UNRAVELING *MLL*-REARRANGED PEDIATRIC ACUTE MYELOID LEUKEMIA

Unraveling *MLL*-rearranged Pediatric Acute Myeloid Leukemia

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UNRAVELING *MLL*-REARRANGED PEDIATRIC ACUTE MYELOID LEUKEMIA

Het ontrafelen van acute myeloïde leukemie
met een *MLL*-gen herschikking in kinderen

Proefschrift

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Chapter 1

General Introduction

HEMATOPOIESIS AND LEUKEMIA

Blood cells are derived from hematopoietic stem cells (HSC) that reside in the bone marrow. HSC's are multipotent and have the capacity to differentiate into the cells of all blood lineages, i.e. erythrocytes, platelets, neutrophils, eosinophils, basophils, monocytes, T and B lymphocytes, natural killer cells, and dendritic cells (Figure 1).
Leukemia is cancer of the blood in which the normal population of blood cells in the bone marrow is replaced by immature blood cells (blasts). These immature cells occur due to an imbalance in differentiation and proliferation. Accumulation of these malignant cells in the bone marrow results in decrease of normal mature blood cells, like white blood and red blood cells and platelets.

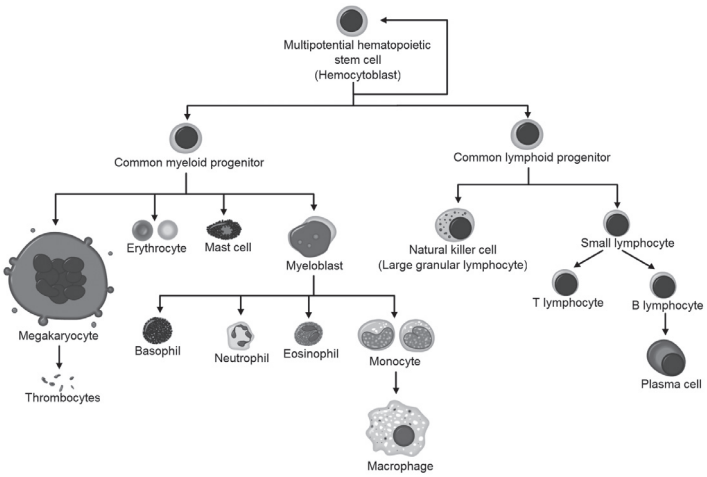

Figure 1: The development of different blood cells from haematopoietic stem cell to mature cells

ACUTE MYELOID LEUKEMIA

Leukemias can be divided in several groups. Acute leukemias are characterized by the rapid increase of blast cells in the bone marrow, whereas the hallmark of chronic leukemias is the presence of mature but abnormal blood cells. A further subdivision can be made depending on which cell type is affected. Lymphoblastic leukemias are derived from the lymphoid lineage, whereas in myeloid leukemias are derived from the myeloid lineage.
In children, acute leukemias are the most frequent type of cancer[1]. In the Netherlands every year around 140 new children are diagnosed with leukemia of which 80% have acute lymphoblastic leukemia (ALL), whereas only 15% present with acute myeloid leukemia (AML). The incidence of AML increases with age and in adults it accounts for 80% of the acute leukemias with a median age of approximately 65 years[2].

AML is a heterogeneous disease reflected by differences in morphologic subtype, immunophenotype, cytogenetic and molecular abnormalities. In the past, AML was mainly classified on morphology according to the French-American-British (FAB) system into 8 categories, i.e. M0 to M7, based on the type of cell from which the leukemia developed and its degree of maturity[3]. More recently, the World Health Organization (WHO) introduced a classification which attempts to be more clinically useful and includes morphology, immunophenotype, genetics, and clinical features (Table 1)[4].

OUTCOME AND TREATMENT OF PEDIATRIC AML

Over the last decades outcome in pediatric AML has improved from only 5% up to 60% with the most recent treatment protocols (Table 2)[5-11]. This has been achieved by improved chemotherapy schedules, improvements in supportive care and by better risk-group stratification. The latter is mainly based on cytogenetics and early response to treatment. In order to survive, children need very intensive chemotherapy, which harbors the risk of severe toxicity and even death during or after treatment (5-10% of the cases). Currently, most treatment protocols consist of 4 to 5 blocks of intensive chemotherapy, using a cytarabine and anthracycline backbone. The use of stem-cell transplantation after complete remission has currently been abandoned by most study groups, since it did not improve survival. Since early-deaths and long-term side effect of treatment play an important role, current treatment strategies cannot be intensified. Therefore, the aim for the future is to design subgroup directed treatment protocols based on the genetic characteristics of pediatric AML to increase survival and reduce toxicity and late effects.

TWO-HIT MODEL IN DE LEUKEMOGENESIS OF AML

Kelly and Gilliland proposed a model in which two classes of abnormalities cooperate to cause AML[12]. Type-I mutations confer a proliferative signal, which results in a survival advantage for the leukemic cells, whereas type-II mutations induce impairment of hematopoietic differentiation (Figure 2).

TYPE-I MUTATIONS IN PEDIATRIC AML

Most type-I mutations are involved in kinases or key-players of the RAS-pathway. The most important mutations are found in the FLT3-gene, a receptor tyrosine kinase. These aberrations are internal tandem duplications of *FLT3* (*FLT3*-ITD) and mutation in the

Table 1: WHO-classification 2008 of AML and related precursor neoplasms.

AML with recurrent genetic abnormalities	AML with t(8;21)(q22;q22); RUNX1-RUNX1T1
	AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11
	APL with t(15;17)(q22;q12); PML-RARA
	AML with t(9;11)(p22;q23); MLLT3-MLL
	AML with t(6;9)(p23;q34); DEK-NUP214
	AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1
	AML (megakaryoblastic) with t(1;22)(p13;q13); RBM15-MKL1
	Provisional entity: AML with mutated NPM1
	Provisional entity: AML with mutated CEBPA
AML with myelodysplasia-related changes	Previously documented MDS or MDS/MPN
	Specific myelodysplasia-related cytogenetic abnormalities
	Dysplasia in 50% or more of the cells in 2 or more myeloid lineages
Therapy-related myeloid neoplasms	
Acute myeloid leukemia, not otherwise specified	AML with minimal differentiation
	AML without maturation
	AML with maturation
	Acute myelomonocytic leukemia
	Acute monoblastic/monocytic leukemia
	Acute erythroid leukemia
	Acute megakaryoblastic leukemia
	Acute basophilic leukemia
	Acute panmyelosis with myelofibrosis
Myeloid sarcoma	Extramedullary proliferation of blasts of one or more of the myeloid lineages that disrupts the normal architecture of the tissue
Myeloid proliferations related to Down syndrome	Transient abnormal myelopoiesis
	Myeloid leukemia associated with Down syndrome
Blastic plasmacytoid dendritic cell neoplasm	Blastic NK-cell lymphoma

Table 2: Outcome in pediatric AML from most recent international large studies.

Study	N	5y-pEFS	5y-EFS	ED
AML-BFM98	473	50%	62%	3.2%
NOPHO-AML93	219	49%	64%	3%
MRC12	455	56%	66%	4%
LAME91	262	47%	61%	4%
AIEOP AML 2002/01	205	55%	70%	4.3%
CCG-2961 (regimen C)	406	46%	57%	12%
St. Jude AML97	78	49%	54%	4%

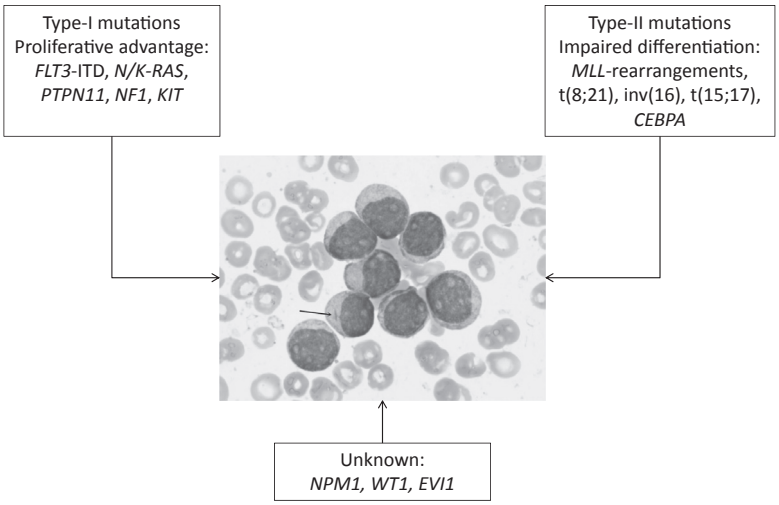

Figure 2: The different type of aberrations identified in AML

tyrosine kinase domain that confer a poor prognosis in pediatric AML[13]. Other aberrations that lead to a proliferative advantage of the leukemic cell are mutations in *KIT* and genes involved in the RAS-pathway, i.e. *N/KRAS* and *PTPN11*. *KIT* is a receptor tyrosine kinase that belongs to the same family as *FLT3*[14] and mutations in *KIT* confer survival and growth advantage to the leukemic cells[15]. The RAS family consists of 3 G-Proteins, i.e. *NRAS*, *KRAS* and *HRAS* and mutations in these genes lead to activation of the RAS-MAPK-ERK pathway, which induces proliferation and cell survival. Recently in 5% of the pediatric AML cases a mutation in *PTPN11*, a tyrosine phosphate, was identified[16], that lead to activation of the same RAS-MAPK-ERK pathway. *KIT* and RAS-pathway mutations are not restricted to AML, but are found in various types of cancer.

TYPE-II MUTATIONS IN PEDIATRIC AML

Type-II mutations in pediatric AML are often chromosomal aberrations, which are detected by conventional karyotyping, FISH and/or RT-PCR. The most common translocation leading to a differentiation arrest are 11q23/MLL-rearrangements, t(8;21)(q22;q22) [*AML1-ETO*], inv(16)(p13q22)[*CBFB-MYH11*] and t(15;17)(q21;q22) [*PML-RARA*]. Pediatric AML cases with a t(8;21)(q22;q22), inv(16)(p13q22) and t(15;17)(q21;q22) have a favorable outcome. Treatment strategies against t(15;17)(q21;q22) include next to chemotherapy, all-trans retinoic acid (ATRA), which activates the retinoic acid receptor (*RARA*) and causes the leukemic cells to differentiate[17].

More recently other molecular aberrations were discovered in cytogenetically normal AML (CN-AML) which were mutually exclusive with the other type-II aberrations. Mutations in the *CEBPA* gene lead to a block in granulocyte differentiation, and therefore can be considered as a type-II aberration[18]. Also mutations in *NPM1* have been recently reported. Although the exact role of *NPM1* in leukemogenesis is unknown, it is considered as a type-II aberration as it is mutually exclusive to other cytogenetic type-II aberrations. In pediatric AML, mutations in *CEBPA* and *NPM1* have a favorable outcome[19,20].

MLL-REARRANGED AML

8-20% of pediatric AML is characterized by translocations involving the *Mixed Lineage Leukemia* (*MLL)*-gene on chromosome 11q23[21]. The *MLL*-gene encodes a DNA-binding protein that methylates and acetylates histones. *MLL*-rearrangements are likely to deregulate patterns of *HOX*-gene expression in hematopoietic stem cells or progenitors. The *MLL*-gene comprises a so-called breakpoint region-cluster that behaves as a hotspot for chromosomal translocation. More than 60 different fusion partners of the *MLL*-gene have been identified[22]. In AML, the main translocation partners are t(9;11), t(11;19); t(6;11) and t(10;11). It has been suggested that subgroups of *MLL*-rearranged pediatric AML are associated with a better than average outcome compared to other subgroups. For instance, in some studies, the t(9;11) group has been associated with a higher sensitivity to different classes of anti-leukemia drugs, and a better prognosis[8,23-25]. For the other types of *MLL*-rearrangements no data on outcome are available, mainly because these subgroups in pediatric AML are small. Targeting transcription factors, like MLL, has not been successful in experimental models. Searching for novel therapeutic targets in these groups of patients seems to be essential for further improvement of outcome.

OUTLINE OF THIS THESIS

Currently, the cure rate for pediatric AML seems to have reached a plateau. Intensifying current treatment strategies is not possible do to treatment-related deaths and long term side effects. Therefore efforts should be made to identify new therapeutic possibilities, targeting the leukemic clone. To achieve this, the biology of pediatric AML, which is a heterogeneous disease, should be elucidated. In this thesis we used array-CGH and microarrays to identify new biological markers in pediatric AML.

Part One: The genetic and molecular characteristics of pediatric AML

In **Chapter 2**, we give an overview of the current different type-I and type-II mutations in pediatric AML. We screened a large cohort of pediatric AML cases for cytogenetic and molecular aberrations in pediatric AML.

In **chapter 3** we studied whether gene expression profiling can be used to identify known subgroups in AML.

Overexpression of *EVI1* is an independent poor prognostic factor in adult AML and is often associated with 3q26 aberrations. However in pediatric AML 3q26 aberrations are rare and little is known about the role of EVI1. Therefore, in **chapter 4**, we analyzed whether overexpression of *EVI1* has prognostic relevance in pediatric AML.

Part Two: The heterogeneity of *MLL*-rearranged AML

Since *MLL*-rearrangements seem to be the most common aberration in pediatric AML, we have reviewed the biology, outcome and treatment of different types of *MLL*-rearranged AML in **chapter 5**.

A large number of translocation partners of *MLL* have been discovered and in **chapter 6** we show that still new ones are identified.

Chapter 7 describes a large international collaborative study, in which we combined outcome data of *MLL*-rearranged pediatric AML from 11 different study groups, and identified differences based on translocation partner. This shows that *MLL*-rearranged AML is a heterogeneous disease with differences in biological background.

To identify novel biological markers that could explain these differences, we identified in **chapter 8**, with the use of array-CGH, that inactivation of *NF1* plays a role in acute leukemias, including *MLL*-rearranged AML.

Gene expression profiling revealed distinct gene expression signatures for the different translocation partners in *MLL*-rearranged AML, as described in **chapter 9**, We identified *BRE* to be mainly overexpressed in t(9;11)(p22;q23) and that high expression of *BRE* in pediatric AML is an independent favorable prognostic factor for relapse free survival.

In **chapter 10**, we describe a low frequency of *MLL*-PTD in pediatric AML, using MLPA as a novel screening technique.

Chapter 11 summarizes this thesis and discusses the results and their future perspectives.

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Chapter 2

The genetic heterogeneity of pediatric acute myeloid leukemia

B.V. Balgobind[1*], I.H.I.M. Hollink[1*], S.T.C.J.M. Arentsen-Peters[1], M. Zimmermann[2], J. Harbott[3], H.B. Beverloo[4], A. von Bergh[4], J. Cloos[5], G.J.L. Kaspers[5,6], V. de Haas[6], Z. Zemanova[7], J. Stary[7], J. Cayuela[8], A. Baruchel[8], U. Creutzig[9], D. Reinhardt[2], R. Pieters[1], C.M. Zwaan[1#] and M.M. van den Heuvel-Eibrink[1#]

* These authors contributed equally to this manuscript
# These authors contributed equally to this manuscript

[1]Department of Pediatric Oncology/Hematology, Erasmus MC – Sophia Children's Hospital, Rotterdam, the Netherlands; [2]Department of Pediatric Oncology/Hematology, Medical School of Hannover, Hannover, Germany; [3]Department of Pediatric Hematology/Oncology, Justus-Liebig-University, Giessen, Germany;
[4]Department of Clinical Genetics, Erasmus MC, Rotterdam, the Netherlands; [5]Department of Pediatric Oncology/Hematology, VU University Medical Center, Amsterdam, the Netherlands; [6]Dutch Childhood Oncology Group (DCOG), The Hague, the Netherlands; [7]Center of Oncocytogenetics, General University Hospital and Charles University in Prague, 1st Faculty of Medicine, Prague, Czech Republic; [8]Department of Hematology, St. Louis Hospital, Paris, France; [9]Department of Pediatric Hematology/Oncology, University Hospital Muenster, Muenster, Germany.

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Chapter 3

Evaluation of gene expression signatures predictive for cytogenetic and molecular subtypes of pediatric acute myeloid leukemia

B.V. Balgobind[1], M.M. Van den Heuvel-Eibrink[1], R.X. De Menezes[1,2], D. Reinhardt[3], I.H.I.M. Hollink[1], S.T.J.C.M. Arentsen-Peters[1], E.R. van Wering[4], G.J.L. Kaspers[5], J. Cloos[5], E.S.J.M. de Bont[6], J.M. Cayuela[7], A. Baruchel[7], C. Meyer[8], R. Marschalek[8], J. Trka[9], J. Stary[9], H.B. Beverloo[10], R. Pieters[1], C.M. Zwaan[1] and M.L. den Boer[1]

[1]Pediatric Oncology/Hematology, Erasmus MC - Sophia Children's Hospital, Rotterdam, The Netherlands; [2]Center for Human and Clinical Genetics, LUMC, Leiden, The Netherlands; [3]AML-BFM Study Group, Hannover, Germany; [4]Dutch Childhood Oncology Group (DCOG), The Hague, The Netherlands; [5]Pediatric Oncology/Hematology, VU University Medical Center, Amsterdam, The Netherlands; [6]Pediatric Oncology/Hematology, Beatrix Children's Hospital, Groningen, The Netherlands; [7]Hematology, St. Louis Hospital, Paris, France; [8]Institute of Pharmaceutical Biology, ZAFES, Diagnostic Center of Acute Leukemias (DCAL), Frankfurt, Germany; [9]Pediatric Hematology/Oncology, [2]nd Medical School, Charles University, Prague, Czech Republic; [10]Clinical Genetics, Erasmus MC, Rotterdam, The Netherlands.

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ABSTRACT

Background

Pediatric acute myeloid leukemia is a heterogeneous disease characterized by non-random genetic aberrations related to outcome. The genetic subtype is currently detected by different diagnostic procedures which differ in success rate and/or specificity.

Design and Methods

We examined the potential of gene expression profiles to classify pediatric acute myeloid leukemia. Gene expression microarray data of 237 children with acute myeloid leukemia were collected and a double-loop cross validation approach was used to generate a subtype-predictive gene expression profile in the discovery cohort (n=157) which was then tested for its true predictive value in the independent validation cohort (n=80). The classifier consisted of 75 probe sets, representing the top 15 discriminating probe sets for *MLL*-rearranged, t(8;21)(q22;q22), inv(16)(p13q22), t(15;17)(q21;q22) and t(7;12)(q36;p13)-positive acute myeloid leukemia.

Results

These cytogenetic subtypes represent approximately 40% of cases of pediatric acute myeloid leukemia and were predicted with 92% and 99% accuracy in the discovery and independent validation cohort, respectively. However, for *NPM1, CEBPA, MLL(*-PTD*), FLT3(*-ITD*), KIT, PTPN11* and *N/KRAS* gene expression signatures had limited predictive value. This may be caused by a limited frequency of these mutations and by underlying cytogenetics. This latter is exemplified by the fact that different gene expression signatures were discovered for *FLT3*-ITD in patients with normal cytogenetics and in those with t(15;17)(q21;q22)-positive acute myeloid leukemia, which pointed to *HOXB*-upregulation being specific for *FLT3*-ITD$^+$ cytogenetically normal acute myeloid leukemia.

Conclusions

In conclusion, gene expression profiling correctly predicted the most prevalent cytogenetic subtypes of pediatric acute myeloid leukemia with high accuracy. In clinical practice, this gene expression signature may replace multiple diagnostic tests for approximately 40% of pediatric acute myeloid leukemia cases whereas only for the remaining cases (predicted as 'acute myeloid leukemia-other') additional tests are indicated. Moreover, the discriminative genes reveal new insights into the biology of acute myeloid leukemia subtypes that warrants follow-up as potential targets for new therapies.

INTRODUCTION

Pediatric acute myeloid leukemia (AML) is a heterogeneous disease that accounts for 15-20% of the acute leukemias in children[1] and is classified according to the WHO classification, which is based on non-random genetic aberrations[2]. Over recent decades the outcome of pediatric AML has improved and current overall survival rates range from 50% to 70%[3]. The most important prognostic factors in pediatric AML are response to induction therapy and the cytogenetic and molecular subtype of the disease[4-5].

Gilliland *et al.* postulated that the pathogenesis of AML requires both type-I and type-II mutations[6]. Type-II mutations are often chromosomal rearrangements of transcription factors leading to impaired differentiation of the hematopoietic cell, such as 11q23/*MLL*-rearranged, t(8;21)(q22;q22)[*RUNX1-RUNXT1*], inv(16)(p13q22)[*CBFB-MYH11*], or t(15;17)(q21;q22)[*PML-RARA*]. Patients with t(8;21)(q22;q22), inv(16)(p13q22) and t(15;17)(q21;q22)-positive AML have a favorable prognosis in contrast to *MLL*-rearranged cases. Type I mutations often reflect molecular mutation hotspots in specific genes (*FLT3*, *KIT*, *NRAS*, *KRAS*, *PTPN11* and *NF1*) involved in the proliferation of hematopoietic cells[7-8]. In adult and pediatric AML *FLT3*-internal tandem duplications (*FLT3*-ITD) and *KIT* mutations have been correlated with an inferior outcome[9-11].

In approximately 20% of the pediatric AML cases no chromosomal aberrations have yet been discovered. These patients with apparent cytogenetically normal (CN) AML are currently treated as a homogeneous group with an intermediate risk factor. However, point mutations and small deletions in *CEBPA* and *NPM1* as well as partial tandem duplications in *MLL* (*MLL*-PTD) are found in both pediatric and adult CN-AML. The frequency of these mutations is lower in children than in adults. Moreover, the prognostic impact differs between children and adults[10,12-15]. These observations highlight the genetic heterogeneity within AML as well as between adults and children with AML and the need for separate studies in pediatric AML to demonstrate the value of mutations for stratification in contemporary pediatric AML treatment protocols.

A new case of AML is currently primarily identified by cytomorphology and immunophenotyping. Further characterization needed for risk-stratification includes the detection of chromosomal aberrations by conventional karyotyping and molecular cytogenetics of specific genetic lesions, for instance by fluorescence in situ hybridization (FISH) and/or reverse transcriptase (RT) polymerase chain reaction (PCR). However, it can be difficult to obtain a karyogram since this requires successful induction of in vitro cellular proliferation to obtain metaphases for analysis of chromosomal changes. In addition, FISH and RT-PCR procedures may also yield inconclusive results, for example due to poor interphase preparations (FISH), limitations in signal detection (FISH), sub-clonality (FISH and RT-PCR) and sequence variations in probe/primer-hybridizing regions (FISH and RT-PCR).

Microarray-based gene expression profiling studies showed that pediatric and adult AML can be accurately classified into cytogenetically distinct subtypes[16-20]. In the Microarray Innovations-in-LEukemia (MILE) study, gene expression profiles accurately classified over 3000 cases with acute and chronic leukemia[21].

We recently showed that a double-loop cross-validation classification approach yielded a highly stable and accurate classifier with high predictive value for subtypes of pediatric acute lymphoblastic leukemia (ALL) in both the cross-validation cohort as well as in a totally independent cohort of pediatric ALL[22]. In the current study we used this double-loop cross-validation method to determine whether gene expression signatures can predict prognostically relevant specific cytogenetic subtypes (11q23/*MLL*-rearranged, t(8;21)(q22;q22), inv(16)(p13q22), t(15;17)(q21;q22), t(7;12)(q36;p13) and CN-AML) as well as cases with molecular aberrations in *NPM1*, *CEBPA*, *FLT3*-ITD, *N/KRAS*, *KIT* and *PTPN11* in pediatric AML.

DESIGN AND METHODS

Patients

Viably frozen bone marrow or peripheral blood samples from 237 children with de novo AML, 33 with relapsed and 8 with secondary AML were provided by the Dutch Childhood Oncology Group, 'Berlin-Frankfurt-Münster' AML Study Group, Czech Pediatric Hematology and St. Louis Hospital in Paris, France. Informed consent was obtained from patients, after Institutional Review Board approval according to national law and regulations. Leukemic cells were isolated by sucrose density centrifugation and non-leukemic cells were eliminated as previously described[23]. All processed samples contained more than 80% leukemic cells, as determined morphologically using cytospins stained with May-Grünwald-Giemsa (Merck, Darmstadt, Germany). Subsequently, a minimum of 5×10^6 leukemic cells were lysed in Trizol reagent (Gibco BRL, Life Technologies, Breda, the Netherlands). Genomic DNA and total RNA were isolated according to the manufacturer's protocol, with minor modifications[24].

Cytogenetics

Leukemic samples were routinely investigated for cytogenetic aberrations by standard chromosome-banding analysis, and screened by the above mentioned study groups for recurrent non-random genetic aberrations characteristic for AML as described by the WHO 2008 classification of myeloid neoplasms and acute leukemia[2], including *MLL*-rearrangements, inv(16)(p13q22), t(8;21)(q22;q22) and t(15;17)(q21;q22), using RT-PCR and/or FISH. In the case of incomplete data, the Erasmus MC group performed RT-PCR to detect inv(16)(p13q22), t(8;21)(q22;q22) and t(15;17)(q21;q22) and split-signal

FISH to detect rearrangements of the *MLL*-gene using standardized primers and probe combinations as previously described[25-26]. In three cases, predicted as *MLL*-rearranged AML, screening for an *MLL*-rearrangement was performed with long-distance inverse (LDI) PCR as previously described[27]. In addition, all patients under the age of 18 months were screened for t(7;12)(q36;p13) by FISH. The probes used were five cosmid clones covering the breakpoints in the *ETV6* gene and a PAC clone (RP5-1121A15) containing the *HLXB9* gene, as previously described[28].

Mutation analysis

Samples were screened for hotspot mutations in *NPM1*, *CEPBA*, *FLT3*-ITD, *NRAS*, *KRAS*, *PTPN11*, *KIT* and *MLL*-partial tandem duplication (*MLL*-PTD) as previously described[7,29-32]. If positive for *MLL*-PTD, this was confirmed by multiplex ligation-dependent probe amplification (MLPA) analysis (MRC Holland, Amsterdam, The Netherlands). The reaction mix for MPLA-analysis contained probes for exon 2 to 13 of *MLL* for *MLL*-PTD detection and exon 17 of *MLL* as internal control. A probe in the *serpinB2* gene was used as external control[33]. Data were analyzed using GeneMarker v1.5 (Softgenetics, State College, USA).

Microarray

Integrity of total RNA was checked using the Agilent 2100 Bio-analyzer (Agilent, Santa Clara, USA). cDNA and biotinylated cRNA was synthesized and hybridized to the Affymetrix Human Genome U133 Plus 2.0 Array *(*Affymetrix, Santa Clara, USA*)* according to the manufacturer's guidelines. Data were acquired using 'expresso' (Bioconductor package 'Affy'), and probe-set intensities were normalized using the variance stabilization normalization procedure (Bioconductor package 'VSN') in the statistical data analysis environment R, version 2.2.0. The original data files have been submitted to the GEO database (GSE17855).

Statistics

To find signatures for the different cytogenetic and molecular subtypes an empirical Bayes linear regression model was used to compare samples from each group to all other samples[34]. This model takes advantage of the large number of probe sets to yield better estimates for the gene-specific standard error, producing more powerful tests for differential expression even if small sample sizes are involved. Moderated T-statistics p-values were corrected for multiple testing using the False Discovery Rate (FDR) method defined by Benjamini and Hochberg[35]. The top 50 most significant probe sets for each subtype were used as a starting point to construct the classifier.

The construction of the classifier

Samples were divided at random in a discovery cohort of 157 cases that were used for the double-loop cross validation approach and an independent cohort of 80 cases, which was only tested once and served as a true independent validation cohort. In all following 100 cycles of the double-loop cross-validation approach, the sample distribution of the discovery cohort reflected the distribution of cytogenetic subtypes as seen in the total cohort of 237 cases. The double-loop cross-validation method was used to build a support-vector machine-based classifier predictive for the known cytogenetic subtypes of pediatric AML. This approach avoids over-fitting of gene expression profiling data[36] and has proven to yield a stable classifier with high accuracy to predict subtypes of pediatric acute lymphoblastic leukemia, as previously showed[22]. The double-loop cross-validation method was only applied to the discovery cohort of 157 cases. This consists of an inner loop containing 2/3 of cases in which the minimal number of probe sets yielding the highest prediction sensitivity is being determined (100 iterations for each selected number of probe sets) and an outer loop containing the remaining 1/3 of cases serving to validate the obtained results from the inner loop (also 100 iterations per list of probe sets) (Supplementary Figure S1).

In each of 100 runs of the inner loop, patients were randomly assigned to the inner-training (9/10) and inner-test (1/10) group (10-fold cross-validation). To start, the top-50 probe sets most discriminative for each subtype were selected by rank of P-values obtained by applying an empirical Bayes linear regression model (LIMMA) to the inner-training group. These probe sets were used to construct a support vector machine-based classifier which was then tested for predictive sensitivity on the inner-test group of remaining 1/10 of cases (100 iterations). Next, the minimum number of probe sets that optimally classified the patients in the inner loop was obtained by backwards selection starting with 250 probe sets (50 probe sets x 5 subtypes) using a global test for ranking the significance of probe sets in each iteration in order to reduce multiple testing errors as previously described[22]. The optimal number of probe sets determined in the inner loop was used to construct a classifier for which the median sensitivity was estimated via 3-fold cross-validation by applying the trained classifier to the remaining 1/3 of cases of the outer loop (100 iterations; Supplementary Figure S1). The final gene expression classifier, trained on all 157 cases in the discovery cohort, was used to determine the prediction accuracy in the independent group of 80 cases (Supplementary Figure S1).

The same approach was used to select probe sets predictive for the most frequent molecular aberrations, i.e. *NPM1*, *CEBPA*, *MLL*-PTD, *FLT3*-ITD, *KIT*, and combined mutations in the *RAS*-pathway (*NRAS*, *KRAS* and *PTPN11*), Since *NPM1*, *CEBPA* and *MLL*-PTD were mutually exclusive from the other cytogenetic subgroups, these abnormalities could be simultaneously included in one model together with the known cytogenetic subgroups, for which the prediction accuracy was estimated as described

above. In addition, we performed an analysis in which the most discriminative probe sets for type-I mutations in *FLT3*-ITD, *KIT* and *RAS*-pathway were identified after adjusting for the underlying cytogenetic aberrations.

Software

R (version 2.2.0 and version 2.5.0) and the R packages affy, vsn, e1071, globaltest, limma, multtest and marray were used to run the above-mentioned analyses[34,37-42]. Hierarchical clustering analysis was performed in Genemaths XT (Applied Maths, Austin, USA).

RESULTS

Patients characteristics

Gene expression profiles were generated from 237 newly diagnosed pediatric AML cases. Non-random cytogenetic subgroups of pediatric AML with a sufficient number of cases were included, i.e. *MLL*-rearranged AML, t(8;21)(q22;q22), inv(16)(p13q22), t(15;17)(q21;q22) and CN-AML. In addition, seven t(7;12)(q36;p13)-positive infant AML cases were included (Table 1). For the other cytogenetic groups, e.g. t(6;9)(p23;q34) (n=7), monosomy 7 (n=4), trisomy 8 (n=1) and complex karyotype (n=11), no significant discriminative genes were found and these cases were therefore combined into a single group annotated with 'remaining cytogenetics'. No karyotype was available for 25 cases but since these cases were negative for *MLL*-rearrangements, t(8;21)(q22;q22), inv(16)(p13q22), t(15;17)(q21;q22) and t(7;12)(q36;p13), these cases were included in the 'unknown other cytogenetics' category.

Definition of subgroups

Using an empirical Bayes linear regression model many discriminative probe sets with high statistical significance ($p<1.0^{-08}$) were found for *MLL*-rearranged, t(8;21)(q22;q22), inv(16)(p13q22), t(15;17)(q21;q22) and t(7;12)(q36;p13)-positive AML (Supplementary Table S2). In contrast, only a limited number of discriminative probe sets were found significant at lower p-value ($p<1.0^{-04}$) for CN-AML, cases with remaining genetic aberrations or unknown other cytogenetics (Supplementary Table S2). Hereafter, this mixed group is referred to as the 'AML-other' group. Based on an equal distribution of these groups, the overall cohort was divided into a discovery cohort (n=157) to construct the classifier and an independent validation cohort (n=80).

Probe set selection for classifier estimated with the discovery cohort

The classifier was constructed by selecting the most statistically significantly discriminative probe sets for each of the 5 cytogenetic subtypes *MLL*-rearranged, t(8;21)(q22;q22), inv(16)

Table 1: Cytogenetic and molecular characteristics of pediatric AML patients in this study.

	Discovery cohort (n=157)		Validation cohort (n=80)		Total (n=237)	
	N	**%**	**N**	**%**	**N**	**%**
Cytogenetic subtypes						
MLL-rearrangements	31	*20*	16	*18*	47	*20*
t(8;21)(q22;q22)	18	*11*	10	*11*	28	*12*
inv(16)(p13q22)	17	*11*	10	*11*	27	*11*
t(15;17)(q21;q22)	14	*9*	5	*6*	19	*8*
t(7;12)(q36;p13)	5	*3*	2	*2*	7	*3*
AML-other[#]						
CN-AML[a]	24	*15*	15	*17*	39	*16*
remaining cytogenetics[a]	33	*21*	12	*14*	45	*19*
Unknown other cytogenetics[a]	15	*10*	10	*11*	25	*11*
Molecular subtypes						
NPM1 [b]	9	*5*	8	*9*	18	*7*
MLL-PTD [b]	3	*2*	3	*3*	6	*2*
CEBPA [b]	10	*6*	6	*7*	16	*6*
FLT3-ITD	30	*18*	18	*21*	48	*19*
KIT	12	*7*	6	*7*	18	*7*
N/KRAS	23	*14*	18	*21*	41	*16*
PTPN11	4	*2*	1	*1*	5	*2*

[#] Including 3 cases predicted as *MLL*-rearranged AML and confirmed with LDI-PCR
[a] Together forming the AML-other subtype used throughout this classification study. All samples were negative for MLL-rearrangements, t(8;21)(q22;q22), inv(16)(p13q22), t(15;17)(q21;q22) and t(7;12)(q36;p13)
[b] These subtypes were only observed in samples in the AML-other subtype

(p13q22), t(15;17)(q21;q22) and t(7;12)(q36;p13)-positive AML. A double-loop cross-validation approach was used that also included a backward selection procedure to keep the number of probe sets needed for most accurate classification to a minimum in order to avoid over-fitting of data, as previously described[22]. In the inner-loop the minimum number needed for highest predictive sensitivity of 100% was determined to be 75 probe sets (Table 2, Supplementary Table S3), i.e. 15 probe sets per cytogenetic subtype, whereas randomly selected probe sets only yielded a median sensitivity of 60% (Supplementary Figure S2). Some of these probe sets represented the same gene, e.g. four probe sets presented *RUNX1T1* for the t(8;21)(q22;q22) subtype (Table 2). The constructed classifier took into account the expression levels of all 75 probe sets, including the 60 probe sets that were not selected for a particular subtype. The classifier built with these 75 probe sets yielded a median accuracy of 92% in the outer loop. Notably, all inv(16)(p13q22), t(15;17)(q21;q22) and t(7;12)(q36;p13)-

Table 2: Overview of discriminative genes used in the classification of pediatric AML.

MLL-rearranged AML	**t(8;21) (q22;q22)**	**inv(16) (p13q22)**	**t(15;17) (q21;q22)**	**t(7;12) (q36;p13)**
WHAMML1 (2)	RUNX1T1 (4)	MYH11 (3)	HGF (2)	TP53BP2
ITM2A (2)	IL5RA (2)	LPAR1 (2)	STAB1 (2)	chr14q23.1
C10orf140 (2)	POU4F1 (2)	NT5E (2)	FAM19A5 (2)	DYX1C1
C10orf114	SIPA1L2 (2)	NRP1	ANXA8	EDIL3
CES1	TRH	TM4SF1	LGALS12	LIN28B
TBC1D12	PGAM5	LRP4	SIX3	BAMBI
PHACTR3	SIPA1L2	CLIP3	PGBD5	MAF
LOC84989	CACNA2D2	MN1	C2orf82	FAM171B
ZNF91	Unknown (chr8q21.3) (2)	SPARC	FGF13	AGR2
ZNF329		AK5	MST1	Unknown (chr2q14.3)
Unknown (chr13q22.1)		Unknown (chr17p13.3)	TNFRSF4	CRISP3
Unknown (chr10p12.31)			IGDCC4	MNX1
				CTTNBP2
				KRT72
				MMP9

In parentheses the number of probe sets representing the same gene. In total 75 probe sets were included in the classifying model, which represent 59 unique genes.

positive cases were correctly predicted in each of the 100 iterations (100% sensitivity, specificity, positive predictive value and negative predictive value) (Table 3A).

Hierarchical clustering showed that the cytogenetic subtypes formed distinct clusters according to the gene expression signature using these 75 probe sets (Figure 1A, Supplementary Table S3). Only three AML-other patients were misclassified as having *MLL*-rearranged AML. In these cases, *MLL* involvement could not be confirmed with FISH, but LDI-PCR revealed that all three samples did indeed harbor an *MLL*-rearrangement (Supplementary Table S4). These three samples were, therefore, included as true positive *MLL*-rearranged cases in the construction of the final classifier in the discovery cohort. As expected, including these cases as *MLL*-rearranged AML improved the diagnostic values of the 3-fold cross-validation in the outer loop (Table 3B).

Table 3: Diagnostic test values for the classification of pediatric AML by a gene expression signature consisting of 75 probe sets.

A)

	Discovery cohort[a]				
	3-fold cross-validation (100 iterations)				
	% sensitivity	**% specificity**	**% PPV**	**% NPV**	**% accuracy**
***MLL*-rearranged**	80 (70-90)	95 (95-98)	83 (78-89)	95 (93-97)	93 (92-95)
t(8;21)(q22;q22)	100 (83-100)	100 (100-100)	100 (100-100)	100 (98-100)	100 (98-100)
inv(16)(p13q22)	100 (100-100)	100 (100-100)	100 (100-100)	100 (100-100)	100 (100-100)
t(15;17)(q21;q22)	100 (100-100)	100 (100-100)	100 (100-100)	100 (100-100)	100 (100-100)
t(7;12)(q36;p13)	100 (100-100)	100 (100-100)	100 (100-100)	100 (100-100)	100 (100-100)
AML-other	92 (92-96)	92 (88-96)	92 (88-96)	93 (92-96)	92 (90-94)
All groups	92 (88-96)	92 (92-96)	93 (92-96)	92 (88-96)	92 (90-94)

[a] Values represent the median and 25th-75th percentiles (in parentheses) obtained by 3-fold cross-validation using the discovery cohort of 157 cases (100 iterations).

B)

	Discovery cohort[a]				
	3-fold cross-validation (100 iterations)				
	% sensitivity	**% specificity**	**% PPV**	**% NPV**	**% accuracy**
***MLL*-rearranged[#]**	90 (82-100)	97 (97-100)	92 (89-100)	98 (95-100)	96 (94-98)
t(8;21)(q22;q22)	100 (83-100)	100 (100-100)	100 (100-100)	100 (98-100)	100 (98-100)
inv(16)(p13q22)	100 (100-100)	100 (100-100)	100 (100-100)	100 (100-100)	100 (100-100)
t(15;17)(q21;q22)	100 (100-100)	100 (100-100)	100 (100-100)	100 (100-100)	100 (100-100)
t(7;12)(q36;p13)	100 (100-100)	100 (100-100)	100 (100-100)	100 (100-100)	100 (100-100)
AML-other	96 (96-100)	94 (89-96)	94 (88-96)	96 (96-100)	96 (92-98)
All groups	94 (89-96)	96 (96-100)	96 (96-100)	94 (88-96)	96 (92-98)

[a] Values represent the median and 25th-75th percentiles (in parentheses) obtained by 3-fold cross-validation using the discovery cohort of 157 cases (100 iterations).
[#] In contrast to Table 2A, the *MLL*-rearranged category in Table 2B now includes the 3 novel *MLL*-rearranged cases that were predicted by gene expression profiling and confirmed by LDI-PCR.

Independent validation of the classifier

The true accuracy of the classifier was tested in the independent validation cohort of 80 patients. The true sensitivity, specificity, positive predictive value, negative predictive value and accuracy in this validation cohort was 98%, 100%, 100%, 97% and 99%, respectively (Table 3C). Only one *MLL*-rearranged AML case was misclassified as AML-other (Table 4). Hierarchical cluster analysis also demonstrated the discriminative value of the selected probe sets in the independent validation cohort (Figure 1B). At this point, only

Table 3: Continued

C)

	Validation cohort				
	independent validation group, N=80				
	% sensitivity	**% specificity**	**% PPV**	**% NPV**	**% accuracy**
***MLL*-rearranged**	94	100	100	98	99
t(8;21)(q22;q22)	100	100	100	100	100
inv(16)(p13q22)	100	100	100	100	100
t(15;17)(q21;q22)	100	100	100	100	100
t(7;12)(q36;p13)	100	100	100	100	100
AML-other	100	98	97	100	99
All groups	98	100	100	97	99

patients at initial diagnosis of AML had been included. Next, we addressed whether the classifier was also suitable for predicting the subtype of 33 relapsed and eight secondary AML cases. All nine *MLL*-rearranged cases (3 secondary and 6 relapsed AML cases), all five t(8;21)(q22;q22) relapses and all 27 other relapse and secondary AML cases were correctly predicted by our classifier (Supplementary Table S5).

Comparison with other gene expression profiles in pediatric and adult acute myeloid leukemia

Ross *et al.* demonstrated that children with AML could be classified using gene expression profiles generated by Affymetrix U133A microarrays containing 22,283 probe sets[17]. An overall accuracy of 93% was achieved using 150 probe sets to classify *MLL*-rearranged AML, t(8;21)(q22;q22), inv(16)(p13q22), t(15;17)(q21;q22) and acute megakaryoblastic leukemias (M7). In that study, t(7;12)(q36;p13)-positive AML cases were not included. The 150 probe sets of Ross *et al.* were used to construct a classifier in our discovery cohort which was then applied to the independent validation cohort, exactly as done for testing our own 75 probe set-based classifier. Five out of 80 patients were misclassified, yielding an overall predictive accuracy of 94% for the Ross set compared to 99% for the 75 probe sets selected in this study (Supplementary Table S6). The misclassified cases included two *MLL*-rearranged and two t(7;12)(q36;p13)-positive cases which were assigned to the AML-other category and one AML-other case which was predicted as an *MLL*-rearranged case. Since only 40 out of our 75 probe sets were present on the U133A microarray used by Ross *et al.*, the reciprocal comparison of our list of selected probe sets on the Ross' dataset was not informative.

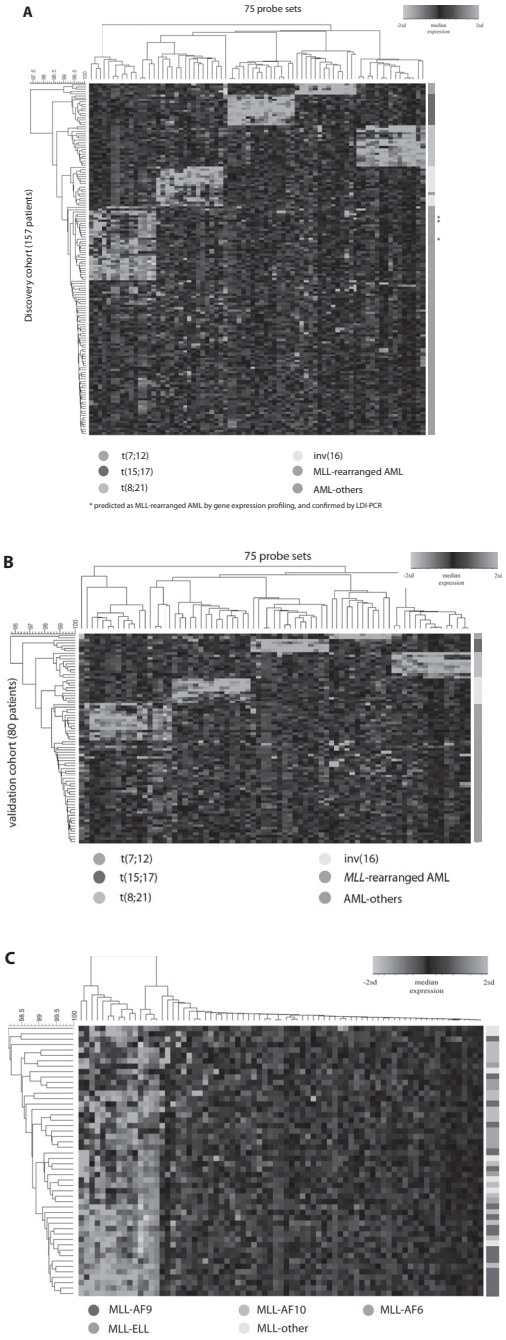

Figure 1: Hierarchical clustering of the cytogenetic subtypes of pediatric AML by gene expression profiling. a) Hierarchical clustering of 157 patients in discovery cohort by gene expression signature derived from 75 classifying probe sets (Supplementary Table S2). (b) Validation of gene expression pattern in 80 patients of the independent validation cohort (c) *MLL*-rearranged AML cases do not separate in distinct clusters based on similarity in expression pattern related to the translocation partner using the 75 classifying probe sets.

Table 4: Prediction of the classifier on the independent validation cohort.

		Subtype according to cytogenetic screening					
		MLL	**t(8;21)**	**inv(16)**	**t(15;17)**	**t(7;12)**	**AML-Other**
SVM Predicted Subtype	**MLL-rearranged**	15	0	0	0	0	0
	t(8;21)(q22;q22)	0	10	0	0	0	0
	inv(16)(p13q22)	0	0	10	0	0	0
	t(15;17)(q21;q22)	0	0	0	5	0	0
	t(7;12)(q36;p13)	0	0	0	0	2	0
	AML-Other	1	0	0	0	0	37

The sensitivity, specificity, positive predictive value, negativew predictive value and accuracy were respectively 98% (51/52), 100% (37/37), 100% (52/52), 97% (37/38) and 99% (79/80)

Valk *et al.* described 16 different subgroups in adult AML using 2856 probe sets present on Affymetrix U133A microarrays[18]. A classifier built with these 2856 probe sets resulted in an overall accuracy of 94% when applied to our independent pediatric validation cohort (Supplementary Table S6). Three *MLL*-rearranged cases and one t(7;12)(q36;p13)-positive case were misclassified as AML-others. One AML-other patient was misclassified as an *MLL*-rearranged AML.

Potential type-II molecular aberration: *NPM1*, *CEBPA* and *MLL*-PTD

Mutations in *NPM1* and *CEBPA* and partial tandem duplications in *MLL* (*MLL-PTD)* might be considered as various type-II mutations and were only observed in samples belonging to the AML-other group (Supplementary Table S7). Moreover, these molecular abnormalities were mutually exclusive reflecting heterogeneity amongst the AML-other cases. For *CEBPA*, 852 probe sets were found to be statistically discriminative between mutated and germ-line cases whereas only 12 probe sets were found to be discriminative for *MLL*-PTD at the same cut-off value of $p<0.05$ (FDR-corrected; Supplementary Table S8). 3-fold cross-validation with the top 15 most discriminative probe sets for mutations in *NPM1*, *CEBPA* and *MLL* (i.e. *MLL*-PTD) revealed a median sensitivity and accuracy in the outer loop of 43% and 92% respectively and a sensitivity, specificity, positive predictive value, negative predictive value and accuracy of 18%, 98%, 75%, 82% and 81%, respectively, in the independent validation cohort (Table 5A, Supplementary Table S9). In the independent validation cohort 7/8 cases with an *NPM1* mutation, 4/6 cases with a *CEBPA* mutation and all three *MLL*-PTD cases were misclassified. Moreover, when adding these three molecular subtypes to the previously used five cytogenetic subtypes, the accuracy of 99% based on the five cytogenetic subtypes dropped to 78% in the validation cohort (Supplementary Table S10). All misclassified cases were assigned to the AML-other category.

Table 5: Diagnostic test values for the prediction of mutations in *NPM1*, *CEBPα* and *MLL*-PTD for the independent validation cohort by gene expression signature consisting of 45 probe sets (A) and for the prediction of type-I molecular subtypes for the independent validation cohort by gene expression signature consisting of 30 probe sets (B).

A)

	Validation cohort				
	independent validation group, N=80				
	% sensitivity	**% specificity**	**% PPV**	**% NPV**	**% accuracy**
NPM1	13	99	50	91	90
***MLL*-PTD**	0	100	ND	96	96
CEBPA	33	100	100	95	95
Remaining cases	98	18	82	75	81
All groups	18	98	75	82	81

B)

	Validation cohort				
	independent validation group, N=80				
	% sensitivity	**% specificity**	**% PPV**	**% NPV**	**% accuracy**
***FLT3*-ITD**	72	100	100	93	94
KIT	33	99	66	95	94
Remaining cases	89	63	86	94	88
All groups	63	89	94	86	88

Type-I mutations: *FLT3*-ITD, *KIT*, *N/KRAS* and *PTPN11*

Internal tandem duplication in *FLT3 (FLT3*-ITD), mutations in *KIT* and mutations in genes involved in the RAS-pathway (*NRAS* (n=34), *KRAS* (n=7) and *PTPN11* (n=5)) were observed in 44% of all cases. In contrast to *FLT3*-ITD and *KIT* aberrations, no discriminative probe sets were found for *N/KRAS* and only a limited number for *PTPN11* (Supplementary Table S8). Combining the aberrations in the RAS-pathway into one group still did not identify discriminative probe sets. Therefore, we only included *FLT3*-ITD and *KIT* into a classification model for the prediction of type-I mutations. However, the 30 most discriminative probe sets for these subtypes resulted in a classifier with limited predictive value. The highest predictive values were found for *FLT3*-ITD, with a positive predictive value and negative predictive value of 100% and 93%, respectively (Table 5B, Supplementary Table S11). Inclusion of the top 15 discriminative probe sets for aberrations in the RAS-pathway (although with $p>0.05$) did not result into prediction of this subtype (Supplementary Table S12).

Although a large number of discriminative genes were found for *FLT3*-ITD (Supplementary Table S8), many probe sets were similar to those found for t(15;17)(q21;q22) instead of being specific for *FLT3*-ITD. This is in line with the fact that *FLT3*-ITD is often found in t(15;17)(q21;q22)-positive cases. To correct for these cytogenetic effects, we applied the Bayes linear regression model while adjusting for cytogenetic subtype. In this multivariate analysis, unique gene expression signatures specific for *FLT3*-ITD positive cases were found that differ between t(15;17)(q21;q22)-positive and CN-AML cases (Figure 2, Supplementary Table S13). Specifically, the genes of the *HOXB* cluster were over-expressed in all patients with a *FLT3*-ITD positive CN-AML and not in *FLT3*-ITD negative CN-AML or t(15;17)(q21;q22) patients (Figure 2, Supplementary Figure S3). The same multivariate approach for *KIT* and RAS-pathway mutations did not result in cytogenetic subtype specific gene expression signatures.

When adding the 15 probe sets discriminative for t(15;17)(q21;q22)/*FLT3*-ITD and the 9 most discriminative probe sets [the 6 other probe sets showed overlap with the t(15;17)(q21;q22) subgroup] for CN-AML/*FLT3*-ITD from the multivariate analysis to our classifier, we still could not accurately predict all *FLT3*-ITD cases. Although numbers were small in these subgroups, the accuracy in the independent validation cohort dropped to 86% due to misclassification of cases, especially CN-AML/*FLT3*-ITD cases (Supplementary Table S14).

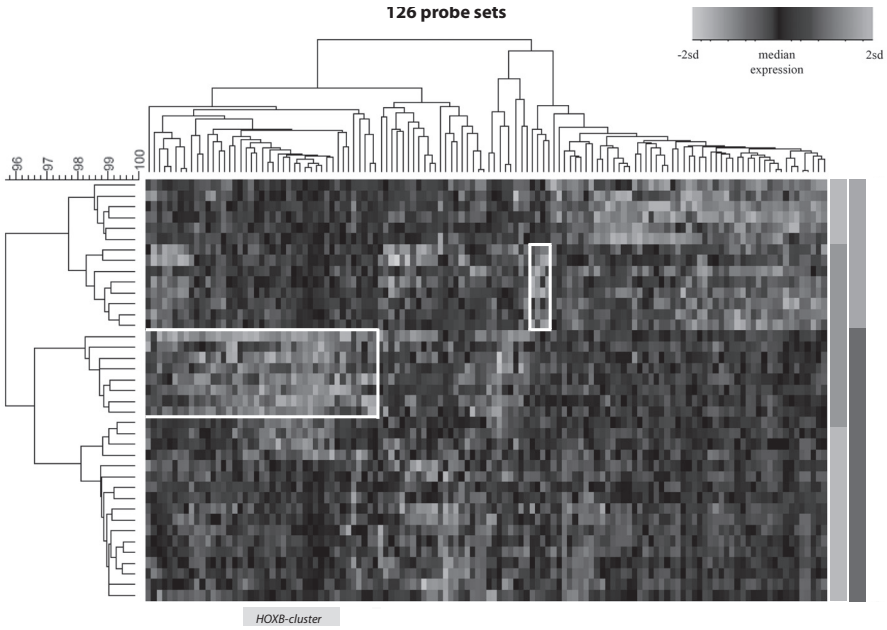

Figure 2: Hierarchical clustering of *FLT3*-ITD positive cases in t(15;17)(q21;q22) and CN-AML. Hierarchical clustering for *FLT3*-ITD in t(15;17)(q21;q22) and CN-AML based on 126 probe sets selected by multivariate analysis including molecular and cytogenetic subtype (Supplementary Table S12). Highlighted boxes represent probe sets for *FLT3*-ITD and the specific cytogenetic subtype. The *HOXB* cluster probe sets are represented in yellow.

DISCUSSION

Cytogenetic aberrations have prognostic value in pediatric AML and, hence, genetic subtypes are used for risk stratification in most current pediatric AML treatment protocols and are part of the current WHO classification of myeloid neoplasms and acute leukemia[2]. In the present study we explored the possibility of microarray-based gene expression profiling to identify the cytogenetic and molecular subtypes in pediatric AML. A gene expression signature of 75 probe sets predicted the most important non-random cytogenetic aberrations in an independent pediatric AML cohort with 99% accuracy and a positive and negative predictive value of 100% and 97%, respectively. In addition, unique gene-expression signatures were found for *FLT3*-ITD AML which differed between cytogenetic subtypes.

Gene expression profiling has been shown to predict the major cytogenetic subgroups in both pediatric and adult AML[17-19]. The sensitivity and specificity of such a signature should be addressed in an independent and representative cohort, since microarray data analysis can easily result into over-interpretation of data[43]. Recently, the MILE study group, using an independent validation cohort of 1152 cases, robustly showed that gene expression profiles can be used to classify different types of (mainly adult) myelodysplastic syndrome and chronic and acute leukemia cases into known cytogenetic subtypes[21]. This study mainly included adult cases (whose disease may differ in etiology from that of children) and did not address the prediction of molecular abnormalities (e.g. *FLT3*-ITD and *RAS* mutations) by gene expression profiles. In the present study we specifically addressed the value of gene expression profiles for prediction of and classification based on cytogenetic and molecular subtypes of children at initial diagnosis of AML. We previously showed the predictive value of a double-loop cross-validation approach to select classifying probe sets and validate these using an independent validation cohort in pediatric ALL[22], and therefore used the same unbiased approach in this childhood AML study.

