Altered Gene & MiRNA Regulation in Pediatric Acute Myeloid Leukemia

JENNY E. KATSMAN-KUIPERS

De foto's op de omslag en tussen de hoofdstukken zijn genomen op het strand bij Oostkapelle, Zeeland. Karakteristiek voor deze provincie zijn de zogenaamde paalhoofden. De auteur vindt paalhoofden zijn de ideale wegwijzer om vooruit te kijken, naar de horizon (en willicht zelfs daar voorbij).

Both the photograph on the cover as well as the one between chapters have been taken on the beach near Oostkapelle, Zeeland. The so-called *paalhoofden* are characteristic for this part of the Netherlands. The author thinks these *paalhoofden* are the ideal guide to look forward, to the horizon (and maybe even beyond).

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"Cheshire- Puss, would you tell me, please, which way I ought to go from here?"

"That depends a good deal on where you want to get to," **said the Cat.**

"I don't much care where —," **said Alice.**

"Then it doesn't matter which way you go." **said the Cat.**

"— so long as I get **somewhere,"** **Alice added as an explanation.**

"Oh, you're sure to do that," **said the Cat,** *"if you only walk long enough."*

— Lewis Carroll, Alice in Wonderland

Voor alle kinderen met leukemie en hun ouders

CONTENTS

1

INTRODUCTION

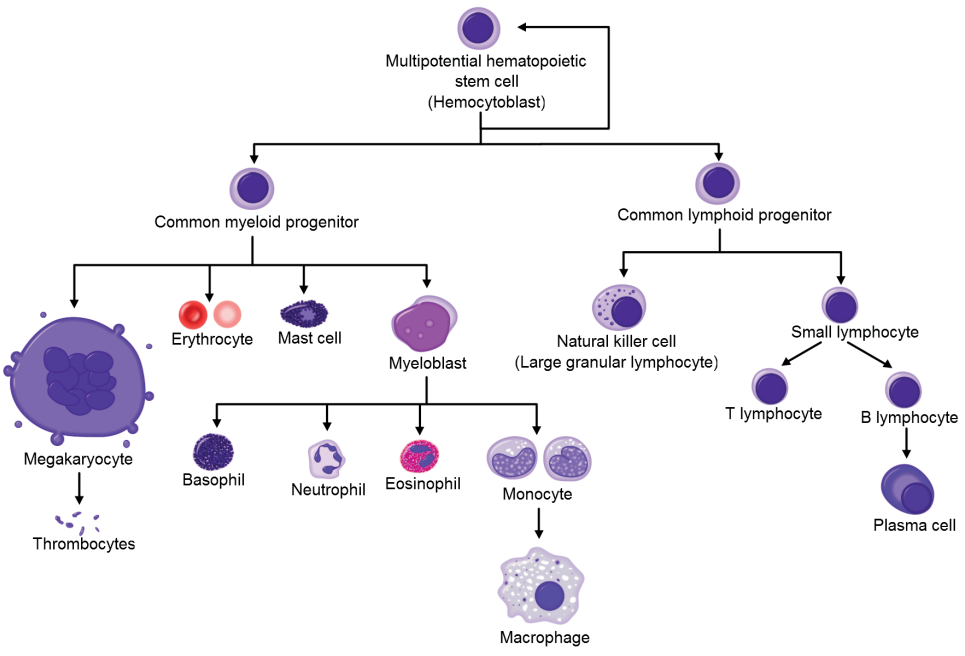
NORMAL HEMATOPOIESIS

The life-span of blood cells differs from very long – a maximum of 120 days for erythrocytes- to very short -8 hours for granulocytes-. Hence, to preserve the cell numbers required for normal function, hematopoiesis is a continuous process of blood cell maturation and proliferation.

Hematopoiesis occurs in different organs and tissues during the first months of embryonic life. It starts in the yolk sac during early developmental stages of an embryo, is taken over mainly by the liver and in a very limited extent by the spleen during the second trimester of pregnancy while the bone marrow is the most important organ involved in hematopoiesis in the third trimester of pregnancy. The bone marrow will take over hematopoiesis as most important organ from birth onwards, retaining this function throughout life. In children, the bone marrow of the long bones such as the femur and the tibia is the most important in hematopoiesis, while in adults hematopoiesis occurs mainly in flat bones: the pelvis, cranium, vertebrae and sternum.

All hematopoietic cells originate from the pluripotent hematopoietic stem cell (Figure 1). Normal function of blood cells can be roughly divided in three categories: delivery of oxy-

Figure 1: Development of blood cells from hematopoietic stem progenitor cells



Schematic representation of normal hematopoiesis. The multipotent hematopoietic stem cell possesses self-renewal capacity and can differentiate into the different mature blood cells. Source: A. Rad, Wikipedia Commons

Table 1: FAB-classification of Acute Myeloid Leukemia

Type	Name	% of pediatric AML	Chromosomal rearrangements
M0	Minimally differentiated AML	2-5%	
M1	AML without maturation	10-15%	
M2	AML with maturation	25-30%	t(8;21)(q22;q22), t(6;9)(p23q34)
M3	Acute promyelocytic leukemia (APL)	5-10%	t(15;17)(q22;q21)
M4	Acute myelomonocytic leukemia	15-25%	inv(16)(p13q22), del(16q)
M4eo	M4, together with bone marrow eosinophilia	10%	inv(16)(p13;q22), t(16;16)(p13;q21)
M5	Acute monoblastic(M5a) or monocytic(M5b) leukemia	15-25%	11q23 rearrangements
M6	Acute erythroid leukemia	1-3%	
M7	Acute megakaryoblastic leukemia	5-10%	t(1;22)(p13;q13)

gen to tissues by red blood cells, inflammation and wound healing by neutrophils, monocytes, lymphocytes, eosinophils, basophils, and fibroblasts, and continuous surveillance of blood vessels, blood clot formation and initiation of tissue repair by platelets.

PATHOGENESIS OF LEUKEMIA

Leukemia develops when proliferation is enhanced and maturation of blood cells is arrested. Depending on the lineage of the maturation arrest this results in lymphoid leukemia or myeloid leukemia. As the myeloid progenitor sires thrombocytes, erythrocytes, basophils, neutrophils, eosinophils and monocytes, myeloid leukemia is a heterogeneous disease. The practically unlimited growth of the leukemic clone that is derived from one progenitor of the myeloid lineage interferes with the formation of other cells, causing the symptoms fitting the initial presentation of leukemia. These include pallor and tiredness resulting from anemia —if erythrocytes are superseded—, easy bruising and spontaneous bleeding —by trombocytopenia—, and fever and infections resulting from a relative lack of granulocytes or monocytes. Organomegaly occurs due to blasts infiltrating organs such as the liver, spleen and testes, as well as by extramedullary hematopoiesis.

Leukemia is classified into four major categories: acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphoblastic leukemia (ALL) and chronic lymphoblastic leukemia (CML) [1]. Acute leukemia is the most frequent type of cancer in children[2]. In the Netherlands, 120-140 children are annually diagnosed with leukemia,15-20% of them is diagnosed with AML, whereas the vast majority is diagnosed with ALL (80%)[3,4].

Table 2: WHO classification of AML and related neoplasms (2008)

Acute myeloid leukemia with recurrent genetic abnormalities	AML with t(8;21)(q22;q22); RUNX1-RUNX1T1
	AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11
	APL with t(15;17)(q22;q12); PML-RARA
	AML with t(9;11)(p22;q23); MLLT3-MLL
	AML with t(6;9)(p23;q34); DEK-NUP214
	AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1
	AML (megakaryoblastic) with t(1;22)(p13;q13); RBM15-MKL1
	Provisional entity: AML with mutated NPM1
	Provisional entity: AML with mutated CEBPA
Acute myeloid leukemia with myelodysplasia-related changes	
Therapy-related myeloid neoplasms	
Acute myeloid leukemia, not otherwise specified	AML with minimal differentiation
	AML without maturation
	AML with maturation
	Acute myelomonocytic leukemia
	Acute monoblastic/monocytic leukemia
	Acute erythroid leukemia
	Pure erythroid leukemia
	Erythroleukemia, erythroid/myeloid
	Acute megakaryoblastic leukemia
	Acute basophilic leukemia
	Acute panmyelosis with myelofibrosis
Myeloid sarcoma	
Myeloid proliferations related to Down syndrome	Transient abnormal myelopoiesis
	Myeloid leukemia associated with Down syndrome
Blastic plasmacytoid dendritic cell neoplasm	
Acute leukemias of ambiguous lineage	
Acute undifferentiated leukemia	Mixed phenotype acute leukemia with t(9;22)(q34;q11.2); BCR-ABL1
	Mixed phenotype acute leukemia with t(v;11q23); MLL rearranged
	Mixed phenotype acute leukemia, B-myeloid, NOS
	Mixed phenotype acute leukemia, T-myeloid, NOS

EPIDEMIOLOGY OF CHILDHOOD AML

The incidence of acute myeloid leukemia increases with age, most patients are older than 50[5]. In children, incidence peaks in infants whereafter incidence is low during childhood only to rise again in adolescence. In the period 2002-2012 the mean incidence of AML in 0-4 year old children was 1.2 per 100.000, this was significantly lower in 5-9 year old children with 0.4 per 100.000, as well as in adolescents: 0.7 per 100.000 in 10-14 year olds and 15-19 year olds[6]. AML usually occurs *de novo*, but it can also arise from clonal evolution of preleukemic myeloproliferative diseases like myelodysplastic syndrome (MDS) or juvenile myelomonocytic leukemia (JMML). Rarely, AML occurs in children with underlying genetic diseases such as chromosomal-breakage syndromes (Fanconi anemia, Bloom syndrome), or other congenital syndromes (severe congenital neutropenia, Diamond-Blackfan anemia, dyskeratosis congenita), or as a secondary neoplasm after previous radiotherapy or chemotherapy[7–10]. Environmental factors —reported to be of importance in adult AML— are likely to be less relevant in children as exposure time is usually much shorter[11,12].

Familial occurrence of AML has been reported incidentally, and recent investigations associate germ-line mutations in several genes (for instance *tumor protein p53 (TP53)*, *GATA binding protein 2 (GATA2)*, *runt-related transcription factor 1 (RUNX1)*, and *CCAAT/enhancer binding protein (C/EBP), alpha (CEBPA)*) with this[13–18]. Patients with Down's syndrome that are born with a transient myeloproliferative disorder comprise a special patient group. In those patients, a mutation in the *GATA binding protein 1 (GATA1)* gene is a characteristic hit towards AML[19].

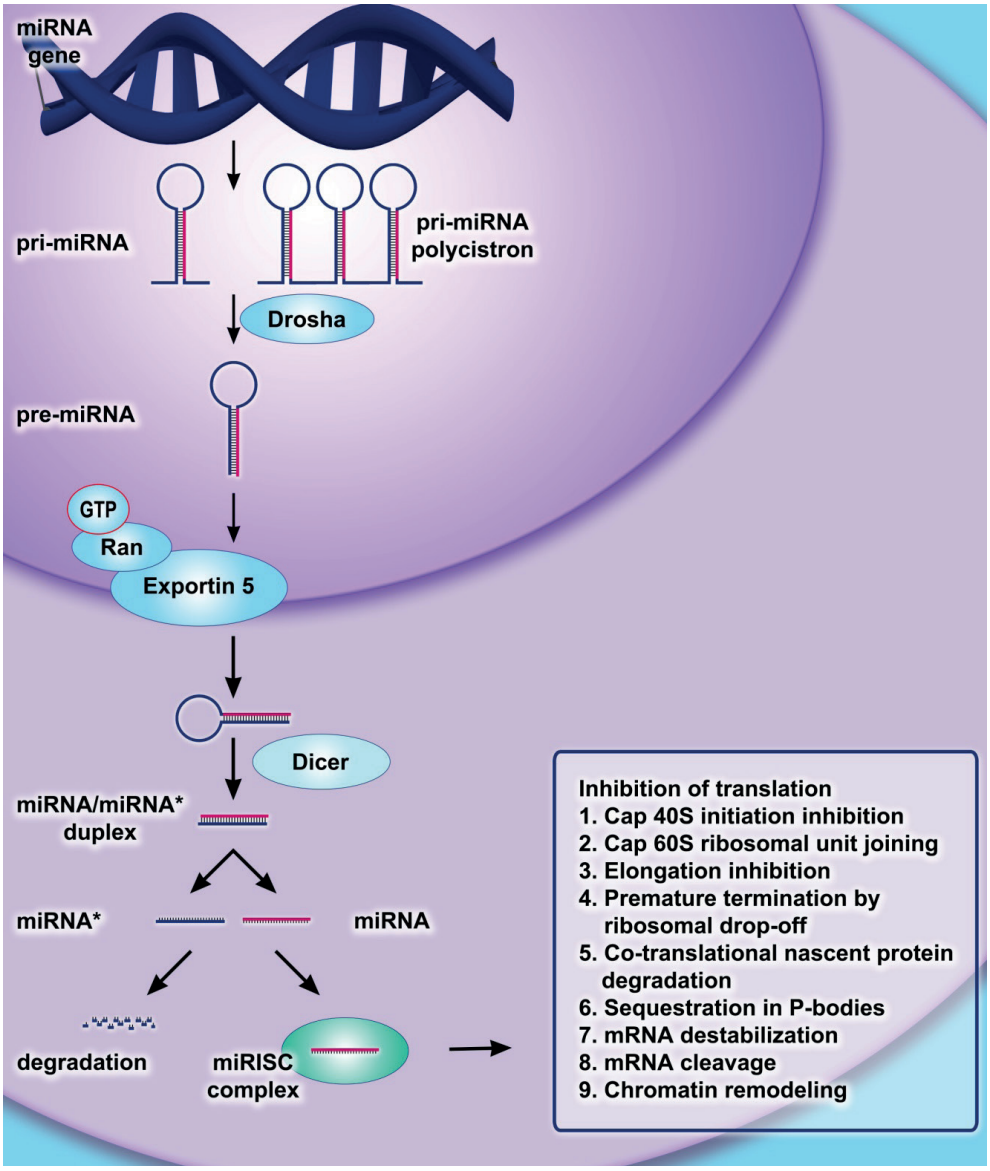
DIAGNOSIS AND CLASSIFICATION OF CHILDHOOD AML

AML classification is based on morphology, cytochemistry and immunophenotyping according to the French, American, and British (FAB) schema that includes 8 major subtypes: M0-M7 (Table 1). This classification represents the cell line of which the AML originated. The various genetic events in AML often occur specifically in cells of common origin: for example t(15;17)(q22;q21) is associated with FAB-M3.

In 2000, the World Health Organization (WHO) introduced a classification system that was based on the FAB-classification. The new classification adapted the number of blasts required for diagnosis (20% instead of 30%) and the incorporation of molecular, morphologic and clinical features. The presence of cytogenetic aberrations was used as an important determinant of leukemia-type[20]. The WHO classification of (adult) AML was updated in 2008 to include a larger number of recurrent genetic abnormalities, it also introduced a stringently defined subclass of acute leukemias of ambiguous lineage; that included the presence of t(9;22)(q34;q11.2), and presence of t(v;11q23)/*MLL*-rearrangements (Table 2)[21].

At the moment, the WHO classification identifies several subtypes of AML based on cyto-

Figure 2: miRNA biogenesis and action



Primary miRNAs (pri-miRs) are transcribed from the DNA and trimmed into premature miRNAs (pre-miRs) by a microprocessor complex that includes Drosha and DGCR8. Exportin 5 and Ran-GTP subsequently export the pre-miR from the nucleus to the cytoplasm where it is processed by the enzyme complex Dicer to a miRNA duplex. Generally, one of the two strands is degraded (the miRNA* strand), while the other, the 'guide strand', is incorporated into the miRNA inducing silencing complex (RISC)65. The RISC binds to the 3'-UTR of a specific mRNA by (im)perfect base pairing that uses bases 2-8 from the total ~22 nucleotides of the miRNA, the 'seed sequence'. This action results in inhibition of translation, through one or more of nine different mechanisms.

genetic aberrations (mainly chromosomal translocations) as well as single gene mutations to create uniform subgroups defined by unifying molecular targets. The aim of this ongoing additional characterization of AML blasts is to facilitate therapeutic stratification[21].

The WHO 2008 classification does not discriminate between pediatric and adult AML. Although it can categorize about 60-70% of pediatric AML patients, the hallmark aberration for about 30% of pediatric cases is either recently discovered and not acknowledged in the WHO classification, like t(7;12)(q36;p13), t(7;12)(q32;p13), t(1;22)(p13;q13), and t(5;11)(q35;p15.5), or not identified yet[22,23]. Furthermore, diagnosis of AML in adults requires only 20% of blasts in the bone marrow, whereas this threshold in children lies at 30%. If the blast percentage is 20-30, the percentage should be determined after an interval of approximately 2 weeks to allow for differentiation between AML and advanced MDS[23].

TREATMENT AND OUTCOME OF CHILDHOOD AML

Outcome of pediatric AML has radically improved over the last 4 decades. Most recent data (2004-2010) indicate 5-year event free survival rates up to 50-60% and up to70% 5-year overall survival.[24–27].

In general, chemotherapeutic regimens have a backbone that consists of 4-5 cycles of intensive cytarabine therapy combined with an anthracycline. For treatment of acute promyelocytic leukemia (APL), the addition of ATRA improves survival by inducing leukemic cell differentiation and reducing early death rates[28]. The use of allogenic stem cell transplantation in first complete remission is no longer recommended by most study groups, except for selected high-risk patients[29].

Current treatment stratification is based on the combination of cytogenetic aberrations and early treatment response, which is usually measured as the morphological complete remission rate after one or two courses of therapy, often confirmed by flow cytometry. The clinical benefit of minimal-residual disease monitoring, by morphology, immunophenotyping or quantification of molecular aberrations and gene expression levels, is still under investigation[25,26,30–32].

Part of the improved survival is due to improvements in supportive care[33–37]. Still, a significant number of patients (5-10%) suffers from early death or treatment related mortality[38–41]. Considering this, further treatment intensification is generally not considered feasible[38,42]. Further exploration of new therapeutic options could be guided by results from genetic and epigenetic studies.

GENE DEREGULATION IN CHILDHOOD AML

AML arises from at least two somatic genetic events affecting gene function: one that confers a proliferative advantage (type I mutations), the other inducing differentiation arrest (type II mutations)[43]. Type I abnormalities often involve activating mutations of genes involved in signal transduction pathways like *FLT3*, *C-KIT*, *N-RAS*, *K-RAS* and *PTPN11*[44,45]. Type II abnormalities mainly result from genetic alterations of hematopoietic transcription factors, often caused by AML-specific translocations like t(15;17)(q22;q21)/*PML-RARA,* t(8;21)(q22;q22)/*AML1-ETO,* inv(16)(p13;q22)/*CBFB-MYH11,* 11q23/*MLL*-rearrangements, or from mutations in genes such as *NPM1*, *GATA1, WT1*, and *CEBPA*[46–50]. Recent reports indicate that, aside from altered gene function, altered gene expression profiles are characteristic for patient groups of pediatric AML[51]. Expression of some deregulated genes relates to outcome, like *BRE, EVI1*, *IGSF4*, *BAALC* and *ERG*[52–55].

EPIGENETIC REGULATION OF GENE EXPRESSION

Epigenetic events influence gene expression in the cell without altering the genetic code and involves processes such as DNA methylation, histone modification, nucleosome remodeling and RNA-mediated targeting of mRNA (for example by miRNAs)[56]. Most research in AML has focused on alterations in DNA hypermethylation resulting in silencing of tumor suppressors, as this mechanism represents an immediate opportunity of therapeutic intervention by demethylating drugs such as decitabine or azacitidine. Thus, DNA-methylation profiles, the significance of recurrent mutations in epigenetic modifying genes (type III mutations) like *TET2, IDH/ IDH2,* and *DNMT3A*, as well as the effects of DNA-demethylases have been studied in AML [57,58].

RNA-MEDIATED GENE TARGETING BY MIRNAS

RNA mediated gene targeting by miRNAs is another epigenetic mechanism that has been extensively studied the last years. MiRNAs are circa 22 nucleotide long non-coding RNAs that were discovered in roundworms in 1993 and inhibit gene expression through antisense miRNA-RNA interaction[59]. Mature miRNAs are formed from primary miRNA genes in a process consisting of several steps (Figure 2). These includes trimming of the primary miRNA into premature miRNAs, export from the nucleus to the cytoplasm for further modification and processing by the enzyme complex Dicer to a miRNA duplex [60–64]. Either one of these strands can be incorporated into the miRNA inducing silencing complex (RISC-complex) to facilitate (im)perfect base pairing to the 3'-UTR of a specific mRNA (target-mRNA)[65]. This results in inhibition of translation, through one or more of nine different mechanisms[66]. Disturbance of mRNA stability by miRNAs resulting in less mRNA expression was the avenue of research that was pursued in this thesis[67]. Acquiring this information was important as miRNAs can inhibit both proto-oncogenes and tumor suppressor

genes.

Each individual miRNA affects multiple target genes. In turn, 60% of human genes are miRNA targets. This implies a broad spectrum of possible functions for each miRNA. Indeed, miRNAs influence many physiological processes like apoptosis, proliferation, differentiation, and ageing[68–73]. Deregulation of miRNA expression contributes to leukemogenesis through interaction with tumor suppressor genes involved in proliferation and differentiation[71,72,74–85].

In the course of 2008, the first results of miRNA expression in adult AML patients were reported, identifying miRNA expression profiles related to AML subtype[86,87]. Later on, miRNAs predicting clinical outcome were identified [78,83,88–91]. However, information regarding miRNA expression and function in pediatric AML was unavailable at the time this thesis project started.

OUTLINE OF THIS THESIS

The aim of this PhD project was to provide insight into altered gene expression and relevant regulating molecular mechanisms, with a special focus on identifying MiRNAs contributing to leukemogenesis and outcome of pediatric acute myeloid leukemia.

In **Chapter 2**, we studied differential expression of genes in pediatric AML with *MLL*-rearrangements, and focused specifically on prognostic relevant subgroups as defined by *MLL* translocation partner. For the most significant gene, *IGSF4*, the mechanism influencing differential expression and its prognostic relevance in pediatric AML was assessed. **Chapter 3** describes a study on the occurrence of mutations in two specific genes, *RUNX1* and *CSF3R,* in both *de novo* and secondary pediatric AML.

Chapter 4 to 7 focus on epigenetic deregulation in pediatric AML. **Chapter 4** describes a study of three candidate miRNAs (i.e. miR-155, miR-196a/b, and miR-29a) in pediatric AML patients that initiated further miRNA expression profiling studies in a large international cohort of pediatric AML samples containing relevant subtypes of pediatric AML. These miRNA expression profiles, as well as sensitivity and specificity of classification based on miRNA expression profiles, and correlations between miRNA expression and predicted prognosis are described in **Chapter 5**. **Chapter 6** continues this line of research, studying the specific miR signature in t(8;21) positive pediatric AML and its consequences for differentiation and cell survival. Additionally, mutation analysis of candidate epigenetic regulator genes is described in **chapter 7**.

Chapter 8 summarizes this thesis while **Chapter 9** comprises the general discussion of and future perspectives.

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HIGH *IGSF4* EXPRESSION IN PEDIATRIC M5 ACUTE MYELOID LEUKEMIA WITH T(9;11) (P22;Q23)

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CHAPTER 2: HIGH IGSF4 EXPRESSION IN T(9;11) M5 AML

ABSTRACT

Pediatric mixed-lineage leukemia (*MLL*)–rearranged acute monoblastic leukemia with t(9;11)(p22;q23) has a favorable outcome compared with other *MLL*-rearranged AML. The biologic background for this difference remains unknown. Therefore, we compared gene expression profiles (GEPs; Affymetrix HGU133 + 2.0) of 26 t(9;11)(p22;q23) patients with 42 other *MLL*-rearranged AML patients to identify differentially expressed genes. *IGSF4*, a cell-cell adhesion molecule, was found to be highly expressed in t(9;11)(p22;q23) patients, which was confirmed by real-time quantitative polymerase chain reaction and Western blot. *IGSF4* expression within t(9;11)(p22;q23) patients was 4.9 times greater in French-American-British morphology classification (FAB)–M5 versus other FAB-types (P= .001). Methylation status investigation showed that high *IGSF4*-expressing t(9;11)(p22;q23) patients with FAB-M5 have no promoter hypermethylation, whereas all other cases do. Cell-line incubation with demethylating agent decitabine resulted in promoter demethylation and increased expression of *IGSF4*. Down-regulation of *IGSF4* by siRNA did not affect proliferation or drug sensitivity. In a cohort of 79 *MLL*-rearranged AML cases, we show significant better overall survival for cases with high *IGSF4* expression (5-year overall survival 0.70 vs. 0.37, P= .03) In conclusion, we identified *IGSF4* overexpression to be discriminative for t(9;11)(p22;q23) patients with FAB- M5, regulated partially by promoter methylation and resulting in survival benefit.

INTRODUCTION

Pediatric acute myeloid leukemia (AML) is a heterogeneous disease. Currently, apart from response to treatment, the most important prognostic factor is cytogenetic aberrations. Well-known cytogenetic abnormalities that predict differences in survival are t(15;17)(q22;q21) (PML-RARa), t(8;21)(q22;q22) (RUNX1-RUNX1T1), inv(16)(p13q22) (CBF-MYH11), and mixed-lineage leukemia (*MLL*)-rearranged AML.[1-3] Intensive chemotherapy has improved survival rate during the past decades (5-year event-free survival [EFS] 60%). Future therapeutic strategies should be directed toward outcome as well as toward limitation of short- and long-term toxicity.[4] It is anticipated that such strategies can be determined by the molecular targeting of abnormally expressed genes in specific genetic types of pediatric AML.[5]

In recent years, more than 60 different translocation partners of the *MLL* gene have been described.[6] In pediatric *MLL*-rearranged AML, the most common translocations are t(9;11)(p21;q23) (*MLL*-AF9), which occurs in approximately 50% of patients; t(10;11)(p12;q23) (*MLL-AF10*), t(6;11)(q27;q23) (*MLL-AF6*); and t(11;19)(q23;p13.3) (*MLL-ENL*).[1,2] Of interest is that t(9;11) has been linked with favorable outcome.[7-9] Recently, we identified that superior prognosis in the t(9;11) cases was restricted to those with French-American British morphology classification (FAB) M5 phenotype.[10]

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

METHODS

Patients

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

The samples included 33 pediatric *MLL*-rearranged cases with t(9;11)(p22;q23) and 52

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

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

Values reflect number of cases (%) unless otherwise specified. AML indicates acute myeloid leukemia; FAB, French-American-British morphology classification; GEP, gene expression profiling array; MLL, mixed-lineage leukemia; RT-qPCR, quantitative real-time polymerase chain reaction; and WBC, white blood cell count.

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

Materials

Leukemic cells were isolated and enriched as previously described.[11,12] All resulting samples contained 80% or more leukemic cells, as determined morphologically by May-Grünwald-Giemsa (Merck)–stained cytospins. A minimum of 5 x 10^6 leukemic cells was lysed in Trizol reagent (Gibco BRL/Life Technologies) and stored at -80°C. Isolation of genomic DNA and total cellular RNA was performed as described previously.[13] Leukemic samples were routinely investigated for *MLL*-rearrangements by standard chromosome-banding

analysis and/or fluorescence *in situ* hybridization. If translocation with one of the common partners (*MLL-AF9*, *MLL-AF10*, *MLL-AF6*, *MLL-ENL*, and *MLL-ELL*) was suspected, reverse transcriptase polymerase chain reaction (PCR) was performed. (Primers are described in supplemental Table 1) Of the 85 *MLL*-rearranged cases, 33 harbored a t(9;11)(p22;q23), 19 a t(10;11)(p12;q23), and 15 a t(6;11)(q27;q23). The remaining 18 cases were confirmed with long- distance inverse PCR as *MLL* other.

Gene expression profiling

Gene expression profiling (GEP) was performed on the RNA of a cohort of 237 *de novo* and 8 secondary pediatric AML samples. We included 14 additional cases of *MLL*-rearranged AML (5 of which carried a t(9:11)) for GEP to increase group size. Integrity of total RNA was checked with the use of the Agilent 2100 Bio-analyzer (Agilent). cDNA and biotinylated cRNA was synthesized, hybridized, and processed on the Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix) according to the manufacturer's guidelines. Data acquisition was performed by the use of Expresso (Bioconductor package Affy), and probe-set intensities were normalized by the use of the variance stabilization normalization (Biocon- ductor package VSN) in the statistical data analysis environment R, version 2.7.0.[14,15] Expression levels were log-transformed during this normalization. An empirical Bayes linear regression model was used to compare the signatures for the t(9;11) cases to all other *MLL*-rearranged AML cases.[16] Moderated T-statistics P values were corrected for multiple testing by use of the false discovery rate method defined by Benjamini and Hochberg.[17] *IGSF4* was identified from a top-50 differentially expressed gene list. For the expression analysis of *IGSF4*, probe set 209031_at was used.

Quantitative real-time PCR

Quantitative real-time PCR (RT-qPCR) was performed on cDNA of 95 pediatric AML patient samples, selected on availability of remaining cDNA, produced as previously described.[18] Within this group, 57 were classified as *MLL*-rearranged leukemia, of which 24 harbored a t(9;11) (Table 1). An ABI PRISM 7900HT sequence detector (Applied Biosystems) was used to validate the GEP results. Primers used for *IGSF4* are described in supplemental Table 1. For expression analysis of *IGSF4*, SYBRgreen (Finnzymes) was used. The expression of the genes was compared with glyceraldehyde 3-phosphate dehydrogenase, with primers and probe as previously described (sequences are shown in supplemental Table 1).[18] The average cycle threshold (Ct) value was used to calculate mRNA expression levels of *IGSF4* relative to the expression level of the reference gene (glyceraldehyde 3-phosphate dehydrogenase) by use of the comparative cycle time (ΔCt) method.[19]

Western blot

For Western blot, 10 leukemia samples were selected on the basis of the availability of material, of which 3 harbored a t(9;11), 3 harbored another *MLL*-translocation, and

4 had a karyotype other than *MLL* (AML-other, containing a case with t(8;21), one with inv(16), one with t(15;17), and one with a normal karyotype). Cell pellets stored at -80°C were quickly thawed and resuspended in 100 µL of lysis buffer composed of 25mM Tris (tris[hydroxymethyl]aminomethane) buffer, 150mM NaCl, 5mM EDTA (ethylenediaminetetraacetic acid), 10% glycerol, 1% Triton X-100, 10mM sodium pyrophosphate, 1mM sodium orthovanadate, 10mM glycerolphos- phate, 1mM dithiothreitol, 1mM phenylmethylsulfonyl fluoride, 1% aprotinin (Sigma-Aldrich), 10mM sodium fluoride, and 20 µL of freshly prepared sodium pervanadate. Subsequently, cell lysis was allowed for 30 minutes on ice. Cell lysates were cleared by centrifugation for 15 minutes at 10 000g (13 000 rpm) and 4°C. Protein concentration was determined by use of the bicinchoninic acid protein assay (Pierce Biotechnology) with different concentrations of bovine serum albumin as standards. Cell lysates containing 20 ng of protein were separated on 10% polyacrylamide gels and transferred onto nitrocellulose membranes (Schleicher & Schuell). Western Blots were probed with goat polyclonal IgG anti-TSLC1 (synonym of IGSF4, sc-25 077; Santa Cruz Biotechnology) and mouse antibeta-actin (ab6276; Abcam) antisera. Subsequently, the blots were labeled with peroxidase-conjugated antigoat antibody (sc-2020; Santa Cruz Biotechnol- ogy) or antimouse antibody (DAKO). Chemiluminescence (SuperSignal West Femto Maximum Sensitivity Substrate; Pierce Biotechnology) was used to detect luminescence using the Syngene chemigenius (Syngene).

Methylation-specific PCR

To investigate the methylation status of *IGSF4* methylation-specific PCR (MS-PCR) was used. Fourteen leukemia samples were selected, 5 samples with a t(9;11) on the basis of their high *IGSF4* expression with GEP and RT-qPCR. These samples with t(9;11) were compared with 5 *MLL*-rearranged samples with other translocation partners and 4 other AML samples. The primers as described by Overmeer et al[20] were used; 3 different areas of the promoter (designated 1 M/U, 5 M/U, and 9 M/U) were used (sequences are shown in supplemental Table 1). Unmodified genomic DNA was used to test the specificity of the primers for bisulfite-converted DNA. One DNA sample was first treated with DNA methylase SSS1 and methyl donor SAM (M0226S; New England Biolabs) and then bisulfite converted, creating a hypermethylated sample (M) as a control for the methylation specific primers. As a control for the unmethylated specific primers, bisulfate-converted DNA of healthy, adult male donors was used (U). The specificity of the methylation (M)- and unmethylation (U)-specific primers was tested on a dilution range with a mix of M and U DNA (supplemental Figure 1). The dilution ranges indicated that the combination of 9M and 9U is the most specific.

Demethylating agents

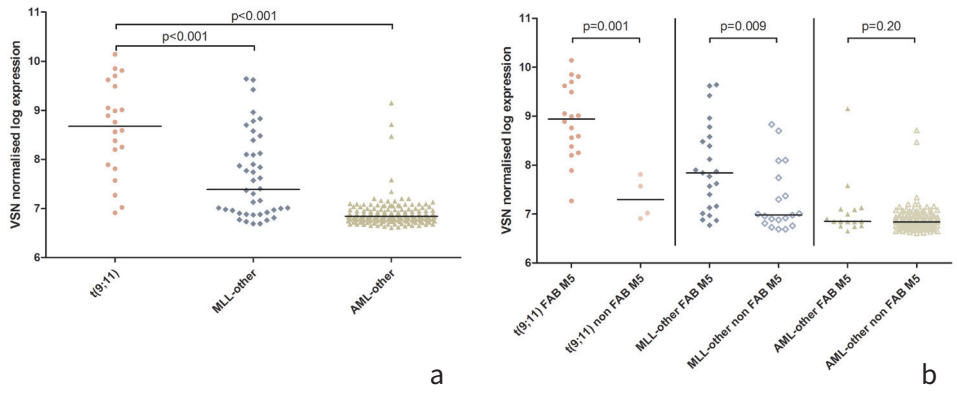
Cell lines ML-2 (*MLL* t(6;11)), HL-60 (AML-other), and MONO-MAC-1 (*MLL* t(9;11); DSMZ

GmbH) were cultured with and without demethylating agent decitabine. ML-2 and HL-60 were selected for their low expression of *IGSF4* on RT-qPCR, and MONO-MAC-1 was used as a control because it shows high *IGSF4* expression. Decitabine concentration was chosen after an in vitro drug assay with decitabine concentrations ranging from 0.125 µM to 4.0 µM and was determined for each cell line to be the approximate 50% lethal concentration. ML-2 was cultured with a concentration of decitabine of 2 µM, HL-60, and MONO-MAC-1 with a concentration of 4 µM. Decitabine and culturing medium (RPMI 1640 with L-alanyl-L-glutamine; Invitrogen); 10% fetal calf serum (FCS; Integro); and penicillin 100 U/mL, streptomycin 100 µg/mL, and fungizone 0.125 µg/mL (PSF; Invitrogen) were refreshed daily. The experimental condition started with 100 x 10^6 cells. Cell counts were determined on a daily basis, and cells were maintained in culture at a concentration of 0.5 x 10^6 cells/mL. Cell samples of both test and control conditions were taken from the medium every other day for the first 6 days and daily thereafter. They were washed with phosphate-buffered saline, and samples for protein studies were frozen at -80°C as dry cell pellets, and for DNA and RNA extraction cells were lysed in Trizol reagent and stored at -80°C. The experiment ended as soon as all remaining experimental cells were apoptotic.

siRNA transfection

The MONO-MAC-1, ie, t(9;11) and NOMO-1, ie, t(9;11) cell line (DSMZ) were cultured in RPMI-1640 medium supplemented with 10% FCS and PSF and grown as suspension cul-

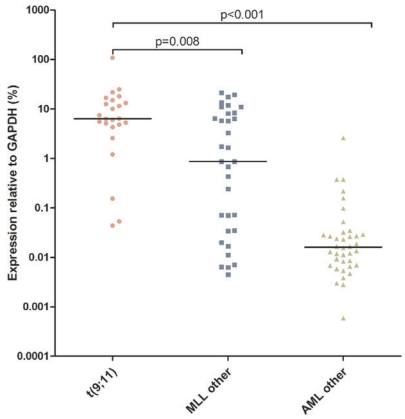
Figure 1. *IGSF4* gene expression in pediatric AML as determined by gene expression arrays.



Graphs showing the expression of probe set 209031_at, representing the *IGSF4* gene, after log transformation. Bars represent the median expression in each group. (A) Significant differences are shown between patients with a t(9;11) (n = 26) and patients with another *MLL* rearrangement (MLL other, n = 42; 8.8 vs 7.4, P < .001) or AML patients without an *MLL* rearrangement (AML other, n = 192; 8.8 vs 6.8, P < .001). (B) Expression of probe set 209031_at with all groups divided on the basis of morphology, ie, FAB M5 versus other FAB-types (non-FAB M5). All cases with unknown FAB type were excluded from this analysis (t(9;11) n = 1, AML-other n = 10). We detected a significant difference for median expression within the patients with a t(9;11) (n = 21 vs n = 4; 8.9 vs 7.3, P = .001) and the patients with other MLL rearrangements (n = 23 vs n = 19; 7.8 vs 7.0, P = .009). This difference was not detected in the remaining patients without an *MLL* rearrangement (AML other, n = 16 vs n = 166; 6.8 vs 6.8, P = .20).

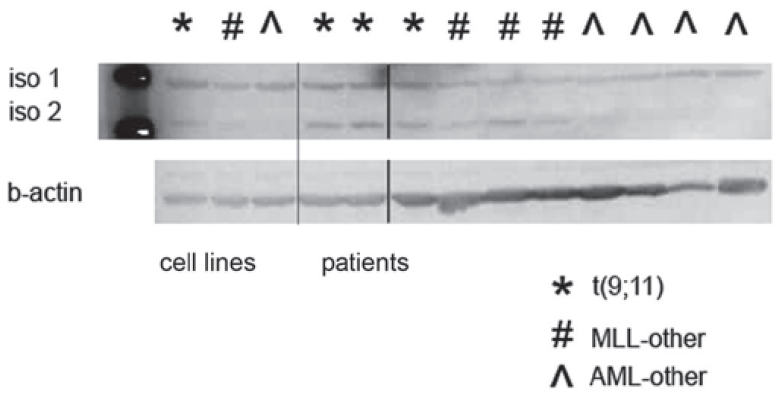
tures at 37°C in humidified air containing 5% CO2. Cells from both cell lines (10 x 10^6) were transfected by electroporation in 400 µL of RPMI 1640 with L-alanyl-L-glutamine (Invitrogen) containing 250nM of either a mix of equal amounts of *IGSF4* siRNAs (Dharmacon ON-TARGETplus LQ-016565; Thermo Fisher Scientific) or Nontargeting siRNA (Dharmacon ON-TARGETplus D-001810-01-05; Thermo Fisher Scientific), in 4-mm electroporation cu-

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



Graph showing the expression of *IGSF4* on mRNA level measured with RT-qPCR. Bars represent the median expression in each group. Significant differences are observed between patients with a t(9;11) (n = 24) and patients with another *MLL*-rearrangement (*MLL* other, n = 33; (6.4 vs 0.9, P = .008) or AML patients without a *MLL* rearrangement (AML other, n = 38; 6.4 vs 0.02, P < .001).

Figure 3. Protein expression analysis of IGSF4 with Western blot.



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

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

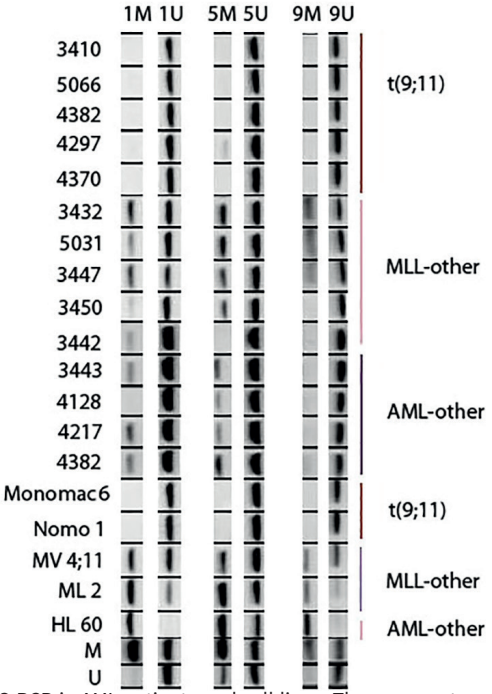
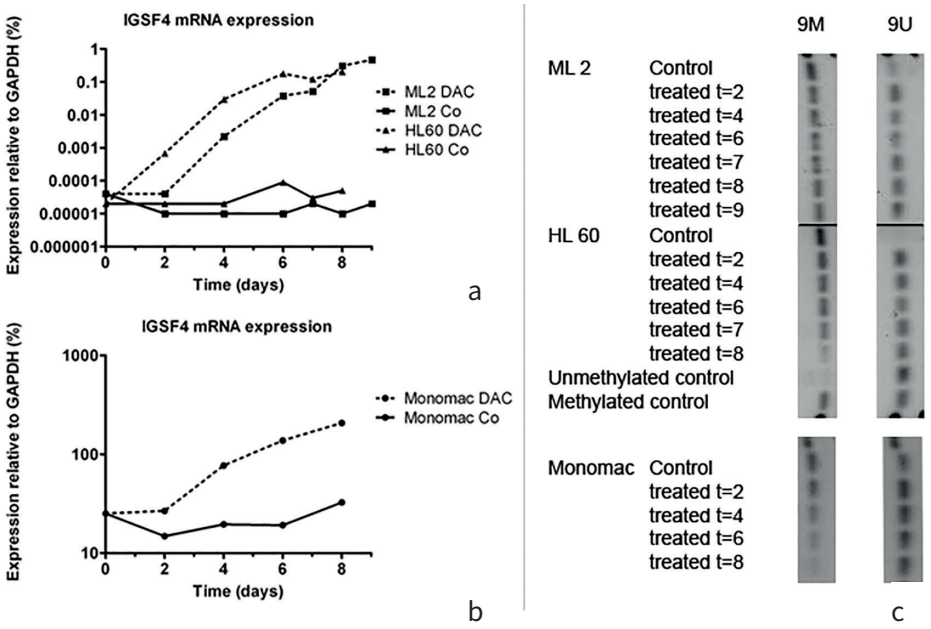


Figure showing results of MS-PCR in AML patients and cell lines. Three separate regions of the promoter were investigated; region 1, 5, and 9. Each column shows results for a specific primer pair (M, methylated; U, unmethylated). The top of the figure shows the *IGSF4* methylation status of several patients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

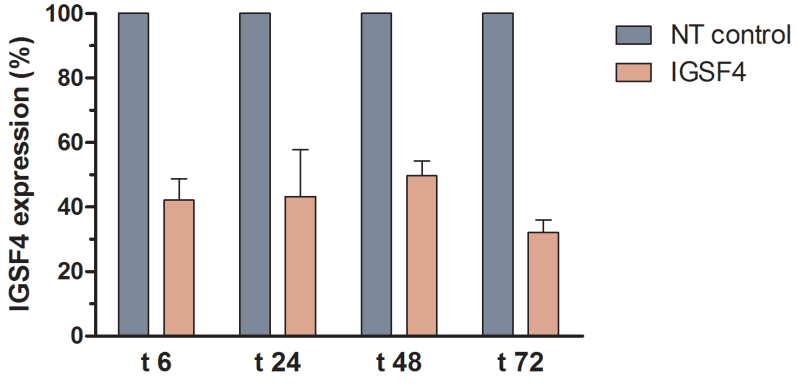
vettes (Bio-Rad; sequences are described in supplemental Table 1). Electroporation was performed by the use of an EPI 2500 gene pulser (Fischer) by applying a rectangle pulse of 300 V for 10 milliseconds. To compensate for the amount of cell death induced merely as a consequence of the electroporation procedure, control cells were electroporated in the absence of siRNA. After incubating for 15 minutes at room temperature, the cells were diluted in 10 mL pf RPMI 1640 supplemented with 10% FCS and PSF and incubated at 37°C and 5% CO2. They were maintained in culture for 72 hours. Cell counts were determined daily (t = 6 hours, t = 24 hours, t = 48 hours, and t = 72 hours). Cell samples of both test and control conditions were taken from the medium at every time point. They were washed with phosphate-buffered saline and lysed in Trizol reagent and stored at -80°C. DNA content and cell-cycle phase were assessed with the use of propidium iodide staining and measured by flow cytometry with a FACSCalibur (Becton Dickinson).

