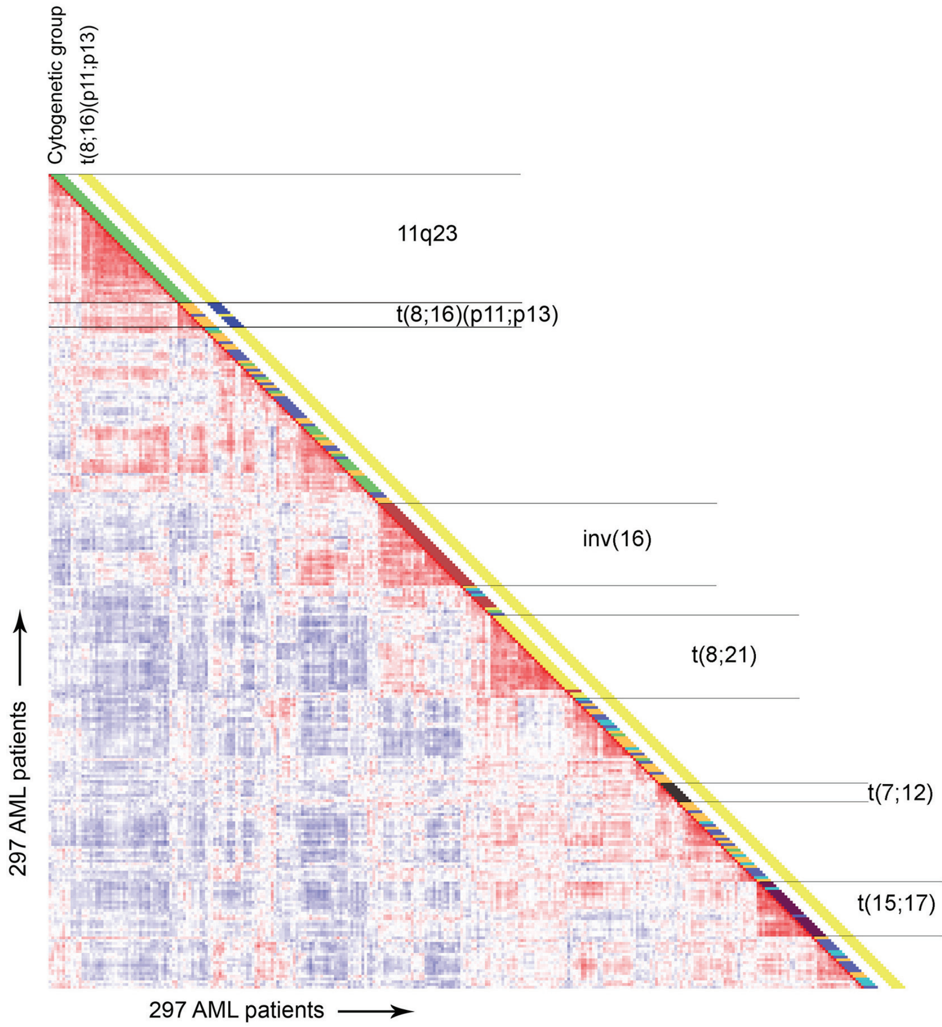
Chapter 5 Supplementary Figure S1

Chapter 7 Figure 1

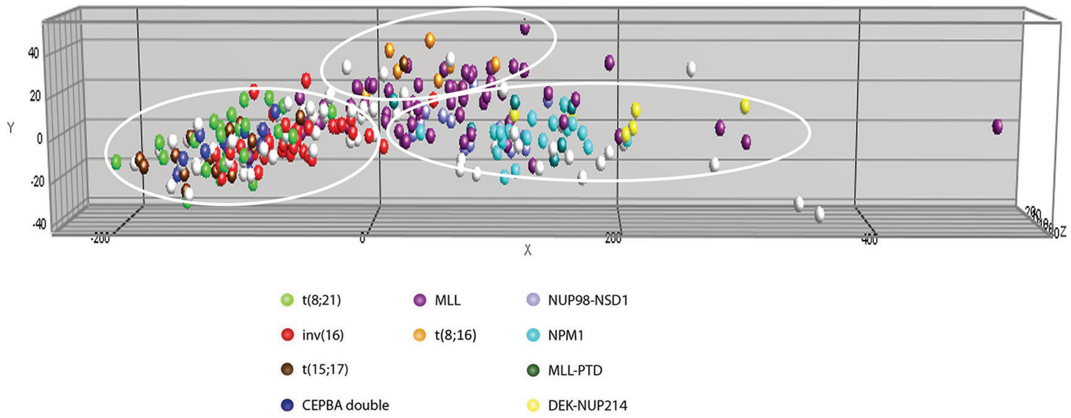


Chapter 7 Figure 2

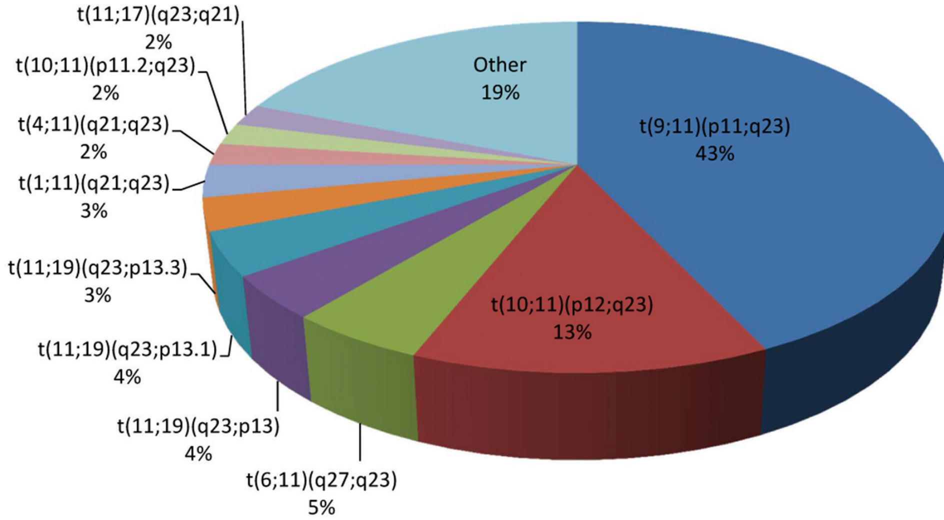