In the present study we identified a gene expression signature of 75 probe sets, representing 15 probe sets for each subgroup, to predict *MLL*-rearranged, t(8;21)(q22;q22), inv(16)(p13q22), t(15;17)(q21;q22) and t(7;12)(q36;p13) positive AML. When applied to our independent validation cohort the sensitivity, specificity, negative and positive predictive values of this signature were 98%, 100%, 100% and 97%, respectively. The prediction of *MLL*-rearranged AML, in particular, was better with the newly selected probe sets compared to the previously used probe sets compiled by Ross *et al.*[17] and Valk *et al.*[18]. Since 35 probe sets were not present on the Affymetrix U133A microarrays used in both former studies, these new probe sets are perhaps decisive for correct prediction of cytogenetic subgroups in pediatric AML.

The 75 probe sets harbored probe sets for genes involved in the specific translocations, e.g. the probe sets for *RUNX1T1/ETO* were highly discriminative for t(8;21)(q22;q22),

those for *MYH11* for inv(16)(p13q22) and the probe set for *HLXB9* for t(7;12)(q36;p13). The high expression of these probe sets is probably related to specific hybridization to the fusion transcript as suggested by Kohlmann *et al.*[44]. Remarkably, six out of 15 probe sets discriminative for *MLL*-rearranged AML (Table 2) were located in non-protein coding regions of the genome. Four of these probe sets were located in a relative small (<40 Kb) region on chromosome 10. This is of interest, since nowadays these regions cannot be considered as junk DNA, but might be involved in the regulation of other genes, such as miRNAs[45].

In parallel to the present study we found that expression of one of the discriminative genes for *MLL*-rearranged AML, i.e. brain and reproductive organ-expressed gene (*BRE*), was highly associated with a favorable prognosis in cases with t(9;11)(p22;q23). Functional studies with *BRE* did not reveal that the proliferation, apoptosis or sensitivity towards drugs was altered upon re-expression in AML cells, suggesting that BRE itself has no anti-proliferative function [46]. Besides *BRE*, other new prognostic genes have recently been identified using gene expression profiling. An example is the angiogenic factor *VEGFC*, for which high level expression was associated with an unfavorable clinical outcome in both childhood and adult AML[47]. According to gene expression profiling, the *EVI1* gene had no prognostic impact in children with AML, in contrast to the situation in adult, emphasizing the need for separate analysis of pediatric and adult AML[48]. In adult AML gene expression profiling showed that high expression of *ERG* and *MN1* was related to outcome, although their role in malignant transformation remains unknown[49-50]. More recently a unique gene expression signature was identified for the prognostically relevant mutation in *IDH2*, which may lead to unravel the role of *IDH2* in the biology of AML[51]. Thus, gene expression profiling identifies new genes linked to subtypes and/or prognosis of AML and may provide important information about the biology of disease when further functional studies have been performed. This knowledge is needed for the rational development and optimization of treatment protocols; moreover, affected genes and pathways may serve as targets for new therapies ('targeted therapy').

Three AML-other cases that were negative by MLL-split signal FISH were initially thought to be misclassified as *MLL*-rearranged AML. However, more detailed analysis of the *MLL*-gene using LDI-PCR confirmed that the *MLL*-gene was indeed rearranged, indicating the higher sensitivity of our gene expression signature than the routine diagnostic FISH procedure in the detection of *MLL*-rearranged cases. Cryptic *MLL*-rearrangements can also be detected using single nucleotide polymorphism (SNP)-array platforms e.g. t(6;11)(q27;q23)[52-53]. These finding illustrate the high potential of advanced methods, such as gene expression profiles, LDI-PCR and SNP-arrays, for detecting rearrangements of the *MLL*-gene in clinical AML samples.

The cytogenetic subgroups that could be correctly predicted in our study represent approximately 40% of the pediatric AML cases (Supplementary Table S1). The remaining

patients in this study had CN-AML or other than the five cytogenetic subgroups tested in this study. Recently the subgroup with CN-AML has been further characterized by recurrent molecular aberrations in *NPM1*, *CEBPA* and *MLL* (-PTD). The heterogeneity of pediatric AML is further illustrated by molecular aberrations detected in different cytogenetic subgroups, i.e. *FLT3*-ITD, *KIT* and mutations in the RAS-pathway. Our results show a less accurate prediction of these molecular aberrations by gene expression signatures compared to signatures predictive for cytogenetic subtypes, as was also observed in adult AML[19,54]. Only *FLT3*-ITD could be predicted with a high negative predictive value (93%) and positive predictive value (100%) in the independent validation cohort, but showed lower sensitivity, as described in adult AML cases with *FLT3*-ITD[55]. The low predictive value for the molecular subtypes including mutations in *NPM1*, *MLL*-PTD, *KIT* and *PTPN11* can be explained by the limited number of discriminative probe sets found for each of these aberrations (Supplementary Table S8 versus Supplementary Table S2). This may be caused by both a limited sample size for each molecular subtype and underlying cytogenetic lesions that have a differential effect on gene expression signatures. A limited sample size itself does not, *per se*, hamper the accuracy of classification if the number of statistically significant probe sets and the fold-change in expression levels of discriminative probe sets is relative high (exemplified by the high accuracy to predict t(7;12)(q36;p13) positive cases despite only five cases being included in the discovery cohort, Supplementary Table S2). However, in combination with heterogeneity in underlying cytogenetic abnormalities (or other genetic lesions) the number of highly discriminative probe sets becomes limited when sample size is also limited.

Since some of these mutations are not mutually exclusive or are restricted to distinct cytogenetic subtypes, we also selected discriminative probe sets for *FLT3*-ITD using a multivariate approach including cytogenetic subtype. For the other molecular subtypes probe sets could not be identified in a multivariate setting, presumably because of the limited frequency of occurrence of these mutations. Moreover, overlapping signaling pathways between subgroups, no effect of the mutation on transcription level, or different mutations per gene can make it difficult to predict these molecular aberrations correctly by gene expression profiles[15,56].

Depending on the cytogenetic background, the specific genetic aberrations may play different roles in the leukemogenesis of pediatric AML. Interestingly, the genes of the *HOXB* cluster were over-expressed in all patients with a *FLT3*-ITD positive CN-AML, but not in those with a *FLT3*-ITD positive t(15;17)(q21;q22), which is in concordance with differences in prognostic relevance between these two subgroups. In adult AML, some of these *HOXB* genes were also identified as discriminating genes for patients with a *FLT3*-ITD[19]. In pediatric AML, *HOXB* upregulation has been correlated with *NPM1* mutations in CN-AML[57]. Here we show that *HOXB* overexpression is not restricted to *NPM1* mutated cases, but is also found in all patients with a *FLT3*-ITD positive CN-AML.

In conclusion, a specific gene expression signature existing of 75 probe sets could accurately identify five cytogenetic subgroups in pediatric AML. Molecular aberrations were hard to predict, which could be due to the low frequency of some of these aberrations and/or gene expression signatures being affected by the underlying cytogenetic abnormality. It remains to be determined whether underlying but yet unknown genetic aberrations in the remaining cases of AML will result in distinct gene expression patterns that can be used for classification. Classification by gene expression profiling may reduce the number of cases for which multiple diagnostic procedures (cytomorphology, FISH, RT-PCR, karyotyping) are performed by at least 40%. In order to use gene expression signatures as new diagnostic tool, prospective studies are needed that determine the feasibility of obtaining sufficient high quality RNA for successful gene expression profiling in clinical practice. Importantly, gene expression profiles may give more insight into the biology and the patho-physiology of the different subtypes of AML which may than point to new ways to treat these patients more effectively.

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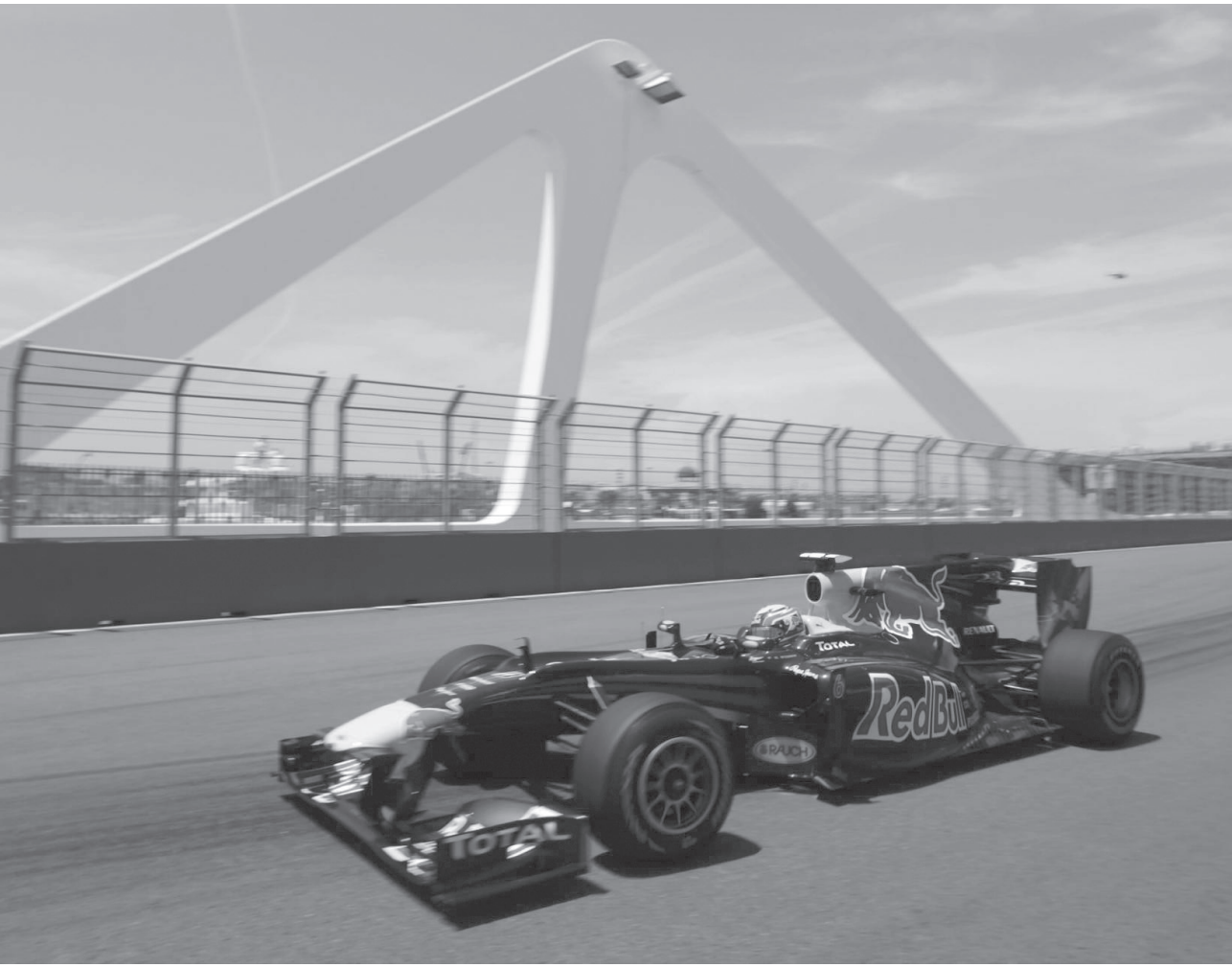
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Chapter 4

EVI1 overexpression in distinct subtypes of pediatric acute myeloid leukemia

B.V. Balgobind[1], S. Lugthart[2], I.H. Hollink[1], S.T.J.C.M Arentsen-Peters[1], E.R. van Wering[3], S.S.N. de Graaf[3,4], D. Reinhardt[5], U. Creutzig[6], G.J.L. Kaspers[7], E.S.J.M. de Bont[8], J. Stary[9], J. Trka[9], M. Zimmermann[5], H.B. Beverloo[10], R. Pieters[1], R. Delwel[2], C.M. Zwaan[1], M.M. van den Heuvel-Eibrink[1]

[1]Department of Pediatric Oncology/Hematology, Erasmus MC – Sophia Children's Hospital, Rotterdam, The Netherlands. [2]Deparment of Hematology, Erasmus MC, Rotterdam, The Netherlands. [3]Dutch Childhood Oncology Group (DCOG), The Hague, The Netherlands. [4]Department of Pediatric Oncology/Hematology, University Medical Center St Radboud, Nijmegen, The Netherlands. [5]AML-BFM Study Group, Pediatric Hematology/Oncology, Medical School Hannover, Hannover, Germany. [6]AML-BFM Study Group, Pediatric Hematology/Oncology, University Hospital, Munster, Germany. [7]Department of Pediatric Oncology/Hematology, VU University Medical Center, Amsterdam, The Netherlands. [8]Department of Pediatric Oncology/Hematology, Beatrix Children's Hospital, University Medical Center Groningen, Groningen, The Netherlands. [9]Department of Pediatric Hematology/Oncology, [2]nd Medical School, Charles University, Prague, Czech Republic. [10]Department of Clinical Genetics, Erasmus MC Rotterdam, The Netherlands.

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ABSTRACT

Overexpression of the ecotropic virus integration-1 (*EVI1*) gene (*EVI1*+), localized at chromosome 3q26, is associated with adverse outcome in adult acute myeloid leukemia (AML). In pediatric AML, 3q26-abnormalities are rare, and the role of *EVI1* is unknown. We studied 228 pediatric AML samples for *EVI1*+ using gene expression profiling and RQ-PCR. *EVI1*+ was found in 20/213 (9%) of children with *de novo* AML, and in 4/8 with secondary-AML. It was predominantly found in *MLL*-rearranged AML (13/47), monosomy 7 (2/3), or FAB M6/7 (6/10), and mutually exclusive with core binding factor AML, t(15;17), and *NPM1*-mutations. Fluorescent *in situ* hybridization (FISH) was performed to detect cryptic 3q26-abnormalities. However, none of the *EVI1*+ patients harbored structural 3q26-alterations. Although significant differences in 4-years pEFS for *EVI1*+ and *EVI1*- pediatric AML were observed (28%±11 vs. 44%±4, p=0.04), multivariate analysis did not identify *EVI1*+ as an independent prognostic factor. We conclude that *EVI1*+ can be found in ~10% of pediatric AML. Although *EVI1*+ was not an independent prognostic factor, it was predominantly found in subtypes of pediatric AML that are related with an intermediate to unfavorable prognosis. Further research should explain the role of *EVI1*+ in disease biology in these cases. Remarkably, no 3q26-abnormalities were identified in *EVI1*+ pediatric AML.

INTRODUCTION

In various myeloid malignancies 3q26-rearrangements can be found[1]. These abnormalities are often associated with overexpression of the ecotropic virus integration-1 (*EVI1*) gene, which is localized at 3q26[2]. The *EVI1* gene encodes for a DNA-binding protein with two zinc-finger domains[3]. It has been shown to play an essential role in early development, since inactivation of *EVI1* in mice embryos is lethal within 8 days after conception[4]. More information on the role of *EVI1* in leukemogenesis was gained in murine leukemia studies using retroviral insertion[5]. Both in mouse and human myeloid progenitors, overexpression of *EVI1* is suggested to impair granulocytic differentiation in hematopoietic stem cells, and hence to result in maturation arrest[2]. Interestingly, myeloid malignancies associated with *EVI1* overexpression often show dysplastic megakaryopoiesis[1,6]. Although the *EVI1* function is not fully understood, recent studies suggest that this gene is involved in chromatin remodeling, through interactions with H3K9 methyltransferases[7-8].

Five splice variants of *EVI1* have been reported, that is *EVI1*-1A,-1B,-1C,-1D and -3L, as well as the *MDS1*/*EVI1* intergenic splice variant[9-10]. The *MDS1* gene is located upstream of *EVI1* and its function is currently unknown. In the *MDS1/EVI1* transcripts the first two exons of *MDS1* have been fused to exon 2 of *EVI1*, resulting in a so-called PR domain containing the EVI1 protein[10]. The PR domain is highly correlated to the SET domain, which has been shown to have a critical role in chromatin-mediated gene expression histone-methyltransferases[11]. In cells that express *MDS1/EVI1* transcripts, the *EVI1* transcripts are normally expressed as well.

In adult acute myeloid leukemia (AML) overexpression of *EVI1* is found in particular in patients with a 3q26-rearrangement, such as inv(3)(q21q26) or t(3;3)(q21;q26). However, high *EVI1* levels have also been discovered in a separate subgroup of AML patients without 3q26-rearrangements[12-13]. In clinical studies in adult AML, overexpression of *EVI1* has shown to be an independent prognostic factor, irrespective of harboring typical 3q26-rearrangements. It was recently shown that high *EVI1* expression can also occur in the absence of the *MDS1/EVI1* transcript in patients with cryptic 3q26-rearrangements involving the *EVI1* gene[13]. In contrast, patients with high expression of both *EVI1* and *MDS1*/*EVI1* were frequently found in adult *MLL*-rearranged AML cases[13].

In children with AML, 3q26-rearrangements have not been frequently described and the role of *EVI1* is unknown[14]. Therefore, we studied the occurrence and the role of *EVI1* overexpression in a large cohort of 228 children with AML.

MATERIALS & METHODS

Patients

Viably frozen bone marrow or peripheral blood samples from 221 patients with newly diagnosed AML, comprising 213 with *de novo* and 8 with secondary-AML, were provided by the Dutch Childhood Oncology Group (DCOG), the 'Berlin-Frankfurt-Münster' AML Study Group (AML-BFM-SG), and the Czech Pediatric Hematology. In addition, 7 relapse samples (no paired samples were included) of AML patients were included. Informed consent was obtained from all patients, after Institutional review Board approval according to national law and regulations. As a control for *EVI1* expression, normal bone marrow of 2 children and 6 adults with informed consent was available at the Erasmus MC - Sophia Children's Hospital in Rotterdam, The Netherlands. Each study group performed central review of the morphology, according to the WHO/FAB (French-American-British) classification[15]. They also provided data on the clinical follow-up of these patients. Survival analysis was restricted to a subset of 198 *de novo* AML patients who were treated using AML-BFM-98, AML-BFM 2004, DCOG-BFM-87, DCOG 92/94, DCOG 97 protocols. Details of the treatment protocols included in the survival analysis and overall outcome data have been previously published, with the exception of study AML-BFM 2004, which is ongoing [16-18]. Because of a selection based on material availability, the survival rates of this patient cohort studied for survival analysis (n=198) are slightly different from the studies published earlier[16-18]. Treatment consisted of 4 to 5 blocks of intensive chemotherapy, using a standard cytarabine and anthracycline backboneLeukemic cells were isolated and enriched from these samples as described earlier[19]. All resulting samples contained ≥80% leukemic cells, as determined morphologically by May-Grünwald-Giemsa (Merck, Darmstadt, Germany)-stained cytospins. A minimum of 5×10^6 purified leukemic cells were lysed in Trizol reagent (Gibco BRL, Life Technologies, Breda, The Netherlands) and stored at -80°C. Genomic DNA and total cellular RNA were isolated as described earlier[20].

Cytogenetic and molecular analysis

Leukemic samples were routinely investigated for cytogenetic abnormalities by standard chromosome-banding analysis, and screened for recurrent non-random genetic abnormalities characteristic for AML, including t(15;17), inv(16), t(8;21) and *MLL*-rearrangements, using either real-time polymerase chain reaction (RT-PCR) and/or fluorescent *in situ* hybridization (FISH) by each study group. *NPM1*, *CEBPA*, *MLL*-PTD, *NRAS*, *KRAS*, *PTPN11*, *KIT*, *FLT3* mutational screening was performed as described earlier[21-26].

Microarray-based gene expression profiling

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[27]) and probe-set intensities were normalized using the variance stabilization normalization (Bioconductor package VSN[28]) in the statistical data analysis environment R, version 2.2.0[29].

Identification of *EVI1* overexpression with gene expression profiling

Four probe sets are positioned within the *EVI1* gene (Supplementary Figure 1). Normalized intensities of these probe sets were extracted from the complete dataset and further clustering analysis was performed with Genemaths XT (Applied Maths, Austin, TX, USA). Of each probe set, the standard deviation using the median as cut off was calculated for all patients. As the probe sets *243277_x_at* and *215851_at* were located in introns of the *EVI1* gene, only the probe sets *221884_at* and *226420_at* were used for hierarchical clustering using the Euclidean distance. Samples were considered to have an abnormal *EVI1* expression (*EVI1+*) based on the hierarchical clustering dendrogram.

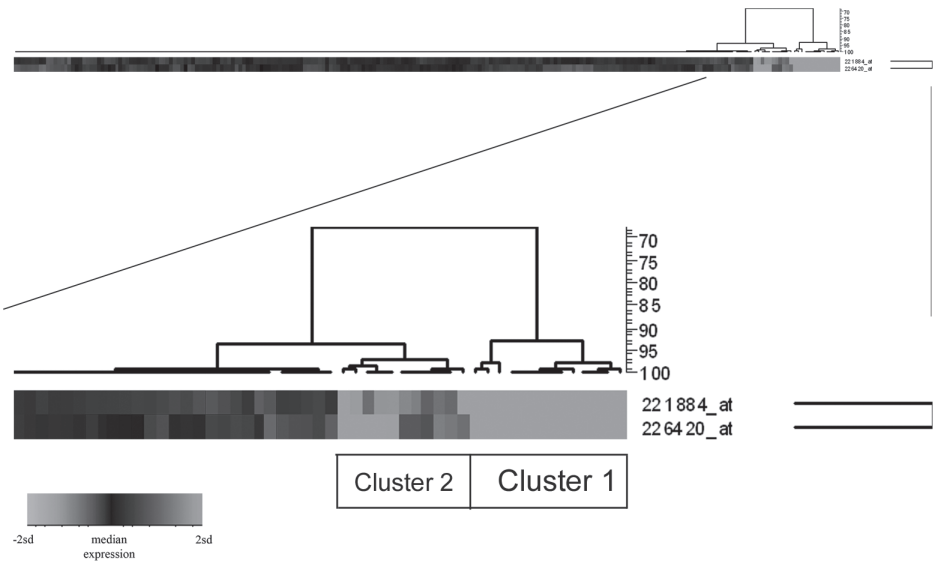

Figure 1: Hierarchical clustering using the gene expression of the 3 probe sets representing *EVI1* in 228 pediatric AML samples reveals a subclustering of 24 *EVI1+* cases.

Hierarchical clustering of 228 pediatric AML samples with probe sets 221884_at and 226420_at representing the *EVI1* gene. Red represents high expression; black intermediate expression; and green low expression for the specific probe set.

Gene expression signatures for *EVI1*+ cases

To find gene expression signatures for *EVI1*, an empirical Bayes linear regression model was used (R package limma)[30]. Moderated T-statistics p-values were corrected for multiple testing using the false discovery rate (FDR) method defined by Benjamini and Hochberg[31]. This was performed using models without and with correction for the different cytogenetic subgroups (*MLL*-rearranged AML, t(8;21), inv(16), t(15;17), normal-, remaining-, and unknown cytogenetics)[30]. In addition, gene expression signatures were generated for *EVI1*+ in specific subsets of AML (that is *MLL*-rearranged AML and non *MLL*-rearranged AML).

RT quantitative PCR and Fluorescence in situ hybridization (FISH)

In 179 samples, including 22 samples with *EVI1* overexpression based on microarray analysis, the RNA expression could be validated by RT quantitative PCR (RQ-PCR). For the other 49 samples, no additional RNA was available to perform RQ-PCR. The relative expression of the *EVI1* transcripts (*EVI1*-1A, -1B, -1D, and -3L) and *MDS1*/*EVI1* transcript was calculated using the comparative cycle time (ΔCt) method, with *GAPDH* as the house-keeping gene[32]. Primer en probe sequences are shown in Supplementary Table 1. A sample was considered *EVI1*+ with RQ-PCR if the cumulative relative expression *EVI1*-1A, -1B and -3L to *GAPDH* was above 1.5%. This showed the highest correlation with *EVI1*+ cases based on gene expression profiling and all normal bone marrow samples were below this threshold.

Interphase cytospins of cases with a high *EVI1* expression were screened with FISH for cryptic 3q26 abnormalities with the Poseidon™ Repeat Free™ EVI t(3;3), inv(3) Break probe (Kreatech, Amsterdam, The Netherlands) according to manufacturer's protocol.

Additional Statistical analysis

Statistical analysis was performed with SPSS 11.0 (SPSS Inc. Chicago, IL, USA) and SAS (SAS-PC, version 9.1). Different variables were compared with the Chi-square test or the Mann-Whitney U test. Probabilities of overall survival (pOS) and event-free survival (pEFS, events: no CR, relapse, secondary malignancy, death from any cause) were estimated by the method of Kaplan and Meier. Correlation between microarray gene expression and the RQ-PCR of the different transcript of *EVI1* was measured with the Spearman correlation coefficient. The Cox Proportional hazards model analysis was applied to determine the association of *EVI1*+ with pOS, pEFS, adjusted for prognostic factors. All tests were two-tailed and a P-value of <0.05 was considered significant.

Table 1: Patients characteristics and expression levels of *EVI1* transcripts

ID	AML type	Karyotype	FAB	MLL-FISH positive	RQ-PCR					FISH inv(3)
					EVI1-A %[1]	EVI1-B %[1]	EVI1-3L %[1]	EVI1-D %[1]	MDS/EVI1 %[1]	
#1	s-AML	*46,XX,t(9;11)(p22;q23)[41]/47,XX,idem,+8[2]*	M5	yes	3.44	1.70	0.48	0.023	0.000	neg
#2	p-AML	*46,X,t(X;6)(p1?2;q2?1)*	M6	no	3.65	2.46	0.66	0.068	0.246	neg
#3	r-AML	*NA*	M1	no	1.89	0.76	0.16	0.004	0.001	neg
#4	p-AML	*46,XY,t(6;11)(q27;q23)*	M5	yes	35.04	7.29	13.53	0.072	0.232	neg
#5	p-AML	*47,XY,+21*	M5	no	15.25	7.81	2.73	0.183	0.086	neg
#6	p-AML	*46,XY,t(9;11)(p22;q23)*	M7	yes	76.43	48.25	11.94	0.997	1.285	neg
#7	p-AML	*46,XY,t(9;11)(p22;q23)/48,idem,+8,+mar*	M5	yes	6.94	4.24	3.12	0.031	0.150	neg
#8	p-AML	*46,XX,t(11;20)(p15;q1?2)[20]*	M4	no	31.59	7.26	10.31	0.195	0.028	neg
#9	p-AML	*46,XX,t(6;11)(q27;q23)*	M4	yes	21.28	8.49	8.42	0.085	1.275	neg
#10	p-AML	*46,XX,t(11;19)(q23;q11)*	M2	yes	2.45	1.41	0.84	0.014	0.033	neg
#11	s-AML	*46,XX,t(11;17;?)(q23;q?21;?)*	M4	no	6.75	3.57	1.66	0.079	0.000	neg
#12	p-AML	*46,XX,inv(9)(p11q13),t(11;17)(q23;q12)*	M5	yes	11.62	3.11	0.89	0.051	0.034	neg
#13	s-AML	*46,XX,t(9;11)(p22;q23)[4]/46,XX[7]*	M5	yes	11.61	1.96	2.65	0.042	0.066	neg
#14	p-AML	*42~44,XY,t(6;11)(q27;q23)[cp2]/ 51,idem,+X,+der(6)t(6;11)(q27;q23),+8,+19,+21[5]*	M5	yes	31.59	7.26	10.31	0.195	0.028	neg
#15	p-AML	*46,XX*	M5	no	9.35	10.68	2.15	0.065	0.052	neg
#16	p-AML	*NA*	M7	no	2.90	5.55	2.99	0.046	0.090	neg
#17	p-AML	*46,XY,add(11)(q23),inc*	M1	yes	5.13	0.87	0.92	0.012	0.101	neg
#18	p-AML	*NA*	M6	no	2.26	1.99	0.24	0.017	0.000	neg
#19	p-AML	*46,XX,t(8;13)(q22;q1?4) [8]/48,idem,+6,+mar[4]/46,XX[8]*	M7	no	9.23	8.10	16.50	0.017	0.339	neg
#20	s-AML	*45,XY,-7[8]/49,XY,-7,+9,+10,+14,+21[12]*	M2	no	0.17	0.20	0.19	0.007	0.128	neg
#21	p-AML	*46,XX,t(9;11)(p22;q23)[5]*	M4	yes	NA	NA	NA	NA	NA	neg
#22	p-AML	*46,XY[20]*	M5	yes	NA	NA	NA	NA	NA	neg
#23	p-AML	*45, XX,inv(2)(p24q14),-7*	M4	no	0.80	0.85	0.43	0.008	0.009	neg
#24	p-AML	*NA*	M7	no	1.01	2.47	8.35	0.002	0.022	neg
#25	p-AML	*46,XY,t(6;11)(q27;q23)[15]*	M1	yes	7.26	3.48	2.70	0.034	0.533	neg
-		*normal bone marrow (median relative expression)*	-	-	0.04	0.09	0.03	0.001	0.000	neg

1) Relative expression to GAPDH 2) no material available NA: not available neg: negative ND: not determined
p-AML = primary-AML, s-AML = secondary-AML, r-AML = relapsed-AML

RESULTS

EVI1 overexpression in pediatric AML as determined by gene expression profiling.
Four probe sets on the Affymetrix Human Genome U133 Plus 2.0 Array were present within the *EVI1* gene (Supplementary Figure 1). These probe sets were located in regions common for all isoforms of *EVI1*. However, *243277_x_at* was found in intron 2-3 and *215851_at* was found partly in intron 15-16. As these short probe sets also showed more random variation of expression, they were not included in the hierarchical clustering analysis. By means of hierarchical clustering of the gene expression profiling data, 3 separate clusters could be identified: one cluster with high expression of *EVI1* (cluster 1), one cluster with an intermediate to high expression of *EVI1* (cluster 2) and one large cluster with low expression of *EVI1*. Therefore, 24 cases in the clusters with intermediate and high expression were considered *EVI1*+ based on the dendrogram (Figure 1). These cases included 19/213 (9%) patients with *de novo* AML, 4/8 (50%) patients with secondary AML, and one patient of whom only relapse material was available.

Validation of _EVI1_ expression and 3q26-aberrations by real-time quantitative PCR and FISH.
Of the 24 patients with a high *EVI1* expression, 22 could be investigated for the various *EVI1* and the *MDS1/EVI1* transcripts using RQ-PCR. Of two patients the amount of available mRNA was not sufficient. In addition, RQ-PCR was also performed on 150 of the 197 remaining pediatric AML samples for which additional mRNA material was available (Figure 2). Normal bone marrow samples of eight individuals (six adults and two pediatric) were used as a control for normal expression of the different transcripts.
Twenty-one of the 22 patients identified by gene expression profiling had a cumulative relative expression to GAPDH of at least 1.5% for one of the *EVI1* transcripts (Table 1). The other patient (#20), whom lacked a cumulative relative expression of at least 1.5%, did show overexpression of *EVI1*-1D and *MDS1/EVI1*, and was considered *EVI1*+. In addition, one patient with FAB-M7 (#24) whom showed low expression of *EVI1* on gene expression profiling, did show abnormal expression of *EVI1* with RQ-PCR. All remaining samples did not show *EVI1* overexpression (Figure 2). Although only 22/23 *EVI1*+ cases could be identified with gene expression profiling in comparison with RQ-PCR, still RQ-PCR for these four *EVI1* isoforms showed high correlation with the gene expression data. Spearman correlation coefficients were 0.68 for *EVI1*-1A, 0.63 for *EVI1*-1B, 0.52 for *EVI1*-3L and 0.78 for *EVI1*-1D. Moreover, microarray analysis showed a sensitivity of 95%, a specificity of 100%, a positive predictive value of 100%, and a negative predictive value of 99%.
On the base of conventional cytogenetics, none of the patients harbored a 3q26 aberration. In addition, only three *EVI1*+ patients lacked the *MDS1/EVI1* transcript, which

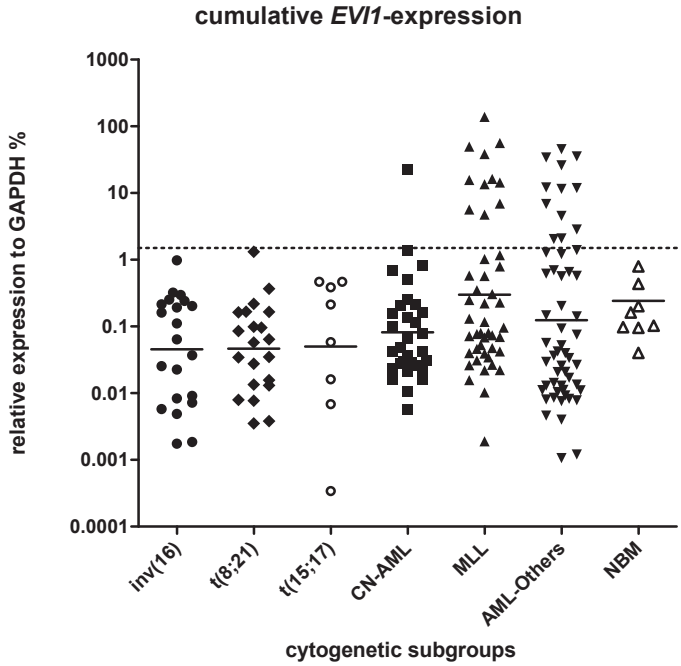

Figure 2: *EVI1* expression in different cytogenetic subgroups.
Cumulative mRNA expression levels of the *EVI1*-1A, -1B, -3L, relative to *GAPDH* (%) for 179 samples in different cytogenetic subgroups in pediatric AML and in 8 normal bone marrow samples (NBM). A cumulative relative expression of 1.5% for one of the transcripts was considered positive.

has previously shown to be a marker to detect cryptic 3q26 aberrations[13]. However, in all *EVI1*+ cases, cryptic 3q26-rearrangements were not detected by FISH (Table 1). Thus, combining data of the gene expression profiling and RQ-PCR, 25 *EVI1*+ cases were identified and none of them harbored a 3q26-aberration.

Clinical characteristics of *EVI1*+ in pediatric AML

EVI1+ pediatric AML was not correlated with sex, white blood cell count or age. When studying the relationship between *EVI1*+ and conventional classification criteria such as morphology (FAB classification) and cytogenetic data (Table 2), a higher frequency of *EVI1* overexpression was detected in patients with (1) *MLL*-rearrangements (n=13/47 cases), including all t(6;11) cases (n=4); (2) acute megakaryoblastic leukemia (AML-M7) (n=4/7 cases); (3) acute erythroblastic leukemia (AML-M6) (n=2/3 cases); and (4) monosomy 7 (n=2/3 cases). The 6 remaining cases were patients with normal karyotype (n=1), other cytogenetics (n=4) or unknown karyotype (n=1), but none of them harbored a 3q26-rearrangement (Table 1 and 2). Overexpression of *EVI1* was not found in the prognostically favorable types of AML, which are t(8;21), inv(16) and t(15;17).

Table 2: Clinical characteristics of *EVI1* positive patients in relation to clinical parameters, morphology and cytogenetics.

	No. of *EVI1* negative patients (%)	No. of *EVI1* positive patients (%)	*p-value*
Sex			*0.181* [#]
male	119 (58)	11 (44)	
female	84 (42)	14 (56)	
Age (years, median)	7.2	9.6	*0.095* [§]
<2 years	30 (15)	6 (24)	*0.273* [#]
2-10 years	77 (38)	11 (44)	
>10 years	96 (47)	8 (32)	
WBC x 10^9/L (median)	39.7	42.2	*0.772* [§]
FAB			***<0.001*** [#]
M0	12 (6)	0 (0)	
M1	25 (12)	3 (12)	
M2	49 (24)	2 (8)	
M3	18 (9)	0 (0)	
M4	49 (24)	5 (2)	
M5	41 (20)	9 (36)	
M6	1 (1)	2 (8)	
M7	3 (2)	4 (16)	
other/unknown	5 (3)	0 (0)	
Cytogenetic abnormalities			***<0.001*** [#]
***MLL*-rearrangements**	34 (17)	13 (52)	
t(8;21)(q22;q22)	28 (14)	0 (0)	
inv(16)(p13q22)	27 (13)	0 (0)	
t(15;17)(q22;q21)	16 (8)	0 (0)	
t(7;12)(q36;p13)	7 (3)	0 (0)	
monosomy 7	1 (0)	2 (8)	
normal cytogenetics	41 (20)	1 (4)	
others/unknown[1]	49 (25)	9 (36)	

[1] See Table 1
[#] Chi-square test
[§] Mann-Whitney-U testv

We also studied *EVI1+* in relation to single-gene mutations. Of interest, 3 patients (#14, #15 and #22) showed a mutation in the *RAS*-gene. In addition, one *EVI1+* patient (#8) had a *CEBPA* mutation. One *EVI1+* patient (#10) had a *FLT3*-ITD, whereas 40/203 *EVI1-* patients had a *FLT3-ITD* (respectively 4.0% vs. 19.7%, p=0.05). *EVI1+* was not found in patients with *NPM1, MLL-PTD* and *KIT* mutations.

Gene expression signature differences within *EVI1+* cases

To get insight into the biology of *EVI1+*, we analyzed our dataset to identify a specific gene expression signature for *EVI1+* pediatric AML. Using an empirical Bayes linear regression model[30], 2103 discriminative probe sets for *EVI1+* were identified. However, within the *MLL*-rearranged AML subtype a different gene expression signature was observed compared to the *EVI1+* cases in other AML subtypes (Supplementary Figure 2). After applying correction for cytogenetic subtype, the amount of discriminating probe sets decreased drastically from 2103 to 253 and only 88 of these 253 probe sets were detected to be strongly significant in both groups, that is *MLL*-rearranged AML and other AML subtypes. Therefore, these 88 probe sets were considered to be highly discriminative for *EVI1+* (FDR-corrected p<0.001) independent of their cytogenetic background. The top 4 probe sets represented overexpression of the *EVI1* and *MDS1* gene themselves. Interestingly, some other probe sets represented genes that have been reported to play a role in hematopoiesis and/or the development of leukemias, for example *PBX1* and *RUNX2*[33-34] (Supplementary Table 2).

Survival analysis and prognosis of *EVI1+* in pediatric AML

Survival data were available for 198 patients, including 18 *EVI1+* cases. Patients with *EVI1* overexpression had a significantly worse 4 years pEFS (28%±11 vs. 44%±4; p=0.04) as compared to patients without *EVI1* overexpression. However, the OS was not significantly different between both groups (56%±12% vs. 64%±4%; p=0.34) (Figure 3A and B). Within the *MLL*-rearranged AML group (n=40) no significant difference for 4 years pEFS (20%±13% vs. 40%±9%; p=0.44) nor for 4 years pOS (50%±16% vs. 47%±9%; p=0.68) between *EVI1+* and *EVI1-* patient was found (Figure 3C and D). The fact that *EVI1* overexpression did not influence outcome in pediatric AML was confirmed with multivariate analysis, including favorable karyotype, age and WBC, and showed that *EVI1+* lacked independent prognostic significance for pEFS (hazard ratio (HR) 1.2; p=0.67) and for pOS (HR 1.0; p=0.97) (Table 3).

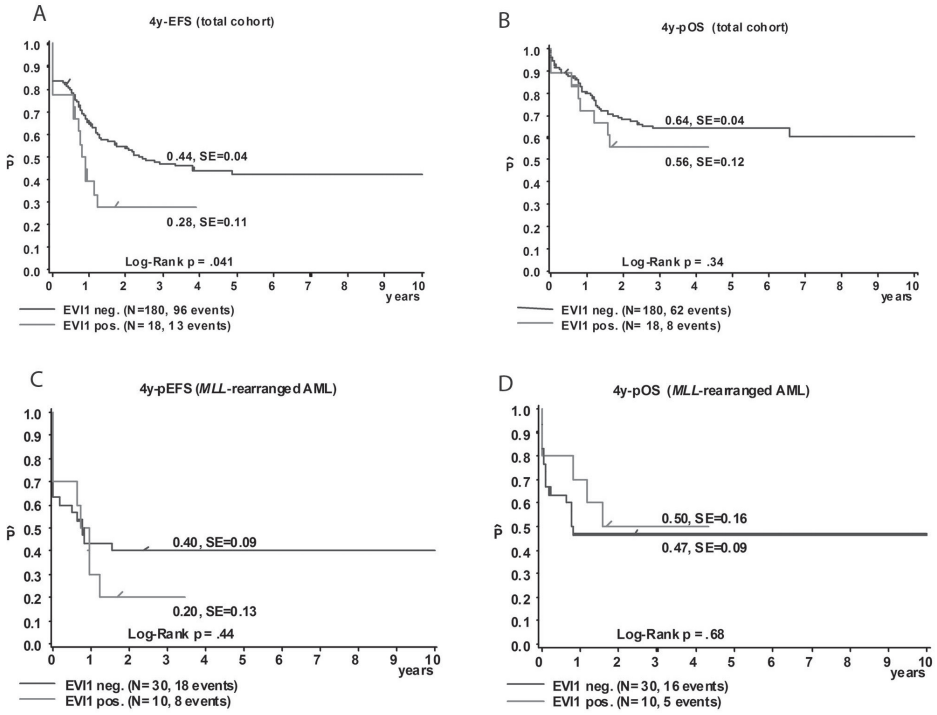

Figure 3: Survival outcome for high *EVI1* expression in pediatric AML.
(a) Kaplan-Meier estimates for pEFS in the total cohort between *EVI1*+ and *EVI1*- patients.
(b) Kaplan-Meier estimates for pOS in the total cohort between *EVI1*+ and *EVI1*- patients.
(c) Kaplan-Meier estimates for pEFS in the cohort of *MLL*-rearranged AML between *EVI1*+ and *EVI1*- patients.
(d) Kaplan-Meier estimates for pOS in the cohort of *MLL*-rearranged AML between *EVI1*+ and *EVI1*- patients.

Table 3: Multivariate analysis of high *EVI1* expression in pediatric AML for event-free survival (EFS) and overall survival (OS).

	EFS			OS		
	HR	**(95% CI)**	**p-value**	**HR**	**(95% CI)**	**p-value**
EVI1+	1.2	(0.6-2.2)	0.67	1.0	(0.5-2.1)	0.97
Favorable karyotype[1]	0.4	(0.2-0.6)	**<0.001**	0.2	(0.1-0.5)	**<0.001**
WBC >50[¶]	1.0	(0.7-1.5)	0.97	1.3	(0.8-2.1)	0.36
Age >10[§]	1.0	(0.6-1.4)	0.87	0.8	(0.5-1.3)	0..34

[1] t(8;21), inv(16) and t(15;17)
[¶] WBC= White blood cell count above 50 x 10^9/l
[§] Children older than 10 years

DISCUSSION

Evi1 was detected by Morishita *et al.* as a common integration site by retroviral insertion in a murine model system, leading to *Evi1* overexpression and leukemia, which suggests a role for *EVI1* as an oncogene[5]. Subsequently, the human *EVI1* gene was detected in the breakpoint region of chromosome 3q26-rearrangements in different myeloid malignancies[1]. Additional evidence that *EVI1* may act as on oncogene comes from studies by Laricchia-Robbio and Nucifora and Kilbey *et al.* who showed that aberrant overexpression of *EVI1* results in loss of cell-cycle control and increased self-renewal[35-36]. Moreover, adult AML patients with high *EVI1* expression, irrespective of harboring a 3q26 abnormality, have a poor prognosis[13].

Until now, no information was available on the role of the *EVI1* gene in pediatric AML, in which cytogenetically detectable 3q26 abnormalities do not seem to occur frequently[14]. In the present study cohort, without cytogenetically detectable 3q26 abnormalities, we discovered *EVI1* overexpression in 20/213 (9%) of the children with *de novo* AML, and in 4/8 (50%) patients with secondary-AML. Moreover, we showed a strong association between *EVI1* overexpression and specific genetic and morphologic subtypes of AML. In contrast to adult AML, we did not find any evidence for chromosome 3q26 aberrations, nor for cryptic 3q26 rearrangements in *EVI1*+ cases. However, we did identify *EVI1*+ in FAB-M6/M7 cases, which has not been reported in adults so far. In addition, we identified *EVI1*+ in subgroups that are considered to have an intermediate or poor prognosis, that is pediatric AML with *MLL*-rearrangements and monosomy 7, which included 3 of the 4 secondary-AML cases. Overexpression of *EVI1* was also identified in *MLL*-rearrangements and monosomy 7 in adult AML[14,37-38]. Interestingly, *EVI1* expression was mutually exclusive with CBF-AML, t(15;17) and *NPM1* mutations which represent favorable types of pediatric AML.

Gilliland *et al.* hypothesized that the initial development of AML results from both type-I and type-II mutations. Type-I mutations induce enhanced proliferation of the hematopoietic cells, whereas type-II mutations lead to impaired differentiation and maturation arrest[39]. Non-random associations between specific mutations have been shown for various other subtypes in AML, such as t(8;21) or inv(16) and *KIT*, supporting the Gilliland hypothesis[40]. We found that *EVI1* was overexpressed in various morphologic and genetic subtypes of childhood AML, and even in homogeneous subgroups *EVI1* overexpression was often only detectable in a subset of patients. Therefore, we assume that *EVI1* overexpression is a secondary and not an initiating event that may occur later in leukemogenesis. Moreover, it is not clear whether *EVI1* is a driver rather than a bystander effect in our cases with *EVI1*+.

Several findings, however, may support a role for *EVI1*+ in leukemogenesis in these specific cases. For instance, all *MLL-AF6* and a significant proportion of monosomy 7

cases are clearly associated with *EVI1*+, not only in pediatric but also in adult AML[13]. Moreover, *in vivo* studies with an *MLL-AF9* mouse-model showed overexpression of *Evi1* after the leukemic transformation[41]. In addition, a recent report in pediatric AML patients with monosomy 7 showed a higher incidence of 3q26-rearangements, and a role for *EVI1* was already suggested by these investigators[37]. We know from Fanconi anemia that patients with 3q26 aberrations have a higher risk of developing AML and if monosomy 7 develops, this occurs in the 3q26 aberrant clone as a second event[42]. Therefore, as both *MLL-AF6* and monosomy 7 are associated with poor outcome in pediatric AML[37-38,43], this may underscore that *EVI1* has a role in these leukemias. However, direct evidence demonstrating an oncogenic effect of *EVI1*+ overexpression in these types of leukemia could not be derived from our study and further evidence is currently lacking. Clearly, further studies need to be performed to unravel the exact biological role of *EVI1*+ in these leukemias.

There is also supporting evidence for a role of *EVI1*+ in the development of AML FAB-M6 and -M7. For instance, adult myeloid malignancies with 3q26 abnormalities show increased numbers of dysplastic megakaryocytes. Other *in vitro* studies show that *EVI1* overexpression leads to impaired erythroid and megakaryocytic differentiation by GATA-1 inactivation[44-46]. However, *in vivo*, no abnormalities of erythroid cells were observed in *Evi1* transgenic mice, although they did show a significant reduction in the number of erythroid colony-forming units, implying a defect of erythroid hematopoiesis affecting erythroid progenitor cells[47]. Therefore, overexpression of *EVI1* might be involved in the development of AML of both acute erythroid and megakaryoblastic leukemia.

Unsupervised cluster analyses (data not shown) did not identify a specific cluster for *EVI1*+ cases, as previously reported in adult AML. These cases were often split among different clusters harboring *MLL*-rearranged AML (adult and pediatric AML), monosomy 7/3q26 aberrations (adult AML), or FAB-M6/M7 (pediatric AML)[48]. However, by supervised clustering, we found that within the subgroup of *MLL*-rearranged AML, *EVI1*+ pediatric patients revealed a different gene expression signature as compared with the *EVI1*+ patients in the other cytogenetic subtypes. Interestingly, methylation array profiles in adult AML identified different subgroups within *EVI1*+ patients, indicating the heterogeneity of this subgroup[49].

Although these data strongly suggest differences in biology between subgroups of *EVI1*+, still probe sets were identified to be discriminative for *EVI1*+, independent of their cytogenetic or morphologic background after multivariate analysis. This could indicate a specific role for *EVI1* overexpression in the development of leukemia in these cases, especially as some of these probe sets included genes that have been previously been reported to play a role in hematopoiesis and/or the development of leukemias, for example *PBX1* and *RUNX2*[33-34]. Moreover, recent analysis of the Pbx1 promoter region in

mice revealed that Evi-1 upregulates Pbx1 transcription[50]. This emphasizes that *PBX1* is a possible target gene of *EVI1* involved in the leukemogenesis of *EVI1*+ patients.
Although differences in EFS and OS for *EVI1*+ and *EVI1*- pediatric AML were observed in our study, we showed that *EVI1*+ has no independent prognostic value for pediatric AML, which is in contrast to adult AML[12-13]. The latter may mainly be caused by differences in frequency of 3q26 abnormalities, but also by differences in therapy and prognosis between adults and children.
In this first study on the relevance of *EVI1* overexpression in pediatric AML, we conclude that *EVI1*+ is found in 9% of *de novo* pediatric AML. *EVI1* is overexpressed in specific cytogenetic (*MLL*-rearrangements and monosomy 7) and morphologic (FAB-M6/7) subtypes. However, the typical *EVI1*+ associated 3q26 aberrations reported in adult AML were not identified, indicating that there may be a difference for the role of *EVI1*+ in adult AML as compared to pediatric AML. Although *EVI1*+ was not an independent prognostic factor, it was predominantly found in types of pediatric AML that are related with an intermediate to unfavorable prognosis, for example *MLL-AF6* and monosomy 7. This underscores the need for further studies to identify the biological role of *EVI1* in the pathogenesis of childhood leukemia.

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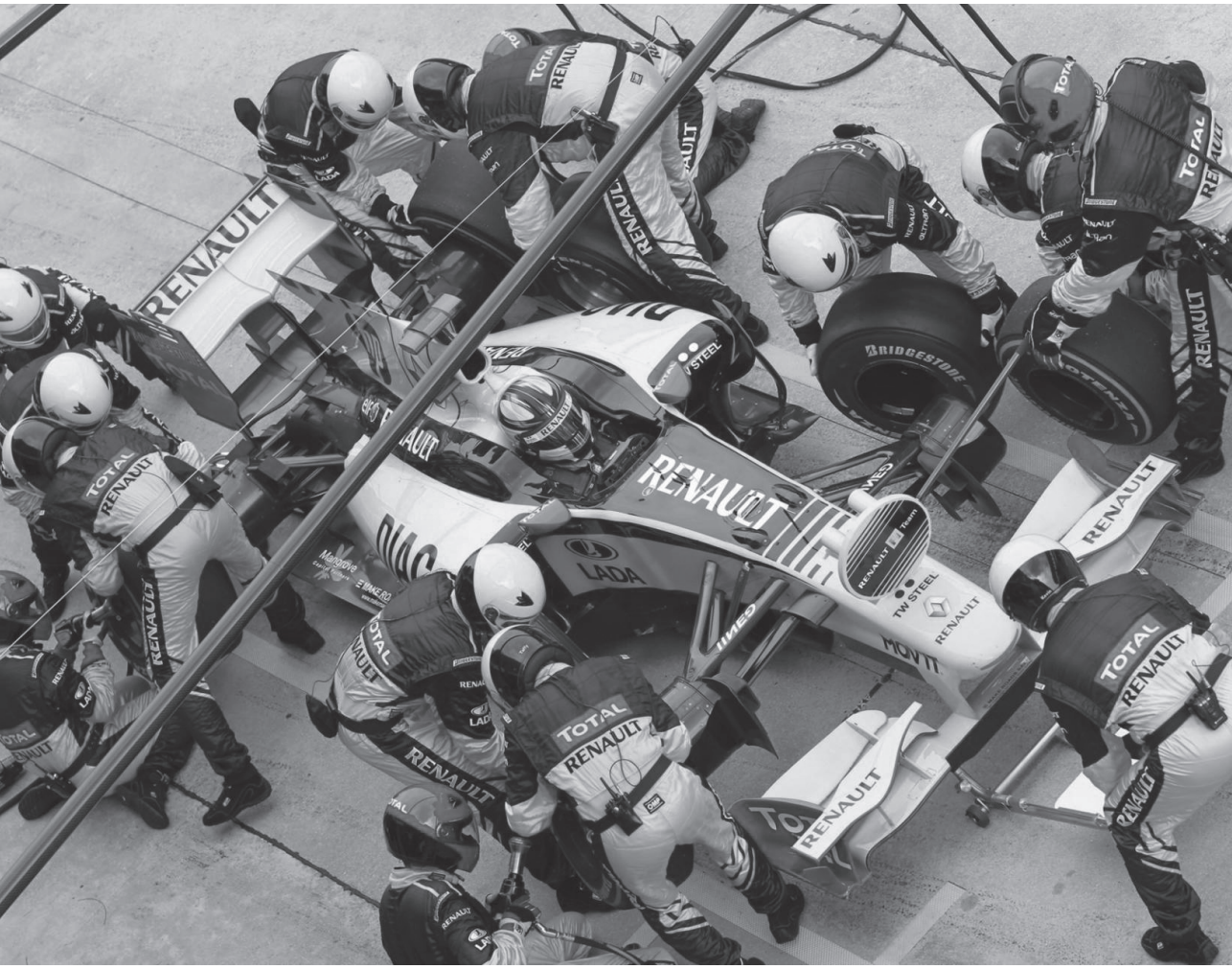
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Chapter 5

The heterogeneity of pediatric *MLL*-rearranged acute myeloid leukemia

B.V. Balgobind[1], C.M. Zwaan[1], R. Pieters[1], and M.M. Van den Heuvel-Eibrink[1].

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

Leukemia, in press

INTRODUCTION

Acute myeloid leukemia (AML) accounts for 15-20% of childhood leukemias[1]. Despite intensive chemotherapy, only 60% of the patients with AML are cured[2]. The heterogeneity of AML is reflected by differences in morphology, immunophenotype, as well as cytogenetic and molecular abnormalities. Recurrent (cyto)genetic aberrations and response to treatment are important prognostic factors in AML and are therefore used for risk group stratification. The cytogenetic subgroups t(8;21), inv(16) and t(15;17) have been consistently associated with favorable outcome; currently reaching 5-year overall survival rates of approximately 80%[3]. The *mixed lineage leukemia* (*MLL*)-rearrangements are related to an intermediate to poor outcome[4-6].

In 1991, it was discovered that the different 11q23-rearrangements involved one and the same unique locus at 11q23. This locus shows homology to sequences within the Drosophila 'trithorax' gene, a developmental regulator[7,8]. Since these aberrations were found in AML as well as acute lymphoblastic leukemia (ALL), the gene was called *Mixed Lineage Leukemia*[9]. Recent studies show that the *MLL*-group itself is genetically and clinically heterogeneous, as more than 60 different translocation partners of the *MLL*-gene with differences in outcome have been described to date[6,10]. The biological background of these differences remains unknown.

In addition to being involved in translocations, the *MLL*-gene is also involved in other aberrations such as partial tandem duplications (*MLL*-PTD), consisting of an in-frame repetition of *MLL* exons[11]. In pediatric AML, *MLL*-rearranged AML presents with a distinct, unique, expression profile as compared to *MLL*-PTD[12,13]. Therefore, *MLL*-rearranged AML and *MLL*-PTD are considered 2 different entities.