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



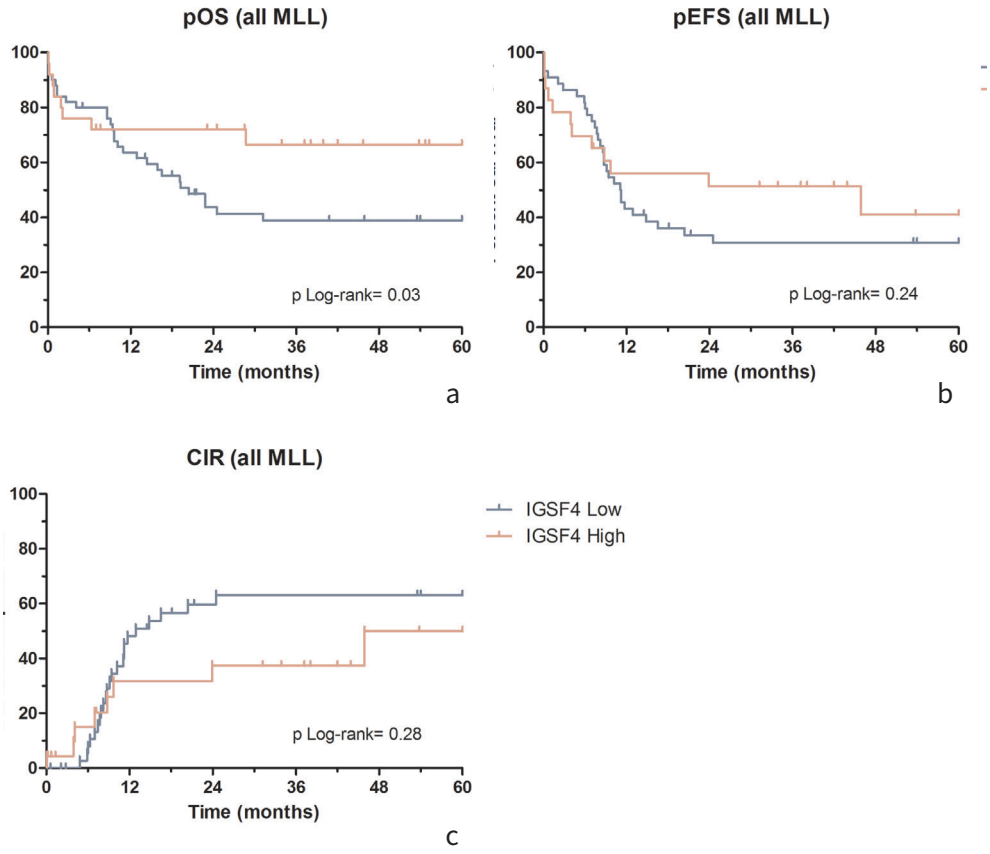
Graph showing *IGSF4* mRNA expression in cell lines ML-2, HL-60, and MONO-MAC-1 at different time points during culture with demethylating agent decitabine (DAC). (A) Solid lines correspond to untreated conditions of cell lines ML-2 (t(6;11)) and HL-60 (AML-other), and dotted lines reflect values from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

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



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

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



Plots showing overall survival (pOS) (A), EFS (pEFS) (B), and cumulative incidence of relapse (CIR; C) for all *MLL*-rearranged patients (n = 26 [*IGSF4* high] vs n = 53 [*IGSF4* low]). Median expression in the t(9;11) group was chosen as a cut-off for the division in high and low *IGSF4* expression. The solid line represents patients with high *IGSF4* expression, the dotted line represents patients with low *IGSF4* expression.

In vitro drug resistance

After transfection, in vitro drug resistance for daunorubicin (Cerubidine; Sanofi Aventis), cytosine arabinoside (Cytosar; Pharmacia), cladribine (2-chlorodeoxyadenosine; Leustatin), and etoposide (Pharmachemie) and as a control also vincristine (Pharmachemie), L-asparaginase (Paronal; Nycomed Christiaens), prednisolone (Bufa Pharmaceutical Products), and dexamethasone (Erasmus MC) was determined by use of the 2-, 3-, and 4-day 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays as described previously.[21] Six concentrations of each drug were tested in duplicate. The ranges of the final concentrations of these drugs were as follows: daunorubicin, 0.002-2.0 J.g/mL; cytosine arabinoside, 0.01-10 J.g/ mL; cladribine, 0.0004-4 J.g/mL; etoposide, 0.05-50 J.g/mL;

vincristine, 0.05-50 J.g/mL; L-asparaginase, 0.003-10 IU/mL; prednisolone, 0.008- 250 J.g/mL; and dexamethasone, 0.0002-6 J.g/mL.

Statistical analyses

Statistical workup of GEP data are described in "Gene expression profiling." For comparison of the gene expression in different groups, the Mann-Whitney U test was used. For assessment of correlation of the results from gene expression profiling and RT-qPCR, Spearman Correlation coefficient was used. All *MLL*-rearranged *de novo* AML cases with available follow-up data were included for survival analysis. Probabilities of overall survival, EFS (events: nonremitter, relapse, secondary malig- nancy, death from any cause), and cumulative incidence of relapse (events: nonremitter, relapse) were estimated by the method of Kaplan and Meier and compared by use of the log-rank test. Median *IGSF4* expression in the t(9;11) group was used to split all *MLL* cases in high and low *IGSF4* expression. The Cox proportional hazards model analysis was applied to determine the association of *IGSF4* overexpression with overall survival and EFS adjusted for prognostic factors as described for pediatric AML (white blood cell count, age, and karyotype). All analyses were performed with SPSS Statistics Version 16.0 (SPSS Inc). All used tests were 2-tailed, and a P value of less than .05 was considered significant.

RESULTS

Gene expression profiling

In a comparison of t(9;11)(*MLL*-AF9) AML with other *MLL*-rearranged AML cases, *IGSF4* was among the highly differentially expressed genes. Recent literature described *IGSF4* as a tumor suppressor gene in solid tumors, but so far no data are available about the function of *IGSF4* in leukemia.[22-24] Because this gene was never described to be expressed in pediatric AML, we decided to further study *IGSF4* specifically.

We chose probe set 209031_at, which revealed the most significant differences, to compare differential expression in AML subgroups. Patients with t(9;11), n= 26, had a 4.1-fold greater median *IGSF4* mRNA expression compared with patients with other *MLL*-rearrangements (n= 42; 8.8 arbitrary units [AU] vs 7.4 AU, P< .001; Figure 1A). *IGSF4* expression was also significantly greater in t(9;11) compared with non-*MLL*–rearranged AML cases with other karyotypes (n= 192; fold change 7.0, median expression of 8.8 vs 6.8 AU, P< .001; Figure 1A). Within the t(9;11) group, expression of *IGSF4* was 4.9-fold greater in FAB-M5 (n= 21) versus other FAB-types (n= 4; median: 8.9 AU vs 7.3 AU, P= .001; Figure 1B). This difference, associated with FAB-classification, was also present in *MLL*-rearranged AML patients with other translocation partners (n= 23 vs n= 19; median 7.8 vs 7.0, fold change 2.4, P= .009), but not in the AML-other group (n= 16 vs n= 166; median 6.8 vs 6.8, P= .20; Figure 1B). All cases with unknown FAB-type were excluded from these analyses (t(9:11)

n= 1, AML-other n= 10).

RT-qPCR

Gene expression results were confirmed with RT-qPCR in 78 cases. An additional 17 cases of which no GEP data were available were used to expand the number of cases. The median relative expression of *IGSF4* in patients with t(9;11) was 7.4-fold greater compared with *MLL*-rearranged patients with another translocation (6.4% vs 0.9%, P= .008; Figure 2). Relative mRNA expression of *IGSF4* in other AML patients was 396-fold lower than in t(9;11) patients (0.02% vs 6.4%, P< .001).

A correlation coefficient was calculated comparing the expression of *IGSF4* by GEP and RT-qPCR. Because of the use of SYBRgreen in the RT-qPCR reaction, Ct values > 32 can be considered noise. The remaining 56 pairs resulted in a highly correlated Spearman R = 0.839 (P= .01; supplemental Figure 2).

Western blot

The IGSF4 antibody specifically identified 2 different isoforms that have previously been described.[25] We did not find a difference in isoform 1 expression, but the expression of isoform 2 was greater in t(9;11) positive patients than in the other patients (Figure 3).

MS-PCR

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

Treatment with demethylating agent

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

Transfection

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

In vitro drug resistance

No significant differences in drug toxicity were found after 2, 3, or 4 days of consecutive culturing of transfected MONO-MAC-1 cells with the most commonly used cytostatic drugs in AML and ALL (supplemental Figures 4-5).

Outcome

Five-year overall survival of *MLL*-rearranged patients with high *IGSF4* expression was 70%, which is significantly better than an overall survival of 37% in *MLL*-rearranged patients with low *IGSF4* expression (n= 79, P= .03; Figure 7A-C). This group included 28 patients with t(9;11). When analyzed separately, this group proved to be too small to show significant survival differences (data not shown). With the use of the Cox proportional hazards model, we found that no correlation with outcome could be shown after adjustment for known prognostic factors (white blood cell count, age; data not shown).

DISCUSSION

In pediatric *MLL*-rearranged AML, t(9;11) is the most common genetic aberration. Recently we showed that prognosis of this patient group largely depends on the morphologic FAB classification, that is, patients with t(9;11) with FAB M5 had a significantly better prognosis than patients with other FAB types.10 However, so far the biologic background for this survival difference is largely unknown. To study differentially expressed genes in this specific group, we performed GEP and identified *IGSF4* as a discriminative gene.

In nonmalignant cells, *IGSF4* is known to play a role in cell-cell adhesion, cell polarity, and as a signaling molecule for natural killer- and T-cell cytotoxicity.[23,26] Recently, Kawano et al[27] showed that *IGSF4* participates in the *ErbB2/ErbB3* pathway as a competitive antagonist of *ErbB2* in complex formation with *ErbB3*. Loss of *IGSF4* expression resulted in *AKT* pathway stimulation, which resulted in improved cell movement and survival.[27] We could not confirm similar pathway activation in our pediatric AML dataset by using microarray analysis (data not shown). In nonsmall cell lung carcinoma, *IGSF4* was found to be located in an area with a common loss of heterozygosity. Transferring this gene in A549 cells (nonsmall cell lung carcinoma cell line) inhibited tumor formation in nude mice.[22] In

neuroblastoma and cervical carcinoma, aberrant promoter methylation of *IGSF4* influenced tumor growth.[20,24]

We found a high *IGSF4* mRNA expression in *MLL*-rearranged pediatric patients with t(9;11) that was associated with increased protein expression, in combination with a hypomethylated promoter region of *IGSF4*. This finding indicates that indeed epigenetic regulation plays a major role in the expression of *IGSF4* in pediatric AML, as was further illustrated by cell line studies with demethylating agents. The expected effect of *IGSF4* on proliferation could not be shown in the siRNA experiments in a t(9;11) cell line. Our in vitro studies, however, do not represent the normal cell environment. Future studies that use a design including environmental factors (such as homing assays) are more potent to show proliferative advantage caused by differential expression of this cell surface protein. We are not the first group to report gene silencing by methylation in pediatric AML. CCAAT/enhancer binding protein is a well-known gene that is linked to mutations as well as methylation differences and whose expression predicts survival in AML.[28,29] In this context, we might consider designing clinical studies to assess whether the outcome of patients with epigenetic silencing can be improved by adding demethylating agents.

So far only the authors of one study in AML cell lines reported on *IGSF4*, showing hypermethylation of its promoter region in *MLL*-rearranged AML cell lines versus cell lines without an *MLL* rearrangement.[30] In adult T-cell leukemia, *IGSF4* overexpression resulted in a proliferation advantage.[31] However, the precise role of *IGSF4* in hematopoiesis and leukemogenesis is currently unknown. It remains to be determined whether cell-cell adhesion plays a role in *IGSF4*$^+$ leukemia like it does in solid tumors. The finding by Boles et al[26] that expression of *IGSF4* protein on the cell surface is a trigger for natural killer cell– and CD8$^+$ T cell–mediated cytotoxicity, might support our finding of overexpression of *IGSF4* in a group with a more favorable outcome. Normally, circulating leukocytes do not express high levels of *IGSF4*. If Boles' hypothesis proves to be true in pediatric AML, we would expect blasts with high *IGSF4* expression to be more easily recognized by the immune system. Blasts with low *IGSF4* expression are able to evade this mechanism. Because low expression is often derived from promoter hypermethylation, these patients might benefit from demethylating agents. In conclusion, we hypothesize that silencing of *IGSF4* could be considered as a secondary event, causing the leukemic blasts to be immunologically silent and thereby allowing longer survival. The *ErbB-RAC-AKT* pathway, influenced by *IGSF4* interaction with *ErbB3*, could also be of interest for leukemias, because this pathway is linked to proliferation and apoptosis.[27]

We found that *IGSF4* was mainly and most apparently expressed in monoblastic t(9;11)-rearranged patients. Because this subgroup of *MLL*-rearranged pediatric AML has recently been identified as important prognostic group,[10] the role of *IGSF4* deserves further attention. Interestingly, the *IGSF4* expression also seems to be determined by the cell type (M5) in which the maturation arrest occurs. This reflects a unique novel collaboration of a

specific (epi-)genetic aberration and type II mutations (ie, *MLL*) together with maturation status in pediatric AML.

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

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

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SUPPLEMENTAL TABLES

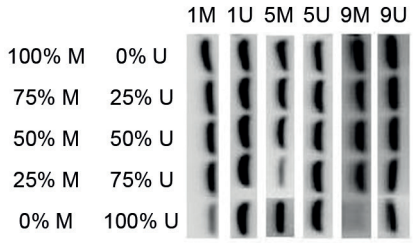
Supplemental Table 1. Primer and probe sequences

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

siRNA	Target sequence
IGSF4 siRNA J-016565-05	CGAAAGACGUGACAGUGAU
IGSF4 siRNA J-016565-06	GUAAUCUGAUGAUCGAUAU
IGSF4 siRNA J-016565-07	AAAGCUCACUCGGAUUAUA
IGSF4 siRNA J-016565-058	GCGCUUGAGUUAACAUGUG

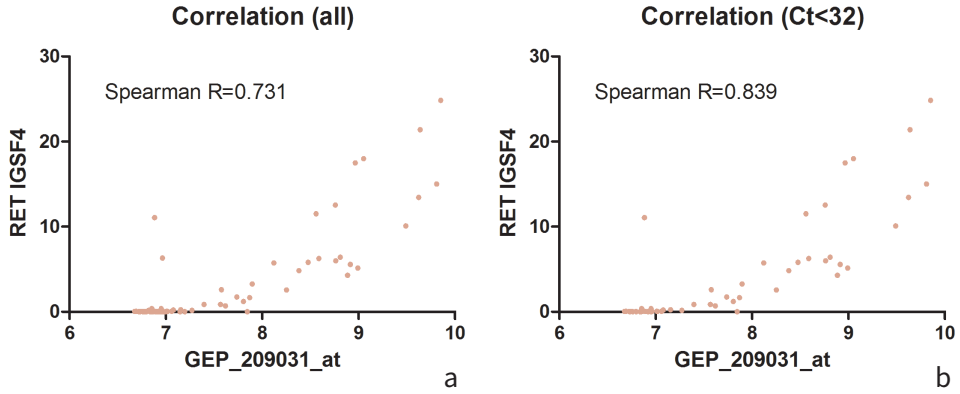
SUPPLEMENTAL FIGURES

Figure S1. MS-PCR primer test *IGSF4*



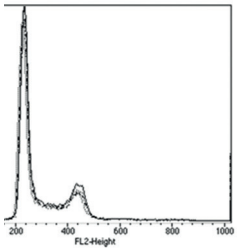
The MS-PCR primers targeting *IGSF4* were tested for specificity with a dilution range of a mix with methylated (M) and unmethylated (U) bisulfate converted DNA. On the left the composition of the used DNA mix, on the top the different primer pairs.

Figure S2. Correlation of *IGSF4* expression measured by GEP and RQ-PCR



These figures show the correlation of *IGSF4* expression measurements by GEP and RQ-PCR. When all acquired data were used, 76 data pairs could be analyzed, resulting in a Spearman R of 0.665 with p=0.01(panel a). When the pairs that had raw ct-values >32 were excluded, 56 pairs could be analyzed, resulting in Spearman R=0.848 with p=0.01 (panel b).

Figure S3. PI-staining showing cell cycle phase and apoptosis 48 hours after transfection



	N0	Electroporation	NT	IGSF4
Apoptosis	8.85%	17.47%	17.18%	14.21%
G0/1	52.91%	50.55%	48.90%	52.09%
S	13.96%	11.39%	12.75%	12.16%
G2/M	18.66%	14.81%	15.09%	15.00%

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

Figure S4. Drug response profile after transfection: AML drug panel

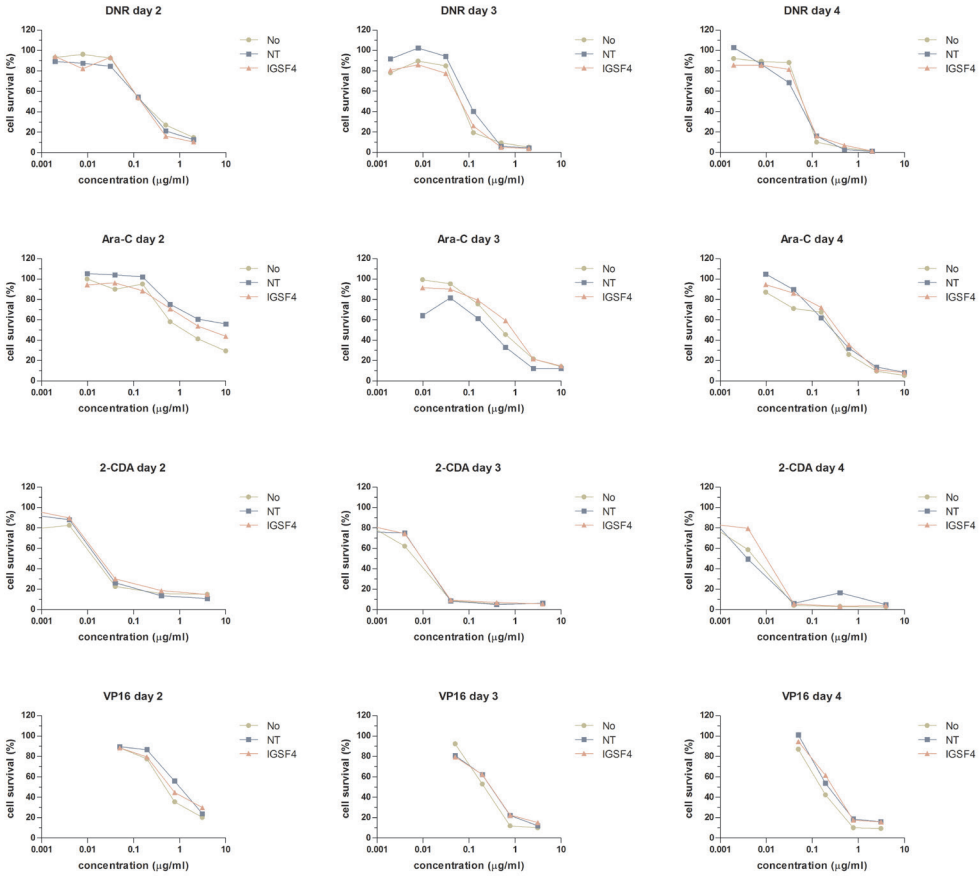


Figure showing results of drug resistance test for cell line MONO-MAC-1 after transfection with siRNA. No: control condition, NT: transfection with non-targeting control siRNA, *IGSF4*: transfection with *IGSF4* siRNAs. Cells were incubated for 2, 3 and 4 days respectively. DNR: daunorubicine, Ara-C: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

Figure S5. Drug response profile after transfection: ALL drug panel

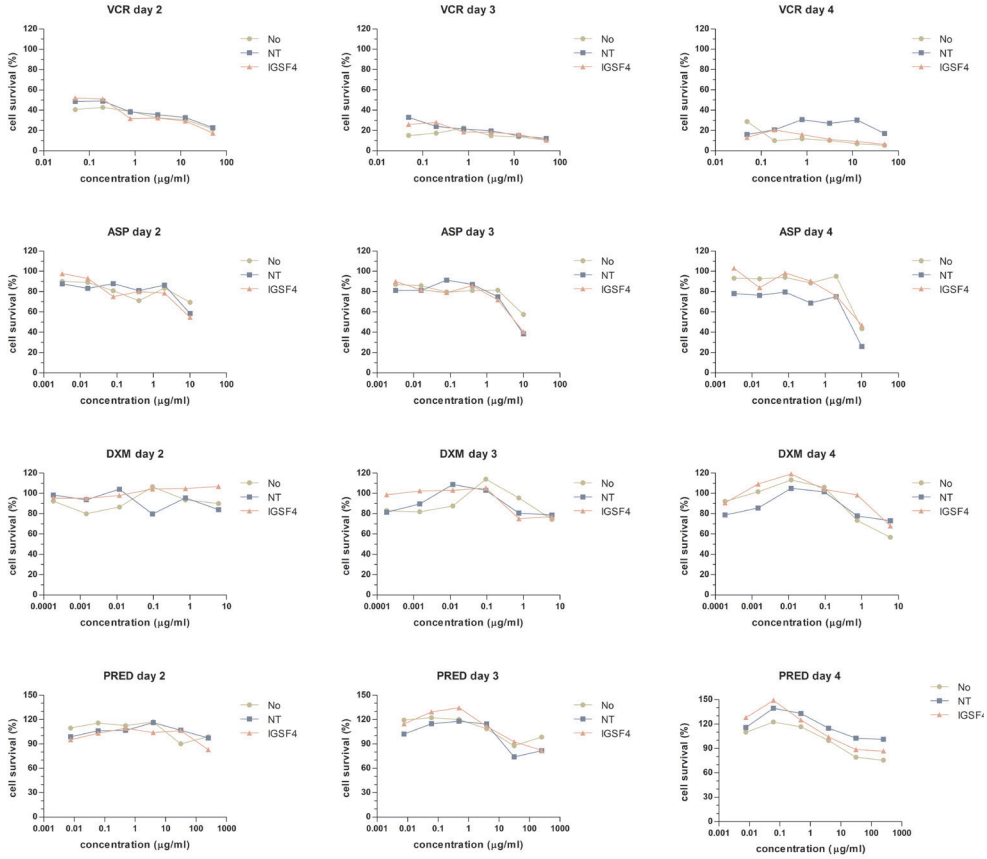


Figure showing results of drug resistance test for cell line MONO-MAC-1 after transfection with siRNA. No reflects control condition, NT transfection with non-targeting control siRNA and *IGSF4* transfection with *IGSF4* siRNAs. Cells were incubated for 2, 3 and 4 days respectively. VCR: vincristine,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

3

COOPERATIVITY OF *RUNX1* AND *CSF3R* MUTATIONS IN SEVERE CONGENITAL NEUTROPENIA: A UNIQUE PATHWAY IN MYELOID LEUKEMOGENESIS

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CHAPTER 3: ASSOCIATED RUNX1 AND CSF3R MUTATIONS IN CN/AML

ABSTRACT

Severe congenital neutropenia (CN) is a preleukemic bone marrow failure syndrome with a 20% risk of evolving into leukemia or myelodysplastic syndrome (MDS). Patterns of acquisition of leukemia-associated mutations were investigated using next-generation deep-sequencing in 31 CN patients who developed leukemia or MDS. Twenty (64.5%) of the 31 patients had mutations in *RUNX1*. A majority of patients with *RUNX1* mutations (80.5%) also had acquired *CSF3R* mutations. In contrast to their high frequency in CN patients who developed leukemia or MDS, *RUNX1* mutations were found in only 9 of 307 (2.9%) patients with *de novo* pediatric acute myeloid leukemia. A sequential analysis at stages prior to overt leukemia revealed *RUNX1* mutations to be late events in leukemic transformation. Single-cell analyses in 2 patients showed that *RUNX1* and *CSF3R* mutations were present in the same malignant clone. Functional studies demonstrated elevated granulocyte colony-stimulating factor (*G-CSF*)–induced proliferation with diminished myeloid differentiation of hematopoietic CD341 cells coexpressing mutated forms of *RUNX1* and *CSF3R*. The high frequency of cooperating *RUNX1* and *CSF3R* mutations in CN patients suggests a novel molecular pathway of leukemogenesis: mutations in the hematopoietic cytokine receptor (*G-CSFR*) in combination with the second mutations in the downstream hematopoietic transcription fator (*RUNX1*). The detection of both *RUNX1* and *CSF3R* mutations could be used as a marker for identifying CN patients with a high risk of progressing to leukemia or MDS.

INTRODUCTION

Congenital neutropenia (CN) is a heterogeneous bone marrow failure syndrome characterized by severe neutropenia (blood neutrophil counts <0.5*10^9/l) and maturation arrest of myelopoiesis at the level of the promyelocytes/myelocytes.[1] Autosomal-dominant and sporadic CN cases are predominantly attributable to mutations in *ELANE*, the gene encoding neutrophil elastase.[2] Several other genetic mutations, including those in *HAX1* (HCLS1-associated protein X-1), *G6PC3* (glucose 6 phosphatase, catalytic, 3), *GFI1* (growth factor independent 1 transcription repressor), and *WAS* (Wiskott-Aldrich syndrome gene), have been described in patients with CN.[3-6] The majority of CN patients benefit from treatment with granulocyte colony-stimulating factor (G-CSF).[7] Common pathological mechanisms for the maturation arrest of myeloid development in these patients include the lack of myeloid-specific transcription factors such as *LEF-1* (lymphoid enhancer-binding factor 1) and *CEBPA* (CCAAT/enhancer binding protein a), and defective *G-CSF* signaling.[8] CN is a preleukemic syndrome with a cumulative incidence of leukemia of >20% after 20 years.[9] Approximately 70% to 80% of CN patients who develop acute myeloid leukemia (AML) or myelodysplastic syndrome (MDS) acquire heterozygous G-CSF receptor (*CSF3R*) mutations, independent of the genetic subtype, suggesting that these mutations are involved in leukemogenesis.[10-12] This pattern is distinct from *de novo* childhood AML in which CSFR3 mutations are very rare. Current evidence indicates that *CSF3R* gene mutations are not sufficient for leukemic transformation. As Welch et al[13] reported, in many cases of myeloid leukemias, only 1 or 2 cooperating mutations are needed to generate the malignant founding clone. In our study, our hypothesis is that the initial driver mutations are the *CSF3R* mutations, and cell clones harboring *CSF3R* mutations have a growth advantage and acquire additional cooperating mutations (in the majority of patients *RUNX1* mutations) that contribute to AML initiation and disease progression.

Point mutations in *RUNX1* (runt-related transcription factor 1) have been described in *de novo* AML or AML secondary to MDS, radiation exposure, or chemotherapy at frequencies of 6% to 33%.[14-18] High incidence of *RUNX1* mutations has been associated with monosomy 7, trisomy 21, or trisomy 13.[17,19,20] Most of the *RUNX1* mutations are acquired heterozygous point mutations, predominantly located in the Runt homology/DNA binding (RHD) or transactivation (TAD) domains. They are associated with poor prognosis in AML.[14-17,19-21] *RUNX1* germline mutations, with or without acquired *RUNX1* mutations, have also been described in a familial platelet disorder with a predisposition to AML.[22,23]

The goal of the present study was to identify the steps of leukemia progression in CN by analyzing genomic profiles of patients during the course of the disease using next-generation DNA deep-sequencing. Specifically, we sought to determine if CN patients acquired leukemia-associated mutations in the course of the development of myeloid leukemia and to assess the clinical significance of these findings.

MATERIALS AND METHODS

Patients

Thirty-one patients with CN who developed leukemia or MDS were analyzed. All patients had suffered from neutropenia and recurrent infections from birth and were subsequently started on treatment with G-CSF. They developed MDS or leukemia at 2 to 38 years of age. The mutation subtypes were as follows: *ELANE* (n=18), *HAX1* (n=6), *WAS* (n=4), *ELANE* plus *GFI1* (n=2), and G6PT/SLC37A4 (n=1). Two patients were negative for *ELANE*, *HAX1*, *G6PC3*, *GFI1*, and *WAS* mutations. We collected bone marrow or blood samples in association with annual follow-up as recommended by the Severe Chronic Neutropenia International Registry and French Severe Chronic Neutropenia Registry.[24] This study was performed with the informed consent of all subjects obtained through the respective institutional review boards and was approved by the Hannover Medical School Institutional Review Board. This study was conducted in accordance with the Declaration of Helsinki.

Pediatric AML samples were obtained from the Dutch Childhood Oncology Group (The Hague, The Netherlands), the AML-BFM Study Group (Hannover, Germany), as well as from the University Hospital in Prague (Czech Republic) and the St. Louis Hospital (Paris, France). Clinical and centrally reviewed cytogenetic and other cell-biological characteristics were made available by these cooperative groups/hospitals. All recurrent cytogenetic groups known in pediatric AML were represented among the 307 *de novo* pediatric AML patients (Table 2).

Mutation screening

Mutation analyses of the known leukemia-associated genes (*RUNX1*, *NPM1*, *FLT3-ITD*, *FLT3-TKD*, *CEBPA*, *NRAS*, *KRAS*, *CBL*, *TET2*, *IDH1*, *IDH2*, *DNMT3A*, *SUZ12*, *EP300*, and *CSF3R*) were performed using a sensitive next-generation amplicon deep-sequencing assay (454 Life Sciences, Branford, CT) or a SeqCap EZ library (Roche Nimblegen, Madison, WI) followed by sequencing on the Hisequation 2000 sequencing system (Illumina, San Diego, CA). Minimum coverage was at least 467-fold, and sequences were confirmed by ABI Sanger sequencing when supporting reads of mutated alleles were in excess of 20%. For sequential analyses of the timecourse of occurrence of *RUNX1* and *CSF3R* mutations, mutated regions of *RUNX1* and *CSF3R* genes were amplified using polymerase chain reaction (PCR), and PCR products were sequenced using a SOLiD 5500XL ligation-based sequencing system or the SeqCap EZ library (Roche Nimblegen) followed by sequencing on the Hisequation 2000 system (Illumina). In the 302 *de novo* AML-samples, the protein coding sequence of the *RUNX1* gene (exon 3-8) (*RUNX1*-002, NM_001754.4) was PCR-amplified using specific primers and subsequently sequenced in forward and reverse direction. Purified PCR products were directly sequenced from both strands. Primer sequences are available upon request. The sequence data were analyzed using CLC Workbench, version 3.5.1 (CLC

Table 1. Clinical and cytogenetic characteristics as well as types of mutations of CN patients who progressed to leukemia or MDS

Patient number	AML subtype	Karyotype	Inherited mutations	Acquired *RUNX1* mutations*	Acquired *CSF3R* mutations†	Acquired AML-associated mutations
6	AML M5	45,XX,-7	*ELANE* (L152P)	R80S	Q726X	
7	MDS/ AML M1	46,XY-7, 121	*ELANE* (S126L)	R135K	Q726P	
14	AML M1	45,XY,-7[9];46,XY[11] (2010) 47,XY 121[13];46,XY[2] (2011)	*ELANE* (C151Y)	R139G M240I	Q718X	
15	AML M1	t(p1;q3)	*ELANE* (C151Y)	R139X	Q731X	
16	AML M4	46,XY	*ELANE* (G214R)	R174X	Q720X	
18	AML FAB NA	46, XY, t(9;11)	*ELANE* (N113K)	R64P	Q718X	
22	AML M1	46,XY	*ELANE* (IVS411G.T)	K83Q	Q718X	
30	pre-B ALL	48,XX,del(5)(q21q34),þ21, þ 22(16)/46,XX[8]	*ELANE* (G185R)	A160T S114X	Q702X	
31	RAEBT/AML FAB NA	47,XY, 121 [14] /46, XY [4]	*ELANE* (G174R)	D171N	Q718X, Q726X	*SUZ12*, *EP300*‡
21	AML M2	47,XX 1mar[8], 47, idem, del(10)(q32)	*ELANE* (G214R), *GFI1*	R174X	Q739X	
4	AML M0	45,XX,-7[12];46,XX[11]	*WAS* (S478I)	Intron 4, c.415_427d-up6 Intron 4, c.421_427dup7	Q707L	*SUZ12* (S154X) *EP300* (R2263X)
20	MDS RAEB	46,XX,add(2)(q37),add(7)(q22)	*WAS*	Q370X	Y729X	*CBL* (splice site c.1096-1G.C (Intron 7)
26	AML FAB NA	45,XY,-7	*WAS* (L270P)	R80S	Y729X	CREBBP (I2329M)
10	MDS RAEB	45,XY 27 [10], 46XY [5]	*HAX1* (V44X)	F13TrpfsX14 R139ProfsX47	Q726P	*FLT3-ITD*
19	MDS	46,XX	*HAX1* (V44X)	L29S, R64P	Y729X	
12	AML M2	47,XX,121	GPT1	K83Q	Q720X	
25	MDS RAEB-2	46,XX,dup(21)(q22.1q22.3)[19]	Neg	S114P Y380_G394delinsC	Q726X	
11	AML/B-ALL	46,XY,add(21q)	*ELANE* (A57V)	R174L	neg	
13	AML FAB NA	46,XX	*ELANE* (A79VfsX9)	R139X V137D	neg	*FLT3-ITD*
17	MDS	46,XY	*HAX1* (V44X)	I22K	neg	*EP300* (C369F)
1	AML M2/M4	47,XY,1 8	*ELANE* (D230MfsX1)	Neg	Q716X, Q726X	
9	AML M2	46,XX, del 7q [9], 46XX [1]	*ELANE* (S126X)	Neg	Q731X	
24	AML M5	5Q-deletion, a translocation of chr.I and 21	*ELANE* (Y228X)	Neg	Y729X	
27	MDS/AML FAB NA	47,XY, 27, 121, 121 [9]/46, XY [5]	*ELANE* (L92P)	Neg	Q718X	
5	MDS RAEB-T	46,XY	*HAX1* (V44X)	Neg	Q726X	
3	MDS RAEB	45,XX,-7,del(18)(q22)[11/45],idem,der (6)t(3,6)(q13;p24) [2]/ 45,XX,-7, del(13)(q13q33) [2]	*WAS* (I331M)	Neg	Q716X	*NRAS*§

NA, not available; pre-B ALL, pre-B acute lymphoblastic leukemia.

*For all patients except patient 17 amino acid positions according to the *RUNX1* transcript variant Q01196 (www.uniprot.org) was used; for patient 17 amino acid positions according to the *RUNX1* transcript variant Q2TAM6 (www.uniprot.org) was used.

†Amino acid positions were assigned as has been reported by Dong *et al*[10] or by UniProtKB Q99062 minus 23 amino acids of signal peptide.

‡See Beekman *et al.*[25]

§N-RAS mutations were measured according Nakao M. *et al.*[26]

Bio, Aarhus, Denmark). In case of a suspected mutation, the fragment was re-amplified and sequenced in both directions.

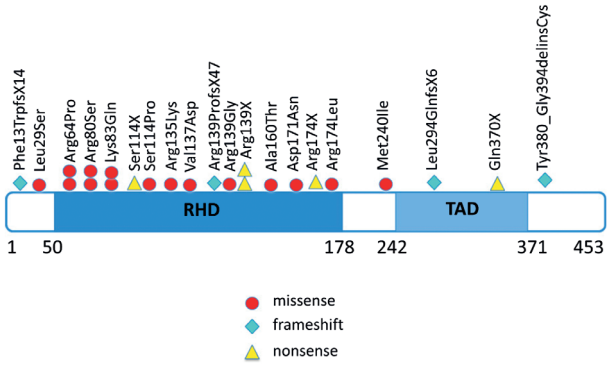
All *RUNX1* mutated samples were also screened for *CSF3R* and *ELANE* mutations. Briefly, if cDNA was available, *CSF3R* cDNA was amplified and sequenced in forward and reverse direction. When only genomic DNA was available, *CSF3R* was amplified and sequenced to detect mutations and deletions around 2 previously reported *CSF3R* hotspot mutations. For *ELANE*, the protein coding exons (exons 1-5) were amplified and subsequently sequenced in forward and reverse direction on genomic DNA. Primer sequences are available upon request.

RESULTS

High frequency of *RUNX1* gene mutations in CN patients who developed leukemia

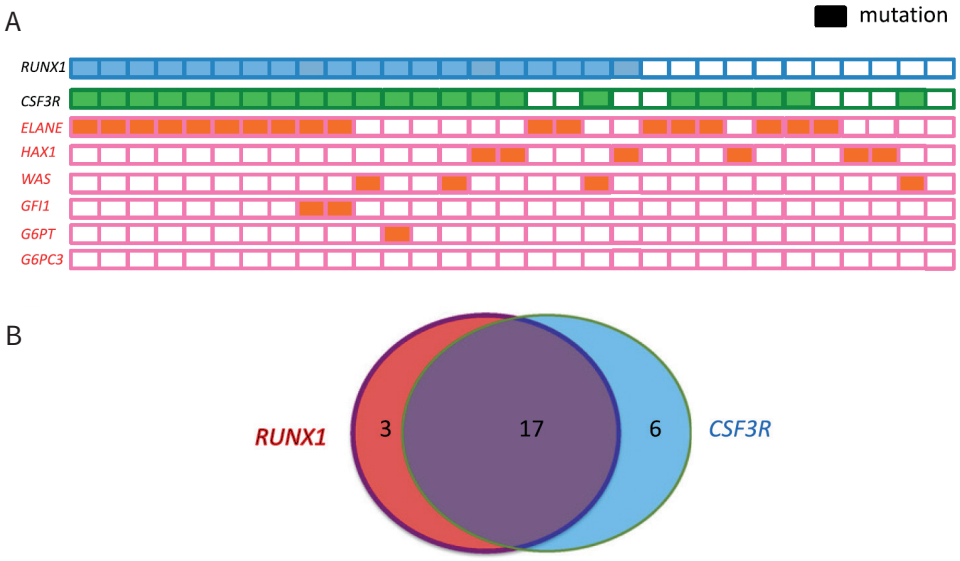
We included 31 CN patients who developed MDS or leukemia in this study (Table 1) using materials from all of the patients available to us though our international collaborations. Twenty-one patients developed leukemia and 10 patients MDS. Of the leukemic patients, 16 patients had AML, 3 had AML after MDS, 1 had biphenotypic leukemia, and one had acute lymphoblastic leukemia (ALL). Of these 31 patients, 20 (64.5%) had heterozygous *RUNX1* mutations; these included missense mutations (n=16), nonsense mutations (n=5), frameshift mutations (n=4), and mutations in the splice-acceptor site of intron 4 (n=2) (Figure 1; Table 1). The most frequently affected position was Arg139, resulting in p.Arg139Gly, p.Arg139X (n=2), and p.Arg139ProfsX47 (n=4). Additionally, p.Arg64Pro, p.Arg80Ser, p.Lys83Gln, and Arg174 (p.Arg174X and p.Arg174Leu) mutations were identified, each

Figure 1. Localization of *RUNX1* mutations in CN patients who developed leukemia or MDS.



The position of *RUNX1* mutations found in CN patients, with affected amino acids numbered. Amino acid positions correspond to the *RUNX1* transcript variant Q01196 (www.uniprot.org). Locations of the functionally important RHD and TAD are shown. Each symbol represents 1 patient.

Figure 2. Frequency and distribution of acquired *RUNX1* and *CSF3R* mutations and inherited *ELANE*, *WAS*, *GFI1*, *GPT*, and *HAX1* mutations within a cohort of CN patients who developed leukemia or MDS.



(A) Each patient with a *RUNX1* mutation is represented by a blue rectangle, and each patient with a *CSF3R* mutation is represented by a green rectangle. Patients with inherited mutations are represented by orange rectangles. Open rectangles correspond to patients without mutations. (B) Venn diagram illustrating the relationship among *RUNX1* and *CSF3R* mutations in CN patients who developed leukemia (n=31). Diameters of each circle are roughly proportional to the number of mutations.

in 2 patients. Of the 27 *RUNX1* mutations, 18 were localized within the RHD, 4 within or proximal to the TAD and 2 in splice-sites.

Simultaneous occurrence of 2 distinct heterozygous *RUNX1* mutations

In 8 patients, we detected 2 distinct heterozygous *RUNX1* mutations. In one patient, 2 insertion mutations were found at the splice-acceptor site of intron 4, predicted to affect the splicing of exons 3 and 4, which encode the RHD of *RUNX1*. In 3 patients, 2 mutations were present solely in the RHD or were present in both RHD and TAD (1 each). In 4 patients, 1 of 2 mutations was localized to the RHD. In 2 patients, we could perform allele-specific analysis of *RUNX1* mutations. Patient 10 incurred deletions leading to frame shifts on both alleles (p.Phe13TrpfsX14 and p.Arg139ProfsX47). In patient 14, 2 single missense substitutions were detected on the same allele; the first was inherited from the mother (p.Met240Ile) and is located 2 amino acids upstream of the TAD, and the second acquired mutation (p.Arg139Gly) is in the RHD of *RUNX1* (supplemental Figure 1, available on the Blood Web site).

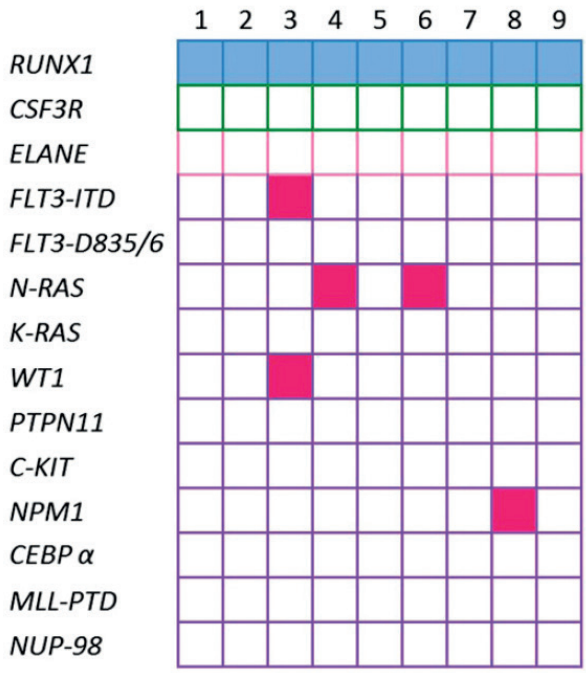
Association between *RUNX1* mutations, clinical characteristics, and cytogenetics in CN patients who developed leukemia or MDS

No correlation was seen between age at progression to leukemia and *RUNX1* mutations (13.8 years in *RUNX1* mutated vs 10.2 years in *RUNX1* wild-type, (WT) groups). No gender correlation was found; 10 male and 10 female patients acquired *RUNX1* mutations, as opposed to 5 males and 6 females without *RUNX1* mutations. Among 20 patients carrying mutated *RUNX1*, one developed AML FAB M0, 4 developed M1, 2 developed M2, 1 developed M4, and 1 developed M5. A cytogenetic analysis revealed that *RUNX1* mutations were associated with monosomsy 7 in 6 patients (30%), with trisomy 21 in 6 patients (30%), and with monosomy 7/trisomy 21 in 2 patients (Table 1).

Distribution of *RUNX1* mutations based on CN-specific germline mutations

Of the 20 patients with *RUNX1* mutations, 19 were positive for known CN-causing mutations: *ELANE* (n=12 patients, 66%), *WAS* (n=3, 75%), *HAX1* (n=3, 50%), both *ELANE* and *GFI1* (n=1), and G6PT/SLC37A4 (n=1). Conversely, 5 of 17 patients harboring *ELANE* mutations, 3 of 6 patients with *HAX1* mutations, and 1 of 4 patients with *WAS* mutations tested negative

Figure 3. Mutational status of leukemia in *de novo* pediatric AML patients with *RUNX1* aberrations.



Identified gene mutations in 9 *de novo* pediatric AML patients with *RUNX1* aberrations; each mutation is depicted by a blue rectangle. Patients with inherited mutations are represented by pink rectangles. Open rectangles correspond to patients without mutations.

Table 2. Characteristics of pediatric AML patients with *RUNX1* aberrations

	Gender	Age	FAB-type	Karyotype sample	Other mutations	*RUNX1* mutation	Effect	RUNX1 Protein domain	Event1	Time dx-event	Time dx-death	Cause of death
1	M	5.0	M1	45,XY,-7[13]/45,idem,der(18)t(8;18)(q21;q22)[2]	-	c.179 ins GG	FS	NRDBn/NRHn	non-remitter	0.0	alive	-
2	M	2.6	M5	46,XY,t(16;21)(p11;q22)/46,idem,i(22)(q10)/46,XY	-	c.292delC	FS	RUNT	relapse	15.8	26.3	leukemia
3	M	12.3	M3	46,XY[17]	FLT3-ITD & WT1	c.328 A>C	MS	RUNT	non-remitter	0.0	15.1	infection
4	M	3.9	M1	46,XY, aberrant(pseudodiploid)[9] ISH:MLL&inv(16) not aberrant	N-RAS	c.424_425 insGG	FS	RUNT	relapse	11.4	14.9	leukemia
5	M	13.8	M1	46,XY,add(8)(q22),add(16)(p13.1)[3]/ 46,XY,add(8)(q22)[13]	-	c.424_425 insGGG	IF	RUNT	relapse	2.3	7.0	leukemia
6	F	14.1	M4	46,XX,-7[21]	N-RAS	c.497G>A	MS	RUNT	relapse	25.3	43.3	toxic, neurological syndrome
7	F	3.9	M1	46,XX,del(5)(q31q34),del(16)(?q22)[1]/ 46,XX[4]	-	c.507_508 ins11	FS	RUNT	non-remitter	0.0	3.2	leukemia
8	F	15.4	M1	46,XX	NPM1	c.1085 C>T	MS	TAD	early death	0.0	0.0	leukemia
9	M	2.1	M7	49,XY,+16,del(17)(p11),+19,+21,+21,-22[5]/ 46,XY [22]	-	c.1190 A>G	MS	TID	none	-	alive	-

F=female, M=male, age at diagnosis in years, dx=diagnosis, FS=frameshift, MS=missense, iF- in frame, time dx-event1 and time dx-death in months

for *RUNX1* mutations (Table 1; Figure 2A).