Chapter 8 Figure 3



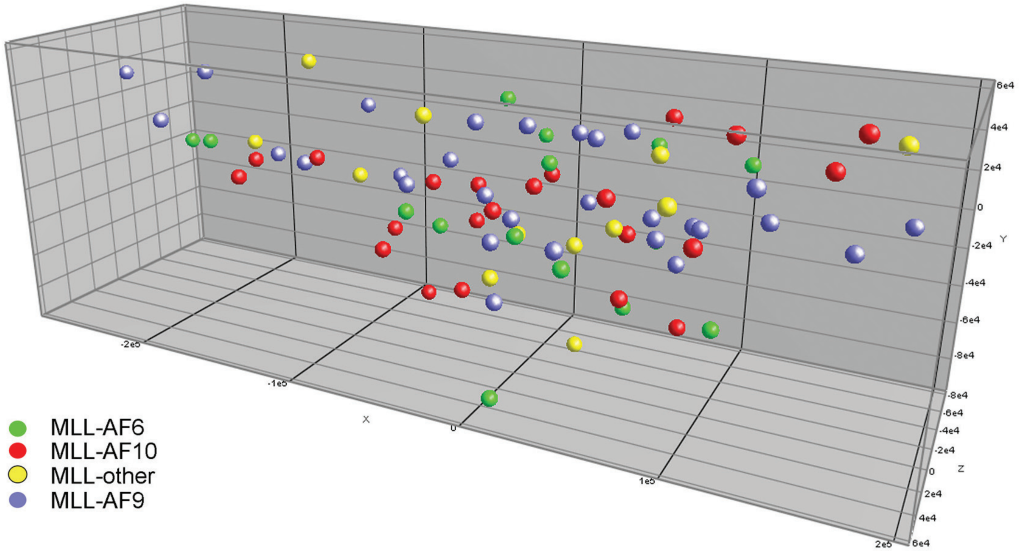
Chapter 8 Supplementary Figure S1



Chapter 9 Figure 1



Chapter 9 Figure 2



Chapter 9 Figure 3