ETIOLOGY OF *MLL*-REARRANGEMENTS

A prenatal origin for *MLL*-rearranged AML has been shown in neonatal bloodspots taken from Guthrie cards of infants who later developed AML[14,15]. Environmental factors to which the fetus is exposed in utero may play an important part in the development of *MLL*-rearranged AML, which seem to occur due to inappropriate non-homologous end joining of double strand breaks[16]. The 11q23 locus is particularly sensitive to cleavage after treatment with topoisomerase-II inhibitors. Since DNA topoisomerase-II seems to be highly expressed in developing fetuses[17], exposure to DNA topoisomerase-II inhibitors could induce *MLL*-rearrangements in utero. The most abundant environmental source of DNA topoisomerase-II inhibitors is diet. For example flavonoids, such as quercetin (in some fruits and vegetables) and genistein (in soy); and catechins (in tea, cacao, and red wine) inhibit DNA topoisomerase-II[18]. Further evidence for a role of

DNA topoisomerase-II inhibitors is provided by the fact that exposure to bioflavonoids can induce cleavage of the *MLL*-gene in human myeloid and lymphoid progenitor cells[19]. A large case-control study of maternal diet and infant leukemia showed that the amount of maternal consumption of foods containing DNA topoisomerase-II inhibitors was correlated to the risk of developing *MLL*-rearranged AML[20]. Another case-case study found a significantly elevated risk for *MLL*-rearranged AML associated with maternal use of pesticide, as well DNA damaging drugs[21].

The role of topoisomerase-II inhibitors is further strengthened by the high frequency of *MLL*-rearrangements in therapy-related AML for patients who have been treated with DNA topoisomerase-II inhibitors, e.g. etoposide[22]. Interestingly, identical to a short latency period to develop de *novo MLL*-rearranged AML in children, the latency period to develop therapy-related AML is much shorter for *MLL*-rearrangements compared to the latency period of therapy-related AML with unbalanced aberrations, e.g. monosomy 5 or monosomy 7[23].

THE BIOLOGY OF *MLL*-REARRANGEMENTS AND DEVELOPMENT OF AML

The *MLL*-gene encodes a DNA binding protein with an N-terminal DNA binding domain and a C-terminal SET domain. MLL was found to be part of a large chromatin modifying complex in which the SET-domain of MLL has histone methyltransferase and histone acetyltransferase activity[24] (Figure 1). During the formation of this complex, MLL is cut by taspase into an N-terminal (MLL^N) and a C-terminal (MLL^C) fragment[25]. MLL^C then associates with at least 4 proteins, i.e. the histone acetyltransferase MYST1(MOF)[26], and WDR5, RBBP5 and ASH2L to ensure histone modification and methyltransferase activity[27]. The MLL^N fragment has a binding site for MEN1 at the amino terminal end and both recruit PSIP1(LEDGF)[28]. PSIP1 then contacts the chromatin by a PWWP domain. MLL^N also contains a CxxC domain, which specifically binds to unmethylated DNA[29]. Transcription factors like p53 and CTNNB1(β-catenin) are most likely to recruit the MLL complex to initiate RNA synthesis[26,30] and specific genes, like the *HOX* genes, seem to be more dependent on the MLL complex for chromatin modification during transcription than others[31,32].

In *MLL*-rearrangements the breakpoint in *MLL* is highly conserved and all fusion partners are fused in frame leading to a gain of function of the MLL-complex. The MLL-fusion most likely disrupts the MLL-complex, leading to inappropriate expression of specific *HOX* genes in pediatric *MLL*-rearranged AML, i.e. *HOXA4*, *HOXA5*, *HOXA9*, and *HOXA10*[13]. In addition, the *HOX* cofactor *MEIS1* is upregulated in *MLL*-rearranged AML[13]. In general, *HOX* gene expression plays a key role in the regulation of hematopoietic development. Overexpression of *HOXA9* results in increased numbers of

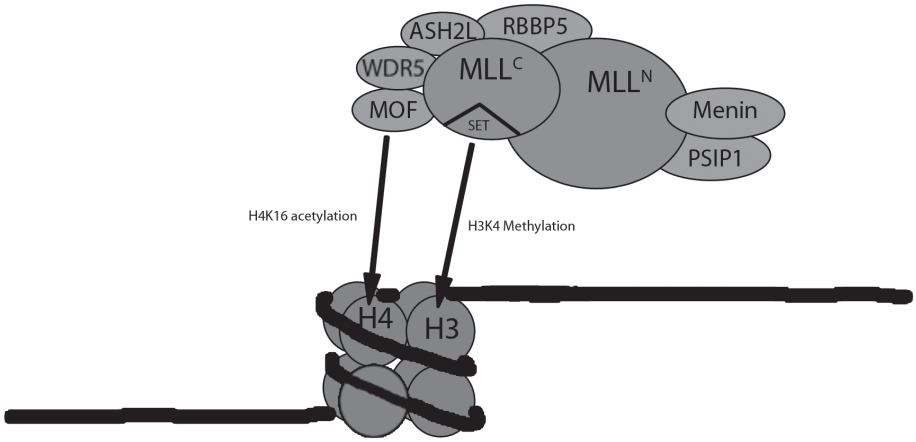

Figure 1: The MLL-complex. MLL is part of a large chromatin modifying complex in which the SET-domain of MLL has histone methyltransferase and histone acetyltransferase activity.During the formation of this complex, MLL is cut into an N-terminal (MLL^N) and a C-terminal (MLL^C) fragment. MLL^C associates with the histone acetyltransferase MYST1, and the WDR5, RBBP5 and ASH2L to ensure histone modification and methyltransferase activity. The MLL^N fragment has a binding site for MEN1 at the amino terminal end and both recruit PSIP1. PSIP1 then contacts the chromatin by a PWWP domain.MLL^N also contains a CxxC domain, which specifically binds to unmethylated DNA

self-renewing hematopoietic stem cells. Therefore, a failure to down regulate high *HOX* expression can inhibit maturation and trigger leukemogenesis in *MLL*-rearranged AML. Although *MLL*-rearrangements are predominantly found in AML, they are also detected in 6% of pediatric ALL cases. The association of *MLL*-rearrangements with different hematopoietic lineages and the requirement of *HOX* expression in early development of hematopoietic cells suggest that *MLL*-rearrangements occur in an early progenitor with lymphoid and myeloid potential. Although differences in translocation partner AML and ALL suggest a role for lineage commitment, *MLL-MLLT1(ENL)* consistently generated AML in mice[33], whereas *MLL-MLLT1* is found in both AML and ALL in humans. *MLL-GAS7* induced AML, ALL and acute biphenotypic leukemia in mice[34], indicating that it is conceivable that in addition to the *MLL*-fusion, a secondary event or a specific microenvironment is necessary for lineage commitment.

EPIDEMIOLOGY OF *MLL* ABERRATIONS IN PEDIATRIC AML

MLL-rearrangements are the most common recurrent cytogenetic aberration in pediatric AML, in contrast to adult AML, where less than 3% of the cases have an *MLL*-rearrangement[35]. In a large German study the highest frequency of *MLL*-rearranged AML was found in the children younger than 2 years of age[36]. In a large collaborative retrospective analysis, the median age at diagnosis of *MLL*-rearranged pediatric AML

was 2.2 years. This relation with age is probably due to the prenatal origin and short latency. However, age differed greatly between the different translocation partners, since cases with t(6;11)(q27;q23) and t(11;17)(q23;q21) were older compared to children with other *MLL*-rearrangements (Figure 2A). Hence, the incidence of *MLL*-rearranged AML decreases with age. This is in contrast to the frequency of *MLL*-PTD which is equally low in pediatric AML, ranging from 1-10%, depending on the screening method[13,37-39], as well as in adult AML, in the range of 5-10%[37,40-47].

THE DIFFERENT TRANSLOCATION PARTNERS OF THE REARRANGED *MLL* GENE

So far, more than 60 different fusion partners of *MLL* have been identified. Approximately 50% of the pediatric AML cases with an *MLL*-rearrangement consist of t(9;11)(p22;q23). The other 50% predominantly include t(6;11)(q27;q23), t(10;11)(p12;q23), t(11;19)(q23;p13.1) and t(11;19)(q23;p13.3) (Figure 3, Table 1)[48]. This distribution is

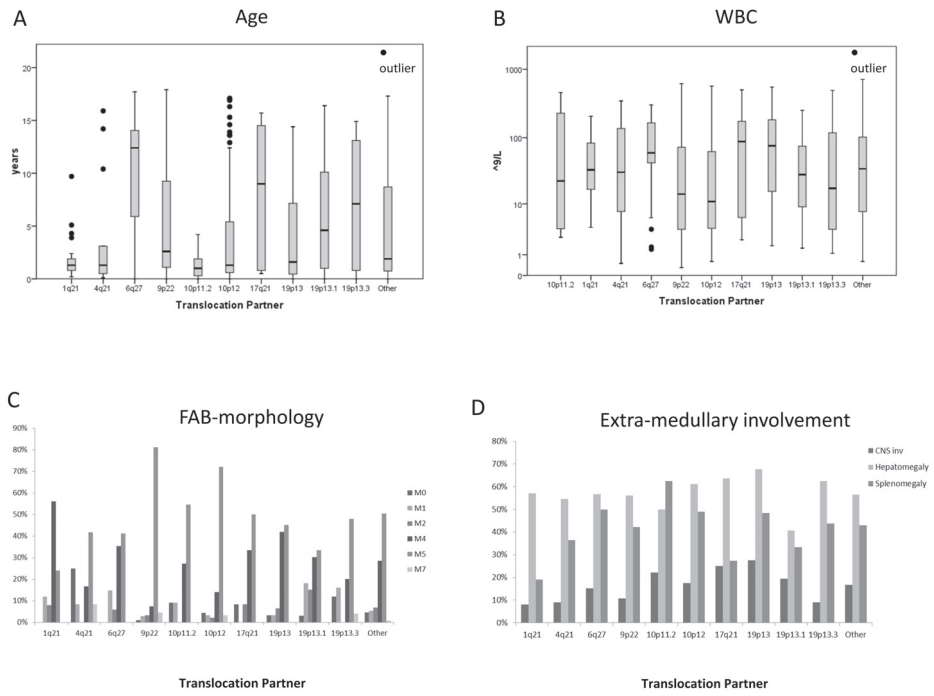

Figure 2: Clinical characteristics of *MLL*-rearranged AML per translocation partner. Differences are identified for age (A), WBC (B) and FAB-morphology (C) based on the translocation partner, but not for extramedullary disease (D)

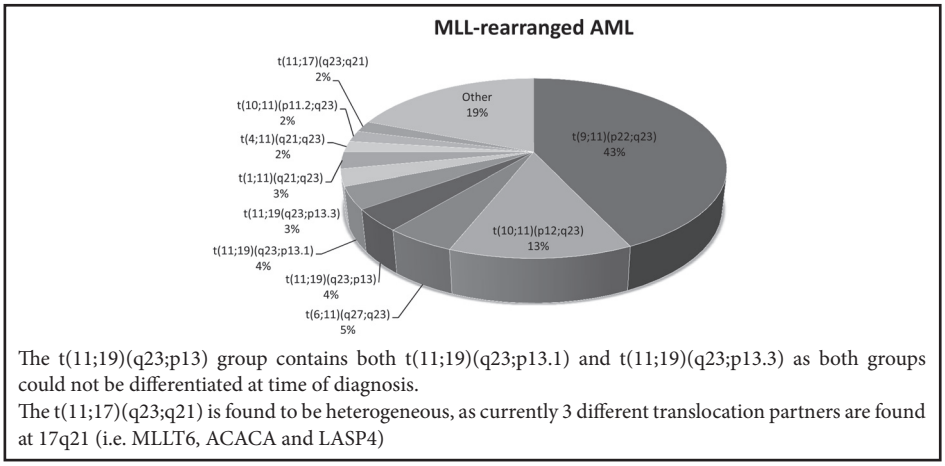

Figure 3: The distribution of translocation partners of *MLL* in pediatric AML. The most common translocation in *MLL*-rearranged AML is t(9;11)(p22;q23), accounting for almost 50% of the cases. In addition t(10;11)(p12;q23), t(6;11)(q27;q23), t(11;19)(q23;p13) and t(1;11)(q21;q23) are also more frequently found accounting for 13%, 5%, 11% and 3% of the cases respectively.

Table 1: The most common translocation partners in *MLL*-rearranged AML

***MLL*-rearrangement**	**translocation partner**	**alias**
t(9;11)(p22;q23)	*MLLT3*	*AF9*
t(10;11)(p12;q23)	*MLLT10*	*AF10*
t(6;11)(q27;q23)	*MLLT4*	*AF6*
t(11;19)(q23;p13.1)	*ELL*	
t(11;19)(q23;p13.3)	*MLLT1*	*ENL*
t(1;11)(q21;q23)	*MLLT11*	*AF1q*
t(4;11)(q21;q23)	*AFF1*	*AF4*
t(10;11)(p11.2;q23)	*ABI1*	
t(11;17)(q23;q21)	*MLLT6*	*AF17*

almost identical in adult AML, with the exception of t(6;11)(q27;q23), which has a higher relative frequency in adult *MLL*-rearranged AML[35,49].

Most of the translocation partners of MLL can be classified into cytosolic/membrane proteins and nuclear proteins[50-53]. Interestingly, the most frequent translocation partners of MLL encode nuclear proteins (e.g. AFF1(AF4), MLLT3(AF9), MLLT1 and MLLT10(AF10)) and are most likely not randomly selected. Rather they are part of a protein network serving common functional processes capable of binding to histones. For example, direct binding interactions have already been described between AFF1, MLLT3 and MLLT1 and AFF1/MLLT10, which play a functional role in leukemogenesis[54,55]. The complex of these proteins, which is called the MLLT1/ENL associated protein complex

(EAP)[55], was also linked to DOT1L, which methylates H3K79[56], and pTEFb, which is necessary to convert "promoter-arrested" RNA polymerase II into an "elongating" RNA polymerase II[57]. In addition it has been demonstrated that MLLT10 binds DOT1L and that binding of DOT1L was necessary for the transforming activity of MLL-MLLT10[56]. These findings strongly suggest that MLL fusion proteins MLL–MLLT1, MLL–MLLT3 and MLL–MLLT10 recruit the EAP complex leading to inappropriate histone methylation and transcriptional elongation[50,51,53,58]. Indeed, aptamere peptides that were able to disrupt the MLLT3/AFF1 complex proved to be toxic for leukemic cells with MLL-AFF1 translocations but not for blast cells of different etiology[59].

The MLL-fusion with translocation partners that encode for cytosolic/membrane proteins (e.g. MLLT11(AF1q) and MLLT4(AF6)) seem to have different pathways leading to oncogenic activity of the MLL-fusion gene. However, it is thought that dimerization of these proteins is contributing to the activation of target genes[60]. However, it is still unknown how dimerization of these MLL-fusion proteins inappropriately activates target genes. It is possible that cytosolic proteins do enter the nucleus due to the fusion to MLL and can play a role in the pathways found for the nuclear fusion proteins. Indeed when the ABI1 protein, a fusion partner of MLL, is imported into the nucleus, it interacts with MLLT1[61].

In gene expression profiling studies *MLL*-rearranged cases clustered together as one group in AML as well as in ALL[13,62-64]. However our laboratory showed that within *MLL*-rearranged infant ALL each type of *MLL* translocation is associated with a translocation-specific gene expression signature[65]. We identified a specific gene expression signature for the total group of *MLL*-rearranged AML cases[12], but were also able to identify a specific signature for t(9;11)(p22;q23)[66]. This indicates that in addition to the common pathways in *MLL*-rearranged AML, there are other pathways which seem to be more dependent on the fusion partner of *MLL*.

CLINICAL CHARACTERISTICS OF *MLL*-REARRANGED PEDIATRIC AML

MLL-rearranged AML is correlated with the morphological subtypes acute myelomonoblastic leukemia and monoblastic leukemia, which represent the French-American-British (FAB) classification subtypes FAB-M4 and –M5 respectively[67]. *MLL*-rearranged AML patients often present with high tumor load, which includes organomegaly in ~50% of the cases, a high median WBC (20.9x10^9/l) and CNS-involvement in 14% of the cases[6,35,48,68] (Figure 2B-D). However, the clinical characteristics differ for the different translocation partners. For example patients with a t(6;11)(q27;q23) had a higher median WBC than other *MLL*-rearranged AML. These characteristics illustrate that *MLL*-rearranged AML is a clinically heterogeneous disease, primarily based on the differences in translocation partner.

PROGNOSTIC FACTORS AND OUTCOME OF *MLL*-REARRANGED PEDIATRIC AML

In general, *MLL*-rearranged AML is associated with a poor outcome. However, optimized intensive treatment regimens for AML have also improved outcome for *MLL*-rearranged AML. Table 2 shows that patients with *MLL*-rearranged AML have an intermediate outcome with a 5y-EFS ranging from 32% to 54% and a 5y-OS ranging from 42% to 62%[4,5,69-77]. Currently, hematopoietic stem cell transplantation is no longer advised in first remission.
Recently, we identified the t(1;11)(q21;q23) subgroup as a new prognostic subgroup in pediatric AML. This type of AML has an excellent clinical outcome (5y-pEFS of 92%, and a 5y-pOS of 100%)[6]. The biological background for this favorable outcome is poorly understood and conflicting data on the overexpression of *MLLT11*, the translocation partner of *MLL* in t(1;11)(q21;q23) have been reported. In cell lines overexpression of *MLLT11* was associated with enhanced doxorubicin-induced apoptosis[78], whereas another study showed that high *MLLT11* expression in AML was independently associated with poor survival[79]. In contrast, the t(10;11)(p12;q23) and t(6;11)(q27;q23) subgroup have poor prognosis with a 5y-pEFS of 31% and 11% and a 5y-pOS of 45% and 22% respectively. Also in adult AML patients with a t(6;11)(q27;q23) have a poor outcome[80].
In the past, some studies have shown that t(9;11)(p22;q23) had a favorable outcome and a higher sensitivity to drugs compared with the other *MLL*-rearrangements[68,81-83]. However, within the t(9;11)(p22;q23) subgroup, prognosis appeared to be related to morphology, as the group with acute monoblastic leukemia (FAB-M5) and t(9;11)(p22;q23) had a significantly better outcome than those with other FAB-types[6]. This could explain the favorable outcome for t(9;11)(p22;q23) in the study by Rubnitz *et al.*, since 21/23 cases had FAB-M5[68].
In addition to translocation partners, other variables like WBC (>100x10^9/L), age (>10 years) and additional cytogenetic aberrations were of prognostic relevance in pediatric *MLL*-rearranged AML[6]. Hence, with current intensive chemotherapy *MLL*-rearranged pediatric AML cases have an overall intermediate outcome. Translocation partners, WBC, age and additional chromosomal aberrations are independent prognostic factors within this disease, once again underscoring the fact that *MLL*-rearranged AML is a heterogeneous disease.

THE DETECTION OF *MLL*-REARRANGEMENTS

The true incidence of *MLL*-rearrangements in pediatric AML is considered to be in the range of 15-25% according to the latest trials[4,5], since cryptic *MLL*-rearrangements were not always identified in the past with conventional karyotyping only. In addition, newly identified prognostic groups such as t(1;11)(q21;q23), t(6;11)(q27;q23) and t(10;11)(p12;q23) are not identified with current screening procedures,

Table 2: Outcome of *MLL*-rearranged AML per study group compared with outcome in the total group of pediatric AML.

Study Group/protocol	Year	pediatric *MLL*-rearranged AML		Year	pediatric AML		*ref*
		5y-pEFS (%, SE)	5y-pOS (%, SE)		5y-pEFS (%, SE)	5y-pOS (%, SE)	
		(Balgobind et al. 2009)[6]					
BFM	1993-2004	40 (4)	54 (4)	1978-2001	51 (3)	58 (2)	*69*
DCOG	1993-2004	54 (9)	64 (9)	1983-1998	42 (4)	42 (4)	*70*
NOPHO	1993-2004	50 (7)	63 (7)	1984-2001	50 (3)	60 (3)	*71*
LAME	1993-2004	61 (7)	74 (6)	1988-1998	48 (4)	62 (4)	*72*
AIEOP	1995-2004	47 (9)	53 (9)	1982-2001	54 (4)	60 (4)	*73*
POG	1993-1999	36 (5)	45 (5)	1981-2000	32 (2)	42 (2)	*74*
St. Jude	1993-2004	55 (8)	60 (8)	1980-2000	44 (15)	57 (11)	*75*
CCG	1995-2002	36 (4)	51 (5)	1979-1995	34 (4)	47 (4)	*76*
BFM98	1998-2003	34 (5)	49 (5)	1998-2003	50 (2)	62 (3)	*5*
MRC12	1988-2002	59¥	62¥	1988-2002	49¥	58¥	*4*

Abbreviations: 5y-pEFS, probability of event-free survival at 5 years from diagnosis; 5y-pOS, probability of overall survival at 5 years from diagnosis; y, years;
¥10-y survival rates

Since Southern Blot is an out-dated method and it has its limitations such as the requirement of large amounts of DNA and the laborious procedure, other techniques are used to detect *MLL*-rearrangements, including FISH and PCR [84-86]. FISH screening for *MLL*-rearrangements at diagnosis has become the standard approach in many AML protocols. However, gene expression profiling (GEP) has illustrated that current diagnostic techniques do not guarantee 100% sensitivity for the detection of *MLL*-rearrangements [12]. For 3 cases that clustered together with those with an *MLL*-rearrangement, LDI-PCR revealed that an *MLL*-rearrangement was present which had not been detected by split-signal FISH. This also shows the advantage of LDI-PCR, which can detect all translocation partners of *MLL* on only a little amount of DNA, if harvesting RNA for RT-PCR or GEP is not successful[10].

Since GEP is not yet feasible for diagnostic purposes and LDI-PCR for *MLL*-rearrangements is currently performed at one center, FISH is currently the state-of-the-art method for the detection of *MLL*-rearranged leukemia, but could lead to false-negative results. Therefore we would suggest that FISH and LDI-PCR are the methods of choice to detect MLL-rearrangements. However, since prognosis in *MLL*-rearranged AML depends on the translocation partner, these groups should be screened in future AML protocols for further risk stratification (Figure 4).

MOLECULAR ABNORMALITIES IN *MLL*-REARRANGED AML

As with other types of leukemia, the cause of *MLL*-rearranged AML is unknown. It has been postulated by Gilliland *et al.* that the pathogenesis of AML requires both type I and type II mutations[87]. Type-II mutations are often chromosomal rearrangements of transcription factors leading to impaired differentiation of the hematopoietic cell, such as *MLL*-rearrangements. Type I mutations mainly reflect molecular mutation hotspots in specific genes (*FLT3*, *KIT*, *NRAS*, *KRAS* and *PTPN11*) involved in the proliferation of hematopoietic cells. We previously showed that only 50% of the *MLL*-rearranged AML cases harbored a known type-I mutation, and most of these mutations were identified in genes involved in the RAS-pathway, including mutations in *NRAS*, *KRAS*, *PTPN11* and *NF1*[88] (Figure 5). Mutations in *RAF* and *SOS1*, which are also part of the RAS-pathway, are rarely found in AML[89-91].

Mutations in *FLT3* and *KIT* are rare in *MLL*-rearranged AML[88,92]. Recently, *novel* molecular aberrations were found in pediatric AML, i.e. mutations in *NPM1*, *CEBPA* and *WT1*. Of these mutations, only *WT1* mutations were found in *MLL*-rearranged AML and these even at a low frequency[93-96]. In more than 60% of the cases a molecular aberration has not been identified, which could indicate that an *MLL*-rearrangement on its own could be sufficient to induce leukemia. However, 40% do harbor a secondary aberration

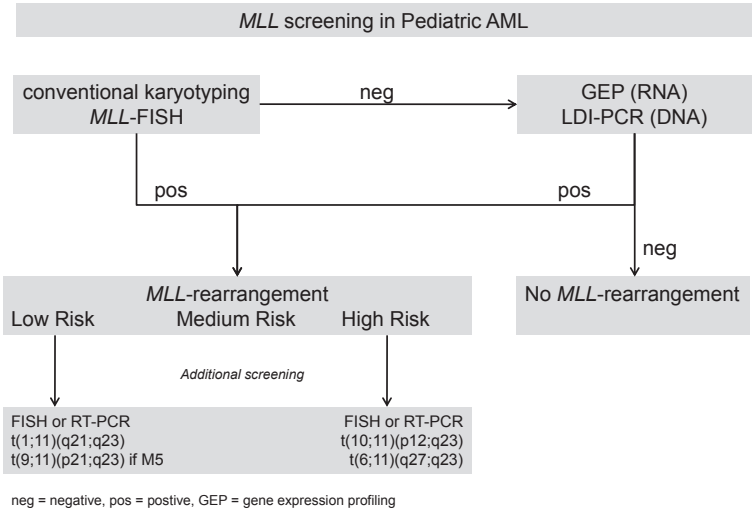

Figure 4: Future recommendations to detect *MLL*-rearrangements in pediatric AML. AML cases are routinely screened for *MLL*-rearrangements by conventional karyotyping and FISH. However these techniques do not guarantee 100% sensitivity. Therefore gene expression profiling and LDI-PCR could be used to identify cases missed with FISH, although these techniques are currently used in research setting only. Since, outcome is also dependent on translocation partner we recommend that all *MLL*-rearranged AML cases should be screened for the favorable prognostic groups t(1;11)(q21;q23) and t(9;11)(p22;q23) with FAB-M5 and the poor prognostic groups t(10;11)(p12;q23) and t(6;11)(q27;q23) for further risk stratification.

and therefore it is conceivable that Gilliland's hypothesis is still a valid model for at least the large subset of *MLL*-rearranged pediatric AML. This is supported by the fact that in MLL-AF9 transgenic mice *NRAS* expression contributed to acute leukemia maintenance by suppressing apoptosis and reducing differentiation of leukemia cells[97]. Also the introduction of *FLT3*-ITD in *MLL-MLLT3* overexpressing mice accelerated onset of AML[98]. Although *FLT3*-ITD is not a frequent event in *MLL*-rearranged AML, overexpression of *FLT3* and a higher sensitivity to *FLT3*-inhibitors was found in *MLL*-rearranged ALL[99]. In adult AML, the highest *FLT3* expression was detected in *MLL*-rearranged AML[100]. We recently confirmed this high expression of *FLT3* in a large cohort of pediatric AML cases (unpublished data).

Another gene which shows differential expression in *MLL*-rearranged AML cases is *EVI1*. In adult AML overexpression of *EVI1* is an independent poor prognostic factor, and in half of these cases an *MLL*-rearrangement was identified[101,102]. We confirmed this in pediatric AML, where overexpression of *EVI1* is mainly associated with a subset of *MLL*-rearranged AML [103]. All cases with a t(6;11)(q27;q23), that carry a very poor outcome, showed overexpression of *EVI1*, consistent with adult t(6;11)(q27;q23) cases. This suggests a role for *EVI1* in leukemogenesis in these specific cases. Indeed, *in vivo* studies with an *MLL-MLLT3* mouse-model showed overexpression of *Evi1* after the leukemic

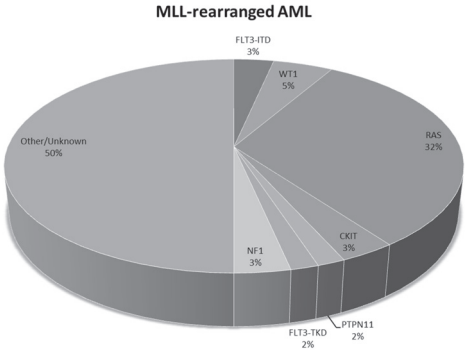

Figure 5: Frequency of type-I aberrations in pediatric *MLL*-rearranged AML. In 50% of the *MLL*-rearranged AML cases a type-I aberration can be identified. The most common type-I aberrations are linked to the RAS pathway, i.e. mutations in *NRAS*, *KRAS*, *PTPN11* and *NF1*. The aberrations account for 37% of the *MLL*-rearranged AML cases.

transformation[104]. However, direct evidence demonstrating an oncogenic effect of *EVI1* in these types of leukemia is currently lacking.

Using gene expression profiling, we identified specific genes involved in *MLL*-rearranged AML with t(9;11)(p22;q23), such as high expression of *BRE* which is associated with a favorable outcome[66]. In addition, overexpression of *IGSF4*, by epigenetic regulation, was discovered in cases with t(9;11)(p22;q23) and FAB-M5 morphology, a subgroup with a relatively favorable outcome[105]. The exact role of these genes in leukemogenesis of *MLL*-rearranged AML needs to be further elucidated. It would be of importance to identify mutations or aberrant expression of specific genes or signaling pathways that are involved in those subtypes with a poor outcome, e.g. t(6;11)(q27;q23).

TOWARDS TARGETED THERAPY

Over the last decades, outcome in pediatric AML has improved significantly, and up to 60% of children suffering from AML currently survive[3]. However, improving the outcome in pediatric AML with current treatment protocols is hampered by the treatment-related deaths and long term side effects. Therefore, to improve outcome in pediatric AML, development of leukemia-specific targeting drugs is an important challenge.

MLL-rearranged AML cases are currently treated with standard treatment , where there is sufficient evidence that *MLL*-rearranged AML is heterogeneous in biology and outcome. New agents should target specific biological markers in *MLL*-rearranged AML that play a crucial role in the development of leukemia and are related to outcome. This strategy has proven to be worthwhile in other types of leukemia. For instance, using ATRA has become the standard for acute promyelocytic leukemia (APL) as adjuvant therapy. In addition, imatinib is the first choice of treatment in chronic myeloid leuke-

mia (CML), rather than interferon and/or cytarabine. Moreover, imatinib is also used as adjuvant therapy in Ph+ ALL[106]. Since abnormal activation of tyrosine kinases predominantly leads to cell proliferation in different types of cancer, these are suitable targets for therapy. Here we will discuss the possible use of recently developed tyrosine kinase inhibitors (TKI's), other inhibitors and the possibility of targeting the MLL-complex or the downstream targets.

FLT3, a tyrosine kinase, has been reported to be highly expressed in *MLL*-rearranged ALL[99], and co-expression of *FLT3* with *MLL-AF9* in AML shortened latency in mice significantly[98]. In *MLL*-rearranged AML, high expression of *FLT3* was found in almost all cases. FLT3-inhibitors, such as PKC412, have already shown to induce apoptosis in *MLL*-rearranged ALL and *FLT3*-ITD/AML cells *in vitro* and in mouse models[107-110]. PKC412 showed potential in phase I/II trials in adult AML[111]. An international pediatric relapsed AML phase II trial with PKC412 is currently ongoing.

One other possible kinase to be targeted in *MLL*-rearranged AML is GSK3, a serine/threonine kinase. *MLL*-rearranged leukemic cells are dependent on GSK3, since GSK3 inhibitors induce proliferation arrest in these cells [112]. Inhibition of GSK3 with lithium already showed potential in these studies, but further research is warranted since GSK3 slows other malignancies, e.g. colon cancer[113], and induces chromosomal instability[114].

RAS pathway signaling plays a significant role in leukemogenesis in AML. In fact, in *MLL*-rearranged AML this is currently the only pathway with known mutations. Therefore patients harboring these mutations could benefit from RAS pathway inhibition. RAS activation depends on post-translational farnesylation and inhibitors of farnesyltransferase (FTI) could be a potential targeted therapy. Unfortunately, the activity of tipfarnib, a FTI, did not show any correlation with RAS mutations nor with pathway dependent activation in adult AML, indicating that the antileukemic activity of tipfarnib may be due to other mechanisms than RAS inhibition[115]. RAF can be inhibited by Sorafenib. Sorafenib also targets FLT3 mutated AML cells[116] and current phase I/II trials are ongoing in AML[117,118]. Inhibitors of MEK have been developed which sensitize leukemic blast cells to other drugs, e.g. arsenic trioxide and demethylating agents[119-121]. Current RAS pathway inhibitors probably will not fully block the leukemic transformation in *MLL*-rearranged AML, but may have an additive effect with current treatment strategies by targeting the proliferative advantage of these leukemic cells[122].

Another possibility is to directly target the MLL-complex[123]. The MLL-complex requires the proteins MEN1 and PSIP1 to interact with chromatin. Indeed excision of *Men1*, potently inhibits proliferation of MLL-MLLT3-transformed cells and *Hoxa9* expression in mice[124], and similarly, knock-down of Psip1 impaired Hoxa9 expression[28]. This raises the possibility to block the function of Men1 or PSIP1 as a new targeted therapy in *MLL*-rearrangements. The MLL protein also contains a CxxC-domain, which is conserved in all MLL-fusion proteins and specifically binds unmethylated CpG-dinucleotides.

Induced mutations of several residues in the CxxC-domain have been shown to abolish both DNA binding and prevent myeloid transformation[125].
In addition, proteins recruited by the MLL-complex could be potential targets. Thus far, MLLT1 and MLLT10 have been associated with DOT1L. Compounds could be developed to inhibit the histone methyltransferase activity of DOT1L[126]. However, safety studies are warranted, since genetic disruption in mice results in embryonic lethality[127].
Several downstream targets of the MLL-fusion complex could be possible targets of *MLL*-rearranged AML. Upregulation of *HOX* genes is one of the most important hallmarks of *MLL*-rearranged leukemias. Mouse models have shown that MLL-fusion proteins are at least partially dependent on Hox function[128]. *MEIS1*, a co-factor of *HOX*, is consistently highly expressed in *MLL*-related leukemias. In fact, induction and maintenance of *MLL*-induced leukemogenesis in a murine model required Meis1[129]. Strategies based on down-regulation of these genes might prove to be of benefit in the treatment of *MLL*-rearranged AML.

CONCLUSION & FUTURE PERSPECTIVES

MLL-rearrangements are typically found in younger children with AML. In the past, *MLL*-rearranged AML has been related to poor outcome despite intensive chemotherapy. However recent studies showed that outcome in *MLL*-rearranged AML is dependent on different factors, e.g. translocation partner, age, WBC and additional cytogenetic aberrations. Cases with a t(1;11)(q21;q23) have an excellent outcome and may benefit from less intensive treatment, whereas cases with a t(6;11)(q27;q23) or t(10;11)(p21;q23) have a poor outcome and do need adjusted and alternative treatment strategies to improve outcome. Although cooperating events are a hallmark of developing AML, additional genetic aberrations in *MLL*-rearranged AML are hardly identified. To achieve further improvements in outcome, unraveling the biology of *MLL*-rearranged AML is warranted.

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Chapter 6

Novel prognostic subgroups in childhood 11q23/*MLL*-rearranged acute myeloid leukemia: results of an international retrospective study

B.V. Balgobind[1], S.C. Raimondi[2, 3*], J. Harbott[4*], M. Zimmermann[5], T.A. Alonzo[3], A. Auvrignon[6], H.B. Beverloo[7, 8], M. Chang[9], U. Creutzig[10], M.N. Dworzak[11], E. Forestier[12], B. Gibson[13], H. Hasle[14], C.J. Harrison[15], N.A. Heerema[3, 16], G.J.L. Kaspers[17, 18, 19], A. Leszl[20], N. Litvinko[21], L. Lo Nigro[22], A. Morimoto[23, 24], C. Perot[6], R. Pieters[1], D. Reinhardt[5], J.E. Rubnitz[2], F.O. Smith[3, 25], J. Stary[26], I. Stasevich[21], S. Strehl[11], T. Taga[23, 27], D. Tomizawa[23, 28], D. Webb[18, 29], Z. Zemanova[30], C.M. Zwaan[1, 17**] and M.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, USA; [3] Children's Oncology Group (COG), Arcadia, CA, USA; [4] AML-BFM Study Group, Department of Pediatric Hematology and Oncology, University of Giessen, Germany; [5] AML-BFM Study Group, Pediatric Hematology/Oncology, Medical School Hannover, Hannover, Germany; [6] French Leucémie Aique Myeloide Enfant (LAME), Hopital Trousseau, Paris, France; [7] Dutch Childhood Oncology Group (DCOG), 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 (COG), Data Centre, Gainesville, FL, USA; [10] AML-BFM 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 (NOPHO),Department of Clinical Science, Pediatrics, Umeå University Hospital, Sweden; [13] Department of Pediatric Oncology/Hematology, Royal Hospital for Sick Children, Glasgow, United Kingdom; [14] Nordic Society for Pediatric Hematology and Oncology (NOPHO),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, The Ohio State University, Columbus, OH, USA; [17] Dutch Childhood Oncology Group (DCOG), The Hague, The Netherlands; [18] AML Committee I-BFM-SG; [19] Department of Pediatric Oncology/Hematology, VU University Medical Center, Amsterdam, The Netherlands;.[20] Italian Association of Pediatric Hematology Oncology (AIEOP), Clinica Pediatrica, Università Padova, Padova, Italy; [21] Research Center for Pediatric Oncology and Hematology, Minsk, Belarus; [22] Italian Association of Pediatric Hematology Oncology (AIEOP), Clinica Pediatrica, Università Catania, Catania, Italy; [23] Japanese Pediatric Leukemia/Lymphoma Study Group (JPLSG); [24] Department of Pediatrics, Jichi Medical University, Tochigi, Japan; [25] Hematology/Oncology and Pediatrics, Cincinnati Children's Hospital Medical Center and the University of Cincinnati College of Medicine, Cincinnati, OH, USA; [26] Czech Pediatric Hematology/Oncology (CPH), University Hospital Motol and [2]nd 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; [30] Center of Oncocytogenetics, General University Hospital and First Faculty of Medicine, Charles University, Prague, Czech Republic.

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ABSTRACT

Translocations involving chromosome 11q23 frequently occur in pediatric AML and are associated with poor prognosis. In most cases, the *MLL*-gene localized at 11q23 is involved, and more than 50 translocation partners have been described. Clinical outcome data of the 11q23-rearranged subgroups are scarce, because most collaborative group 11q23 series are too small for meaningful analysis of subgroups, although some studies suggest that patients with t(9;11)(p22;q23) have a more favorable prognosis. We retrospectively collected outcome data of 756 children with 11q23- or *MLL*-rearranged AML from 11 collaborative groups to identify differences in outcome based on translocation partners. All karyotypes were centrally reviewed before assigning patients to subgroups. The event-free survival of 11q23/*MLL*-rearranged pediatric AML at 5 years from diagnosis was 44% (±5%), with large differences across subgroups (11%±5% to 92%±5%). Multivariate analysis identified the following subgroups as independent predictors of prognosis: t(1;11)(q21;q23), (hazard ratio [HR] 0.1, p=0.004); t(6;11)(q27;q23), (HR 2.2, p<0.001); t(10;11)(p12;q23), (HR 1.5, p=0.005); and t(10;11)(p11.2;q23), (HR 2.5, p=0.005). We could not confirm the favorable prognosis of the t(9;11)(p22;q23) subgroup. We identified large differences in outcome within 11q23/*MLL*-rearranged pediatric AML and novel subgroups based on translocation partners that independently predict clinical outcome. Screening for these translocation partners is needed for accurate treatment stratification at diagnosis.

INTRODUCTION

Acute myeloid leukemia (AML) is a heterogeneous disease. Currently, response to therapy and cytogenetic abnormalities, including abnormalities such as t(8;21), inv(16), and t(15;17), which together are defined as good-risk AML, are the main prognostic factors[1]. Translocations involving chromosome 11q23 is composed of 15% to 20% of all pediatric AML cases and are, in general, associated with a poor outcome[2-4]. In more than 95% of cases involving 11q23 rearrangements, the *mixed lineage leukemia* (*MLL*) gene is involved, which requires confirmation with molecular analyses such as fluorescence in situ hybridization (FISH)[5]. The heterogeneity of *MLL*-rearranged AML is further reflected by the identification of more than 50 different fusion partners of this gene[6].

In AML, the most common 11q23 rearrangements are t(9;11)(p22;q23) (in ~50% of cases), t(11;19)(q23;p13.1), t(11;19)(q23;p13.3), t(6;11)(q27;q23), and t(10;11)(p12;q23)[2,3]. Most AML samples with 11q23 rearrangements are morphologically classified as FAB (French-American British morphology classification)-M4 or FAB-M5[7].

In some studies, the t(9;11)(p22;q23) subgroup has been associated with a better prognosis, which may at least partially be the result of enhanced sensitivity to different classes of drugs, as demonstrated for this subgroup[4,8-10]. In the current treatment protocols of St. Jude Children's Research Hospital (St. Jude) and the Nordic Society for Pediatric Hematology and Oncology (NOPHO), this has resulted in low-risk stratification of these patients. Studies on the clinical outcome of various 11q23 rearrangements in pediatric acute lymphoblastic leukemia (ALL) showed age-specific outcome differences. For instance, in children older than 1 year, t(4;11)(q21;q23) showed an adverse outcome compared with that of children who carried other 11q23 rearrangements[11], which is not the case in infants[12]. However, clinical outcome data of pediatric patients with AML expressing other 11q23 rearrangements are extremely scarce, because most collaborative group series are too small for meaningful outcome analysis of these rare subgroups.

Therefore, a very large international study was undertaken to collected clinical outcome data of 756 children with 11q23 or *MLL*-rearranged pediatric AML from 11 collaborative study groups from 15 countries. Our aim was to identify differences in clinical outcome based on the various 11q23 or *MLL* rearrangements, which may result in better risk-group stratification and risk-group–directed therapy for these patients.

PATIENTS, MATERIALS AND METHODS

Patients

Data on 756 patients with 11q23- or *MLL*-rearranged pediatric AML were collected from 11 collaborative study groups, including the Berlin-Frankfurt-Münster Study Group (BFM,

Germany and Austria; n=160), the Japanese Pediatric Leukemia/Lymphoma Study Group (JPLSG, Japan; n=75), the Leucémies Aiguës Myéloblastiques de l'Enfant Cooperative Group (LAME, France; n=61), the Czech Pediatric Hematology Working Group (CPH, Czech republic; n=18), the St. Jude (USA; n=44), the Associazione Italiana Ematologia Oncologia Pediatrica (AIEOP, Italy; n=34); Belarus (n=25), the Children's Oncology Group (COG, USA; both including the Children's Cancer Group (CCG) and the Pediatric Oncology Group (POG) studies; n=215), the NOPHO (Denmark, Finland, Iceland, Norway and Sweden; n=59), the Dutch Children's Oncology Group (DCOG, The Netherlands; n=34), and 2 centers of the Medical Research Council (MRC, United Kingdom; n=31).

The patients were identified by the study groups reviewing their karyotypic records and including those with 11q23- or *MLL*-rearranged AML, as determined by G-, Q-, or R-banded karyotyping, FISH, or RT-PCR. For the purpose of this study, these data were grouped as 11q23/*MLL*-rearranged AML. A predefined set of data was collected for each case and checked for consistency and completeness. This set of data consisted of clinical data obtained at initial diagnosis, including sex, age, white blood cell count (WBC), hemoglobin, platelets, extramedullary disease and FAB-morphology. In addition, we collected data on treatment, such as therapy protocol, including hematopoietic stem cell transplantation (HSCT), and al events (including non-responders, relapse, second malignancy, or death) during follow-up. Only patients younger than 18 years at diagnosis were included in the analysis. Patients were eligible when diagnosed between January 1, 1993 and January 1, 2005. The CCG, however, included patients diagnosed between January 1, 1995 and January 1, 2003; the POG included patients diagnosed between January 1, 1993 and January 1, 2000 (Supplementary Table 1). Exclusion criteria consisted of secondary AML following congenital bone marrow (BM) failure disorders (e.g., Fanconi anemia, severe congenital neutropenia, and Shwachman syndrome), aplastic anemia, prior chemotherapy or radiotherapy for other diseases, and prior myelodysplastic syndrome.

Patients were treated on national/collaborative group AML trials[4,13-22]. The treatment protocols were approved according to local law and guidelines, including the Declaration of Helsinki. Patients received anthracycline- and cytarabine-based induction courses, followed by consolidation courses that included high-dose cytarabine (Supplementary Table 2). Different anthracyclines were used. To calculate the cumulative anthracycline dose prescribed in the various protocols, we used a ratio of 1:5 (in mg/m^2) for daunorubicine:idarubicin or mitoxantrone and a ratio of 1:1 for daunorubicin:doxorubicin[23]. Hundred and eight patients received allogeneic HSCT in first CR, whereas 59 patients underwent autologous HSCT; these patients were included in the "chemotherapy only" group[24].

Categories of 11q23 or *MLL* rearrangements

Most patients were entered into the study based on available karyotypic reports showing 11q23/*MLL* rearrangements. Assignment to 11q23/*MLL*-rearranged subgroups based on

Table 1: Characteristics of pediatric patients with 11q23/*MLL*-rearranged AML grouped on the basis of translocation partners.

	Total	t(9;11) (p22;q23)	t(10;11) (p12;q23)	t(6;11) (q27;q23)	t(11;19) (q23;p13)	t(11;19) (q23;p13.1)	t(11;19) (q23;p13.3)	t(1;11) (q21;q23)	t(4;11) (q21;q23)	t(10;11) (p11.2;q23)	t(11;17) (q23;q21)	Other	*p
N	756	328	98	35	31	34	25	25	13	12	12	143	
Sex (N=756)													
Male, N (%)	367 (48.5)	157 (47.9)	55 (56.1)	19 (54.3)	13 (41.9)	15 (44.1)	11 (44.0)	9 (36.0)	3 (23.1)	6 (50)	7 (58.3)	72 (50.3)	0.495
Median age (years) (N=756)	2.2	2.6	1.3	12.4	1.6	4.6	7.1	1.3	1.3	1.0	9.0	1.9	
													<0.001
<2 y, N (%)	358 (47.4)	137 (41.8)	61 (62.2)	3 (8.6)	18 (58.1)	14 (41.2)	9 (36.0)	19 (76.0)	8 (61.5)	9 (75.0)	4 (33.3)	72 (50.3)	
2-9 y, N (%)	227 (30.0)	117 (35.7)	22 (22.4)	12 (34.3)	8 (25.8)	11 (32.4)	5 (20.0)	6 (24.0)	2 (15.4)	3 (25.0)	3 (25.0)	41 (28.6)	
≥10 y, N (%)	171 (22.6)	74 (22.6)	15 (15.3)	20 (57.1)	5 (16.1)	9 (26.5)	11 (44.0)	-	3 (23.1)	-	5 (41.7)	30 (21.0)	
Median WBC, x10^9/L (N=754)	20.9	13.7	10.9	59.8	76.0	7.0	17.5	33.4	20.0	17.2	87.8	17.2	
													<0.001
<20 x 10^9/L, N (%)	371 (49.1)	180 (54.9)	58 (59.2)	8 (22.9)	10 (32.3)	15 (44.1)	14 (56.09	9 (36.0)	6 (46.2)	6 (50.0)	4 (33.3)	61 (42.7)	
20-<100 x 10^9/L, N (%)	206 (27.2)	82 (25.0)	22 (22.4)	12 (34.3)	9 (29.0)	13 (38.2)	3 (12.0)	11 (44.0)	3 (23.1)	2 (16.7)	3 (25.0)	46 (32.2)	
≥100 x 10^9/L, N (%)	177 (23.4)	65 (19.8)	18 (18.4)	15 (42.9)	12 (38.7)	6 (17.6)	8 (32.0)	5 (20.0)	4 (30.8)	4 (33.3)	5 (41.7)	35 (24.5)	
FAB-type, N (%) (N=722)													<0.001
FAB-M0	23 (3.2)	3 (1.0)	4 (4.3)	-	1 (3.2)	1 (3.0)	3 (12.0)	-	3 (25.0)	1 (9.1)	1 (8.3)	6 (4.5)	
FAB-M1	40 (5.5)	9 (2.9)	3 (3.2)	5 (14.7)	1 (3.2)	6 (18.2)	4 (16.0)	3 (12.0)	1 (8.3)	1 (9.1)	-	7 (5.3)	
FAB-M2	33 (4.6)	10 (3.2)	2 (2.2)	2 (5.9)	2 (6.5)	5 (15.2)	-	2 (8.0)	-	-	1 (8.3)	9 (6.8)	
FAB-M3	-	-	-	-	-	-	-	-	-	-	-	-	
FAB-M4	137 (19.0)	23 (7.3)	13 (14.0)	12 (35.3)	13 (41.9)	10 (30.3)	5 (20.0)	14 (56.0)	2 (16.7)	3 (27.3)	4 (33.3)	38 (28.6)	
FAB-M5	462 (64.0)	254 (81.2)	67 (72.0)	14 (41.2)	14 (45.2)	11 (33.3)	12 (48.0)	6 (24.0)	5 (41.7)	6 (54.5)	6 (50.0)	67 (50.4)	
FAB-M6	-	-	-	-	-	-	-	-	-	-	-	-	
FAB-M7	20 (2.8)	14 (4.5)	3 (3.2)	-	-	-	1 (4.0)		1 (8.3)	-	-	1 (0.8)	
FAB-unspecified	7 (1.0)	-	1 (1.1)	1 (2.9)	-	-	-	-	-	-	-	5 (3.8)	

Table 1: Continued

	Total	t(9;11) (p22;q23)	t(10;11) (p12;q23)	t(6;11) (q27;q23)	t(11;19) (q23;p13)	t(11;19) (q23;p13.1)	t(11;19) (q23;p13.3)	t(1;11) (q21;q23)	t(4;11) (q21;q23)	t(10;11) (p11.2;q23)	t(11;17) (q23;q21)	Other	*p
Median blast in BM (%) (N=653)	80.0	82.0	82.0	88.5	72.0	70.0	86.0	69.0	72.0	45.0	85.0	79.0	
CNS-involvement, N (%) (N=693)	100 (14.4)	33 (11.0)	16 (17.6)	5 (15.2)	8 (27.6)	6 (19.4)	2 (9.1)	2 (8.0)	1 (9.1)	2 (22.2)	3 (25.0)	22 (17.1)	0.272
Hepatomegaly, N (%) (N=634)	371 (58.8)	163 (57.0)	55 (63.2)	17 (63.0)	21 (67.7)	11 (40.7)	10 (62.5)	12 (57.1)	6 (60.0)	4 (50.0)	7 (63.6)	65 (59.1)	0.782
Splenomegaly, N (%) (N=641)	278 (43.4)	123 (43.2)	44 (50.0)	15 (55.6)	15 (48.4)	9 (34.6)	7 (43.8)	4 (20.0)	4 (20.0)	5 (62.5)	3 (27.3)	49 (45.0)	0.347
Additional cytogenetic aberrations, N (%) (N=736)	375 (51.0)	164 (51.4)	42 (43.8)	19 (54.3)	21 (66.7)	21 (61.8)	12 (48.0)	18 (75.0)	5 (38.5)	5 (41.7)	8 (66.7)	60 (44.4)	0.062

*P-values indicate whether the differences are significant among the subgroups.

Abbreviations: y, years; WBC, white blood cell count; FAB, French-American-British morphology classification; BM, bone marrow; CNS, central nervous system; N, number

translocation partners took place after independent central review by the co-authors SR and JH of the breakpoint in the corresponding chromosome and followed the International System for Human Cytogenetic Nomenclature (ISCN2005)[25]. Molecular confirmation of an *MLL* rearrangement with FISH and/or RT-PCR was not required, because conventional cytogenetics methods, RT-PCR, and FISH are usually complementary and allow reliable identification of 11q23/*MLL* gene rearrangements in leukemic clones. Patients with an *MLL* rearrangement detected by FISH and an unknown translocation partner were classified in the "11q23/*MLL*-other" group. At least 10 patients had to be included per subgroup; otherwise the cases were allocated to the 11q23/*MLL*-other group. There were 328 (43%) patients with t(9;11)(p22;q23), 98 (13%) with t(10;11)(p12;q23), 35 (5%) with t(6;11)(q27;q23), 34 (5%) with t(11;19)(q23;p13.1), 25 (3%) with t(11;19)(q23;p13.3), 31 (4%) with t(11;19)(q23;p13)-without ascertained subband, 25 (3%) with t(1;11)(q21;q23), 13 (2%) with t(4;11)(q21;q23), 12 (2%) with t(10;11)(p11.2;q23), and 12 (2%) with t(11;17)(q23;q21). The remaining 143 (19%) patients, including 4 patients with t(9;11) and another breakpoint on chromosome 9, were assigned to the 11q23/*MLL*-other subgroup (Table 1).

Statistical analyses

Complete remission (CR) was defined as less than 5% blasts in the BM, with regeneration of trilineage hematopoiesis plus absence of leukemic cells in the cerebrospinal fluid or elsewhere[26]. Early death was defined as any death within the first 6 weeks of treatment. Treatment of patients who did not obtain CR was considered a failure on Day 0. Overall survival (OS) was measured from the date of diagnosis to the date of last follow-up or death due to 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. For OS and EFS 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 (5y-pOS) and EFS (5y-pEFS), and survival estimates were compared using the log-rank test. Cumulative incidence functions of relapse (with other events and death while in CR as competing events) were constructed by the method of Kalbfleisch and Prentice and compared by Gray's test. For multivariate analysis, the Cox proportional-hazard regression model was used; allogeneic HSCT was included as a time-dependent covariate. Continuous variables known to be of prognostic value in AML where categorized according to cutoff points (e.g., age older than 1, 2, 5 or 10 years, WBC less than 20×10^9/L or greater than 100×10^9/L). The χ^2 or Fisher's Exact test was used to compare differences in percentages of variables among subgroups; the Mann-Whitney U test was used for continuous variables. All p-values are descriptive and explorative and were considered significant if $p \leq 0.01$. Statistical analyses were conducted using SAS software (SAS-PC, version 9.1).

Table 2. Survival estimates of prognostic factors in 11q23/*MLL*-rearranged AML, including subgroups based on translocation partners.

	5y-pEFS (%, SE)	*P-value (Log rank)	5y-pCIR (%, SE)	*P-value (Gray)	5y-pOS (%, SE)	*P-value (Log rank)
***MLL*-rearrangement**						
All	44 (2)		35 (2)		56 (2)	
t(1;11)(q21;q23)	92 (5)	*<0.001*	4 (4)	*<0.001*	100 (0)	*<0.001*
t(9;11)(p22;q23)	50 (3)		29 (3)		63 (3)	
t(11;19)(q23;p13)	49 (9)		24 (8)		49 (10)	
t(11;19)(q23;p13.1)	46 (9)		42 (9)		61 (9)	
t(11;19)(q23;p13.3)	46 (10)		21 (9)		47 (11)	
t(11;17)(q23;q21)	42 (14)		41 (15)		67 (14)	
t(10;11)(p12;q23)	31 (5)		52 (5)		45 (5)	
t(4;11)(q21;q23)	29 (13)		56 (16)		27 (13)	
t(10;11)(p11.2;q23)	17 (11)		50 (16)		27 (13)	
t(6;11)(q27;q23)	11 (5)		54 (9)		22 (7)	
Other	39 (4)		40 (5)		54 (5)	
Additional cytogenetic aberrations						
No	48 (3)	*0.01*	31 (3)	0.03	61 (3)	*0.002*
Yes	39 (3)		39 (3)		48 (3)	
White blood cell count						
<20 x 10^9/L, N (%)	48 (3)	*0.004*	45 (3)	0.6	59 (3)	*0.004*
20-<100 x 10^9/L, N (%)	43 (4)		42 (4)		57 (4)	
≥100 x 10^9/L, N (%)	36 (4)		46 (4)		47 (4)	
Age						
<10 years	46 (2)	*0.006*	43 (3)	0.048	60 (2)	*0.001*
≥10 years	34 (4)		51 (4)		42 (4)	

RESULTS

11q23 or *MLL* rearrangements

Of the 756 cases, 376 (50%) were evaluated by conventional cytogenetics only. 380 (50%) cases were evaluated by conventional cytogenetics and in addition an *MLL* rearrangement was confirmed with either FISH or RT-PCR. We did not detected differences in sex distribu-

tion, age at diagnosis and diagnostic WBC between these two groups. No differences were also detected for outcome between the cases for whom only conventional cytogenetics were available and the cases that were molecularly evaluated (pEFS 42.9%±2.6% vs. 44.3%±2.6%, p=0.70). Moreover, since conventional cytogenetic methods are usually complementary to RT-PCR and FISH and allow reliable identification of *MLL* gene rearrangements in leukemic clones, we addressed the total group as 11q23/*MLL*-rearrangements.