High frequency of cooperating *RUNX1* and *CSF3R* mutations in CN patients who developed leukemia or MDS

To evaluate possible cooperating effects of "leukemogenic" gene mutations in combination with *RUNX1* mutations, we performed targeted deep-sequencing of candidate genes known to be mutated in *de novo* AML, namely *NPM1*, FLT3, *CEBPA*, *NRAS*, *KRAS*, *CBL*, *TET2*, *IDH1*, *IDH2*, *DNMT3A*, *SUZ12*, *EP300*, and *CSF3R*. In 19 of 20 patients, we detected cooperating *RUNX1* mutations. Intriguingly, 17 of 20 patients (80.5%) exhibited both *RUNX1* and *CSF3R* mutations (Figure 2B), 3 had both a *RUNX1* and an *EP300* mutation, 2 had a *RUNX1* mutation and FLT3, and 1 had a *RUNX1* and a *CBL* mutation. Six patients with acquired *CSF3R* mutations had WT *RUNX1*. One patient with unaffected *RUNX1*, *ELANE*, or *CSF3R* had an activating *NRAS* mutation in codon 61. This may represent a unique type of CN/MDS and could suggest that *NRAS* and *CSF3R*, or *RUNX1*, mutations are mutually exclusive (Table 1). No mutations were found in *CEBPA*, *DNMT3A*, *IDH1*, *IDH2*, *NPM1*, or *TET2*. All *CSF3R* mutations were C-terminal truncated mutations, which are localized between amino acids 680 and 780. Intriguingly, all 6 patients who were negative for *RUNX1* and *CSF3R* mutations developed MDS only, whereas in the group of patients with cooperative *RUNX1*

and *CSF3R* mutations, 11 developed AML and only 6 MDS/AML.

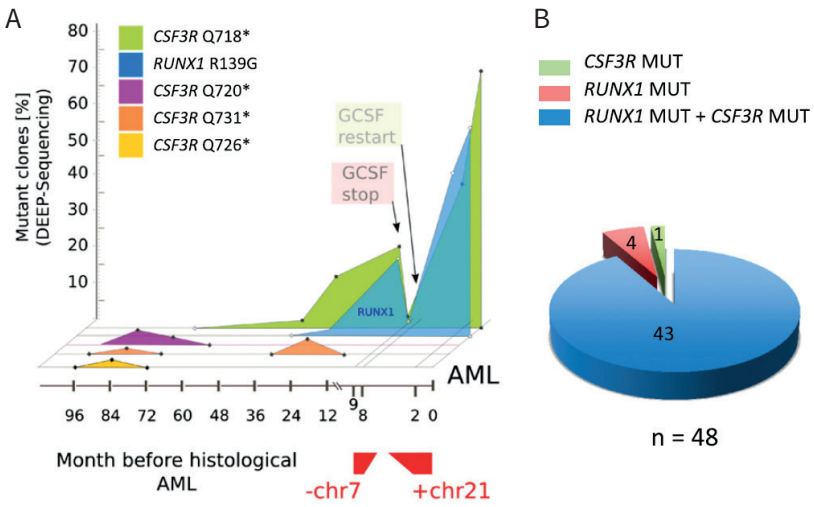
Molecular karyotyping by array-CGH (comparative genomic hybridization)

To elucidate the underlying molecular mechanisms of cancer susceptibility and progression in secondary acute leukemia or MDS, we conducted a comprehensive genome-wide characterization of genomic aberrations in the transformed cells from several patients with inherited bone marrow syndromes. When available, samples at different time points of disease progression were analyzed. Thirty-one CN patients were analyzed. A summary of the genomic aberrations identified by array-CGH is given in supplemental Table 1. Large genomic alterations, namely, monosomy 7/-7q, 121q, or 13q, were associated with leukemia. Apart from common copy number variants, such as *UGT2B*, *GSTT1*, and *HEATR4*, no microdeletions or microduplications were detected in primary or secondary diseases.

RUNX1 mutations in *de novo* pediatric AML

To evaluate whether *de novo* pediatric AML also has high frequency of *RUNX1* mutations, we sequenced *RUNX1* in *de novo* pediatric AML samples. We detected *RUNX1* mutations in 9 of 307 (2.9%) pediatric patients with *de novo* AML (Table 2): one deletion, 4 insertions, and 4 missense mutations (single-nucleotide substitutions) for which single-nucleotide polymorphisms have not been described. We found 6 N-terminal *RUNX1* mutations,

Figure 4. Cooccurrence of *RUNX1* and *CSF3R* mutations in combination with monosomy 7 and trisomy 21 in a leukemic clone of a CN/AML patient.



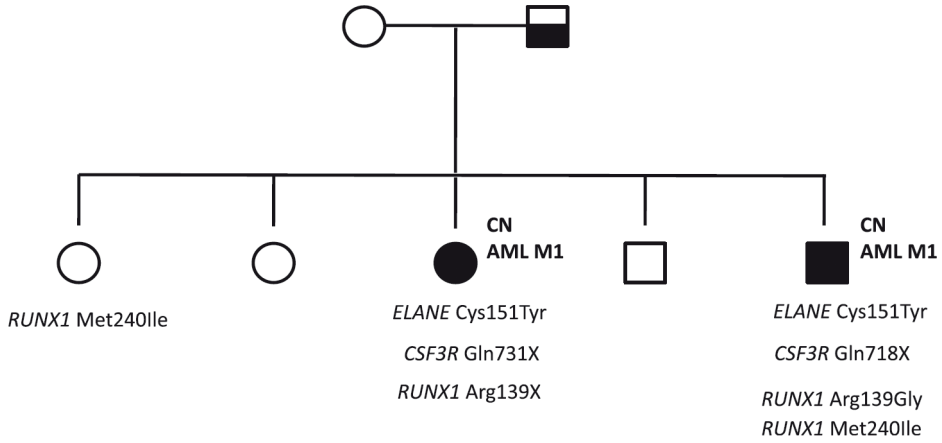
(A) Graphic presentation of a mutational analysis of deep-sequencing data for *RUNX1* and *CSF3R* genes in CN/AML patient 14. Results of sequencing DNA samples from different time points prior to overt AML (x-axis) and the percentage of mutant clones (y-axis) are presented. (B) Diagram of the distribution of *RUNX1* and *CSF3R* mutations in DNA isolated from single clones (n=48) of AML samples from CN patient 14.

1 C-terminal mutation, and 2 mutations outside the RHD and TAD. No co-occurrence of *RUNX1* mutations with *CSF3R* or *ELANE* mutations was found in these patients (Figure 3).

The median age was the same for patients with mutated and wild-type *RUNX1*. The frequency of immature phenotype (FAB M1) was higher in patients with mutated *RUNX1* (56%) than in patients with WT *RUNX1* (10%) (Fischer's exact test P=.0019). Interestingly, mutations were not found in patients without *MLL*-rearrangements, t(8;21) inv(16), t(7;12) or t(15;17) whereas concomitant mutations in *FLT3-ITD*, *NRAS*, *NPM1*, and WT1 were found in 5 of 9 patients (supplemental Figure 2).

RUNX1 mutations were mainly found in pediatric AML patients with an adverse prognosis. Although 4 patients received allogeneic stem cell therapy, none survived. Further, we did not find a strong gene expression signature, suggestive of specific driver alterations, in *RUNX1*-mutated pediatric AMLs (data not shown).

Figure 5. Mutation analysis of a CN/AML pedigree.

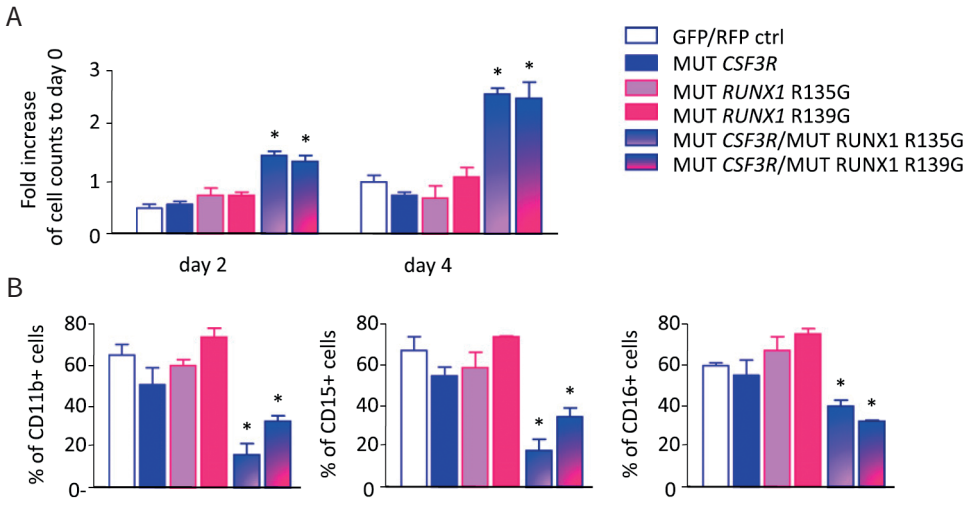


Squares indicate males and circles indicate females. Open symbols represent unaffected persons; the half-filled square represent the father with a mosaicism for *ELANE* mutation and mild neutropenia; closed symbols represent 2 persons affected by CN, both of whom also developed AML-M1. Inherited mutations in *ELANE* (p.Cys151Tyr) and *RUNX1* (p.Met240Ile) as well as acquired *RUNX1* (p.Arg139Gly; p.Arg139*) and *CSF3R* (p.Gln718X; p.Gln731*) mutations are indicated for each affected person.

RUNX1 mutations are late events in the leukemic transformation of CN

To evaluate the time points and sequence of acquisition of *RUNX1* and *CSF3R* mutations, we performed a consecutive analysis of both mutations in 10 CN/AML patients. DNA from bone marrow cells collected at different time points prior to leukemia was analyzed. In 6 of 10 patients, a *CSF3R* mutation occurred prior to *RUNX1* mutations (Figure 4A and supplemental Figure 3), consistent with previous data demonstrating that acquisition of *CS-*

Figure 6. Enhanced proliferation and diminished myeloid differentiation of hematopoietic cells.



CD341 bone marrow cells from healthy individuals were transduced with lentivirus-based green fluorescent protein (GFP)-tagged *RUNX1* mutants (p.Arg135Gly or p.Arg139Gly) or red fluorescent protein (RFP)-tagged *CSF3R* mutant (d715) alone, cotransduced with *RUNX1* and *CSF3R* mutants, or cotransduced with control GFP- and RFP-tagged lentiviral constructs. Transduced cells were treated with 10 ng/mL of G-CSF. (A) Cell number was evaluated on days 2 and 4 of culture by estimation of RFP1/GFP1 cells using fluorescence-activated cell sorter. (B) G-CSF–triggered myeloid differentiation was evaluated on day 8 of culture. Data represent means 6 standard deviations and are derived from 2 independent experiments, each in triplicate (*P<.05).

F3R mutations is an early event in the leukemic transformation in CN.[11,12] In 2 patients, both *RUNX1* and *CSF3R* mutations were detected in the earliest available DNA samples prior to leukemia/MDS. Two patients had no *CSF3R* mutations and acquired *RUNX1* mutations 2 months prior to AML or at the time point of AML progression. Interestingly, monosomy 7 or trisomy 21 appeared after acquisition of *RUNX1* mutations.

The time course of mutational events in patient 14 is shown in Figure 4A. The transient appearance of cell clones with different *CSF3R* mutations was detected as early as 8 years prior to leukemia. Three different *CSF3R* mutations (p.Q720X, p.Q726X, and p.Q731X) were detected at the age of 12 years (8 years prior to leukemia). All 3 clones fell below the detection level within 2-3 years, and one clone (p.Q731X) reappeared for a short period of time 3 years prior to leukemia. One additional clone with a p.Q718X mutation was detected 5 years prior to leukemia at the age of 15 years. Intriguingly, this clone acquired an additional *RUNX1* mutation 2 years prior to leukemia. The mutations in *RUNX1* and *CSF3R* preceded monosomy 7, which was observed nine months prior to leukemia. After acquisition of monosomy 7, treatment with G-CSF was discontinued and, intriguingly, the clone with mutated *RUNX1* and *CSF3R* was no longer detectable. Because of the life-threatening infection status of the patient, G-CSF therapy was restarted, which was rapidly followed by expansion of the cell clone with *RUNX1* and *CSF3R* mutations, an additional trisomy 21,

and a bone marrow morphology that revealed overt AML.

To evaluate whether *RUNX1* and *CSF3R* mutations were present in the same transformed cell clone or whether 2 different clones carried *RUNX1* and *CSF3R* mutations, we performed colonyforming assays using leukemia blasts and CD341/CD331 bone marrow cells isolated 7 years prior to leukemia development of one CN/AML patient. We also isolated DNA from single colonies and analyzed samples for the presence of *RUNX1* and *CSF3R* mutations by Sanger sequencing. Both *RUNX1* and *CSF3R* p.Q718X mutations were detected in 43 of 48 leukemic colonies, whereas no mutations were found in cells isolated 7 years prior leukemia (Figure 4B).

Analysis of familial CN/AML cases

A mutation analysis of *RUNX1* was also performed in a family in which 2 siblings with *ELANE*-CN developed AML M1. The father was mosaic for the *ELANE* mutation and had a mild, asymptomatic neutropenia. One of the affected siblings had a germline *RUNX1* variation (p.Met240Ile) that was inherited from the healthy mother and which has not been previously reported as a common single-nucleotide polymorphism. A healthy sister also carried this p.Met240Ile *RUNX1* alteration. Both affected siblings had acquired distinct *RUNX1* mutations in Arg139: a p.Arg139Gly missense mutation in the brother and a p.Arg139X nonsense mutation in the sister. Both affected siblings also had *CSF3R* nonsense mutations: p.Q718X and p.Q731X in the brother and sister, respectively. None of the healthy siblings had acquired *RUNX1* or *CSF3R* mutations, or inherited *ELANE* mutations (Figure 5).

Enhanced proliferation of CD341 cells cotransduced with mutated *RUNX1* and mutated *CSF3R*

To test the effects of coexpression of *RUNX1* and *CSF3R* mutations, we cotransduced CD341 hematopoietic cells with cDNAs encoding the truncated *CSF3R* mutant (d715) or *RUNX1* variants carrying missense mutations within the RHD (Arg135Gly or Arg139Gly). We treated transduced cells with G-CSF and evaluated proliferation and myeloid differentiation. Proliferation of cells expressing with the *CSF3R* mutant in combination with either *RUNX1* Arg135Gly or *RUNX1* Arg139Gly mutants was elevated relative to cells transduced with control vectors, the *CSF3R* mutant alone, or *RUNX1* mutants alone (Figure 6A). In parallel, we observed diminished myeloid differentiation of cells transduced with mutated *CSF3R* and *RUNX1*, as assessed by surface expression of the myeloid-specific surface markers, CD11b, CD15 and CD16 (Figure 6B; supplemental Figure 4).

DISCUSSION

CN is a preleukemic bone marrow failure syndrome with a high risk of progression to AML or MDS.[9] Rare cases of ALL associated with CN have also been described.[27] The genetic

changes involved in the evolution of CN to leukemia are still largely unknown. The frequency of acquired *CSF3R* mutations in CN/AML patients is ~80%, substantially higher than that in patients who have not yet progressed to leukemia (~20%), suggesting that *CSF3R* mutations are drivers of leukemogenesis.[10,11] Previous studies have demonstrated a clonogenic proliferative advantage of hematopoietic cells carrying a truncated CSF3R.[40,41] However, *CSF3R* mutations alone are not sufficient for leukemia development, and additional genetic events are required.[28-30] Moreover, the first case of leukemia in a CN patient was described in 1969, many years before G-CSF was used for the treatment of CN.[31,32] This suggests that G-CSF treatment is not causative for leukemic transformation.

Here, we provide the first report of the high frequency of *RUNX1* mutations in CN/AML patients. It is important to mention that there is no other clinical entity with this high frequency of *RUNX1* mutations. In contrast, we found that *RUNX1* mutations are a rare event in *de novo* childhood AML (frequency <3%) and are not associated with the acquisition of *CSF3R* mutations. Interestingly, *RUNX1* mutations were found in pediatric AML patients mutually exclusive of *MLL*-rearrangements, t(8,21), inv(16), t(7,12), or t(15,17) whereas concomitant mutations in the *FLT3*, *NRAS*, *NPM1*, and WT1 genes were found in 5 of 9 patients. Intriguingly, patients with CN associated with glycogen storage disease type Ib, or those harboring *ELANE*, *HAX1*, *WAS*, or *GFI1* mutations who developed secondary leukemia, acquired *RUNX1* mutations. This observation demonstrates that *RUNX1* is a crucial driver of leukemogenesis, independent of the underlying diverse inherited aberrations. The vast majority of *RUNX1* mutations in CN/AML and *de novo* AML patients impair RUNX1 function (loss of DNA binding or transactivation capacity) and would be predicted to have dominant negative effects or to generate a null allele (no protein or extremely truncated protein).[33-37]

The high frequency of combined *RUNX1* and *CSF3R* mutations and the presence of both mutations in the same leukemic cell clones clearly suggest that these 2 mutations cooperate as drivers in leukemogenesis. Our hypothesis is that the initial driver mutations are the *CSF3R* mutations and cell clones harboring *CSF3R* mutations having a growth advantage acquire in the majority of patients *RUNX1* mutations that contribute to AML initiation and disease progression.

In contrast, the association between *RUNX1* and *CSF3R* mutations was not found in *de novo* pediatric AML, suggesting a distinct and specific mechanism in CN. Only 2 patients with a *RUNX1* mutation had no *CSF3R* mutation; conversely, 6 patients with a *CSF3R* mutation had no *RUNX1* mutations. Intriguingly, 5 patients who were negative for *CSF3R*, and *RUNX1* mutations had MDS only and no overt AML, suggesting that *CSF3R*/*RUNX1* mutations are rather a feature of AML and less frequent of MDS. There was no evidence from a copy number analysis that the chromosomal region 21q22.1 containing *RUNX1* was lost in these latter 6 cases, but the loss of *RUNX1* expression (eg, through DNA or histone methylation) cannot be excluded. CN patients require lifelong treatment with high therapeutic

doses of G-CSF.[1] Most of the *CSF3R* mutations described in CN patients lead to a truncated CSF3R protein lacking the intracellular domain responsible for the termination of proliferative signals.[10-12,38,39] Moreover, we demonstrated the transitory appearance of clones carrying different *CSF3R* mutations in CN patients many years prior to AML (Figure 4A). These data are in line with our previously published findings showing more than one mutation in *CSF3R* in a CN patient who developed AML.[42] Our data suggest that as soon as a cell clone carrying a *CSF3R* mutation is hit by an additional mutation in *RUNX1*, the inevitable fate of this clone is transformation into leukemic cells. It has been reported that a *RUNX1* loss-of-function mutation per se is not sufficient to cause leukemia but does block myeloid differentiation.[43] Our patient data taken together with *in vitro* studies strongly support the hypothesis that, by markedly elevating proliferation and diminishing myeloid differentiation of hematopoietic cells, *CSF3R* and *RUNX1* mutations are the major drivers of leukemogenesis in patients carrying both mutated proteins.

The importance of *RUNX1* mutations in leukemic transformation was substantially strengthened by the analysis of a unique family with 2 siblings suffering from CN that subsequently transformed into AML. In both children, cooperating *RUNX1* and *CSF3R* mutations were detected that were not present in healthy family members. Inherited *RUNX1* mutations have been described in familial platelet disorder-AML, another inherited preleukemic bone marrow failure syndrome with a high propensity to develop into AML.[22,23] Cryptic *RUNX1* lesions (translocations, deletions, or mutations) have also been observed in Fanconi anemia patients who developed AML or MDS.[44] These data, together with our observations, suggest that *RUNX1* mutations could represent an important common event in triggering secondary leukemia in patients with bone marrow failure syndromes.

In conclusion, our findings may improve monitoring of the possible leukemic transformation in CN patients in which *CSF3R* mutations are already present. We recommend yearly bone marrow examinations. Patients with both *RUNX1* and *CSF3R* mutations appear to be at high risk for rapid evolution to leukemia and should be considered candidates for stem cell transplantation.

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Authorship

Contribution: J.S. and D.S. collected and analyzed data, supervised experiments, and wrote the manuscript; J.S. also provided financial support; J.E.K.-K., J.O., C.M.Z., D.R., and M.M.v.d.H.-E. collected and analyzed data on *de novo* pediatric AML patients and wrote the part of the manuscript describing *de novo* AML patients data; C.Z. provided patient materials; O.K. performed in vitro experiments with CD341 cells; M.K. performed DNA sample preparation, analysis of sequenced data, sequencing of single CFU clones, and allele-specific PCR; M.U¨ . and S.K. analyzed deep-seq data of *CSF3R*; R.B., K.B., and C.S. made lentiviral constructs with WT and MUT *RUNX1*; S.S. and A.K. performed deep-seq of *RUNX1* and other leukemia-associated mutations and wrote manuscript; M.G.V., R.H., and I.P.T. performed *RUNX1* and *CSF3R* analysis of some CN/AML patients; I.P.T. wrote the manuscript; V.M., D.C.D., P.V., and J.D. provided some patient materials; D.C.D. wrote the manuscript; G.G. and B.S. provided some patient materials and cytogenetic analysis; M.S. wrote the manuscript; and K.W. provided patients material, supervised experiments, analyzed data, wrote the manuscript, and provided financial support.

Conflict-of-interest disclosure:

D.C.D. is a consultant and receives research support from Amgen, the manufacturer of G-CSF mentioned in the paper and used to treat cyclic and CN. The remaining authors declare no competing financial interests.

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SUPPLEMENTAL MATERIALS AND METHODS

DNA isolation

We isolated DNA from bone marrow, peripheral blood or bone marrow smears of CN patients who developed leukemia using the DNA isolation Kit from Qiagen (Qiagen, Hilden, Germany) or the Ultra Clean DNA Blood Isolation Kit from Dianova (Hamburg, Germany). In some cases, DNA was amplified using the REPLI-g Mini Kit according the manufacturer's protocol (Qiagen, Hilden, Germany) or the Illustra GenomiPhi V2 DNA Amplifications Kit (GE Healthcare, München, Deutschland).

Mutational analysis

For sequential analyses of the time-course of occurrence of *RUNX1* and *CSF3R* mutations, mutated regions of *RUNX1* and *CSF3R* genes were amplified using polymerase chain reaction (PCR) and PCR products were sequenced using a SOLiD 5500XL ligationbased sequencing system or the SeqCap EZ library (Roche Nimblegen) followed by sequencing on the Hiseq 2000 system (Illumina). In the 302 de novo AML-samples, the protein coding sequence of the *RUNX1* gene (exon 3-8) (*RUNX1*-002, NM_001754.4) was PCR-amplified using specific primers and subsequently sequenced in forward and reverse direction. Purified PCR products were directly sequenced from both strands. Primer sequences are available upon request. The sequence data were analyzed using CLC Workbench version 3.5.1 (CLC Bio, Aarhus, Denmark). In case of a suspected mutation, the fragment was re-amplified and sequenced in both directions. All *RUNX1* mutated samples were also screened for *CSF3R* and *ELANE* mutations. Briefly, if cDNA was available, *CSF3R* cDNA was amplified and sequenced in forward and reverse direction. When only genomic DNA was available, *CSF3R* was amplified and sequenced to detect mutations and deletions around two previously reported *CSF3R* hotspot mutations: a missense mutation at position 595 and a nonsense mutation at position 716 (NM_000760.3). For *ELANE*, the protein coding exons (exons 1-5) were amplified and subsequently sequenced in forward and reverse direction on genomic DNA. Primer sequences are available upon request.

Karyotyping

Molecular karyotyping using high-resolution aCGH by means of oligo-arrays (180k/400k, Agilent Technologies, Waldbronn, Germany) was performed following the manufacturers instructions (Oligonucleotide Array-Based CGH for Genomic DNA Analysis v. 4.0). The direct labeling protocol was used with 1.0 µg DNA as starting material. Scanning was done using Agilent scanner G2505C at a resolution of 2 µm. Image analysis was done with Feature Extraction Software (Agilent Technologies) with the standard protocol and data processing was performed with Genomic-Workbench (Agilent Technologies). The real resolution under the given filter settings (at least 10 probes with mean log2-ratio = 0.5) was

around 50 kb. Standard karyotyping was performed on metaphases of bone marrow or blood cells according to standard procedures. Karyotypes were described according to the 2009 International System for Human Cytogenetic Nomenclature.[1]

Cell purification and separation

We isolated bone marrow (BM) or peripheral blood mononuclear cells by Ficoll- Hypaque gradient centrifugation (Amersham Biosciences). In some cases CD34+ or CD33+ leukemia blasts were isolated by sequential immunomagnetic labeling with corresponding MACS beads (Miltenyi Biotech, Bergisch Gladbach, Germany). Cells were counted and viability was assessed by trypan blue dye exclusion. Purity of sorted CD34+ and CD33+ cells was more than 96% as assessed by FACS analysis and by staining with May-Grünwald-Giemsa.

Colony-forming units (CFUs) assay

CD34+ cells or AML blasts were plated in 1 mL methylcellulose medium (5 x 10^3/dish) (Methocult H4230; StemCell Technologies) supplemented with cytokine cocktail (IL-3 (20 ng/ml), IL-6 (20 ng/ml), TPO (20 ng/ml), SCF (50 ng/ml), FLT3-L (50ng/ml). After 14 days of culture DNA from single colonies was isolated using the UltraClean® Blood DNA Isolation Kit (Non-Spin) (Mo Bio Laboratories Inc.). DNA was precipitated by addition of isopropanol and washed in 70% ethanol.

Allele-specific PCR

We isolated total RNA from blasts of CN/AML patients, used random hexamer primed cDNA to amplify PCR products containing both mutations, ligated PCR products in PCR cloning vector pGEM-T, transformed competent cells with pGEM-T vector containing PCR product, isolated DNA from single bacterial colonies and performed Sanger sequencing with primers specific to vector sequence. The presence of both mutations in the same sequence indicates that they were located on the same allele.

Whole-genome DNA amplification

Whole-genome amplification by isothermal stand displacement was performed using the GenomiPhi V2 Amplification Kit (GE Healthcare), as described in the manufacturer's protocol. Concentration of the amplified DNA was measured by Fluorometric Qubit™ dsDNA HS Assay Kits (Molecular Probes, Invitrogen). 100 ng of amplified DNA were taken for each sequencing reaction.

Screening of inherited CN-causing as well as of RUNX1 and CSF3R mutations

Inherited ELANE, HAX1, G6PC3, WAS and GFI1 mutations were analyzed using ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit on a 3500 Genetic Analyzer (Applied

Biosystems) and 4peaks and Chromas softwares or using the SeqCap EZ library (Roche Nimblegen, Madison, WI) followed by sequencing on the Hiseq2000 (Illumina, San Diego, CA). Primers are available upon request. Analysis of CSF3R mutations was as described previously.[2]

Lentiviral transduction of CD34+ cells

We used HEK 293T cells for lentiviral supernatant production. 293T cells were maintained in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% FCS, 100 U/ml penicillin/streptomycin, and 2 mM glutamine. The day before transfection, $5x10^6$ 293T cells were plated on a 10-cm dish. For transfection, the medium was exchanged and 25 μM chloroquine (Sigma-Aldrich, Munich, Germany) was added. 8 μg transfer vector DNA (pRRL.PPT.SF.DsRedEx.CSF3Rd715.pre or Lego-iG/Puro-Runx1-R135G-CTAP or Lego-iG/Puro-Runx1-R139G-CTAP or pRRL.PPT.SF.DsRedEx.CTRL.pre or LegoiG/ Puro-ctrl-CTAP) and 5 μg of a VSVg (glycoprotein of the vesicular stomatitis virus) envelope plasmid were used. In addition, 12 μg of a lentiviral gag/pol plasmid (pcDNA3 g/p 4xCTE) and 5 μg of a Rev plasmid (pRSV-Rev, kindly provided by T. Hope, Chicago) was cotransfected using the calcium phosphate technique. Medium was changed after 10-12 h. Transfection efficiency was controlled by flow cytometry analysis. Supernatants containing the viral particles were collected 24-72 h after transfection, filtered through a 0.22-μm filter, concentrated using Lenti-X concentrator from Clontech and stored at -80°C until usage. The virus titers averaged and typically ranged from 1 to 5×10^8 IU/ml after concentration. We transduced CD34+ cells from healthy donors (2×10^5/well) with lentiviral supernatants with a multiplicity of infection MOI of 1-2 and assessed transduction efficiency after 72 h as the percentage of GFP- or RFP-positive cells. Vector information is available upon request.

In vitro proliferation and granulocytic differentiation experiments

For transduction we cultured $1x10^5$ transduced CD34+ cells in Stemline II hematopoietic stem cell expansion medium (Sigma) supplemented with 20 ng/ml of interleukin-3 (IL-3), 20 ng/ml of interleukin-6 (IL-6), 20 ng/ml of thrombopoietin, 50 ng/ml of stem cell factor and 50 ng/ml of FLT3 ligand (R&D Systems) for 3 days. Transduced cells were cultured in the same medium for granulocytic differentiation, we cultured $1x10^5$ of transduced CD34+ cells in supplemented RPMI 1640–10% FCS medium in the presence of G-CSF (10 ng/ml) for 8 days. We characterized granulocytic differentiation by FACS analysis of cells stained with allophycocyanin-conjugated CD15-specific (BD Pharmingen, cat. 561716), CD11b-specific (BD Pharmingen, cat. 553312) and CD16-specific antibody (BD Pharmingen, cat. 561304). To assess proliferation we counted viable cells by trypan blue dye exclusion and estimated percentage of RFP+, or GFP+ or RFP+GFP+ cells on day 2 and on day 4 of culture.

Analysis of de novo AML samples

Sporadic pediatric AML samples (n=307) were screened for recurrent genetic abnormalities characteristic for AML, including t(15;17), inv(16), *MLL*-rearrangements, t(7;12) and t(8;21) by the national study groups as described previously.[3,4] Samples without any cytogenetic aberrations detectable by standard chromosome banding technique are designated "cytogenetically normal". In addition to cytogenetic analysis, and apart from *RUNX1*, *GSF3R* and *ELANE* mutation screening, samples were also screened for non randomly occurring mutations in *NPM1*, *CEBPA*, *WT1*, *NRAS* and *KRAS*, *MLL-PTD*, *IDH1/2*, *DNMT3A* and *FLT3* as previously described.[5-12]

Gene expression analysis

Gene expression was analyzed in a subset of 253 sporadic pediatric AML samples with available material as described previously.[3] Briefly, cDNA and cRNA was synthesized of RNA with an RNA integrity number >8. Hybridization and processing on the Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA, USA) was performed according to the manufacturer's guidelines. Data was acquired using Expresso (Bioconductor package Affymetrix) and probe-set intensities were normalized according to the variance stabilization normalization (Bioconductor package VSN) in the data analysis environment R, version 2.12. Gene expression profiles (GEP) were compared between 8 RUNX1 mutated samples and 98 wildtype RUNX1 without *MLL*-rearrangements, t(8;21), inv(16), t(15;17), or t(7;12) according to the empirical Bayes linear regression model using the package for statistical analyses Limma. Moderated T-statistics P values were corrected for multiple testing by the false discovery rate method.[13]

Messenger RNA expression

To determine mRNA expression levels, quantitative real-time PCR (qRT-PCR) was performed on cDNA produced on an ABI PRISM 7900HT sequence detector (Applied Biosystems). Primers for target genes RUNX1 and housekeeping gene GAPDH are available upon request. The average cycle threshold (Ct) values were used to calculate RUNX1 mRNA expression relative to GAPDH mRNA expression by the comparative cycle time method.

Statistical analysis

We performed statistical analysis using the SPSS V. 9.0 statistical package (SPSS).

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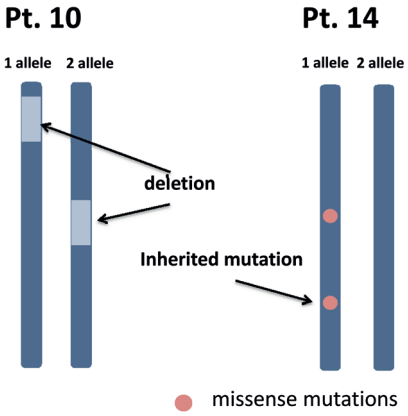
SUPPLEMENTAL TABLES

Supplemental Table 1: Aberration summary reports of array-CGH analyses
Aberration summary reports of array-CGH analysis performed on samples 9G, 11G (two timepoints), 14G (three timepoints, 16G and 2G: in rows the chromosomal regions, with number of chromosome, cytoband, start and stop in basepairs according to hg_19, number of probes on the array indicating the alteration, log2 ratios indicating amplification (>0.4) or losses (<-0.4), p-value for alteration, genes targeted by alteration and CNVs for alterations as reported in the database of genomic variants (projects.tcag.ca/variation).

Due to the nature of this table, it is not printed in this thesis. It can be found on the Blood website.

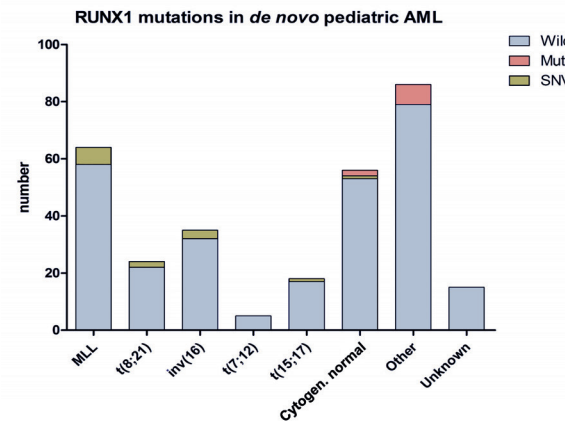
SUPPLEMENTAL FIGURES

Supplemental figure 1. Sequential analysis of acquisition of *RUNX1* and *CSF3R* mutations prior to progression to AML or MDS



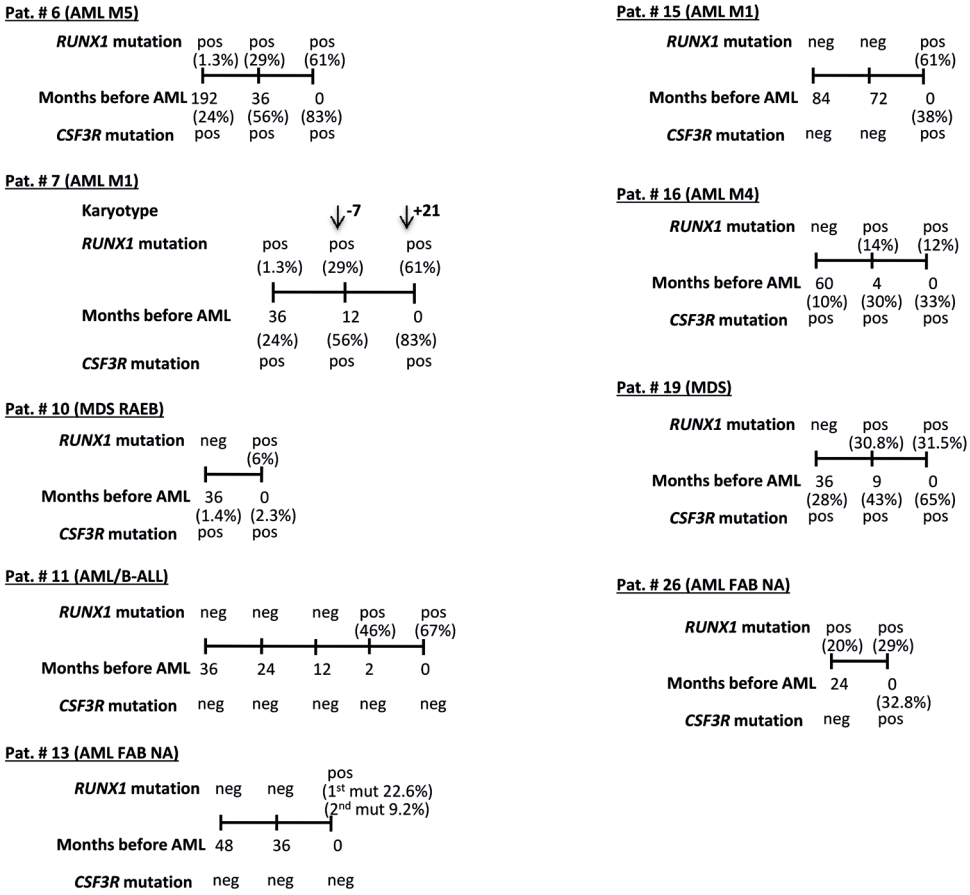
Schematic presentation of the allele-specific PCR analysis of *RUNX1* mutations in two CN patients who developed AML. White boxes represented deletions and red cycles missense mutations.

Supplemental figure 2. Mutational status of leukemia in de novo pediatric AML patients with RUNX1 aberrations.



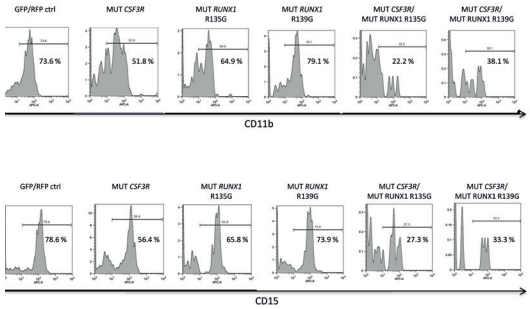
Distribution of RUNX1 abnormalities among de novo pediatric AML patients according to cytogenetic class. Pink bars indicate mutations; blue bars indicate single-nucleotide variants and SNPs.

Supplemental figure 3. Time course of *RUNX1* and *CSF3R* mutations in leukemia progression in CN



Schematic presentation of the deep-sequencing analysis of *RUNX1* and *CSF3R* mutations in the bone marrow of 9 CN patients who developed leukemia or MDS. Karyotype (if available) and a percentage of cells with mutated *RUNX1* and *CSF3R* at different time points prior to leukemia progression are shown for each patient.

Supplemental figure 4. Diminished myeloid differentiation of hematopoietic cells.



CD34+ bone marrow cells from healthy individuals were transduced with lentivirus-based GFP-tagged *RUNX1* mutants (p.Arg135Gly or p.Arg139Gly) or RFP-tagged *CSF3R* mutant (d715) alone, co-transduced with *RUNX1* and *CSF3R* mutants, or co-transduced with control GFP- and RFP-tagged lentiviral constructs. Transduced cells were treated with 10 ng/ml of G-CSF. G-CSF-triggered myeloid differentiation was evaluated on day 8 of culture. Representative FACS histograms are depicted.

4

DIFFERENTIALLY EXPRESSED MIRNAS IN CYTOGENETIC AND MOLECULAR SUBTYPES OF PEDIATRIC ACUTE MYELOID LEUKEMIA

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CHAPTER 4: MIRNAS IN PEDIATRIC ACUTE MYELOID LEUKEMIA

ABSTRACT

Background.
MiRNAs regulate gene expression, and thus play an important role in critical cellular processes. Aberrant miRNA expression patterns have been found in various types of cancer. So far, information about the expression of miRNAs in pediatric acute myeloid leukemia is limited.

Procedure.
We studied expression of miR-29a, -155, -196a, and -196b by stem-loop based RT-qPCR in 82 pediatric acute myeloid leukemia patients selected to represent relevant cytogenetic and molecular subgroups.

Results.
High miR-196a and -b expression was observed in patients carrying *MLL* gene rearrangements (P< 0.001), *NPM1* mutations (P< 0.001), or *FLT3-ITD* in a cytogenetically normal background (P ≤ 0.02), compared to all other patients. In contrast, *CEBPA* mutated cases had a low expression of miR-196a and -b (P ≤ 0.001). Expression of miR-196a and -b was correlated with expression of neighboring *HOXA* and *HOXB* genes (Spearman's r = 0.46–0.82, P< 0.01). Expression of miR-155 was not related to cytogenetic features but high expression of miR-155 was observed in *FLT3-ITD* (P= 0.001) and *NPM1*-mutated cases (P= 0.04). Lower miR-29a expression was mainly observed in *MLL*-rearranged pediatric acute myeloid leukemia, specifically in cases carrying t(10;11) (P< 0.001).

Conclusions.
We show aberrant expression of specific miRNAs in clinically relevant cytogenetic and molecular subgroups of pediatric acute myeloid leukemia, suggesting a role for these miRNAs in the underlying biology in these specific subgroups.

INTRODUCTION

Acute myeloid leukemia (AML) is a heterogeneous disease involving many different cytogenetic and molecular abnormalities[1–3]. In AML, leukemogenesis is characterized by a combination of differentiation arrest and proliferative advantage of myeloid progenitors. Pediatric AML has distinct features compared to adult AML, reflected in differences in response to therapy and prognosis[2]. The 5-year survival of children with AML is currently around 60%[4–7] and relapse is the main reason for therapy failure. In addition, current intensive chemotherapy protocols have reached their limits due to direct and late toxic side effects. Therefore, more knowledge of the biology of pediatric AML is necessary to develop novel treatment strategies that are urgently required. Apart from therapy response, cytogenetic and molecular aberrations are the most important prognostic factors in pediatric AML[2,3,8]. Patients with t(8;21)/*AML1-ETO*, inv(16)/t(16;16)/*CBFB-MYH11*, and t(15;17)/*PML-RARA* represent favorable prognostic subgroups, whereas, for example, patients with monosomy 7 and complex karyotype have a poor outcome[3]. In addition, mutations in *NPM1*, *WT1*, *CEBPA*, and *FLT3* genes are important with respect to survival and prognosis in pediatric AML[8,9]. Nevertheless, relapses occur in both favorable and unfavorable subgroups, and the biological determinants for therapy failure are still largely unknown. Genes involved in AML biology are not only regulated by genetic but also by epigenetic events. Recently, it became clear that specific miRNAs regulate important genes in cancer biology.

MiRNAs are small, non-coding RNAs that regulate protein expression by inhibition of translation or degradation of mRNA transcripts, and thus play an important role in critical cellular processes like proliferation, differentiation and apoptosis. Aberrant expression of miRNAs has been observed in many types of cancer, displaying either tumor suppressive or oncogenic activity. miRNA expression profiles are able to classify specific tumors[10]. For example, a miRNA expression signature has been defined that can discriminate acute lymphoblastic leukemia (ALL) from AML[11]. In addition, specific miRNA genes are found in chromosomal regions that are deleted, amplified, or involved in translocations in cancer. For example, miR-15a and-16-1, two miRNAs that target the oncogene *BCL2*, are located in a small region at 13q14 that is often deleted in chronic lymphocytic leukemia[12]. Moreover, the miR-29b-1/29a cluster is located at 7q32, a region often found to be deleted in therapy-related AML and myelodysplastic syndromes (MDS)[13].

Several studies have addressed the role of miRNAs in adult AML[14–19]. For example, miR-181a is elevated in FAB M1 and M2 morphological subtypes[15]. Also, various miRNAs have been shown to be involved in the biology of subgroups of adult AML. MiR-29a has been shown to be down-regulated in adult patients with AML with an *MLL*-rearrangement compared to patients with AML without *MLL*-rearrangements[17]. Overexpression of miR-29a in hematopoietic stem cells in a mouse model resulted in myeloproliferative disease (MPD)/AML[20]. In addition, miR-29 family members are known to target the oncogenes TCL1 and

MCL1[21,22]. Overexpression of miR-155 in hematopoietic stem cells resulted in a myeloproliferative disorder in a mouse model[23,24]. MiR-155 has been found to be up-regulated in *FLT3-ITD* positive adult AML[17,18]. Expression of miR-196a/b was found to be deregulated in adult *MLL*-rearranged AML[17,18]. We have reported that miR-196b is highly expressed in children with *MLL*-gene rearranged ALL and *HOXA* activated T-ALL[25]. The miR-196a and -b genes are located in the *HOX* gene cluster, known to be upregulated in *MLL*-rearranged leukemia, and miR-196a has been shown to target *HOXB8*[26]. In addition, some miRNAs (e.g., miR-191 and -199a) have been reported to be associated with outcome in adult AML[17,19].

Thus far, knowledge on the role of miRNAs in pediatric AML is limited. To determine whether specific miRNAs are differentially expressed in subgroups of pediatric AML, we used stem-loop-based RT quantitative PCR (RT-qPCR) to determine the expression levels of miR-29a, -155, -196a, and -196b in pediatric patients with *de novo* AML representing different cytogenetic subtypes. These miRNAs were selected based on results from *in vitro* and *in vivo* studies[19–25] and their reported subtype-specific expression in adult AML[14,17,18]. This study shows the relation between expression of miRNAs and clinically relevant molecular and cytogenetic abnormalities in pediatric AML.

Table I: Clinical and cytogenetic/molecular patient characteristics

	Partial cohort	Full cohort
number of patients	57	82
Age at diagnosis (years)		
Median	10.0	10.6
Range	0.1-16.8	0.1-18.5
WBC count (x 10^9/L)		
Median	41.3	40.2
Range	3.7-496	2.5-469
Blast percentage		
Median	92.4	91.8
Range	81-100	74-100
Sex		
male	30 (57%)	45 (58%)
female	23 (43%)	33 (42%)
Karyotype		
MLL t(9;11)	9 (16%)	9 (11%)
MLL t(10;11)	8 (14%)	8 (10%)
t(8;21)	9 (16%)	9 (11%)
inv(16)	10 (18%)	10 (12%)
t(15;17)	8 (14%)	11 (13%)
Normal[a]	13 (23%)	26 (32%)
Other[b]	0 (0%)	9 (11%)
Molecular aberration		
FLT3-ITD	11 (19%)	18 (22%)
NPM1	5 (9%)	9 (11%)
CEBPA	3 (5%)	10 (12%)
RAS	13 (23%)	19 (23%)
WT1	3 (5%)	7 (9%)
FAB classification		
M0	0 (0%)	2 (3%)
M1	3 (6%)	8 (11
M2	13 (24%)	23 (30%)
M3	9 (17%)	12 (16%)
M4	14 (26%)	15 (20%)
M5	14 (26%)	15 (20%)
M6	0 (0%)	0 (0%)
M7	1 (2)	1 (1)

[a]Cytogenetically normal (CN): without cytogenetic aberrations, [b]Other karyotype: with miscellaneous cytogenetic aberrations

METHODS

Patient Samples

Viably frozen diagnostic bone marrow or peripheral blood samples of 82 patients with newly diagnosed pediatric AML were selected to represent different cytogenetic and

molecular subgroups. Patient numbers and characteristics are listed in Table I. Samples were obtained from the Dutch Childhood Oncology Group (DCOG; The Hague, The Netherlands), the AML-''Berlin-Frankfurt-Münster'' Study Group (AML-BFM-SG; Hannover, Germany, and Prague, Czech Republic) and the St. Louis Hospital (Paris, France). Samples were enriched to contain approximately 80% leukemic cells as previously described[27]. Blast percentages were determined morphologically by May-Grünwald-Giemsa (Merck, Darmstadt, Germany)— stained cytospins. As reference material, Ficoll-separated bone marrow mononuclear cells were collected from two children without malignant tumors and bone marrow abnormalities (Sophia Children's Hospital, Rotterdam). Informed consent was obtained according to local law and regulations.

RNA Isolation

Total cellular RNA was isolated using Trizol reagent (Invitrogen, Breda, The Netherlands) as previously described [32]. RNA quality was checked using the RNA 6000 nanoassay on the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). Samples with RNA degradation (RIN < 6) or DNA contamination were excluded.

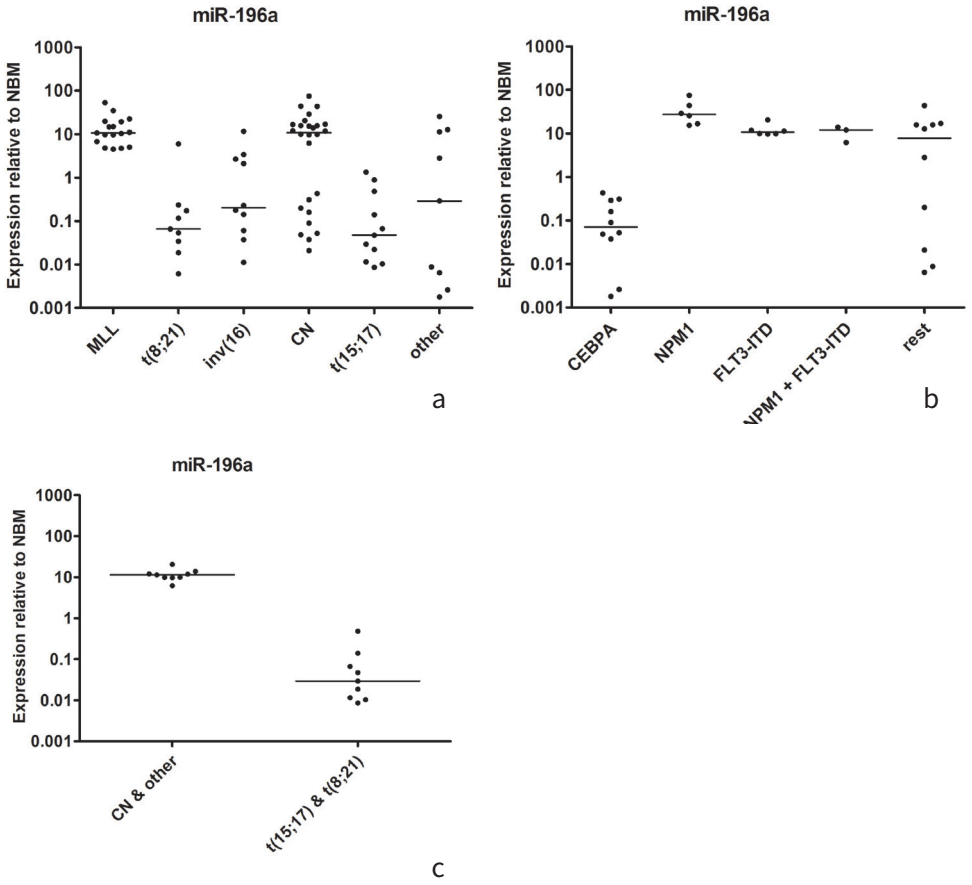
Cytogenetic and Molecular Analysis

Leukemic samples were routinely investigated for cytogenetic abnormalities by standard chromosome-banding analysis. Screening for recurrent nonrandom genetic abnormalities characteristic for AML included t(15;17), inv(16), t(8;21), and *MLL*-gene rearrangements, using either RT-qPCR and/or fluorescent in situ hybridization. Patients lacking cytogenetic abnormalities are designated as cytogenetically normal (CN) and patients with miscellaneous cytogenetic abnormalities are designated as ''other.'' Mutational screening of *NPM1*, *CEBPA*, *WT1*, NRAS,KRAS, and *FLT3* was performed as previously described[9,28–31].

miRNA Expression

Four miRNAs were selected based on their known deregulation in adult AML[14,15,17,18]. Expression of target miRNAs (miR-29a, -155, -196a, and -196b) and reference snoRNA-1 (RNU24) was measured by stem-loop based RT-qPCR. This technique detects mature miRNAs but not precursor miRNAs, using specific (stem-loop RT) primers and probes of the single tube TaqMan miRNA assays (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. RT-qPCR was performed in duplicate on an Applied Biosystems 7900HT RT-PCR system. The threshold was manually set at 0.15, the average cycle threshold (Ct) value was used to calculate miRNA expression levels of the targets relative to the expression level of the reference snoRNA using the comparative Ct method with snoRNA-1 as a housekeeping gene. Expression levels were calculated relative to expression in normal pediatric bone marrow.

Fig. 1. Expression of miR-196a is associated with specific cytogenetic and molecular aberrations in AML.



The relative expression of miR-196a as compared to NBM was determined by RT-qPCR. A: Eighty-two patients selected to represent the variety of different cytogenetic subgroups. B: Expression in relation to different molecular aberrations in 35 patients belonging to the CN or ''other'' cytogenetic subgroups. Rest: other mutations than in *NPM1*, *CEBPA*, or *FLT3-ITD*, or no molecular aberrations. C: expression of miR-196a in 18 patients carrying *FLT3-ITD* in different cytogenetic subgroups. Horizontal lines indicate the median expression.

mRNA Expression

In order to evaluate the influence of miRNAs on gene expression, microarray analysis was performed on 79 patient samples using the Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA) as described previously[33]. Subsequently, RT-qPCR was used to validate gene expression of *HOXA9*, *HOXA10*, and *HOXB9*. Primers and probes are summarized in Supplemental Table I. *GAPDH* was used as an internal reference, using primers and probes as previously described[34]. RT-qPCR was performed in duplicate on an Applied Biosystems 7900HT RT-PCR system. The threshold was manually set at 0.06.

Table II: Expression of miR-196a in cytogenetic or molecular subgroups of AML

		miR-196a		
	n	median expression (range)[a]	fold expression vs. rest[b]	P-value[c]
cytogenetic abnormalities				
MLL	17	10.63 (4.51-53.04)	45.10	*<0.001*
t(8;21)	9	0.07 (0.01-5.95)	0.01	*0.012*
inv(16)	10	0.20 (0.01-11.65)	0.04	0.167
CN[d]	26	10.82 (0.02-75.02)	15.84	*0.006*
t(15;17)	11	0.05 (0.01-1.34)	0.01	*0.002*
other[e]	9	0.29 (0.00-25.55)	0.09	0.202
molecular abnormalities				
CEBPA	10	0.07 (0.00-0.43)	0.01	*0.004*
FLT3-ITD	18	3.34 (0.01-20.53)	1.22	0.443
- CN, other	9	11.37 (6.20-20.53)	23.55	*0.020*
- t(15;17), t(8;21)	9	0.03 (0.01-0.48)	0.01	*0.001*
NPM1	9	16.62 (6.20-75.02)	34.42	*<0.001*
WT1	8	0.71 (0.01-43.71)	0.18	0.421
N/K-RAS	19	9.65 (0.00-75.02)	7.23	0.328

[a] Median expression relative to normal bone marrow, determined by RT-qPCR, [b] Median fold expression of specific subgroup compared to all other patients, [c] Determined by Mann-Whitney test, significant P-values (<0.05) in italic, [d] CN: cytogenetically normal, [e] Other: with miscellaneous cytogenetic abnormalities

The relative expression was calculated using the comparative Ct method with *GAPDH* as housekeeping gene.

Statistical Analysis

The difference in miRNA expression between patient groups was calculated using the Mann–Whitney test. Spearman's correlation was used for assessment of correlation between miRNA expression, mRNA microarray, and RT-qPCR data. All statistical analysis was performed using the SPSS 15.0 software package, all used test were two-sided, P-values <0.05 were regarded as significant.

RESULTS

Pronounced Differences in miR-196a Expression Between and Within Cytogenetic and Molecular Subgroups

Expression of miR-196a was found to be highly variable among pediatric patients with AML (n=82), with a >500-fold difference between high and low expressing groups (P< 0.001, median expression range 0.03–16.6 compared to normal bone marrow). MiR-196a expression was clearly associated with specific non-random cytogenetic abnormalities (Fig. 1A, Table II). The highest expression of miR-196a was found in patients with *MLL*-re-

Table III: Expression of miR-29a and miR-155 in cytogenetic or molecular subgroups of AML[a]

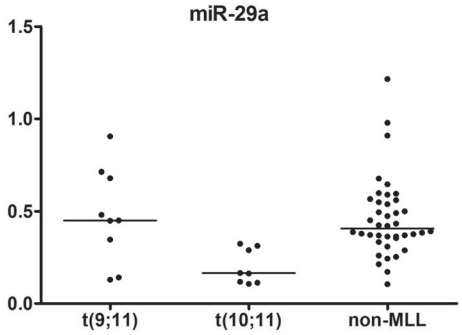
	miR-29a				miR-155			
	n	median expression (range)[a]	fold expression vs. rest[b]	p-value[c]	n	median expression (range)[a]	fold expression vs. rest[b]	p-value[c]
cytogenetic abnormalities								
MLL	17	0.31 (0.11-0.90)	0.77	*0.039*	17	0.50 (0.16-1.16)	0.92	0.721
t(8;21)	9	0.50 (0.33-0.60)	1.34	0.108	9	0.81 (0.13-1.38)	1.59	0.376
inv(16)	10	0.37 (0.17-0.54)	0.95	0.564	10	0.34 (0.20-0.65)	0.60	0.055
CNd	13	0.38 (0.11-1.22)	0.98	0.783	13	0.60 (0.09-3.12)	1.24	0.186
t(15;17)	8	0.53 (0.29-0.91)	1.42	*0.045*	8	0.51 (0.08-1.31)	1.00	0.973
molecular abnormalities[d]								
FLT3-ITD	11	0.47 (0.26-0.91)	1.28	0.070	11	0.94 (0.37-3.12)	2.03	*0.001*
NPM1	5	0.59 (0.38-1.22)	1.58	*0.025*	5	0.68 (0.60-3.12)	1.39	0.038
N/K-RAS	13	0.37 (0.11-1.22)	0.91	0.300	13	0.58 (0.09-1.38)	1.15	0.977

Median expression relative to normal bone marrow, determined by RT-qPCR. [b] Median fold expression of specific subgroup compared to all other patients. [c] Determined by Mann-Whitney test, significant P-values (<0.05) in italics. [d] CN: cytogenetically normal. [e] WT1 and CEBPA were not analyzed due to small patient numbers (n=3)

arrangements versus all other patients (45.1-fold expression, P< 0.001), irrespective of the *MLL*-translocation partner. A relatively low expression compared to all other patients was found in patients with t(8;21) (0.01-fold expression, P= 0.012), inv(16) (0.04-fold expression, P= 0.17), and t(15;17) (0.01-fold expression, P= 0.002). Among patients with AML with a normal (CN) or ''other'' karyotype two groups were observed with either high or low miR-196a expression, which could not be attributed to specific cytogenetic abnormalities. Therefore, we determined whether differences in expression among these patients could be correlated with molecular abnormalities. In the total group (n= 82), high miR-196a expression was associated with mutations in *NPM1* (median fold expression 34.4, P< 0.001) or *FLT3-ITD* (median fold expression 23.6, P= 0.02), whereas low expression was related to *CEBPA* mutations (median fold expression 0.01, P= 0.004) (Fig. 1B, Table II). *FLT3-ITD* also occurs in patients with t(15;17) or t(8;21). Interestingly, within the latter two subgroups, *FLT3-ITD* was associated with low expression of miR-196a (median fold expression 0.01, P< 0.001) (Fig. 1C, Table II). Presence of *WT1* or *N/K-RAS* mutations did not correlate with miR-196a/b expression.

The mature miRNA sequence of miR-196a closely resembles that of miR-196b, differing only in one nucleotide. Therefore, we determined whether the miR-196b expression pattern was similar to that of miR-196a, using a RT-qPCR-based assay specific for miR-196b (Applied Biosystems) in 71 patients with AML for whom these data and material were available. The expression levels of miR-196b and -a were highly concordant in all patients (Spearman's r = 0.87, P< 0.0001). Correspondingly, for miR-196b expression similar associations were found with cytogenetic and molecular abnormalities (Supplemental Table II). No correlations were found between miR-196a/b expression and the clinical charac-

Fig. 2. Differential expression of miR-29a in cytogenetic subgroups of pediatric AML.



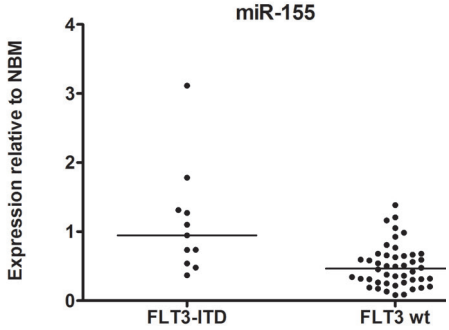
The relative expression of miR-29a as compared to NBM was determined in 57 patients by RT-qPCR. Horizontal lines indicate the median expression.

teristics age, sex, and white blood cell count. In addition, no significant correlations were found between expression of miR-196a/b and outcome (overall survival, OS), using either the 25th and 75th percentiles or an expression of 1 relative to normal bone marrow as cut off points.

Correlation of miR-196a/b Expression With *HOX* Gene Expression

MiR-196a is located in the *HOXB* gene cluster, and miR-196b in the *HOXA* gene cluster. Therefore, we determined whether expression of miR-196a and -b correlates to expression of *HOX* genes in pediatric AML. *MEIS1*, a homeobox gene involved in myeloid transformation but not located in the *HOX* gene cluster, and Annexin A1 (*ANXA1*), a known target for miR-196a, were also included in this analysis. Gene expression profiling was performed on 79 out of 82 patients analyzed for miR-196a, and on 69 out of 71 patients analyzed for miR-196b. Several genes of the *HOXA* and *HOXB* cluster correlated significantly (Spearman's r= 0.46–0.82) with both miR-196a and -b expression, of which *HOXA*3, *A4*, *A5*, *A7*, *A9*, *A10*, and *HOXB*9 were most significantly overexpressed (Supplemental Table III). We also found a similar correlation between *MEIS1* and miR-196a/b expression (Supplemental Table IV). RT-qPCR performed in a subset of patients with AML representing different molecular aberrations (n= 15–19) confirmed the strong correlation between *HOXA9*, *HOXA10*, and miR-196a/b expression (Supplemental Table V). Intriguingly, in RT-qPCR *HOXB9* expression correlated well with miR-196b, but only moderately with miR-196a (Supplemental Table V), which is located close to the *HOXB9* gene. There was hardly any positive correlation between genes located in the *HOXC-D* clusters and miR-196a/b expression. A negative correlation was found between miR-196b expression and *HOXD8*, a predicted target for both miR-196a and -b according to the Miranda algorithm [35] (Spearman's r = -0.31, P= 0.01) (Supplemental Table IV). Thus far, two targets for miR196a have been described in literature, *HOXB8* and *ANXA1* [26,36]. We did not observe a correlation between expression

Fig. 3. Differential expression of miR-155 in molecular subgroups of pediatric AML.



The relative expression of miR-155 was determined in 57 patients by RT-qPCR. Horizontal lines indicate the median expression.

of miR-196a/b and *HOXB8*, but found a negative correlation between miR-196a expression and *ANXA1* (Spearman's r = -0.27, P= 0.02) (Supplemental Table IV).

Decreased Expression of miR-29a in t(10;11) Positive AML

The median expression of miR-29a in 57 newly diagnosed pediatric patients with AML as compared to normal bone marrow was 0.38 (25th–75th percentile = 0.29–0.54). MiR-29a expression varied only slightly among patients, but was associated with specific cytogenetic abnormalities (Table III). Highest expression was found in patients carrying t(15;17) (n= 8, 1.42-fold expression, P= 0.045), and lowest in *MLL*-rearranged patients (n= 17, 0.77-fold expression, P= 0.039), compared to all other patients. We next determined whether the low expression in *MLL*-rearranged patients was associated with specific *MLL* translocation partners. Indeed, we observed that the expression in patients with t(10;11) (n= 8) was significantly lower as compared to all other patients (0.39-fold expression, P< 0.001), whereas the miR-29a level in patients with t(9;11) (n= 9) did not differ significantly from all other patients (1.19-fold expression, P= 0.40) (Fig. 2). Among groups of patients characterized by molecular abnormalities, patients with mutations in *NPM1* (n= 5) had significantly higher miR-29a levels compared to all other patients (1.58-fold expression, P= 0.025). No relation was found between miR-29a expression and *FLT3-ITD* or *N/K-RAS* mutations. For *WT1* and *CEBPA* mutations the patients numbers were too small (n= 3) to analyze. No significant relation was found between miR-29a expression and OS, using the 25th and 75th percentiles as cut-off points.

MiR-155 Expression Is Increased in Patients Carrying *FLT3-ITD*

MiR-155 expression (median expression 0.51 compared to normal bone marrow, 25th–75th percentile = 0.31–0.74) was not correlated with specific cytogenetic abnormalities (Table III). A significant, twofold expression of miR-155 was observed in patients with

FLT3-ITD (n= 11, P= 0.001) compared to all other patients (Fig. 3). In addition, *NPM1* mutations associated with a 1.4-fold miR-155 expression (n= 5, P= 0.038). We did not observe a relation between miR-155 expression and *N/K-RAS* mutations (n= 13). The relation with *WT1* or *CEBPA* mutations was not analyzed due to small numbers (n= 3). No significant association was found between miR-155 expression levels and outcome (OS), using the 25th and 75th percentiles as cut off points.

DISCUSSION

In this pilot study we used RT-qPCR to demonstrate for the first time differential expression of miRNAs in a large group of well-characterized pediatric patients with AML. The non-random distribution of miRNAs between clinically relevant genetic entities that AML is comprised of suggests that these miRNAs are involved in the biology of pediatric AML.

We observed high expression of miR-196a/b in *MLL*-rearranged and *NPM1*-mutated AML. Both miR-196a and -b are located in the *HOX* gene cluster, which is known to be upregulated in *MLL*-rearranged and *NPM1*-mutated AML[15,37,38]. The association between high miR-196a/b expression and *FLT3-ITD* was only observed in patients without recurrent cytogenetic abnormalities, in whom *HOX* genes are also upregulated [33], but not in cases with *FLT3-ITD* and a t(8;21) or t(15;17). Here, we show a high correlation of miRNA 196a/b expression with expression of various *HOXA* and *HOXB* genes. These include *HOXA9* and *HOXA10*, in between which miR-196b is located, and *HOXB9*, next to which miR-196a is located. Consistent with these findings, expression of miR-196a/b has been reported to correlate with *HOX* gene expression in adults[15]. In addition, similar correlations were found between miR-196b expression and *HOX* genes in pediatric ALL[25]. Expression of miR-196a/b appears to be co-regulated with *HOX* genes, and may therefore be an epiphenomenon in genesis of *MLL*-rearranged ALL. However, two studies in AML have recently demonstrated that exogenous expression of miR-196b leads to a partial block of myeloid differentiation and increased proliferation of bone marrow progenitor cells *in vitro*[39,40], supporting the hypothesis that increased expression of miR-196a/b in specific subtypes of AML plays a role in the underlying biology. Further studies are required to determine whether deregulation of miR-196a/b in AML are primary or secondary events in leukemogenesis.

MiR-29 family miRNAs are known to target the oncogenic proteins Mcl-1 and Tcl-1[21,22], and are thought to act as tumor suppressors in AML[41]. On the other hand, ectopic expression of miR-29a in bone marrow progenitor cells resulted in MPD and AML in mice[20], suggesting a role as oncogene. In the latter study, miR-29a was overexpressed in human AML and hematopoietic stem cells, and down-regulated in hematopoietic progenitors. In the present study we found expression of miR-29a to be slightly lower in pediatric AML compared to normal bone marrow mononuclear cells. MiR-29a was especially down-regulated in t(10;11) cases, which have a poor prognosis compared to t(9;11) cases[42]. In addition we observed increased miR-29a expression in patients with *NPM1* mutations, which are re-

lated to a better prognosis[30]. However, no significant association between miR-29a expression and survival was observed in our small series of patients.

In contrast to miR-29a and -196a/b, no predominance was found for miR-155 expression in any of the cytogenetic subgroups. However, a significant high expression of miR-155 was found in patients characterized by *FLT3*-ITD or NPM1-mutations. Similar results were found in two independent studies on adult AML[17,18]. The biological role of miR-155 in AML is still unclear. MiR-155 ectopic expression in mouse models results in both MPD and B-cell malignancies[23,24]. In addition, miR-155 has been shown to play a regulatory role in normal hematopoiesis[43]. Taken together, these data are suggestive of a role for deregulated miR-155 expression in AML.

In conclusion, we demonstrate aberrant expression of miR-29a and -196a/b, but not miR-155, in clinically relevant cytogenetic subgroups of pediatric AML. In addition, expression of miR-29a, -196a/b, and -155 was correlated with the presence of molecular abnormalities, in particular *FLT3-ITD*, *NPM1*, and *CEBPA* mutations. However, we did not observe prognostic significance of miRNA expression differences in this study. This may be due to relatively small numbers of patients in subgroups of AML in this pilot study, and warrants further study using a larger cohort. Our results are mostly but not always consistent with reports on adult AML. The observed differences could be caused by differences in methodology but could also point to relevant differences in miRNA expression between children and adults. Therefore, to determine the role of miRNAs in pediatric AML, it will be important to perform large-scale miRNA profiling in a cohort of pediatric patients, representative of the different cytogenetic and molecular subgroups. Our results support a possible future application of miRNA expression in addition to currently used diagnostic tools, in order to further delineate pediatric AML subtypes. MiRNA signatures have been reported to predict subtypes of adult AML to a similar extent as gene expression signatures[18]. As miRNAs regulate gene expression not only via degradation of mRNAs but also through translational inhibition, they are likely to provide additional information to gene expression profiles, which are based on mRNA levels. Further studies including larger panels of miRNAs are required to confirm this. Furthermore, differential expression of miRNAs between subgroups of AML suggests a role of individual miRNAs in the underlying biology. Therefore, functional studies of aberrantly expressed miRNAs may point out new pathways involved in leukemogenesis, as well as new targets for therapy.

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SUPPLEMENTAL DATA

Supplemental tables

Supplemental Table I: primer and probe sequences for RT-qPCR

Gene	Forward Primer(5'-3')	Reverse Primer(5'-3')	Probe(5'-3')
HOXA9	CAGGGTCTGGTGTTTTGTAT	ACGCTTGACACTCACACTTT	ATGCTTGTGGTTCTCCTCCAGTTG
HOXA10	TCCGAGAGCAGCAAAG	CCGCTCTCGAGTAAGGTA	TGAAAACGCAGCCAA
HOXB9	CCGTGCTGTCTAATCAAAG	CTGGTATTTGGTGTAGGGAC	AGAGGCCGGATCAAACCAAC

Supplemental Table II: Expression of miR-196b in cytogenetic or molecular subgroups of AML

	n	median expression (range)[a]	fold expression vs. rest[b]	p-value[c]
cytogenetic abnormalities				
MLL	15	12.40 (4.82-46.7)	211.79	*0.001*
t(8;21)	8	0.03 (0.01-0.06)	0.01	0.007
inv(16)	8	0.20 (0.02-6.03)	0.04	0.495
CN[d]	20	13.70 (0.01-101.7)	81.55	0.098
t(15;17)	11	0.03 (0.00-0.63)	0.01	*0.002*
other[e]	9	0.26 (0.01-89.12)	0.17	0.710
molecular abnormalities				
CEBPA	8	0.02 (0.01-0.26)	0.00	*0.001*
FLT3-ITD	16	0.04 (0.00-101.7)	0.02	0.549
- CN, other	7	22.05 (12.39-101.7)	108.14	*0.001*
- t(15;17), t(8;21)	9	0.01 (0.00-0.05)	0.00	*<0.001*
NPM1	10	22.33 (11.68-55.07)	159.50	*<0.001*
WT1	5	0.03 (0.01-21.78)	0.01	0.196
N/K-RAS	17	10.14 (0.01-101.7)	22.77	0.309

[a] Median expression relative to normal bone marrow, determined by RT-qPCR, [b] Median fold expression of specific subgroup compared to all other patients, [c] Determined by Mann-Whitney test, significant P-values (<0.05) in italics, [d] CN: cytogenetically normal, [e] Other: with miscellaneous cytogenetic abnormalities

Supplemental Table III: Correlations between miR-196a/b expression and HOX gene expression determined by microarray

	miR-196a	miR-196b
miR-196a	1	.869**
miR-196b	.869**	1
HOXA3 235521_at	.777**	.775**
HOXA4 206289_at	.712**	.730**
HOXA5 213844_at	.754**	.760**
HOXA7 206847_s_at	.685**	.720**
HOXA9 209905_at	.780**	.775**
HOXA9 214651_s_at	.778**	.792**
HOXA10 213150_at	.773**	.818**
HOXA10 213147_at	.768**	.802**
HOXB9 216417_x_at	.648**	.639**
HOXB9 226461_at	.457**	.567**

Correlations depicted as Spearman's correlation coefficient (n=67-78),** P-value<0.01

Supplemental Table IV: Spearman's correlation coefficients for correlation between miR-196a/b and *HOX*, *MEIS1* and *ANXA1*

* P-value<0.05, ** P-value<0.01

		miR-196a n=78	miR-196b n=68
miR-196a	Correlation Coefficient	1.000	.869**
	Sig. (2-tailed)		<0.001
miR-196b	Correlation Coefficient	.869**	1.000
	Sig. (2-tailed)	<0.001	-
HOXA1 214639_s_at	Correlation Coefficient	.594**	.590**
	Sig. (2-tailed)	<0.001	<0.001
HOXA10 213150_at	Correlation Coefficient	.773**	.818**
	Sig. (2-tailed)	<0.001	<0.001
HOXA10 213147_at	Correlation Coefficient	.768**	.802**
	Sig. (2-tailed)	<0.001	<0.001
HOXA11 208493_at	Correlation Coefficient	.258*	.251*
	Sig. (2-tailed)	0.023	0.039
HOXA11 213823_at	Correlation Coefficient	.464**	.494**
	Sig. (2-tailed)	0.190	0.186
HOXA13 231786_at	Correlation Coefficient	0.077	0.090
	Sig. (2-tailed)	0.505	0.464
HOXA13 238571_at	Correlation Coefficient	0.014	0.025
	Sig. (2-tailed)	0.900	0.839
HOXA13 238808_at	Correlation Coefficient	-0.004	0.070
	Sig. (2-tailed)	0.975	0.569
HOXA2 214457_at	Correlation Coefficient	.434**	.422**
	Sig. (2-tailed)	0.734	<0.001
HOXA2 1557050_at	Correlation Coefficient	.381**	.278*
	Sig. (2-tailed)	0.001	0.022
HOXA3 208604_s_at	Correlation Coefficient	0.188	0.105
	Sig. (2-tailed)	0.100	0.394
HOXA3 230080_at	Correlation Coefficient	0.106	0.094
	Sig. (2-tailed)	0.357	0.447
HOXA3 235521_at	Correlation Coefficient	.777**	.775**
	Sig. (2-tailed)	<0.001	<0.001
HOXA3 242528_at	Correlation Coefficient	-0.063	0.006
	Sig. (2-tailed)	0.583	0.961
HOXA4 206289_at	Correlation Coefficient	.712**	.730**
	Sig. (2-tailed)	<0.001	<0.001
HOXA5 213844_at	Correlation Coefficient	.754**	.760**
	Sig. (2-tailed)	<0.001	<0.001
HOXA6 208557_at	Correlation Coefficient	.522**	.522**
	Sig. (2-tailed)	<0.001	0.050
HOXA6 239915_at	Correlation Coefficient	.333**	.379**
	Sig. (2-tailed)	0.003	0.001
HOXA7 206848_at	Correlation Coefficient	0.017	0.014
	Sig. (2-tailed)	0.883	0.911
HOXA7 206847_s_at	Correlation Coefficient	.685**	.720**
	Sig. (2-tailed)	<0.001	<0.001

		miR-196a n=78	miR-196b n=68
HOXA9 209905_at	Correlation Coefficient	.780**	.775**
	Sig. (2-tailed)	<0.001	<0.001
HOXA9 214651_s_at	Correlation Coefficient	.778**	.792**
	Sig. (2-tailed)	<0.001	<0.001
HOXB1 208224_at	Correlation Coefficient	0.024	-0.075
	Sig. (2-tailed)	0.835	0.546
HOXB13 209844_at	Correlation Coefficient	-0.043	-0.184
	Sig. (2-tailed)	0.707	0.134
HOXB13 230105_at	Correlation Coefficient	-0.142	-0.195
	Sig. (2-tailed)	0.215	0.111
HOXB2 205453_at	Correlation Coefficient	.331**	.416**
	Sig. (2-tailed)	0.003	<0.001
HOXB3 208414_s_at	Correlation Coefficient	.357**	.307*
	Sig. (2-tailed)	0.001	0.011
HOXB3 228904_at	Correlation Coefficient	.498**	.558**
	Sig. (2-tailed)	0.349	<0.001
HOXB3 241958_at	Correlation Coefficient	0.027	0.038
	Sig. (2-tailed)	0.814	0.757
HOXB4 231767_at	Correlation Coefficient	.435**	.509**
	Sig. (2-tailed)	6.867	0.092
HOXB5 205600_x_at	Correlation Coefficient	.545**	.535**
	Sig. (2-tailed)	<0.001	0.026
HOXB5 205601_s_at	Correlation Coefficient	.475**	.404**
	Sig. (2-tailed)	1.105	0.001
HOXB6 205366_s_at	Correlation Coefficient	.455**	.472**
	Sig. (2-tailed)	2.889	0.480
HOXB6 205365_at	Correlation Coefficient	0.178	0.104
	Sig. (2-tailed)	0.120	0.399
HOXB6 239791_at	Correlation Coefficient	.266*	.287*
	Sig. (2-tailed)	0.019	0.018
HOXB7 204778_x_at	Correlation Coefficient	.575**	.558**
	Sig. (2-tailed)	<0.001	<0.001
HOXB7 204779_s_at	Correlation Coefficient	.353**	.436**
	Sig. (2-tailed)	0.002	<0.001
HOXB7 216973_s_at	Correlation Coefficient	.456**	.564**
	Sig. (2-tailed)	2.771	<0.001
HOXB8 221278_at	Correlation Coefficient	0.046	0.058
	Sig. (2-tailed)	0.687	0.641
HOXB8 230114_at	Correlation Coefficient	0.098	0.155
	Sig. (2-tailed)	0.394	0.207
HOXB8 229667_s_at	Correlation Coefficient	0.122	.249*
	Sig. (2-tailed)	0.286	0.041
HOXB9 216417_x_at	Correlation Coefficient	.648**	.639**
	Sig. (2-tailed)	<0.001	<0.001
HOXB9 226461_at	Correlation Coefficient	.457**	.567**
	Sig. (2-tailed)	2.615	<0.001

		miR-196a n=78	miR-196b n=68
HOXC10 (218959_at)	Correlation Coefficient	0.044	0.008
	Sig. (2-tailed)	0.699	0.947
HOXC11 (206745_at)	Correlation Coefficient	0.131	-0.043
	Sig. (2-tailed)	0.254	0.726
HOXC12 (1553512_at)	Correlation Coefficient	-0.014	0.043
	Sig. (2-tailed)	0.902	0.730
HOXC13 (219832_s_at)	Correlation Coefficient	0.082	0.132
	Sig. (2-tailed)	0.476	0.282
HOXC4 (206194_at)	Correlation Coefficient	0.154	0.003
	Sig. (2-tailed)	0.178	0.983
HOXC5 (206739_at)	Correlation Coefficient	0.211	.346**
	Sig. (2-tailed)	0.064	0.004
HOXC6 (206858_s_at)	Correlation Coefficient	-0.003	-0.034
	Sig. (2-tailed)	0.981	0.785
HOXC6 (239612_at)	Correlation Coefficient	0.080	0.147
	Sig. (2-tailed)	0.487	0.232
HOXC6 (1556753_s_at)	Correlation Coefficient	-0.156	-0.231
	Sig. (2-tailed)	0.172	0.058
HOXC8 (221350_at)	Correlation Coefficient	0.112	0.045
	Sig. (2-tailed)	0.328	0.715
HOXC9 (231936_at)	Correlation Coefficient	-0.049	-0.023
	Sig. (2-tailed)	0.669	0.852
HOXD1 (205975_s_at)	Correlation Coefficient	-0.086	0.063
	Sig. (2-tailed)	0.452	0.611
HOXD1 (205974_at)	Correlation Coefficient	-0.172	-0.188
	Sig. (2-tailed)	0.133	0.125
HOXD10 (207373_at)	Correlation Coefficient	-0.075	-0.098
	Sig. (2-tailed)	0.511	0.427
HOXD10 (229400_at)	Correlation Coefficient	0.110	0.184
	Sig. (2-tailed)	0.340	0.133
HOXD11 (214604_at)	Correlation Coefficient	-0.100	-0.211
	Sig. (2-tailed)	0.382	0.084
HOXD12 (221411_at)	Correlation Coefficient	0.189	0.194
	Sig. (2-tailed)	0.097	0.114
HOXD13 (207397_s_at)	Correlation Coefficient	0.047	0.096
	Sig. (2-tailed)	0.682	0.434
HOXD13 (207398_at)	Correlation Coefficient	.336**	.285*
	Sig. (2-tailed)	0.003	0.018
HOXD13 (236681_at)	Correlation Coefficient	0.117	0.096
	Sig. (2-tailed)	0.308	0.436
HOXD3 (206601_s_at)	Correlation Coefficient	0.009	0.004
	Sig. (2-tailed)	0.939	0.974
HOXD3 (206602_s_at)	Correlation Coefficient	0.122	0.108
	Sig. (2-tailed)	0.287	0.379
HOXD4 (205522_at)	Correlation Coefficient	-0.067	-0.227
	Sig. (2-tailed)	0.558	0.063

		miR-196a n=78	miR-196b n=68
HOXD4 (1552337_s_at)	Correlation Coefficient	-0.026	-0.020
	Sig. (2-tailed)	0.822	0.874
HOXD8 (231906_at)	Correlation Coefficient	-0.134	-.309*
	Sig. (2-tailed)	0.244	0.010
HOXD9 (205604_at)	Correlation Coefficient	0.021	-0.090
	Sig. (2-tailed)	0.857	0.463
HOXD9 (205605_at)	Correlation Coefficient	0.166	0.113
	Sig. (2-tailed)	0.145	0.359
MEIS1 (204069_at)	Correlation Coefficient	.679**	.701**
	Sig. (2-tailed)	<0.001	<0.001
MEIS1 (242172_at)	Correlation Coefficient	.708**	.723**
	Sig. (2-tailed)	<0.001	<0.001
MEIS1 (1559477_s_at)	Correlation Coefficient	.708**	.749**
	Sig. (2-tailed)	<0.001	<0.001
ANXA1 (201012_at)	Correlation Coefficient	-0.105	-0.066
	Sig. (2-tailed)	0.361	0.591
ANXA1 (233011_at)	Correlation Coefficient	-.269*	-0.236
	Sig. (2-tailed)	0.017	0.053

Supplemental Table V: Correlation between expression of miR-196a/b and *HOX* genes determined by RT-qPCR

	miR-196a	miR-196b
miR-196a	1	.700**
miR-196b	.700**	1
HOXA9	.758**	.829**
HOXA10	.691**	.754**
HOXB9	.422	.723**

Correlations depicted as Spearman's correlation coefficient (n=15-19),** P-value<0.01

CLASSIFICATION OF PEDIATRIC ACUTE MYELOID LEUKEMIA BASED ON MIRNA EXPRESSION PROFILES

5

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Submitted

CHAPTER 5: CLASSIFICATIONO F PEDIATRIC AML BY MIR EXPRESSION

ABSTRACT

Pediatric acute myeloid leukemia (AML) is a heterogeneous disease with respect to biology as well as outcome. A combination of response to induction therapy and molecular markers is used to stratify patients for therapy regimens. We investigated whether known biological subgroups of pediatric AML are reflected by a common microRNA (miR) expression pattern. To this end, expression of 665 miRs were measured in a well-documented set of 165 pediatric AML samples. To identify clusters of patients with common miR expression profiles, unsupervised clustering was performed. We identified fourteen clusters, seven of which had a known (cyto) genetic denominator. For all clusters with at least 10 patients and a known molecular aberration, i.e. 11q23-rearrangements, t(8;21)(q22;q22), inv(16)(p13q22), t(15;17)(q21;q22), *NPM1* and *CEBPA* mutations, we performed classification. The classifier successfully stratified these samples based on expression of 47 miRs. In the 11q23-rearrangements, t(8;21)(q22;q22), inv(16)(p13q22) and t(15;17)(q21;q22) subtypes the classifier achieved high sensitivities (84-94%). In the *NPM1* and *CEBPA* subtypes, however, lower sensitivities were observed (32% and 66%). Specificity was high in all groups, ranging from 87-100%. Target genes of the miRs signatures involved those in the AKT and RAS signaling pathways such as AKT2, KRAS, MAPK1, MAP2K1, PIK3C2A, and TCF4. We conclude that cytogenetic subtypes of pediatric AML have distinct miR expression patterns, whereas molecular subgroups of AML do not. This study indicates frequently deregulated miRs in pediatric AML and may also provide plausible pathways for therapeutic targeting.

INTRODUCTION

Pediatric AML patients are currently stratified into risk categories according to response to induction therapy and genetic abnormalities, as defined in the WHO 2008 classification[1]. Although outcome has increased over the past decades, overall survival rates are in the 60-70% range and the relapse rate is still too high, at approximately 30%[2–9]. To further improve outcome, biological studies aim to identify leukemogenic drivers and/or signaling pathways that can be directly targeted. This is necessary as further chemotherapy intensification may result in an even higher frequency of early and late side effects, including therapy-related mortality. Alternatively, prognosis may be improved by refining the risk-group classification by better defining uniform subgroups by (epi-) genetic and molecular aberrations, which may also contribute to the design of targeted therapy.[10,11] The identification of hallmark aberrations and their accompanying molecular targets for the 15-20% of pediatric AML that currently remains unclassified, is therefore the subject of ongoing biological studies[10].

MiRs —non-coding RNAs of approximately 22 nucleotides— influence gene expression by suppression of translation of genes that have sequences complementary to the miR in their 3'UTR. Because of the many potential target genes, miRs influence many cellular processes like apoptosis, proliferation, differentiation, and ageing as well as hematopoietic differentiation[12–21]. The epigenetic effect of deregulation of miR expression on gene expression contributes to leukemogenesis through interaction with tumor suppressor genes involved in proliferation and differentiation[15,16,22–32]. Although cancer-promoting miRs have been described in adult AML, studies on miR expression and function in pediatric AML are rare[33–36]. Zhang et al described miR-expression differences between FAB-M1, FAB-M2 and FAB-M3 groups of pediatric AML[36]. We have described non-random distribution of miR-29a, miR-155, and miR-196a/b expression between clinically relevant genetic entities of pediatric AML[33]. Daschkey et al showed clustering of pediatric samples with t(8;21), t(15;17) and *MLL*-rearrangements by miR-expression pattern[34]. We also described miR-expression profiles specific for inv(16) and other genetic aberrations in pediatric AML. Moreover, miR-9, specifically low expressed in t(8;21) pediatric AML was identified to act as a tumor-suppressor in cooperation with let-7 family members in a stringent cell-context dependent manner[35]. Another study identified high expression of miR-99a in pediatric AML with FAB M1-M5 phenotype. Overexpression of miR-99a in K562 and HL60 cell lines promoted proliferation and inhibited apoptosis, possibly by regulation of tumor suppressor genes CTDSPL and TRIB2[37]. In the current study we investigated in a large cohort of patients whether genetic and molecular subtypes of childhood AML have specific miR expression profiles.

MATERIALS AND METHODS

Viably frozen diagnostic bone marrow or peripheral blood samples from 165 *de novo* pediatric AML cases were provided by the Dutch Childhood Oncology Group, the AML 'Berlin-Frankfurt-Münster' Study Group, the Czech Pediatric Hematology Group and the St. Louis Hospital in Paris, France. Samples represented the most common and relevant cytogenetic groups and were selected based on the availability of high-quality RNA. Informed consent was obtained from all patients, after Institutional Review Board approval, according to national law and regulations. Samples were enriched to contain at least 80% leukemic cells as previously described[38]. Routine analysis of recurrent non-random cytogenetic aberrations was performed by standard chromosome banding analysis, RT-PCR, and split-signal FISH (*MLL*-rearrangements, inv(16)(p13q22), t(8;21)(q22;q22), t(15;17)(q21;q22), t(7;12)(q36;p13), t(8;16)(p11;p13), and *NUP98*-rearrangements) and hotspot mutations in genes (*NPM1*, *CEBPA*, *FLT3-ITD*, *NRAS*, *KRAS*, *PTPN11*, c-*KIT*, and *MLL-PTD*) as described previously[3940,41 42–46].

MicroRNA expression profiling was performed by Taqman® Array MicroRNA Cards v2.0 (Applied Biosystems, Foster City, CA, USA). Raw Ct-values were analyzed, summarized and exported using SDS 2.3 (Applied Biosystems). All further biostatistical analyses including

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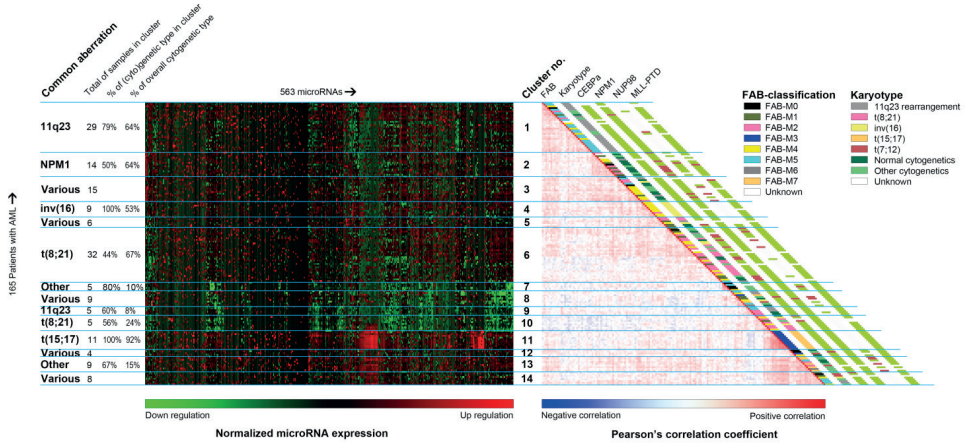
Figure 1: unsupervised clustering of pediatric AML based on miR expression



This combination of heatmaps shows the miR expression profiles of 165 pediatric AML patients divided 14 clusters found through semi-unsupervised clustering based on miR expression of 563 miRs. The selected miRs provided the clearest division between clusters. Left-hand side: normalized miR expression values with green meaning down regulation and red up regulation. The rows were ordered based on the correlation plot on the right. Columns were ordered by hierarchical clustering. Right-hand side: Pairwise correlation between 165 pediatric patients with acute myeloid leukemia. The cells are colored by Pearson's correlation coefficient values ranging from dark blue (negative correlation) to dark red (positive correlation). FAB-type, Karyotype, *CEBPA* mutation status, *NPM1* mutation status, NUP98 rearrangements and *MLL*-PTD are plotted alongside the graph. For the clinical characteristic that have no plotted legend, green means the characteristic is absent, red means that it is present and white means that the sample was not tested for this characteristic.