Clinical characteristics

The patient characteristics of the various 11q23/*MLL* subgroups are described in detail in Table 1. There were no differences in sex distribution among the 11q23/*MLL* subgroups; however, significant differences were seen in age at diagnosis, diagnostic WBC, and FAB types. The median age at diagnosis for the entire cohort was 2.2 years (range, 0-17.9 years). In most 11q23/*MLL* subgroups, the median age at diagnosis was younger than 5 years; however, in the t(6;11)(q27;q23) and t(11;17)(q23;q21) subgroups, the median ages were 12.4 and 9.0 years, respectively (Table 1). Patients with a t(6;11)(q27;q23), t(11;17)(q23;q21), or t(11;19)(q23;p13)-without ascertained subband had a higher median WBC than other subgroups (59.8×10^9/L, 87.8×10^9/L, and 76.0×10^9/L, respectively; Table 1). Most of the AML cases were classified as FAB-M5 (64%) or -M4 (19%); however, in some subgroups another FAB-type distribution was found. For instance, in the t(1;11)(q21;q23) subgroup, only 25% of patients presented with FAB-M5 morphology, and 56% presented with FAB-M4 (Table 1).

11q23/*MLL* rearrangements and survival

The median follow-up time of survivors was 4.8 years. In 99% of the patients, therapy was administered. The overall CR rate was 85% with no significant differences in CR rates among the subgroups (p=0.64). For the cohort of 11q23/*MLL*-rearranged patients, 5y-EFS was 44%±2%; 5y-OS was 56%±2%; and the cumulative incidence of relapse at 5 years from diagnosis was 35%±2% (Figure 1).

Interestingly, the outcome of patients with different 11q23/*MLL* rearrangements varied significantly (Table 2 and Figure 2A and B). The 24 patients with a t(1;11)(q21;q23) had excellent outcome with a 5y-pEFS of 92%±5%, and an 5y-pOS of 100%±0%, as the 2 patients with an event were successfully rescued at relapse. On the other hand, the 35 patients with a t(6;11)(q27;q23) had the worst outcome: 5y-EFS was 11%±5%, and 5y-OS was 22%±7%. In addition, the 11q23 subgroups t(10;11)(p11.2;q23), t(4;11)(q21;q23), and t(10;11)(p12;q23) showed poor outcome (5y-pEFS was 17%±11%, 29%±13%, and 32%±5%, respectively); OS rates were in agreement with the 5y-pEFS data.

When analyzing the type of events there were significant differences in the cumulative incidence of relapse between the various 11q23/*MLL* subgroups (pGray <0.001; Table 2), whereas the 5y-cumulative incidence of death in CR (pGray = 0.468) did not differ significantly among the subgroups.

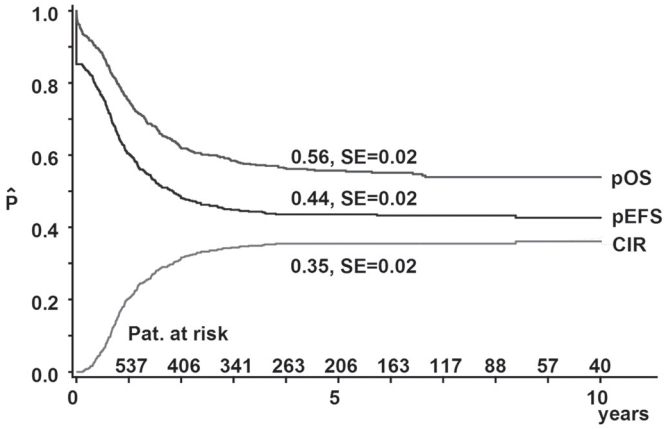

Figure 1. Survival curves of the 756 pediatric patients with 11q23/*MLL*-rearranged AML included in this study. The probability of overall survival (pOS) at 5 years from diagnosis was 56%±2% (312 events); the probability of event-free survival (pEFS) was 44%±2% (417 events); and the probability of cumulative incidence of relapse (pCIR) was 35%±2% (257 events).

Other prognostic factors

Patients with a high WBC (>100×10^9/L) had a significantly worse 5y-pEFS than did patients presenting with a low WBC (<20×10^9/L) (36%±4% vs. 48%±3%; p=0.001). After evaluating various cut-off points for age, patients older than 10 y had significantly worse 5y-pEFS than patients younger than 10 y (34%±4% vs. 46%±2%; p=0.006). Also patients with additional cytogenetic aberrations had significantly worse 5y-pEFS than other patients (39%±3% vs. 48%±3%; p=0.01) (Table 2). Also differences in outcome were seen across the study groups with 5y-pEFS ranging from 29%±10% to 61%±7% (Supplementary Table 3).

Prognostic factors in the t(9;11)(p22;q23) subgroup

The 5y-pEFS of patients with a t(9;11)(p22;q23) was 50%±3%. Within this subgroup, patients with FAB-M5 morphology had a 5y-pEFS of 56%±3%, and those with other FAB subtypes had 31%±7% (p=0.002; Supplementary Table 4). No differences in outcome were detected according to age.

When comparing the outcome of the t(9;11)(p22;q23) subgroup with that of other 11q23 subgroups within the various collaborative study groups, we found in the NOPHO protocols only that patients with a t(9;11)(p22;q23) did better than those with other 11q23/*MLL* rearrangements (5y-pEFS, 77%±8% vs. 38%±9%, p log-rank = 0.006).

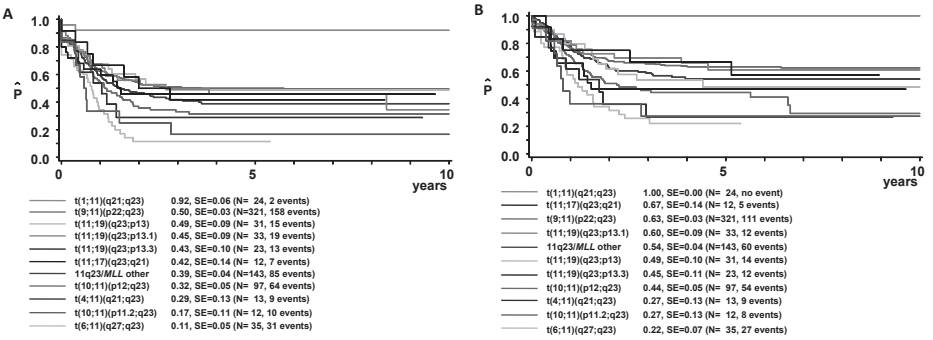

Figure 2. Survival curves for patients with 11q23/*MLL*-rearranged pediatric AML grouped on the basis of different translocation partners. Panel **A** shows event-free survival curves, and panel **B** shows overall survival. Assignment to 11q23-rearranged subgroups was based on translocation partners, as identified after central review of karyotyping. Some patients were assigned to 11q23 subgroups based on RT-PCR results only. If an *MLL* rearrangement was determined by FISH and the translocation partner was unknown, the patient was included in the "11q23/*MLL*-other" group. At least 10 patients had to be included to create a subgroup; otherwise the cases were allocated to the 11q23/*MLL*-other group. Patients with a t(1;11)(q21;q23) showed independent favorable outcome with overall survival at 5 years of 100%±0%, and an event-free survival of 92%±5%. Several rearrangements were identified as predictors of poor clinical outcome, including t(6;11)(q27;q23), t(10;11)(p11.2;q23), t(4;11)(q21;q23), and t(10;11)(p12;q23).

Multivariate analysis of the total cohort of patients with 11q23/*MLL* rearrangements

Cox-regression analysis of EFS from diagnosis revealed the subgroups t(6;11)(q27;q23) (hazard ratio [HR] 2.3, p <0.001), t(10;11)(p12;q23) (HR 1.5, p=0.004), and t(10;11)(p11.2;q23) (HR 2.4, p=0.007) as independent predictors of poor prognosis (Table 3). With an HR of 0.1 (p=0.004), the t(1;11)(q21;q23) subgroup was associated with a favorable outcome. In addition, WBC greater than 100×10^9/L was an independent predictor of poor prognosis (HR 1.4, p=0.003). All of these factors also predicted OS from diagnosis. Allogeneic HSCT as a time-dependent variable or treatment according to study group were not independent predictors of EFS or OS. When the analysis was restricted to the poor prognostic subgroups t(10;11)(p12;q23) or t(6;11)(q27;q23), allogeneic HSCT as time-dependent variable again did not predict for EFS or OS.

Multivariate Cox-regression analysis limited to the subgroup of patients (n=328) with a t(9;11)(p22;q23) identified FAB-M5 as an independent favorable prognostic factor for EFS and OS from diagnosis (both HR 0.4, p=<0.001). In addition, WBC less than 20×10^9/L was an independent favorable prognostic factor for EFS (HR 1.6, p=0.003). Within the t(9;11) subgroup, allogeneic HSCT as a time-dependent variable did not predict OS (HR 1.0, p=0.99; Supplementary Table 5).

Table 3. Multivariate analysis of survival parameters of pediatric patients with 11q23/*MLL*-rearranged AML.

	pEFS			pOS		
	Hazard Ratio	**95% CI**	**p-value**	**Hazard Ratio**	**95% CI**	**p-value**
***MLL*-translocation**						
t(9;11)(p22;q23)	1.0	Reference		1.0	Reference	
t(10;11)(p12;q23)	1.5	1.1-2.1	*0.004*	1.9	1.3-2.6	*<0.001*
t(6;11)(q27;q23)	2.3	1.5-3.4	*<0.001*	2.5	1.6-4.0	*<0.001*
t(11;19)(q23;p13)	1.0	0.6-1.7	0.91	1.4	0.8-2-4	0.27
t(11;19)(q23;p13.1)	1.1	0.7-1.8	0.77	0.9	0.5-1.7	0.83
t(11;19)(q23;p13.3)	1.1	0.6-1.9	0.85	1.5	0.8-2.7	0.17
t(1;11)(q21;q23)	0.1	0.0-0.6	*0.007*	- *		
t(4;11)(q21;q23)	1.6	0.8-3.2	0.15	2.3	1.2-4.6	0.02
t(10;11)(p11.2;q23)	2.4	1.3-4.6	*0.007*	3	1.5-6.3	*0.003*
t(11;17)(q23;q21)	1.0	0.5-2.2	0.94	1	0.4-2.6	0.94
Other	1.3	1.0-1.7	0.05	1.3	0.9-1.8	0.11
Additional cytogenetic aberrations						
No	1.0	Reference		1.0	Reference	
Yes	1.2	1.0-1.5	0.05	1.3	1.1-1.7	0.02
WBC						
<100 x 10^9/L	1.0	Reference		1.0	Reference	
≥100 x 10^9/L	1.4	1.1-1.7	*0.003*	1.4	1.1-1.8	*0.007*
Age						
<10 y	1.0	Reference		1.0	Reference	
≥10 y	1.3	1.0-1.6	0.04	1.4	1.1-1.8	*0.01*
Allogeneic BMT						
No	1.0	Reference		1.0	Reference	
Yes	0.9	0.6-1.3	0.59	0.9	0.6-1.2	0.41

*no events
Abbreviations: pEFS, probability of event-free survival; pOS, probability of overall survival; WBC, white blood cell count; y, years; HSCT, hematopoietic stem cell transplantation

DISCUSSION

In this large international retrospective study, we identified novel prognostic subgroups in pediatric 11q23/*MLL*-rearranged AML. Patients with a t(1;11)(q21;q23) showed favorable outcome independent of other risk factors, whereas those with a t(6;11)(q27;q23),

t(10;11)(p12;q23), or t(10;11)(p11.2;q23) showed poor clinical outcome independent of other factors. These results underscore the importance of international collaboration in the investigation of rare diseases or subgroups.

This study is the first to identify t(1;11)(q21;q23) as subgroup with excellent clinical outcome. The biological background for this favorable outcome is currently poorly understood, and conflicting results have been reported. Co *et al.* performed *AF1q* overexpression and knockdown experiments in cell lines and concluded that overexpression resulted in enhanced doxorubicin-induced apoptosis mediated through the BAD pathway, whereas knockdown of *AF1q* expression resulted in the reverse[27]. In another study, in 64 unselected pediatric AML samples, high *AF1q* expression was independently associated with poor survival[28]. Whether *MLL-AF1q* causes different expression levels of *AF1q* remains unknown, hence further studies should address the underlying biology. Given the excellent outcome of this rare subgroup of patients, we suggest that direct screening for this translocation be incorporated in future pediatric AML treatment protocols and that patients with *MLL-AF1q* should be allocated to the low-risk group (or treatment arm) of these protocols.

In this study, we identified t(6;11)(q27;q23) as an independent predictor of poor prognosis in pediatric AML. In adult AML, the poor outcome of patients with a t(6;11)(q27;q23) has been reported before, and adults with this specific translocation do equally bad (2-year OS, 13%)[29]. Of interest, children with a t(6;11)(q27;q23) have a higher WBC and older age at presentation than other *MLL*-rearranged cases. We could not prove that allogeneic HSCT was of benefit in these patients, but numbers were limited. Given the dismal outcome, studies are urgently needed to elucidate the biology of the *MLL-AF6* fusion gene, which may lead to the development of new treatment regimens for this subgroup. Meanwhile, screening for this translocation should become part of the standard screening procedures in pediatric AML, because it will allow appropriate allocation of these patients to the high-risk treatment arm of pediatric AML protocols and prospectively validate our results.

The cytogenetic abnormalities t(10;11)(p12;q23) and t(10;11)(p11.2;q23) were also identified as independent predictors of an unfavorable prognosis in this study. The t(10;11)(p12;q23) subgroup is the second most common translocation detected in pediatric 11q23/*MLL*-rearranged AML. An accurate diagnosis of patients with this translocation requires specific screening with FISH, because these patients often show heterogeneity in their chromosomal breakpoints and are, therefore, not identified by conventional karyotyping[30]. Similar to patients with t(6;11)(q27;q23), these patients should be allocated to high-risk arm of pediatric AML treatment protocols.

The present study did not confirm the favorable outcome of patients with a t(9;11)(p22;q23), when compared with other 11q23/*MLL*-rearranged subgroups, as previously described in smaller pediatric and adult series.[4,9,31] However, patients with t(9;11)(p22;q23) included in NOPHO protocols still had significantly better outcome than other 11q23/*MLL*-rearranged patients treated on NOPHO protocols. Previously, it was suggested that a

high cumulative dosage of cytarabine administered in NOPHO and St Jude protocols contributed to this better outcome, but this relationship could not be confirmed in the present study[4,9]. Moreover, no differences in cumulative dosages of anthracyclines or etoposide, which could explain the improved outcome of t(9;11)(p22;q23) on NOPHO protocols, were detected. Therefore, the factor(s) that determines the relatively specific benefit of NOPHO protocols for patients with a t(9;11)(p22;q23) remains unknown. One hypothesis may be that the NOPHO study group has a more ethnically homogeneous population than the other European and American study groups and the outcomes in t(9;11)(p22;q23) may be determined, to some extent, by ethnicity-related genetic factors[32,33].

Of interest, within the t(9;11)(p22;q23) subgroup, prognosis was related to the cell-type from which the leukemia originated, as patients with acute monoblastic leukemia did significantly better than those with other FAB-types. The 5y-pEFS of the group of non–FAB-M5 t(9;11)(p22;q23) was only 31%, which was similar to that of the other 11q23/*MLL* poor-risk groups discussed earlier. These results are in accordance with the previously reported prognostically favorable outcome of t(9;11)(p22;q23) of the St. Jude, where a high frequency (21/23 cases) of FAB-M5 was detected and the 2 cases without FAB-M5 did not achieve CR and eventually died[9]. In an adult study by Mrozek *et al.* a favorable outcome was detected for t(9;11)(p22;q23) and possibly addressed to intensive post-remission therapy[31]. In the latter study, 84% of the adult patients with a t(9;11)(p22;q23) presented with a FAB-M5. This may indicate that the favorable outcome of t(9;11)(p22;q23) in these studies is dominated by the frequency of FAB-M5 patients and stressed the importance of including FAB-type for risk stratification.

The frequency of 11q23/*MLL*-rearranged AML may have been underestimated in the included study cohorts, as well as in other studies performed in the past due to cryptic *MLL* rearrangements that cannot be detected by conventional karyotyping. Because the 11q23/*MLL*-rearranged group is associated with poor outcome, FISH screening for *MLL* rearrangements at diagnosis has become the standard approach in many AML protocols. The results from this study confirm that this approach is worthwhile, but they also demonstrate the need to screen for specific translocation partners to allow appropriate treatment stratification. This is not possible by using FISH analysis alone; therefore, additional methods such as RT-PCR should become part of standardized screening procedures to correctly identify patients with specific low- or high-risk *MLL* rearrangements.

In conclusion, this unique and very large international retrospective study identified several novel, independent prognostic 11q23/*MLL*-rearranged subgroups, including the favorable-risk subgroup with a t(1;11)(q21;q23) and the poor-risk subgroups with a t(10;11)(p12;q23), t(10;11)(p11.2,q23), or t(6;11)(q27;q23). We recommend that these subgroups be included in the risk-group stratification process in current and future pediatric AML protocols. The patients with a t(9;11)(p22;q23) represent a heterogeneous subgroup requiring the inclusion of FAB-type for accurate risk-group stratification. In addition, the

biological background of the various subgroups of 11q23/*MLL*-rearranged AML should be further investigated, as these specific subgroups may benefit from specific and targeted treatment components, especially those subgroups with an adverse prognostic outcome, despite current intensive therapy.

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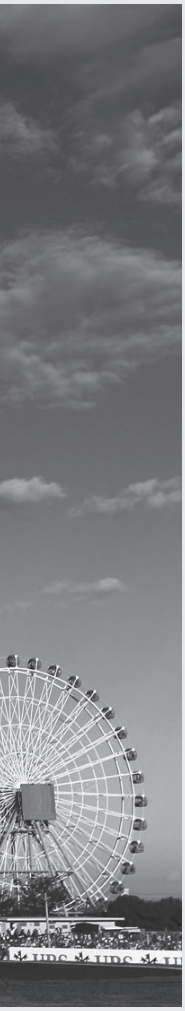
RedBull

Chapter 7



NRIP3: A novel translocation partner of *MLL* detected in a pediatric AML with complex chromosome 11 rearrangements

B.V. Balgobind[1], C.M. Zwaan[1], C. Meyer[3], R. Marschalek[3], R. Pieters[1], H.B. Beverloo[2] and M.M. Van den Heuvel-Eibrink[1]

[1]*Pediatric Oncology/Hematology, Erasmus MC / Sophia Children's Hospital, Rotterdam, The Netherlands*
[2]*Clinical Genetics, Erasmus MC, Rotterdam, The Netherlands*
[3]*Institute of Pharmaceutical Biology, ZAFES, Diagnostic Center of Acute Leukemias (DCAL), Frankfurt, Germany*

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TO THE EDITOR

MLL-rearranged acute myeloid leukemia (AML) is associated with an adverse outcome in most treatment protocols. The *MLL*-gene exhibits an 8-kb breakpoint cluster region, which behaves as a hotspot for chromosomal translocations. So far more than 50 different fusion partners of the *MLL*-gene have been identified[1]. In pediatric AML, the main translocations are: t(9;11), t(11;19); t(6;11) and t(10;11) accounting for almost 15-20% of the cases[2]. Here, we report on a *novel* translocation partner of *MLL* on chromosome 11 in a pediatric AML case

A 5-month old boy presented with AML FAB-M5 and a white blood cell count of $9.8x10^9$/L. CSF analysis showed CNS involvement with a cell count of $1.4x10^6$ cells/mm^3 and with 93% blasts. Immunophenotyping of bone marrow and peripheral blood further confirmed a monoclonal population in at least 50% of which showed the following aberrant phenotype: CD34-, CD117+, CD13+, CD33+, CD15s+, CD14-, CD4+, CD45+ and MPO+. The child responded well to chemotherapy according to the DCOG-AML 97 protocol. The patient is in continuous complete remission 7 years after diagnosis

RBA and QFQ-banded karyotyping and fluorescence in situ hybridization (FISH) showed a double inversion on chromosome 11 in combination with a rearrangement involving chromosome 3 (Figure 1A). At the time of diagnosis the karyotype was *46,XY,der(3)t(3;11)(p21;q23)ins(3;11)(q23;p12p15),der(11) del(11)(p12p15)inv(11)(p1?2q1?1)inv(11)(q1?1q23)t(3;11)(p21;q23)*. FISH using the *MLL* Dual Color, Break Apart Rearrangement Probe (Vysis/Abbott, Des Plaines, IL, USA) confirmed the *MLL* gene rearrangement showing the 3'probe on the short arm of the der(3) and the 5' probe high on the long arm of the der(11).

Long distance inverse (LDI)-PCR as previously described [1] revealed that 5'*MLL* (intron 9) was fused to *NRIP3* (intron 1) located on chromosome 11p15. Moreover, 3'*MLL* was fused to sequences from chromosome 3q21.3 (*FLJ40473*). Subsequently, the *MLL-NRIP3* fusion gene was identified with RT-PCR (Figure 2), using an MLL exon 8 specific forward primer (5'-CGTCGAGGAAAAGAGTGA-3') combined with a *NRIP3* exon 3 specific reverse primer (5'-CAGGCCAAAGAGATGAGAT-3')

Since these results were not in concordance with the observed karyotype, additional FISH analysis was performed. A paint for chromosome 11 showed that there were chromosome 11 sequences present on the short arm of the der(3), and on a small region on the der(3)(q). The pericentric inversion of chromosome 11 was confirmed using probes on both sides of the centromeric region (Figure 1B). The 11p telomeric probe was present on the top of the der(11)(p). The paracentric inversion of 11q was confirmed using probes on 11q13 and 11q21.The 11q telomeric probe was present on the top of the der(3)(p) confirming the t(3;11)(p21;q23). The break apart probes for NUP98 (11p15.4)[3] were unexpectedly detected on the top of the der(3)(p), near the 3'*MLL* localization. Subsequent

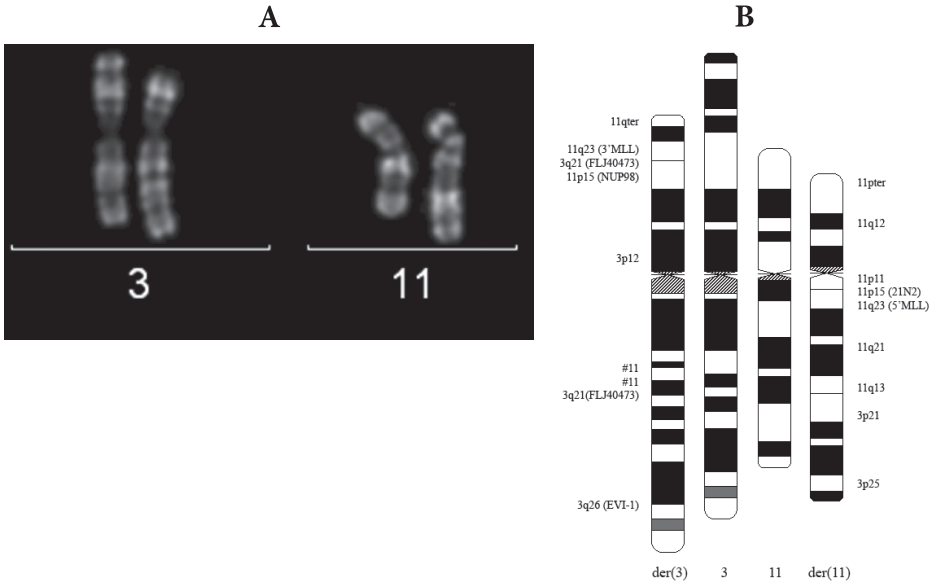

Figure 1:Karyogram of chromosome 3 and 11

(a) Partial RBA-banded karyogram showing chromosomes 3 and 11. The der(3) and the der(11) are shown on the right.

(b) Schematic representation of the normal chromosome 3 and 11 (middle) and the der(3) (left) and the der(11) (right). The band designations provided were all observed using specific FISH probes. The designation #11 is where the whole paint for chromosome 11 showed hybridization to the der(3). Used probes were (from pter to qter) for chromosome 3: RP11-438J1 (3p25), RP11-969E9 (3p21), RP11-451E6 (3p12.3), RP11-79F5 (3p12.1), RP11-456K4 (3q21), RP11-82C9 (3q26); for chromosome 11: RP11-120E20/348A20 (NUP98, 11p15.4), RP11-21N2 (11p15.4), RP11-102E22 (11p11.2), pLC11a (centromere 11), RP11-

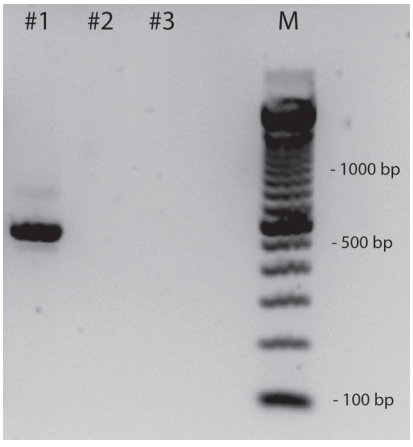

Figure 2: Transcript of the *MLL-NRIP3* fusion gene

Detection of *MLL-NRIP3* transcript (522 bp) in our patient (#1) but not in two patients with another *MLL*-rearrangement (#2 and #3)

hybridization using a more centromeric probe on 11p15.4 (RP11-21N2), which is 5 Mb telomeric to *NRIP3*, showed the signal on the der(11)(q) near the probe for 5'*MLL* based on inverted DAPI banding pattern. A probe covering part of the *FLJ40473* region showed in addition to the normal location on 3q21, a weaker signal on the top of the der(3)(p) near the location of 3'*MLL* and NUP98 (Figure 1B). We were not able to determine the origin of the small insertion of chromosome 11 sequences on the der(3)(q). However, FISH results clearly demonstrated that there were many more chromosome 11 and 3 rearrangements present than expected on basis of the conventional karyotyping.

In this case with complex rearrangements of chromosome 3 and 11 a novel translocation partner of the *MLL*-gene was detected. We have shown that the translocation partner was found on chromosome 11 with LDI-PCR. This technique revealed the *NRIP3* gene on 11p15 as a novel translocation partner of *MLL* in pediatric AML, while the 3' part of *MLL* was translocated to chromosome 3. The latter is thought not to be of importance since the reciprocal *MLL* translocations are often not expressed. Furthermore, it has been suggested that the *MLL* translocation partners are not randomly selected but that they are part of a protein network serving common functional processes. For example, interactions have already been described between AF4 and AF9 and ENL and AF4/AF10, which play a functional role in leukemogenesis[4-5]. So far, the function of *NRIP3* is not known, although it is one of the genes to be frequently hypermethylated in non-small cell lung cancer, hence it may potentially play a role in the pathogenesis of other cancers[6]. As this is the first case in which *NRIP3* is involved as a translocation partner for *MLL*, no conclusions can be drawn with respect to the clinical relevance and prognostic value. However, our patient is in continuous complete remission for more than 7-years.

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Chapter 8

Leukemia-associated *NF1* inactivation in patients with pediatric T-ALL and AML lacking evidence for neurofibromatosis

B.V. Balgobind[1,7], P. Van Vlierberghe[1,7], A.M.W. van den Ouweland[2], H.B. Beverloo[2], J.N.R. Terlouw-Kromosoeto[2], E.R. van Wering[3], D. Reinhardt[4], M. Horstmann[5], G.J.L. Kaspers[6], R. Pieters[1], C. M. Zwaan[1], M.M. Van den Heuvel-Eibrink[1,8] and J.P.P. Meijerink[1,8]

[1]Department of Pediatric Oncology/Hematology, Erasmus MC / Sophia Children's Hospital, Rotterdam, The Netherlands; [2]Department of Clinical Genetics, Erasmus MC, Rotterdam, The Netherlands; [3]Dutch Childhood Oncology Group (DCOG), The Hague, The Netherlands; [4]AML-BFM Study Group, Hannover, Germany; [5]German Co-operative study group for childhood acute lymphoblastic leukemia (COALL), Hamburg, Germany; [6]Department of Pediatric Oncology/Hematology, VU University Medical Center, Amsterdam, The Netherlands; [7]These authors contributed equally to this study; [8]These authors can be considered equally as last authors

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ABSTRACT

Neurofibromatosis type 1 (NF1) is an autosomal dominant genetic disorder caused by mutations in the *NF1* gene. Patients with NF1 have a higher risk to develop juvenile myelomonocytic leukemia (JMML) with a possible progression toward acute myeloid leukemia (AML). In an oligo array comparative genomic hybridization–based screening of 103 patients with pediatric T-cell acute lymphoblastic leukemia (T-ALL) and 71 patients with *MLL*-rearranged AML, a recurrent cryptic deletion, del(17)(q11.2), was identified in 3 patients with T-ALL and 2 patients with *MLL*-rearranged AML. This deletion has previously been described as a microdeletion of the *NF1* region in patients with NF1. However, our patients lacked clinical NF1 symptoms. Mutation analysis in 4 of these del(17)(q11.2)-positive patients revealed that mutations in the remaining *NF1* allele were present in 3 patients, confirming its role as a tumor-suppressor gene in cancer. In addition, *NF1* inactivation was confirmed at the RNA expression level in 3 patients tested. Since the NF1 protein is a negative regulator of the RAS pathway (RAS-GTPase activating protein), homozygous *NF1* inactivation represent a novel type I mutation in pediatric *MLL*-rearranged AML and T-ALL with a predicted frequency that is less than 10%. *NF1* inactivation may provide an additional proliferative signal toward the development of leukemia.

INTRODUCTION

Neurofibromatosis type 1 (NF1) is an autosomal genetic disorder that is clinically characterized by cafe-au-lait spots and frequent fibromatous tumors of the skin and tumors of the central nervous system. The NF1 disorder is caused by genetic heterozygous mutations in the *NF1* gene on chromosome 17q11.2. Most *NF1* mutations are intragenic and have been found over the complete gene. They comprise a diversity of mutation types, where splicing mutations are particularly prevalent given the number of exons. This result into truncation for a large percentage of cases, thereby inactivating the encoded protein neurofibromin[1]. Another genetic aberration includes microdeletions affecting the entire *NF1* locus. Patients with these *NF1* microdeletions display a more severe NF1 phenotype, characterized by mental retardation, facial dysmorphism, and increased risk for developing malignant tumors, including leukemias[2,3]. To this end, NF1 has also been associated with juvenile myelomonocytic leukemia (JMML), with a risk of progression towards acute myeloid leukemia (AML). These malignancies are associated with loss of the wild-type allele, either through deletions or the acquisition of point mutations. In JMML, it has also frequently been reported that the wild-type allele is replaced by the mutant allele as an effect of recombinational events leading to uniparental disomy (UPD)[4-6]. Previously, it was shown that bi-allelic inactivation of *NF1* are found as somatic abnormalities in JMML patients that lack clinical evidence of NF1[7]. Somatic inactivation of *Nf1* in hematopoietic cells results in a progressive myeloproliferative disorder in mice[8], confirming that *NF1* acts as a tumor suppressor gene[5]. The *NF1* gene protein product, neurofibromin, is a GTPase-activating protein (GAP) that inhibits RAS signaling by hydrolysis of active RAS-GTP into inactive RAS-GDP[1,9]. Therefore *NF1* deficiencies act as functional equivalents of activational mutations in *RAS*. Indeed, *NF1* inactivation and *RAS* mutations have been found in a mutually exclusive manner in JMML[7].

AML is a heterogeneous disease, in which early treatment response and cytogenetic abnormalities are the most important prognostic factors. In AML, genetic aberrations can be classified as type I or type II mutations. One hypothesis about the development of AML is the co-existence of both type I and type II mutations which confer proliferative signals (type I mutations affecting the *FLT3, KIT, NRAS, KRAS* or *PTPN11* genes) in combination with type II differentiation impairing mutations (such as *PML-RARA, AML-ETO, CBFB-MYH11* or *MLL*-rearrangements)[10].

MLL-rearrangements account for 8-20% of all cytogenetic abnormalities in pediatric AML[11,12]. *HOX*-genes are the prime targets of MLL fusion products and regulate cellular differentiation in normal hematopoietic development. However, Eguchi et al point to another role of MLL fusion products in *MLL*-rearranged leukemias through the alteration of cell-cycle arrest and apoptosis[13]. Most of these *MLL*-positive AML samples are morphologically classified as FAB-M4 and FAB-M5 and it has been suggested that *MLL*

rearrangements in pediatric AML are associated with a poor outcome. Interestingly, in some studies the t(9;11) subgroup has been associated with a higher sensitivity to different classes of drugs and a better prognosis[14-16]. In addition, many of these *MLL*-rearranged AML patients lack mutations in *FLT3, KIT, NRAS, KRAS* and *PTPN11*, indicating that the type I mutations remains to be elucidated. High-resolution genomic screening of *MLL* rearranged AML patients could provide us with further insight into novel genetic aberrations with prognostic significance or new type I mutations in *MLL*-rearranged AML.

T-cell acute lymphoblastic leukemia (T-ALL) represents about 15% of pediatric ALL cases and is characterized by a rapid progression of disease and a 30% relapse rate within the first 2 years after diagnosis[17]. In the last decade, a large number of new genomic aberrations were identified in T-ALL, including chromosomal translocations, deletions, amplifications, and mutations[18-20]. All these genetic defects target different cellular processes, including the cell-cycle, T-cell differentiation, proliferation, and survival. Cooperation of these genetic events initiates leukemic transformation of thymocytes[18]. *RAS* mutations have been found in less than 5% of patients with T-ALL, showing that proliferative hits affecting the RAS pathway remain rare[18]. On the other hand, more than 50% of the patients with T-ALL are characterized by activating mutations in the NOTCH1 pathway, including the *NOTCH1* gene itself[21,22] or the NOTCH1 regulating U3-ubiquitin ligase *FBXW7*[23,24].

In this study, we used oligo array-comparative genomic hybridization (array-CGH) and identified somatic *NF1* microdeletions as a cryptic genetic abnormality in children with T-ALL and children with *MLL*-rearranged AML that lack symptoms of neurofibromatosis. We present further evidence for the role of *NF1* inactivation as a functional equivalent to activated RAS signaling, and suggest that this can be considered as a new type I mutation in *MLL* rearranged AML and a proliferative hit in T-ALL.

MATERIAL AND METHODS

Patients

Viably frozen diagnostic bone marrow or peripheral blood samples from 103 patients with pediatric T-ALL patients and 71 patients with pediatric *MLL*-rearranged AML were provided by the Dutch Childhood Oncology Group (DCOG), the German Cooperative study group for Childhood Acute Lymphoblastic Leukemia (COALL) and the 'Berlin-Frankfurt-Münster' AML Study Group (AML-BFM-SG). Informed consent was obtained according to local law and regulations and in accordance with the Declaration of Helsinki. Leukemic cells were isolated and enriched from these samples as previously described[25]. All resulting samples contained 90% or more leukemic cells, as determined

morphologically by May-Grünwald-Giemsa (Merck, Darmstadt, Germany)-stained cytospins. These leukemic cells were used for DNA and RNA extraction, and a minimum of 5×10^6 leukemic cells were lysed in Trizol reagent (Gibco BRL, Life Technologies, Breda, The Netherlands) and stored at -80°C. Genomic DNA and total cellular RNA were isolated as described before[25]. From the patients with a deletion of *NF1,* remission and relapse material was only available for patient no. 2736.

Oligo array-CGH

Oligo array-CGH analysis was performed on the human genome CGH Microarray 44k-A (Agilent Technologies, Palo Alto, CA, USA) according to the manufacturer's protocol using a dye-swap experimental design to minimize false positive results, as previously described[25,26].

MLPA

Multiplex ligation-dependent probe amplification (MLPA) analysis was performed using the SALSA P081/082 MLPA assay (MRC Holland, Amsterdam, The Netherlands). SALSA P081/082 consists of 2 reaction mixes containing probes for all constitutive *NF1* exons except for exons 5, 7, 17, 19A, 45, and 47. The exact localization of the MLPA probes can be downloaded from the MRC Holland Web site[27]. The 2 reactions contain 15 and 13 control probes in other regions of the genome, respectively. The patients' samples were analyzed with MLPA according to the manufacturer's protocol[28,29]. Data were analyzed using GeneMarker v1.5 (Softgenetics, State College, PA, USA).

Mutation analysis

For the detection of *NF1* mutations, DNA was subjected to 40 cycles of polymerase chain reaction (PCR) of 15 minutes at 95°C and 1 minute at 60°C, using specific primers for all *NF1* exons, which are being used in *NF1* diagnostics (Department of Clinical Genetics, Erasmus MC, Rotterdam, The Netherlands, A.M.W.O. manuscript in preparation, primers are available on request at *a.vandenouweland@erasmusmc.nl*). *RAS*, *PTPN11* and *KIT* mutation screening was performed as described in Table S1. *NOTCH1* and *FLT3* mutational screening were done as previously described[21,30,31]. PCR products were purified by standard methods and directly sequenced from both strands. The sequence data were analyzed using Seqscape V2.5 (Applied Biosystems, Foster City, CA, USA).

***NF1* expression analysis**

NF1 expression was calculated based upon non-normalized gene expression array data, performed on the human genome U133 Plus 2.0 array (Affymetrix, Santa Clara, CA, USA), as previously described[32], which were available for 3 del(17)(q11.2)-positive and 7 del(17)(q11.2)-negative leukemia patients. For the *NF1* probe-sets, the expression was

normalized to the median expression of *GAPDH* (6 probe sets) for each patient sample. The difference in relative gene expression levels between patients with and without the del(17)(q11.2) was evaluated using the Mann-Whitney-U test.

RESULTS

High resolution genomic screening of a selected subgroup of 103 patients with pediatric T-ALL and 71 patients with pediatric *MLL*-rearranged AML using a 44K oligo array-CGH platform led to the identification of a cryptic deletion, del(17)(q11.2). This deletion was recurrently observed in 3 patients with T-ALL and 2 patients with AML (Figure 1A, B; Table 1). These deletions were about 1.2 Mb in size and covered the *NF1* gene. For all patients, the telomeric breakpoints were situated in the *JJAZ1* gene, whereas the centromeric breakpoints clustered in its pseudogene *JJAZ1P* (Figure 1C, D). The deletion area in these samples was equivalent to those observed in patients with NF1. Genetic and clinical patient characteristics for all del(17)(q11.2)-positive leukemia patients are summarized in Table 1. One of the 3 patients with T-ALL and at least one of the two patients with AML relapsed.

To further confirm the deletion breakpoints, 4 of 5 del(17)(q11.2)-positive leukemia patients and 15 del(17)(q11.2)-negative controls (7 with T-ALL and 8 with AML) were analyzed using an *NF1* locus-specific MLPA assay[28,29]. No residual material was available for patient no. 6421. These analyses confirmed that one copy of the *NF1* locus was lost in all these 4 cases (Figure 2, only T-ALL patient no. 2736 is shown); whereas all control patients retained both copies of the *NF1* gene (only AML control no. 3339 is shown).

In order to investigate complete *NF1* inactivation in our patients, we performed mutation analysis on all exons and exon/intron boundaries of the *NF1* gene in the 4 del(17)(q11.2)-positive patients with leukemia, and in an additional group of 39 patients without a deletion involving chromosomal band 17q11.2 (including 21 patients with *MLL*-rearranged AML and 18 patients with T-ALL). Small frameshift mutations disrupting the *NF1* coding region were only detected in 3 of 4 del(17)(q11.2)-positive patients (Table 1, Figure 3), leading to bi-allelic inactivation of NF1 in these patients. One patient with T-ALL and 2 patients with *MLL*-rearranged AML without a del(17)(q11.2) had a mono-allelic mutation in non-functional domains, possibly reflecting rare polymorphisms. Furthermore, *NF1* expression in the del(17)(q11.2)-positive patients with T-ALL and *MLL* rearranged AML leukemias was significantly lower in 3 patients tested, as compared with 7 T-ALL and AML patient samples that are wild-type for *NF1* (Figure 4).

To further verify a somatic rather than a genetic origin of *NF1* inactivation, we screened relapse and remission material of T-ALL patient no. 2736, for whom material was

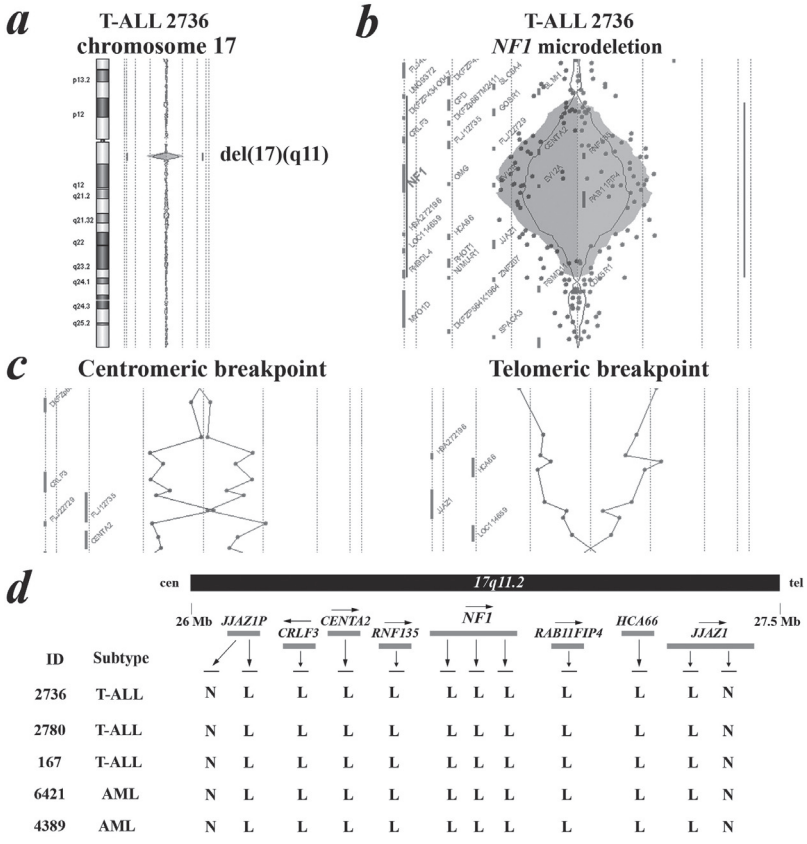

Figure 1. *NF1* microdeletions in pediatric acute leukemias.

(a) Chromosome 17 ideogram and corresponding oligo array-CGH plotof patient DNA/control DNA ratios (blue tracing) versus the dye-swap experiment (red tracing) for T-ALL patient no. 2736.

(b) Detailed visualization of the *NF1* microdeletion at chromosomal band 17q11 in T-ALL patient no. 2736. Hybridization signals around the –2X or +2X lines represent loss of the corresponding region in the patient DNA.

(c) Detailed analysis of the centromeric(left panel) and telomeric (right panel) breakpoint of the *NF1* microdeletion in patient no. 2736.

(d) Overview of oligo array-CGH results in the chromosomal region 17q11.2 for 3 patients with T-ALL and 2 patients with AML with del(17)(q11.2). The 60-mer oligos present on the DNA array and located in this genomic area, as well as the specific genes located in this region with their transcription direction, are shown. Arrows above the indicated genes represent the direction of transcription

Abbreviations: N; normal, L; loss, cen: centromere, tel: telomere.

available. At relapse, the *NF1* microdeletion and *NF1* mutation on the other allele were present, while in the remission sample both mutations remained undetected.

Since *NF1* deficiency could act as a novel type I mutation, we screened all del(17)(q11.2)-positive patients with leukemia for activational mutations in *RAS*. Although *NRAS* or *KRAS* mutations have been described in *MLL*-rearranged AML and T-ALL, no somatic *NRAS* or *KRAS* mutations were found in our 5 del(17)(q11.2)-positive patients with

Table 1. Patient characteristics and truncating *NF1* mutations in pediatric T-ALL and AML

ID	Subtype	Sex	Age (yrs.)	WBC (x 10^9 / l)	Relapse CCR, (# months)	Genetic Subtype	Karyotype	***NF1*** del	***NF1*** Mutation analysis		***Additional mutation analysis***			
									Nucleotide level		*FLT3-ITD or D835 PM*	*C-KIT*	*RAS*	*PTPN11*
									exon	mutation				
2736	T-ALL	M	10.1	13	Relapse, 15	*CALM-AF10*	46,XY,t(2;9)(q21;q34),?t(8,8)(?q22;q?24)	yes	22	c.3734delCinsGGTTTATGGTTT	WT	WT	WT	WT
2736 Remission							46,XY	no		WT	WT	WT	WT	WT
2736 Relapse							46,XY,t(2;9)(q21;q34),?t(8,8)(?q22;q?24)	yes	22	c.3734delCinsGGTTTATGGTTT	WT	WT	WT	WT
2780	T-ALL	F	5	140	CCR, 50+	*TLX3*	46,XX	yes	4	c.333dupA	WT	WT	WT	WT
167	T-ALL	M	16.4	170.9	CCR, 56+	Unknown	NA	yes		WT	WT	WT	WT	WT
4389	AML	F	18.8	41.7	NA	*MLL-AF9*	46,XX,t(9;11)(p22;q23)[4]/46,XX [7]	yes	16	c.2849_2850insTT	WT	WT	WT	WT
6421	AML	F	6.5	NA	Relapse, 68	*MLL-AF10*	47,XX,t(10;11)(p13;q23),+19	yes		ND	WT	WT	WT	WT

WBC: White blood cell count; CCR: continued complete remission; WT: Wild-type; ND: Not determined; NA: Not available PM: Point-mutation

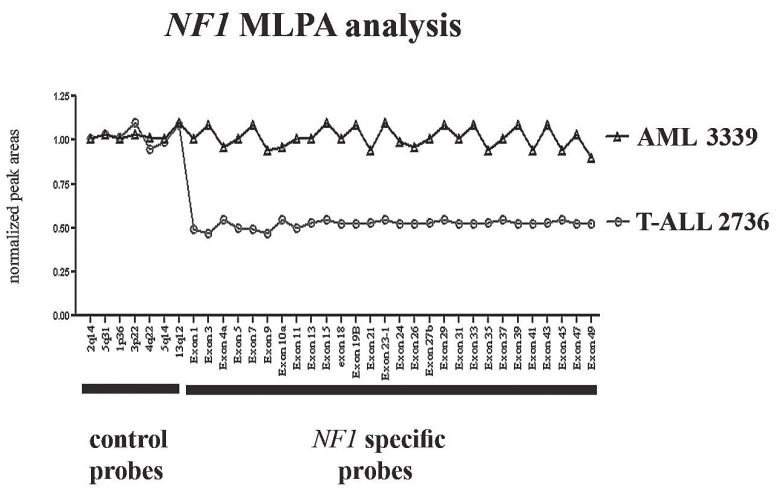

Figure 2. MLPA analysis of *NF1* in selected cases.
MLPA analysis of the *NF1* locus in T-ALL patient no. 2736 and AML patient no.3339. Normalized peak areas around 0.5 represent monoallelic loss of the corresponding genomic region.

leukemia. In addition, both *MLL*-rearranged AML patients with a *NF1* deletion lacked other type I mutations in *FLT3*, *KIT* or *PTPN11* in their leukemic cells**.** The frequency of these mutations in the 71 *MLL*-rearranged AML samples was low, as expected. Only 35% had one of these mutations, and all these mutations were mutually exclusive. Furthermore, the del(17)(q11.2)-positive patients with T-ALL were screened for rearrangements at the *TAL1*, *HOX11L2*, *HOX11*, *CALM-AF10*, *MLL* and *cMYC* loci or the presence of *NOTCH1* mutations. One patient (no. 167) lacked rearrangements of any of the loci mentioned above, whereas a *HOX11L2* translocation (no. 2780) and a *CALM-AF10* fusion gene (no. 2736) were detected in 2 other cases. *NOTCH1* mutations were identified in patients no. 2780 (heterodimerization domain; L1601P) and no. 167 (PEST domain; 2445insLL).

DISCUSSION

Genetic events that lead to leukemogenesis by activating uncontrolled cell proliferation remain to be elucidated in most patients with pediatric T-ALL and *MLL* rearranged AML. We used oligo array-CGH to identify new abnormalities and found somatic *NF1* microdeletions as a cryptic genetic abnormality in patients lacking clinical symptoms of neurofibromatosis. This array-CGH study is currently expanded to other subtypes of leukemias. Recent single nucleotide polymorphism (SNP) array analysis of pediatric ALL by investigators from St Jude Children's Research Hospital showed that this microdeletion in *NF1* may be present at low frequencies in other types of acute leukemia as well.[33]

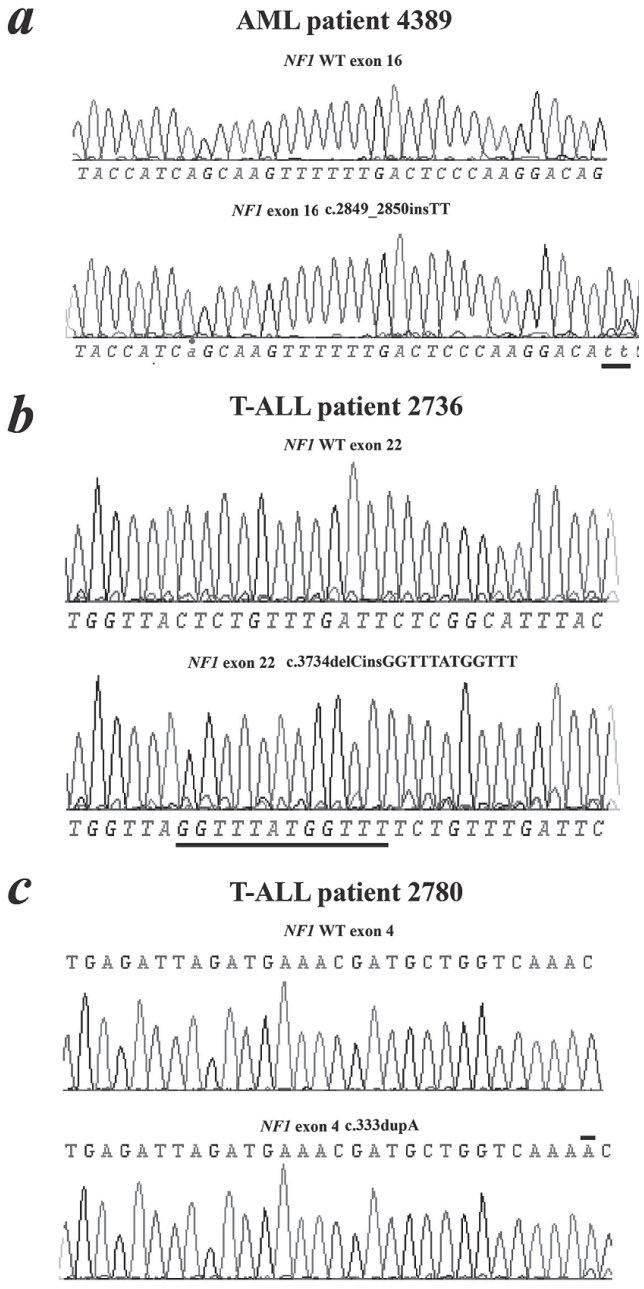

Figure 3. Truncating *NF1* mutations in pediatric T-ALL and AML.

(a) Sequence analysis of patient no. 4389 (AML) showing a *c.2849_2850insTT* mutation in the remaining *NF1* allele

(b) Sequence analysis of patient no. 2736 (T-ALL) showing a *c.3734delCinsGGTTTATGGTTT* mutation in the remaining *NF1* allele

(c) Sequence analysis of patient no. 2780 (T-ALL) showing a *c.333dupA* mutation in the remaining *NF1* allele

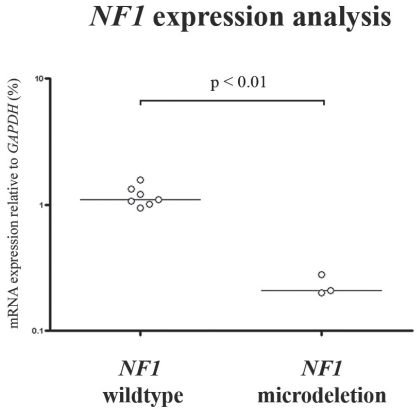

Figure 4. *NF1* expression analysis in pediatric T-ALL and *MLL*-rearranged AML.
NF1 mRNA expression data relative to *GAPDH* (%), based upon gene expression array data, which were available for 3 del(17)(q11.2)-positive (patient no. 2736, no. 2780 and no. 167) and 7 del(17)(q11.2)-negative patients with leukemia.

NF1 microdeletions are observed in about 5% to 20% of patients with NF1[1]. The majority of these patients with NF1 have a 1.4 Mb *NF1* microdeletion due to interchromosomal homologous recombination between the low-copy repeats of the *WI-12393* gene flanking *NF1* and sequences with homology to chromosome 19 during meiosis[34]. A second type of *NF1* microdeletions of about 1.2 Mb in size is due to a mitotic intrachromosomal recombination between the *JJAZ1* and the homologous *JJAZ1P* pseudogene[35,36]. The *NF1* microdeletions in our leukemia patients seemed identical to this 1.2Mb microdeletion type[34]. However, in contrast to NF1 patients with similar *NF1* deletions, our leukemia patients did not meet the clinical criteria for NF1, lacking cafe-au-lait spots, mental retardation, and/or facial dysmorphism. This suggests that the *NF1* deletion in our patients is somatic and leukemia specific, rather than of constitutional genetic origin, although molecular diagnostics for NF1 was not performed in these patients.

Deletion of one allele of *NF1* and further inactivation of the other *NF1* allele in 3 patients through the acquisition of point mutations further confirms the role of *NF1* as a tumor-suppressor gene in the pathogenesis of both pediatric *MLL* rearranged AML and T-ALL. This point was further strengthened by the finding of clonal stability in one of the del(17)(q11.2) patients, where the deletion of *NF1* on one allele and the point-mutation in the other *NF1* allele were both present at diagnosis and relapse while absent in the remission sample. Therefore, the *NF1* abnormalities were of somatic origin in at least patient no. 2736 and were only present in the leukemic cells. Similar findings have been described for JMML patients[7], explaining why these patients did not have any clinical symptoms of neurofibromatosis.