Table 1: classification of AML by miRNA expression

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
MLL(n=36)	93	87	79	97	89
t(8;21)(q22;q22)(n=21)	94	95	84	99	95
inv(16)(p13q22)(n=17)	84	97	86	97	95
t(15;17)(q21;q22)(n=12)	85	100	97	98	98
CEPBA.dm(n=11)	66	100	97	96	96
NPM1(n=11)	32	98	46	93	91

PPV: positive predictive value, NPV: negative predictive value

Table 2: miRs used for classification

MLL	NPM1	T(8;21) (Q22;Q22)	Inv(16) (p13q22)	T(15;17) (Q21;Q22)	CEBPA
let-7f	let-7c	miR-126	let-7c	let-7f	let-7c
miR-126	let-7f	miR-126*	miR-126	miR-181c	miR-139-5p
miR-126*	miR-126	miR-130a	miR-126*	miR-195	miR-149
miR-130a	miR-196b	miR-139-5p	miR-130a	miR-199a-3p	miR-181a
miR-133a	miR-320	miR-149	miR-139-5p	miR-203	miR-181a*
miR-139-5p		miR-196b	miR-199a-3p	miR-29a	miR-181c
miR-149		miR-200c	miR-203	miR-369-3p	miR-196b
miR-181a		miR-26a-1*	miR-26a	miR-369-5p	miR-203
miR-181a*		miR-27a*	miR-26a-1*	miR-374b	miR-222
miR-181c		miR-320	miR-27a*	miR-409-5p	miR-26a-1*
miR-195		miR-582-5p	miR-30b	miR-485-5p	miR-27a*
miR-196b		miR-885-5p	miR-30d	miR-625	miR-340-5p
miR-199a-3p		miR-9	miR-335	miR-654-3p	miR-363
miR-200c		miR-9*	miR-363	miR-654-5p	miR-500a
miR-203			miR-500a		miR-502-3p
miR-222			miR-766		miR-660
miR-26a			miR-944		miR-9
miR-26a-1*					miR-9*
miR-29a					
miR-30b					
miR-30d					
miR-335					
miR-340-5p					
miR-362-5p					
miR-363					
miR-374b					
miR-500*					
miR-500a					
miR-502-3p					
miR-582-5p					
miR-625					
miR-660					
miR-766					
miR-885-5p					
miR-9					
miR-9*					
miR-944					

Red text: miR upregulated in this group vs all other samples
Green text: miR downregulated in this group vs all other samples

quality control, coercion of data, data normalization, and filtering were performed using R 2.11.147.

Unsupervised hierarchical clustering of samples was performed using 563 miRs. These miRs were 6-fold higher or lower expressed compared to the geometric mean of the cohort in at least one patient sample (Pearson correlation and Ward distance) within the R 3.0.3 statistical environment.

A classifier was constructed for the groups identified by unsupervised clustering with at least ten samples. We used the classification strategy described in Balgobind *et al.* which claimed to generate robust classification signatures[39].

The biological function of identified miRs was explored via Ingenuity Pathway Analysis (Ingenuity, Redwood City, CA, USA) using their target genes. Further details are given in the Supplementary Document.

RESULTS

Unsupervised clustering based on miR expression separates cases by type II aberration

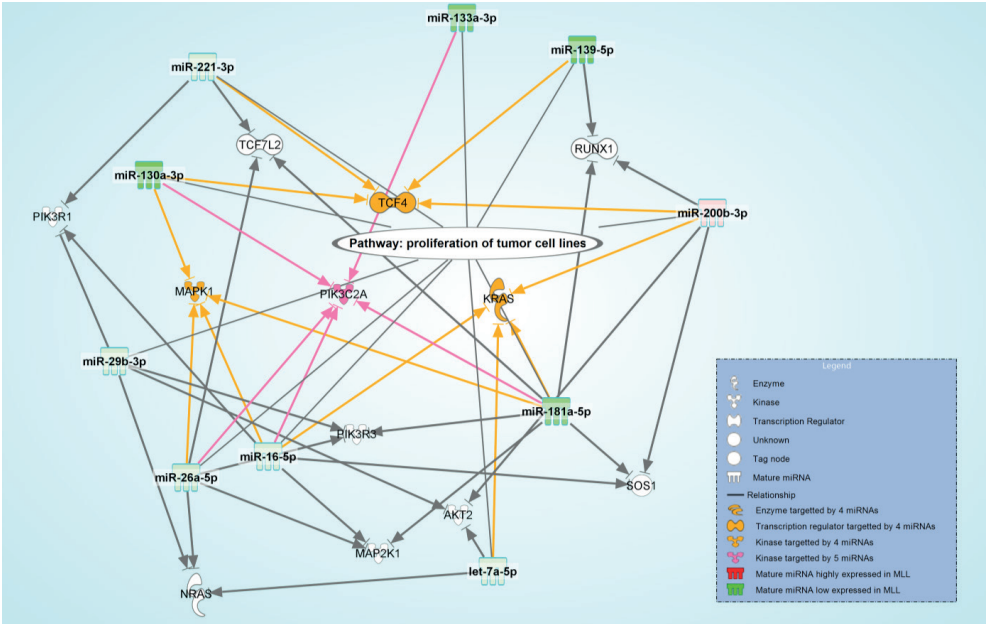
We performed hierarchical clustering of patients using only those miRs that were 6-fold

Figure 2: expression of classifying miRs reflects disturbed pathways

	11q23	NPM1	inv(16)	t(8;21)	CEBPAdm	t(15;17)
proliferation of tumor cell lines	0.79		-1.46	0.12	0.43	-2.00
proliferation of hepatoma cell lines	2.00	0.00	0.00			-2.00
apoptosis of hepatoma cell lines	-1.97	0.00	0.00			1.97
apoptosis of tumor cell lines	-0.82		0.00		-0.51	2.42
cell death	-1.12		2.20		-0.18	
proliferation of cells	1.04		-1.66			
invasion of tumor cell lines	1.29		0.00			
cell movement of tumor cell lines	-0.30		-0.95	0.00		
cell viability of tumor cell lines	-1.03					
apoptosis	-1.03					
necrosis	-0.92					
migration of tumor cell lines	-0.85		0.00			
cell movement				-0.08		

Overview of the pathways strongest associated with miRs used to classify each group. For the miRs used in each group see Table 2. Red: miR expression in that group is predicted to activate this pathway. Blue: miR expression in that particular group represses the pathway.

Figure 3A: deregulation of proliferation by MLL-classifying miRs



the miRs in 'proliferation of tumor cells' that have common target genes (only mRNAs shown that are targeted by more than 2 miRs). MiRs are colored by expression ratio, green meaning downregulation and red upregulation of expression in *MLL*-rearranged samples when compared to non-*MLL*-rearranged samples. This pathway is predicted to be activated in *MLL*-rearranged samples.

Figure 3B: Proliferation of cells is relatively repressed by inv(16)(p13q22)-classifying miRs in inv(16)(p13q22) AML

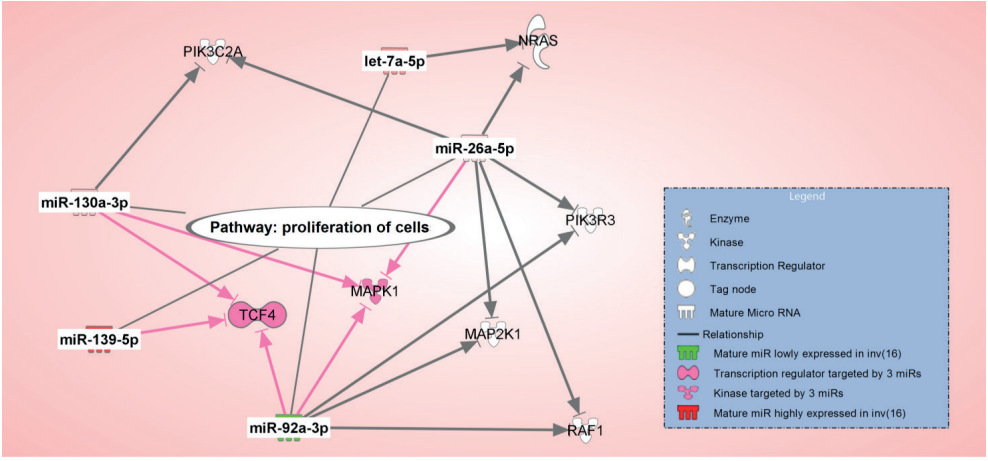


Figure depicting the miRs in 'proliferation of cells' that have common target genes (only mRNAs shown that are targeted by more than one miR). MiRs are colored by expression ratio, green meaning downregulation and red upregulation of expression in samples with inv(16)(p13q22) when compared to non-inv(16)(p13q22). This pathway is predicted to be repressed in samples with inv(16)(p13q22), with repression of MAPK1, and TCF4 through overexpression of targeting miRs as probable important mechanism.

higher or lower expressed compared to the geometric mean of the cohort in at least one patient sample This threshold resulted in selection of 563 miRs. Hierarchical clustering was performed using Person correlation and Ward's minimum variance method of linkage. The heatmap resulting from this clustering is shown in Figure 1. Clusters were analyzed for enrichment of cytogenetic and molecular aberrations, FAB-classification, and mean age and white blood cell count (Supplemental Table 1). We observed that expression of miRs correlate with specific cytogenetic and molecular aberrations, especially with *MLL*-rearrangements, *NPM1* mutations, inv(16)(p13q22), t(8;21)(q22;q22), and t(15;17)(q21;q22). Hierarchical clustering groups were not enriched in occurrence of type I aberrations in this analysis.

Samples containing *MLL*-rearrangements appeared to be separated into two clusters: cluster 1 and cluster 9. This separation was not related to the *MLL*-translocation partner. Half of the samples characterized by *NPM1*-mutations were found adjacent to the samples with *MLL*-rearrangements in cluster 2. Four samples with *NPM1* mutations were scattered in cluster 1 (n=1), cluster 3 (n=2) and cluster 8 (n=1).

Fifty-three percent of the samples with inv(16)(p13q22) were found in cluster 4 and the rest were scattered over clusters 3, 5, 6, 8, 10, and 14. Samples with t(8;21)(q22;q22) grouped closely together in cluster 6 and cluster 10, two cases were found in the heterogeneous cluster 5 that encompasses samples with various aberrations.

We presumed that the distribution of samples with t(8;21)(q22;q22) in two clusters might be due to differences in type I or type II mutations or FAB-classification. However, no significant difference between these clusters was observed based on the afore mentioned characteristics (Supplemental Table 2). All samples with *CEBPA* double mutations were clustered together with t(8;21)(q22;q22) samples.

Eleven out of twelve samples with t(15;17)(q21;q22), were present in cluster 11. We observed no specific biological characteristic of the outlying sample.

Cluster 7 and 13 consisted of over 60% of samples with "other" cytogenetics but no known common cytogenetic or molecular denominator was found in these cases.

For three groups of samples, t(7;12)(q36;p13, t(8;16)(p11;p13) and NUP98-rearrangements, no homogeneous cluster was found.

Classification of samples based on miR expression

To investigate the potential of miRs in predicting known Type II aberration subtypes, we performed classification using samples with *MLL*-rearrangements, t(8;21)(q22;q22), inv(16)(p13q22), t(15;17)(q21;q22), *CEBPA* double mutations and *NPM1* mutations. We generated miR signatures specific to each genetic subtype using Support Vector Machine (SVM). Our strategy to extract signatures is similar the one used in Balgobind *et al*[39]. This strategy, double-loop-cross-validation (DLCV), avoids over-fitting and leads to stable sig-

natures with highest prediction accuracy.

The mean prediction accuracy of 100 DLCV iterations is given in Table 1. We observed that the classifier rendered high prediction sensitivities in the subtypes of *MLL*-rearrangements, t(8;21)(q22;q22), inv(16)(p13q22) and t(15;17)(q21;q22), ranging from 84-94%. In the groups with *CEPBA* double mutations and *NPM1* mutations, low prediction sensitivities were observed (66% and 32%). The specificities in all subtypes were equally high (87-100%). The optimal set of miRs signatures and their expression relative to the other subtypes are given in Table 2, this set includes 47 miRs in total to generate the highest prediction sensitivities.

Samples with *MLL*-rearrangements were classified with 93% sensitivity using 27 miR expression signatures; 14 were high expressed and 13 were low expressed compared to the samples without *MLL*-rearrangements. Among the 13 downregulated miRs , seven are known tumor suppressors: *let-7f*, *miR-126*, *miR-181a*, *miR-181c*, *miR-195*, *miR-26a*, and *miR-29a*[48]. Three miRs were only used to classify *MLL*-rearranged samples: low expressed *miR-133* and *miR-362-5p*, and high expressed *miR-500**. The miRs classifying *MLL*-rearranged samples are (mostly) involved in activation or repression of 12 pathways (Figure 3A). Since most deregulated miRs were involved in the network 'proliferation of tumor cell lines'. This network is predicted to be activated,with loss of repression of MAPK1, TCF4, KRAS and PIK3A2 through an absence of targeting miRs as potential important mechanism.

Five miR signatures were used to classify cases with *NPM1* mutations, of which *let-7c, let-7f,* and *miR-196b* were high expressed and *miR-126* and *miR-320* low expressed.

Samples carrying inv(16)(p13q22)were characterized by seventeen miRs; three were low expressed, 14 were high expressed. Some of these 17 miRs were also characteristic for other groups. The miR expression signature seem to repress the 'proliferation of tumor cell lines', 'proliferation of cells' and 'cell movement' networks, and surprisingly activate the 'cell death' network. High expressed *let-7a-5p*, *miR-130a-3p*, *miR-139-5p* and *miR-26a*-5p and low expressed *miR-9*2a-3p target two genes of the network 'proliferation of cells'; TCF4 and MAPK1(Figure 3B).

Fourteen miRs were specific to samples carrying t(8;21)(q22;q22). Among them eight were low expressed and six were high expressed. The 14 miR expression signatures largely overlapped with the signatures specific to inv(16) subtype.

The classifier generated 18 miR expression signatures specific to samples with *CEBPA* double mutations among them 12 were low expressed and six were high expressed. *CEBPA* specific miRs seem to activate the 'proliferation of tumor cell lines' and repress the networks 'apoptosis of tumor cell lines' and 'cell death' networks. In the 'cell death' network, we identified two genes that might be targets of several of the miRs. TCF4 and MAP2K1 are targeted by upregulated *miR-181a*-5p and *miR-221-3p* and downregulated *miR-203*-3p and *miR-9*2a-3p (Figure 3C). The influence of the deregulated miRs on the 'cell death' network

Figure 3C: CEBPA classifying miRs are involved in repressing cell death in CEBPA-mutated AML

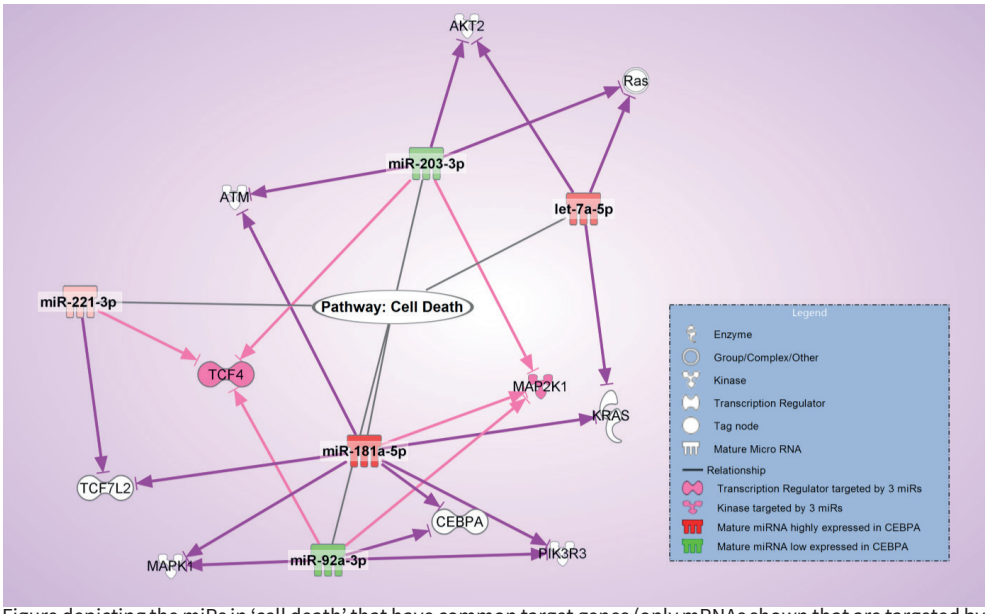


Figure depicting the miRs in 'cell death' that have common target genes (only mRNAs shown that are targeted by more than one miR). MiRs are colored by expression ratio, green meaning downregulation and red upregulation of expression in samples with *CEBPA* mutations when compared to non-*CEBPA* mutations.

Figure 3D: The pathway "Apoptosis of tumor cells" is relatively activated in t(15;17)(q21;q22) AML through overexpression of t(15;17)(q21;q22) classifying miRs

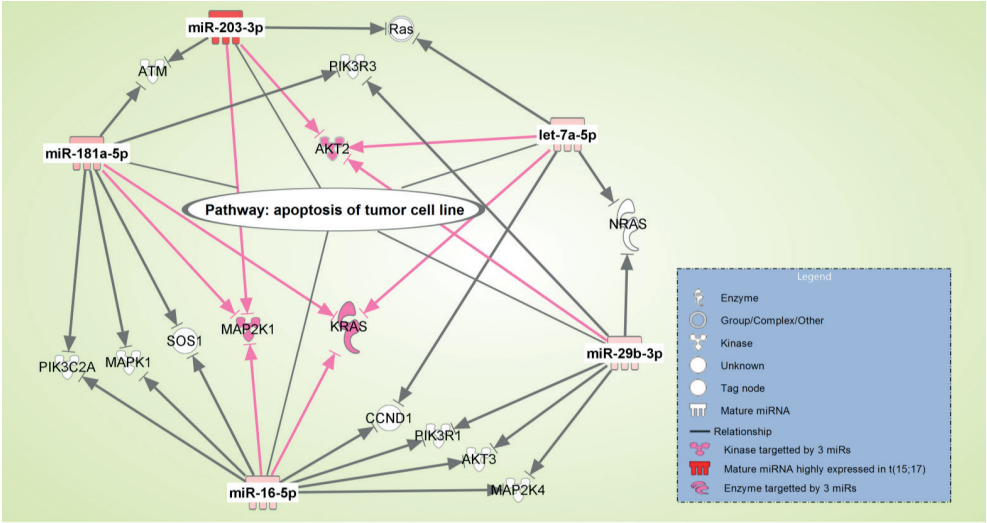


Figure depicting the miRs in 'apoptosis of tumor cells' that have common target genes (only mRNAs shown that are targeted by more than two miRs). MiRs are colored by expression ratio, green meaning downregulation and red upregulation of expression in samples with t(15;17)(q21;q22) when compared to samples without t(15;17)(q21;q22). This pathway is predicted to be activated in samples with t(15;17)(q21;q22).

is hard to predict with three over expressed miRs that might down regulate the network and relative low expression of two miRs that most likely causes activation of the network.

Samples with t(15;17)(q21;q22) were classified by 14 upregulatedmiRs, eight of them were also identified in other groups. Only six miRs, *miR-369-3p*, *miR-369-5p*, *miR-409-5p*, *miR-485-5p*, *miR-654-3p*, and *miR-654-5p,* were specific to t(15;17)(q21;q22) samples. The miR signature might cause repression 'proliferation of tumor cell lines' and activation of 'apoptosis of tumor cells'. High expressed *let-7a-5p*, *miR-16-5p*, *miR-181a*-5p and *miR-203*-3p are able to target MAP2K1, KRAS and AKT2 (Figure 3D).

DISCUSSION

By using real-time RT-qPCR analyzing expression of 665 human miRNAs, unsupervised clustering analysis revealed strong miR expression profiles for samples with cytogenetic abnormalities t(15;17)(q21;q22), *MLL*-rearrangements, inv(16)(p13q22), and t(8;21)(q22;q22), which may reflect the relative homogeneous biology of these subtypes. These results are mostly concordant with previous studies showing strong miR expression signatures for pediatric AML with inv(16)(p13q22) and t(8;21)(q22;q22[34]. The specific miR profile of *MLL*rearranged AML as seen in our study was not previously reported for pediatric nor for adult AML. In addition, one cluster contained 64% of all samples with *NPM1* mutations, indicating a specific miR expression profile, which is in agreement with studies published on AML in adults[49]. So far, other clustering studies in adult AML reported strong signatures for t(15;17)(q21;q22) and the CBF-leukemias as well[50–53].

CEBPA-mutated or *NPM1* mutated cases did not show specific miR expression patterns, which might be due to the number of included samples. Overall, classification by miR expression profiles did not exceed the classification by cytogenetics as previously showed for classification by gene expression profiles[39]. Both methods thus do not seem to be superior to the methods that are currently used to classify patients.

The inferior accuracy of classification of certain cytogenetic subgroups by miR-based classification vs mRNA-based classification has also been reported in two large classifying studies of adult AML [51,53,54]. Relevant miRs commonly found in both adults, by Jongen-Lavrencic *et al*, and children are: *miR-485-5p* for t(15;17)(q21;q22), *miR-126*, miR-196b*, and *miR-9* for t(8;21)(q22;q22), *miR-126*, miR-30b,* and *miR-335* for inv(16)(p13q22), *miR-149, miR-181a, miR-181c, miR-196b,* and *miR-9* for *CEBPA* double mutated cases. In contrast to our results, in adults, classifying miRs for *MLL*-rearranged AML were not identified. MiRs could be used for class prediction of t(15;17)(q21;q22), t(8;21)(q22;q22) and inv(16)(p13q22), although the authors regarded clinical utility limited as well. Li *et al.* used 7 miRs for class prediction of CBF-leukemia (inv(16)(p13q22) and t(8;21)(q22;q22) together), t(15;17)(q21;q22) and *MLL*-rearrangements, which resulted in an accuracy of >94% in a cohort of 52 adults using bead arrays[53]. We confirmed the classifying potential

of *miR-126*/*miR-126** overexpression in CBF leukemias.

MiR-485-5p was the only miR used to classify both adult and pediatric AML with t(15;17)[51,53]. *MiR-485-5p* is encoded on chromosome 14q32.31, and overexpression of several miRs located adjacent to each other on this location has been reported in adult. However, these miRs were not used in a classifier for adult AML with t(15;17). Interestingly, all of the miRs in our classifier used specifically to classify t(15;17)(q21;q22) are encoded on chromosome 14q32.3150. The mechanism behind this overexpression and the possible function of most of these miRs in AML has not been elucidated. However, this repeated finding might provide clues regarding the distinct biology of t(15;17)(q21;q22) positive AML.

Deregulated expression of *miR-126, miR-196b*, and *miR-9* was found in both pediatric and adult t(8;21)(q22;q22) AML. We recently described that down regulated *miR-9* acts as a tumor suppressor in pediatric t(8;21)(q22;q22) AML and induced differentiation through targets HMGA2 and LIN28B in cooperation with the let-7 family. It might be worthwhile to explore the therapeutic potential by artificial overexpression of *miR-9* expression in t(8;21)(q22;q22) positive AML[35].

Expression of miR-126*, miR-30b, miR-30d, and miR-335 was used in both our pediatric classifier as well as in adult classifiers of adult inv(16)(p13q22) AML[51,53]. Deregulated expression of miR-30b has been previously described in AML; overexpression of oncogenic MYBL2 associated with lower expression of miR-30b and poor prognosis[55].

We identified *MiR-196b* and *miR-320* to classify pediatric samples with *NPM1* mutations. These miRs were also identified to classify adult samples with *NPM1* mutations[51]. Although expression of *miR-10a* and *miR-10b* has been described to classify adult AML with *NPM1* mutations, neither miR classified pediatric AML with NPM1 mutations in our cohort[49,56.] This might reflect differences in leukemogenic pathways between children and adults or may also be due to the method of miR-selection. In addition, the miR expression profile of *NPM1*-mutated samples resemble the expression profile of *MLL*-rearranged samples. As the latter were not classified based on miR-expression before, this might partly explain their absence in our classifier.

Expression of five miRs in pediatric AML with CEBPA mutations were also reported in adult AML with *CEBPA* mutations: *miR-149*, *miR-181a*, *miR-181c*, *miR-196b*, and *miR-9*[51,57]. Expression of tumor suppressive *miR-181* family members was found to be high in *CEBPA*-mutated cases and has been correlated to treatment response and better clinical outcome in AML patients. Treatment of AML-blasts carrying *CEBPA*-mutations with lenalidomide sensitized AML cells to chemotherapy and increased *CEBPA*-p30 protein levels and *miR-181a* expression [58].

We studied the possible functional relevance of the miRs that were identified by pathway analysis. Central genes that might be targeted by the classifying miRs are TCF4, MAPK1, KRAS, MAP2K1, and AKT2. Twelve classifying miRs; *let-7a, miR-130a-3p, miR-133a-3p,*

miR-139-5p, miR-16-5p, miR-181a-5p, miR-200b-3p, miR-203-3p, miR-221-3p, miR-26a-5p, miR-29b-3p, and *miR-320b,* target these genes and might induce activation of the RAS/AKT pathway. Interestingly, these miRs were used to classify several of the cytogenetic types and thus not specifically used to classify MLL-rearranged AML, in which deregulation of the RAS/AKT pathway is known[59]. Those miRs and networks might provide direction for further research towards mechanisms underlying pediatric AML. Modification of expression of these miRs might provide future therapeutic options, with deregulation of oncogenic miR-181a-5p and mir-26a-5p as possible starting points for further functional research[34,35].

This study indicates that miR expression profiles are specific for pediatric AML with *MLL*-rearrangements, t(8;21)(q22;q22), inv(16)(p13q22), t(15;17)(q21;q22). Classification of samples with *NPM1*-mutations and *CEBPA*-mutations resulted in low sensitivity. Some patients in which the underlying common genetic or molecular denominator is unknown cluster together by miR expression profile. Further investigations focusing on the common factor in these clusters might reveal new relevant subgroups of pediatric AML. Using expression of 47 miRs, samples could be classified to their cytogenetic group. Pathway analysis revealed that these miRs were involved in regulation of genes in the RAS/AKT pathway, which is deregulated in AML.

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SUPPLEMENTAL METHODS

MiRNA expression measurements

To measure expression of 665 miRs, two cards, A and B, were loaded with RNA from each sample. Per card type, Ct-values were imported (readCtData) into a qPCR-set using the package HTqPCR and correlation plots were called by the plotCtCor command. The number of failed measurements (undetermined, Ct>38, or flagged in any way by SDS software) per card type was summarized with the featureCategory command. Of this number the mean, standard deviation and 95% confidence interval of failed measurements were calculated to obtain the limits of the number of failed measurements. In the R environment, the median expression of each control per card was calculated. We calculated the mean value for all cards per control using these values. In addition, the standard deviation and 95% confidence interval were calculated to obtain the upper limits of expression. Cards were retained for further analyses if it was OK on visual inspection, the number of failed measurements was within 95% confidence interval of the median of failed measurements, and the value of the internal controls was within the 95% confidence interval of the group median for 2 out of 3 controls. If both card A and B for one sample were retained after going through the steps in our flowchart, expression values were merged into one dataset.

MiRNA data normalization

The normalized expression of 664 miRs underwent a filtering in order to make each value in the dataset strictly positive. Shortly; 26 was added to each miR expression value, successively the geometric mean expression was calculated for each miR. This cut-off was chosen as it resulted in the most coherent clustering pattern with a limited number of clusters (range from 2- to 16-fold change).

Classification

The previously described mRNA classification was modified because of sample numbers: five groups were used in the outer loop and three groups in the inner loop (ten and five groups in the mRNA classification). In addition, the present study lacks an independent validation cohort, as no other pediatric miRNA dataset resulting from the same method of expression profiling is available.

Clustering

The clustering result was visualized using Heatmapper. In addition, correlation between miRs was calculated and visualized using the R-package gplots.

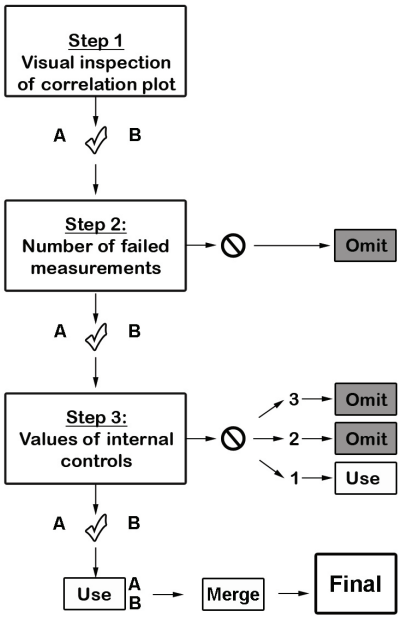
Pathway Analysis

Pathway information was combined with information on mRNAs targets and relationships were indicated (Figure 3A). For clarity, some molecules were taken out of the figure; the mRNAs targeted by less than two of the miRs and miRs that did not have any target mRNAs in common with the other miRs in the pathway. This left nine downregulated miRs, one upregulated miR, and twelve target mRNAs.

Except for the following changes, the core analysis was performed with the default settings: Data Source - ingenuity expert only, Confidence - experimentally observed and high (predicted), Species - mammal. In addition, possible deregulated target genes for the miRs used for classification were found through the construction of a miRNA Target Filter for each group. Filtering was done based on relationship confidence (High (predicted) or Experimentally Observed) and signaling pathway (Acute Myeloid Leukemia Signaling). Information on miR-expression of miRs involved selected pathways was combined with the mRNAs found to be targeted by those same miRs. We used Path Explorer to evaluate the downstream interactions of miRs on mRNAs, using only direct and high confidence/ experimentally observed relationships.

SUPPLEMENTAL FIGURES

Supplemental Figure 1: Flowchart for large scale HT-qPCR Quality Control



Three-step flowchart for quality control of large scale HT-qPCR experiments as used in our pediatric AML cohort. Failed measurements: measurements are considered failed when flagged by the SDS-program or when the Ct values are higher than 38 or not acquired (NA).

SUPPLEMENTAL TABLES

Supplemental Table 1: patient characteristics

	N	(%)
Gender		
Male	86	(52.1)
Female	79	(47.9)
FAB		
M0	11	(6.7)
M1	22	(13.3)
M2	35	(21.1)
M3	13	(7.9)
M4	41	(24.8)
M5	31	(18.8)
M6	2	(1.2)
M7	6	(3.6)
Unknown	4	(2.4)
Type 2		
MLL-rearrangement	36	(21.8)
t(8;21)(q22;q22)	21	(12.7)
inv(16)(p13q22)	17	(10.3)
t(15;17)(q21;q22)	12	(7.3)
t(7;12)(q36;p13)	5	(3.0)
t(6;9)	4	(2.4)
t(8;16)(p11;p13)	1	(0.6)
MLL-PTD	2	(1.2)
NUP98-NSD1	5	(3.0)
NUP98-JARID1A	2	(1.2)
NUP98-TOP1	1	(0.6)
CEBPA homozygous	11	(6.7)
NPM1	11	(6.7)
None	35	(21.2)
Other	2	(1.2)
	Median	**Range**
Age (years)	9.8	0.0-18.4
WBC (x10^9/L)	205	1-490

Supplemental Table 2: patient characteristics per cluster

	cluster 1 n=29		cluster 2 n=14		cluster 3 n=15		cluster 4 n=9		cluster 5 n=6		cluster 6 n=32		cluster 7 n=5	
Gender														
male	17	*59%*	6	*43%*	11	*73%*	5	*56%*	3	*50%*	19	*59%*	2	*40%*
female	12	*41%*	8	*57%*	4	*27%*	4	*44%*	3	*50%*	13	*41%*	3	*60%*
FAB-type														
FAB-M0	1	*3%*	1	*7%*	2	*13%*	0	*0%*	0	*0%*	1	*3%*	3	*60%*
FAB-M1	1	*3%*	5	*36%*	1	*7%*	0	*0%*	3	*50%*	7	*22%*	0	*0%*
FAB-M2	2	*7%*	4	*29%*	3	*20%*	1	*11%*	2	*33%*	13	*41%*	0	*0%*
FAB-M3	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
FAB-M4	5	*17%*	2	*14%*	7	*47%*	8	*89%*	1	*17%*	9	*28%*	1	*20%*
FAB-M5	19	*66%*	0	*0%*	2	*13%*	0	*0%*	0	*0%*	1	*3%*	1	*20%*
FAB-M6	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
FAB-M7	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
Unknown	1	*3%*	2	*14%*	0	*0%*	0	*0%*	0	*0%*	1	*3%*	0	*0%*
Karyotype														
23q11	23	*79%*	1	*7%*	2	*13%*	0	*0%*	1	*17%*	2	*6%*	0	*0%*
CN	1	*3%*	9	*64%*	7	*47%*	0	*0%*	2	*33%*	6	*19%*	0	*0%*
inv(16)	0	*0%*	0	*0%*	1	*7%*	9	*100%*	1	*17%*	2	*6%*	0	*0%*
Other	5	*17%*	4	*29%*	5	*33%*	0	*0%*	0	*0%*	8	*25%*	4	*80%*
t(15;17)	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
t(7;12)	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
t(8;21)	0	*0%*	0	*0%*	0	*0%*	0	*0%*	2	*33%*	14	*44%*	0	*0%*
Unknown	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	1	*20%*
Type 2 aberration														
11q23	23	*79%*	1	*7%*	2	*13%*	0	*0%*	1	*17%*	2	*6%*	0	*0%*
CEBPA dm	0	*0%*	0	*0%*	0	*0%*	0	*0%*	1	*17%*	7	*22%*	0	*0%*
inv(16)	0	*0%*	0	*0%*	1	*7%*	9	*100%*	1	*17%*	2	*6%*	0	*0%*
MLL-PTD	0	*0%*	1	*7%*	1	*7%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
None	3	*10%*	2	*14%*	5	*33%*	0	*0%*	1	*17%*	6	*19%*	5	*100%*
NPM1	1	*3%*	7	*50%*	2	*13%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
NUP98-JARID1A	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
NUP98-NSD1	0	*0%*	2	*14%*	2	*13%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
NUP98-TOP1	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	1	*3%*	0	*0%*
Other	1	*3%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
t(15;17)	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
t(6;9)	0	*0%*	1	*7%*	2	*13%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
t(7;12)	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
t(8;16)	1	*3%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
t(8;21)	0	*0%*	0	*0%*	0	*0%*	0	*0%*	2	*33%*	14	*44%*	0	*0%*
Type 1 aberration														
FLT3-TKD	1	*3%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
FLT3-ITD	1	*3%*	6	*43%*	2	*13%*	0	*0%*	0	*0%*	2	*6%*	0	*0%*
FLT3-ITD&WT1	0	*0%*	1	*7%*	2	*13%*	0	*0%*	0	*0%*	1	*3%*	0	*0%*
WT1	1	*3%*	0	*0%*	2	*13%*	0	*0%*	0	*0%*	2	*6%*	0	*0%*
WT1&RAS	0	*0%*	0	*0%*	1	*7%*	0	*0%*	0	*0%*	1	*3%*	2	*40%*
RAS	9	*31%*	1	*7%*	2	*13%*	1	*11%*	0	*0%*	6	*19%*	0	*0%*
PTPN11	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
C-KIT	1	*3%*	0	*0%*	0	*0%*	4	*44%*	1	*17%*	3	*9%*	0	*0%*
none.or.N.A	16	*55%*	5	*36%*	5	*33%*	4	*44%*	5	*83%*	16	*50%*	3	*60%*
FLT3.ITD&TKD	0	*0%*	0	*0%*	1	*7%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
Other	0	*0%*	1	*7%*	0	*0%*	0	*0%*	0	*0%*	1	*3%*	0	*0%*
MLL type														
MLL-AF9	10	*34%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
MLL-AF10	6	*21%*	0	*0%*	1	*7%*	0	*0%*	1	*17%*	1	*3%*	0	*0%*
MLL-AF6	1	*3%*	1	*7%*	1	*7%*	0	*0%*	0	*0%*	1	*3%*	0	*0%*
MLL-ELL	1	*3%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
MLL-ENL	1	*3%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
Other	4	*14%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
Nono	6	*21%*	13	*93%*	13	*87%*	9	*100%*	5	*83%*	30	*94%*	5	*100%*
Type 3 aberration														
IDH1	1	*3%*	4	*29%*	0	*0%*	0	*0%*	1	*17%*	2	*6%*	0	*0%*
DNMT3A	0	*0%*	0	*0%*	1	*7%*	0	*0%*	0	*0%*	2	*6%*	0	*0%*
TET2	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
CEPBA.sm	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	1	*3%*	0	*0%*
none.or.N.A	28	*97%*	10	*71%*	14	*93%*	9	*100%*	5	*83%*	27	*84%*	5	*100%*
Age														
Median	1.9		12.9		10.3		12.9		13.95		12.3		9.7	
Min	0.3		7.5		1.6		10.3		8.2		0.1		6.3	
Max	14.6		18.4		15.9		17.3		17		16.8		11.3	
WBC														
Median	83		50.1		73.85		110.5		27.4		32.8		2.9	
Min	2.5		5		7.4		33		2.8		2		2.4	
Max	475		230		196		233.5		93.2		354		196	

	cluster 8 n=9		cluster 9 n=5		cluster 10 n=9		cluster 11 n=11		cluster 12 n=4		cluster 13 n=9		cluster 14 n=8	
Gender														
male	3	*33%*	3	*60%*	3	*33%*	3	*27%*	2	*50%*	4	*44%*	5	*63%*
female	6	*67%*	2	*40%*	6	*67%*	8	*73%*	2	*50%*	5	*56%*	3	*38%*
FAB-type														
FAB-M0	0	*0%*	0	*0%*	0	*0%*	0	*0%*	2	*50%*	0	*0%*	1	*13%*
FAB-M1	2	*22%*	0	*0%*	1	*11%*	0	*0%*	0	*0%*	0	*0%*	2	*25%*
FAB-M2	0	*0%*	3	*60%*	5	*56%*	0	*0%*	0	*0%*	2	*22%*	0	*0%*
FAB-M3	1	*11%*	0	*0%*	0	*0%*	11	*100%*	1	*25%*	0	*0%*	0	*0%*
FAB-M4	2	*22%*	0	*0%*	3	*33%*	0	*0%*	1	*25%*	0	*0%*	2	*25%*
FAB-M5	2	*22%*	2	*40%*	0	*0%*	0	*0%*	0	*0%*	1	*11%*	3	*38%*
FAB-M6	1	*11%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	1	*11%*	0	*0%*
FAB-M7	1	*11%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	5	*56%*	0	*0%*
Unknown	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
Karyotype														
23q11	2	*22%*	3	*60%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	2	*25%*
CN	2	*22%*	2	*40%*	1	*11%*	0	*0%*	2	*50%*	2	*22%*	0	*0%*
inv(16)	1	*11%*	0	*0%*	2	*22%*	0	*0%*	0	*0%*	0	*0%*	1	*13%*
Other	2	*22%*	0	*0%*	1	*11%*	0	*0%*	1	*25%*	6	*67%*	3	*38%*
t(15;17)	1	*11%*	0	*0%*	0	*0%*	11	*100%*	0	*0%*	0	*0%*	0	*0%*
t(7;12)	1	*11%*	0	*0%*	0	*0%*	0	*0%*	1	*25%*	1	*11%*	2	*25%*
t(8;21)	0	*0%*	0	*0%*	5	*56%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
Unknown	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
Type 2 aberration														
11q23	2	*22%*	3	*60%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	2	*25%*
CEBPA dm	0	*0%*	0	*0%*	2	*22%*	0	*0%*	0	*0%*	0	*0%*	1	*13%*
inv(16)	1	*11%*	0	*0%*	2	*22%*	0	*0%*	0	*0%*	0	*0%*	1	*13%*
MLL-PTD	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
None	2	*22%*	1	*20%*	0	*0%*	0	*0%*	3	*75%*	6	*67%*	1	*13%*
NPM1	1	*11%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
NUP98-JARID1A	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	2	*22%*	0	*0%*
NUP98-NSD1	0	*0%*	1	*20%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
NUP98-TOP1	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
Other	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	1	*13%*
t(15;17)	1	*11%*	0	*0%*	0	*0%*	11	*100%*	0	*0%*	0	*0%*	0	*0%*
t(6;9)	1	*11%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
t(7;12)	1	*11%*	0	*0%*	0	*0%*	0	*0%*	1	*25%*	1	*11%*	2	*25%*
t(8;16)	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
t(8;21)	0	*0%*	0	*0%*	5	*56%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
Type 1 aberration														
FLT3-TKD	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
FLT3-ITD	2	*22%*	1	*20%*	0	*0%*	8	*73%*	0	*0%*	0	*0%*	1	*13%*
FLT3-ITD&WT1	1	*11%*	1	*20%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
WT1	0	*0%*	0	*0%*	0	*0%*	1	*9%*	0	*0%*	0	*0%*	1	*13%*
WT1&RAS	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
RAS	1	*11%*	1	*20%*	0	*0%*	0	*0%*	1	*25%*	0	*0%*	1	*13%*
PTPN11	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	1	*11%*	0	*0%*
C-KIT	0	*0%*	0	*0%*	4	*44%*	0	*0%*	0	*0%*	1	*11%*	0	*0%*
none.or.N.A	5	*56%*	2	*40%*	5	*56%*	2	*18%*	3	*75%*	7	*78%*	5	*63%*
FLT3.ITD&TKD	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
Other	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
MLL type														
MLL-AF9	1	*11%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
MLL-AF10	1	*11%*	2	*40%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	1	*13%*
MLL-AF6	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	1	*13%*
MLL-ELL	0	*0%*	1	*20%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
MLL-ENL	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
Other	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
Nono	7	*78%*	2	*40%*	9	*100%*	11	*100%*	4	*100%*	9	*100%*	6	*75%*
Type 3 aberration														
IDH1	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	1	*11%*	0	*0%*
DNMT3A	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
TET2	0	*0%*	1	*20%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*	0	*0%*
CEPBA.sm	1	*11%*	0	*0%*	0	*0%*	0	*0%*	1	*25%*	0	*0%*	0	*0%*
none.or.N.A	8	*89%*	4	*80%*	9	*100%*	11	*100%*	3	*75%*	8	*89%*	8	*100%*
Age														
Median	9.6		8.7		8.4		11.9		3.1		1.4		5.7	
Min	0.8		6.2		0.7		4.1		0.8		0		0.3	
Max	15.2		12.8		15		16.9		4.9		12.6		14.2	
WBC														
Median	21.8		135.4		24		22.5		31.1		31.6		15.05	
Min	1.4		2.5		2.3		7		15.6		3		2.3	
Max	105		324		84.8		247		226.8		217.9		181.5	

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MIR-9 IS A TUMOR SUPPRESSOR IN PEDIATRIC AML WITH T(8;21)

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CHAPTER 6: MIR-9 IN PEDIATRIC AML

ABSTRACT

MicroRNAs (miRNAs) play a pivotal role in the regulation of hematopoiesis and development of leukemia. Great interest emerged in modulating miRNA expression for therapeutic purposes. In order to identify miRNAs, which specifically suppress leukemic growth of acute myeloid leukemia (AML) with t(8;21), inv(16) or mixed lineage leukemia (*MLL*) rearrangement by inducing differentiation, we conducted a miRNA expression profiling in a cohort of 90 cytogenetically characterized, *de novo* pediatric AML cases. Four miRNAs, specifically downregulated in *MLL*-rearranged, t(8;21) or inv(16) AMLs, were characterized by their tumor-suppressive properties in cell lines representing those respective cytogenetic groups. Among those, forced expression of miR-9 reduced leukemic growth and induced monocytic differentiation of t(8;21) AML cell lines *in vitro* and *in vivo*. The tumor-suppressive functions of miR-9 were specifically restricted to AML cell lines and primary leukemic blasts with t(8;21). On the other hand, these functions were not evident in AML blasts from patients with *MLL* rearrangements. We showed that miR-9 exerts its effects through the cooperation with let-7 to repress the oncogenic *LIN28B*/*HMGA2* axis. Thus, miR-9 is a tumor suppressor-miR which acts in a stringent cell context dependent manner.