Since *NF1* deficiency leads to the activation of the RAS signaling pathway[9], and none of the del(17)-positive patients with leukemia had mutations in *NRAS* or *KRAS*, *NF1* microdeletions

presumably provide an alternative mechanism for RAS activation in both *MLL*-rearranged myeloid and T-lymphoid leukemias, thereby representing a novel type I abnormality. These leukemia patients may potentially benefit from additional treatment with RAS inhibitors like farnesylthiosalicylic acid[37] or downstream inhibitors.

Both del(17)-positive patients with AML were further screened for any of the other currently known type I mutations in AML. As expected, no other type I mutations were detected, indicating that *NF1* microdeletions could act as a novel type I mutation which cooperate with the *MLL* translocation (type II mutation) in the pathogenesis of AML.

The idea of a multi-step pathogenesis in T-cell leukemia is widely accepted[18-20,38]. Cooperative genetic events affect cell-cycle, T-cell differentiation, proliferation, and survival. We identified a number of cooperative aberrations in the del(17)(p11.2)-positive T-ALL samples. *NOTCH1* mutations, generally present in about 50% of T-ALL[21], were identified in 2 out of 3 del(17)(p11.2)-positive T-ALL samples. In addition, genetic aberrations that induce a T-cell differentiation arrest were identified in patient no. 2780 (*HOX11L2* translocation) and patient no. 2736 (*CALM-AF10* translocation). These data further suggests that loss of *NF1* can be involved in the development of T-ALL, as one of the genetic hits in multistep oncogenesis.

In this study, we identified 3 patients with a deletion of *NF1* and an inactivational mutation on the remaining allele. We could not identify homozygous somatic *NF1* mutations in 21 patients with *MLL*-rearranged AML and 18 patients with T-ALL without a microdeletion. This suggests that the frequency of bi-allelic inactivation, until now the only mechanism described for oncogenesis, is less than 10% in these groups.

Other mechanisms of NF1 inactivation, such as inactivation through the duplication of the mutated (UPD) *NF1* allele at the expense of the remaining wild-type allele, as observed in NF1 patients with JMML, may have been missed[6]. Of interest, recent SNP array analysis of pediatric ALL, and JMML without underlying NF1, showed that there was no UPD involved in the *NF1* region[6,33]. In addition, in adult AML approximately 20% have large regions of UPD, but none of them involves the *NF1* locus[39,40]. Hence, UPD of the NF1 locus may be a rare event in leukemias of somatic origin compared to leukemias which originate from patients with clinical evidence of NF1. Therefore, the frequency of bi-allelic *NF1* inactivation in pediatric *MLL*-rearranged AML and T-ALL as we reported here may be underestimated. Future studies should be extended by sequencing the *NF1* locus, including the promoter region and the 3' untranslated region (UTR) and look for abnormalities in NF1 protein expression.

In conclusion, we report the identification of *NF1* microdeletions in patients with pediatric T-ALL and *MLL* rearranged AML without clinical evidence of NF1. We confirmed *NF1* inactivation by reduced *NF1* expression levels, and bi-allelic *NF1* mutations in 3 out of 5 patients, confirming the role of *NF1* as a tumor suppressor gene in cancer. *NF1* inactivation is a novel type I mutation in *MLL* rearranged AML and a new proliferative hit in T-ALL.

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POTENZA
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MONTE CARLO

Chapter 9

High *BRE* expression in pediatric *MLL*-rearranged AML is associated with favorable outcome

B.V. Balgobind[1], C.M. Zwaan[1], D. Reinhardt[2], T.J.C.M. Arentsen-Peters[1], I.H.I.M. Hollink[1], V. de Haas[3], G.J.L. Kaspers[4], E.S.J.M. de Bont[5], A. Baruchel[6], J. Stary[7], C. Meyer[8], R. Marschalek[8], U. Creutzig[9], M.L. de Boer[1], R. Pieters[1], and M.M. Van den Heuvel-Eibrink[1].

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

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ABSTRACT

Translocations involving the *MLL*-gene, localized at 11q23, frequently occur in pediatric acute myeloid leukemia (AML). We recently reported differences in prognosis between the different translocation partners, suggesting differences in biological background. To unravel the latter, we used microarrays to generate gene expression profiles of 245 pediatric AML cases, including 53 *MLL*-rearranged cases. Thereby, we identified a specific gene expression signature for t(9;11)(p22;q23), and identified *BRE (brain and reproductive organ-expressed)* to be discriminative for t(9;11)(p22;q23) ($p<0.001$) when compared with other *MLL*-subtypes. Patients with high *BRE* expression showed a significantly better 3-year relapse free survival (pRFS) (80±13 vs. 30±10%, $p=0.02$) within *MLL*-rearranged AML cases. Moreover, multivariate analysis identified high *BRE* expression as an independent favorable prognostic factor within pediatric AML for RFS (HR=0.2, $p=0.04$). No significant differences were identified for 3-year event free survival nor for 3-year overall survival. Forced expression of *BRE* did not result in altered cell proliferation, apoptosis or drug sensitivity, which could explain the favorable outcome. In conclusion, overexpression of the *BRE* gene is predominantly found in *MLL*-rearranged AML with t(9;11)(p22;q23). Although further investigation for the role of BRE in leukemogenesis and outcome is warranted, high *BRE* expression is an independent prognostic factor for pRFS in pediatric AML.

INTRODUCTION

Acute myeloid leukemia (AML) is a heterogeneous disease. Currently, initial response to therapy and cytogenetic abnormalities are the main prognostic factors[1]. Translocations involving chromosome 11q23 comprise 15% to 20% of all pediatric AML cases. In more than 95% of the cases with 11q23 rearrangements, the *mixed lineage leukemia* (*MLL*) gene is involved. The heterogeneity of *MLL*-rearranged AML is reflected by the identification of more than 60 different fusion partners of this gene[2]. In AML, the most common 11q23 rearrangements are t(9;11)(p22;q23)(MLL-AF9) (approximately 50% of cases), t(11;19)(q23;p13.1)(MLL-ENL), t(11;19)(q23;p13.3)(MLL-ELL), t(6;11)(q27;q23)(MLL-AF6), and t(10;11)(p12;q23)(MLL-AF10)[3-4]. Most AML samples with 11q23 rearrangements are morphologically classified as FAB (French-American British morphology classification)-M4 or FAB-M5[5].

We recently showed by a large retrospective international collaborative study that t(1;11)(q21;q23)(MLL-AF1q) was associated with a favorable outcome, whereas t(10;11)(p12;q23), t(10;11)(p11.2,q23)(MLL-ABI1), or t(6;11)(q27;q23) were associated with a poor outcome[6]. In some studies, t(9;11)(p22;q23) had been associated with a better prognosis, which may at least partially be due to enhanced sensitivity to different drugs[7-10]. We found that within the t(9;11)(p22;q23) cases, prognosis was related to the cell-type from which the leukemia originated, as patients with FAB-M5 showed a significantly better outcome than those with other FAB-types[6]. These outcome differences between the various translocation partners may point at differences in biological background.

In gene expression profiling studies in *MLL*-rearranged AML and acute lymphoblastic leukemia (ALL), *MLL*-rearranged cases clustered together as a single entity[11-14]. However Stam *et al.* showed that within *MLL*-rearranged infant ALL each type of *MLL* translocation is associated with a translocation-specific gene expression signature[15]. In the present study, we performed a supervised analysis of gene expression profiles in a large cohort of pediatric AML cases (n=245) to identify and analyze differentially expressed genes between the various *MLL*-rearranged AML cases stratified by translocation partners, to elucidate potential genes of interest which are related to the observed differences in outcome. This led to the identification of high expression of a *novel* gene of interest related to t(9;11)(p22;q23), i.e. *BRE (brain and reproductive organ-expressed)*, which is further described in this paper.

MATERIAL AND METHODS

Patients

Viably frozen diagnostic bone marrow or peripheral blood samples from 237 *de novo* and 8 secondary pediatric AML patients, including 53 pediatric *MLL*-rearranged 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. Informed consent was obtained after Institutional Review Board approval according to local law and regulations. Each study group performed central morphology review. In addition, the collaborative study groups also provided data on the clinical follow-up of these patients. Survival analysis was restricted to the subset of 205 pediatric AML patients who were treated according to the BFM and Dutch pediatric AML protocols (studies AML-BFM 98, AML-BFM 04, DCOG-BFM 87, DCOG 92/94 and DCOG 97). Details of the treatment protocols included in the survival analysis and overall outcome data have been previously published, with the exception of study AML-BFM 2004, which is ongoing[16-18].

Leukemic cells were isolated by sucrose density centrifugation and non-leukemic cells were eliminated as previously described[19]. All processed samples contained more than 80% leukemic cells, as determined morphologically using cytospins stained with May-Grünwald-Giemsa (Merck, Darmstadt, Germany). Subsequently, a minimum of $5x10^6$ leukemic cells were lysed in Trizol reagent (Gibco BRL, Life Technologies, Breda, The Netherlands). Genomic DNA and total RNA were isolated according to manufacturer's protocol, with minor modifications[20].

Cytogenetic and molecular analysis

Leukemic samples were routinely investigated for *MLL*-rearrangements by standard chromosome-banding analysis and/or fluorescent *in situ* hybridization. If necessary, RT-PCR was performed for the common translocations *MLL-AF9, MLL-AF10, MLL-AF6, MLL-ENL* and *MLL-ELL* (Primers are described in Supplementary Table S1). Of the 53 cases, 21 harbored a t(9;11)(p22;q23), 16 a t(10;11)(p12;q23) and 5 a t(6;11)(q27;q23). The remaining 11 cases were confirmed with LDI-PCR to have another translocation partner and were considered as *MLL*-others[21]. *NPM1, CEBPA, WT1, NRAS*, *KRAS*, *PTPN11*, *KIT*, *FLT3* hotspot mutational screening was performed as previously described[22-26]. Overexpression of *EVI1* was previously established by gene expression profiling and real-time quantitative (RQ)-PCR[27].

Microarray-based gene expression profiling

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 pro-

cessed 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 2.2.0[28-29].

To find a gene expression signature characteristic for the t(9;11)(p22;q23) group, which were mostly FAB-M5 cases, an empirical Bayes linear regression model was used to compare samples from this group to all other samples[30]. Moderated T-statistics p-values were corrected for multiple testing using the False Discovery Rate (FDR) method defined by Benjamini and Hochberg.

RQ-PCR for *BRE*

In 45 of the 53 *MLL*-rearranged AML samples, the RNA expression could be validated by real-time quantitative RT-PCR (RQ-PCR). The relative expression of *BRE* was calculated using the comparative cycle time (ΔCt) method, with GAPDH as the house-keeping gene[31]. Primer and probe sequences are described in Supplementary Table S1.

Protein extraction and Western blot analysis

In 11 of the 53 *MLL*-rearranged AML samples material for protein extraction and Western blot analysis was available. Western blots were probed with mouse anti-BRE (kindly provided by Dr. Y.L. Chui[32]). Further detail on protein extraction and Western blot is described in the Supplementary Material & Methods.

Cell culture, transfection and cell-cycle proliferation

Different cell lines with an *MLL*-rearrangement, i.e. Monomac-1, THP1, NOMO1, MV4;11 and ML2 (DSMZ, Braunschweig, Germany), were tested for *BRE*-expression. However, none of them showed overexpression of *BRE*. Since a cell line model was not available the Monomac-1 cell line was transfected to overexpress *BRE* and cell viability, transfection efficiency and cell-cycle proliferation were measured as described in the Supplementary Material & Methods.

In vitro drug resistance assay

In vitro drug resistance for the different cytostatic agents as described in the Supplementary Material & Methods was determined using the 4-day 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously[33].

Additional statistical analysis

Statistical analysis was performed with SPSS 15.0 (SPSS Inc. Chicago, IL, USA). Different variables were compared with the χ^2 test, *t*-statistics test or the Mann-Whitney-U test.

Probabilities of overall survival (pOS), event-free survival (pEFS, events: no CR, relapse, secondary malignancy, death from any cause) and relapse free survival (pRFS, events: relapse) were estimated by the method of Kaplan and Meier. The Cox Proportional hazards model analysis was applied to determine the association of overexpression of *BRE* with pOS, pEFS, and pRFS adjusted for prognostic factors as described for pediatric AML [white blood cell count (WBC) >50x10^9/l, age >10 years, favorable karyotype, i.e. t(8;21), inv(16) and t(15;17), and t(9;11)(p22;q23)]. All tests were two-tailed and a p-value of less than 0.05 was considered significant.

RESULTS

High *BRE* expression in t(9;11)(p22;q23)

From the microarray-based gene expression profiles of 245 pediatric AML cases, the 21 t(9;11)(p22;q23) cases were compared with the 32 other *MLL*-rearranged cases, and a specific gene expression signature for t(9;11)(p22;q23) was identified (Figure 1). Interestingly, 5 of the top 50 most discriminative probe sets for the t(9;11)(p22;q23) group were probe sets for the *BRE*-gene (Supplementary Table S2). The VSN-normalized mean average intensity of 3 of these probe sets (205550_s_at, 211566_x_at and 212645_x_at) was 3.7-fold higher for t(9;11)(p22;q23) compared with the other *MLL*-rearranged AML cases ($p<0.001$) (Figure 2A). In the total cohort of pediatric AML cases (n=245) high expression of BRE was only identified in *MLL*-rearranged AML cases, and in 1 infant AML harboring a t(8;16)(p11;p13) (Figure 2B).

The gene expression data were validated with RQ-PCR in 45/53 *MLL*-rearranged AML cases. A correlation between the log-transformed gene expression profiling data and the log-transformed RQ-PCR data was found ($r^2=0.6$, $p<0.001$) (Supplementary Figure S1). The median relative *BRE* expression to *GAPDH* for t(9;11)(p22;q23) was 56% compared with 8% for the other *MLL*-rearranged AML cases ($p<0.001$) (Figure 2C).

In a previous pediatric AML study by Ross *et al.*, gene expression profiling was performed with the Affymetrix Human Genome U133A microarray, which had one probe set representing the *BRE* gene. We re-analyzed their *MLL*-rearranged AML cases (n=23) for validation purposes and confirmed that high *BRE* expression was predominantly found in the t(9;11)(p22;q23) cases (Supplementary Figure S2).

Clinical and genetic characteristics of patients with high *BRE* expression

Based on the VSN-normalized mean average intensity (712 A.U.) of the 3 most significant differentially expressed probe sets within the *MLL*-rearranged AML cases, a distinction was made into a group with high *BRE* expression (> 712 A.U.) and a group with low expression (< 712 A.U.).

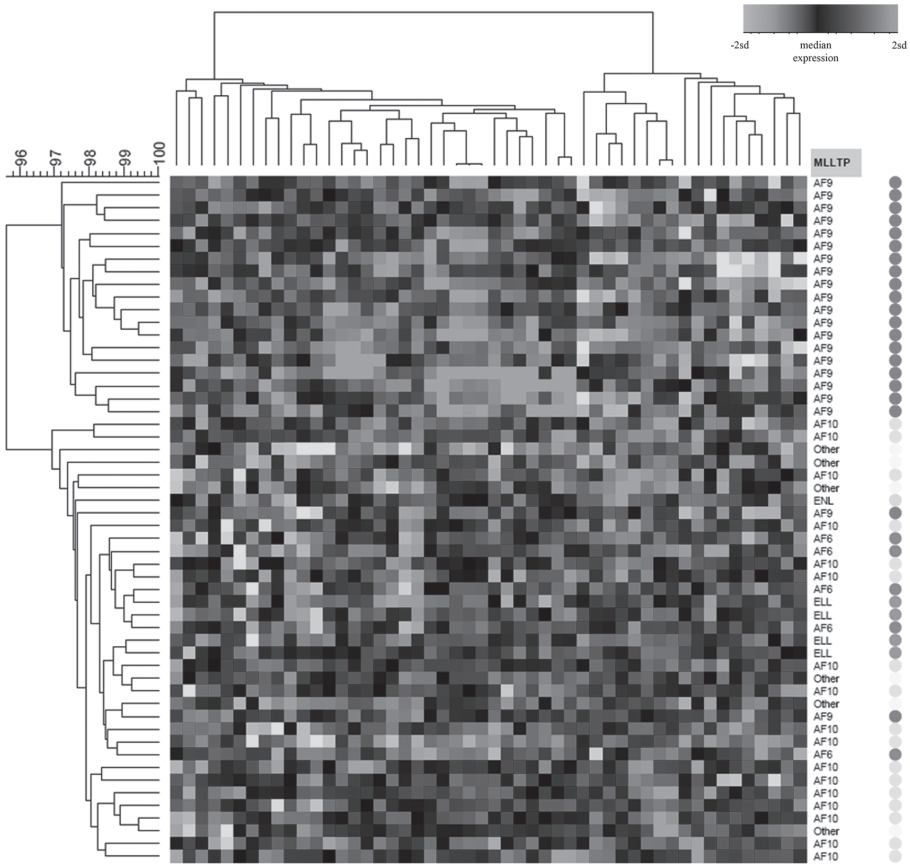

Figure 1: Hierarchical clustering based on the top 50 most discriminative genes for t(9;11)(p22;q23). Hierarchical clustering of 53 *MLL*-rearranged AML cases based on the top 50 most discriminative genes showed for t(9;11)(p22;q23) after supervised analysis (Supplementary Table S2).

Within the total cohort of pediatric AML, high *BRE* expression was found in 19 patients, including 18/53 with *MLL*-rearranged AML, and one case with a t(8;16)(p11;p13). Seventeen of the 19 cases with high *BRE* expression were FAB-M5. The remaining two cases were FAB-M4 and an unknown FAB type, respectively ($p<0.001$). In addition, patients with high *BRE* expression were significantly younger than those with low *BRE* expression (2.8 vs. 9.8 years, $p=0.007$). None of the cases with high *BRE* expression had a *FLT3*-ITD ($p=0.03$) (Table 1A). However, the differences in morphology, age and *FLT3*-ITD were determined by the significantly high frequency of *MLL*-rearranged AML in patients with high *BRE* expression.

Within the *MLL*-rearranged cases, the group with high *BRE* expression consisted of 15/21 with t(9;11)(p22;q23) and 3/16 with t(10;11)(p12;q23). No differences for age, sex, white blood cell count (WBC), and FAB morphology nor for mutations in *WT1*, *FLT3*-ITD,

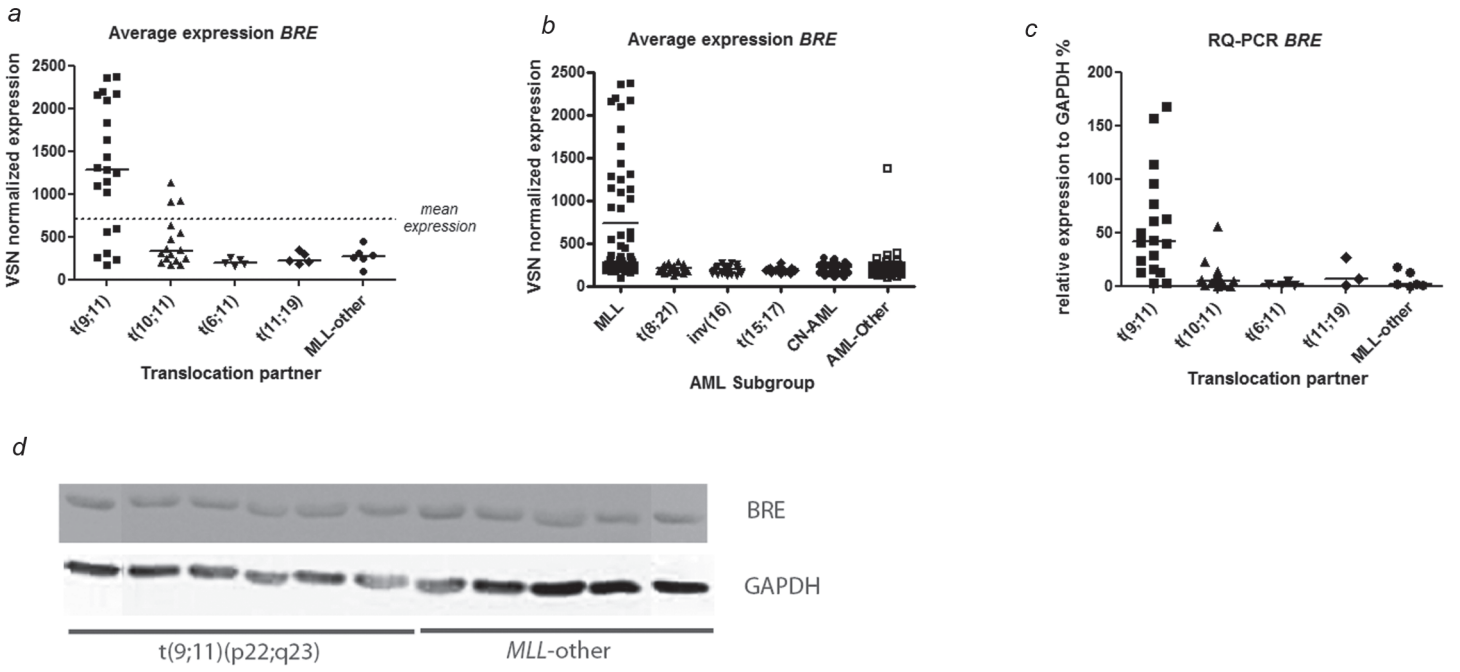

Figure 2: Expression of *BRE* in pediatric AML. *BRE*+ was predominantly found in t(9;11)(p22;q23). Using gene expression profiling, 18 *MLL*-rearranged AML cases showed an expression higher than the mean average expression and were considered *BRE*+ (A). In the remaining cohort of 245 pediatric AML cases also one case with a t(8;16)(p11;p13) showed high expression of BRE (B). RQ-PCR confirmed the gene expression data (C). Protein analysis in 10 *MLL*-rearranged AML cases did not show differences in expression (D).

Table 1A. Clinical characteristics of patients with high expression of BRE within pediatric AML.

	No. of BRE- patients (%)	No. of BRE+ patients (%)	*p-value*
Sex (N=245)			*0.822* [#]
male	125 (55)	10 (53)	
female	101 (45)	9 (47)	
Age (median, range, years, N=245)	9.8 (0-18.0)	2.8 (0.3-17.7)	***0.007*** [§]
WBC x 10^9/L (median, range, N=219)	40.2 (0.0-483)	57.6 (5.9-469)	*0.077* [§]
FAB (N=245)			***<0.001*** [#]
M0	14 (6)	0 (0)	
M1	28 (12)	0 (0)	
M2	54 (24)	0 (0)	
M3	20 (9)	0 (0)	
M4	55 (24)	1 (5)	
M5	36 (16)	17 (90)	
M6	3 (1)	0 (0)	
M7	8 (4)	0 (0)	
other/unknown	8 (4)	1 (5)	
Cytogenetics (N=245)			***<0.001*** [#]
***MLL*-rearrangements**	35 (16)	18 (95)	
t(8;21)	28 (12)	0 (0)	
inv(16)	27 (12)	0 (0)	
t(15;17)	18 (8)	0 (0)	
CN-AML	41 (18)	0 (0)	
AML-other/unknown	77 (34)	1 (5)*	
***EVI1*-overexpression (N=245)**	30 (13)	1 (5)	*0.482* [#]
***WT1* mutations (N=238)**	21 (10)	1 (5)	*1.000* [#]
***CEBPA* mutations (N=236)**	16 (7)	0 (0)	*0.376* [#]
***NPM1* mutations (N=240)**	17 (8)	0 (0)	*0.374* [#]
***FLT3-ITD* (N=245)**	46 (20)	0 (0)	***0.029*** [#]
***CKIT* mutations (N=245)**	17 (8)	1 (5)	*1.000* [#]
***N/K-RAS* mutations (N=243)**	45 (20)	3 (16)	*0.773* [#]
***PTPN11* mutations (N=243)**	3 (1)	1 (5)	*0.279* [#]

*This case harbored a t(8;16)(p11;p13)

[#] Chi-square test

[§] Mann-Whitney-U test

KIT, *N/K-RAS* were found between cases with high and low *BRE* expression. In contrast, cases with high *BRE* expression showed an inverse correlation with *EVI1* overexpression (p=0.02) (Table 1B). In fact, 4 of the 5 patients with *EVI1* overexpression and a t(9;11)(p22;q23) had the lowest expression of *BRE*. Within the patients with a t(9;11)(p22;q23), 13/15 patients with high *BRE* expression had a FAB-M5, which was not significantly different compared to those with low *BRE* expression (p=0.31). Within the patients with

Table 1B. Clinical characteristics of patients with high expression of BRE within *MLL*-rearranged AML.

	No. of BRE- patients (%)	No. of BRE+ patients (%)	*p-value*
Sex (N=53)			*0.268* [#]
male	23 (66)	9 (50)	
female	12 (34)	9 (50)	
Age (median, range, years, N=53)	5.8 (0-18.0)	2.8 (0.3-17.7)	*0.778* [§]
WBC x 10^9/L (median, range, N=49)	68.3 (1.1-443)	42.3 (5.9-469)	*0.737* [§]
FAB			*0.192* [#]
M0	3 (9)	0 (0)	
M1	2 (6)	0 (0)	
M2	1 (3)	0 (0)	
M3	0 (0)	0 (0)	
M4	6 (17)	1 (6)	
M5	21 (60)	16 (89)	
M6	0 (0)	0 (0)	
M7	2 (6)	0 (0)	
other/unknown	0 (0)	1 (6)	
***MLL*-rearrangement**			***<0.001*** [#]
t(9;11)(p22;q23)	6 (17)	15 (83)	
t(10;11)(p12;q23)	13 (37)	3 (17)	
t(6;11)(q27;q23)	5 (14)	0 (0)	
t(11;19)(q23;p13)	5 (14)	0 (0)	
***MLL*-other**	6 (17)	0 (0)	
***EVI1*-overexpression (N=53)**	13 (37)	1 (6)	***0.020*** [#]
***WT1* mutations (N=52)**	1 (3)	1 (6)	*1.000* [#]
***FLT3-ITD* (N=53)**	2 (0)	0 (0)	*0.543* [#]
***CKIT* mutations (N=52)**	0 (0)	1 (6)	*0.346* [#]
***N/K-RAS* mutations (N=52)**	11 (32)	3 (17)	*0.329* [#]
***PTPN11* mutations (N=53)**	0 (0)	1 (6)	*0.340* [#]

[#] Chi-square test
[§] Mann-Whitney-U test

a t(10;11)(p12;q23), numbers were too small to identify significant correlations between cases with high and low *BRE* expression.

Favorable disease free survival in pediatric AML patients with high *BRE* expression

Within the total cohort of pediatric AML, follow-up data were available for 205 patients, including 17 cases with high *BRE* expression. No differences were found for 3y-pEFS between cases with high and low *BRE* expression (53±12% vs. 43±4%, p=0.61) and for 3y-pOS (64±12 vs. 62±4%, p=0.77). However, the cases with high *BRE* expression had a higher 3 years pRFS as compared to cases with low expression (82±12 vs. 52±4%, p=0.01) (Figure 3A).

Within the *MLL*-rearranged cases, high *BRE* expression suggested a better 3y-pEFS compared to those with a low BRE expression (50±13 vs. 21±8%, p=0.12) and a better 3y-pOS (62±13 vs. 32±9%, p=0.19), but the results were not statistically significant. However, again cases with high BRE expression had a significantly better 3y-pDFS compared to cases with low expression (80±13 vs. 32±11%, p=0.03) was found (Figure 3B).

Multivariate analysis within the total cohort of pediatric AML, including prognostic factors such as age, WBC favorable karyotype and t(9;11)(p22;q23), showed that next to favorable karyotype, high *BRE* expression was an independent favorable prognostic factor for RFS (HR 0.2, p=0.04), but not for EFS (HR 0.4, p=0.06) and OS (HR 0.5, p=0.29) (Table 2).

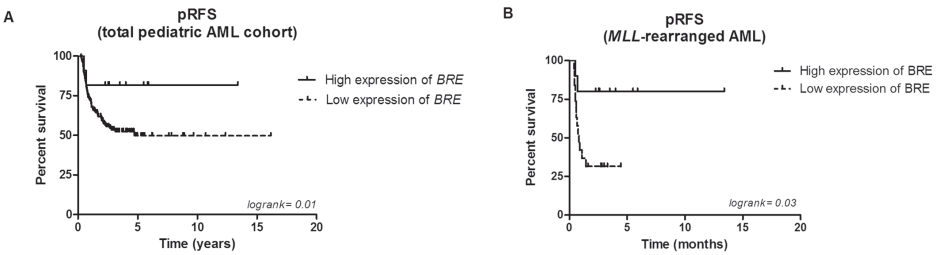

Figure 3: Relapse free survival outcome for *BRE* expression in pediatric AML. Survival analysis showed that high expression of *BRE* was related to a better 3-y pRFS in all pediatric AML cases (A), and in the *MLL*-rearranged AML cases (B).

Functional assays after transfection

Functional studies were performed to find an explanation for the association of high *BRE* expression and favorable outcome. To explore a possible role for BRE in cell proliferation, the Monomac-1 cell line, harboring a t(9;11)(p22;q23), was transiently transfected with pLNCX-BRE by means of electroporation. However, a maximum transfection efficiency of only 25-30% was achieved with pLNCX-EGFP. At different time points an increased expression of BRE on mRNA was measured (Supplementary Figure 3A), but not evi-

Table 2. Multivariate analysis for event free survival (EFS), relapse free survival (RFS) and overall survival (OS).

	EFS			RFS			OS		
	HR	**(95% CI)**	**p-value**	**HR**	**(95% CI)**	**p-value**	**HR**	**(95% CI)**	**p-value**
***BRE*+**	0.4	(0.1-1.0)	0.064	0.2	(0.0-0.9)	**0.036**	0.5	(0.2-1.7)	0.287
t(9;11) (q22;q23)	1.6	(0.6-4.2)	0.358	1.6	(0.4-6.6)	0.527	1.6	(0.5-4.7)	0.417
Favorable karyotype[1]	0.3	(0.2-0.6)	**<0.001**	0.3	(0.2-0.6)	**<0.001**	0.2	(0.1-0.5)	**<0.001**
WBC >50[¶]	1.0	(0.6-1.7)	0.890	0.9	(0.5-1.7)	0.858	1.1	(0.6-2.1)	0.659
Age >10[§]	0.9	(0.6-1.3)	0.578	0.8	(0.5-1.2)	0.264	0.9	(0.5-1.4)	0.545

[1]including t(8;21), inv(16) and t(15;17)
[¶] WBC= White blood cell count above 50 x 10^9/l
[§] Children older than 10 years

dently on protein level (Supplementary Figure 3B), which could be explained by the low transfection efficiency. This could explain that we did not detect significant differences in cell-cycle proliferation between *BRE*-transfected cells and those transfected with an empty vector (Supplementary Figure 3C), nor an apoptotic effect of *BRE* (Supplementary Figure 3D), In addition *in vitro* drug sensitivity for cells overexpressing *BRE* showed no significant differences in drug sensitivity compared with cell transfected with an empty vector or non-transfected cell line (Supplementary Table 3).

BRE protein expression in *MLL*-rearranged AML

To identify a correlation between protein and mRNA expression levels, we performed Western blot analysis on 11 *MLL*-rearranged AML cases, of whom protein was available in our cell bank. No differences in BRE protein expression were found between t(9;11) (p22;q23) (n=6) and the other *MLL*-rearranged AML cases (n=5) (Figure 2D). The *BRE* transfected Monomac-1 cell line was used as positive control (Supplementary Figure 3B), indicating the specificity of the antibody against BRE.

DISCUSSION

Pediatric AML is a heterogeneous disease and currently response to therapy and cytogenetic abnormalities are the main prognostic factors. Interestingly, for the *MLL*-rearranged group, we previously showed in a large international retrospective study that prognosis mainly depends on the translocation partner of *MLL*[6]. AML patients with

t(1;11)(q21;p23) and t(9;11)(p22;q23) with FAB-M5 were found to have a favorable prognosis, whereas patients with t(10;11)(p12;q23) and t(6;11)(q27;q23) were found to have a unfavorable prognosis. This diversity in outcome indicates differences in leukemogenesis within *MLL*-rearranged AML. However, to date, the factors that play a role in these biological differences are largely unknown.

In the present study, we performed gene expression profiling on a large cohort of pediatric AML samples, with the aim to detect such differences in biology. Supervised clustering analysis identified a specific gene expression signature for t(9;11)(p22;q23), the most common translocation partner in *MLL*-rearranged AML. High expression of the *BRE* gene was one of the strongest components of this signature. High *BRE* expression was not restricted to t(9;11)(p22;q23), but also 3 cases with a t(10;11)(p12;q23) and one case with a t(8;16)(p11;p13) showed high *BRE* expression. Interestingly, t(8;16)(p11;q23) has been linked to *MLL*-rearranged AML based on gene expression profiles in adult AML[34]. This particular t(8;16)(p11;p13) case occurred in an infant with a FAB-M5 AML. All other pediatric AML cases did not show high *BRE* expression, indicating that particularly MLL-fusions may lead to high *BRE* expression and hence play a role in leukemogenesis in AML. Interestingly, high *BRE* expression was not found in t(9;11)(p22;q23) (n=10) or other *MLL*-rearranged precursor B or infant ALL (n=71) cases in two large GEP studies performed at our center, indicating it specific role in AML[15,35].

Recently, overexpression of *BRE* has been described in hepatocellular and esophageal carcinomas[32,36]. However, to date *BRE* has never been associated with hematological malignancies. This study shows that *BRE* plays a role in pediatric AML and mainly in patients with t(9;11)(p22;q23). Moreover, high *BRE* expression was an independent favorable prognostic factor due to a reduced relapse rate in remission. To explain the observed difference in relapse risk we examined the effect of *BRE* expression *in vitro* on altered cell proliferation, apoptosis and drug sensitivity in AML.

The BRE protein is mainly localized in the cytoplasm, but nuclear localization has also been reported as a subunit of the holoenzyme complex BRCC, which contains BRCA1, BRCA2, RAD51, and BARD1[37]. BRCC enhances cellular survival after DNA damage by ubiquitin E3 ligase activity. Interestingly, association of BRE to this complex further enhances the E3 ligase activity. After death-receptor stimulation also an increased binding of cytosolic BRE to ubiquitinated proteins was found, suggesting that BRE plays an important role in post-translational modification of proteins. Intriguingly, *BRE* transcription has been shown to be down-regulated after DNA damage and retinoic acid treatment[38]. Li *et al.* showed that BRE is a Death Receptor-associated anti-apoptotic protein, inhibiting the mitochondrial apoptotic pathway[39]. We could not find evidence that overexpression of *BRE* influenced apoptosis in a *MLL*-rearranged AML with the current cell line studies. This may be due to the fact that only a transfection rate of only 30% was achieved despite rigorous efforts. Alternatively, other mechanisms could play a

role, and the role in apoptosis may be tissue dependent. Alternative approaches, such as stable transduction with a selectable vector and manipulation of primary hematopoietic progenitors could elucidate this.

Previous studies demonstrated that overexpression of BRE enhances tumor growth, rather than initiating tumor formation *in vivo*[40]. However, *BRE* overexpression did not lead to cell proliferation *in vitro*[41]. Tang *et al.* showed conflicting data, i.e. a decreased *in vitro* cell proliferation by overexpression of BRE and upregulation of p53, prohibitin and proteins involved in the nuclear factor-kB signalling[42]. In a *MLL*-rearranged AML cell line, we could not show such a role for BRE in cell proliferation. It is conceivable that stable transfection cell line models or *in vivo* models could give more insight whether BRE overexpression influences proliferation or apoptosis of AML cells similar to what was previously shown in hepatocellular carcinomas models.

Using *in vitro* assays, we could not find that *BRE* overexpression was related to higher drug sensitivity, although the low transfection rate could have influenced these results. Li *et al.* showed that knock-down of endogenous *BRE* has little, if any, modulatory effect on apoptosis induced by etoposide in contrast to TNF-α induced apoptosis[39]. This seems to be in line with our results, since etoposide induced apoptosis was not influenced by overexpression of *BRE* in AML. Since we could not explain the observed difference in relapse risk in our series, further investigation is required to elucidate the role of *BRE* overexpression in the leukemogenesis of t(9;11)(p22;q23). MLL-fusion proteins are involved in inappropriate transcriptional activation, and a specific role for MLL-AF9 in the transcription of *BRE* is most likely.

In this study, protein analysis of BRE did not correlate with mRNA expression and differences in outcome were therefore only related to mRNA expression levels. However differences between mRNA and BRE expression are commonly observed and Greenbaum *and al.* suggested that, next to translation regulation and differences of *in vivo* protein half-lives, a significant amount of experimental error could be the lack of perfect correlation between protein and mRNA[43]. This could have influenced the results, since we used cell-bank material instead of fresh samples. We did not further investigate other translation or post-translation regulations of BRE, since this was beyond the scope of this study. However further research is warranted, as the differences in *BRE* expression with microarray data were confirmed with RQ-PCR. Moreover these differences in the transcription of *BRE* seem to influence outcome in pediatric AML.

This study uncovers a small part of the biological background that could explain the recently discovered clinical relevant heterogeneity of *MLL*-rearranged AML based on translocation partner. The majority of cases with high *BRE* expression were discovered in the prognostically favorable group with a t(9;11)(p22;q23) and FAB M5. Within the unfavorable prognostic group t(10;11)(p12;q23) only 3/16 cases had a high *BRE* expression, whereas in the most unfavorable subtype t(6;11)(q27;q23) no cases were found.

Furthermore, high *BRE* expression is inversely correlated with the overexpression of *EVI1* in *MLL*-rearranged AML. Overexpression of *EVI1* is a poor prognostic factor in adult AML. In pediatric AML, it was predominantly found in groups with a poor outcome, including FAB M7 and t(6;11)(q27;q23)[27]. Therefore, high *BRE* expression seems to be part of a favorable signature in *MLL*-rearranged AML.

In conclusion, our study shows that overexpression of *BRE* is predominantly found in *MLL*-rearranged AML with a t(9;11)(p22;q23). Moreover, high *BRE* expression is an independent favorable prognostic factor due to a reduced relapse rate in remission in pediatric AML. So far, we could not elucidate the exact underlying mechanism. Further research is warranted to explore the role of *BRE* in AML and to identify the link between MLL-AF9 and the transcription of *BRE*.

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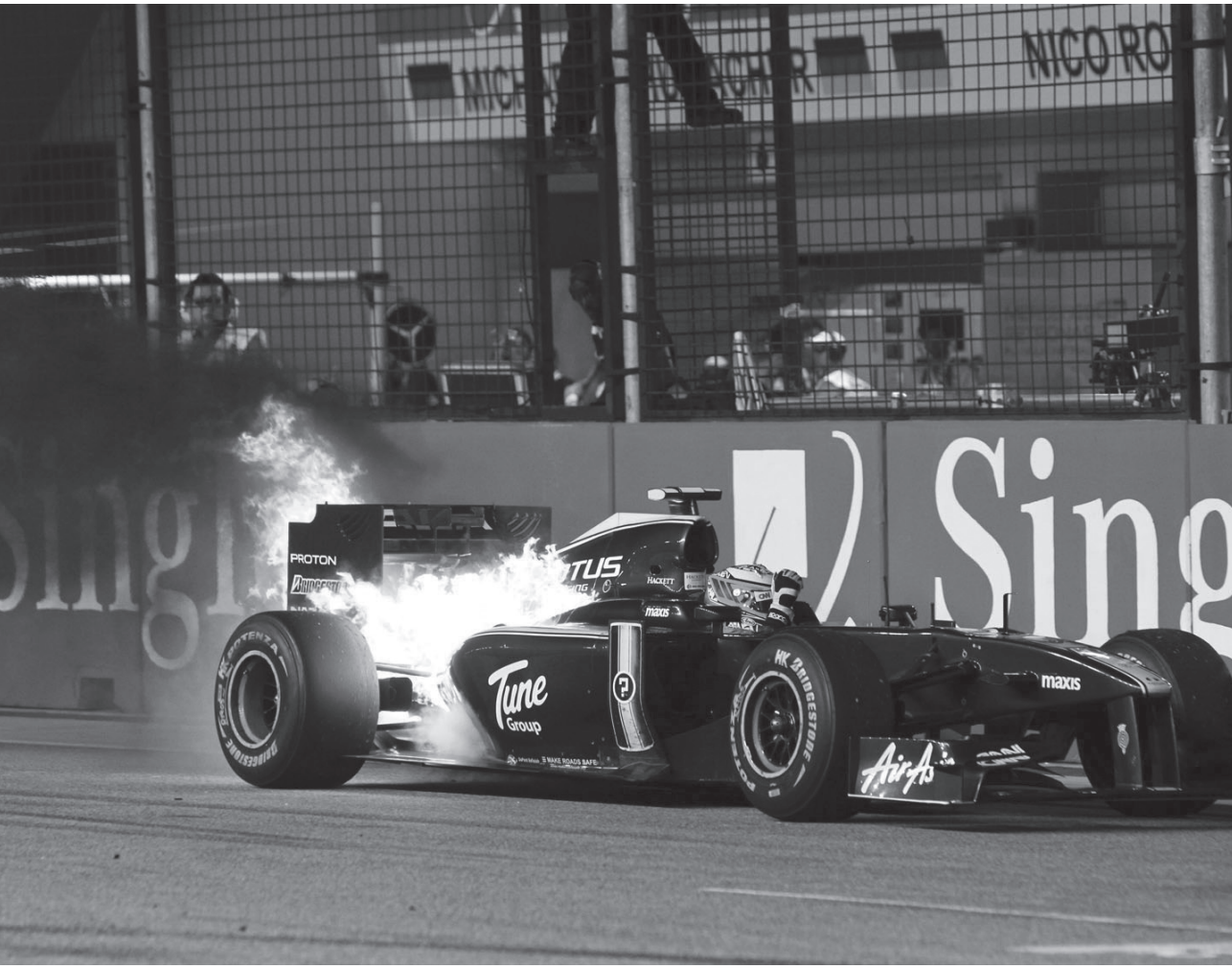
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Chapter 10

Low frequency of *MLL*-Partial Tandem Duplications in pediatric acute myeloid leukemia using MLPA as a *novel* DNA screenings technique

B.V. Balgobind[1], I.H.I.M. Hollink[1], D. Reinhardt[2], E.R. van Wering[4], S.S.N. de Graaf[4,7], A. Baruchel[5], J. Stary[6], H.B. Beverloo[8], G.E. de Greef[9], R. Pieters[1], C.M. Zwaan[1] and M.M. van den Heuvel-Eibrink[1]

[1]Paediatric Oncology/Hematology, Erasmus MC/Sophia Children's Hospital, Rotterdam, The Netherlands; [2]AML-BFM Study Group, Hannover, Germany; [4]DCOG, The Hague, The Netherlands; [5]Haematology, St. Louis Hospital, Paris, France; [6]Paediatric Hematology/Oncology, University Hospital Motol and [2]nd Medical School, Charles University, Prague, Czech Republic; [7]Paediatric Oncology/Hematology, University Medical Center St Radboud, Nijmegen, The Netherlands [8]Clinical Genetics, Erasmus MC, Rotterdam, The Netherlands [9]Haematology, Erasmus MC, Rotterdam, The Netherlands.

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ABSTRACT

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

INTRODUCTION

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

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

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

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

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

It is likely that the difference in detection methods that were used contributes to the reported differences in frequency of *MLL*-PTD. *MLL*-PTD was initially discovered using Southern Blot (SB) analysis, but in most subsequent studies detection was performed with mRNA and DNA RT-PCR. Screening of *MLL*-PTD with SB has its limitations as large amounts of DNA are required to perform SB, and the procedure is laborious. On the other hand, using a nested mRNA RT-PCR approach to detect pathogenic *MLL*-

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

Study	no. of patients	adult/pediatric	*MLL*-PTD (%)	screening method
Schnittger *et al.*(2000)	387	adult	3.4	genomic XL PCR
Shiah *et al.* (2002)	81	adult	11.0	mRNA RT-PCR + Southern Blot
Steudel *et al.* (2003)	956	adult	5.0	mRNA RT-PCR + Southern Blot
Libura *et al.* (2003)	185	adult	3.2	Southern Blot
Munoz *et al.* (2003)	93	adult	10	genomic XL PCR
Ozeki *et al.* (2004)	181	adult	10.9	mRNA RT-PCR
Bacher *et al.* (2005)	1881	adult	5.8	genomic XL PCR
Olesen *et al.* (2005)	250	adult	4.0	mRNA RQ-PCR
Shih *et al.* (2006)	865	adult	6.4	multiplex PCR
Ross *et al. (2004)*	130	pediatric	10.0	mRNA RT-PCR
Shih et al. (2006)	123	pediatric	0.9	multiplex PCR
Shimada *et al. (2008)*	158	pediatric	13.0	mRNA RT-PCR
Balgobind *et al. (2009)*	276	pediatric	2.5	DNA-MLPA + mRNA RT-PCR

PTD has shown to be not usable, since it detects *MLL*-PTD in healthy individuals and single-round mRNA RT-PCR may also yield false positive results[22-23]. Another genomic screening method of potential value is the multiplex ligation-dependent probe amplification of DNA (DNA-MLPA), which is already being used as a stand-alone test to rapidly detect aneuploidy in amniotic fluid cells with a high specificity and sensitivity[24], and reliably detects HER-2/neu amplification in breast cancer [25]. Furthermore DNA-MLPA only needs as little as 100 ng of DNA per patient and is less time-consuming. In addition, a large number of patients can be screened at once.
In this study, we screened the largest cohort of pediatric AML cases so far, using both mRNA RT-PCR and DNA-MLPA to accurately detect the occurrence of *MLL*-PTD and the association with other genetic events and prognosis.

MATERIAL AND METHODS

Patients

Viably frozen diagnostic bone marrow or peripheral blood samples from 276 newly diagnosed pediatric AML 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. Informed consent was obtained after Institutional Review Board approval according to local law and regulations. Each study group performed central morphological review[26]. The collaborative study groups also provided data on the clinical follow-up of these patients.
After thawing, leukemic cells were isolated by depletion of contaminating cells as previously described[27]. All resulting samples contained >80% leukemic cells, as determined morphologically by May-Grünwald-Giemsa (Merck, Darmstadt, Germany)-stained cytospins. The purified leukemic cell samples were used for DNA and RNA extraction, and a minimum of $5x10^6$ leukemic cells were lysed in Trizol reagent (Gibco BRL, Life Technologies, Breda, The Netherlands) and stored at -80°C. Genomic DNA and total cellular RNA were isolated as described before[28].

Detection of *MLL*-PTD

We designed a probe mix for DNA-MLPA analysis containing adjacent probes within exon 2 to 5 and exon 7 to 13 of the *MLL* gene for detection of common and rare types of *MLL*-PTD. Exon 17 of the *MLL* gene was used as an internal control. A probe set in the *serpinB2* gene, which is located in a region for which only one copy number variation has been described (*http://projects.tcag.ca/variation*), was used as external control according to the manufacturer's protocol (MRC Holland, Amsterdam, the Netherlands) (Table 2). The patient samples were analyzed according to the manufacturer's protocol.

Table 2: Primer sequences DNA-MLPA *MLL*-PTD

exon 13 FW	**5'-GGGTTCCCTAAGGGTTGGACACAGTGGTCTCATGATTTCT-3'**
exon 13 RV	5'-CACTGTGTCATGATTGCGCCA**TCTAGATTGGATCTTGCTGGCAC**-3'
exon 8 FW	**5'-GGGTTCCCTAAGGGTTGGAGTGGCTCCCCGCCCAAGTATCC-3'**
exon 8 RV	5'-CTGTAAAACAAAAACCAAAAGAAA**TCTAGATTGGATCTTGCTGGCAC**-3'
exon 17 FW	**5'-GGGTTCCCTAAGGGTTGGAGATATTGTGAAGATCATTCAAGCAG-3'**
exon 17 RV	5'-CCATTAATTCAGATGGAGGACAGCC**TCTAGATTGGATCTTGCTGGCAC**-3'
exon 10 FW	**5'-GGGTTCCCTAAGGGTTGGAGGGAGATGGGAGGCTTAGGAATCTTGA-3'**
exon 10 RV	5'-CTTCTGTTCCTATAACACCCAGGGTGG**TCTAGATTGGATCTTGCTGGCAC**-3'
exon 2 FW	**5'-GGGTTCCCTAAGGGTTGGAGCAATTCTTAGGTTTTGGCTCAGATGAAG-3'**
exon 2 RV	5'-AAGTCAGAGTGCGAAGTCCCACAAGGTCT**TCTAGATTGGATCTTGCTGGCAC**-3'
exon 3 FW	**5'-GGGTTCCCTAAGGGTTGGAGGAAAAAGGGATCAGAAATTCAGAGTAGTTC-3'**
exon 3 RV	5'-TGCTTTGTATCCTGTGGGTAGGGTTTCCAAA**TCTAGATTGGATCTTGCTGGCAC**-3'
SerpinB2 FW	**5'-GGGTTCCCTAAGGGTTGGACAGAGAACTTTACCAGCTGTGGGTTCATGCAGC-3'**
SerpinB2 RV	5'-AGATCCAGAAGGGTAGTTATCCTGATGCGATTT**TCTAGATTGGATCTTGCTGGCAC**-3'
exon 4 FW	**5'-GGGTTCCCTAAGGGTTGGACGAGGACCCCGGATTAAACATGTCTGCAGAAGAGC-3'**
exon 4 RV	5'-AGCTGTTGCCCTTGGCCGAAAACGAGCTGTGTTTC**TCTAGATTGGATCTTGCTGGCAC**-3'
exon 5 FW	**5'-GGGTTCCCTAAGGGTTGGAGAAGATGCTGAACCTCTTGCTCCACCCATCAAACCAA-3'**
exon 5 RV	5'-TTAAACCTGTCACTAGAAACAAGGCACCCCAGGAACC**TCTAGATTGGATCTTGCTGGCAC**-3'
exon 7 FW	**5'-GGGTTCCCTAAGGGTTGGAGCCAGCACTGGTCATCCCGCCTCAGCCACCTACTACAGG-3'**
exon 7 RV	5'-ACCGCCAAGAAAAGAAGTTCCCAAAACCACTCCTAGTGA**TCTAGATTGGATCTTGCTGGCAC**-3'
exon 9 FW	**5'-GGGTTCCCTAAGGGTTGGAGAAAAACCACCTCCGGTCAATAAGCAGGAGAATGCAGGCAC-3'**
exon 9 RV	5'-TTTGAACATCCTCAGCACTCTCTCCAATGGCAATAGTTCTA**TCTAGATTGGATCTTGCTGGCAC**-3'
exon 11 FW	**5'-GGGTTCCCTAAGGGTTGGACCAAGTCTGTTGTGAGCCCTTCCACAAGTTTTGTTTAGAGGAG-3'**
exon 11 RV	5'-AACGAGCGCCCTCTGGAGGACCAGCTGGAAAATTGGTGTTGTC**TCTAGATTGGATCTTGCTGGCAC**-3'
exon 12 FW	**5'-GGGTTCCCTAAGGGTTGGAGCTGGAGTGTAATAAGTGCCGAAACAGCTATCACCCTGAGTGCCT-3'**
exon 12 RV	5'-GGGACCAAACTACCCCACCAAACCCACAAAGAAGAAGAAAGTCTG**TCTAGATTGGATCTTGCTGGCAC**-3'

FW=forward probe, RV = reverse probe

Briefly, genomic DNA was denaturated and hybridized overnight with a mix of all probes. The adjacent probes were then ligated, so only these sequences were amplified during RT-PCR. Subsequently, these amplified products were separated using capillary electrophoresis. Using Gene Marker v1.5, the peak patterns obtained were compared to that of 3 negative controls to calculate the relative allelic ratios. No inter-assay variability was detected after performing a triple experiment in one assay and for all 3 controls (Supplementary Figure 1).
We also performed mRNA RT-PCR to detect *MLL*-PTD transcripts, to allow comparison with the DNA-MLPA results, using *MLL*-654c (*AGGAGAGAGTTTACCTGCTC*) as forward primer and *MLL*-5.3 (*GGAAGTCAAGCAAGCAGGTC*) as reverse primer[29].

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

Cytogenetic and molecular analysis
The pediatric samples were routinely investigated for cytogenetic aberrations by standard chromosome-banding analysis by the collaborative study groups. Moreover, they were screened for recurrent non-random genetic aberrations characteristic for AML, including t(15;17), inv(16), t(8;21) and *MLL*-rearrangements, using either mRNA RT-PCR and/or fluorescent in-situ hybridization (FISH). *NPM1*, *CEBPA*, *NRAS*, *KRAS*, *PTPN11*, *C-KIT* and *FLT3* mutational screening were done as previously described, and included mutational hotspots only[31-35].

Statistical analysis
Statistical analysis was performed using SPSS 15.0 (SPSS Inc. Chicago, USA). Different variables were compared with the χ^2 test or the Mann-Whitney-U test. All tests were two-tailed and a p-value less than 0.05 was considered significant.

RESULTS

Frequency of *MLL*-PTD using DNA-MLPA in pediatric AML and comparison with mRNA RT-PCR
Using DNA-MLPA, which has a 92% accuracy as compared to SB, we detected *MLL*-PTD in 6/275 patients (2.2%). In all 6 patients, *MLL*-PTD mRNA expression was

confirmed with RT-PCR, showing high expression levels of *MLL*-PTD. The patients showed an average relative allelic ratio of the amplified region of at least 1.3 compared to the controls. In one additional patient, DNA-MLPA analysis could not be performed since no DNA was available. However, this patient's sample was considered to harbor an *MLL*-PTD, since mRNA RT-PCR demonstrated *MLL*-PTD transcripts, and SB analysis, which was performed at diagnosis, showed an abnormal *MLL* pattern. In addition, an *MLL*-rearrangement was excluded using split signal FISH analysis (data not shown). Therefore the total number of patients with *MLL*-PTD was 7/276 (2.5%).
In 226/276 samples mRNA RT-PCR screening for *MLL*-PTD was performed. In 6 cases both DNA-MLPA and mRNA RT-PCR were positive for *MLL*-PTD as described above. In 7 patients, mRNA RT-PCR detected *MLL*-PTD transcripts without evidence for *MLL*-PTD using DNA-MLPA. Interestingly, these transcripts were only observed in *MLL*-rearranged AML, and not encountered in any of the other 213 AML samples without an *MLL*-rearrangement.

Characteristics of patients with *MLL*-PTD

The characteristics of the 7 patients with *MLL*-PTD are described in tables 3 and 4. None of the patients harboring an *MLL*-rearrangement, t(8;21), inv(16) or t(15;17) revealed a *MLL*-PTD. In one patient with an *MLL*-PTD a trisomy 11 was found, while the other 6 cases were found in patients with normal cytogenetics (CN-AML) (n=3) or in patients in whom cytogenetic analysis failed (n=3).
FLT3-ITD was present in 4 out of 7 patients with *MLL*-PTD, while one patient showed a mutation in the kinase domain of *FLT3* and another showed a mutation in *NRAS*. There was a significantly higher frequency of *FLT3*-ITD in patients with an *MLL*-PTD than in those without *MLL*-PTD (p=0.016) (Table 3). The age of patients with *MLL*-PTD was not different from patients without *MLL*-PTD (median 7.5 years and 9.8 years, respectively; p=0.72). Patients with *MLL*-PTD tended to have higher white blood cell counts (WBC) at initial diagnosis than those without *MLL*-PTD (median WBC 97 x 10^9 /l vs. 40 x 10^9 /l respectively; p=0.07). Two *MLL*-PTD patients had a relative allelic ratio of more than 2.0. They presented with a WBC of 133.0 x 10^9 /l and 169.0 x 10^9 /l, respectively.

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

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

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

	MLL-PTD negative patients	*MLL*-PTD positive patients	*p-value*
Sex, N (%) (n=276)			*0.704* [#]
male	150 (56)	3 (43)	
female	119 (44)	4 (57)	
Age, years (median, range) (n=271)	9.8 (0.1-18.8)	7.5 (4.8-18.0)	*0.740* [§]
WBC x 10^9/L (median, range) (n=231)	40 (0-585)	97 (45-170)	*0.076* [§]
FAB, N (%) (n=276)			*0.146* [#]
M0	13 (5)	1 (14)	
M1	27(10)	3 (43)	
M2	55 (20)	1 (14)	
M3	17 (6)	0 (0)	
M4	69 (26)	2 (29)	
M5	71 (26)	0 (0)	
M6	0 (0)	0 (0)	
M7	5 (2)	0 (0)	
other/unknown	12 (5)	0 (0)	
Cytogenetic abnormalities, N (%) (n=276)			***<0.001*** [#]
***MLL*-rearrangements**	69 (26)	0 (0)	
t(8;21)	33 (12)	0 (0)	
inv(16)	29 (11)	0 (0)	
t(15;17)	16 (6)	0 (0)	
normal cytogenetics	41 (14)	3 (43)	
trisomy 11	0 (0)	1 (14)	
other/unknown **	81 (30)	3 (43)	
***FLT3*-ITD, N (%) (n=253)**			***0.016*** [#]
no	208 (85)	3 (43)	
yes	38 (16)	4 (57)	

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

Table 4: Patient characteristics *MLL*-PTD

patient ID	age	sex	WBC	FAB	Karyotype	MLL-PTD	allelic ratio	Sec abnormality
AML_DE62	7	male	68,1	M1	47,XY,+11	ex2-ex8	1,5	NRAS
AML_DE129	7	male	120,4	M1	46,XY	ex2-ex7	1,5	FLT3-ITD
AML_NL97	14	female	44,8	M1	46,XX	ex2-ex8	1,5	FLT3-ITD
AML_NL182	11	female	72,9	M2	46, XX	SB, RT-PCR pos	ND	FLT3-TKD
AML_DE68	7	female	169,9	M4	NA	ex2-ex8	2	FLT3-ITD
AML_DE15	4	female	NA	M0	NA[1]	ex2-ex9	1,5	-
AML_FR11	18	male	133	M4	NA[1,2]	ex2-ex8	2,5	FLT3-ITD

NA = not available, SB= Southern Blot, ND= not determined
[1]) no MLL-rearranged AML or t(8;21) detected
[2]) no inv(16) detected

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

	***MLL*-rearranged patients**	***MLL*-PTD positive patients**	***p-value***
Sex, N (%) (n=75)			*0.695* [#]
male	38 (56)	3 (43)	
female	30 (44)	4 (57)	
Age (median, range) (n=75)	6.2 (0.1-18.8)	7.5 (4.8-18.0)	*0.074* [§]
WBC x 10^9/L (median, range) (n=64)	61 (1.2-585)	97 (45-170)	*0.345* [§]
FAB, N (%) (n=71)			***<0.001*** [#]
M0	3 (4)	1 (14)	
M1	2 (3)	3 (43)	
M2	1 (1)	1 (14)	
M3	0 (0)	0 (0)	
M4	9 (13)	2 (29)	
M5	50 (72)	0 (0)	
M6	0 (0)	0 (0)	
M7	1 (1)	0 (0)	
other/unknown	3 (4)	0 (0)	
FTL3-ITD, N (%) (n=70)			***<0.001*** [#]
no	65 (97)	3 (43)	
yes	2 (3)	4 (57)	

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

Clinical outcome in pediatric AML with *MLL*-PTD

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

DISCUSSION

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

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

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

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

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

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

Although the role of *MLL*-PTD in leukemogenesis is not clear these patients could benefit from treatment with DNA methyltransferase (DNMT) inhibitors and/or histone deacetylase (HDAC) inhibitors. A recent study has shown that $Mll^{PTD/WT}$ knockin mice are fully viable with modest developmental defects, have aberrant gene expression and altered hematopoiesis, but do not develop leukemia[9]. However leukemic blast cells of

adult patients with *MLL*-PTD, which are present on only one allele, do not express the wild type (WT) MLL, which is based on epigenetic silencing of the normal allele[42]. This is in contrast to *MLL*-rearranged AML, which does express wild type MLL. Interestingly, treatment of *MLL*-PTD positive cases with DNMT and HDAC inhibitors resulted in induction of the WT-MLL and selective sensitivity to cell death compared with *MLL-PTD* negative cases with normal expression of WT-MLL [42].

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

In conclusion, the frequency of *MLL*-PTD in pediatric AML is low and may have been overestimated in earlier studies. In this study, we screened the largest pediatric AML cohort so far, using DNA-MLPA as a novel screening method for *MLL*-PTD in combination with mRNA RT-PCR, and revealed a frequency of only 2.4% in pediatric AML. Larger prospective studies are necessary to further define the prognostic relevance of *MLL*-PTD in pediatric AML.

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Summary and Discussion


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Chapter 11

Summary

PART ONE: THE GENETIC AND MOLECULAR CHARACTERISTICS OF PEDIATRIC AML

Acute myeloid leukemia (AML) accounts for 15-20% of childhood leukemias and despite intensive chemotherapy, only 60% of the patients with AML are cured. Moreover, the heterogeneity of AML is reflected by differences in morphology, immunophenotype, as well as cytogenetic and molecular abnormalities. Recurrent (cyto)genetic aberrations and response to treatment are important prognostic factors in AML.

In **chapter 2** we present an overview of different type-I and type-II aberrations in pediatric AML, including non-random associations between these aberrations, as well as associations with clinical characteristics and outcome, with the aim to detect specific groups of pediatric AML. The heterogeneity of pediatric AML is reflected by the presence of these type-I and type-II aberrations, and their prognostic impact. Several non-random associations between genetic aberrations are present, and specific subgroups are identified e.g. *c-KIT* mutations with CBF-AML and *FLT3*-ITD with t(15;17)(q22;q21) or CN-AML. Furthermore, major differences exist between AML in children younger and older than two years at diagnosis. Very young children with AML were characterized by a high frequency of MLL-rearrangements, t(7;12)(q36;p13) and t(8;16)(p11;p13). In contrast, t(8;21)(q22;q22), t(15;17)(q22;q21) and CN-AML were predominately found in children above the age of 2 years.

In **chapter 3**, we focused on the potential of gene expression profiles (GEP) to classify pediatric AML, since the genetic subtype is currently being detected by different diagnostic procedures which differ in success rate and/or specificity. Gene expression microarray data of 237 children with AML were generated and a double-loop cross validation approach was used to generate a subtype-predictive GEP. A specific gene expression signature existing of 75 probe sets could accurately identify 5 cytogenetic subgroups, i.e. *MLL*-rearranged, t(8;21), inv(16), t(15;17) and t(7;12)-positive AML. In contrast, molecular aberrations were hard to predict, which could be due to the low frequency of some of these aberrations and/or gene expression signatures being affected by the underlying cytogenetic abnormality. The latter is exemplified by the fact that different gene expression signatures were discovered for *FLT3*-ITD in CN-AML and t(15;17)-positive AML. *FLT3*-ITD+/CN-AML was related to *HOXB*-upregulation. It remains to be determined whether underlying but yet unknown genetic aberrations in the remaining cases of AML will result into distinct gene expression patterns that can be used for classification. This is important, as classification by gene expression profiling may reduce the number of cases for which multiple diagnostic procedures (cytomorphology, FISH, RT-PCR, karyotyping) are required with at least 40%.

The study in **chapter 4** describes the relevance of *EVI1* overexpression (*EVI1*+) in pediatric AML. Overexpression of the *EVI1*, localized at chromosome 3q26, is associated

with adverse outcome in adult AML. *EVI1*+ is found in 9% of de novo pediatric AML. *EVI1* is overexpressed in specific cytogenetic (*MLL*-rearrangements and monosomy 7) and morphologic (FAB-M6/7) subtypes. However, the typical *EVI1*+ associated 3q26 aberrations reported in adult AML were not identified, indicating that there may be a difference for the role of *EVI1*+ in adult AML as compared to pediatric AML. Although *EVI1*+ was not an independent prognostic factor, it was predominantly found in types of pediatric AML that are related with an intermediate to unfavorable prognosis, e.g. *MLL-AF6* and monosomy 7.

PART TWO: THE HETEROGENEITY OF *MLL*-REARRANGED AML

Translocations involving chromosome 11q23 frequently occur in pediatric AML and are associated with poor prognosis. In most cases, the *MLL*-gene localized at 11q23 is involved, and more than 50 translocation partners have been described.

Chapter 5 presents a review on the heterogeneity of *MLL*-rearranged AML. In this chapter de etiology, clinical characteristics and prognosis are summarized for *MLL*-rearranged AML. In addition the future possibilities to target this group of pediatric AML are discussed.

In **chapter 6** a unique and very large international retrospective study was undertaken to collected clinical outcome data of 756 children with 11q23 or *MLL*-rearranged pediatric AML from 11 collaborative study groups from 15 countries. Our aim was to identify differences in clinical outcome based on the various 11q23 or *MLL* rearrangements. We identified several novel, independent prognostic 11q23/*MLL*-rearranged subgroups, including the favorable-risk subgroup with a t(1;11)(q21;q23) and the poor-risk subgroups with a t(10;11)(p12;q23), t(10;11)(p11.2,q23), or t(6;11)(q27;q23). We recommend that these subgroups should be included in the risk-group stratification process in current and future pediatric AML protocols. The patients with a t(9;11)(p22;q23) represent a heterogeneous subgroup requiring the inclusion of FAB-type for accurate risk-group stratification.

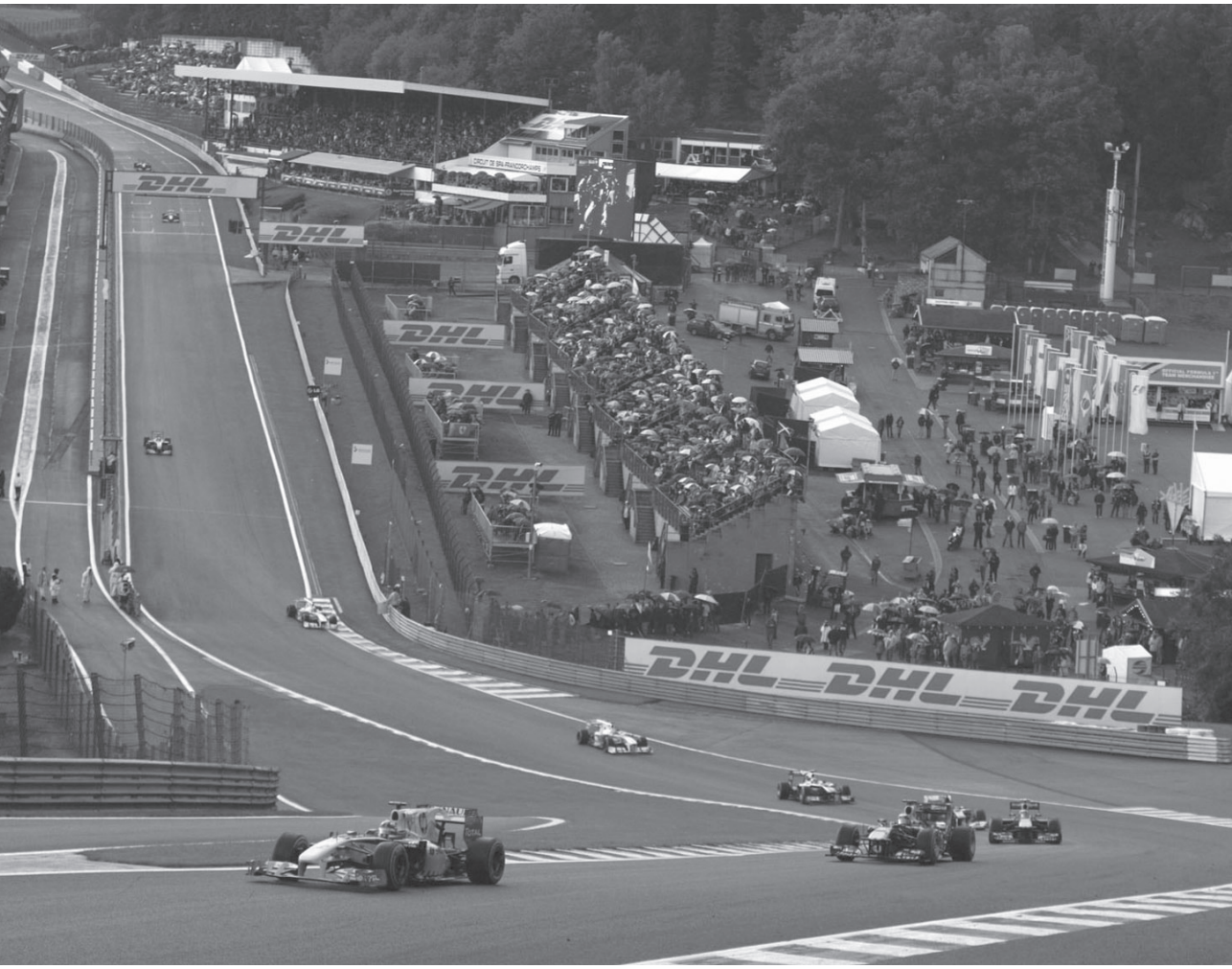
In **chapter 7** we report on a novel translocation partner of *MLL* on chromosome 11 in a pediatric AML case. In this case with complex rearrangements of chromosome 3 and 11 a novel translocation partner of the *MLL*-gene was detected. We have shown that the translocation partner was found on chromosome 11 with LDI-PCR. This technique revealed the *NRIP3* gene on 11p15 as a novel translocation partner of *MLL* in pediatric AML, while the 3' part of *MLL* was translocated to chromosome 3.

In **chapter 8**, we report the identification of somatic *NF1* microdeletions as a cryptic genetic abnormality in *MLL*-rearranged AML patients that lack symptoms of neurofibromatosis, by the use of oligo array-comparative genomic hybridization (array-CGH).

We present further evidence for the role of *NF1* inactivation as a functional equivalent to activated RAS signaling. We confirmed NF1 inactivation by reduced *NF1* expression levels, and bi-allelic *NF1* mutations in 3 out of 5 patients, confirming the role of *NF1* as a tumor suppressor gene in cancer.

In **chapter 9** gene expression profiles of 53 *MLL*-rearranged AMLs among 245 pediatric AML cases were used to identify and analyze differentially expressed genes between the various *MLL*-rearranged AML cases stratified by translocation partners and to elucidate potential genes of interest which are related to the observed differences in outcome. Thereby, we identified a specific gene expression signature for t(9;11)(p22;q23), and identified *BRE* (brain and reproductive organ-expressed) to be discriminative for t(9;11)(p22;q23) ($p<0.001$) when compared with other *MLL*-subtypes. Overexpression of *BRE* was predominantly found in *MLL*-rearranged AML with a t(9;11)(p22;q23). Moreover, high *BRE* expression is an independent favorable prognostic factor due to a reduced relapse rate in remission in pediatric AML. So far, we could not elucidate the exact underlying mechanism.

In addition to rearrangements with a large variety of different partner genes, *MLL* rearrangements can be found within *MLL* as the result from partial tandem duplication (*MLL*-PTD), consisting of an in-frame repetition of *MLL* exons. *MLL*-PTD is found in 3-5% of adult acute myeloid leukemia (AML), and is associated with poor prognosis. The study described in **chapter 10** shows that the frequency of *MLL*-PTD in pediatric AML is low and may have been overestimated in earlier studies. In this study, we screened the largest pediatric AML cohort so far, using DNA-MLPA as a novel screening method for *MLL*-PTD in combination with mRNA RT-PCR, and revealed a frequency of only 2.4% in pediatric AML. Larger prospective studies are necessary to further define the prognostic relevance of *MLL*-PTD in pediatric AML.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Chapter 12

Discussion and future perspectives

THE DEVELOPMENT OF AML

Like other cancers, the origin of acute myeloid leukemia (AML) is unknown, but a multi-step mechanism is necessary to develop AML. In normal hematopoiesis, immature stem-cells proliferate and mature in the bone marrow to form mature white blood cells, red blood cells and thrombocytes. In contrast, leukemic cells are characterized by a maturation arrest and by enhanced proliferation, resulting in suppression of normal hematopoiesis.

The combinations of cytogenetic and molecular aberrations in pediatric AML led to the hypothesis that for the development of AML at least two different hits are needed, as postulated by Gilliland et al. One hit results in a proliferative advantage, the type-I aberrations, and the other hit leads to a differentiation stop, the type-II aberrations[1]. This hypothesis was supported by functional studies. For instance, introducing *FLT3*-ITD in primary hematopoietic cells using a murine bone marrow transplant assay induced a myeloproliferative phenotype similar to that observed in chronic myeloid leukemia[2], and a "knock-in" strategy to generate mice containing a single allele of t(8;21)(q22;q22) led to the generation of dysplastic hematopoietic progenitors[3]. However, both models did not generate the full phenotype of AML, which was also shown in several other studies for other aberrations such as inv(16)(p13q22) and t(15;17)(q22;q21)[4,5]. In addition, several mouse model studies showed that co-operating events did lead to the development of frank leukemia[6-8]. In agreement with these data from murine models, Wang et al. showed that patients with a t(8;21)(q22;q22) and an additional *KIT* aberration at diagnosis, remain t(8;21)(q22;q22) positive at continuous complete remission, whereas the *KIT* mutation was lost, suggesting again that both mutations were necessary to develop human AML[9]. More evidence for this 'two-hit' hypothesis is found in the studies from Guthrie cards. Wiemels *et al.* and McHale *et al.* showed that cytogenetic aberrations such as t(8;21)(q22;q22), inv(16)(p13q22) and t(15;17)(q22;q21) were already present in utero, but the development of AML showed a latency of up to 12 years, indicating that the this period is needed to acquire additional aberrations[10-12]. However, a limitation of this 'two-hit' hypothesis has been the lack of identifiable proliferative and maturation arrest inducing mutations in most AML cases. Moreover, *MLL*-rearranged AML maybe an exception, since introduction of *MLL*-rearrangements in murine models led to the development of AML without requiring additional aberrations[13-16]. Therefore, we focused on further unraveling the heterogeneity of pediatric AML, with the aim to identify aberrations which could support the 'two-hit' hypothesis in those cases without known type-I or type-II aberrations, or which might otherwise play an important role in the leukemogenesis of AML. Furthermore, we specially put an emphasis on *MLL*-rearranged AML, which seem to behave differently and are mainly found in pediatric AML compared to other types of AML.

DIFFERENCES IN MOLECULAR-GENETIC ABERRATIONS ILLUSTRATE THE HETEROGENEITY OF PEDIATRIC AML

In chapter 2 we showed that the most common type-II aberrations in pediatric AML are cytogenetic aberrations, i.e. *MLL*-rearranged AML, t(8;21), inv(16), and t(15;17)[17]. Moreover, mutations in *NPM1* and *CEBPA* were identified in cytogenetically normal pediatric AML (CN-AML), which were mutually exclusive with each other, and with other type-II aberrations. In addition, several non-random associations were identified between type II and type I aberrations (mutations in *FLT3*, *N/K-RAS*, *PTPN11* and *KIT*), i.e. *KIT* mutations with CBF-AML, *FLT3*-ITD with t(15;17)(q22;q21) and CN-AML, in agreement with the 2-hit hypothesis mentioned above, and with the literature.
However, in respectively 41% and 37% of pediatric AML samples studied in chapter 2 we were not able to find a known type-I or type-II aberrations, suggesting that in a large number of pediatric AML these aberrations still need to be discovered[17]. However, other aberrations were identified, e.g. *WT1* mutations and *EVI1* overexpression, which were not mutually exclusive with other type-1 or type-II aberrations[18-20], and hence may represent an additional category of abnormalities involved in leukemogenesis, although their exact role on the functional level still needs to be elucidated. This indicates that in some pediatric AML cases, aberrations leading to the development of AML cannot be easily classified as type-I or type-II aberrations. Probably, more than 2 aberrations are necessary to develop AML. Furthermore, the functional role of the aberrations sometimes dependent on the cell lineage of origin and it should be elucidated if they are a driver rather than a bystander effect. For example, the role of *FLT3*-ITD seems to be different in pediatric cases with t(15;17)(q22;q21) than cases with CN-AML, since we identified different gene-expression profiles between both groups correcting for their cytogenetic background. In addition, whereas *FLT3*-ITD in CN-AML is related to a poor prognosis, *FLT3*-ITD in t(15;17)(q22;q21) did not influence the favorable outcome.
In chapter 3, we studied whether gene expression profiling of pediatric AML could correctly predict the different known cytogenetic and molecular subtypes of AML by supervised clustering analysis, hence analyzing its potential to replace current diagnostic techniques by GEP[21]. We showed that specific cytogenetic subtypes of AML could be diagnosed correctly using GEP, whereas this was not the case for the known molecular aberrations in AML. Therefore, 50% of the cases could not be correctly predicted using GEP-based analysis, questioning the use of GEP for use in routine diagnostics. In pediatric ALL, where most of the subtypes, 65% of all cases, were correctly predicted, and where the BCR-ABL-like subtype was identified based on this same analysis[22].
Apart from using GEP for diagnostic purposes an important goal of this high-throughput technique is the potential to detect novel abnormalities, using another statistical approach (unsupervised clustering analysis) as already shown for adult AML by Valk *et al.*[23]. They

identified several clusters in a large set of adult AML samples, based on cytogenetic and molecular aberrations, such as t(8;21), inv(16), t(15;17), *CEBPA*-mutations and *EVI1*-overexpression, but also novel clusters, including one cluster with cases of AML with a poor treatment outcome. Currently, we are in the process of analyzing our GEP data in a similar fashion to identify *novel* aberrations in pediatric AML, and to analyze whether the same clusters are present as in adult AML as well as identifying specific clusters for pediatric AML[24].

Over the last decade, the development of other high-throughput techniques, e.g. genomic profiling (array-CGH, SNP-arrays) or sequencing the kinome, have led to the identification of novel aberrations in AML[25-27]. Using array-CGH, we identified aberrations associated with AML such as *WT1* aberrations and *NF1*-deletions, as described in chapter 8[20,28]. In large genome-wide studies of pediatric AML, chromosomal aberrations, such as recurrent amplifications or deletions could not be identified[26,28]. Moreover, the frequency of deletions and amplifications was low, which is in contrast to pediatric ALL[29,30], and which suggest that pediatric and adult AML are genomically stable.

The aberrations leading to AML may be found in other mechanisms leading to leukemogenesis, such as point-mutations, differences in methylation status of genes or differences in mi-RNA expression or in protein expression. Recently the first whole genome sequencing of a CN-AML sample led to the identification of mutations in the *IDH1* and *DNMT3A* genes, which were absent in the germ-line[31,32]. Screening a large cohort of adult AML cases identified a frequency of 8% for *IDH1* and 22% for *DNMT3A* mutations. The frequency and biological role of these aberrations in pediatric AML needs to be further explored. Of interest, Ho *et al.* did not identify *IDH1* mutations in 257 pediatric AML cases[33].

Apart from mutations, disruption of other mechanisms could lead to the development of AML. Several other regulating mechanisms are present that prevent inappropriate growth of blood cells. For instance, some of the recently identified mi-RNAs have a specific role in the development of cancer. Mi-RNAs are post-transcriptional regulators that bind to complementary sequences on target messenger RNA transcripts (mRNAs), usually resulting in translational repression and gene silencing. In AML, the expression of several mi-RNAs has been linked to the development of specific subtypes of AML[34]. Furthermore, several mi-RNAs, e.g. miR-126 and miR-29b, have been functionally linked to a role in the leukemogenesis of AML[35,36]. Within our study group, in collaboration with the Hannover Medical School, efforts are being made to identify which mi-RNAs play a role in the development of pediatric AML, and moreover if targeting these mi-RNAs could lead to cell death in these leukemic cells[37]. Another possible mechanism to develop AML is aberrant methylation patterns of tumor-suppressor genes or tumor oncogenes. In adult AML large differences in gene methylation status have been identified[38]. Although numbers are low, we already identified that *CEBPA*-silenced cases by

promoter hypermethylation seem to be related with a poor outcome in pediatric acute myeloid leukemia[24]. In our lab, different methylation profiles were found for the different translocation partners of *MLL*-rearranged ALL[39], and research is ongoing to elucidate their role in leukemogenesis. Moreover, we aim to identify the methylation patterns in *MLL*-rearranged AML as well as other genetic groups of pediatric AML. In the future, specific subtypes might benefit from the use of demethylating agents.

In conclusion, large differences in cytogenetic and molecular aberrations show that pediatric AML is a heterogeneous disease. Evidence suggests that a multi-step mechanism is necessary to develop AML, which may include not only cytogenetic aberrations and molecular mutations, but also inappropriate expression of mi-RNAs or aberrant methylation of genes, which may be either subgroup and/or case dependent.

MOLECULAR CHARACTERIZATION OF PEDIATRIC AML IS AGE DEPENDENT

AML represents 15-20% of childhood leukemias, and increases to 30% in adolescents, and 50% in adult leukemia. Differences in genetic subtypes were also found between younger and older children with AML. Very young children with AML are characterized by different frequencies of cytogenetic and molecular aberrations as compared to older children with AML[17] (chapter 2: Figure 5). The increasing incidence of CN-AML by age in childhood (approximately 15%) is continued into adulthood, in which CN-AML is present in approximately 45% of AML cases, whereas *MLL*-rearrangements are rare in adult AML but are found in 15-20% of childhood AML, and t(7;12)(q36;p13) is limited to pediatric AML[40,41]. Furthermore, infants with AML are characterized by a high tumor load, such as high white blood cell count, and more frequent extra-medullary involvement, as well as a high percentage of acute monoblastic leukemia cases. This is also reflected by the differences in cytogenetics, e.g. a higher frequency of *MLL*-rearrangements in infants (~40% versus ~20% in older children). Thus, further studies are needed to analyze why specific aberrations are related to development of AML in early life, whereas others develop later on. In contrast to pediatric ALL, where infants are now treated on different protocols compared to older children, no differences in outcome between infants and older children have been identified in pediatric AML[42]. Although this does not suggest a benefit of different treatment strategies based on age, it is conceivable that the biological differences related to age may lead to different treatment strategies in the future, when targeted therapy options become available.

Next to differences in frequency of CN-AML and *MLL*-rearranged AML, also core-binding factor AMLs, i.e. t(8;21) and inv(16), decrease with increasing age into adulthood[43]. Moreover, with increasing age an antecedent hematologic disorder including myelodysplastic syndrome (MDS) is more often reported in cases with AML. In adult AML, *EVI1*

overexpression is an independent poor prognostic factor[44], whereas in pediatric AML we could not relate *EVI1* overexpression to poor outcome, which could be influenced by the fact that aberrations of chromosome 3q26, such as inv(3), are not present in pediatric AML in contrast to adult AML[45]. In addition, outcome of *NPM1* in adult AML seems to be influenced by *FLT3*-ITD[46], which was not the case in pediatric AML, possibly based on the type of mutation in *NPM1*[47]. Thus, although the cytogenetic and molecular aberrations found in pediatric AML are almost identical to those found in adults, frequencies of subtypes of AML most often seem to be different and some subtypes show differences in outcome.

OUTCOME AND TREATMENT OF PEDIATRIC AML

Overall survival of pediatric AML is in the range of 60% with current treatment strategies. The prognosis in AML is determined by several factors. High WBC, response to treatment and cytogenetic and molecular aberrations are independent prognostic factors. Established favorable prognostic cytogenetic groups in pediatric and adult AML include t(8;21), inv(16) and t(15;17), whereas monosomy 7 and t(6;9) are related to poor prognosis in pediatric as well as adult AML. The type-II *NPM1* mutations and *CEBPA* double mutations are associated with a favorable outcome in pediatric AML[47,48], and *WT1* mutations and *FLT3*-ITD with a poor outcome in pediatric AML (Table 1)[17,20,24,47-56]. Currently, novel pediatric AML cases are screened for cytogenetic aberrations with conventional karyotyping. Only specific cytogenetic subgroups, such as *MLL*-rearrangements, t(8;21), inv(16) and t(15;17), are screened with molecular techniques such as FISH and PCR. Most study protocols have started to stratify their patients in standard- or high-risk, based on cytogenetics [t(8;21), and inv(16)] and response to treatment after first induction. However, pediatric AML can be further stratified based on the different molecular aberrations, which show differences in outcome. Therefore, future diagnostic strategies should include screening for these molecular aberrations, e.g. *NPM1*, *CEPBA*, *WT1* and *FLT3*-ITD. It would be of interest to screen all these aberrations in one assay. In chapter 3 we explored the possibility to use gene expression profiling as a diagnostic assay for all cytogenetic and molecular aberrations. This was possible for all cytogenetic subgroups, but unfortunately not for all molecular aberrations in pediatric AML. The latter could be the results of small sample size of these subtypes in combination with a weak signature. The development of a single gene expression profiling assay is ongoing in collaboration with the adult AML study groups.

Within current pediatric AML treatment protocols almost all patients receive anthracycline- and cytarabine-based induction courses, followed by consolidation courses that include high-dose cytarabine. Of interest, AML cases with t(15;17) are successfully

Table 1: Outcome of cytogenetic and molecular aberrations in pediatric AML

	5-y EFS	5-y OFS
Cytogenetic subtypes		
t(8;21)(q22;q22)		
Von Neuhoff et al. (2010)	84%	91%
Harrison et al. (2010) #	69%	80%
inv(16)(p13q22)		
Von Neuhoff et al. (2010)	70%	87%
Harrison et al. (2010) #	74%	81%
t(15;17)(q22;q21)		
Von Neuhoff et al. (2010)	73%	92%
***MLL*-rearrangements**		
Von Neuhoff et al. (2010)	34%	49%
Harrison et al. (2010) #	59%	61%
Balgobind et al. (2009)	44%	56%
Monosomy 7		
Von Neuhoff et al. (2010)	17%	25%
Harrison et al. (2010) #	29%	32%
Hasle et al. (2007)	28%	35%
t(6;9)(p23;q34)		
Harrison et al. (2010) #	10%	50%
Abnormality of 5q		
Von Neuhoff et al. (2010)	50%	49%
Harrison et al. (2010) #	18%	27%
Molecular subtypes		
***FLT3*-ITD**		
Zwaan et al. (2003)	29%	32%
Meshinchi et al. (2008)	31%	33%
***KIT*-mutations**		
Goemans et al. (2005)	45%	60%
Balgobind et al. (this thesis)	53%	69%
***RAS*-mutations**		
Goemans et al. (2005)	59%	67%
Balgobind et al. (this thesis)	51%	70%

	5-y EFS	5-y OFS
***CEBPA*-double mutations**		
Hollink et al. (2011)	58%	79%
Ho et al. (2009)	70%	83%
***NPM1*-mutations**		
Hollink et al. (2009)	80%	85%
Brown et al. (2007)	50%	55%
***WT1*-mutations**		
Hollink et al. (2009)	22%	35%
Ho et al. (2010)	28%	41%

5y-EFS: 5 year event free survival; 5y-OS: 5 year overall survival
#10y-EFS and 10y-OS

treated by adding ATRA to conventional chemotherapy, which mainly reduces the early mortality as previously found in this subtype of AML[57]. In addition, children with Down syndrome and AML (DS-AML) are treated with reduced-intensity treatment protocols, based on the remarkable sensitivity of DS-AML cells as demonstrated by in-vitro cell-culture assay. This also results less deaths due to treatment related mortality, together resulting in increasing survival rates of over 90%[58]. This reflects the unique subtype of leukemia which occurs in these children, and which is characterized by a mutation in the *GATA1*-gene, a hematopoietic transcription factor localized on the X-chromosome[59].
To improve outcome in pediatric AML, several novel treatment strategies have been adopted or are under investigation, mainly in refractory or relapsed AML. This includes new formulations of old drugs, such as liposomal daunorubicin, which is assumed to be less cardiotoxic compared to conventional anthracyclines, but also nucleoside analogues such as clofarabine or fludarabine, which inhibit DNA synthesis[60]. Since AML cells express CD33 in more than 90% of the cases, the use of Gemtuzumab ozogamycin (GO), a monoclonal anti-CD33, has also been explored, but is currently limited by its withdrawal from the therapeutic market in the USA[61-63].
Overall survival with current treatment strategies has reached a plateau since further intensification of chemotherapy will lead to a higher treatment-related mortality. Therefore, the future of treating pediatric AML has to focus on targeted therapy if outcome has to be improved without adverse effects. The prime example of such targeted treatment is the activity of imatinib in both chronic myeloid leukemia and Philadelphia positive-ALL, the latter mainly in combination with chemotherapy. FLT3-inhibitors, such as PKC412, have already shown to induce apoptosis in *FLT3*-ITD/AML cells *in vitro* and in mouse models[64-67]. In addition, PKC412 showed potential in phase I/II trials in adult AML[68], although responses were limited, probably reflecting the fact that AML leukemogenesis is driven by many different activated pathways, which results in diminished oncogenic

addition when one pathway is shut down. A pivotal randomized trial in adults in combination with chemotherapy is ongoing, which should show whether the drug is able to induce synergistic cell-death together with regular chemotherapy. There is also an international pediatric relapsed AML phase I trial with PKC412. At St. Jude Children's Research Hospital sorafenib is tested for its potential to inhibit FLT3-aberrant leukemias. Most likely, this will be the first of many trials with specific inhibitors to treat AML, and will hopefully improve outcome.

Next to genetic targeting, several agents have been developed which reverse epigenetic changes in leukemic cells. Various combinations of small-molecule HDAC-inhibitors (e.g., vorinostat and entinostat) and DNA methyltransferases (e.g., azacitidine and decitabine) are being studied extensively in adult AML[69-71]. Since these studies show promising initial results, pediatric phase I trials are being planned.

Therefore, to improve outcome, it will be necessary to develop novel therapeutic strategies. This includes specific targeted treatment of the leukemic cell. However, the first results are not as spectacular as in CML with imatinib. Further research is warranted to successfully use targeted therapy in AML.

MLL-REARRANGED PEDIATRIC AML: A HETEROGENEOUS DISEASE

MLL-rearrangements are the most frequent cytogenetic aberrations in pediatric AML. The true incidence of *MLL*-rearrangements in pediatric AML is in the range of 15-25% according to the latest trials, with a high incidence of ~50% in infants and a lower incidence in children who are older. Recent studies show that the *MLL*-group itself is also genetically heterogeneous, as more than 60 different translocation partners of the *MLL*-gene are described[72]. Approximately 50% of the pediatric AML cases with an *MLL*-rearrangement consist of t(9;11)(p22;q23). The other 50% predominantly include t(6;11)(q27;q23), t(10;11)(p12;q23), t(11;19)(q23;p13.1) and t(11;19)(q23;p13.3)[49]. Moreover, in *MLL*-rearranged pediatric AML still new translocation partners are found, for instance we recently discovered 2 new translocation partners of *MLL*, i.e. NRIP3 and KIAA1524[73,74].

The heterogeneity of *MLL*-rearranged pediatric AML is further reflected by differences in additional genetic and molecular aberrations. We showed that 50% of the *MLL*-rearranged pediatric AML cases harbored a molecular aberration, and ~70% of these mutations were identified in genes involved in the RAS-pathway, including mutations in *NRAS*, *KRAS*, *PTPN11* and *NF1*. This suggests that RAS-pathway aberrations play an important role in the development of *MLL*-rearranged pediatric AML. In the other 50% of cases a second aberration could not be identified, and therefore we used gene expression profiling to identify new molecular markers in *MLL*-rearranged pediatric AML. In

addition, it will be of interest to see if the RAS-pathway is also aberrantly expressed in those cases without mutations identified in genes involved in the RAS-pathway, indicating a pivotal role in the leukemogenesis of *MLL*-rearranged AML. Therefore, further research on protein-level is ongoing

A gene which showed differential expression in *MLL*-rearranged AML cases is *EVI1*. We showed that overexpression of *EVI1* is mainly associated with a subset of *MLL*-rearranged pediatric AML, i.e. t(6;11)(q27;q23)[44,45]. Although this suggests an important role for *EVI1* in the leukemogenesis in these specific cases, direct evidence of an oncogenic effect of *EVI1* in this leukemia is currently lacking. Furthermore, we found high expression of *BRE* in *MLL*-rearranged AML with t(9;11)(p22;q23)[75], and overexpression of *IGSF4*, by epigenetic regulation, in t(9;11)(p22;q23) with FAB-M5 morphology[76]. The exact role of these genes in leukemogenesis of *MLL*-rearranged AML needs to be further elucidated.

Epigenetic differences were found in *MLL*-rearranged ALL based on translocation partner[39]. Further unraveling pediatric AML based on genetic, epigenetics, mi-RNAs and functional studies may lead to specific therapeutic targets in *MLL*-rearranged AML.

DIFFERENT OUTCOME IN *MLL*-REARRANGED AML BASED ON TRANSLOCATION PARTNER

In the past, *MLL*-rearranged AML was associated with a poor outcome. However, optimized intensive treatment regimens for AML have also improved outcome for *MLL*-rearranged AML[49]. In the past, some study groups showed that t(9;11)(p22;q23) had a favorable outcome and a higher sensitivity to cytostatic drugs, such as cytarabine, compared with the other *MLL*-rearrangements[77-80]. Within a large intergroup analysis we showed that within the t(9;11)(p22;q23) subgroup, prognosis appeared to be related to the cell of origin[49].

We identified the t(1;11)(q21;q23) as a new favorable prognostic subgroup in pediatric AML with an excellent clinical outcome[49]. This indicates that this subtype of *MLL*-rearranged AML should be treated with less intensive chemotherapy to avoid side effects due to cytotoxicity in these children. In contrast, the t(10;11)(p12;q23) and t(6;11)(q27;q23) subgroup carries a very poor prognosis. These large differences in outcome, mainly based on translocation partner, suggest differences in biology between these subtypes of *MLL*-rearranged AML. Identifying the role of specific genes or biological pathways within each subtype, mainly those with a poor outcome, could lead to targeted treatment and improvement of outcome in the future. Since prognosis in *MLL*-rearranged AML depends on the translocation partner, specific abnormalities which have a strong influence on outcome, such as t(1;11(q21;q23), t(10;11)(p12;q23) and t(6;11)(q27;q23), should specifically be screened for in future AML protocols for further risk group stratification.

This would mean that standard *MLL*-screening with FISH is not sufficient and additional standardized screening methods should be developed.

NOVEL TREATMENT STRATEGIES FOR *MLL*-REARRANGED AML

Improving the outcome in pediatric *MLL*-rearranged AML with current intensive treatment protocols is hampered by higher treatment-related mortality, as previously also mentioned for pediatric AML in general. Therefore, to improve outcome, development of novel therapeutic strategies is an important challenge. In *MLL*-rearranged AML, high expression of *FLT3* was found in almost all cases, indicating that FLT3-inhibitors, such as PKC412, may be an effective class of drugs, although clinical responses to this drug are limited [64-67].

In *MLL*-rearranged AML the RAS/RAF/MEK-pathway is currently the only pathway with known mutations. Therefore patients harboring these mutations could benefit from RAS pathway inhibition. RAS activation depends on post-translational farnesylation and inhibitors of farnesyltransfarase could be suitable targeted drugs. Unfortunately, the activity of tipfarnib did not show any correlation with RAS mutations nor with pathway dependent activation in adult AML, indicating that the anti-leukemic activity of tipfarnib may be due to other mechanisms than RAS inhibition[81]. RAF can be inhibited by sorafenib that also targets FLT3 mutated AML cells[82], and current phase I/II trials with this drug are ongoing in AML[83,84]. Sorafenib in children with de novo *FLT3*-ITD and relapsed/refractory AML inhibited aberrant tyrosine kinase signaling in leukemic cells, indicating that further research is warranted[85]. Inhibitors of MEK have been developed that sensitize leukemic blast cells to other drugs, e.g. arsenic trioxide and demethylating agents[86-88]. Current RAS pathway inhibitors probably will not fully block the leukemic transformation in *MLL*-rearranged AML, but may have additive effect with current treatment strategies by targeting the proliferative advantage of these leukemic cells[89].

Finally, it would be of importance to identify mutations or aberrant expression of specific genes or signaling pathways that are involved in those subtypes with a poor outcome, e.g. t(6;11)(q27;q23). Liedtke *et al.* identified a highly conserved Ras association (RA1) domain at the amino-terminus of AF6 as the minimal region sufficient for hematopoietic transformation and leukemogenesis in MLL-AF6, the fusion protein in t(6;11)(q27;q23)[90]. It would be of interest to study the functional effect of targeting this domain in MLL-AF6. Using gene expression profiling we were also able to identify a specific gene expression signature for t(6;11)(q27;q23). Further studies will be necessary to identify the role of the individual genes that determine this signature in the leukemogenesis of t(6;11)(q27;q23)-AML.

CONCLUSIONS

In conclusion, the heterogeneity of pediatric AML is reflected by differences in cytogenetic and molecular aberrations. However, current treatment protocols consider pediatric AML as a homogeneous disease. Moreover, further intensification of chemotherapy is not possible as it results in even higher morbidity and mortality. Therefore, future treatment strategies have to focus on the development of targeted therapy to further improve survival in specific subgroups of AML. Eventually, this could lead to personalized treatment strategies in pediatric AML based on the differences in biological background and cooperating events leading to the development of AML.

The *MLL*-rearranged pediatric AML group, which comprises 15-20% of pediatric AML cases, is heterogeneous in itself, mainly based on translocation partner and molecular markers. Although cooperating events are a hallmark of developing AML, additional genetic aberrations in *MLL*-rearranged AML have hardly been identified. To achieve further improvements in outcome, further dissection of the biology of *MLL*-rearranged AML and pediatric AML as a whole is warranted.

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Chapter 13

Nederlandse samenvatting

DEEL I: DE GENETISCHE EN MOLECULAIRE EIGENSCHAPPEN VAN AML IN KINDEREN

Van alle soorten leukemie bij kinderen gaat het in 15-20% om acute myeloïde leukemie (AML). Ondanks intensieve chemotherapie, overleeft momenteel slechts 60-70% van de kinderen met AML. Daarnaast is AML een heterogene ziekte, met verschillen in morfologie (FAB-type), immunofenotype en cytogenetische en moleculaire afwijkingen. Cytogenetische en moleculaire afwijkingen en de respons op de behandeling zijn de belangrijkste prognostische factoren bij kinderen met AML.

In **hoofdstuk 2** presenteren we een overzicht van de verschillende type-I en type-II afwijkingen in kinderen met AML. Type-I afwijkingen zijn afwijkingen die leiden tot ongeremde groei (proliferatie) van de leukemiecel, terwijl type-II afwijkingen leiden tot het niet uitrijpen van deze cellen (differentiatie stop). De heterogeniteit van AML in kinderen wordt weerspiegeld door de verschillende soorten type-I en type-II afwijkingen en bijbehorende verschillen in prognose. Tevens, hebben we vaste associaties gevonden tussen moleculaire afwijkingen en cytogenetische subgroepen, zoals *KIT* mutaties met CBF-AML en *FLT3*-ITD met t(15;17)(q22;q21) of normaal karyotype. Opvallend is dat er verschillen zijn in moleculaire en genetische afwijkingen bij kinderen met AML jonger en ouder dan twee jaar bij diagnose. Zeer jonge kinderen met AML worden namelijk gekenmerkt door een hoge frequentie van *MLL*-gen herschikkingen, t(7;12)(q36;p13) en t(8;16)(p11;p13), terwijl t(8;21)(q22;q22), t(15;17); (q22;q21), CN-AML en de moleculaire afwijkingen *CEBPA*, *NPM1*, *WT1* en *FLT3*-ITD voornamelijk worden gevonden boven de leeftijd van 2 jaar.

In **hoofdstuk 3** hebben we ons gericht op de mogelijkheden van gen expressie profilering (GEP) met het doel verschillende genetische subgroepen van kinderen met AML correct te voorspellen om zo de diagnostiek te versimpelen. Momenteel zijn er namelijk verschillende diagnostische methoden (zoals cytomorfologie, FISH, RT-PCR en karyotypering) nodig om deze groepen te karakteriseren. Met behulp van microarrays werden gen expressie profielen gegenereerd van 237 kinderen met AML, en werd gekeken of de meest belangrijke groepen in kinderen met AML hiermee voorspeld konden worden. Uiteindelijk bleek dat met 75 probe sets 5 cytogenetische subgroepen [*MLL*-gen herschikkingen, t(8;21), inv(16), t(15;17) en t(7,12)] correct konden worden voorspeld. Het was echter niet mogelijk om de bekende moleculaire afwijkingen (zoals *NPM1*, *WT1* en *FLT3*-ITD) met prognostische waarde in kinderen met AML te voorspellen. Dit zou mogelijk kunnen komen door de lage frequentie van sommige van deze afwijkingen, maar eventueel ook door de invloed van de onderliggende cytogenetische afwijking. Dit laatste was het geval voor de *FLT3*-ITD subgroep, aangezien we verschillende gen expressie profielen vonden voor *FLT3*-ITD in AML met een normaal karyotype, welke gerelateerd waren aan upregulatie van de *HOXB*-genen in vergelijking met *FLT3*-ITD in

t(15;17). De conclusie is dat gen expressie profilering kan leiden tot het aanpassen van de diagnostische procedure in ten minste 40% van de kinderen met AML.
In **hoofdstuk 4** beschrijven we dat overexpressie van het *EVI1*-gen in kinderen met AML te vinden is in 9% van de patiënten, en gerelateerd is aan specifieke cytogenetische (*MLL*-gen herschikkingen en monosomie 7) en morfologische (FAB-M6/7) subgroepen. Echter, typische chromosoom 3q26 afwijkingen met overexpressie van het *EVI1*-gen zoals gevonden in volwassenen met AML konden in kinderen met AML niet worden geïdentificeerd. Dit zou kunnen betekenen dat er verschillen zijn in de rol van *EVI1* overexpressie in volwassenen met AML ten opzichte van kinderen. Hoewel *EVI1* overexpressie, in tegenstelling tot volwassen, geen onafhankelijk prognostische factor was bij kinderen, werd het wel voornamelijk gevonden in subgroepen van pediatrische AML die gepaard gaan met een ongunstige prognose, zoals *MLL-AF6* en monosomie 7.

DEEL II: DE HETEROGENITEIT VAN *MLL*-GEN HERSCHIKKINGEN IN KINDEREN MET AML

Translocaties van chromosoom 11q23, waar het *MLL*-gen op gelokaliseerd is, worden gezien in 15-20% van de kinderen met AML, en zijn in het verleden vaak geassocieerd met een slechte prognose. Tot nu toe zijn er al meer dan 50 verschillende translocatie partners van het *MLL*-gen beschreven. Het *MLL*-gen codeert voor een DNA bindend eiwit dat de expressie van specifieke genen reguleert. Door *MLL*-gen herschikkingen wordt dit proces verstoord, waardoor disregulatie van specifieke genen die een rol hebben in de ontwikkeling van bloedcellen, zoals de *HOX*-genen, kan leiden tot het niet uitrijpen van deze cellen.
Hoofdstuk 5 is een review van de heterogeniteit van *MLL*-gen herschikkingen in kinderen met AML. In dit hoofdstuk wordt de etiologie, klinische kenmerken en de prognose op basis van de huidige literatuur samengevat. Daarnaast bespreken we de toekomstige therapeutische mogelijkheden voor deze groep kinderen met AML.
In **hoofdstuk 6** wordt een unieke en omvangrijke internationale retrospectieve studie beschreven, uitgevoerd in samenwerking met 11 onderzoeksgroepen uit 15 landen, waarin klinische en prognostische data van 756 kinderen met AML en een 11q23/*MLL*-gen herschikking werden verzameld. Dit leidde tot de identificatie van nieuwe, onafhankelijke prognostische subgroepen binnen *MLL*-gen herschikte AML in kinderen. Dit zijn t(1;11)(q21;q23) met een gunstige prognose (overleving 100%), en t(10;11)(p12;q23), t(10;11)(p11.2;q23) en t(6;11)(q27;q23) met een slechte prognose (overleving minder dan 45%). Daarom adviseren we dat deze subgroepen onderdeel zouden moeten uitmaken van de risicostratificatie in toekomstige AML protocollen bij kinderen. Dit houdt tevens in dat de aanwezigheid van deze afwijkingen specifiek onderzocht moet worden bij de initiële

diagnose. De patiënten met t(9;11)(p22;q23) hadden een gemiddelde prognose, echter ook deze groep bleek onderlinge heterogeniteit te vertonen, waarbij verschillen in morfologie (FAB-type) samen ging met verschillen in prognose.
Nog steeds worden nieuwe translocatie partners van het *MLL*-gen gevonden. In **hoofdstuk 7** beschrijven we een nieuwe translocatie partner van het *MLL*-gen op chromosoom 11 in een kind met AML met een complexe herschikking van chromosoom 3 en 11. Gebruikmakend van een relatief nieuwe techniek, namelijk LDI-PCR uitschrijven, werd het *NRIP3*-gen, gelokaliseerd op chromosoom 11p15, als translocatie partner gevonden, terwijl de rest van het *MLL*-gen gefuseerd was met chromosoom 3.
In **hoofdstuk 8** tonen we microdeleties in het *NF1*-gen aan bij een aantal AML patiënten met *MLL*-gen herschikkingen zonder symptomen van neurofibromatose, door middel van array-CGH, een onderzoeksmethode waarmee kleinere deleties en amplificaties in chromosomen opgespoord kunnen worden. NF1 is een belangrijke regulator van het RAS proliferatie route, welke vaak geactiveerd is in tumoren en leukemie. In deze studie bevestigden we dat *NF1* geïnactiveerd was met verminderde *NF1* expressie niveaus, wat veroorzaakt werd door bi-allelische *NF1* mutaties in 3 van de 5 patiënten. Hierdoor lijkt *NF1* een tumor-suppressor gen, welke geïnactiveerd kan zijn in *MLL*-gen herschikte pediatrische AML.
In **hoofdstuk 9** werd met behulp van GEP van 53 kinderen met AML en een *MLL*-gen herschikking gezocht naar genen die mogelijk de verschillen in prognose konden verklaren tussen de verschillende *MLL*-gen herschikkingen in kinderen met AML. Daaruit bleek het *BRE*-gen hoog tot expressie in t(9;11)(p22;q23) te komen in vergelijking met andere *MLL*-gen subtypen ($p<0,001$). Bovendien bleek hoge *BRE* expressie een onafhankelijke gunstige prognostische factor voor ziektevrije overleving in kinderen met AML. Hoe overexpressie van *BRE* bijdraagt aan de leukemogenesis is echter nog niet duidelijk en zal nog verder uitgezocht moeten worden.
Naast herschikkingen met verschillende genen bestaan er ook herschikkingen in het *MLL*-gen zelf als gevolg van gedeeltelijke tandem duplicatie (*MLL*-PTD), wat inhoud dat bepaalde delen van het *MLL*-gen vermenigvuldigd zijn in het gen zelf. *MLL*-PTD is te vinden in 3-5% van de volwassen patiënten met AML en wordt geassocieerd met een slechte prognose. In **hoofdstuk 10** blijkt dat de frequentie van de *MLL*-PTD bij kinderen met AML zeer laag is en mogelijk is overschat in eerdere studies. Met behulp van DNA-MLPA als een nieuwe screening methode voor het *MLL*-PTD in combinatie met mRNA RT-PCR bleek de frequentie slechts 2,4% bij kinderen met AML.


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About the Author

CURRICULUM VITAE

Brian Vinod Balgobind werd geboren op 30 mei 1979 te Delfzijl, een havenstad gelegen in het Noordoosten van Nederland. In 1997 behaalde hij zijn VWO/Gymnasium diploma aan het Thorbecke Scholengemeenschap/Spectrum College te Utrecht. Hierna heeft hij nog 2 jaar Bio-Farmaceutische Wetenschappen gestudeerd aan de Universiteit van Leiden, waarna hij in 1999 begon aan de opleiding Geneeskunde aan de Universiteit van Utrecht. Tijdens de opleiding heeft hij diverse stages gelopen op het Laboratorium Kinderimmunologie in het Wilhelmina Kinderziekenhuis (supervisor: Dr. J. Frenkel, kinderarts). Na het behalen van zijn arts-examen in 2005 is Brian als promovendus begonnen op de afdeling Kinderoncologie in het Erasmus MC/Sophia Kinderziekenhuis (promotor: Prof. Rob Pieters, kinderoncoloog). Hier vond het onderzoek plaats beschreven in dit proefschrift. Tevens haalde hij in 2008 de NWO Mozaïek grant binnen. Thans werkt Brian als arts-assistent niet in opleiding in het Maasstad Ziekenhuis op de afdeling Kindergeneeskunde (supervisor Dr. C. Lincke, kinderarts) Vanaf 1 juni 2011 zal Brian starten als ANIOS Radiotherapie in het VU Medisch Centrum te Amsterdam, waarna hij na enkele maanden zal starten met de opleiding tot radiotherapeut-oncoloog (supervisor Prof.dr. B.J. Slotman, radiotherapeut-oncoloog).