INTRODUCTION

Pediatric acute myeloid leukemia (AML) is a heterogeneous disease that is morphologically and cytogenetically classified into different subtypes. At present, cytogenetic aberrations along with treatment response are the most important prognostic factors.[1] In AML, wellknown chromosomal aberrations that predict the differences in survival include t(15;17)(q22;q21) (*PML-RARα*), t(8;21)(q22;q22) (*RUNX1-RUNX1T1*), inv(16)(p13q22) (*CBF-MYH11*), t(7;12) and mixed lineage leukemia (*MLL*) rearrangements.[1,2] In addition, molecular changes that cannot be detected by classical karyotyping, such as *WT1*, *NPM1*, *CEBPA* and *DNMT3A* mutations, have an influence on the biology and prognosis in pediatric as well as adult AML.[1,3] Recently, alterations in the expression program caused by transcriptional or post-transcriptional mechanisms have been demonstrated to be responsible for leukemogenesis.[4] Altered transcription can be caused by misdirected epigenetic modifications, including histone methylation and DNA methylation, or dysregulated microRNA (miRNA) expression, which perturbs post-transcriptional gene regulation by interference with mRNAs.[5] MiRNA expression profiles or signatures have been identified in solid tumors as well as hematopoietic malignancies, including adult AML.[6,7] Some miRNAs, such as miR-9, miR-181 and miR-212 have been shown to be able to predict outcome in adult AML.[8–10] Subsequent functional studies revealed their central regulatory role in hematopoietic differentiation and their contribution to leukemic transformation.[4] For instance, miR-196b is aberrantly overexpressed in *MLL*-rearranged AML,[11] and its knockdown by antagomirs overturned *MLL*-fusion-mediated cell immortalization.[12] Similarly, miR-29b and miR-125b were shown to contribute to myeloid leukemogenesis.[13–15] In contrast, higher miR-181a expression is associated with favorable outcome especially in adult AML with normal karyotype,[10] while ectopic expression of miR-181a sensitizes AML cell lines to chemotherapy *in vivo* and *in vitro*.[16] These insights open new avenues for therapeutic intervention: modulation of a deregulated miRNA, which specifically induces differentiation of leukemic blasts while sparing normal hematopoiesis, could be exploited therapeutically. This would mimic the effect of ATRA (alltrans-retinoic acid) treatment in acute promyelocytic leukemia expressing the PML-RARa fusion protein.

However, for pediatric AML, reports on miRNA expression signatures are scarce. Subtle differences in miRNA expression patterns between pediatric and adult samples suggests the importance of performing a large-scale miRNA expression screening in pediatric AML patients.[11]

Here we describe a large-scale miRNA expression profiling in a representative cohort of well-characterized pediatric AML patients in order to identify specific, clinically relevant, tumor-suppressive miRNAs. This study discovered that miR-9 suppresses leukemic growth of t(8;21) AML *in vitro* and *in vivo*, while inducing monocytic differentiation by interfering with the *LIN28B*/let-7/ *HMGA2* axis.

MATERIALS AND METHODS

Patient samples and cell lines

Viably frozen diagnostic bone marrow or peripheral blood samples from a selection of 90 *de novo* pediatric AML cases were provided by the Dutch Childhood Oncology Group (DCOG), the AML 'Berlin-Frankfurt-Münster' Study Group (AML-BFM-SG), the Czech Pediatric Hematology Group (CPH) and the St. Louis Hospital in Paris, France. Samples were chosen to represent the most common and relevant cytogenetic groups, that is, t(8;21), inv(16), t(7;12), t(15;17) as well as the *MLL*-rearranged pediatric AML and were selected based on the availability of high-quality RNA. Normal karyoype and 'other' cytogenetic subtypes of AML were not included in the current analyses based on the gross molecular heterogeneity. Each study group performed central morphological reviews according to the FAB classification. Ficoll-separated bone marrow mononuclear cells were collected from four children without malignant tumors or bone marrow abnormalities (Sophia Children's Hospital, Rotterdam). $CD34^+$ hematopoietic stem and progenitor cells (HSPCs) were obtained from cord blood of healthy donors and isolated using CD34- immunomagnetic microbeads (Miltenyi, Bergisch Gladbach, Germany). Informed consent was obtained from all patients, after Institutional Review Board approval, according to national law and regulations and in accordance to the Declaration of Helsinki. Samples were enriched to contain at least 80% leukemic cells as previously described.[17] Cell lines NB4, THP-1, ME-1, SKNO-1 and KASUMI-1 were obtained from the German National Resource Center for Biological Material (DSMZ, Braunschweig, Germany) and cultured as recommended. Differentiation of KASUMI-1, SKNO-1 and THP-1 cells was induced by 50nM Calcitriol (Sigma-Aldrich, St Louis, MO, USA) and 10 ng/ml GM-CSF (Preprotech, Rocky Hill, NJ, USA) over 7 days. Differentiation of NB4 cells was induced by 1mM ATRA (Sigma-Aldrich) and 10 ng/ml G-CSF (Preprotech).

Table 1. Patient characteristics.

	Total n=90	
Sex	N	(%)
Male	46	(51)
Female	44	(49)
FAB		
M0	3	(3)
M1	3	(3)
M2	20	(22)
M3	13	(14)
M4	27	(30)
M5	23	(26)
M6	0	(0)
M7	1	(1)
Cytogenetics		
MLL	35	(39)
t(8;21)	21	(23)
inv(16)	17	(19)
t(15;17)	12	(13)
t(7;12)	5	(6)
	Median	range
Age (years)	9.9	(0.1-17.3)
WBC (x 109/L)	35.1	(0-475)

Xenograft mouse model

NOD.Cg-Rag1tm1Mom Il2rgtm1Wjl/SzJ (NRG) and Foxn1nu/J (nude) mice (Jackson Laboratory, Bar Harbor, ME, USA) were maintained in a pathogen free environment. All experimental procedures using these mice were performed in accordance with protocols approved by the local authorities (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit). For competitive *in vivo* assay, $1x10^7$ KASUMI-1 cells (ratio 2:1 of transduced vs untransduced cells) were injected i.f. per animal into a cohort of 10 recipients. Recipients were randomly assigned. After 28 days, mice were sacrificed. Leukemic cells were isolated from femurs and analyzed as described.18 In another setting, $1x10^7$ transduced KASUMI-1 cells were injected s.c. into Foxn1nu/J mice, together with Matrigel (BD, Franklin Lakes, NJ, USA). Tumor mass was evaluated after 6 weeks.

MiRNA and mRNA expression profiling and RT-qPCR

Total cellular RNA was isolated using Trizol reagent (Invitrogen, Breda, The Netherlands) as previously described.[19] MiRNA expression was measured on Taqman Array MicroRNA Cards v2.0 using Taqman technology in the 7900 HT Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) according to the manufacturers' protocol. mRNA expression profiling was performed using Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's guidelines. For more details see supplementary Methods. All microarray data have been deposited in NCBI's Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) with GEO Series accession numbers GSE52541.

Lentiviral transduction

MiRNAs were cloned into a modified Lentiviral Gene Ontology (LeGO) as previously described.[20] As control a non-silencing shRNA inside the miR-30 backbone (ThermoFisher Scientific Inc., Waltham, MA, USA) was used. Stable miRNA knockdown was achieved using the sponge technology.[21] In brief, 12 specific target sites of miR-9 as an artificial 30UTR were inserted into the lentiviral pLBid-GIP/C vector22 downstream of the SFFV-driven mCherry cassette, while an antisense CMV-driven GFP-IRES-Puro served as selection marker (Supplementary Figure 4b). The non-silencing sponge (SP-sc) control sequence was adapted from Loya et al. [21] THP-1 cells with higher endogenous miR-9 levels (Supplementary Figure 3) were used to monitor functionality of the SP-9 decoy by decreased mCherry fluorescence (Supplementary Figure 4c). The following shRNAs in the pLKO.1 (ThermoFisher) were used for target validation: *HMGA2*#1 (TRCN0000021964), *HMGA2*#2 (TRCN0000021966), *HMGA2*#3 (TRCN0000021967) and *LIN28B*#1 (TRCN0000122599), *LIN28B*#2 (TRCN0000143619) and *LIN28B*#3 (TRCN0000219859) (Sigma-Aldrich). Transduction and culture of HSPCs and cell lines was performed using lentiviral supernatant, prepared by calcium-phosphate transfection of HEK293T cells as previously described.[14,20]

HSPCs were cultured in RPMI (10% FCS and 1% penicillin streptomycin); FLT3L, IL3, IL6, GM-CSF, M-CSF (all Preprotech) for monocytic *in vitro* differentiation, or in the presence of SCF, IL3, GM-CSF, and G-CSF for granulocytic *in vitro* differentiation. For *in vitro* studies, leukemic blasts were maintained, transduced or used for colony-forming assays (HSC003, R&D Systems, Wiesbaden-Nordenstadt, Germany or Collagen-Cult, SCT, Vancouver, Canada) as described.[18,23]

Flow cytometry and cell sorting

Immunophenotyping of differentiated cells was performed by flow cytometry on a Navios 10/3 device (Beckman Coulter, Krefeld, Germany) as previously described[14] using the following antibodies: anti-CD14-PC5.5, anti-CD13-PC5.5, anti-CD11b-PC7, anti-CD14-APC and anti-CD15-PacB (Beckman Coulter), and anti-CD11b-APC (BD Pharmingen, Heidelberg, Germany). Kaluza Software by Beckman Coulter was used for analyses. Cells were sorted on a MoFlo device (Beckman Coulter) as previously described.[14,23]

Apoptosis, proliferation and colony forming assays

Apoptosis analyses using the Annexin V Apoptosis Detection Kit II were performed according to manufacturer's instructions (BD Pharmingen) and as previously described.[23] Cell viability assays were performed using CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA) on a Glo Max luminometer (Promega) according to the manufacturer's instructions. For growth competition, transduced GFP þ and mCherry þ cells were mixed in 1:1 ratio. The fraction of GFP þand mCherry þcells was determined every third day by flow cytometry. Colony-forming assays with transduced cell lines and HSPCs were performed and analyzed as described previously (HSC001 for cell lines, HSC003 for HSPCs, R&D Systems).[20]

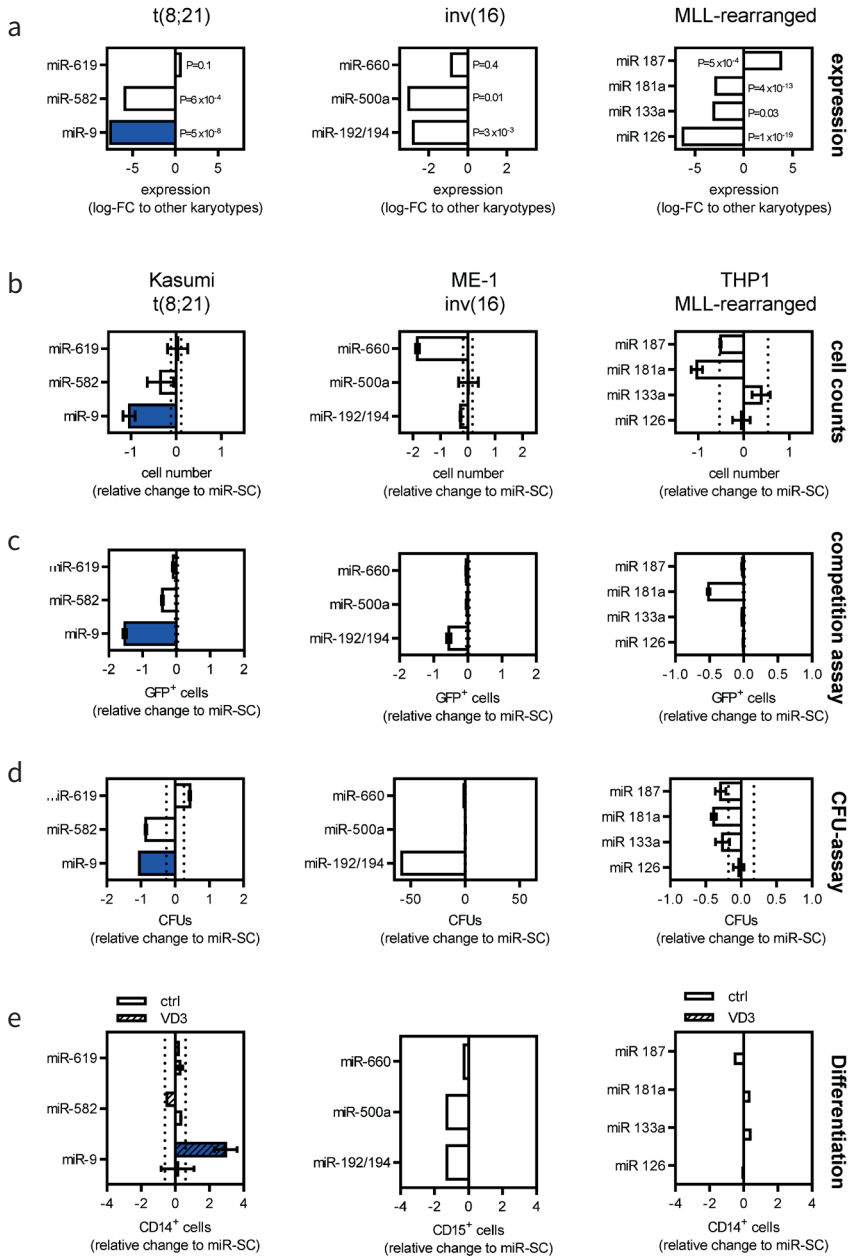
Western blot

Cell lysates were prepared and western blot analyses and immunoprecipitation were performed as described previously.[14] The following antibodies were used: LIN28 (clone 13D4.1), *HMGA2* (DR1073), (Millipore, EMD Millipore Corporation, Billerica, MA, USA), b-ACTIN (C4), anti-mouse IgG HRP, and anti-rabbit IgG HRP (all Santa Cruz, Dallas, TX, USA).

Luciferase reporter assay

For reporter assays, the 30 UTR or intron fragments of the respective mRNAs containing the miR-9 binding site were cloned from cDNA into pMIR-REPORT (Ambion, Paisley, UK). 293T cells (2 x10^4) were plated and co-transfected with 6.25 ng of the firefly luciferase report vector, 0.25 ng of the control vector containing renilla luciferase pGL4.7 (Promega) and 50 ng miRNA expression vector in 96-well plates by using FuGENE HD (Roche, Basel,

Figure 1. Functional screening of seven deregulated miRNAs or miRNA clusters in three AML cell lines.



(a) Expression of indicated miRNAs in respective karyotype groups as log2 fold change over all other karyotypes as measured by TaqMan low-density array (TLDA). (b-e) All readouts are shown as fold change relative to miR-SC where - 1 means one fold reduction (50%), 1 means one fold increase (200%). (b) Number of viable, miRNA- or miR-SC-transduced KASUMI-1, ME-1 and THP-1 cells after 3 days in culture determined by luminescence cell viability assay. (c) Fraction of GFP+ miRNA-transduced cells after 15 days of culture in competition with Cherry+ miR-SC-transduced control cells. (d) Number of CFUs in methylcellulose-based colony-forming assays of miRNA-transduced cells. (e) Percentage of CD14 + or COls+ miRNA transduced cells, respectively, withor without induction of differentiation by calcitriol (VD3; KASUMI-1, THP-1) after 7 days of culture.

Switzerland), according to the manufacturer's protocol. Firefly and renilla luciferase activities were measured consecutively by using dualluciferase assays (Promega), 48 h after the transfection. All experiments were performed in replicates.

Statistical analyses

MiRNA (n= 253) DCT values (zero detection values were set at -25) of AML samples (n= 90) were clustered using correlation (method spearman) hierarchical clustering (method ward) using the hclust function of R (www.R-project.org) and visualized using heatmap.2.[24] Most discriminative miRNAs were uncovered by detecting significantly differently expressed miRNAs (p.FDR <0.05) using linear modeling and Baysian P-value estimation based on t-type statistics using the R package limma.[25] For this purpose, each of five cytogenetically defined subgroups were contrasted against the four others.

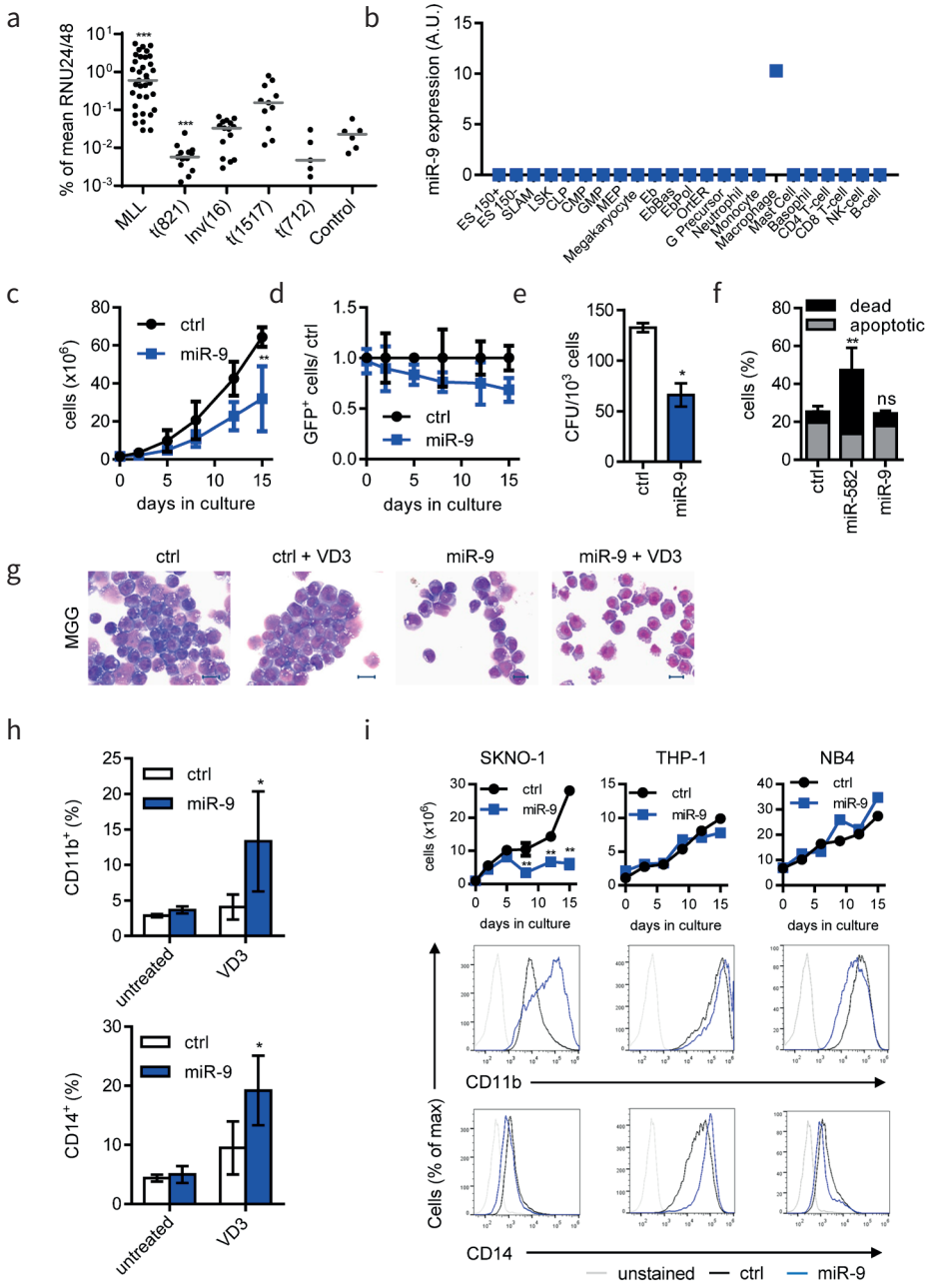
MiR-9 target genes were indicated by miRecords[26] and considered for further validation if they were predicted by three target prediction algorithms: miRTarget2,[27] PITA[28] and TargetScan.[29] Statistical analyses of experiments were performed using the two-tailed Student's t-test for two groups and ANOVA for three or more groups. Analyses were performed with SPSS Statistics Version 19.0 (SPSS Inc., Armonk, NY, USA) or GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA). All used tests were two tailed, and a P-value of <0.05 was considered significant.

RESULTS

In vitro characterization of differentially expressed miRNAs reveals biological significance

AML with t(8;21), inv(16) or *MLL*-rearrangement is characterized by biologically distinct fusion proteins. Together these subgroups represent 44% of the cases.[1] In order to identify miRNAs, which specifically suppress leukemic growth of AML with t(8;21), inv(16) or *MLL*-rearrangement by inducing differentiation, we conducted a miRNA expression profiling in a cohort of 90 cytogenetically characterized, *de novo* pediatric AML cases (Table 1, Supplementary Figure 1-2 and Supplementary Table 1) followed by gain-of-function studies. The decision to further investigate five miRNAs and two miRNA clusters *in vitro* using three cell lines was based on their ranking in the expression screening (specifically downregulated in one karyotype vs all others; Figure 1a and Supplementary Table 1) and on literature review (no reported tumor-suppressive function in AML). The miRNA candidates were cloned in a lentiviral vector system. Ectopic expression of each miRNA was tested in the cell line harboring the respective cytogenetic aberration of the subgroup, where it was downregulated, that is, KASUMI-1 for t(8;21), ME-1 for inv(16) and THP1 for *MLL*-rearranged AML (Figure 1a). To judge the effect, four readouts were used: proliferation was

Figure 2. Mir-9 suppresses proliferation and induces differentiation in KASUMI-1 cells.



(a) Expression of miR-9-5p as measured by TLDA. CD34+ HSPCs were used as a control. Expression is significantly higher in MLL-rearranged AML (Log2-fold change=6.98, P<0.0001) and significantly lower expressed in t(8;21) AML (Log2-fold change=- 7.58, P<0.0001) when compared with all other AML patients. (b) Expression of miR-9 in the indicated hematopoietic stem and/or progenitor cell compartments and mature blood cells.[31] (c) Total cell counts of miR-9 or miR-SC (ctrl) transduced KASUMI-1 cells. (d) Growth competition assay. The fraction of GFP+ miRNA-transduced cells at indicated time points of culture is shown in relation to miR-SC (ctrl).

Legend continues on next page

Legend Figure 2 continued:
(e) Number of CFUs in methylcellulose-based colony-forming assays of miR-9 and miR-SC (ctrl) transduced cells. (f) Percentage of apoptotic (Annexin V+ /7-AAD-) and dead (7-AAD+) cells for miR-9, miR-582 and miR-SC (ctrl) transduced KASUMI-1 cells measured by flow cytometry after 3 days of culture. (g) Representative microscopic images of May–Grünwald–Giemsa (MGG) stained cytospins (scale bars, 50 mm) of miR-9 or miR-SC transduced KASUMI-1 cells with and without VD3 treatment.(h) Percentage of CD11b+ (upper graph) and CD14+ (lower graph) KASUMI-1 cells as determined by flow cytometry. (i) Growth anddifferentiation in SKNO-1, THP-1 and NB4 cells. Upper panel, total cell counts of miR-9- or miR-SC-transduced untreated cells; lower panels, representative histograms of normalized fluorescence intensities from flow cytometric staining of indicated surface markers in SKNO-1 and THP-1 cells treated with VD3 and NB4 cells treated with ATRA. (c–i) Data are presented as mean±s.d. of replicates of two to four independent experiments (*P<0.05; **P<0.01; ***P<0.001).

assessed by cell viability assays after 72 h (Figure 1b), cell competition by relative fluorescence on day 15 (Figure 1c), colony-forming capacity by colony-forming unit (CFU) assays (Figure 1d) and differentiation by flow cytometry with and without induction (Figure 1e). For each karyotype, we included one miRNA as a control, which was either insignificantly changed (miR-619 in t[8;21] KASUMI-1 and miR-660 in inv[16] ME-1) or upregulated (miR-187 in *MLL*-rearranged THP1; Figure 1a).

Using this approach, we identified two miRNAs and two miRNA clusters that reduced cell growth and colony-forming capacity upon ectopic expression (Figure 1b–d): two out of two miRNAs in KASUMI-1 (miR-9, miR-582), one out of two in ME-1 (miR-192/194 bicistron) and one out of three in THP1 (miR-181a/b bicistron). Recently, miR-181a was also defined by others[30] to be tumorsuppressive in *MLL*-rearranged leukemia, showing the validity of our results. None of the miRNAs enhanced cell growth and colonyforming capacity upon their ectopic expression.

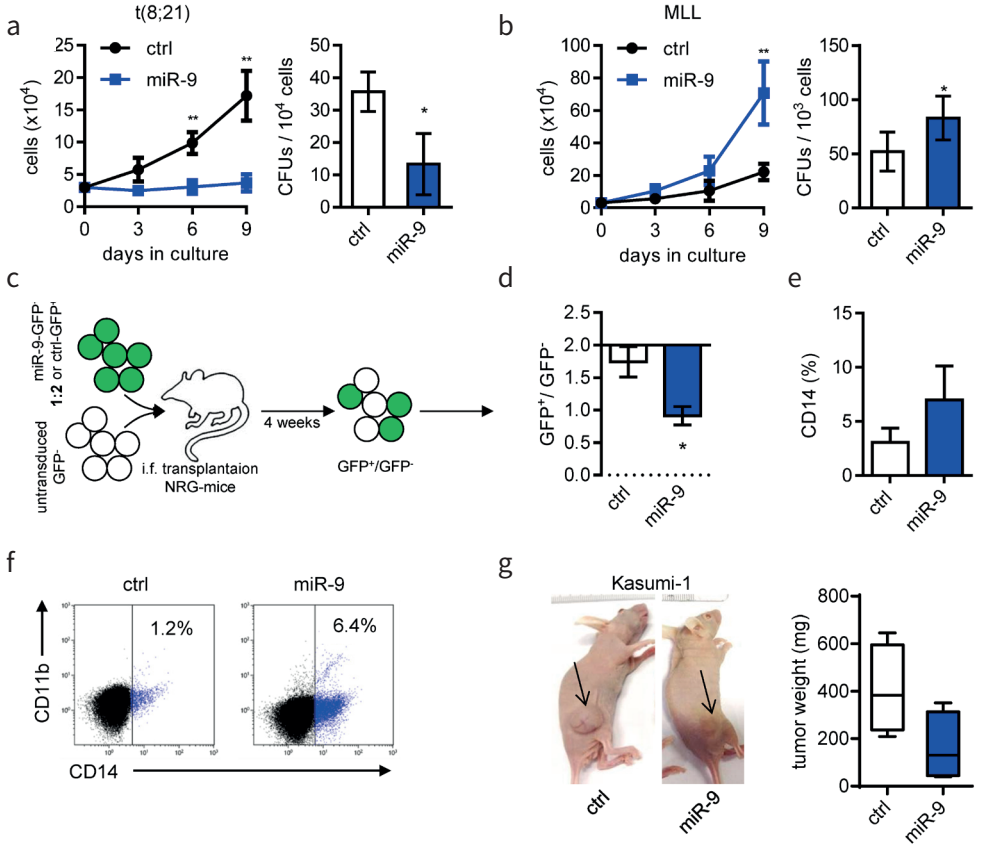
In KASUMI-1 cells, we identified one miRNA, miR-9, which not only reduced cell growth and colony-forming capacity, but also strongly induced monocytic differentiation in concert with calcitriol (that is, vitamin D3 (VD3); Figure 1e). This suggested the further exploration of the function of miR-9 in t(8;21) AML.

Ectopic expression of miR-9 in KASUMI-1 suppresses growth, colony-forming capacity and synergizes with calcitriol in monocytic differentiation

MiR-9 was repressed in AML patients with t(8;21), while it was expressed to a higher extent in patients with *MLL*-rearrangements (Figure 2a and Supplementary Figure 3a). During normal hematopoiesis miR-9 is only expressed in mature macrophages, and not in stem and/or progenitor cells nor in cells of other mature blood lineages (Figure 2b, from Petriv et al. [31]). Those facts, together with the growth arrest and monocytic differentiation upon ectopic miR-9 expression in t(8;21)-positive cell line KASUMI-1, point towards a regulatory function of miR-9 during monopoiesis and an advantage of miR-9-downregulation during t(8;21)-mediated leukemogenesis.

This was further investigated in separate experiments confirming the primary character-

Figure 3. MiR-9 exerts a tumor-suppressive function in primary t(8;21) leukemic cells *in vitro* and in xeno-transplantation models *in vivo*.



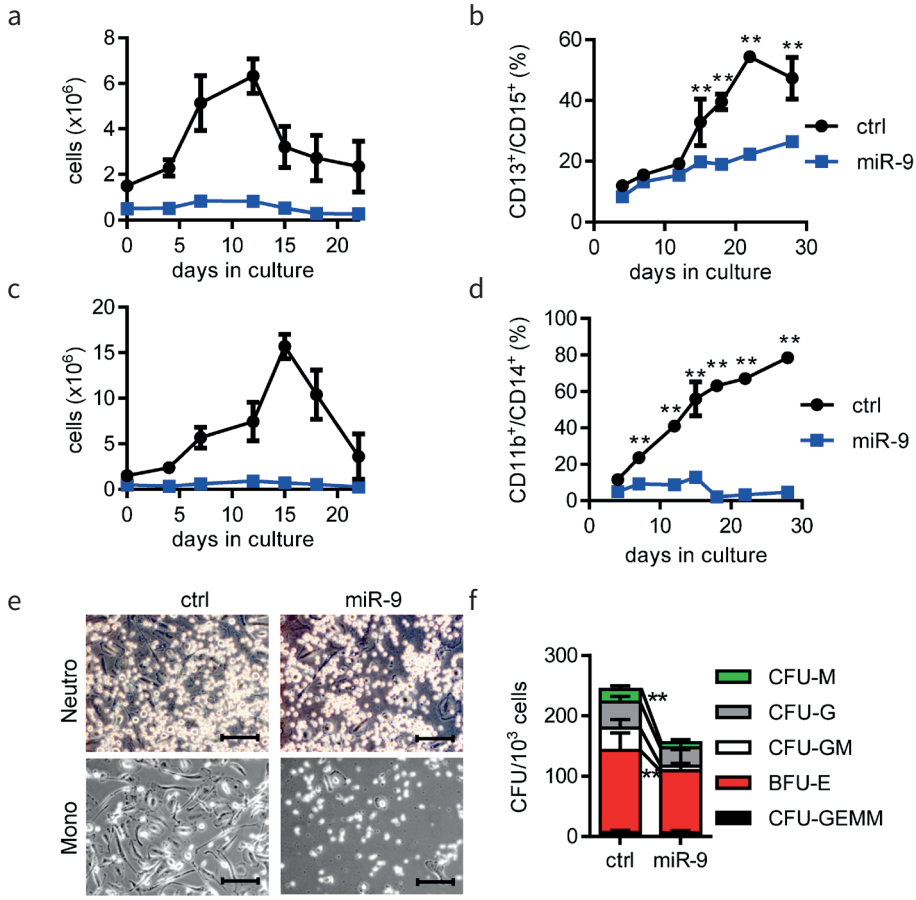
(a and b) Cell counts (left diagram) and number of CFUs in methylcellulose-based colony-forming assays (right diagram) of miR-9 or miR-SC (ctrl)- transduced t(8;21) (a) or MLL rearranged (b) AML patient blasts (N=2 and N=3, respectively). (c) Scheme illustrating the experimental setup of the competitive xenotransplantation assay. (d) Ratio of GFP+ transduced vs GFP- untransduced KASUMI-1 cells in NRG mice after 28 days following i.f. transplantation. (e–f) Percentage of CD14+ (e) and representative flow cytometry plots (f) of GFP+ -transduced KASUMI-1 cells in the same mice as in (d). (g) Photographs (left) and tumor weights (right) of nude mice subcutaneously injected with miR-9 or miR-SC (ctrl) transduced KASUMI-1 cells after 6 weeks. (d–g) Data are presented as mean±s.d. of N=5 mice per group (*P<0.05; **P<0.01).

ization. Consistently, upon ectopic miR-9 expression (137-fold elevation compared with the miR-SC (non-silencing) control, Supplementary Figure 3b) we observed a reduction in growth of KASUMI-1 cells in culture compared with controls (Figure 2c). MiR-9 expression levels upon ectopic expression were within a physiologic range, as miR-9 expression is 302-fold higher in *MLL*-rearranged patients as compared with t(8;21) patients (Supplementary Figure 3a). In the growth competition assay, GFP⁺ miR-9-transduced cells were outcompeted by mCherry⁺ miR-SC-transduced control cells, while the GFP⁺ miR-SC-transduced cells were not (Figure 2d). The colony forming capacity showed a twofold reduction (Figure 2e). This growth reduction by miR-9 was not due to an increased rate of apoptosis

or cell death (Figure 2f). However, this was not the case for miR-582, where the decrease in the number of viable cells was clearly caused by the induction of apoptosis (Figures 1b and 2f).

The reduction of cell growth was in part attributed to monocytic differentiation of the leukemic cells in conjunction with calcitriol. This was evident by the appearance of miR-9 transgenic cells with a lighter cytoplasm and uneven cell membranes exhibiting ruffles and protrusions. In addition to that, an enhanced expression of CD11b and CD14 was detected in comparison with the control cells in culture (Figure 2g-h). The induction of monocytic differentiation and reduction of cell growth by miR-9 was also observed in an-

Figure 4. MiR-9 blocks in vitro cell growth and differentiation of HSPCs.



(a, c) Total cell counts of miR-9- or miR-SC-transduced (ctrl) HSPCs during neutrophilic (a) and monocytic (c) in vitro differentiation. (b, d) Percentage of CD13+/CD15+ cells during neutrophilic (b) and CD11b+/CD14+ cells during monocytic (d) in vitro differentiation. (e) Phase contrast microscopic images of in vitro differentiated HSPCs (scale bars, 200 mm). (f) Number of CFUs in methylcellulose-based colony-forming assays of miR-9 and miR-SC-transduced HSPCs. (a–f) Data are presented as mean±s.d. of replicates from one of two independent experiments (**P<0.01).

other t(8;21) cell line, SKNO-1, but not in THP-1 and NB4 (with t[15;17]) cells (Figure 2i). MiR-9 is marginally expressed in t(8;21) KASUMI-1 and SKNO-1 cells and lowly expressed in t(15;17) NB4 cells, while it is highly expressed in THP-1 cells (Supplementary Figure 4a). MiR-9 repression using a lentiviral sponge system conferred a growth advantage to SKNO-1 cells and growth disadvantage to THP-1 and NB4 cells (Supplementary Figure 4b-d). Thus, miR-9 blocks proliferation and induces differentiation in t(8;21) positive cells but not in *MLL*-rearranged and t(15;17) positive cells.

MiR-9 exerts a tumor-suppressive function in primary t(8;21) leukemic cells *in vitro* and in xenotransplantation models *in vivo*

To investigate if those cell-context specific effects are restricted to cell lines or apply globally to t(8;21) AML, we ectopically expressed miR-9 in primary leukemic blasts using the same lentiviral system. As in the cell lines, miR-9 suppressed leukemic growth and colony forming capacity of t(8;21) AML samples (n= 2; Figure 3a). On the other hand, the opposite effect was observed in *MLL*-rearranged samples (n= 3; Figure 3b). The suppression of leukemic growth in t(8;21) AML was accompanied by the induction of monocytic genes (Supplementary Figure 5a).

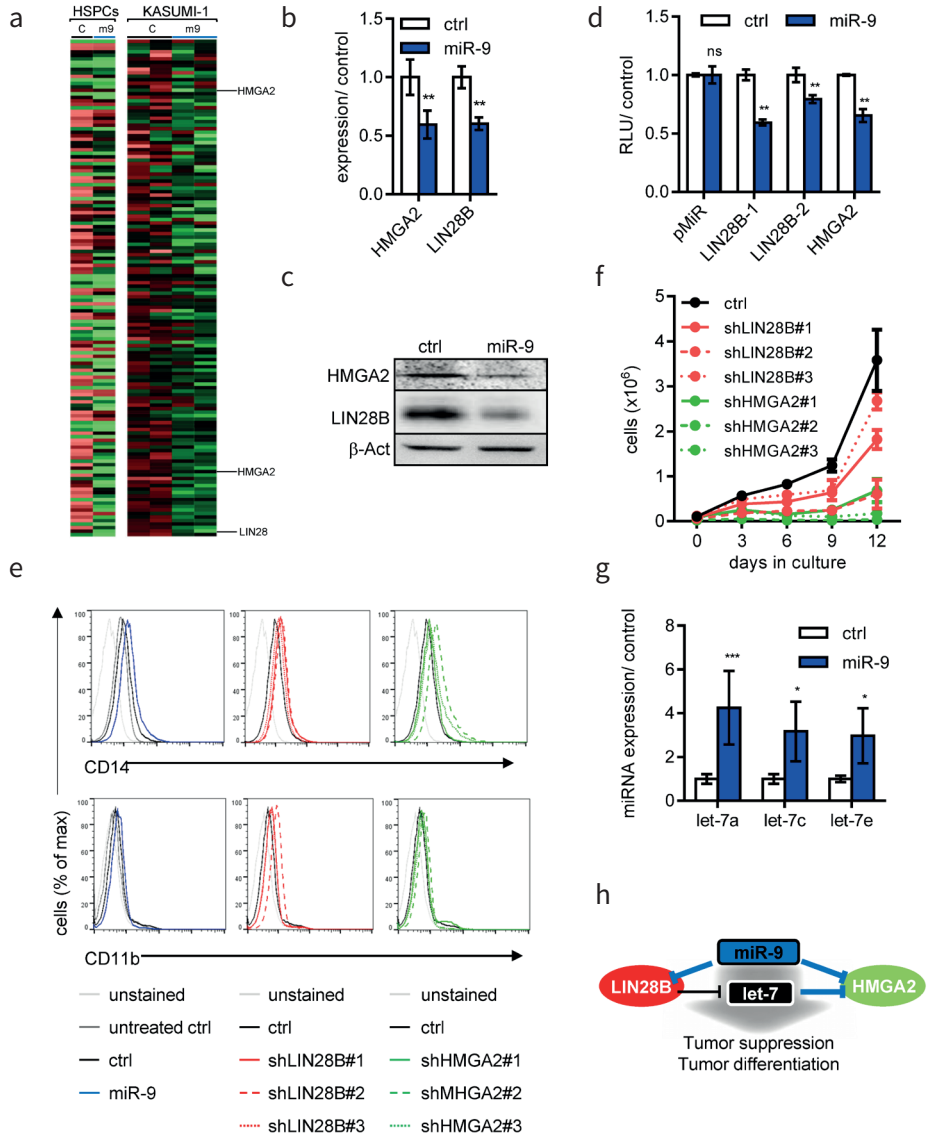
Using two independent xenotransplantation models, we could also prove the tumor-suppressive function of miR-9 *in vivo*. First we performed a competitive transplantation assay, where NRG mice were intrafemorally transplanted with miR-9-or miR-SC transduced KASUMI-1 cells (GFP^+) together with untransduced competitors (GFP^-; GFP^+:GFP-ratio 2:1; Figure 3c). While the GFP^+:GFP^-ratio of 2:1 was mainly unchanged in the bone marrow of non-silencing control recipients after 28 days, it was reduced in the miR-9-recipient mice (Figure 3d). Thus, miR-9-transduced KASUMI-1 cells were outcompeted by their untransduced competitors. Furthermore, we could confirm the induction of monocytic differentiation of t(8;21) KASUMI-1 cells *in vivo* (Figure 3e and f). Similarly, the tumor size of nude mice, subcutaneously injected with transduced KASUMI-1 cells, was reduced by miR-9 (Figure 3g). Thus, miR-9 acts as a tumor suppressor in t(8;21) AML *in vitro* and *in vivo*.

MiR-9 overexpression in CD34 þ-HSPCs and *MLL*-rearranged AML blasts impairs granulocytic and monocytic differentiation

To test if the effects of miR-9 are restricted to t(8;21) leukemic cells or if miR-9 generally induces myelomonocytic differentiation, we transduced and evaluated human CD34 þ-HSPCs and *MLL*-rearranged AML blasts. Monocytic differentiation was suppressed in *MLL*-rearranged AML samples, as indicated by the reduced expression of monocytic genes and the round morphology of the cells (Supplementary Figure 5a and b).

During granulocytic *in vitro* differentiation of $CD34^+$-HSPCs, the miR-9-transduced $CD34^+$-HSPCs ceased to proliferate (Figure 4a). Accordingly, the granulocytic differentiation was diminished, as quantified by the fraction of $CD13^+/CD15^+$ granulocytes by flow cytometry

Figure 5. LIN28B and HMGA2 are miR-9 target genes.



(a) Heatmap displaying 93 validated and predicted miR-9 target genes downregulated in miR-9-transduced (m9) HSPCs (n=1) and KASUMI-1 cells (n=2) compared with the miR-SC-transduced controls (C), respectively; indicated are probe sets for HMGA2 and LIN28B. (b) RT-qPCR of miR-9 target genes in KASUMI-1 cells. (c) Western blot of miR-9 target genes in miR-9- and miR-SC (ctrl)-transduced KASUMI-1 cells. (d) Luciferase reporter assay with indicated 30 UTR fragments in 293T cells. Data are presented as mean±s.d. of replicates of five independent experiments (**P<0.01). (e) Flow cytometry for CD11b and CD14 markers of miR-9-, miR-SC (ctrl), shLIN28 (#1-3)- and shHMGA2 (#1-3)-transduced KASUMI-1 cells. Except for untreated control each sample was treated with 50 nM VD3. (f) Number of viable cells of control- or shRNA-transduced KASUMI-1 cells determined by luminescence cell viability assay. (g) RT-qPCR of let-7 isoforms in miR-9 and miR-SC (ctrl)-transduced KASUMI-1 cells. (h) Molecular model of miR-9 anti-tumor mechanism. MiR-9 inhibits both LIN28B and HMGA2, additionally a double-negative feedback reliefs let-7 from LIN28B suppression and increases reduction of HMGA2 and tumor suppression, overall resulting in enhanced tumor differentiation. (b, f, g) Data are presented as mean±s.d. of replicates of two to four independent experiments (*P<0.05; **P<0.01; ***P<0.001).

(Figure 4b). During the monocytic *in vitro* differentiation, the number of cells was 20-fold lower on day 15 (Figure 4c) and only few $CD11b^+/CD14^+$ monocytes were detected in culture (Figure 4d). The block in proliferation and differentiation was also apparent in phase contrast microscopy (Figure 4e). Especially during monocytic differentiation no fusiform, adherent macrophages were seen. In the CFU assay the number of monocytic and mixed granulocytic–monocytic CFUs was significantly reduced (Figure 4f and Supplementary Figure 6a and b).

Thus, in contrast to t(8;21) AML, miR-9 blocks myelomonocytic differentiation in HSPCs and *MLL*-rearranged AML. However, similar to t(8;21) AML and in contrast to *MLL*-rearranged AML, proliferation of HSPCs was blocked.

MiR-9 exerts its effects by targeting the *LIN28B*/let-7/*HMGA2* axis

To identify target genes which might explain these controversial findings, we performed global gene expression profiling in miR-9-and miR-SC-transduced $CD34^+$-HSPCs and KASUMI-1 cells. In total, 4712 genes were downregulated after miR-9 overexpression in both cell types. Overall, 93 genes out of 219 predicted miR-9 target genes were downregulated (Figure 5a and Supplementary Table 2). Among those genes were known miR-9 targets such as *CDH1*, *NFKB1*, *BACE1* and *REST*.

The transcription factor *MAF* acts in concert with *MAFB* as the two master regulators of macrophage development.[32,33] We could demonstrate an intronic miR-9 binding site in the *MAF* unspliced transcript as being responsive to miR-9 binding in reporter assays (Supplementary Figure 6c). This intron can be retained in *MAF* isoform B (isoB). Both isoforms were strongly repressed during *in vitro* monocytic differentiation (Supplementary Figure 6d). Repression of ten miR-9 target genes – downregulated in the microarray analysis – in transduced HSPCs was validated by RT-qPCR (Supplementary Figure 6e). Among those are *SHC1*,a mediator of SCF/Kit signaling, the oncogene *ATF1*, *PAK4* involved in inhibiting apoptosis and regulating cell motility, *PRUNE* and *CDH1* that play a role in cell migration. These genes give a possible explanation to the block in growth and differentiation and might be responsible for the drastic morphological change in cells.

Expression of High Mobility Group A2 (*HMGA2*) and lin-28 homolog (C. elegans) (*LIN28B*) has been correlated with leukemogenesis and the alteration of hematopoietic differentiation.[34,35] Both genes are predicted targets of miR-9 and were downregulated upon ectopic expression of miR-9 in both $CD34^+$-HSPCs and KASUMI-1 cells (Figure 5a and b). In addition to PCR validation, downregulation of *HMGA2* and *LIN28B* was confirmed by western blotting in KASUMI-1 cells, but not in *MLL*-rearranged patient blasts (Figure 5c and Supplementary Figure 7a). *HMGA2* and *LIN28B* were validated as direct targets by 3'UTR luciferase reporter assays (Figure 5d).