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PHD PORTFOLIO

Summary of PhD training and teaching

Name PhD student: Brian Balgobind	PhD period: 2005-2009
Erasmus MC Department: Pediatric Oncology	Promotor(s): Prof. dr. R. Pieters
Research School: Molecular Medicine	Supervisor: Dr. M.M. van den Heuvel-Eibrink and Dr. C.M. Zwaan

1. PhD training

	Year	Workload (ECTS)
General courses		
- Biomedical English Writing and Communication	2008	4 ECTS
- Statistics: Classical Methods for Data-analysis	2007	5.7 ECTS
Specific courses (e.g. Research school, Medical Training)		
- Basic Course on R	2007	1.4 ECTS
Seminars and workshops		
- 2nd Erasmus Workshop on Molecular Therapeutics in Acute Leukemia, Rotterdam	2005	0.6 ECTS
	2005-2009	0.5 ECTS
- Jaarlijkse Sophia Kindergeneeskunde Onderzoeksdag, Rotterdam	2006-2009	0.8 ECTS
- Annual Molecular Medicine Day, Rotterdam	2006	0.1 ECTS
- Workshop Array-CGH, Leiden	2007	1.4 ECTS
- 4th ASPO Master Class Pediatric Oncology, Noordwijk aan Zee	2007, 2008	1.0 ECTS
- AML-BFM Research Symposium, Hannover, D	2008	0.1 ECTS
- Erasmus MC Wetenschapsdag 2008, Rotterdam	2008	0.2 ECTS
- Dag voor jonge onderzoekers, Nederlandse Vereniging voor Kindergeneeskunde, Veldhoven		
Oral Presentations		
- "Retrospective 11q23 study in pediatric AML", 18th Annual Meeting of the I-BFM Study Group	2007	1 ECTS
- "NF1 Microdeletions in Pediatric MLL-Rearranged AML and T-ALL: A Novel Mechanism for RAS Activation", 49th ASH Annual Meeting	2007	1 ECTS
- "NF1 Microdeletions in Pediatric MLL-Rearranged AML and T-ALL: A Novel Mechanism for RAS Activation", Erasmus MC Wetenschapsdag 2008	2008	1 ECTS
	2008	1 ECTS
- "Differences in cyto- and molecular genetic aberrations between children <2 years and older children with acute myeloid leukemia", 6th Bi-annual Childhood Leukemia Symposium	2008	1 ECTS
- "Differences in cyto- and molecular genetic aberrations between children <2 years and older children with acute myeloid leukemia", 13th EHA Annual Congress	2008	1 ECTS
	2008	1 ECTS
- "Type I/II mutation in pediatric AML, AML-BFM Research Symposium		
- "Cytogenetic subgroups in pediatric acute myeloid leukemia are accurate predicted with gene expression profiling", 40th SIOP Annual Meeting	2008	1 ECTS

- "The clinical significance of EVI1 overexpression in pediatric acute myeloid leukemia", 40th SIOP Annual Meeting	2009	1 ECTS
- "Novel prognostics subgroups in childhood 11q23/MLL-rearranged acute myeloid leukemia as defined by translocation partners: a retrospective international study", 20th Annual Meeting of the I-BFM Study Group	2009	1 ECTS
- Novel prognostics subgroups in childhood 11q23/MLL-rearranged acute myeloid leukemia as defined by translocation partners: a retrospective international study, 14th EHA Annual Congress	2009	1 ECTS
- "Nieuwe inzichten in genetica van AML", Kinderoncologisch Centrum Rotterdam (KOCR) Symposium	2009	1 ECTS
- "MLL-rearranged AML: a heterogeneous disease", Sophia Kindergeneeskunde Onderzoekersdag	2005-2009	4 ECTS
- 6 Oral presentations at Research Meetings Pediatrics/Pediatric Oncology		
(Inter)national conferences		
- 5th Bi-annual Childhood Leukemia Symposium, Noordwijkerhout, NL	2006	0.6 ECTS
- 6th Bi-annual Childhood Leukemia Symposium, Glasgow, GB	2008	0.6 ECTS
- 17th Annual Meeting of the I-BFM Study Group, Noordwijkerhout, NL	2006	0.6 ECTS
- 18th Annual Meeting of the I-BFM Study Group, Brugges, B	2007	0.6 ECTS
- 20th Annual Meeting of the I-BFM Study Group, Bergamo, I	2009	0.6 ECTS
- 48th ASH Annual Meeting, Orlando, USA	2006	1 ECTS
- 49th ASH Annual Meeting, Atlanta, USA	2007	1 ECTS
- 50th ASH Annual Meeting, San Francisco, USA	2008	1 ECTS
- 51th ASH Annual Meeting, New Orleans, USA	2009	1 ECTS
- 13th EHA Annual Congress, Copenhagen, DK	2008	1 ECTS
- 14th EHA Annual Congress, Berlin, D	2009	1 ECTS
- 40th SIOP Annual Meeting, Berlin, D	2008	1 ECTS
Other		
- NWO Mozaiek Grant 2008	2008	3 ECTS
2. Teaching		
	Year	**Workload (ECTS)**
Supervising Medical Students		
- Supervising medical student Jenny Kuipers: The role of IGSF4 and ADAM23 in MLL-rearranged AML	2008-2009	10 ECTS
Total		**54.8 ECTS**

ABBREVIATIONS

AIEOP	Associazione Italiana Ematologia Oncologia Pediatrica
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
APL	acute promyelocytic leukemia
array-CGH	array-comparative genomic hybridization
ATRA	all-trans retinoic acid
AU	arbitrary units
BFM	Berlin-Frankfurt-Münster Study Group
CBF	core binding factor
CIR	cumulative incidence of relapses
CML	chronic myeloid leukemia
CN-AML	cytogenetically normal AML
CNS	central nerve system
COALL	Co-operative study group for Childhood Acute Lymphoblastic Leukemia
COG	children's oncology group
CPH	Czech Pediatric Hematology Working Group
CR	complete remission
DCOG	Dutch Children's Oncology Group
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
EFS	event-free survival
FAB	French-American-British
FDR	false discovery rate
FISH	Fluorescent in situ hybridization
FTI	inhibitors of farnesyltransfarase
GEO	Gene Expression Omnibus
GEP	gene expression profiling
HDAC	histone deacetylase
HR	hazard ratio
HSC	hematopoietic stem cells
HSCT	hematopoietic stem cell transplantation
ITD	Internal tandem duplication
JMML	juvenile myelomonocytic leukemia
JPLSG	Japanese Pediatric Leukemia/Lymphoma Study Group
LAME	Leucémies Aiguës Myéloblastiques de l'Enfant Cooperative Group
LDI	long-distance inverse

MDS	myelodysplastic syndrome
MILE	Microarray Innovations-in-LEukemia
miRNA	microRNA
MLPA	multiplex ligation-dependent probe amplification
MRC	Medical Research Council
NOPHO	Nordic Society for Pediatric Hematology and Oncology
OS	overall survival
PAC	P1-derived artificial chromosome
PTD	partial tandem duplications
RFS	relapse free survival
RNA	Ribonucleic acid
RQ-PCR	real-time quantitative polymerase chain reaction
RT-PCR	reverse transcription polymerase chain reaction
SB	Southern blot
SNP	single nucleotide polymorphism
TKD	tyrosine kinase domain
UPD	uniparental disomy
VSN	variance stabilization normalization
WBC	white blood cell count
WHO	World Health Organization
WT	wild type

Genes:

ABI1	abl-interactor 1
AFF1/AF4	AF4/FMR2 family, member 1
ASH2L	ash2 (absent, small, or homeotic)-like
BAD	BCL2-associated agonist of cell death
BRE	brain and reproductive organ-expressed
CADM1/IGSF4	cell adhesion molecule 1
CALM/PICALM	phosphatidylinositol binding clathrin assembly protein
CBFB	core-binding factor, beta subunit
CEBPA	CCAAT/enhancer binding protein (C/EBP), alpha
DOT1L	DOT1-like, histone H3 methyltransferase
ERG	v-ets erythroblastosis virus E26 oncogene homolog
ETV6	ets variant 6
EVI1	ecotropic viral integration site 1
FBXW7	F-box and WD repeat domain containing 7
FLT3	fms-related tyrosine kinase 3

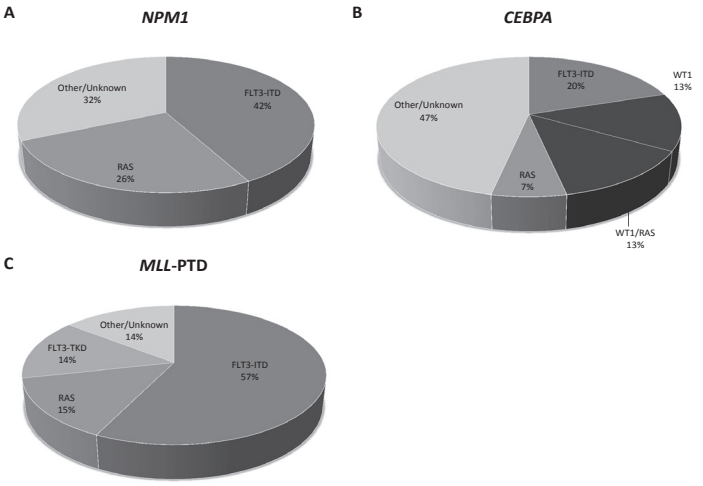
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GATA1	GATA binding protein 1
GSK3	glycogen synthase kinase 3
HOX	homeobox gene
IDH2	isocitrate dehydrogenase 2 (NADP+)
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene
MAPK1/ERK	mitogen-activated protein kinase 1
MDS1	myelodysplasia syndrome 1
MEIS1	Meis homeobox 1
MEN1	multiple endocrine neoplasia I
MLL	mixed-lineage leukemia
MLLT1/ENL	mixed-lineage leukemia; translocated to, 1
MLLT10/AF10	mixed-lineage leukemia; translocated to, 10
MLLT11/AF1q	mixed-lineage leukemia; translocated to, 11
MLLT3/AF9	mixed-lineage leukemia; translocated to, 3
MLLT4/AF6	mixed-lineage leukemia; translocated to, 4
MLLT6/AF17	mixed-lineage leukemia; translocated to, 6
MN1	meningioma (disrupted in balanced translocation) 1
MNX1/HLXB9	motor neuron and pancreas homeobox 1
MYC	v-myc myelocytomatosis viral oncogene homolog
MYH11	myosin, heavy chain 11, smooth muscle
MYST1	MYST histone acetyltransferase 1
NF1	neurofibromin 1
NOTCH1	notch 1
NPM1	nucleophosmin
NRIP3	nuclear receptor interacting protein 3
NUP98	nucleoporin 98kDa
PBX1	pre-B-cell leukemia homeobox 1
PML	promyelocytic leukemia
PSIP1	PC4 and SFRS1 interacting protein 1
PTPN11	protein tyrosine phosphatase, non-receptor type 11
RAF1	v-raf-1 murine leukemia viral oncogene homolog 1
RARA	retinoic acid receptor, alpha
RAS	rat sarcoma viral oncogene homolog
RUNX1/AML1	runt-related transcription factor 1
RUNX1T1/ETO	runt-related transcription factor 1; translocated to, 1
RUNX2	runt-related transcription factor 2
serpinB2	serpin peptidase inhibitor, clade B (ovalbumin), member 2

SUZ12/JJAZ1	suppressor of zeste 12 homolog
TAL1	T-cell acute lymphocytic leukemia 1
TLX3/HOX11L2	T-cell leukemia homeobox 3
VEGFC	vascular endothelial growth factor C
WDR5	WD repeat domain 5
WT1	Wilms tumor 1

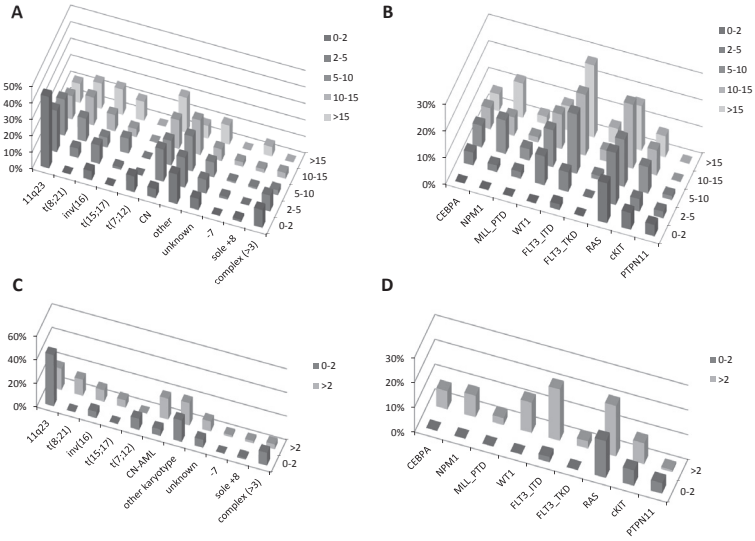
Appendix A

Supplementary data

Chapter 2



Supplementary Figure 1: Distribution of type-I aberrations in *NPM1, CEBPA* double and *MLL-PTD* mutated AML. Different type-I aberrations can be found for the type-II gene aberrations *NPM1* (A), *CEBPA* (B) and *MLL-PTD* (C). *FLT3*-ITD and *WT1* mutations show large differences in frequencies between the subgroups.



Supplementary Figure 2. The different genetic aberrations according to age. Differences were found for cytogenetic (A) and molecular (B) aberrations according to age categories in pediatric AML. The most importance differences were found between children older and younger than 2 years for cytogenetic (C) and molecular (D) differences

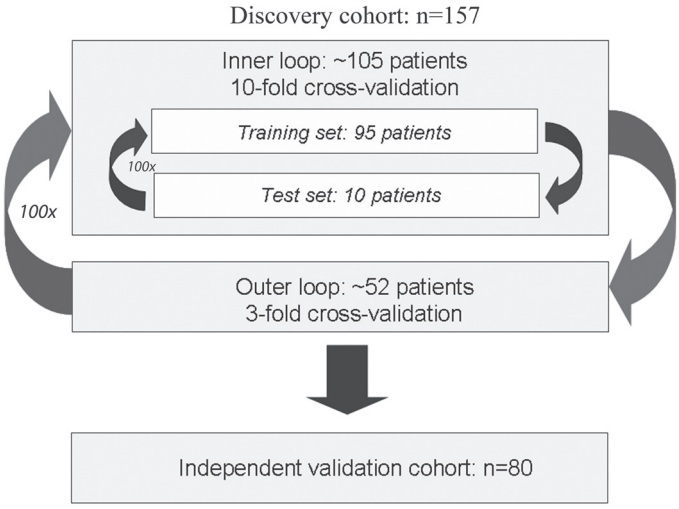
Supplementary Table 1: Comparison of the clinical characteristics of the total cohort (n=442) compared to the AML-BFM-93 and -98 trials respectively.

	This study	AML-BFM 93	AML-BFM 98	p-value
N	442	471	473	
Sex				
Male	250 (57%)	255 (54%)	253 (54%)	0.62
Age				
< 10 yrs	247 (56%)	286 (61%)	259 (55%)	0.14
≥ 10 yrs	195 (44%)	185 (39%)	214 (45%)	
WBC				
< 100 * 10^9/L	229 (76%)	384 (81%)	378 (80%)	0.11
≥ 100 * 10^9/L	75 (24%)	87 (19%)	95 (20%)	
FAB-morphology				
M0	23 (5%)	25 (5%)	21 (4%)	0.16
M1	43 (10%)	55 (12%)	68 (14%)	
M2	94 (21%)	125 (27%)	123 (26%)	
M3	27 (6%)	23 (5%)	30 (6%)	
M4	102 (23%)	91 (19%)	94 (20%)	
M5	113 (26%)	101 (21%)	97 (21%)	
M6	5 (1%)	16 (3%)	13 (3%)	
M7	17 (4%)	31 (7%)	25 (5%)	
Other/unknown	18 (4%)	4 (1%)	1 (0%)	

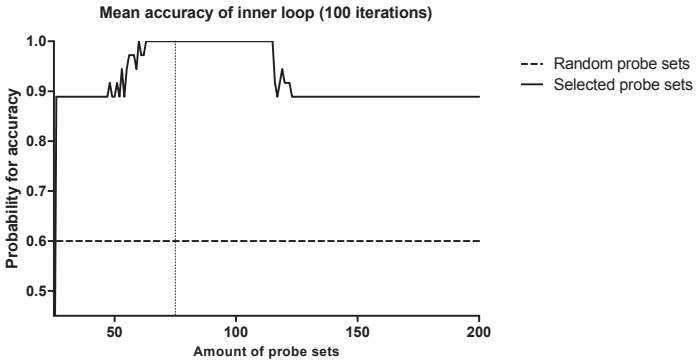
Supplementary Table 1B: Comparison of the clinical characteristics of the cases included in the survival analysis (n=317) compared to the AML-BFM-93 and -98 trials.

	This study	AML-BFM 93	AML-BFM 98	p-value
N	317	471	473	
Sex				
Male	186 (59%)	255 (54%)	253 (54%)	0.32
Age				
< 10 yrs	179 (56%)	286 (61%)	259 (55%)	0.17
≥ 10 yrs	138 (44%)	185 (39%)	214 (45%)	
WBC				
< 100 * 10^9/L	198 (76%)	384 (81%)	378 (80%)	0.19
≥ 100 * 10^9/L	63 (24%)	87 (19%)	95 (20%)	
FAB-morphology				
M0	17 (5%)	25 (5%)	21 (4%)	0.22
M1	30 (10%)	55 (12%)	68 (14%)	
M2	67 (21%)	125 (27%)	123 (26%)	
M3	19 (6%)	23 (5%)	30 (6%)	
M4	73 (23%)	91 (19%)	94 (20%)	
M5	84 (27%)	101 (21%)	97 (21%)	
M6	4 (1%)	16 (3%)	13 (3%)	
M7	14 (4%)	31 (7%)	25 (5%)	
Other/unknown	9 (3%)	4 (1%)	1 (0%)	

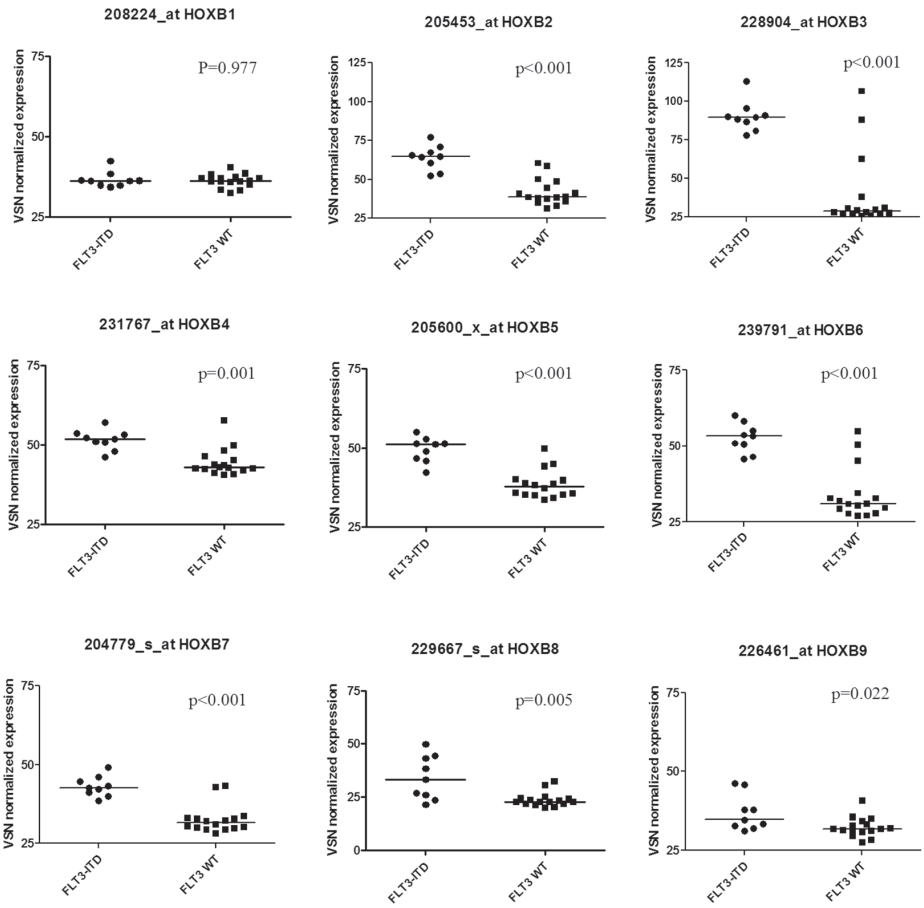
Chapter 3



Supplementary Figure 1: Identification of a gene-expression signature enabling classification of pediatric AML . The discovery cohort was used to estimate the number of probe sets in the inner loop (2/3 of the patients) and the prediction accuracy in the outer loop (remaining 1/3 of the patients). The final classifier was constructed on the total discovery cohort (n=157) and this was tested on the validation cohort (n=80) to determine the true accuracy of the classifier.



Supplementary Figure 2. Estimated sensitivity within the inner loop. The median sensitivity was calculated using 100 iterations. Probe sets selected for the different subgroups yielded a higher sensitivity compared with random selected probe sets. A minimum of 75 probe sets was needed to have a median sensitivity of 100%



Supplementary Figure 3. Gene expressions of the *HOXB* cluster between *FLT3*-ITD+ and *FLT3*-ITD- in CN-AML. Within CN-AML, all patients positive for *FLT3*-ITD show higher expression of *HOXB2* to *HOXB9*. Also 3 patients without *FLT3*-ITD show this expression, including 2 patients with an *NPM1* mutation.

Supplementary Table 1: Distribution of cytogenetic subtypes in current study compared to the average frequency observed in other pediatric AML studies.

	Current Study (%)	Pediatric AML studies (%)
Cytogenetic subtypes		
MLL-rearrangements	*20*	*18*
t(8;21)(q22;q22)	*11*	*13*
inv(16)(p13q22)	*11*	*6*
t(15;17)(q21;q22)	*9*	*4*
t(7;12)(q36;p13)	*3*	*ND*
CN-AML	*15*	*24*

ND = not determined.

Supplementary Table 2: Number of probe sets at different cut-off p-values per cytogenetic subtype (total cohort).

MLL	t(8;21)	inv(16)	t(15;17)	t(7;12)	CN-AML	Remaining cytogenetics	AML-unknown	
FDR adjusted p-values								
p<1.0E-08	1171	247	138	604	31	2	0	0
p<1.0E-06	2031	431	260	1027	59	4	1	0
p<1.0E-04	3886	853	610	2125	118	6	2	0
p<0.001	5829	1431	994	3477	182	12	6	1
p<0.005	7943	2210	1482	5478	325	25	47	2
p<0.01	9164	2752	1843	6586	453	47	93	5
p<0.05	13866	4804	3506	11491	1349	239	525	42

Supplementary Table 3: Overview of 75 probe sets used to classify pediatric AML in the present study.

Probe Set	Gene	Gene Symbol	Chromosome
244536_at	tumor protein p53 binding protein, 2 isoform 2	TP53BP2	chr1q42.12
228740_at	---	---	chr14q23.1
227949_at	phosphatase and actin regulator 3	PHACTR3	chr20q13.32-q13.33
1552665_at	hypothetical LOC84989	LOC84989	chr10q21.3
1567101_at	---	---	chr13q22.1
1557261_at	WAS protein homolog associated with actin, golgi membranes and microtubules-like 1 /// WAS protein homolog associated with actin, golgi membranes and microtubules-like 2 (pseudogene)	WHAMML1 /// WHAMML2	chr15q11.2 /// chr15q13.1
213908_at	WAS protein homolog associated with actin, golgi membranes and microtubules-like 1 /// WAS protein homolog associated with actin, golgi membranes and microtubules-like 2 (pseudogene)	WHAMML1 /// WHAMML2	chr15q11.2 /// chr15q13.1
1559265_at	chromosome 10 open reading frame 140	C10orf140	chr10p12.31
1559266_s_at	chromosome 10 open reading frame 140	C10orf140	chr10p12.31
1555923_a_at	chromosome 10 open reading frame 114	C10orf114	chr10p12.31
239503_at	---	---	chr10p12.31
209616_s_at	carboxylesterase 1 (monocyte/macrophage serine esterase 1)	CES1	chr16q13-q22.1
221858_at	TBC1 domain family, member 12	TBC1D12	chr10q23.33
235273_at	dyslexia susceptibility 1 candidate 1	DYX1C1	chr15q21.3
202746_at	integral membrane protein 2A	ITM2A	chrXq13.3-Xq21.2
202747_s_at	integral membrane protein 2A	ITM2A	chrXq13.3-Xq21.2
206059_at	zinc finger protein 91	ZNF91	chr19p13.1-p12
219765_at	zinc finger protein 329	ZNF329	chr19q13.43
201496_x_at	myosin, heavy chain 11, smooth muscle	MYH11	chr16p13.11
201497_x_at	myosin, heavy chain 11, smooth muscle	MYH11	chr16p13.11
207961_x_at	myosin, heavy chain 11, smooth muscle	MYH11	chr16p13.11
232716_at	lysophosphatidic acid receptor 1	LPAR1	chr9q31.3
241773_at	lysophosphatidic acid receptor 1	LPAR1	chr9q31.3
209386_at	transmembrane 4 L six family member 1	TM4SF1	chr3q21-q25
212850_s_at	low density lipoprotein receptor-related protein 4	LRP4	chr11p11.2-p12
238091_at	---	---	chr17p13.3

Supplementary Table 3: Continued

Probe Set	Gene	Gene Symbol	Chromosome
1553994_at	5'-nucleotidase, ecto (CD73)	NT5E	chr6q14-q21
227486_at	5'-nucleotidase, ecto (CD73)	NT5E	chr6q14-q21
212358_at	CAP-GLY domain containing linker protein 3	CLIP3	chr19q13.12
205330_at	meningioma (disrupted in balanced translocation) 1	MN1	chr22q11|22q12.1
212667_at	secreted protein, acidic, cysteine-rich (osteonectin)	SPARC	chr5q31.3-q32
222862_s_at	adenylate kinase 5	AK5	chr1p31
239519_at	neuropilin 1 isoform a	NRP1	chr10p11.22
203074_at	annexin A8 /// annexin A8-like 1 /// annexin A8-like 2	ANXA8 /// ANXA8L1 /// ANXA8L2	chr10q11.22
230244_at	chromosome 2 open reading frame 82	C2orf82	chr2q37.1
204150_at	stabilin 1	STAB1	chr3p21.1
38487_at	stabilin 1	STAB1	chr3p21.1
223828_s_at	lectin, galactoside-binding, soluble, 12	LGALS12	chr11q13
206634_at	SIX homeobox 3	SIX3	chr2p16-p21
210997_at	hepatocyte growth factor (hepapoietin A; scatter factor)	HGF	chr7q21.1
210998_s_at	hepatocyte growth factor (hepapoietin A; scatter factor)	HGF	chr7q21.1
205110_s_at	fibroblast growth factor 13	FGF13	chrXq26.3
229459_at	family with sequence similarity 19 (chemokine (C-C motif)-like), member A5	FAM19A5	chr22q13.32
229655_at	family with sequence similarity 19 (chemokine (C-C motif)-like), member A5	FAM19A5	chr22q13.32
214228_x_at	tumor necrosis factor receptor superfamily, member 4	TNFRSF4	chr1p36
227870_at	immunoglobulin superfamily, DCC subclass, member 4	IGDCC4	chr15q22.31
205614_x_at	macrophage stimulating 1 (hepatocyte growth factor-like)	MST1	chr3p21
219225_at	piggyBac transposable element derived 5	PGBD5	chr1q42.13
225275_at	EGF-like repeats and discoidin I-like domains 3	EDIL3	chr5q14
229349_at	lin-28 homolog B (C. elegans)	LIN28B	chr6q16.3-q21

Supplementary Table 3: Continued

Probe Set	Gene	Gene Symbol	Chromosome
203304_at	BMP and activin membrane-bound inhibitor homolog (Xenopus laevis)	BAMBI	chr10p12.3-p11.2
206363_at	v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)	MAF	chr16q22-q23
227370_at	family with sequence similarity 171, member B	FAM171B	chr2q32.1
209173_at	anterior gradient homolog 2 (Xenopus laevis)	AGR2	chr7p21.3
243339_at	---	---	chr2q14.3
207802_at	cysteine-rich secretory protein 3	CRISP3	chr6p12.3
214614_at	motor neuron and pancreas homeobox 1	MNX1	chr7q36
232136_s_at	cortactin binding protein 2	CTTNBP2	chr7q31
1564435_a_at	keratin 72	KRT72	chr12q13.13
203936_s_at	matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	MMP9	chr20q11.2-q13.1
210744_s_at	interleukin 5 receptor, alpha	IL5RA	chr3p26-p24
211517_s_at	interleukin 5 receptor, alpha	IL5RA	chr3p26-p24
206622_at	thyrotropin-releasing hormone	TRH	chr3q13.3-q21
1555943_at	phosphoglycerate mutase family member 5	PGAM5	chr12q24.33
216832_at	runt-related transcription factor 1; translocated to, 1 (cyclin D-related)	RUNX1T1	chr8q22
242845_at	---	---	chr8q21.3
216831_s_at	runt-related transcription factor 1; translocated to, 1 (cyclin D-related)	RUNX1T1	chr8q22
205529_s_at	runt-related transcription factor 1; translocated to, 1 (cyclin D-related)	RUNX1T1	chr8q22
205528_s_at	runt-related transcription factor 1; translocated to, 1 (cyclin D-related)	RUNX1T1	chr8q22
228827_at	---	---	chr8q21.3
225056_at	signal-induced proliferation-associated 1 like 2	SIPA1L2	chr1q42.2
233587_s_at	signal-induced proliferation-associated 1 like 2	SIPA1L2	chr1q42.2
204811_s_at	calcium channel, voltage-dependent, alpha 2/delta subunit 2	CACNA2D2	chr3p21.3
206940_s_at	POU class 4 homeobox 1	POU4F1	chr13q31.1
211341_at	POU class 4 homeobox 1	POU4F1	chr13q31.1

Legend: Probe sets are listed in the same order as in Figure 1B of the main paper.

Supplementary Table 4: Detailed cytogenetic data of misclassified cases in the discovery cohort.

ID	Subtype	Predicted	Karyotype	FISH	LDI-PCR
#1	AML-Other	*MLL*	51~53,XX,+2,+4,+6,+10,+add(11)(p?15), +13,-18,+21,+22,inc[cp10]	negative	*t(10;11)*
#2	AML-Other	*MLL*	46,XY	negative	*t(10;11)*
#3	AML-Other	*MLL*	46,XX	negative	*t(11;19)*

Supplementary Table 5. Predictive value of the classifier for relapsed and secondary pediatric AML cases.

		Subtype according to cytogenetic screening					
		MLL	**t(8;21)**	**inv(16)**	**t(15;17)**	**t(7;12)**	**AML-Other**
SVM Predicted Subtype	***MLL*-rearranged**	9	0	0	0	0	0
	t(8;21)	0	5	0	0	0	0
	inv(16)	0	0	0	0	0	0
	t(15;17)	0	0	0	0	0	0
	t(7;12)	0	0	0	0	0	0
	AML-Other	0	0	0	0	0	27

Supplementary Table 6: Diagnostic test values for the classification of cytogenetic subtypes in the validation cohort of pediatric AML cases.

	Independent validation cohort (n=80)		
	Pediatric AML		***Adult AML***
	Balgobind *et al*	Ross *et al* [ref16]	Valk *et al* [ref17]
	75 Probe sets	150 probe sets	2856 probe sets
	%	%	%
***MLL*-rearranged**			
% sensitivity	94	88	81
% specificity	100	98	98
% PPV	100	93	93
% NPV	98	96	95
% accuracy	99	96	95
t(8;21)			
% sensitivity	100	100	100
% specificity	100	100	100
% PPV	100	100	100
% NPV	100	100	100
% accuracy	100	100	100

Supplementary Table 6: Continued

	Independent validation cohort (n=80)		
	Pediatric AML		***Adult AML***
	Balgobind *et al*	Ross *et al* [ref16]	Valk *et al* [ref17]
	75 Probe sets	150 probe sets	2856 probe sets
	%	%	%
inv(16)			
% sensitivity	100	100	100
% specificity	100	100	100
% PPV	100	100	100
% NPV	100	100	100
% accuracy	100	100	100
t(15;17)			
% sensitivity	100	100	100
% specificity	100	100	100
% PPV	100	100	100
% NPV	100	100	100
% accuracy	100	100	100
t(7;12)			
% sensitivity	100	0	50
% specificity	100	100	100
% PPV	100	ND	100
% NPV	100	98	99
% accuracy	100	98	99
AML-Other			
% sensitivity	100	97	97
% specificity	98	91	91
% PPV	97	90	90
% NPV	100	98	98
% accuracy	99	94	94
Overall			
% sensitivity	98	91	91
% specificity	100	97	97
% PPV	100	98	98
% NPV	97	90	90
% accuracy	99	94	94

ND= not determined, since no samples were classified as t(7;12)

Supplementary Table 7: Molecular aberrations found in 237 children with newly diagnosed AML according to cytogenetic subtype.

		Subtype according to cytogenetic screening					
		***MLL* (N=47)**	***t(8;21)* (N=28)**	***inv(16)* (N=27)**	***t(15;17)* (N=19)**	***t(7;12)* (N=7)**	***AML-other* (N=92)**
Molecular aberration	***FLT3-ITD***	1	3	1	12	0	31
	RAS pathway	11	4	6	0	1	24
	KIT	1	8	8	0	1	0
	MLL-PTD	0	0	0	0	0	5
	NPM1	0	0	0	0	0	17
	CEBPA	0	0	0	0	0	16

Supplementary Table 8: Number of probe sets at different cut-off p-values per molecular subtype (total cohort).

	NPM1	***CEBPA***	***MLL*-PTD**	***FLT3*-ITD**	***KIT***	***N/KRAS***	***PTPN11***
FDR adjusted p-values							
p<1.0E-08	7	7	0	54	8	0	1
p<1.0E-06	12	13	0	127	11	0	1
p<1.0E-04	27	66	1	387	46	0	6
p<0.001	46	131	4	933	86	0	10
p<0.005	75	222	7	1732	155	0	12
p<0.01	106	320	9	2308	220	0	19
p<0.05	266	852	12	4896	510	0	57

Supplementary Table 9: Diagnostic test values for the prediction of mutations in *NPM1*, *CEBPA* and *MLL*-PTD by gene expression signature consisting of 45 probe sets.

A)

	Discovery cohort [a]				
	3-fold cross-validation (100 iterations)				
	% sensitivity	**% specificity**	**% PPV**	**% NPV**	**% accuracy**
NPM1	16 (0-33)	100 (100-100)	100 (100-100)	95 (94-96)	94 (94-96)
***MLL*-PTD**	0 (0-0)	100 (100-100)	ND	98 (98-98)	98 (98-98)
CEBPA	67 (67-100)	100 (100-100)	100 (100-100)	98 (98-100)	96 (96-98)
Remaining cases	100 (100-100)	43 (26-43)	92 (90-92)	100 (100-100)	92 (90-92)
All groups	43 (26-43)	100 (100-100)	100 (100-100)	92 (90-92)	92 (90-92)

ND= Not determined

[a] Values represent the median and 25th-75th percentiles (in parentheses) obtained by 3-fold cross-validation using the discovery cohort of 157 cases (100 iterations).

Supplementary Table 9: Continued
B)

	Validation cohort				
	independent validation group, N=80				
	% sensitivity	**% specificity**	**% PPV**	**% NPV**	**% accuracy**
NPM1	13	99	50	91	90
***MLL*-PTD**	0	100	ND	96	96
CEBPA	33	100	100	95	95
Remaining cases	98	18	82	75	81
All groups	18	98	75	82	81

Supplementary Table 10: Diagnostic test values for the prediction of cytogenetic and molecular subtypes (*NPM1*, *CEBPA* and *MLL*-PTD) by gene expression signature consisting of 120 probe sets.

A)

	Discovery cohort [a]				
	3-fold cross-validation (100 iterations)				
	% sensitivity	**% specificity**	**% PPV**	**% NPV**	**% accuracy**
***MLL*-rearranged**	90 (80-90)	97 (95-97)	88 (82-90)	97 (95-98)	94 (92-96)
t(8;21)	100 (100-100)	100 (100-100)	100 (100-100)	100 (100-100)	100 (100-100)
inv(16)	100 (80-100)	100 (100-100)	100 (100-100)	100 (98-100)	100 (98-100)
t(15;17)	100 (100-100)	100 (100-100)	100 (100-100)	100 (100-100)	100 (100-100)
t(7;12)	100 (100-100)	100 (100-100)	100 (100-100)	100 (100-100)	100 (100-100)
NPM1	0 (0-33)	100 (100-100)	100 (50-100)	96 (94-96)	94 (94-96)
***MLL*-PTD**	0 (0-0)	100 (100-100)	ND	98 (98-98)	98 (98-98)
CEBPA	100 (66-100)	100 (100-100)	100 (100-100)	100 (98-100)	100 (98-100)
Remaining cases[#]	91 (88-94)	82 (79-85)	70 (67-74)	95 (93-97)	85 (82-88)
All groups	82 (79-85)	91 (88-94)	95 (93-97)	70 (67-74)	85 (82-88)

ND= Not determined

[a] Values represent the median and 25th-75th percentiles (in parentheses) obtained by 3-fold cross-validation using the discovery cohort of 157 cases (100 iterations).

[#] Including 3 cases predicted as *MLL*-rearranged AML and confirmed with LDI-PCR

Supplementary Table 10: Continued
B)

	Validation cohort				
	independent validation group, N=80				
	% sensitivity	**% specificity**	**% PPV**	**% NPV**	**% accuracy**
***MLL*-rearranged**	94	98	94	98	98
t(8;21)	100	100	100	100	100
inv(16)	100	100	100	100	100
t(15;17)	100	100	100	100	100
t(7;12)	100	100	100	100	100
NPM1	13	99	50	91	90
***MLL*-PTD**	0	100	ND	96	96
CEBPA	33	100	100	95	95
Remaining cases	90	75	55	96	78
All groups	75	90	96	55	78

ND= Not determined

Supplementary Table 11: Diagnostic test values for the prediction of type-I molecular subtypes by gene expression signature consisting of 30 probe sets.

A)

	Discovery cohort [a]				
	3-fold cross-validation (100 iterations)				
	% sensitivity	**% specificity**	**% PPV**	**% NPV**	**% accuracy**
***FLT3*-ITD**	40 (30-53)	95 (93-98)	67 (60-80)	87 (85-90)	85 (83-87)
KIT	25 (0-25)	100 (98-100)	100 (50-100)	94 (92-94)	92 (92-94)
Remaining cases	95 (92-97)	35 (29-43)	80 (78-82)	70 (58-80)	77 (75-81)
All groups	35 (29-43)	95 (92-97)	70 (58-80)	80 (78-82)	77 (75-81)

[a] Values represent the median and 25th-75th percentiles (in parentheses) obtained by 3-fold cross-validation using the discovery cohort of 157 cases (100 iterations).

B)

	Validation cohort				
	independent validation group, N=80				
	% sensitivity	**% specificity**	**% PPV**	**% NPV**	**% accuracy**
***FLT3*-ITD**	72	100	100	93	94
KIT	33	99	66	95	94
Remaining cases	89	63	86	94	88
All groups	63	89	94	86	88

Supplementary Table 12: Diagnostic test values for the prediction of type-I molecular subtypes by gene expression signature consisting of 45 probe sets.

A)

	Discovery cohort [a]				
	3-fold cross-validation (100 iterations)				
	% sensitivity	**% specificity**	**% PPV**	**% NPV**	**% accuracy**
***FLT3*-ITD**	40 (30-50)	95 (93-98)	67 (55-83)	87 (85-89)	85 (83-87)
RAS-pathway[b]	0 (0-0)	95 (95-98)	0 (0-0)	84 (84-85)	82 (81-83)
KIT	25 (0-25)	100 (98-100)	100 (50-100)	94 (92-94)	92 (92-94)
Remaining cases	87 (83-93)	27 (18-32)	62 (59-64)	60 (50-78)	62 (58-65)
All groups	27 (18-32)	87 (83-93)	60 (50-78)	62 (59-64)	62 (58-65)

[a] Values represent the median and 25th-75th percentiles (in parentheses) obtained by 3-fold cross-validation using the discovery cohort of 157 cases (100 iterations).
[b] Includes cases with mutations in *NRAS*, *KRAS* and *PTPN11*.

B)

	Validation cohort				
	independent validation group, N=80				
	% sensitivity	**% specificity**	**% PPV**	**% NPV**	**% accuracy**
***FLT3*-ITD**	61	98	92	90	90
RAS-pathway[a]	5	94	20	76	73
KIT	17	99	50	94	93
Remaining cases	89	35	54	79	60
All groups	30	89	76	52	58

[a] Includes cases with mutations in *NRAS*, *KRAS* and *PTPN11*.

Supplementary Table 13: The 126 probe sets used for the hierarchical clustering of *FLT3*-ITD positive cases in t(15;17) and CN-AML ranked according to hierarchical order in Figure 2 (*HOXB*-genes highlighted in yellow).

Probe Set	Gene	Gene Symbol	Chromosome
219201_s_at	twisted gastrulation homolog 1 (Drosophila)	TWSG1	chr18p11.3
217848_s_at	pyrophosphatase (inorganic) 1	PPA1	chr10q11.1-q24
204429_s_at	solute carrier family 2 (facilitated glucose/fructose transporter), member 5	SLC2A5	chr1p36.2
203710_at	inositol 1,4,5-triphosphate receptor, type 1	ITPR1	chr3p26-p25
216944_s_at	inositol 1,4,5-triphosphate receptor, type 1	ITPR1	chr3p26-p25
225337_at	abhydrolase domain containing 2	ABHD2	chr15q26.1
201432_at	catalase	CAT	chr11p13
211922_s_at	catalase	CAT	chr11p13
201468_s_at	NAD(P)H dehydrogenase, quinone 1	NQO1	chr16q22.1
220658_s_at	aryl hydrocarbon receptor nuclear translocator-like 2	ARNTL2	chr12p12.2-p11.2
228011_at	family with sequence similarity 92, member A1	FAM92A1	chr8q22.1
224204_x_at	aryl hydrocarbon receptor nuclear translocator-like 2	ARNTL2	chr12p12.2-p11.2
228624_at	transmembrane protein 144	TMEM144	chr4q32.1
202890_at	microtubule-associated protein 7	MAP7	chr6q23.3
210145_at	phospholipase A2, group IVA (cytosolic, calcium-dependent)	PLA2G4A	chr1q25
204030_s_at	schwannomin interacting protein 1	SCHIP1	chr3q25.32-q25.33
229309_at	adrenergic, beta-1-, receptor	ADRB1	chr10q24-q26
218445_at	H2A histone family, member Y2	H2AFY2	chr10q22
236738_at	Similar to LOC166075	LOC401097	chr3q25.33
204082_at	pre-B-cell leukemia homeobox 3	PBX3	chr9q33-q34
232088_x_at	hypothetical LOC100271722	hCG_2039027	chr22q13.31
228365_at	copine VIII	CPNE8	chr12q12
204779_s_at	homeobox B7	HOXB7	chr17q21.3
216973_s_at	homeobox B7	HOXB7	chr17q21.3
231767_at	homeobox B4	HOXB4	chr17q21-q22
205453_at	homeobox B2	HOXB2	chr17q21-q22
205600_x_at	homeobox B5	HOXB5	chr17q21.3

Supplementary Table 13: Continued

Probe Set	Gene	Gene Symbol	Chromosome
230743_at	hypothetical LOC404266	LOC404266	chr17q21.32
239791_at	Hypothetical LOC404266	LOC404266	chr17q21.32
228904_at	homeobox B3	HOXB3	chr17q21.3
236892_s_at	---	---	chr17q21.33
1553808_a_at	NK2 transcription factor related, locus 3 (Drosophila)	NKX2-3	chr10q24.2
205366_s_at	homeobox B6	HOXB6	chr17q21.3
205601_s_at	homeobox B5	HOXB5	chr17q21.3
232979_at	---	---	chr17q21.33
242426_at	neuregulin 4	NRG4	chr15q24.2
232424_at	PR domain containing 16	PRDM16	chr1p36.23-p33
242269_at	hypothetical LOC440556	FLJ42875	chr1p36.32
226500_at	zinc finger and BTB domain containing 47	ZBTB47	chr3p22.1
237108_x_at	hypothetical LOC440556	FLJ42875	chr1p36.32
210327_s_at	alanine-glyoxylate aminotransferase	AGXT	chr2q36-q37
204501_at	nephroblastoma overexpressed gene	NOV	chr8q24.1
214321_at	nephroblastoma overexpressed gene	NOV	chr8q24.1
218164_at	spermatogenesis associated 20	SPATA20	chr17q21.33
225097_at	homeodomain interacting protein kinase 2	HIPK2	chr7q32-q34
201618_x_at	glycosylphosphatidylinositol anchor attachment protein 1 homolog (yeast)	GPAA1	chr8q24.3
210338_s_at	heat shock 70kDa protein 8	HSPA8	chr11q24.1
58696_at	exosome component 4	EXOSC4	chr8q24.3
219919_s_at	slingshot homolog 3 (Drosophila)	SSH3	chr11q13.2
227400_at	nuclear factor I/X (CCAAT-binding transcription factor)	NFIX	chr19p13.3
224968_at	coiled-coil domain containing 104	CCDC104	chr2p16.1
238583_at	methionine sulfoxide reductase B3	MSRB3	chr12q14.3
1554127_s_at	methionine sulfoxide reductase B3	MSRB3	chr12q14.3
225790_at	methionine sulfoxide reductase B3	MSRB3	chr12q14.3
230520_at	androgen-induced 1	AIG1	chr6q24.2
205382_s_at	complement factor D (adipsin)	CFD	chr19p13.3
204548_at	steroidogenic acute regulatory protein	STAR	chr8p11.2
222462_s_at	beta-site APP-cleaving enzyme 1	BACE1	chr11q23.2-q23.3

Supplementary Table 13: Continued

Probe Set	Gene	Gene Symbol	Chromosome
205204_at	neuromedin B	NMB	chr15q22-qter
1553723_at	G protein-coupled receptor 97	GPR97	chr16q21
222043_at	clusterin	CLU	chr8p21-p12
208791_at	clusterin	CLU	chr8p21-p12
208792_s_at	clusterin	CLU	chr8p21-p12
214615_at	purinergic receptor P2Y, G-protein coupled, 10	P2RY10	chrXq21.1
236280_at	---	---	chrXq21.1
224964_s_at	guanine nucleotide binding protein (G protein), gamma 2	GNG2	chr14q21
202759_s_at	A kinase (PRKA) anchor protein 2 /// PALM2-AKAP2 readthrough transcript	AKAP2 /// PALM2-AKAP2	chr9q31-q33
226694_at	A kinase (PRKA) anchor protein 2 /// PALM2-AKAP2 readthrough transcript	AKAP2 /// PALM2-AKAP2	chr9q31-q33
219955_at	LINE-1 type transposase domain containing 1	L1TD1	chr1p31.3
238292_at	---	---	chr6q27
242078_at	---	---	chr2q11.2
206682_at	C-type lectin domain family 10, member A	CLEC10A	chr17p13.1
227006_at	protein phosphatase 1, regulatory (inhibitor) subunit 14A	PPP1R14A	chr19q13.1
207675_x_at	artemin	ARTN	chr1p33-p32
208216_at	distal-less homeobox 4	DLX4	chr17q21.33
235434_at	---	---	chr22q13.2
241975_at	Hypothetical gene supported by BX647608	LOC399959	chr11q24.1
201069_at	matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	MMP2	chr16q13-q21
224839_s_at	glutamic pyruvate transaminase (alanine aminotransferase) 2	GPT2	chr16q12.1
200986_at	serpin peptidase inhibitor, clade G (C1 inhibitor), member 1	SERPING1	chr11q12-q13.1
201564_s_at	fascin homolog 1, actin-bundling protein (Strongylocentrotus purpuratus)	FSCN1	chr7p22

Supplementary Table 13: Continued

Probe Set	Gene	Gene Symbol	Chromosome
202718_at	insulin-like growth factor binding protein 2, 36kDa	IGFBP2	chr2q33-q34
207895_at	N-acetylated alpha-linked acidic dipeptidase-like 1	NAALADL1	chr11q12
222693_at	fibronectin type III domain containing 3B	FNDC3B	chr3q26.31
225032_at	fibronectin type III domain containing 3B	FNDC3B	chr3q26.31
230486_at	Poly(rC) binding protein 3	PCBP3	chr21q22.3
242393_x_at	ArfGAP	AGAP10 /// AGAP4 /// AGAP9 /// BMS1P5	chr10q11.22
243937_x_at	ArfGAP	AGAP10 /// AGAP4 /// AGAP9 /// BMS1P5	chr10q11.22
238553_at	ArfGAP	AGAP10 /// AGAP4 /// AGAP9 /// BMS1P5	chr10q11.22
239151_at	hypothetical LOC399753	RP11-144G6.7	chr10q11.22
205110_s_at	fibroblast growth factor 13	FGF13	chrXq26.3
234269_at	---	---	chrXq27.1
227410_at	family with sequence similarity 43, member A	FAM43A	chr3q29
213125_at	olfactomedin-like 2B	OLFML2B	chr1q23.3
207031_at	NK3 homeobox 2	NKX3-2	chr4p16.1
205614_x_at	macrophage stimulating 1 (hepatocyte growth factor-like)	MST1	chr3p21
216320_x_at	macrophage stimulating 1 (hepatocyte growth factor-like)	MST1	chr3p21
205944_s_at	clathrin, heavy chain-like 1	CLTCL1	chr22q11.2|22q11.21
221636_s_at	MOCO sulphurase C-terminal domain containing 2	MOSC2	chr1q41
204537_s_at	gamma-aminobutyric acid (GABA) A receptor, epsilon	GABRE	chrXq28
228285_at	tudor domain containing 9	TDRD9	chr14q32.33
236787_at	---	---	chr2p11.2

Supplementary Table 13: Continued

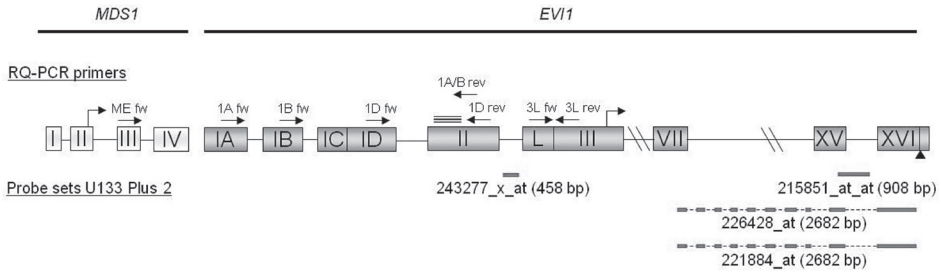
Probe Set	Gene	Gene Symbol	Chromosome
203074_at	annexin A8 /// annexin A8-like 1 /// annexin A8-like 2	ANXA8 /// ANXA8L1 /// ANXA8L2	chr10q11.22
214228_x_at	tumor necrosis factor receptor superfamily, member 4	TNFRSF4	chr1p36
227185_at	hypothetical LOC643988	LOC643988	chr1p36.33
225203_at	protein phosphatase 1, regulatory (inhibitor) subunit 16A	PPP1R16A	chr8q24.3
233072_at	netrin G2	NTNG2	chr9q34
214203_s_at	proline dehydrogenase (oxidase) 1	PRODH	chr22q11.21
228550_at	reticulon 4 receptor	RTN4R	chr22q11.21
206634_at	SIX homeobox 3	SIX3	chr2p16-p21
209815_at	patched homolog 1 (Drosophila)	PTCH1	chr9q22.3
227145_at	lysyl oxidase-like 4	LOXL4	chr10q24
235468_at	hexaribonucleotide binding protein 3	hCG_1776007	chr17q25.3
223828_s_at	lectin, galactoside-binding, soluble, 12	LGALS12	chr11q13
224794_s_at	cerebral endothelial cell adhesion molecule	CERCAM	chr9q34.11
210755_at	hepatocyte growth factor (hepapoietin A; scatter factor)	HGF	chr7q21.1
210997_at	hepatocyte growth factor (hepapoietin A; scatter factor)	HGF	chr7q21.1
210998_s_at	hepatocyte growth factor (hepapoietin A; scatter factor)	HGF	chr7q21.1
230244_at	chromosome 2 open reading frame 82	C2orf82	chr2q37.1
229459_at	family with sequence similarity 19 (chemokine (C-C motif)-like), member A5	FAM19A5	chr22q13.32
229655_at	family with sequence similarity 19 (chemokine (C-C motif)-like), member A5	FAM19A5	chr22q13.32
204150_at	stabilin 1	STAB1	chr3p21.1
38487_at	stabilin 1	STAB1	chr3p21.1
204163_at	elastin microfibril interfacer 1	EMILIN1	chr2p23.3-p23.2
212285_s_at	agrin	AGRN	chr1p36.33
217419_x_at	agrin	AGRN	chr1p36.33

Supplementary Table 14: Diagnostic test values for the prediction of the cytogenetic subtypes and different subgroups of *FLT3*-ITD for the independent validation cohort by gene expression signature consisting of 99 probe sets.

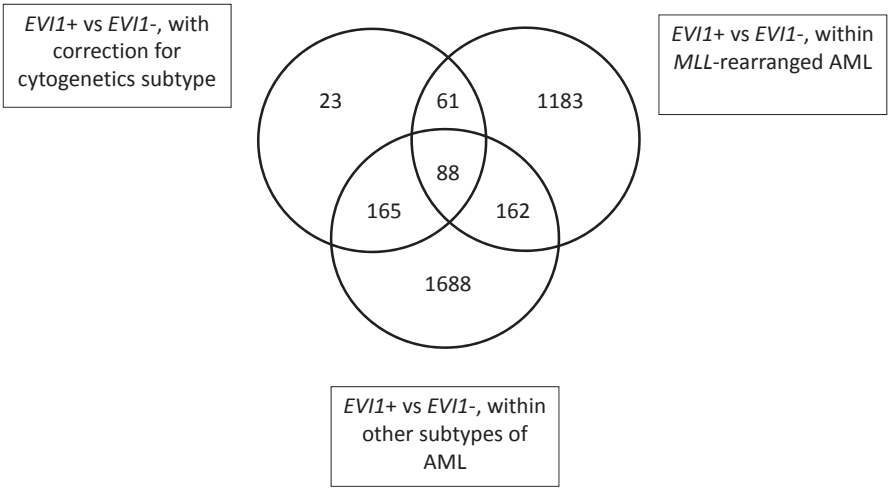
	Validation cohort				
	independent validation group, N=80				
	% sensitivity	**% specificity**	**% PPV**	**% NPV**	**% accuracy**
***MLL*-rearranged**	94	98	94	98	98
t(8;21)(q22;q22)	100	100	100	100	100
inv(16)(p13q22)	100	100	100	100	100
t(15;17)(q21;q22)	0	99	0	99	98
t(15;17)/*FLT3*-ITD	75	99	75	99	98
t(7;12)(q36;p13)	100	100	100	100	100
CN-AML/*FLT3*-ITD	0	100	ND	90	90
AML-other [a]	76	100	100	82	89
All groups	80	97	98	74	86

[a] Without the CN-AML/*FLT3*-ITD cases. ND = not determined

Chapter 4



Supplementary Figure 1: Position of probe sets, primers and probes within the *EVI1* locus. Schematic overview of *EVI1* and *MDS1* primers used RQ-PCR and localization of the probe sets used for gene expression profiling using the Affymetrix U133 Plus2 array. This overview is not drawn in scale. The primers are represented by arrows and probe sets are drawn in red lines. In case *MDS1/EVI1* is present, than the exon II and III of *MDS1* are fused with exon II of *EVI1*.



Supplementary Figure 2: Venn diagram representing the probe sets with an FDR-corrected p-value <0.001 between subsets of *EVI1*+. Gene expression profiles discriminative between *EVI1*+ and *EVI1*- AML samples after correction for cytogenetic abnormalities (left circle), within MLL-rearranged AMLs (right circle) and within cytogenetically 'other' patients (bottom circle) revealed an *EVI1* signature of 88 probe sets, which were found to be in overlap between these different gene expression signatures (FDR-corrected p-value<0.001).

Supplementary Table 1: Primer and probe combinations used for quantitative real-time PCR.

Primer/Probe name	Oligonucleotide sequence (5'- 3')
MDS1/EVI1 forward	GAAAGACCCCAGTTATGGATGG
MDS/EVI1 and EVI1-1D reverse	GTACTTGAGCCAGCTTCCAACA
EVI1-1D forward	CTTCTTGACTAAAGCCCTTGGA
Probe EVI1-1D and MDS1/EVI1	TCTTAGACGAATTTTACAATGTGAAGTTCTGCATAGATG
EVI1-1A forward	TATTGCTGAGTTGAGGCCATAG
EVI1-1B forward	TGCGGTCTGGACACGTCTC
EVI1-1A/1B reverse	CTTCCAACATCTGGTTGACTGG
EVI1-3L forward	GGTATCTTAGTGTATATCTTGCCCTTTGT
EVI1-3L reverse	GCGCAATGTCTGCAACTACTCT
GAPDH forward	GTCGGAGTCAACGGATT
GAPDH reverse	AAGCTTCCCGTTCTCAG
Probe GAPDH	TCAACTACATGGTTTACATGTTCCAA

Supplementary Table 2: 88 probe sets highly discriminative for *EVI1*+.