To investigate whether repression of those two oncogenes at least in part mediates the

effects of miR-9 on t(8;21) AML cells, we knocked down their expression using three independent shRNAs. Knockdown efficiency was confirmed by RT-qPCR (Supplementary Figure 7b and c). When shRNA-transduced KASUMI-1 cells were treated with calcitriol, we observed for each shRNA-construct a pronounced induction of CD11b or CD14 (Figure 5e). While knockdown of *LIN28B* more strongly enhanced expression of CD14, knockdown of *HMGA2* elevated CD11b more efficiently (Figure 5e). Accordingly, cell viability measurement over time revealed a growth disadvantage for all six shRNA constructs (Figure 5f). Likewise, colony-forming capacity was reduced by knockdown of the two genes (Supplementary Figure 7d). Thus, single knockdown of *LIN28B* and *HMGA2* was sufficient to partly recapitulate the miR-9 phenotype in KASUMI-1 cells; that is, induction of monocytic differentiation and suppression of leukemic growth.

LIN28B has been shown to suppress let-7 processing.[36] Interestingly, let-7 is a well-known tumor suppressor[37] and exerts its function at least in part by inhibiting the oncogene *HMGA2*. [38] To test whether this axis is involved in miR-9-mediated suppression of leukemic growth, we quantified expression of mature let-7 homologs let-7a, let-7c and let-7e by RT-qPCR. All three let-7 homologs were upregulated upon miR-9 expression in KASUMI-1 cells (Figure 5g).

Collectively our data demonstrates that miR-9 acts as a growth suppressor and differentiation inducer in t(8;21) AML by inhibiting *HMGA2* and *LIN28B* (Figure 5h). The two target genes are connected via let-7 miRNAs, which accumulate upon downmodulation of *LIN28B*, further inhibiting *HMGA2* and additionally feeding into miR-9 anti-tumor propensities.

DISCUSSION

Here we describe a large scale miRNA expression profiling study in pediatric AML, which revealed specific miRNA expression patterns in *MLL*-rearranged AML, t(8;21) positive AML, t(15;17) positive AML, and inv(16) positive pediatric AML. We combined this expression profiling with a functional characterization of seven candidate miRNAs/miRNA clusters in three cell lines. This led to the discovery that miR-9 suppresses leukemic growth while inducing monocytic differentiation in t(8;21) AML *in vitro* and *in vivo*. This growth inhibitory function was also evident in normal $CD34^+$-HSPCs while differentiation induction was restricted to t(8;21) leukemic cells. Several predicted targets were downregulated upon forced miR-9 expression in cell lines and primary patient samples. We showed that miR-9 exerts its effects in cooperation with let-7 by repressing the oncogenic *LIN28B/HMGA2* axis.

As miR-9 is encoded in three locations in the human genome and is highly conserved among species, it seems that a tightly controlled miR-9 expression is of great importance for cellular integrity. In our cohort of pediatric AML samples, miR-9 expression was significantly lower in t(8;21) positive AML and significantly higher in *MLL*-rearranged AML as

compared with all other AML. In contrast to other studies showing high expression of miR-9 as a predictor of poor survival in adult AML,8 miR-9 expression was not an independent prognostic factor in multivariate analysis in pediatric AML (data not shown). Nevertheless, we clearly showed that ectopic expression of miR-9 in AML cell lines and primary patient samples with t(8;21) conferred a tumor-suppressive phenotype and induced differentiation along the monocytic lineage. Our results are in accordance with a previous study showing that miR-9 releases the differentiation block of oncogenic EVI1. [39] Hence, the findings are clinically relevant since substances or genetic modulators that stop leukemic growth by releasing the differentiation block of leukemic blasts, are warranted in leukemia therapy.[40,41] For example, in acute promyelocytic leukemia, survival increased by introducing ATRA, which specifically induces differentiation in blasts harboring the fusion protein PML-RARa. [42] MiR-9 is exclusively expressed in macrophages during normal hematopoietic differentiation.[31] Therefore, we hypothesize that elevating the expression of miR-9 could provide a specific treatment option for t(8;21) AMLs, but not for *MLL*-rearranged AML, where miR-9 exerts oncogenic effects[43] (Figures 2 and 3).

By interfering with the oncogenic *LIN28B*/let-7/*HMGA2* axis, miR-9 can achieve its tumor-suppressive properties (Figure 5). The developmentally regulated RNA-binding protein *LIN28B* belongs to the group of factors that can be used for reprogramming somatic cells into pluripotent stem cells[44] and is overexpressed in various types of cancer.[36] *LIN28A* and *LIN28B* selectively repress expression of let-7 miRNAs.[45–48] The let-7 miRNA family members act as tumor suppressors by repressing oncogenes including *RAS*, *MYC* and *HMGA2*. In addition to that many tumors are characterized by the downregulation of multiple let-7 miRNAs.[49] In our study, ectopic expression of miR-9 led to downregulation of *LIN28B* and, as a consequence, to upregulation of three tested let-7 family members (Figure 5g). *HMGA2* codes for a small non-histone, chromatin-associated protein that modulates transcription by altering the chromatin architecture[50,51] and serves as a target of let-7 and miR-9.[38] In analogy to *LIN28*, *HMGA2* is expressed in undifferentiated proliferating cells during embryogenesis and in several benign and malignant tumors.[52] In many of these tumors, a truncation of the human *HMGA2* disrupts the binding and negative regulation by let-7, resulting in oncogenic transformation.[53] Thus, miR-9 inhibits two well characterized oncogenes, *LIN28B* and *HMGA2*, while restoring the expression of the linked tumor suppressor let-7. In concert, let7 and miR-9 can even achieve a greater knockdown of oncogenic *HMGA2* (Figure 5h). This may release leukemic cells from their differentiation block and stop their growth.

Alternatively, downregulation of *LIN28B* and *HMGA2* in HSPCs might prevent the initiation of a differentiation program. In normal HSPCs, this failure in the induction of myelomonocytic differentiation upon miR-9 expression is fortified by the concordant repression of *MAF*, an essential transcription factor for myelomonopoiesis and another target of miR-9. We demonstrated that an intronic miR-9 binding site is responsible for miR-9 mediated downregulation of *MAF* isoforms during *in vitro* monocytic differentiation of normal

HSPCs. Moreover, a set of miR-9 target genes, such as *ATF1*, *PAK4*, *PRUNE* and *CDH1*, associated to cell motility and migration, are repressed by ectopic miR-9 expression (shown by microarray analysis and RT-qPCR validation), thus, restricting a key function of terminally differentiated myelomonocytic cells. In contrast, in adult AML Senyuk et al. demonstrated that miR-9 not only induced myelomonocytic differentiation of malignant hematopoietic cells but also of normal murine Lin$^-$-HSPCs.[39] Thus despite the overall concordance between the studies there are some differences, which might be attributed to different vectors systems (lentivirus vs retrovirus), resulting in different expression levels of miR-9, and different cell contexts (human CD34 þ-HSPCs vs murine Lin$^-$-HSCPs) tested.

In conclusion, by performing miRNA expression screening in pediatric AML, we gained novel insights in the biology and development of this disease. The identification of miR-9 as a novel tumor suppressor and differentiation inducer in t(8;21) AML opens the avenue for future therapeutic options.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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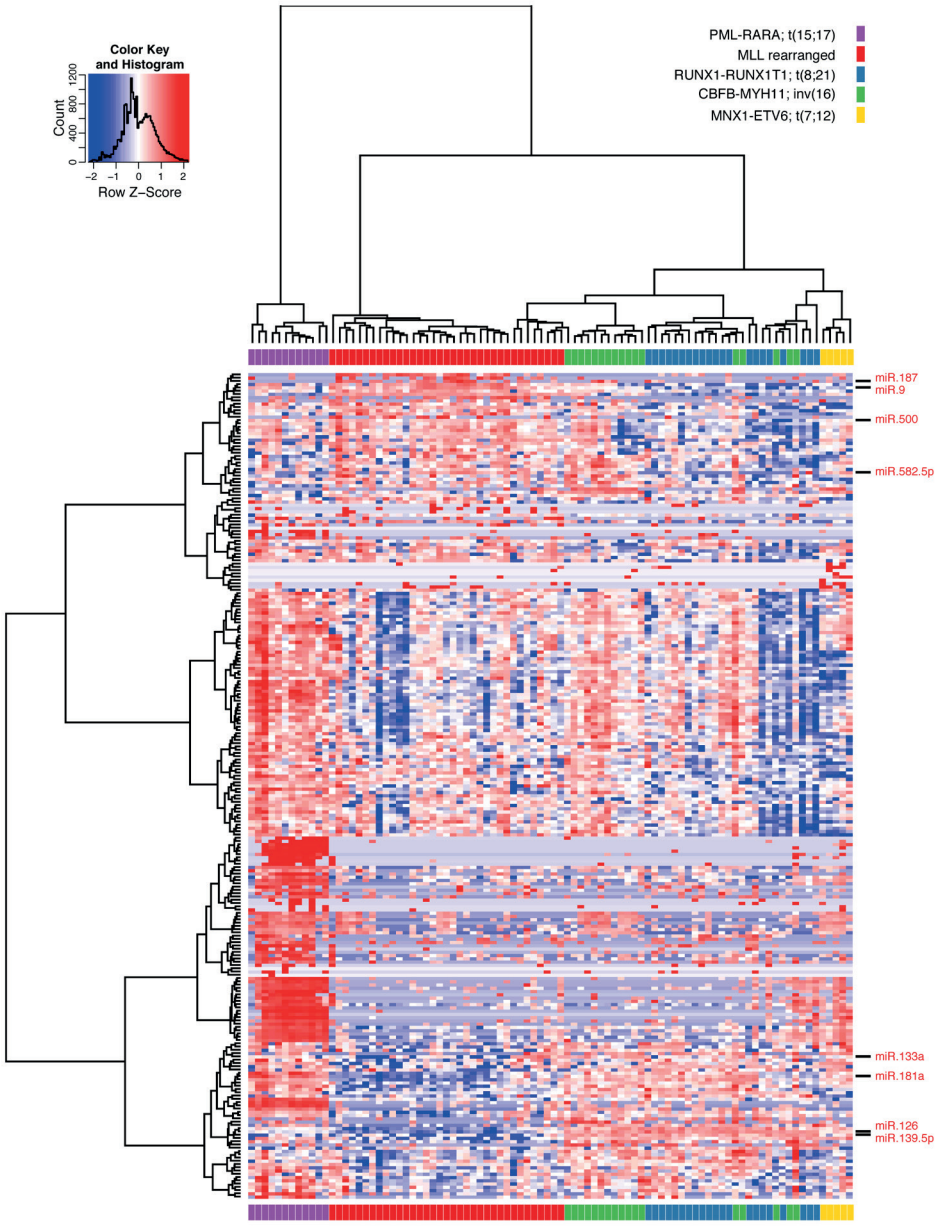
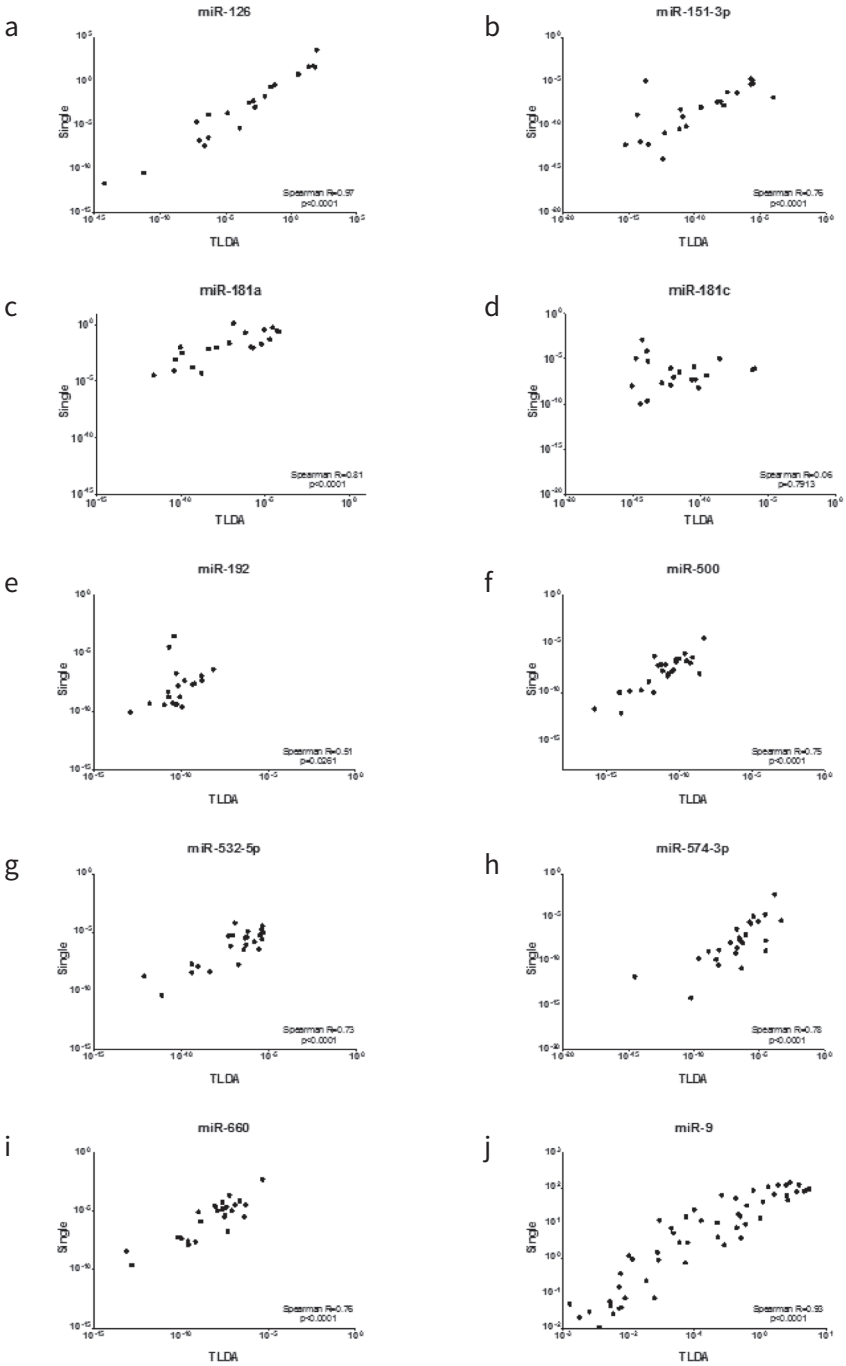
SUPPLEMENTARY FIGURES

Supplementary Figure 1. miRNA expression stratifies different cytogenetic types of pediatric AML.



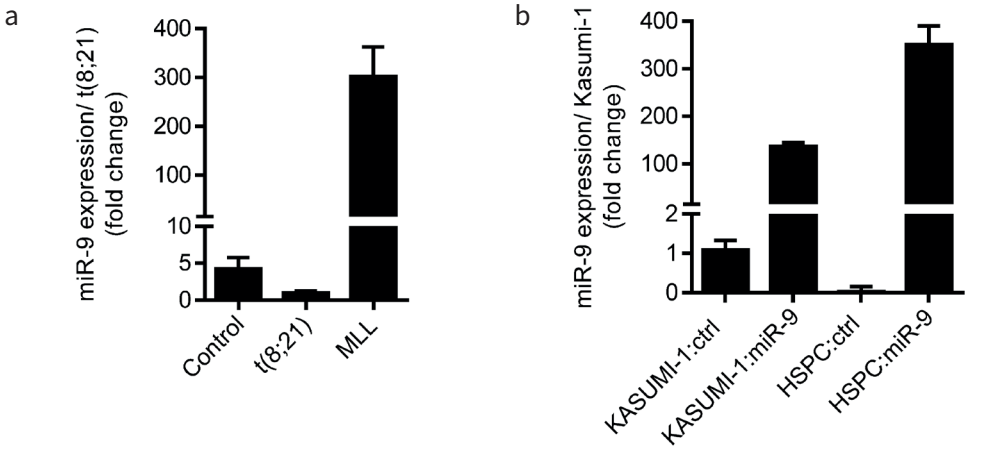
Unsupervised hierarchical correlation clustering of AML samples (n=90) and most discriminative miRNA values (n=253). Colors at the patient sample branches denote AML cytogenetic subgroups as indicated at the top right. Heatmap colors indicate miRNA expression values as indicated at the top left.

Supplementary Figure 2. Correlation between single and multiplex microRNA assays for a selection of 10 microRNAs.



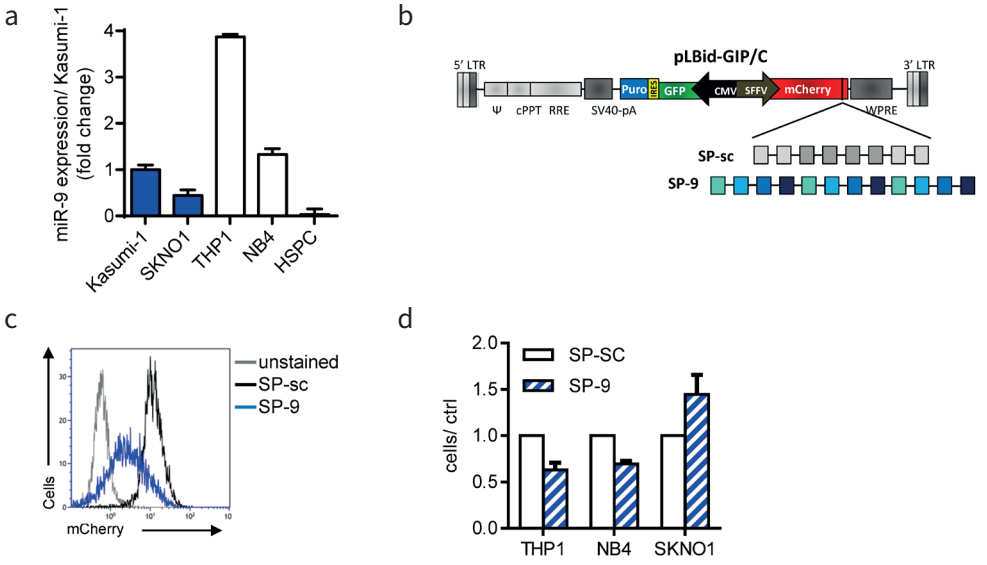
Figures display expression measured by multiplex miRNA assay vs. expression measured by singleplex miRNA assay for 10 microRNAs. Reproducibility of miR-9 measurements is very good: Spearman R=0.91, p<0.0001.

Supplementary Figure 3. Expression of miR-9.



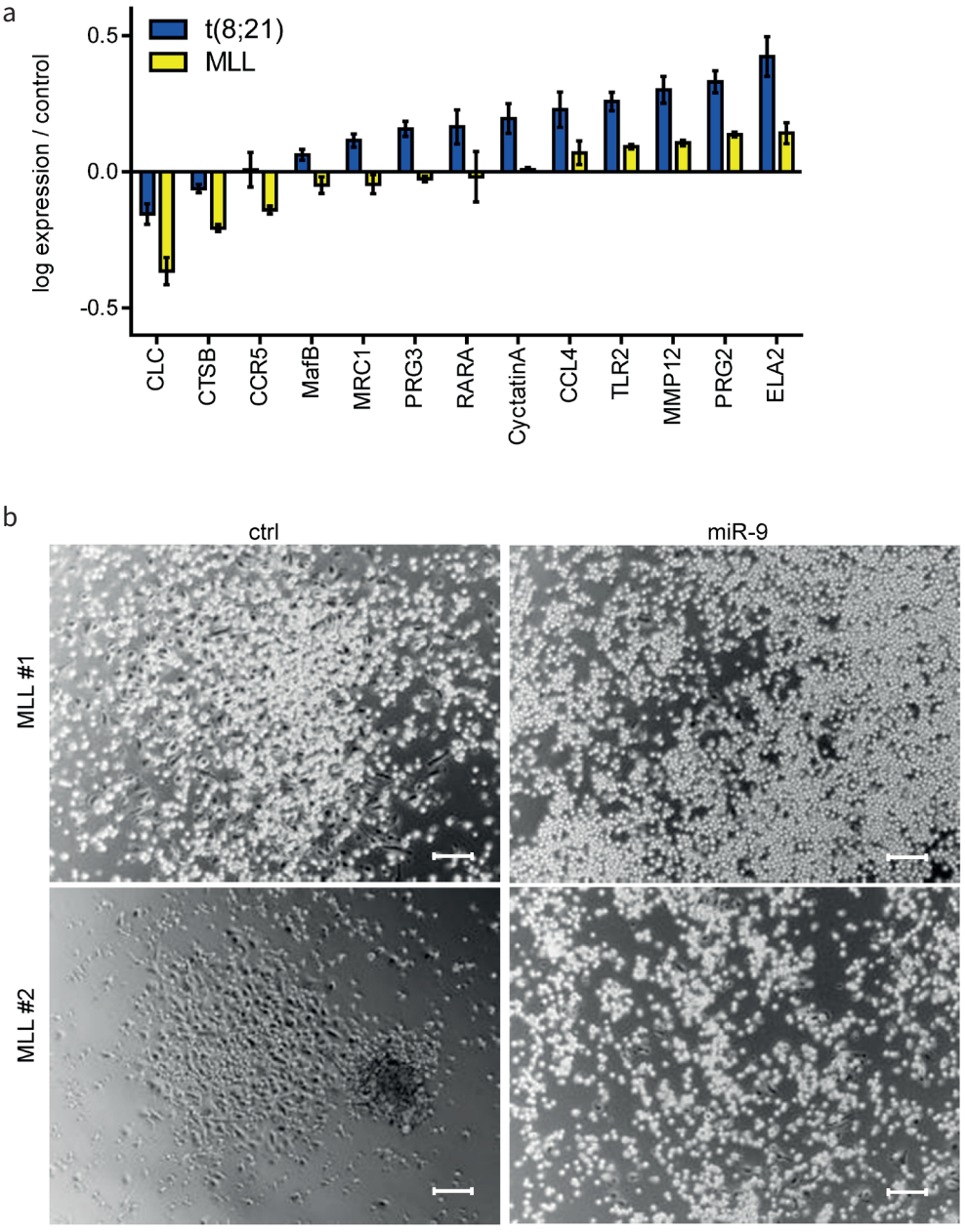
(a) Expression of miR-9-5p as measured by TLDA in CD34+-HSPCs (control) and blasts from patients with t(8;21) and *MLL*-rearrangement. Expression is normalized to t(8;21) patients. (b) Expression of miR-9-5p measured in duplicate by RT-qPCR after transduction of CB-HSPCs and KASUMI-1 with miR-SC (ctrl) or miR-9.

Supplementary Figure 4. Sponge-mediated inhibition of miR-9 in cell lines.



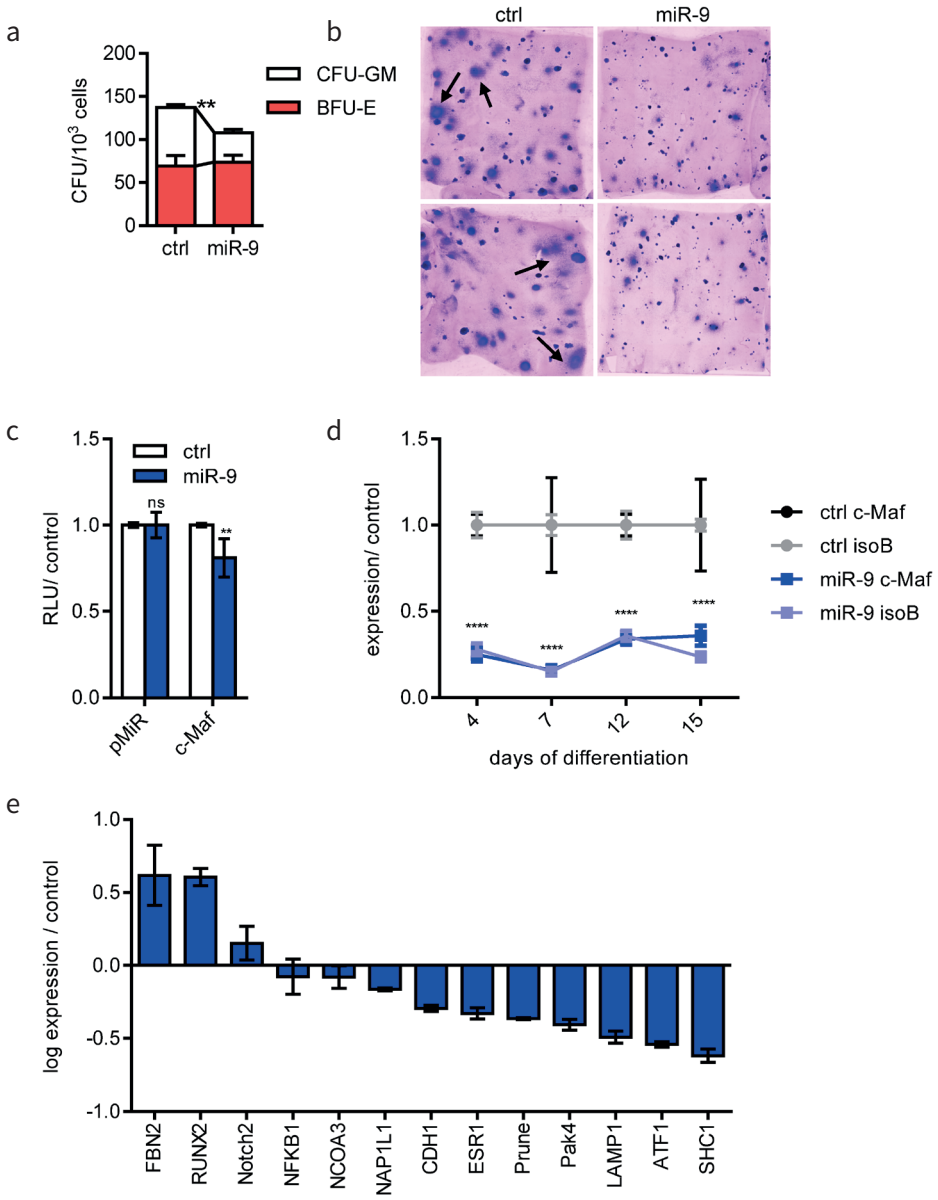
(a) Expression of miR-9-5p normalized to RNU44 measured in duplicate by RT-qPCR of the indicated cell lines. (b) Schematic map of the modified sponge (SP) vector used for miRNA inhibition.[1] For the SP inserts each lined block represents a bulged target site for the respective miRNA; SP-sc sequence was adapted from Loya et al.[2] (c) Fluorescence intensity for mCherry validating miRNA inhibition by SP-9 in THP1 cells. (d) Number of viable miR-9-transduced SKNO1, THP1 and NB4 cells relative to the miR-SC-transduced control on day 7 of culture. Mean±s.d. of replicates from one representative of two independent experiments are shown.

Supplementary Figure 5. RT-qPCR and luciferase reporter assay for target validation of miR-9.



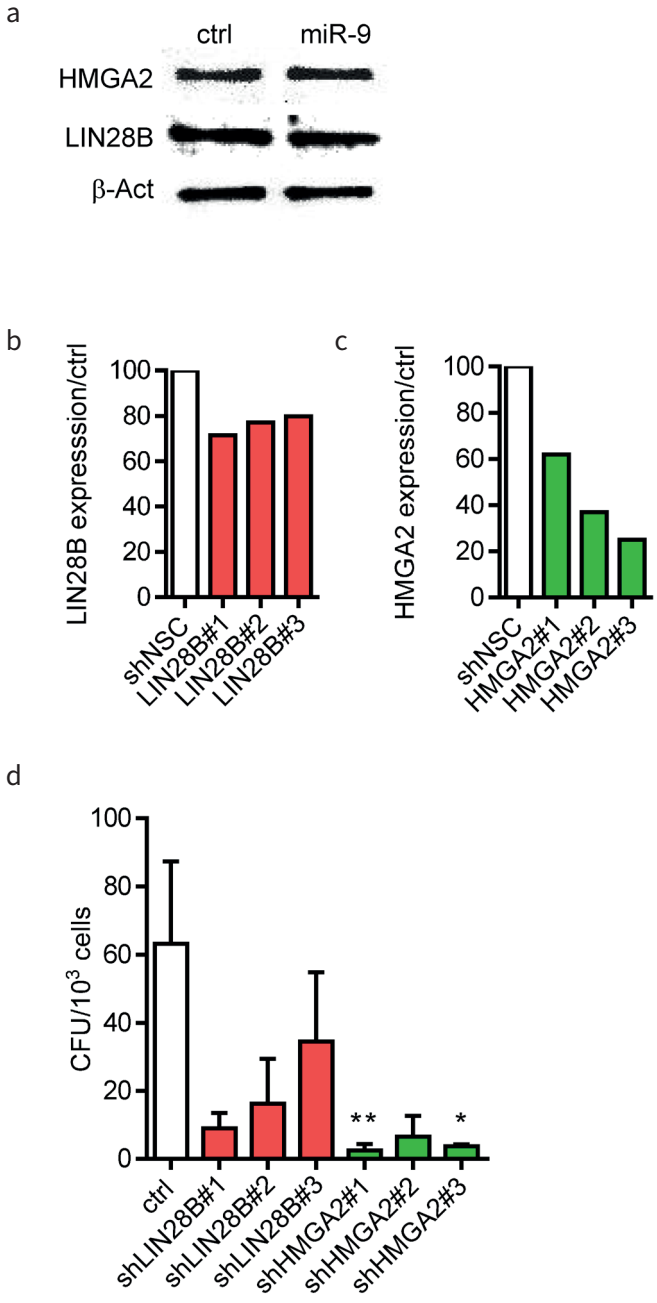
(a) RT-qPCR of granulocytic and monocytic differentiation markers in miR-9-transduced t(8;21) (N=2) and MLL-rearranged (N=2) patient blasts. Data were normalized to housekeeping genes and miR-SC control of the same samples. (b) Representative photomicrographs of transduced MLL-rearranged blasts (scale bars 100µm) showing less adherence upon miR-9 transduction.

Supplementary Figure 6. RT-qPCR and luciferase reporter assay for target validation of miR-9.



(a) Number of CFUs in collagen-based colony-forming assays of miR-9 and miR-SC (ctrl) transduced HSCPs. (b) Representative photographs of the slides of (a); arrows indicate normal sized CFU-GMs in the miR-SC control, missing upon miR-9 expression. (c) Luciferase reporter assay with indicated 3'UTR fragments in 293T cells. Data are presented as mean±s.d. of replicates of five independent experiments (**p<0.01). (d) mRNA levels of MAF and its isoform B (isoB) measured during in vitro monocytic differentiation of miR-9- and miR-SC (ctrl)-transduced CD34+-HSPCs on different time points (****p<0.0001). (e) RT-qPCR of miR-9 targets *FBN2*, *RUNX2*, *NOTCH2*, *NFKB1*, *NCOA3*, *NAP1L1*, *CDH1*, *ESR1*, *PRUNE*, *PAK4*, *LAMP1*, *ATF1*, and *SHC1* in transduced HSPCs on day 7 of monocytic in vitro differentiation. Data were normalized to housekeeping genes and miR-SC sample and are presented as relative change. (a, b, d, e) Data are presented as mean±s.d. of replicates of two independent experiments (*p<0.05; **p<0.01; ***p<0.001).

Supplementary Figure 7. Validation of miR-9 target genes.



(a) Western Blot of LIN28B and HMGA2 in MLL-rearranged patient blasts (N=1); Actin was used as loading control. (b) Expression of LIN28B cDNA 3 days after transduction using three LIN28B shRNAs (#1 - #3) relative to shNSC (%). (c) Expression of HMGA2 cDNA 3 days after transduction using three HMGA2 shRNAs (#1 - #3) relative to shNSC. (d) Methylcellulose colony assay of shRNA-transduced Kasumi-1 cells enumerated on day 14 of culture. (**p<0.01)

SUPPLEMENTARY TABLES

Supplementary Table 1. Differential expression of 253 differentially expressed miRNAs in AML patients.

Group	ID	logFC	t	P.Value	adj.P.Val	B
MLL	hsa.miR.126.4395339	-6.23	-13.06	2.12E-22	1.41E-19	40.49
MLL	hsa.miR.126..4373269	-7.28	-11.92	3.75E-20	1.25E-17	35.39
MLL	hsa.miR.130a.4373145	-5.17	-11.23	9.29E-19	2.06E-16	32.23
MLL	hsa.miR.181a.4373117	-3.06	-9.55	2.67E-15	4.43E-13	24.38
MLL	hsa.miR.196b.4395326	8.05	9.46	4.22E-15	5.60E-13	23.93
MLL	hsa.miR.181c.4373115	-3.53	-8.29	1.11E-12	1.23E-10	18.44
MLL	hsa.miR.26a.1..4395554	-5.79	-8.16	2.03E-12	1.93E-10	17.84
MLL	hsa.miR.9.4373285	6.98	7.97	4.94E-12	4.10E-10	16.97
MLL	hsa.miR.335.4373045	-2.50	-7.71	1.71E-11	1.26E-09	15.75
MLL	hsa.miR.9..4395342	6.75	7.52	4.22E-11	2.80E-09	14.86
MLL	hsa.miR.944.4395300	4.98	7.08	3.13E-10	1.89E-08	12.90
MLL	hsa.miR.222.4395387	-1.19	-6.17	1.96E-08	1.08E-06	8.85
MLL	hsa.miR.181a..4373086	-3.04	-5.67	1.75E-07	8.92E-06	6.71
MLL	hsa.miR.139.5p.4395400	-5.57	-5.62	2.12E-07	1.00E-05	6.53
MLL	hsa.miR.199a.3p.4395415	-1.47	-5.49	3.83E-07	1.68E-05	5.95
MLL	hsa.miR.99b.4373007	-4.41	-5.47	4.05E-07	1.68E-05	5.90
MLL	hsa.miR.374b.4381045	-0.94	-5.33	7.44E-07	2.91E-05	5.31
MLL	hsa.miR.146b.3p.4395472	-5.90	-5.27	9.32E-07	3.44E-05	5.09
MLL	hsa.miR.125a.5p.4395309	-3.09	-5.25	1.04E-06	3.45E-05	4.98
MLL	hsa.miR.200c.4395411	1.10	5.25	1.00E-06	3.45E-05	5.01
MLL	hsa.miR.766.4395177	-0.98	-5.19	1.32E-06	4.18E-05	4.75
MLL	hsa.miR.26a.4395166	-1.42	-5.15	1.56E-06	4.70E-05	4.59
MLL	hsa.miR.502.3p.4395194	3.84	5.12	1.75E-06	5.04E-05	4.48
MLL	hsa.miR.203.4373095	-5.37	-5.04	2.46E-06	6.81E-05	4.15
MLL	hsa.miR.149.4395366	4.78	4.90	4.28E-06	1.14E-04	3.61
MLL	hsa.miR.339.3p.4395295	1.19	4.83	5.54E-06	1.41E-04	3.36
MLL	hsa.miR.511.4373236	-4.28	-4.77	7.03E-06	1.73E-04	3.13
MLL	hsa.miR.660.4380925	1.50	4.68	1.02E-05	2.41E-04	2.78
MLL	hsa.miR.127.3p.4373147	-5.71	-4.59	1.43E-05	3.28E-04	2.45
MLL	hsa.miR.532.3p.4395466	1.31	4.58	1.53E-05	3.39E-04	2.39
MLL	hsa.miR.500..4373225	4.14	4.54	1.74E-05	3.73E-04	2.26
MLL	hsa.miR.501.3p.4395546	3.33	4.43	2.62E-05	5.45E-04	1.87
MLL	hsa.miR.376c.4395233	-5.29	-4.41	2.87E-05	5.77E-04	1.78
MLL	hsa.miR.187.4373307	3.77	4.40	3.01E-05	5.88E-04	1.74
MLL	hsa.miR.625.4395542	-1.25	-4.32	4.01E-05	7.60E-04	1.46
MLL	hsa.miR.320.4395388	-0.59	-4.31	4.13E-05	7.62E-04	1.43
MLL	hsa.miR.139.3p.4395424	-4.05	-4.15	7.65E-05	1.37E-03	0.85
MLL	hsa.miR.495.4381078	-4.39	-4.00	1.29E-04	2.25E-03	0.35
MLL	hsa.miR.340.4395369	-1.08	-3.98	1.42E-04	2.35E-03	0.26
MLL	hsa.miR.362.5p.4378092	1.97	3.98	1.39E-04	2.35E-03	0.28
MLL	hsa.miR.425..4395413	2.40	3.96	1.53E-04	2.48E-03	0.19
MLL	hsa.miR.195.4373105	-1.11	-3.80	2.63E-04	4.05E-03	-0.32
MLL	hsa.miR.874.4395379	-2.82	-3.81	2.56E-04	4.05E-03	-0.30
MLL	hsa.miR.135a.4373140	-3.56	-3.78	2.83E-04	4.27E-03	-0.39

Group	ID	logFC	t	P.Value	adj.P.Val	B
MLL	hsa.miR.500.4395539	2.35	3.77	2.91E-04	4.29E-03	-0.42
MLL	hsa.miR.30d.4373059	-0.74	-3.74	3.22E-04	4.65E-03	-0.52
MLL	hsa.miR.425.4380926	0.55	3.73	3.33E-04	4.70E-03	-0.55
MLL	hsa.miR.501.5p.4373226	3.38	3.72	3.47E-04	4.74E-03	-0.59
MLL	hsa.miR.654.3p.4395350	-2.92	-3.72	3.50E-04	4.74E-03	-0.60
MLL	hsa.miR.582.5p.4395175	4.14	3.71	3.58E-04	4.76E-03	-0.62
MLL	hsa.miR.432.4373280	-3.99	-3.70	3.67E-04	4.78E-03	-0.64
MLL	hsa.miR.30e.4395334	-0.73	-3.67	4.10E-04	5.23E-03	-0.74
MLL	hsa.miR.376a.4373026	-4.64	-3.65	4.35E-04	5.45E-03	-0.80
MLL	hsa.miR.628.5p.4395544	1.01	3.60	5.25E-04	6.45E-03	-0.98
MLL	hsa.miR.655.4381015	-3.62	-3.58	5.59E-04	6.72E-03	-1.04
MLL	hsa.miR.885.5p.4395407	2.31	3.58	5.67E-04	6.72E-03	-1.05
MLL	hsa.miR.200b..4395385	2.09	3.56	6.02E-04	7.01E-03	-1.11
MLL	hsa.miR.29a.4395223	-0.93	-3.54	6.31E-04	7.23E-03	-1.15
MLL	hsa.miR.642.4380995	3.02	3.53	6.64E-04	7.47E-03	-1.20
MLL	hsa.miR.486.5p.4378096	-1.17	-3.50	7.30E-04	8.07E-03	-1.29
MLL	hsa.miR.502.5p.4373227	3.37	3.47	7.97E-04	8.67E-03	-1.37
MLL	hsa.miR.323.3p.4395338	-2.53	-3.46	8.35E-04	8.80E-03	-1.41
MLL	hsa.miR.143..4395257	1.21	3.46	8.33E-04	8.80E-03	-1.41
MLL	hsa.miR.431.4395173	-4.13	-3.45	8.66E-04	8.99E-03	-1.45
MLL	hsa.miR.410.4378093	-3.39	-3.43	9.24E-04	9.44E-03	-1.51
MLL	hsa.miR.136..4395211	-3.93	-3.42	9.41E-04	9.47E-03	-1.52
MLL	hsa.miR.493.4395475	-3.29	-3.40	1.01E-03	1.00E-02	-1.59
MLL	hsa.miR.299.5p.4373188	-3.41	-3.39	1.03E-03	1.00E-02	-1.61
MLL	hsa.miR.451.4373360	-3.92	-3.38	1.08E-03	1.04E-02	-1.65
MLL	hsa.miR.374a.4373028	-0.68	-3.36	1.15E-03	1.09E-02	-1.71
MLL	hsa.miR.135a..4395343	-0.92	-3.35	1.18E-03	1.11E-02	-1.74
MLL	hsa.miR.30a.4373061	-0.73	-3.33	1.26E-03	1.16E-02	-1.79
MLL	hsa.miR.30b.4373290	-0.61	-3.32	1.30E-03	1.17E-02	-1.83
MLL	hsa.miR.889.4395313	-2.99	-3.32	1.29E-03	1.17E-02	-1.82
MLL	hsa.miR.411.4381013	-4.06	-3.30	1.38E-03	1.22E-02	-1.88
MLL	hsa.let.7f.4373164	-1.06	-3.30	1.41E-03	1.23E-02	-1.90
MLL	hsa.miR.181c..4395444	-2.85	-3.26	1.60E-03	1.38E-02	-2.02
MLL	hsa.miR.539.4378103	-3.97	-3.20	1.92E-03	1.63E-02	-2.18
MLL	hsa.miR.26b.4395167	-0.87	-3.19	1.95E-03	1.64E-02	-2.20
MLL	hsa.miR.424.4373201	-3.54	-3.18	2.02E-03	1.67E-02	-2.23
MLL	hsa.miR.661.4381009	-1.39	-3.18	2.04E-03	1.67E-02	-2.24
MLL	hsa.miR.622.4380961	-2.23	-3.17	2.11E-03	1.71E-02	-2.27
MLL	hsa.miR.654.5p.4381014	-2.41	-3.15	2.24E-03	1.79E-02	-2.33
MLL	hsa.miR.125a.3p.4395310	-2.94	-3.14	2.32E-03	1.81E-02	-2.36

Group	ID	logFC	t	P.Value	adj.P.Val	B
MLL	hsa.miR.598.4395179	2.94	3.13	2.35E-03	1.81E-02	-2.38
MLL	hsa.miR.616.4395525	2.62	3.13	2.37E-03	1.81E-02	-2.38
MLL	hsa.miR.768.3p.4395188	-0.57	-3.14	2.31E-03	1.81E-02	-2.36
MLL	hsa.miR.363.4378090	3.29	3.11	2.50E-03	1.89E-02	-2.43
MLL	hsa.miR.224.4395210	-4.22	-3.09	2.64E-03	1.96E-02	-2.48
MLL	hsa.miR.369.5p.4373195	-2.30	-3.09	2.69E-03	1.96E-02	-2.50
MLL	hsa.miR.491.5p.4381053	-0.67	-3.09	2.68E-03	1.96E-02	-2.49
MLL	hsa.miR.339.5p.4395368	1.26	3.07	2.80E-03	2.02E-02	-2.54
MLL	hsa.miR.134.4373299	-3.36	-3.03	3.22E-03	2.30E-02	-2.66
MLL	hsa.miR.200a.4378069	2.89	3.02	3.27E-03	2.31E-02	-2.68
MLL	hsa.miR.28.3p.4395557	0.48	3.01	3.45E-03	2.41E-02	-2.73
MLL	hsa.miR.197.4373102	0.51	3.00	3.54E-03	2.43E-02	-2.75
MLL	hsa.miR.587.4380950	-2.37	-3.00	3.51E-03	2.43E-02	-2.74
MLL	hsa.miR.133a.4395357	-2.85	-2.99	3.63E-03	2.46E-02	-2.77
MLL	hsa.miR.146a.4373132	-3.11	-2.96	3.94E-03	2.64E-02	-2.85
MLL	hsa.miR.409.5p.4395442	-2.14	-2.92	4.38E-03	2.88E-02	-2.95
MLL	hsa.miR.941.4395294	1.86	2.93	4.37E-03	2.88E-02	-2.94
MLL	hsa.miR.518b.4373246	-1.59	-2.90	4.65E-03	3.03E-02	-3.00
MLL	hsa.miR.429.4373203	2.23	2.89	4.89E-03	3.15E-02	-3.05
MLL	hsa.miR.143.4395360	-3.31	-2.87	5.17E-03	3.29E-02	-3.10
MLL	hsa.miR.532.5p.4380928	1.54	2.87	5.20E-03	3.29E-02	-3.10
MLL	hsa.miR.16.4373121	-0.49	-2.84	5.53E-03	3.40E-02	-3.16
MLL	hsa.miR.382.4373019	-3.76	-2.84	5.52E-03	3.40E-02	-3.16
MLL	hsa.miR.628.3p.4395545	1.15	2.84	5.52E-03	3.40E-02	-3.16
MLL	hsa.miR.191.4395410	0.46	2.84	5.61E-03	3.42E-02	-3.17
MLL	hsa.miR.30c.4373060	-0.51	-2.83	5.69E-03	3.43E-02	-3.18
MLL	hsa.miR.29c.4395171	-0.62	-2.81	6.01E-03	3.57E-02	-3.23
MLL	hsa.miR.509.5p.4395346	-2.38	-2.81	6.03E-03	3.57E-02	-3.24
MLL	hsa.miR.933.4395287	1.46	2.80	6.20E-03	3.64E-02	-3.26
MLL	hsa.miR.340..4395370	-1.62	-2.77	6.77E-03	3.94E-02	-3.34
MLL	hsa.miR.193b.4395478	-2.10	-2.76	6.94E-03	3.97E-02	-3.37
MLL	hsa.miR.223.4395406	0.73	2.76	6.94E-03	3.97E-02	-3.36
MLL	hsa.miR.220b.4395317	-1.82	-2.75	7.16E-03	4.06E-02	-3.39
MLL	hsa.miR.497.4373222	-2.22	-2.75	7.30E-03	4.11E-02	-3.41
MLL	hsa.miR.551a.4380929	-2.59	-2.70	8.39E-03	4.68E-02	-3.54
AMLETO	hsa.miR.196b.4395326	-8.17	-7.53	3.92E-11	2.60E-08	15.06
AMLETO	hsa.miR.9.4373285	-7.58	-7.22	1.63E-10	5.42E-08	13.69
AMLETO	hsa.miR.126.4395339	5.13	6.69	1.88E-09	4.16E-07	11.35
AMLETO	hsa.miR.126..4373269	5.46	5.60	2.35E-07	3.90E-05	6.73