Probe set	Gene Symbol	median FC	FDR-corrected p-value[a]
221884_at	*EVI1*	9.39	4.69E-62
226420_at	*EVI1*	7.40	4.12E-60
243277_x_at	*EVI1*	1.97	4.76E-21
237269_at	*MDS1*	1.72	7.29E-15
204497_at	*ADCY9*	0.63	8.66E-08
212224_at	*ALDH1A1*	2.12	1.27E-07
239210_at	*PBX1*	1.20	2.98E-07
236859_at	*RUNX2*	6.30	3.60E-07
244579_at	*TRPS1*	6.74	1.34E-05
236858_s_at	*RUNX2*	4.90	1.43E-05
221912_s_at	*CCDC28B*	1.51	1.89E-05
1553286_at	*ZNF555*	1.26	3.02E-05
231436_at	---	1.14	4.40E-05
230942_at	*CKLFSF5*	1.13	4.46E-05
222717_at	*SDPR*	1.70	5.23E-05
203408_s_at	*SATB1*	0.61	7.09E-05
226961_at	*LOC222171*	1.17	7.09E-05
200965_s_at	*ABLIM1*	2.74	8.34E-05
224367_at	*BEX2*	2.98	8.65E-05
235048_at	*KIAA0888*	1.57	1.12E-04
213288_at	*OACT2*	1.17	1.12E-04
230186_at	*MGC17839*	1.24	1.38E-04
225812_at	*LOC619208*	1.45	1.86E-04
203946_s_at	*ARG2*	1.53	1.87E-04
1558411_at	*LOC93556*	1.12	1.95E-04
232653_at	*TRPS1*	3.16	2.77E-04
223464_at	*OSBPL5*	0.79	2.80E-04
225799_at	*MGC4677 /// LOC541471*	1.80	2.94E-04
205442_at	*MFAP3L*	1.23	2.94E-04
213201_s_at	*TNNT1*	1.53	2.94E-04
1555123_at	*ST6GAL2*	1.11	3.42E-04
230006_s_at	*DKFZp313A2432*	1.31	4.09E-04
224976_at	*NFIA*	1.16	4.10E-04
210715_s_at	*SPINT2*	1.91	4.97E-04
203895_at	*PLCB4*	1.30	5.11E-04

Supplementary Table 2: Continued

Probe set	Gene Symbol	median FC	FDR-corrected p-value[a]
225864_at	*FAM84B*	2.02	5.11E-04
217963_s_at	*NGFRAP1*	14.23	6.23E-04
243128_at	*ZNF175*	1.19	6.23E-04
221690_s_at	*NALP2*	1.48	6.73E-04
1561073_at	*TRPS1*	1.48	7.83E-04
212489_at	*COL5A1*	1.32	8.71E-04
204653_at	*TFAP2A*	1.13	8.77E-04
222513_s_at	*SORBS1*	1.14	9.25E-04
228611_s_at	*C3orf17*	1.26	1.07E-03
208970_s_at	*UROD*	2.06	1.14E-03
224970_at	*NFIA*	1.21	1.18E-03
235721_at	*DTX3*	1.32	1.31E-03
1554679_a_at	*LAPTM4B*	2.08	1.55E-03
226640_at	*LOC221955*	0.67	1.65E-03
240065_at	*FAM81B*	1.28	1.65E-03
224975_at	*NFIA*	1.22	2.19E-03
219998_at	*HSPC159*	1.26	2.33E-03
226747_at	*KIAA1344*	1.55	2.49E-03
235780_at	*PRKACB*	1.48	2.63E-03
226363_at	*ABCC5*	1.50	2.66E-03
205390_s_at	*ANK1*	1.20	2.71E-03
208971_at	*UROD*	1.55	2.71E-03
1554076_s_at	*MGC17839*	1.27	2.84E-03
203803_at	*PCYOX1*	0.87	2.84E-03
208767_s_at	*LAPTM4B*	2.27	2.88E-03
222074_at	*UROD*	1.22	2.91E-03
225832_s_at	*LOC221955*	0.57	3.00E-03
200984_s_at	*CD59*	2.04	3.27E-03
211701_s_at	*TRO*	1.29	3.30E-03
212054_x_at	*KIAA0676*	0.88	3.36E-03
239047_at	*LOC159091*	1.31	3.48E-03
205040_at	*ORM1*	1.20	3.90E-03
202468_s_at	*CTNNAL1*	1.34	3.90E-03
233123_at	*SLC40A1*	1.27	4.68E-03
241869_at	*APOL6*	0.77	4.96E-03

Supplementary Table 2: Continued

Probe set	Gene Symbol	median FC	FDR-corrected p-value[a]
239578_at	*TANC*	1.19	4.96E-03
218795_at	*ACP6*	1.50	5.10E-03
200985_s_at	*CD59*	1.91	5.21E-03
205790_at	*SCAP1*	1.21	5.68E-03
215994_x_at	*KIAA0676*	0.83	5.76E-03
223304_at	*SLC37A3*	1.19	6.49E-03
221957_at	*PDK3*	1.03	6.57E-03
202765_s_at	*FBN1*	1.11	6.57E-03
206431_x_at	*KIAA0676*	0.85	6.61E-03
226413_at	*LOC400027*	1.75	6.62E-03
238164_at	*USP6NL*	1.45	6.72E-03
213822_s_at	*UBE3B*	1.09	6.89E-03
209369_at	*ANXA3*	1.28	7.05E-03
217149_x_at	*TNK1*	1.14	7.36E-03
220911_s_at	*KIAA1305*	1.42	8.58E-03
213978_at	*LOC92154*	1.11	8.60E-03
237958_at	*MCPH1*	1.13	8.72E-03
227307_at	*LOC90139*	1.13	8.91E-03

[a] FDR-corrected p-value after correction for cytogenetics.
FC = Fold change of normalized $\log^2$ expression between EVI1+ and EVI1 negative cases.

Supplementary Table 1. Distribution of 11q23/*MLL*-rearranged AML subgroups by collaborative study group.

MLL-rearrangement	Study Groups, Inclusion Dates, and Number of Patients (%)																								Total
	AIEOP		BFM		CCG		CPH		DCOG		JPLSG		LAME		Belarus		NOPHO		POG		SJCRH		MRC*		
	1995-	2004	1993-	2004	1995-	2002	1999-	2004	1993-	2004	1994-	2004	1993-	2004	1994-	2004	1993-	2004	1993-	1999	1993-	2004	1994-	2004	
	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	
t(9;11)(p22;q23)	12	35.3	72	45.0	55	43.0	7	38.9	15	44.1	29	38.7	36	59.0	9	36.0	26	44.1	28	32.2	20	45.5	19	61.3	**328**
t(10;11)(p12;q23)	7	20.6	21	13.1	12	9.4	6	33.3	7	20.6	7	9.3	6	9.8	8	32.0	7	11.9	6	6.9	9	20.5	2	6.5	**98**
t(6;11)(q27;q23)	3	8.8	9	5.6	5	3.9	3	16.7	1	2.9	3	4.0	1	1.6	2	8.0	2	3.4	5	5.7	1	2.3	-	-	**35**
t(11;19)(q23;p13)	1	2.9	6	3.8	4	3.1	-	-	5	14.7	5	6.7	4	6.6	-	-	6	10.2	-	-	-	-	-	-	**31**
t(11;19)(q23;p13.1)	-	-	3	1.9	8	6.3	2	11.1	-	-	7	9.3	1	1.6	-	-	1	1.7	6	6.9	5	11.4	1	3.2	**34**
t(11;19)(q23;p13.3)	4	11.8	1	0.6	7	5.5	-	-	-	-	1	1.3	1	1.6	1	4.0	1	1.7	9	10.3	-	-	-	-	**25**
t(1;11)(q21;q23)	2	5.9	4	2.5	3	2.3	-	-	-	-	2	2.7	5	8.2	1	4.0	1	1.7	4	4.6	3	6.8	-	-	**25**
t(4;11)(q21;q23)	1	2.9	2	1.3	2	1.6	-	-	1	2.9	-	-	1	1.6	-	-	-	-	1	1.1	2	4.5	3	9.7	**13**
t(10;11)(p11.2;q23)	-	-	1	0.6	5	3.9	-	-	-	-	-	-	-	-	-	-	-	-	3	3.4	2	4.5	1	3.2	**12**
t(11;17)(q23;q21)	-	-	4	2.5	2	1.6	-	-	2	5.9	1	1.3	-	-	-	-	2	3.4	1	1.1	-	-	-	-	**12**
Other	4	11.8	37	23.1	25	19.5	-	-	3	8.6	20	26.7	6	9.8	4	16.0	13	22	24	27.6	2	4.5	5	16.1	**143**
Total	**34**		**160**		**128**		**18**		**34**		**75**		**61**		**25**		**59**		**87**		**44**		**31**		**756**

* Only 2 centers from the MRC participated in this study
For study group abbreviations, see the Methods section. N, number of patients.

Supplementary Table 2. Cumulative dosages of chemotherapy as used in protocols of the various collaborative study groups.

Study Group	Drug			
	Cytarabine (g/m^2)	*Anthracyclines* (mg/m^2)	*Etoposide* (mg/m^2)	*Amsacrine* (mg/m^2)
AIEOP	39	400	800-1500	-
BFM	47	420-450	950	-
CCG	34.8	320	1900	-
CPH	47	420-450	950	
DCOG/MRC	41	500-900	950-1500	500
JPLSG-AML99	59	375	3150	
JPLSG-Other	74	430	-	
LAME	44	160	400	300-450
Belarus	29	335	1200	
NOPHO	49	375-480	1200	-
POG	20.7-55.7	135	600-1000	-
St. Jude	35-67	250-550	800-1500	-

*For study group abbreviations, see the Methods section.
†A ratio of 1:5 was used for daunorubicin and idarubicin or mitoxantrone, and a ratio of 1:1 was used for daunorubicin and doxorubicin.

Supplementary Table 3. Survival analysis for *MLL*-rearranged AML based on study groups.

Study groups	5y-pEFS (%, SE)	*p (logrank)	5y-pCIR (%, SE)	*p (Gray)	5y-pOS (%, SE)	*p (logrank)
		0.005		<0.001		0.03
LAME	61 (7)		29 (7)		74 (6)	
St. Jude	55 (8)		18 (6)		60 (8)	
DCOG	54 (9)		29 (8)		64 (9)	
NOPHO	50 (7)		29 (6)		63 (7)	
AIEOP	47 (9)		30 (8)		53 (9)	
JPLSG	47 (6)		43 (6)		58 (6)	
CPH	42 (12)		30 (13)		61 (12)	
BFM	40 (4)		40 (4)		54 (4)	
POG	36 (5)		54 (5)		45 (5)	
CCG	36 (4)		29 (4)		51 (5)	
Belarus	29 (10)		43 (12)		48 (11)	

Supplementary Table 4. Survival analysis of the t(9;11)(p22;q23) subgroup based on FAB type and differences in diagnostic WBC.

	N	5y-pEFS (%, SE)	*P-value (Logrank)	5y-pOS (%, SE)	*P-value (Logrank)
t(9;11)(p22;q23)					
FAB-M0	3	33 (27)	0.002	33 (27)	0.001
FAB-M1	9	11 (10)		15 (13)	
FAB-M2	10	40 (15)		58 (16)	
FAB-M4	23	34 (10)		48 (11)	
FAB-M5	254	59 (3)		70 (3)	
FAB-M7	14	24 (12)		24 (12)	
FAB M5 with WBC <20 x 10^9/L	129	67 (4)	<0.001	77 (4)	<0.001
FAB M5 with WBC ≥20 x 10^9/L	124	45 (5)		60 (5)	
FAB other with WBC <20 x 10^9/L	48	31 (7)		40 (8)	
FAB other with WBC ≥20 x 10^9/L	20	20 (9)		37 (12)	

*P-values indicate differences among the subgroups

Abbreviations: 5y-pEFS, probability of event-free survival at 5 years after diagnosis; 5y-pOS, probability of overall survival at 5 years after diagnosis; FAB, French-American-British classification system; WBC, white blood cell count

Supplementary Table 5. Multivariate analysis of survival estimates within the t(9;11)(p22;q23) pediatric AML subgroup.

	pEFS			pOS		
	Hazard Ratio	**95% CI**	**p-value**	**Hazard Ratio**	**95% CI**	**p-value**
Additional cytogenetic abberrations						
No	1.0	Reference		1.0	Reference	
Yes	1.2	0.9-1.7	0.2	1.5	1.0-2.2	0.047
WBC						
>=20 x 10^9/L	1.0	Reference		1.0	Reference	
<20 x 10^9/L	0.6	0.4-0.8	*0.001*	0.6	0.4-0.9	0.006
Age						
<10 y	1.0	Reference		1.0	Reference	
≥10 y	1.1	0.7-1.6	0.67	1.1	0.7-1.8	0.6
FAB-type						
FAB other	1.0	Reference		1.0	Reference	
FAB M5	0.4	0.3-0.6	*<0.001*	0.4	0.3-0.6	*<0.001*
Allogeneic BMT						
No	1.0	Reference		1.0	Reference	
Yes	1.0	0.6-1.7	0.99	1.0	0.5-1.9	0.94

Abbreviations: pEFS, probability of event free survival; pOS, probability of overall survival; WBC, white blood cell count; y, years; FAB, French-American-British classification; HSCT, hematopoietic stem cell transplantation

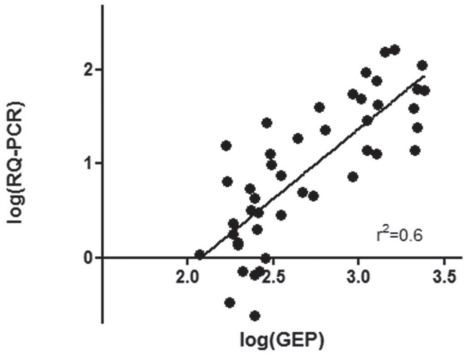
Chapter 8

Supplementary Table 1: Primers used for mutation screening

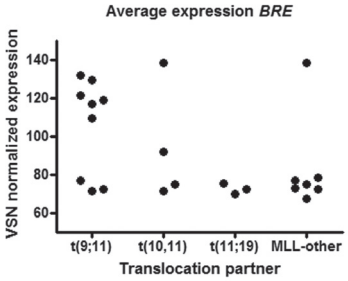
Primer	Primer sequence (5'-3')
NRAS exon 1 Forward	GGG GGT TGC TAG AAA ACT A
NRAS exon 1 Reverse	ATC CGA CAA GTG AGA GAC A
NRAS exon 2 Forward	CCC AGG ATT CTT ACA GAA AA
NRAS exon 2 Reverse	TCC CCA TAA AGA TTC AGA AC
KRAS exon 1 Forward	CGT CGA TGG AGG AGT TT
KRAS exon 1 Reverse	AAC CCA AGG TAC ATT TCA GA
KRAS exon 2 Forward	CCA GCA ATG CAC AAA GAT
KRAS exon 2 Reverse	CCC CCA AGA ACT TCA TTT A
PTPN11 exon 3 Forward	TTG GGT TTC TTT CAA CAC TT
PTPN11 exon 3 Reverse	GCC TTT GGA GTC AGA GAG T
PTPN11 exon 13 Forward	TGG CTC TGC AGT TTC TCT
PTPN11 exon 13 Reverse	CAT TCC GAA ATC AAA CAG TT
C-KIT exon 8 Forward	CCG CCT CCT TGT ACC TT
C-KIT exon 8 Reverse	TTC AGC AAA CAA AAT TAA TGT CTA
C-KIT exon 17 Forward	TCC TCC AAC CTA ATA GTG TAT TC
C-KIT exon 17 Reverse	CAT TCC GAA ATC AAA CAG TT

For the detection of *RAS* and *PTPN11* mutations the purified DNA was subjected to 40 cycles of PCR of 15" at 95°C and 1' at 60°C. For the detection of *C-KIT* mutations, purified DNA was subjected to 14 PCR cycles of 15" at 95°C and 1' at 63°C and 20 cycles of 15" at 95°C and 1' at 56°C.

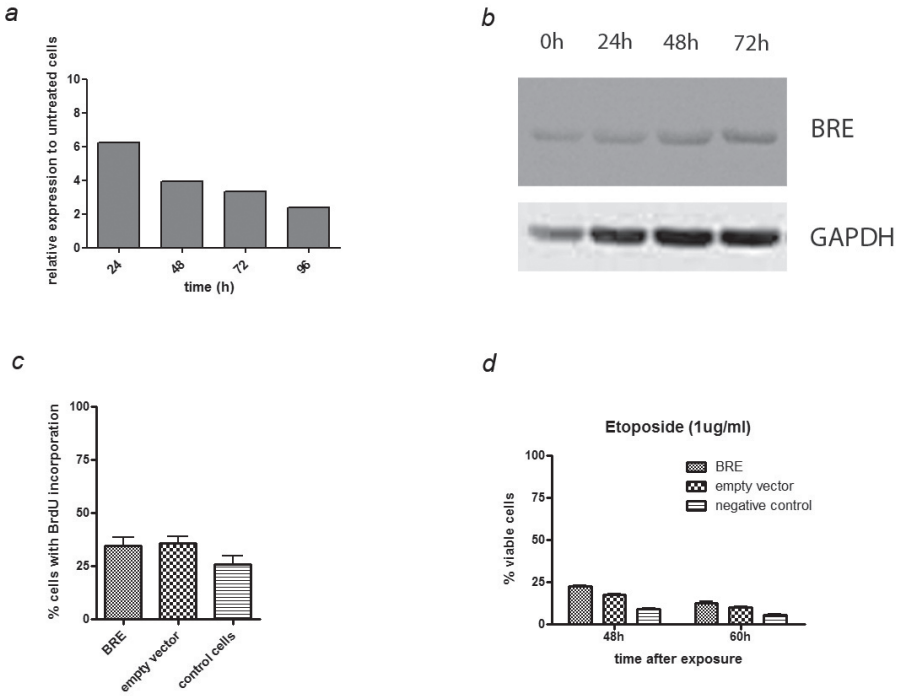
Chapter 9



Supplementary Figure 1: Correlation between gene expression profiling and RQ-PCR. Log transformed data of gene expression profiling and RQ-PCR showed high a high correlation with r^2=0.6



Supplementary Figure 2: Validation of BRE expression in an independent cohort. High expression of BRE was also predominantly found in t(9;11)(p22;q23) in an independent cohort of *MLL*-rearranged AML cases, for whom gene expression profiling previously was performed by Ross *et al.*



Supplementary Figure 3: Effect of overexpression of BRE on cell-proliferation or apoptosis. Monomac-1 was transiently transfected with *BRE*. After 24h a 6-fold higher expression of *BRE* was identified. After 4 days the expression was still higher than untreated cells (A). Moreover, after 3 days an increased expression of the BRE protein was found (B). Overexpression of *BRE* did not influence cell-proliferation. Compared to the negative control BrdU incorporation was identical (C). Exposing cells to VP16 (1ug/ml) showed no anti-apoptotic effect of BRE after 48h (D).

Supplementary Materials & Methods

Protein extraction and Western blot analysis

In 11 of the 53 *MLL*-rearranged AML samples material for protein extraction and Western blot analysis was available. Cell pellets stored at –80°C were briefly 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 orthovanadate, 10 mM glycerolphosphate, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% aprotinin (Sigma, St. Louis, MO, USA), 10 mM sodium fluoride, and 20 µL freshly prepared sodium pervanadate. Accordingly, 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 25 µg protein were separated on 10% polyacrylamide gels topped with 4% stacking gels, and transferred onto nitrocellulose membranes (Schleichler & Schuell, Dassel, Germany). Western blots were probed with mouse anti-BRE (kindly provided by Dr. Y.L. Chui[1]) and with mouse anti-GAPDH (Cell Signaling, Danvers, MA, USA) antiserum. Accordingly, the blots were labeled with peroxidase-conjugated anti-mouse IgG antibodies (DAKO, Glostrup, Denmark) and visualized using SuperSignal West Femto chemiluminescent substrate (Pierce Biotechnology, Rockford, IL, USA).

Cell culture and transfection

Different cell lines with an *MLL*-rearrangement, i.e Monomac-1, THP1, NOMO1, MV4;11 and ML2 (DSMZ, Braunschweig, Germany), were tested for *BRE*-expression. However, none of them showed overexpression of *BRE*. Since a cell line model was not available the Monomac-1 cell line was transfected to overexpress *BRE*.

The Monomac-1 cell line was cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS) (Integro, Zaandam, The Netherlands), 100 IU/mL penicillin, 100 µg/mL streptomycin, and 0.125 µg/mL fungizone (Invitrogen) and grown as suspension cultures at 37°C in humidified air containing 5% CO2.

The *BRE* cDNA clone (MGC Clone ID: 4974, Invitrogen) was cloned into the NotI/SalI site of the eukaryotic expression vector pLNCX2 (Clontech, Mountain View, CA, USA). Monomac-1 cells ($15x10^6$) were transfected by electroporation in 400 µl RPMI 1640 with L-Alanyl-L-Glutamine (Invitrogen) containing 2 µg of either the pLNCX2-BRE construct or pLNCX2 as empty vector (mock transfection), in 4 mm electroporation cuvettes (BioRad, Hercules, CA, USA). 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 vector. Electroporation was performed using an EPI 2500 gene pulser (Fischer, Heidelberg, Germany) applying a rectangle pulse of 570 V for 2 ms. After incubating for 15 min at room temperature, the cells were diluted in 25 ml RPMI-1640 supplemented with 10% FCS (Integro), 100 IU/ml penicillin, 100 µg/ml streptomycin and 0.125 µg/ml fungizone (Invitrogen) and incubated at 37°C and 5% CO2.
Cell viability was assessed by Annexin V/PI staining determined by flow cytometry using a FACSCalibur (Becton Dickinson, San Jose, CA, USA). Flow cytometry was also used to determine transfection efficiency of pLNCX2-EGFP.

BrdU cell-cycle proliferation assay
Monomac-1 cells ($1x10^6$) were incubated 2 days after transfection (with pLNCX2-BRE or an empty vector) with 10uM BrdU for 1 hour. Cells from the same population without BrdU were used as negative staining control. Next, cells were fixed, treated with DNase to expose BrdU epitopes, incubated with FITC-conjugated anti-BrdU antibodies, and total DNA was stained with 7-AAD following instruction of the manual (FITC BrdU Flow Kit, BD Pharmingen, San Diego, CA, USA). The flow cytometry assay was performed using a FACSCalibur.

In vitro drug resistance assay
In vitro drug resistance for daunorubicin (DNR; Cerubidine, Sanofi Aventis, Gouda, The Netherlands), vincristine (VCR; Pharmachemie, Haarlem, The Netherlands), L-asparaginase (ASP; Paronal, Nycomed Christiaens, Breda, The Netherlands), prednisolone (PRED; Bufa Pharmaceutical Products, Uitgeest, The Netherlands), cytosine arabinoside (ARA-C; Cytosar, Pharmacia, Woerden, The Netherlands), Cladribine (2-CDA; Leustatin, Janssen-Cilag, Tilburg, The Netherlands), dexamethasone (DXM; Erasmus MC, Rotterdam, The Netherlands), and etoposide (VP16; Toposin, Pharmachemie, Haarlem, The Netherlands) was determined using the 4-day 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously[2]. Six concentrations 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; VCR: 0.05 - 50 µg/ml; ASP: 0.003 - 10 IU/ml; PRED: 0.008 - 250 µg/ml; ARA-C: 0.01 – 10 µg/ml; 2-CDA: 0.0004 - 4 µg/ml; DXM: 0.0002 – 6 µg/ml and VP16: 0.05 – 50 µg/ml.

1. Chan BC, Ching AK, To KF, et al. BRE is an antiapoptotic protein in vivo and overexpressed in human hepatocellular carcinoma. Oncogene. 2008;27:1208-1217.
2. Pieters R, Loonen AH, Huismans DR, et al. In vitro drug sensitivity of cells from children with leukemia using the MTT assay with improved culture conditions. Blood. 1990;76:2327-2336.

Supplementary Table 1: Primers and probe sequence

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
BRE Forward (exon 9)	GAG GAG GAT GTC TCA TTG A
BRE Reverse (exon 10)	AGC CTT CTG CAT CAT ATT C
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)

Supplementary Table 2: Top 50 most discriminative probe sets for t(9;11)(p22;q23)

Probe ID	Gene Symbol	p-value
1552665_at	MGC14425	4.51E-09
225905_s_at	ST3GAL3	5.28E-09
205550_s_at	BRE	6.18E-09
211566_x_at	BRE	7.55E-09
212645_x_at	BRE	1.17E-08
227877_at	LOC389289	1.75E-08
1555702_a_at	ST3GAL3	1.68E-07
210868_s_at	ELOVL6	1.67E-06
1562260_at	BRE	2.77E-06
238749_at	BRE	5.80E-06
204304_s_at	PROM1	1.01E-05
227491_at	---	1.10E-05
221942_s_at	GUCY1A3	1.35E-05
221840_at	PTPRE	1.53E-05
229530_at	---	1.71E-05
206046_at	ADAM23	1.81E-05
1557278_s_at	TNPO1	3.05E-05
220036_s_at	LMBR1L	3.16E-05
225612_s_at	B3GNT5	3.50E-05
228793_at	JMJD1C	3.69E-05
1555678_at	ST3GAL3	5.25E-05

Supplementary Table 2: Continued

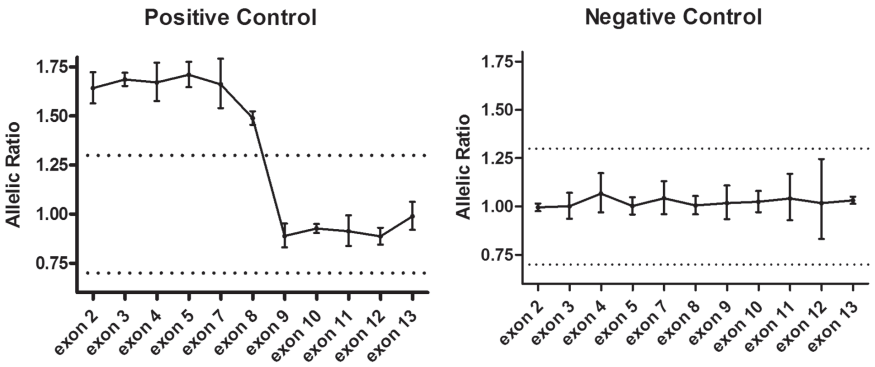
Probe ID	Gene Symbol	p-value
226245_at	KCTD1	5.51E-05
202283_at	SERPINF1	6.27E-05
227407_at	FLJ90013	6.63E-05
201857_at	ZFR	7.20E-05
235291_s_at	---	7.42E-05
239260_at	CORIN	8.69E-05
230245_s_at	LOC283663	8.89E-05
57540_at	RBKS	1.00E-04
243492_at	CTMP	1.01E-04
206257_at	CCDC9	1.02E-04
238158_at	---	1.07E-04
240143_at	ADAM23	1.08E-04
232812_at	LOC401052	1.10E-04
1569366_a_at	ZNF569	1.38E-04
215001_s_at	GLUL	1.42E-04
203604_at	ZNF516	1.45E-04
232645_at	LOC153684	1.45E-04
213808_at	ADAM23	1.49E-04
218421_at	CERK	1.52E-04
222149_x_at	DKFZp434P162	1.58E-04
1559018_at	PTPRE	1.74E-04
214318_s_at	13CDNA73	1.84E-04
226246_at	KCTD1	1.85E-04
201328_at	ETS2	1.86E-04
216614_at	ITPR2	1.90E-04
229253_at	THEM4	1.91E-04
209031_at	IGSF4	2.15E-04
203781_at	MRPL33	2.21E-04
214319_at	13CDNA73	2.49E-04

Supplementary Table 3: MTT-assay lethal concentration (LC-50) values for various cytostatics.

	LC50		
	Monomac-1	Monomac-1 empty vector	Monomac-1 BRE
daunorubicin (µg/ml)	0.07	0.03	0.08
vincristine (µg/ml)	<0.05	0.09	0.12
L-asparaginase (IU/ml)	1.12	1.38	1.13
prednison (µg/ml)	>250	>250	>250
cytarabine (µg/ml)	0.02	0.02	0.02
cladribine (µg/ml)	0.02	0.02	0.02
dexamethasone (µg/ml)	>6	>6	>6
etoposide (µg/ml)	0.47	0.46	0.68

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

Chapter 10



Supplementary Figure 1: DNA-MLPA intra-assay variability was performed in 3-fold for (A) a positive control and (B) for a negative control. All exons show mean and their 95%CI, indicating that false negative and false positive results can be excluded due to intra-assay variability

Supplementary Table 1: Patient characteristics of an independent validation cohort of 27 adult leukaemia's

Disease type	FAB-type	Sex	Age	Karyotype	MLPA *MLL*-PTD	SB
AML		F	33	46,XX[31]	MLPA: neg	neg
AML		M	74	46,XY[53]	MLPA: neg	neg
AML	M5a	F	35	47,XX,+8,t(11;19)(q23;p13)[25]	MLPA: neg	neg
AML	M5	F	52	47,XX,+1,del(1)(p11p35),t(9;11)(p21~22;q23)[21]	MLPA: del exon 10	neg
AML		F	60	46,XX[20]	MLPA: neg	neg
MDS		F	72	47,XX,+11[15]/46,XX[1]	MLPA: neg	neg
AML	M5	F	83	46,XY[30]	MLPA: pos	*MLL*-PTD
T-ALL		M	31	46,XY,t(11;19)(p13q13.4)c[30]	MLPA: neg	neg
AML	M4	M	49	46,XY[30]	MLPA: neg	neg
AML		M	63	46,XY[32]	MLPA: pos	*MLL*-PTD
AML		M	28	46,XY[32]	MLPA: neg	neg
AML	M1	M	73	46,XY[30]	MLPA: neg	neg
AML	M2	F	65	47,XX,+11[31]/46,XX[1]	MLPA: pos	*MLL*-PTD
AML	M5	F	18	46,XX,t(9;11)(p21~22;q23)[57]	MLPA: neg	neg
AML	M5	M	59	46,XY[53]	MLPA: pos	*MLL*-PTD
AML	M1	M	64	46,XY[32]	MLPA: pos	*MLL*-PTD
AML	M1	M	56	47,XY+8[35]/46,XY[2]	MLPA: neg	neg
AML	M2/M4	M	55	47,XY+11[10]/48,idem,+13[13]/46,XX[9]	MLPA: pos	*MLL*-PTD
AML	M5b	M	59	46,XY,t(11;18)(q23;q23)[23]/46,XY[44]	MLPA: del exon 9	neg
AML	M1	M	44	46,XY[67]	MLPA: neg	*MLL*-PTD
Pro B-ALL		M	64	64,X,+X,-Y,+1,+1,+2,+3,+5,+6,+6,add(7)(p1),add(9)(p1),add(9)(p2),+10,+11,+11,+12,-13,add(13)(p1),+14,-15,add(15)(p1),+18,-20,+21,+22,+22,+1-2mar[cp21]/56-65,inc[32]/46,XY[29]	MLPA: neg	neg
AML	M5	F	35	46,XX[44]	MLPA: neg	neg
AML	M4	M	43	47,XY,+11[14]/46,XY[13]	MLPA: pos	*MLL*-PTD
AML	M1	M	52	46,XY[29]	MLPA: pos	*MLL*-PTD
AML	M5	F	49	47,XX,+11[27]/46,XX[5]	MLPA: neg	neg

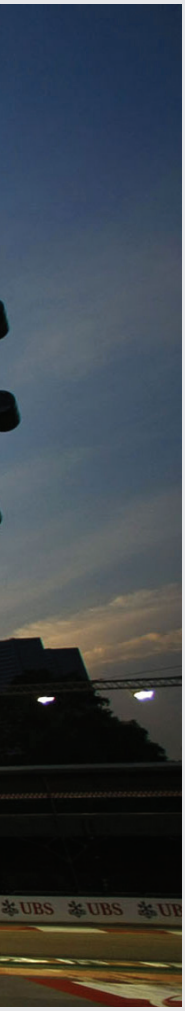
Supplementary Table 1: Continued

Disease type	FAB-type	Sex	Age	Karyotype	MLPA *MLL*-PTD	SB
AML	M6	M	62	45-48,XY,der(1)del(1)(p13p31)inv(1)(p13q24)t(1;7)(q3?2;q3?)[18],idic(4)(q1?2))[17],der(5)t(1;5)(?;?q11)[14],-5[5],-7[18],del(7)(p1?2p2?2)[18],+8[18],+11[14],der(17)ins(17;1)(p;?)t(1;17)(?;q)[17],-17[1],der(20)t(5;20)(?;p1?2)[18],+1-4mar[9][cp19]/46,XY[2]	MLPA: neg	neg
AML	M2	F	75	46,XX[18]	MLPA: neg	*MLL*-PTD
AML	M2	F	60	46,XX[32]	MLPA: pos	*MLL*-PTD

SB = Southern Blot, F= female, M= male, neg = negative for MLL-PTD


SingTel
SingTel
SingTel
UBS
SingTel

Appendix B



Color figures

Chapter 2

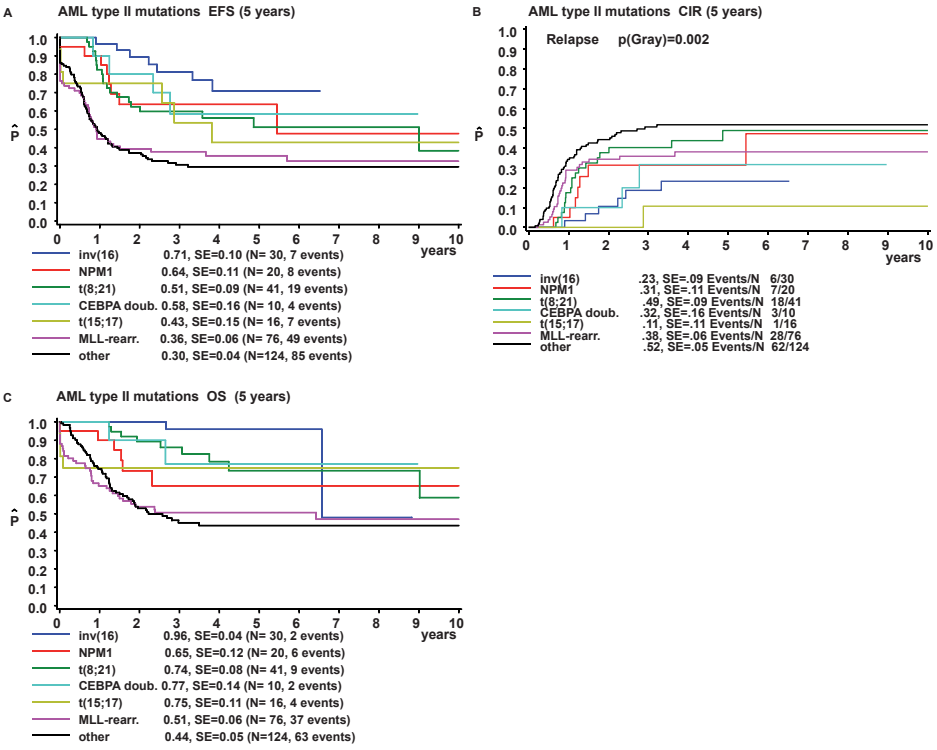


Figure 4: Survival analysis of the type-II aberrations in pediatric AML. Kaplan-Meier estimates for pEFS (A), CIR (B) and pOS (C)for the different type-II aberrations.

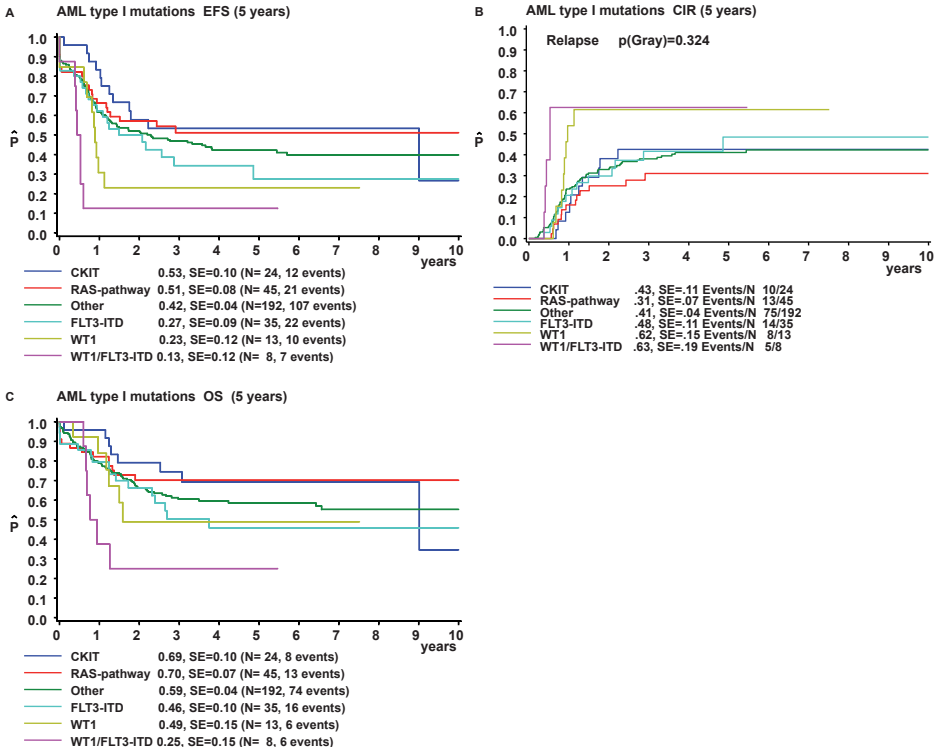
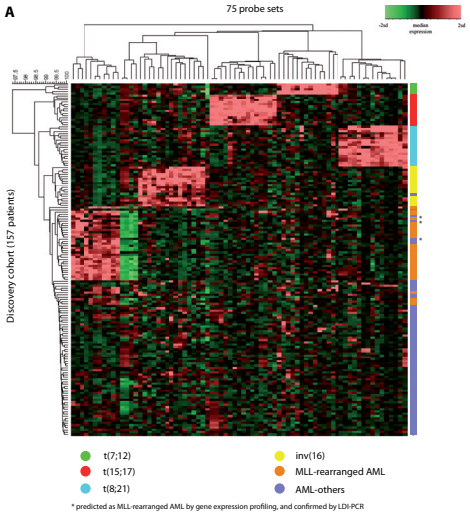


Figure 5: Survival analysis of the type-I aberrations in pediatric AML. Kaplan-Meier estimates for pEFS (A), CIR (B) and pOS (C)for the different type-I aberrations.

Chapter 3



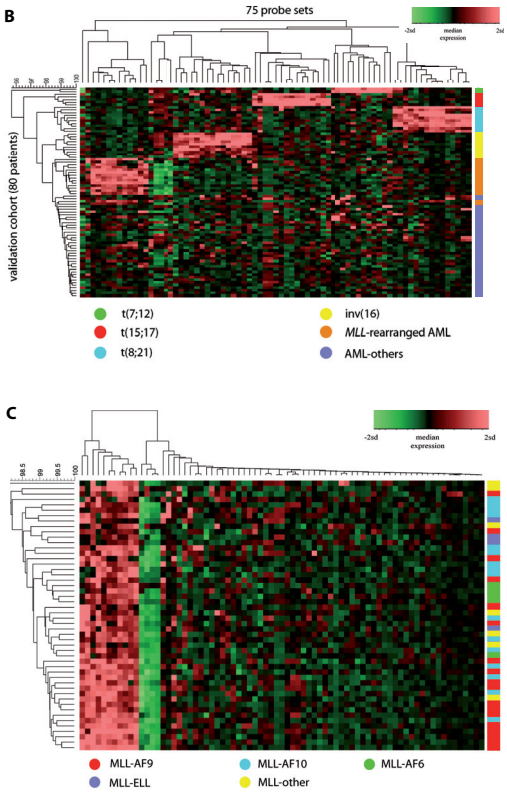


Figure 1: Hierarchical clustering of the cytogenetic subtypes of pediatric AML by gene expression profiling. a) Hierarchical clustering of 157 patients in discovery cohort by gene expression signature derived from 75 classifying probe sets (Supplementary Table S2). (b) Validation of gene expression pattern in 80 patients of the independent validation cohort (c) *MLL*-rearranged AML cases do not separate in distinct clusters based on similarity in expression pattern related to the translocation partner using the 75 classifying probe sets.

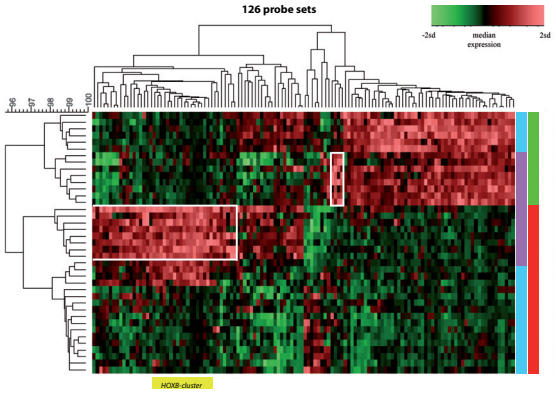


Figure 2: Hierarchical clustering of *FLT3*-ITD positive cases in t(15;17)(q21;q22) and CN-AML. Hierarchical clustering for *FLT3*-ITD in t(15;17)(q21;q22) and CN-AML based on 126 probe sets selected by multivariate analysis including molecular and cytogenetic subtype (Supplementary Table S12). Highlighted boxes represent probe sets for *FLT3*-ITD and the specific cytogenetic subtype. The *HOXB* cluster probe sets are represented in yellow.

Chapter 4

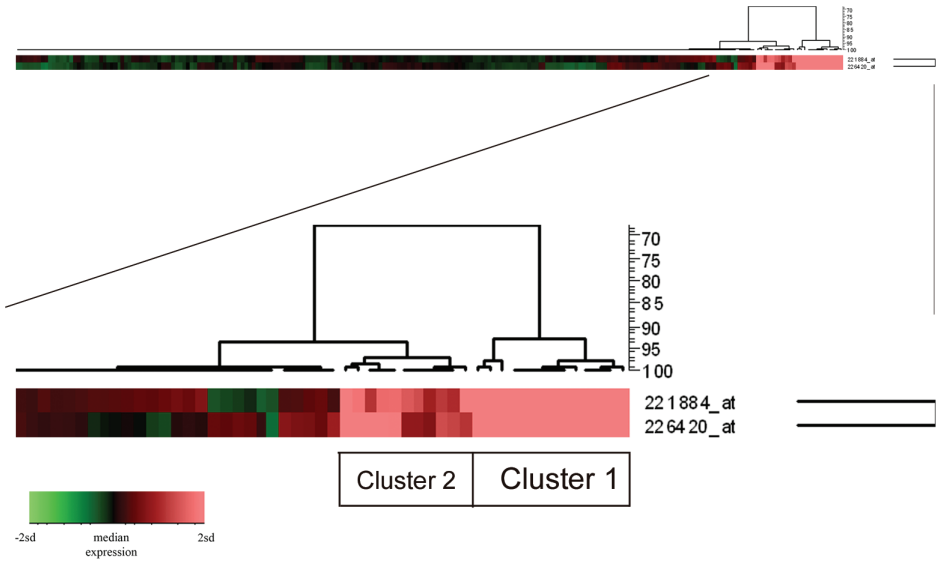


Figure 1: Hierarchical clustering using the gene expression of the 3 probe sets representing *EVI1* in 228 pediatric AML samples reveals a subclustering of 24 *EVI1*+ cases.
Hierarchical clustering of 228 pediatric AML samples with probe sets 221884_at and 226420_at representing the *EVI1* gene. Red represents high expression; black intermediate expression; and green low expression for the specific probe set.

Chapter 6

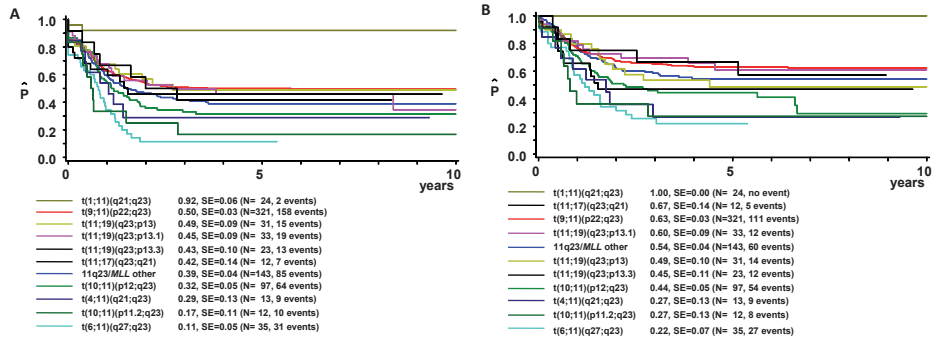


Figure 2. Survival curves for patients with 11q23/*MLL*-rearranged pediatric AML grouped on the basis of different translocation partners. Panel **A** shows event-free survival curves, and panel **B** shows overall survival. Assignment to 11q23-rearranged subgroups was based on translocation partners, as identified after central review of karyotyping. Some patients were assigned to 11q23 subgroups based on RT-PCR results only. If an *MLL* rearrangement was determined by FISH and the translocation partner was unknown, the patient was included in the "11q23/*MLL*-other" group. At least 10 patients had to be included to create a subgroup; otherwise the cases were allocated to the 11q23/*MLL*-other group. Patients with a t(1;11)(q21;q23) showed independent favorable outcome with overall survival at 5 years of 100%±0%, and an event-free survival of 92%±5%. Several rearrangements were identified as predictors of poor clinical outcome, including t(6;11)(q27;q23), t(10;11)(p11.2;q23), t(4;11)(q21;q23), and t(10;11)(p12;q23).

Chapter 8

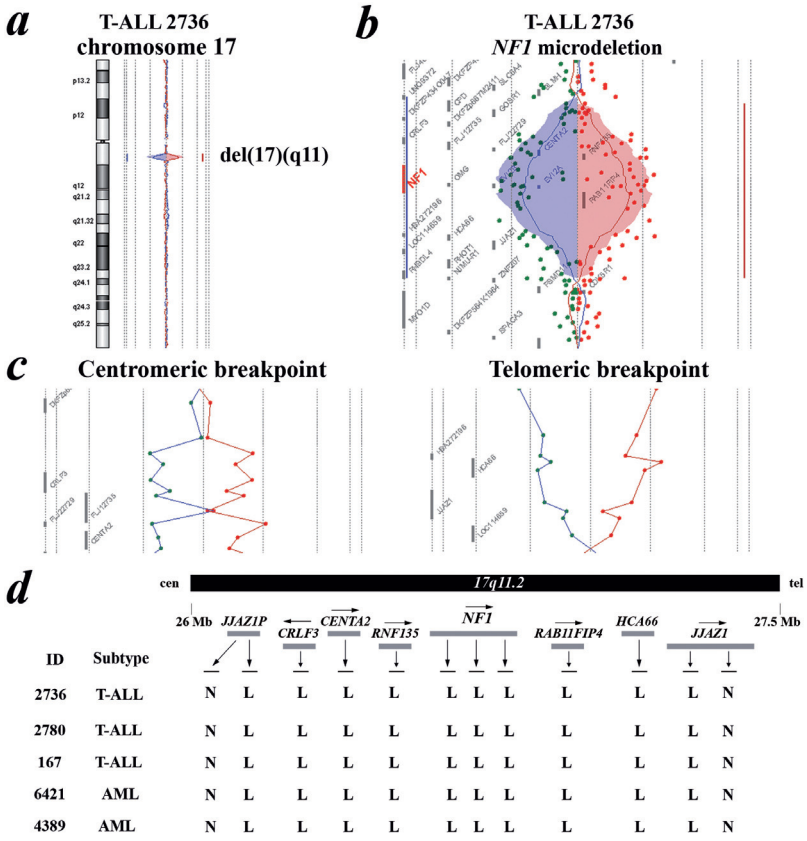


Figure 1. *NF1* microdeletions in pediatric acute leukemias.

(a) Chromosome 17 ideogram and corresponding oligo array-CGH plotof patient DNA/control DNA ratios (blue tracing) versus the dye-swap experiment (red tracing) for T-ALL patient no. 2736.

(b) Detailed visualization of the *NF1* microdeletion at chromosomal band 17q11 in T-ALL patient no. 2736. Hybridization signals around the –2X or +2X lines represent loss of the corresponding region in the patient DNA.

(c) Detailed analysis of the centromeric(left panel) and telomeric (right panel) breakpoint of the *NF1* microdeletion in patient no. 2736.

(d) Overview of oligo array-CGH results in the chromosomal region 17q11.2 for 3 patients with T-ALL and 2 patients with AML with del(17)(q11.2). The 60-mer oligos present on the DNA array and located in this genomic area, as well as the specific genes located in this region with their transcription direction, are shown. Arrows above the indicated genes represent the direction of transcription

Abbreviations: N; normal, L; loss, cen: centromere, tel: telomere.

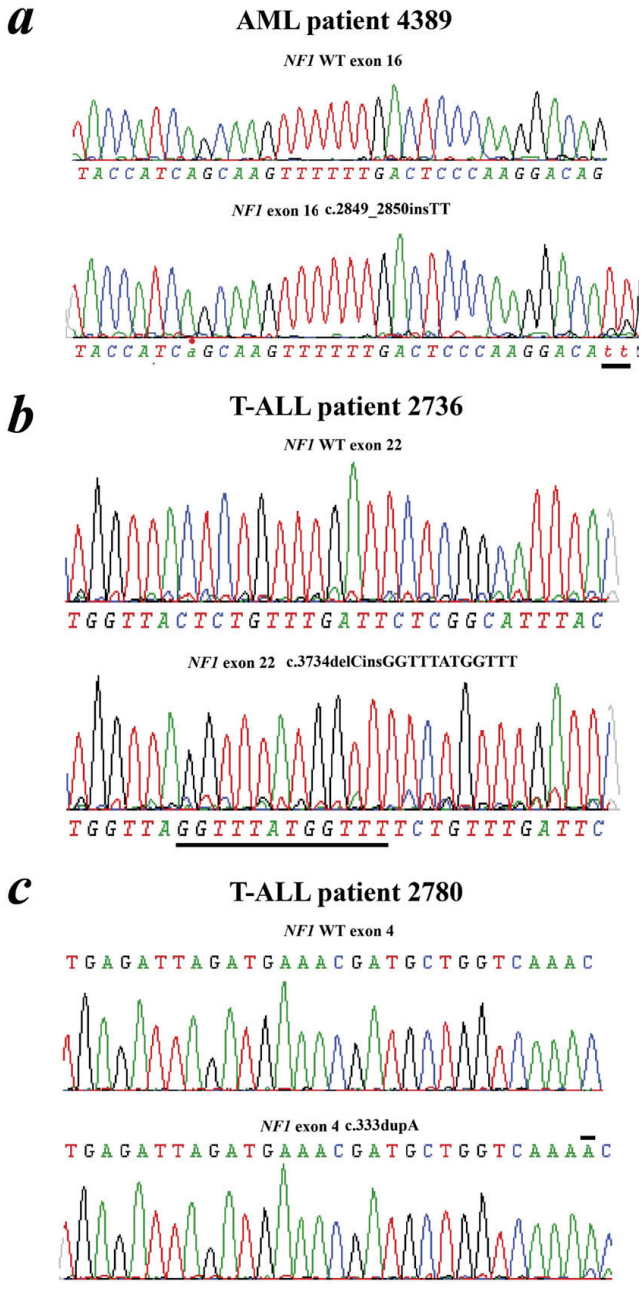


Figure 3. Truncating *NF1* mutations in pediatric T-ALL and AML.

(a) Sequence analysis of patient no. 4389 (AML) showing a *c.2849_2850insTT* mutation in the remaining *NF1* allele

(b) Sequence analysis of patient no. 2736 (T-ALL) showing a *c.3734delCinsGGTTTATGGTTT* mutation in the remaining *NF1* allele

(c) Sequence analysis of patient no. 2780 (T-ALL) showing a *c.333dupA* mutation in the remaining *NF1* allele

Chapter 9

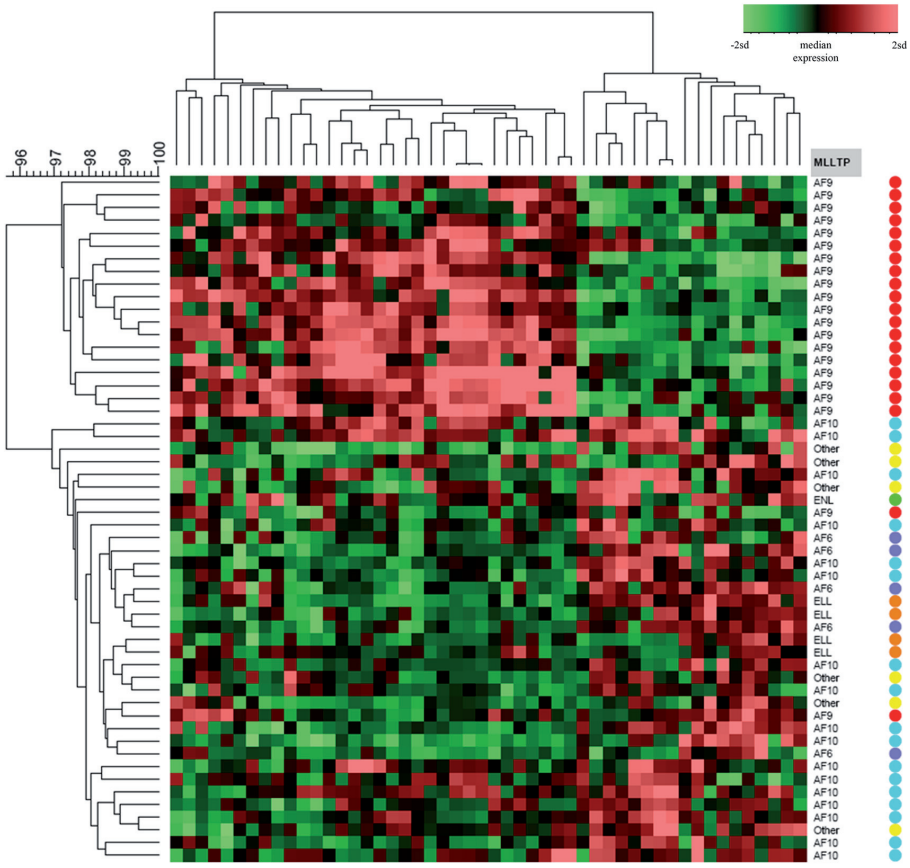


Figure 1: Hierarchical clustering based on the top 50 most discriminative genes for t(9;11)(p22;q23). Hierarchical clustering of 53 *MLL*-rearranged AML cases based on the top 50 most discriminative genes showed for t(9;11)(p22;q23) after supervised analysis (Supplementary Table S2).