Group	ID	logFC	t	P.Value	adj.P.Val	B
AMLETO	hsa.miR.660.4380925	-1.92	-5.34	7.05E-07	9.37E-05	5.68
AMLETO	hsa.miR.532.3p.4395466	-1.62	-5.02	2.64E-06	2.92E-04	4.43
AMLETO	hsa.miR.628.5p.4395544	-1.50	-4.89	4.36E-06	4.14E-04	3.95
AMLETO	hsa.miR.582.5p.4395175	-5.87	-4.76	7.38E-06	6.13E-04	3.45
AMLETO	hsa.miR.125b.4373148	-4.56	-4.56	1.62E-05	1.08E-03	2.71
AMLETO	hsa.miR.9..4395342	-5.44	-4.57	1.57E-05	1.08E-03	2.74
AMLETO	hsa.miR.27a.4373287	-1.59	-4.50	2.08E-05	1.25E-03	2.47
AMLETO	hsa.miR.301a.4373064	-1.26	-4.44	2.54E-05	1.41E-03	2.29
AMLETO	hsa.miR.139.5p.4395400	5.23	4.33	3.91E-05	1.73E-03	1.88
AMLETO	hsa.miR.152.4395170	-3.89	-4.33	3.87E-05	1.73E-03	1.89
AMLETO	hsa.miR.450a.4395414	-5.31	-4.36	3.42E-05	1.73E-03	2.00
AMLETO	hsa.miR.28.3p.4395557	-0.74	-4.20	6.34E-05	2.63E-03	1.43
AMLETO	hsa.miR.223.4395406	-1.18	-4.08	9.90E-05	3.87E-03	1.01
AMLETO	hsa.miR.221.4373077	-1.53	-4.05	1.09E-04	4.03E-03	0.92
AMLETO	hsa.miR.885.5p.4395407	-2.93	-4.00	1.31E-04	4.15E-03	0.75
AMLETO	hsa.miR.99a.4373008	-2.66	-4.01	1.27E-04	4.15E-03	0.78
AMLETO	hsa.miR.27a..4395556	-1.57	-4.01	1.25E-04	4.15E-03	0.79
AMLETO	hsa.miR.574.3p.4395460	-1.26	-3.96	1.48E-04	4.47E-03	0.63
AMLETO	hsa.miR.23a.4373074	-1.94	-3.94	1.61E-04	4.65E-03	0.55
AMLETO	hsa.miR.20b.4373263	-1.27	-3.79	2.76E-04	7.46E-03	0.05
AMLETO	hsa.miR.362.5p.4378092	-2.17	-3.78	2.81E-04	7.46E-03	0.04
AMLETO	hsa.miR.21.4373090	-1.60	-3.77	2.94E-04	7.50E-03	-0.01
AMLETO	hsa.miR.100.4373160	-3.81	-3.76	3.06E-04	7.52E-03	-0.04
AMLETO	hsa.miR.424.4373201	-4.67	-3.72	3.53E-04	8.38E-03	-0.18
AMLETO	hsa.miR.122.4395356	2.28	3.64	4.52E-04	1.03E-02	-0.40
AMLETO	hsa.miR.320.4395388	0.59	3.63	4.66E-04	1.03E-02	-0.43
AMLETO	hsa.miR.532.5p.4380928	-2.15	-3.57	5.80E-04	1.24E-02	-0.64
AMLETO	hsa.miR.502.5p.4373227	-3.96	-3.55	6.13E-04	1.27E-02	-0.69
AMLETO	hsa.miR.548b.5p.4395519	-2.83	-3.54	6.40E-04	1.29E-02	-0.73
AMLETO	hsa.miR.210.4373089	-1.91	-3.49	7.42E-04	1.45E-02	-0.86
AMLETO	hsa.miR.27b.4373068	-2.33	-3.48	7.69E-04	1.46E-02	-0.90
AMLETO	hsa.miR.365.4373194	-1.98	-3.40	9.98E-04	1.84E-02	-1.14
AMLETO	hsa.miR.124.4373295	-1.09	-3.38	1.06E-03	1.91E-02	-1.19
AMLETO	hsa.miR.130a.4373145	2.57	3.30	1.37E-03	2.28E-02	-1.43
AMLETO	hsa.miR.618.4380996	-2.40	-3.30	1.37E-03	2.28E-02	-1.43
AMLETO	hsa.miR.181c..4395444	3.34	3.31	1.37E-03	2.28E-02	-1.42
AMLETO	hsa.miR.200b.4395362	-1.96	-3.27	1.51E-03	2.45E-02	-1.52
AMLETO	hsa.let.7c.4373167	-2.23	-3.19	1.95E-03	3.09E-02	-1.75
AMLETO	hsa.miR.24.4373072	-0.67	-3.18	2.01E-03	3.10E-02	-1.77

Group	ID	logFC	t	P.Value	adj.P.Val	B
AMLETO	hsa.miR.424..4395420	-3.86	-3.14	2.28E-03	3.45E-02	-1.89
AMLETO	hsa.miR.10a.4373153	-3.40	-3.11	2.49E-03	3.68E-02	-1.97
AMLETO	hsa.miR.199b.5p.4373100	-3.27	-3.08	2.72E-03	3.92E-02	-2.05
AMLETO	hsa.miR.149.4395366	-3.68	-3.05	2.98E-03	4.11E-02	-2.14
AMLETO	hsa.miR.193a.5p.4395392	-1.08	-3.04	3.12E-03	4.11E-02	-2.18
AMLETO	hsa.miR.503.4373228	-3.67	-3.04	3.14E-03	4.11E-02	-2.18
AMLETO	hsa.let.7f.1..4395528	-3.59	-3.05	3.01E-03	4.11E-02	-2.14
AMLETO	hsa.miR.190b.4395374	3.31	3.03	3.15E-03	4.11E-02	-2.19
AMLETO	hsa.miR.28.5p.4373067	-0.83	-3.01	3.39E-03	4.24E-02	-2.25
AMLETO	hsa.miR.361.5p.4373035	-1.43	-3.02	3.34E-03	4.24E-02	-2.24
AMLETO	hsa.miR.708.4395452	-2.99	-2.98	3.73E-03	4.59E-02	-2.34
inv16	hsa.let.7c.4373167	4.05	6.05	3.38E-08	2.25E-05	8.60
inv16	hsa.miR.511.4373236	6.10	5.69	1.59E-07	5.30E-05	7.12
inv16	hsa.miR.99a..4395252	6.67	5.59	2.41E-07	5.33E-05	6.74
inv16	hsa.miR.766.4395177	1.27	5.49	3.72E-07	6.17E-05	6.32
inv16	hsa.miR.139.5p.4395400	5.76	4.43	2.72E-05	3.01E-03	2.28
inv16	hsa.miR.192.4373108	-1.05	-4.44	2.62E-05	3.01E-03	2.32
inv16	hsa.miR.126.4395339	3.93	4.25	5.22E-05	3.98E-03	1.68
inv16	hsa.miR.335.4373045	2.04	4.31	4.23E-05	3.98E-03	1.87
inv16	hsa.miR.30d.4373059	1.02	4.24	5.39E-05	3.98E-03	1.64
inv16	hsa.miR.27a..4395556	1.74	4.12	8.33E-05	5.32E-03	1.24
inv16	hsa.miR.30a.4373061	1.09	4.11	8.81E-05	5.32E-03	1.19
inv16	hsa.miR.500.4395539	-3.02	-3.91	1.78E-04	9.85E-03	0.53
inv16	hsa.miR.363.4378090	-4.92	-3.83	2.35E-04	1.20E-02	0.28
inv16	hsa.miR.584.4381026	3.38	3.79	2.73E-04	1.29E-02	0.14
inv16	hsa.miR.944.4395300	-3.76	-3.69	3.89E-04	1.72E-02	-0.19
inv16	hsa.miR.126..4373269	4.03	3.50	7.19E-04	2.98E-02	-0.75
inv16	hsa.miR.194.4373106	-2.79	-3.46	8.20E-04	3.20E-02	-0.87
inv16	hsa.miR.133a.4395357	3.97	3.39	1.06E-03	3.91E-02	-1.10
inv16	hsa.miR.139.3p.4395424	4.17	3.32	1.29E-03	4.50E-02	-1.28
PMLRARA	hsa.miR.409.5p.4395442	9.03	16.70	3.48E-29	2.31E-26	55.66
PMLRARA	hsa.miR.654.5p.4381014	9.22	14.84	8.22E-26	2.73E-23	48.11
PMLRARA	hsa.miR.369.5p.4373195	8.70	13.53	2.55E-23	5.64E-21	42.51
PMLRARA	hsa.miR.654.3p.4395350	9.28	13.18	1.21E-22	2.02E-20	40.99
PMLRARA	hsa.miR.370.4395386	11.89	12.32	6.13E-21	8.14E-19	37.15
PMLRARA	hsa.miR.495.4381078	12.74	12.14	1.37E-20	1.52E-18	36.36
PMLRARA	hsa.miR.369.3p.4373032	6.26	11.98	2.85E-20	2.70E-18	35.64
PMLRARA	hsa.miR.224.4395210	14.67	10.90	4.44E-18	3.68E-16	30.69
PMLRARA	hsa.miR.452.4395440	13.06	10.76	8.63E-18	6.37E-16	30.04

Group	ID	logFC	t	P.Value	adj.P.Val	B
PMLRARA	hsa.miR.431.4395173	12.81	10.45	3.82E-17	2.54E-15	28.58
PMLRARA	hsa.miR.485.5p.4373212	7.28	10.09	2.02E-16	1.22E-14	26.94
PMLRARA	hsa.miR.889.4395313	9.42	10.05	2.53E-16	1.40E-14	26.72
PMLRARA	hsa.miR.134.4373299	11.40	9.89	5.30E-16	2.71E-14	25.99
PMLRARA	hsa.miR.655.4381015	10.43	9.56	2.59E-15	1.23E-13	24.43
PMLRARA	hsa.miR.488.4395468	6.22	9.12	2.15E-14	9.51E-13	22.36
PMLRARA	hsa.miR.376c.4395233	12.21	8.80	9.80E-14	4.07E-12	20.87
PMLRARA	hsa.miR.493..4373218	5.45	8.70	1.54E-13	6.01E-12	20.43
PMLRARA	hsa.miR.127.3p.4373147	12.64	8.64	2.07E-13	7.63E-12	20.14
PMLRARA	hsa.miR.493.4395475	9.40	8.62	2.28E-13	7.96E-12	20.04
PMLRARA	hsa.miR.99a.4373008	5.57	8.26	1.28E-12	4.26E-11	18.35
PMLRARA	hsa.miR.668.4395181	6.02	8.21	1.61E-12	5.10E-11	18.12
PMLRARA	hsa.miR.193b.4395478	7.00	8.13	2.32E-12	7.01E-11	17.76
PMLRARA	hsa.miR.376a.4373026	11.74	7.81	1.06E-11	3.07E-10	16.27
PMLRARA	hsa.miR.487b.4378102	11.10	7.64	2.36E-11	6.53E-10	15.49
PMLRARA	hsa.miR.299.5p.4373188	9.07	7.62	2.57E-11	6.83E-10	15.41
PMLRARA	hsa.miR.539.4378103	11.04	7.51	4.34E-11	1.11E-09	14.89
PMLRARA	hsa.miR.410.4378093	8.83	7.44	6.10E-11	1.50E-09	14.56
PMLRARA	hsa.miR.411.4381013	10.85	7.38	8.12E-11	1.93E-09	14.28
PMLRARA	hsa.miR.136..4395211	10.09	7.28	1.29E-10	2.95E-09	13.83
PMLRARA	hsa.miR.323.3p.4395338	6.41	7.23	1.61E-10	3.56E-09	13.61
PMLRARA	hsa.miR.432.4373280	9.40	7.09	3.12E-10	6.68E-09	12.96
PMLRARA	hsa.miR.365.4373194	4.31	6.98	5.17E-10	1.07E-08	12.47
PMLRARA	hsa.miR.543.4395487	6.92	6.96	5.58E-10	1.12E-08	12.40
PMLRARA	hsa.miR.29c.4395171	1.84	6.91	6.90E-10	1.35E-08	12.19
PMLRARA	hsa.miR.193b..4395477	6.95	6.87	8.31E-10	1.58E-08	12.00
PMLRARA	hsa.miR.29a.4395223	2.22	6.84	9.57E-10	1.77E-08	11.87
PMLRARA	hsa.miR.758.4395180	7.37	6.83	9.91E-10	1.78E-08	11.83
PMLRARA	hsa.miR.374b.4381045	1.59	6.78	1.25E-09	2.14E-08	11.60
PMLRARA	hsa.miR.382.4373019	10.92	6.78	1.26E-09	2.14E-08	11.60
PMLRARA	hsa.miR.181c.4373115	4.43	6.66	2.14E-09	3.55E-08	11.08
PMLRARA	hsa.miR.199a.3p.4395415	2.42	6.66	2.22E-09	3.60E-08	11.04
PMLRARA	hsa.miR.195.4373105	2.44	6.59	3.02E-09	4.66E-08	10.74
PMLRARA	hsa.miR.409.3p.4395443	9.55	6.59	2.98E-09	4.66E-08	10.76
PMLRARA	hsa.miR.374a.4373028	1.63	6.45	5.58E-09	8.42E-08	10.15
PMLRARA	hsa.miR.433.4373205	8.05	6.18	1.90E-08	2.81E-07	8.95
PMLRARA	hsa.miR.181a.4373117	3.34	6.08	2.91E-08	4.21E-07	8.54
PMLRARA	hsa.miR.337.3p.4395268	5.76	5.94	5.46E-08	7.71E-07	7.92
PMLRARA	hsa.miR.491.5p.4381053	1.63	5.88	7.13E-08	9.86E-07	7.67

Group	ID	logFC	t	P.Value	adj.P.Val	B
PMLRARA	hsa.miR.574.3p.4395460	2.13	5.83	8.88E-08	1.20E-06	7.45
PMLRARA	hsa.let.7f.4373164	2.38	5.65	1.88E-07	2.49E-06	6.73
PMLRARA	hsa.miR.220b.4395317	4.80	5.65	1.93E-07	2.52E-06	6.70
PMLRARA	hsa.miR.203.4373095	8.34	5.59	2.48E-07	3.17E-06	6.45
PMLRARA	hsa.miR.503.4373228	7.55	5.54	2.99E-07	3.74E-06	6.27
PMLRARA	hsa.miR.100.4373160	6.46	5.51	3.47E-07	4.27E-06	6.13
PMLRARA	hsa.miR.324.3p.4395272	1.56	5.50	3.64E-07	4.39E-06	6.08
PMLRARA	hsa.miR.770.5p.4395189	6.36	5.47	4.01E-07	4.76E-06	5.99
PMLRARA	hsa.miR.151.3p.4395365	-4.12	-5.36	6.43E-07	7.49E-06	5.53
PMLRARA	hsa.let.7e.4395517	1.91	5.35	6.82E-07	7.81E-06	5.47
PMLRARA	hsa.miR.379.4373349	7.91	5.26	1.00E-06	1.13E-05	5.10
PMLRARA	hsa.miR.381.4373020	4.70	5.20	1.24E-06	1.37E-05	4.90
PMLRARA	hsa.miR.424.4373201	7.55	5.11	1.85E-06	2.02E-05	4.51
PMLRARA	hsa.let.7a.4373169	2.00	5.02	2.66E-06	2.85E-05	4.16
PMLRARA	hsa.miR.504.4395195	5.73	4.95	3.43E-06	3.62E-05	3.92
PMLRARA	hsa.miR.625.4395542	1.97	4.90	4.34E-06	4.50E-05	3.69
PMLRARA	hsa.let.7g.4395393	1.56	4.79	6.54E-06	6.58E-05	3.29
PMLRARA	hsa.miR.19a.4373099	1.65	4.80	6.46E-06	6.58E-05	3.31
PMLRARA	hsa.miR.340.4395369	1.79	4.74	8.20E-06	8.13E-05	3.08
PMLRARA	hsa.miR.494.4395476	2.14	4.69	9.89E-06	9.66E-05	2.90
PMLRARA	hsa.let.7d.4395394	1.92	4.64	1.21E-05	1.16E-04	2.71
PMLRARA	hsa.miR.337.5p.4395267	7.56	4.63	1.23E-05	1.16E-04	2.69
PMLRARA	hsa.miR.542.3p.4378101	6.07	4.63	1.25E-05	1.17E-04	2.67
PMLRARA	hsa.miR.221.4373077	2.12	4.61	1.33E-05	1.22E-04	2.62
PMLRARA	hsa.miR.212.4373087	-4.25	-4.59	1.42E-05	1.29E-04	2.55
PMLRARA	hsa.miR.450a.4395414	6.88	4.59	1.46E-05	1.31E-04	2.52
PMLRARA	hsa.miR.485.3p.4378095	4.67	4.55	1.66E-05	1.47E-04	2.40
PMLRARA	hsa.miR.125b.4373148	5.63	4.52	1.92E-05	1.67E-04	2.26
PMLRARA	hsa.miR.335.4373045	2.37	4.34	3.70E-05	3.19E-04	1.63
PMLRARA	hsa.miR.136.4373173	4.48	4.31	4.19E-05	3.57E-04	1.51
PMLRARA	hsa.miR.656.4380920	4.95	4.27	4.96E-05	4.17E-04	1.36
PMLRARA	hsa.miR.26a.4395166	1.73	4.22	5.84E-05	4.85E-04	1.20
PMLRARA	hsa.miR.744.4395435	1.41	4.18	6.74E-05	5.52E-04	1.06
PMLRARA	hsa.miR.411..4395349	5.39	4.16	7.44E-05	5.97E-04	0.97
PMLRARA	hsa.miR.622.4380961	4.05	4.15	7.47E-05	5.97E-04	0.97
PMLRARA	hsa.miR.329.4373191	2.10	4.14	7.84E-05	6.20E-04	0.92
PMLRARA	hsa.miR.450b.5p.4395318	6.87	3.95	1.56E-04	1.22E-03	0.27
PMLRARA	hsa.miR.19b.4373098	1.17	3.91	1.77E-04	1.37E-03	0.15
PMLRARA	hsa.miR.16.4373121	0.92	3.89	1.96E-04	1.48E-03	0.05

Group	ID	logFC	t	P.Value	adj.P.Val	B
PMLRARA	hsa.miR.542.5p.4395351	5.96	3.89	1.95E-04	1.48E-03	0.06
PMLRARA	hsa.miR.148a.4373130	4.21	3.85	2.25E-04	1.68E-03	-0.08
PMLRARA	hsa.miR.455.3p.4395355	5.19	3.81	2.54E-04	1.87E-03	-0.19
PMLRARA	hsa.miR.103.4373158	1.17	3.79	2.71E-04	1.98E-03	-0.25
PMLRARA	hsa.miR.186.4395396	0.80	3.73	3.37E-04	2.43E-03	-0.46
PMLRARA	hsa.miR.222..4395208	1.42	3.73	3.40E-04	2.43E-03	-0.47
PMLRARA	hsa.miR.423.5p.4395451	1.52	3.72	3.51E-04	2.48E-03	-0.50
PMLRARA	hsa.miR.361.5p.4373035	2.13	3.69	3.92E-04	2.74E-03	-0.60
PMLRARA	hsa.miR.30c.4373060	0.91	3.65	4.41E-04	3.05E-03	-0.71
PMLRARA	hsa.miR.299.3p.4373189	4.88	3.58	5.52E-04	3.78E-03	-0.92
PMLRARA	hsa.miR.128.4395327	2.23	3.57	5.71E-04	3.83E-03	-0.96
PMLRARA	hsa.miR.450b.3p.4395319	2.45	3.57	5.70E-04	3.83E-03	-0.95
PMLRARA	hsa.miR.135a..4395343	1.39	3.56	5.92E-04	3.93E-03	-0.99
PMLRARA	hsa.miR.125b.1..4395489	3.73	3.54	6.41E-04	4.21E-03	-1.06
PMLRARA	hsa.miR.26b.4395167	1.35	3.51	7.03E-04	4.57E-03	-1.15
PMLRARA	hsa.miR.181a..4373086	2.93	3.48	7.90E-04	5.09E-03	-1.26
PMLRARA	hsa.miR.454.4395434	0.97	3.46	8.38E-04	5.35E-03	-1.31
PMLRARA	hsa.miR.222.4395387	1.07	3.45	8.59E-04	5.43E-03	-1.34
PMLRARA	hsa.miR.15b.4373122	1.02	3.40	1.02E-03	6.36E-03	-1.50
PMLRARA	hsa.miR.17.4395419	0.96	3.40	1.02E-03	6.36E-03	-1.50
PMLRARA	hsa.miR.380.4373022	1.90	3.37	1.11E-03	6.85E-03	-1.58
PMLRARA	hsa.miR.210.4373089	2.28	3.34	1.23E-03	7.52E-03	-1.67
PMLRARA	hsa.miR.202.4395474	2.47	3.33	1.25E-03	7.57E-03	-1.69
PMLRARA	hsa.miR.658.4380923	1.00	3.32	1.30E-03	7.77E-03	-1.72
PMLRARA	hsa.miR.455.5p.4378098	4.52	3.30	1.40E-03	8.30E-03	-1.79
PMLRARA	hsa.miR.145.4395389	2.64	3.28	1.49E-03	8.75E-03	-1.85
PMLRARA	hsa.miR.497.4373222	3.73	3.27	1.54E-03	8.95E-03	-1.88
PMLRARA	hsa.miR.106b.4373155	0.99	3.24	1.66E-03	9.58E-03	-1.95
PMLRARA	hsa.miR.196b.4395326	-5.29	-3.24	1.69E-03	9.67E-03	-1.97
PMLRARA	hsa.miR.376a..4395238	2.68	3.16	2.16E-03	1.23E-02	-2.19
PMLRARA	hsa.miR.618.4380996	2.85	3.13	2.36E-03	1.33E-02	-2.27
PMLRARA	hsa.miR.130b.4373144	1.41	3.11	2.50E-03	1.40E-02	-2.33
PMLRARA	hsa.miR.199b.5p.4373100	4.10	3.11	2.52E-03	1.40E-02	-2.34
PMLRARA	hsa.miR.924.4395265	1.23	3.07	2.83E-03	1.56E-02	-2.44
PMLRARA	hsa.miR.487a.4378097	2.17	3.03	3.24E-03	1.77E-02	-2.57
PMLRARA	hsa.miR.23b.4373073	3.96	3.00	3.46E-03	1.87E-02	-2.63
PMLRARA	hsa.miR.20b.4373263	1.28	3.00	3.52E-03	1.89E-02	-2.64
PMLRARA	hsa.miR.15a.4373123	1.07	2.94	4.15E-03	2.19E-02	-2.79
PMLRARA	hsa.miR.548b.5p.4395519	2.98	2.94	4.15E-03	2.19E-02	-2.79

Group	ID	logFC	t	P.Value	adj.P.Val	B
PMLRARA	hsa.miR.768.3p.4395188	0.77	2.93	4.28E-03	2.22E-02	-2.82
PMLRARA	hsa.miR.943.4395299	2.36	2.93	4.28E-03	2.22E-02	-2.82
PMLRARA	hsa.miR.874.4395379	3.19	2.92	4.45E-03	2.29E-02	-2.86
PMLRARA	hsa.miR.518b.4373246	2.29	2.90	4.63E-03	2.37E-02	-2.89
PMLRARA	hsa.miR.301a.4373064	1.07	2.88	5.04E-03	2.56E-02	-2.97
PMLRARA	hsa.miR.7.2..4395425	3.43	2.87	5.14E-03	2.59E-02	-2.99
PMLRARA	hsa.miR.331.3p.4373046	0.54	2.86	5.25E-03	2.62E-02	-3.01
PMLRARA	hsa.miR.130a.4373145	2.79	2.84	5.65E-03	2.80E-02	-3.07
PMLRARA	hsa.miR.139.5p.4395400	-4.47	-2.82	5.89E-03	2.88E-02	-3.11
PMLRARA	hsa.miR.223..4395209	-0.91	-2.82	5.89E-03	2.88E-02	-3.11
PMLRARA	hsa.miR.106a.4395280	0.81	2.81	6.07E-03	2.94E-02	-3.14
PMLRARA	hsa.miR.154.4373270	2.15	2.80	6.30E-03	3.03E-02	-3.17
PMLRARA	hsa.miR.20a.4373286	1.14	2.78	6.54E-03	3.11E-02	-3.21
PMLRARA	hsa.miR.301b.4395503	1.78	2.78	6.61E-03	3.11E-02	-3.22
PMLRARA	hsa.miR.509.3p.4395347	1.42	2.78	6.61E-03	3.11E-02	-3.22
PMLRARA	hsa.miR.124.4373295	1.13	2.77	6.87E-03	3.19E-02	-3.25
PMLRARA	hsa.miR.628.5p.4395544	1.14	2.77	6.84E-03	3.19E-02	-3.25
PMLRARA	hsa.miR.939.4395293	1.04	2.76	7.11E-03	3.28E-02	-3.28
PMLRARA	hsa.miR.99b.4373007	3.53	2.75	7.21E-03	3.30E-02	-3.29
PMLRARA	hsa.miR.187.4373307	-3.57	-2.75	7.30E-03	3.32E-02	-3.31
PMLRARA	hsa.miR.152.4395170	3.23	2.74	7.50E-03	3.39E-02	-3.33
PMLRARA	hsa.miR.204.4373094	2.33	2.70	8.30E-03	3.70E-02	-3.42
PMLRARA	hsa.miR.661.4381009	1.72	2.70	8.25E-03	3.70E-02	-3.42
PMLRARA	hsa.miR.342.3p.4395371	0.71	2.68	8.80E-03	3.90E-02	-3.47
PMLRARA	hsa.miR.101.4395364	1.18	2.67	8.93E-03	3.93E-02	-3.49
PMLRARA	hsa.miR.551a.4380929	3.68	2.67	9.00E-03	3.93E-02	-3.49
PMLRARA	hsa.miR.579.4395509	2.83	2.66	9.21E-03	4.00E-02	-3.51
PMLRARA	hsa.miR.556.5p.4395455	2.27	2.65	9.63E-03	4.15E-02	-3.55
PMLRARA	hsa.miR.877.4395402	0.98	2.64	9.85E-03	4.22E-02	-3.58
PMLRARA	hsa.miR.767.5p.4395182	0.69	2.62	1.04E-02	4.45E-02	-3.63
PMLRARA	hsa.miR.451.4373360	4.43	2.60	1.10E-02	4.63E-02	-3.67
PMLRARA	hsa.miR.25..4395553	-3.26	-2.58	1.17E-02	4.89E-02	-3.73
PMLRARA	hsa.miR.936.4395290	0.45	2.57	1.17E-02	4.89E-02	-3.73
PMLRARA	hsa.miR.18a..4395534	-1.59	-2.57	1.20E-02	4.97E-02	-3.75
T712	hsa.miR.98.4373009	-7.65	-9.64	1.74E-15	1.15E-12	24.55
T712	hsa.miR.525.5p.4378088	1.91	6.20	1.75E-08	5.81E-06	9.22
T712	hsa.let.7d.4395394	-3.44	-5.94	5.33E-08	8.84E-06	8.16
T712	hsa.miR.372.4373029	4.42	5.99	4.28E-08	8.84E-06	8.37
T712	hsa.miR.28.3p.4395557	-1.70	-5.54	2.99E-07	3.98E-05	6.53

Group	ID	logFC	t	P.Value	adj.P.Val	B
T712	hsa.miR.191.4395410	-1.63	-5.21	1.23E-06	1.36E-04	5.19
T712	hsa.miR.574.3p.4395460	-2.79	-4.93	3.71E-06	3.52E-04	4.15
T712	hsa.miR.484.4381032	-1.39	-4.55	1.69E-05	1.32E-03	2.73
T712	hsa.miR.588.4380952	1.93	4.54	1.79E-05	1.32E-03	2.67
T712	hsa.let.7e.4395517	-2.47	-4.48	2.19E-05	1.45E-03	2.48
T712	hsa.miR.518f..4395498	1.08	4.43	2.68E-05	1.62E-03	2.29
T712	hsa.let.7b.4395446	-2.57	-4.31	4.14E-05	2.27E-03	1.89
T712	hsa.miR.345.4395297	-1.63	-4.29	4.59E-05	2.27E-03	1.79
T712	hsa.miR.886.3p.4395305	-5.90	-4.28	4.78E-05	2.27E-03	1.75
T712	hsa.let.7f.4373164	-2.75	-4.12	8.54E-05	3.78E-03	1.21
T712	hsa.miR.197.4373102	-1.44	-4.10	9.23E-05	3.83E-03	1.14
T712	hsa.let.7a.4373169	-2.42	-3.91	1.82E-04	7.12E-03	0.51
T712	hsa.miR.628.5p.4395544	-2.28	-3.87	2.11E-04	7.77E-03	0.38
T712	hsa.miR.199a.5p.4373272	2.89	3.66	4.23E-04	1.48E-02	-0.27
T712	hsa.miR.628.3p.4395545	-3.05	-3.63	4.73E-04	1.57E-02	-0.37
T712	hsa.miR.425.4380926	-1.12	-3.56	5.95E-04	1.77E-02	-0.58
T712	hsa.miR.214..4395404	4.80	3.56	6.06E-04	1.77E-02	-0.60
T712	hsa.miR.612.4380983	3.27	3.55	6.14E-04	1.77E-02	-0.61
T712	hsa.miR.149.4395366	-7.66	-3.49	7.45E-04	2.01E-02	-0.79
T712	hsa.miR.20b..4395422	3.87	3.49	7.56E-04	2.01E-02	-0.80
T712	hsa.let.7g.4395393	-1.74	-3.43	9.27E-04	2.37E-02	-0.99
T712	hsa.miR.550..4380954	-3.25	-3.39	1.05E-03	2.59E-02	-1.10

Supplementary Table 2. Top-25 miR-9 targets downregulated in miR-9-transduced HSPCs (n=1) andKASUMI-1 (n=2).

*p<0.05, **p<0.01, ***p<0.001

probe_ID	Gene Symbol	Mean	miR-sc - miR-9			Average difference	Target	Spearman R miR-9 vs probe in patients
			CB-HSPC	KASUMI-1 batch 3	KASUMI-1 batch 5			
229349_at	LIN28B	9.08	-0.91	-0.61	-0.79	-0.77	Predicted	0.08
202733_at	P4HA2	6.83	-0.91	-0.28	-1.06	-0.75	Predicted	0.24**
204342_at	SLC25A24	9.11	-1.09	-0.49	-0.60	-0.73	Predicted	0.08
201161_s_at	CSDA	10.19	-1.43	-0.36	-0.35	-0.71	Predicted	-0.17*
221553_at	MAGT1	8.29	-1.12	-0.25	-0.47	-0.62	Predicted	0.00
224899_s_at	MAGT1	10.61	-1.07	-0.25	-0.23	-0.52	Predicted	-0.12
204224_s_at	GCH1	7.75	-0.70	-0.33	-0.40	-0.48	Predicted	-0.06
212122_at	RHOQ	6.79	-0.77	-0.38	-0.26	-0.47	Predicted	0.02
214449_s_at	RHOQ	8.04	-0.96	-0.25	-0.18	-0.46	Predicted	-0.03
210596_at	MAGT1	8.16	-0.89	-0.30	-0.18	-0.46	Predicted	-0.05
201552_at	LAMP1	10.69	-0.73	-0.24	-0.39	-0.45	Predicted	0.05
223264_at	MESDC1	8.85	-0.55	-0.41	-0.40	-0.45	Predicted	-0.11
212119_at	RHOQ	9.38	-0.83	-0.27	-0.25	-0.45	Predicted	0.02
201160_s_at	CSDA	12.10	-0.98	-0.19	-0.17	-0.45	Predicted	-0.10
224221_s_at	VAV3	6.63	-0.78	-0.17	-0.36	-0.44	Predicted	-0.15
227131_at	MAP3K3	8.60	-0.72	-0.27	-0.27	-0.42	Predicted	0.00
203397_s_at	GALNT3	8.36	-0.69	-0.21	-0.33	-0.41	Predicted	0.02
201827_at	SMARCD2	9.08	-0.17	-0.42	-0.64	-0.41	Predicted	-0.15
208025_s_at	HMGA2	8.77	-0.31	-0.44	-0.45	-0.40	Predicted	-0.06
208796_s_at	CCNG1	11.94	-0.71	-0.23	-0.24	-0.39	Predicted	-0.27***
212117_at	RHOQ	9.47	-0.72	-0.30	-0.15	-0.39	Predicted	0.05
205486_at	TESK2	7.61	-0.58	-0.25	-0.33	-0.39	Predicted	-0.04
201551_s_at	LAMP1	7.68	-0.68	-0.14	-0.28	-0.37	Predicted	0.00
212120_at	RHOQ	8.80	-0.67	-0.16	-0.27	-0.37	Predicted	0.10
212882_at	KLHL18	8.07	-0.60	-0.17	-0.33	-0.37	Predicted	0.00

SUPPLEMENTARY METHODS

Cytogenetic and molecular analysis

Bone marrow or peripheral blood samples of pediatric patients with AML were routinely investigated for cytogenetic abnormalities by standard chromosome-banding analysis. Screening for recurrent nonrandom genetic abnormalities characteristic for AML included t(15;17), inv(16), t(8;21), t(7;12), and *MLL*-gene rearrangements, using either RT-qPCR and/or fluorescent *in situ* hybridization. Patients lacking cytogenetic abnormalities are designated as cytogenetically normal (CN) and patients with miscellaneous cytogenetic abnormalities are designated as patients with other cytogenetics (OC). The distribution of morphological, cytogenetic and molecular subgroups illustrates a representative pediatric AML cohort which was enriched for t(7;12) cases , as previously described.[3]

miRNA and mRNA expression profiling and RT-qPCR

RNA quality was assessed using the RNA 6000 nano assay on the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). In short, expression of 665 microRNAs was measured using two 384-wells Taqman Low Density Cards. Input was 500ng total RNA per card. Raw Ct-values were analyzed, summarized and exported per plate using SDS 2.3 (Applied Biosystems). Baseline was manually set at 0.15 for all plates. All data mining and analyses were done in R 2.11.1.[4] Per card type, Ct-values were imported (readCtData) into a qPCR-set using the package HTqPCR.[5] The average cycle threshold (Ct) value was used to calculate microRNA expression levels relative to the expression level of the small reference RNAs (mean of (mean) Mamm-U6, RNU44 and RNU48) by use of the comparative cycle time (ΔCt) method.[6]

Validity of the multiplex system was tested by singleplex assays for 10 differentially expressed miRNAs (miR-9, miR-126, miR-151-3p, miR-181a, miR-181c, miR-192, miR-500, miR-532-5p, miR-574-3p, and miR-660) by duplicate RT-qPCR TaqMan MiRNA assays (Applied Biosystems) on an Applied Biosystems 7900HT RT-PCR system. The average cycle threshold (Ct) value was used to calculate microRNA expression levels relative to the expression level of the reference (RNU44) using the comparative Ct method. Correlation between singleplex and multiplex assays was assessed by the calculation of Spearman Correlation Coefficient.

Data acquisition and normalization was performed in the statistical data environment R (version 2.12.0 (2010-10-15)) as described previously.[7] Results were validated by RT-qPCR using FAST SYBR Green dye on a StepOnePlus (Applied Biosystems) according to the manufacturer's instructions. Primers are available upon request. Data analysis was performed with use of geNorm algorithms comparative Ct method.[8]

Analyses for differentially expressed microRNAs were performed on -ΔCt values in the en-

vironment R using the limma package. Non detected miRNAs were substituted by an artificial low level of expression of 25. MicroRNAs were considered differentially expressed when the FDR-adjusted moderated t statistic p-value was smaller than 0.05. For prediction of miR-9 targets, VSN-normalized values of the miR-sc condition were subtracted by the VSN-normalized values of the paired miR-9 condition. Probes downregulated in all three experiments and annotated by hgu133plus2.db (version 2.4.5) were compared to validated and predicted miR-9 target genes as supplied by miRecords.[9]

Quality control for miRNA expression experiments

Quality control was performed using a combination of three approaches in three steps. The first step is visual inspection of correlation plots. Correlation plots were called by the plotCtCor command (HTqPCR). This method provides a quick first impression of the data as well as an opportunity for pattern recognition. The correlation coefficient is based on the number of failed measurements for each card. Measurements are considered failed when flagged by the SDS-program or when the Ct values are higher than 38 or not acquired (NA). Failed measurements can be due to either technical failure of the reaction or expression below the detection level. Cards that correlated poorly to the other cards of the same type (Pearson R<0.6) were omitted from further analyses.

The second step uses the number of failed measurements (undetermined, Ct>38, or flagged in any way by SDS software) per card to determine quality. The number of failed measurements per card was summarized with the featureCategory command (HTqPCR). Of this number the mean, standard deviation and 95% confidence interval were calculated for all A and all B cards to obtain the upper limits of the number of failed measurements (A card n= 265, B card n=285). If the number of failed measurements on a card exceeded the limit, this card was omitted from further analyses.

The third quality measure uses the expression levels of the control reactions to determine card quality. In the R environment, the median Ct-value of each control was calculated per card. With these values, the mean value for all A and B cards was calculated per control. In addition, the standard deviation and 95% confidence interval were calculated to obtain the upper limits of expression per control per card type. If the Ct-value of two out of three control reactions per card exceeded the upper limit of expression, the card was omitted from further analyses.

If both card A and B for one sample were retained after going through the steps of quality control, expression values were merged into one dataset. In total, 28 cards were excluded.

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7

MAPPING EPIGENETIC REGULATOR GENE MUTATIONS IN CYTOGENETICALLY NORMAL PEDIATRIC ACUTE MYELOID LEUKEMIA

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CHAPTER 7: EPIGENETIC REGULATOR MUTATIONS IN PEDIATRIC CN-AML

Acute myeloid leukemia (AML) accounts for approximately 15-20% of all childhood leukemias, and despite dramatic improvements in treatment outcome, only approximately 70% of children with AML are cured.[1] AML results from collaborating genetic aberrations in at least 2 different classes; type-I aberrations, inducing uncontrolled cell proliferation and/or survival, and type-II aberrations inhibiting cell differentiation. However, certain aberrations found in AML do not completely fit into our current definition of type-I and type-II aberrations.[2] These aberrations concern epigenetic modifier genes involved in DNA methylation and histone modification, such as Ten-Eleven Translocation 2 (*TET2*), Isocitrate Dehydrogenase 1 (*IDH1*), *IDH2*, Enhancer of Zeste Homolog 2 (*EZH2*), DNA (cytosine-5)-Methyltransferase 3 Alpha (*DNMT3A*) and Additional Sex Combs Like-1 (*ASXL1*). Most of these genes are frequently mutated in adult AML patients with a prevalence varying between 12% to 35% per gene.[3-7] Reports on prognostic implications of these mutations are ambiguous. It has, however, been shown that patients with *IDH* mutations have a hypermethylation phenotype. This implies that demethylating agents such as decit-

Table 1. Clinical and genetic characteristics of pediatric CN-AML cases carrying an epigenetic regulator gene mutation.

Gene	Patient	Mutation[1]	Protein change[2]	Age (yrs)	Sex	WBC count (x109/uL)	Other mutations	Survival status	Follow up (m)
IDH1	1	c.395G>A	p.R132H	3.6	M	14.1	NUP98-NSD1; K-RAS	Death after relapse	23
IDH1	2	c.395G>A	p.R132H	14.3	F	44.8	FLT3-ITD; MLL-PTD	Alive	50
IDH1	3	c.395G>A	p.R132H	15.1	M	9.6		Alive	104
IDH1	4	c.394C>T	p.R132C	12.6	F	3.0		Death after relapse	46
IDH1	5	c.395G>A	p.R132H	13.0	M	226.0	NUP98-NSD1; FLT3-ITD	Alive	32
IDH2	6	c.419G>A	p.R140Q	7.5	M	120.4	MLL-PTD; FLT3-ITD	Alive	71
IDH2	7	c.419G>A	p.R140Q	6.0	M	N/A	NPM1; FLT3-ITD	Alive	40
DNMT3A	8	c.2146G>T	p.V716F	15.3	M	30.8	NPM1; N-RAS	Alive	65
DNMT3A	9	c.2644C>T	p.R882C	8.4	M	25.0	WT1; FLT3-ITD	Alive	52
DNMT3A	10	c.1450C>T	p.R484W	15.7	F	9.5	CEBPA dm	Alive	6
TET2	11	c.3803+2_3ins T	Unknown	11.1	F	163.0	NPM1; FLT3-ITD	Alive	51
TET2	12	c.3646C>T c.4210C>T p.R1404*	p.R1216*	6.9	F	25.1	N-RAS	Alive	50
TET2	13	c.1599G>A	p.M533I	14.3	M	2.2		Alive	34

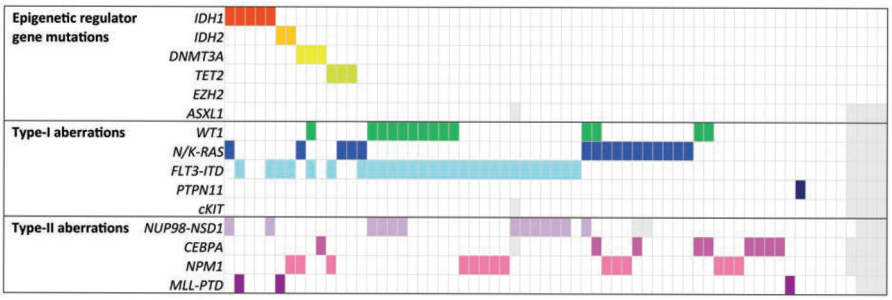
yrs: years; m: months; F: female; M: male; N/A: not available; WBC: white blood cell count; dm: double mutant. 1The nucleotide sequence variations are designated according to the recommen- dations of the Human Genome Variation Society (HGVS, http:// www.hgvs.org/mutnomen/). Numbering of nucleotides is done by using the first base of the translated part of the gene as nucleotide [1].Transcripts used as a reference are: NM_005896.2 for IDH1, NM_002168.2 for IDH2, NM_175629.2 for DNMT3A and NM_001127208.2 for TET2 (National Center for Biotechnology Information). [2]The protein changes are theoretically deduced and designated according to the recommendations of the HGVS.

abine and azacytidine may be of use in the treatment of *IDH*-mutated AML. In addition, a favorable outcome in elderly AML patients with *DNMT3A* mutations after treatment with decitabine has been reported.[7,8]

These epigenetic regulator genes were also studied in pediatric AML. Most of these studies focused on the relevance of mutations in a single gene and mutations were detected in 1-4% of the patients.[9-13] Although no significant statements can be made since study cohorts were quite heterogeneous, most mutations seemed to occur in cytogenetically normal (CN) AML, a subgroup that comprises 20-25% of all pediatric AML. This distribution corresponds to adult AML data in which most epigenetic regulator gene mutations were also identified in CN patients. So far, no larger study focusing on pediatric CN-AML is available and identifying these genetic aberrations may lead to improved risk stratification and a rationale for the use of demethylating agents in these patients. Therefore, we decided to study the mutation prevalence of epigenetic regulator genes *EZH2*, *IDH1*/2, *DNMT3A*, *ASXL1* and *TET2* in a well-documented, international cohort of 65 de novo pediatric CN-AML patients.

Patient material was obtained from the Dutch Childhood Oncology Group (DCOG), the AML-Berlin-Frankfurt-Münster Study group (AML-BFM), the Czech Pediatric Hematology Group, and the Saint-Louis Hospital in Paris, France. A minor selection of the discovered mutations had been included in previous reports, but not with a focus on CN-AML and never within one cohort.[11-13] Exon 4 of *IDH1*/2, exon 13 of *ASXL1*, the SET-domain of *EZH2* and the entire coding region of *TET2* were screened using Sanger sequencing of genomic DNA. The mutational status of *DNMT3A* was assessed by screening cDNA and aberrations were verified in genomic DNA. The prevalence of cytogenetic and molecular aberrations was assessed as previously described.[14]

Figure 1. Distribution of somatic mutations in 65 pediatric CN-AML patients.



Patients are represented vertically (patient number 1 to num- ber 65 from left to right, 1 column per patient) and molecular aberrations horizontally. Each mutated patient is represented by a colored rectangle, a wild-type patient by a white rectangle and when data are not available due to lack of patient material, the rectangle is gray. CEBPA is considered mutated in case of a double mutation only.

In our study cohort, aberrations in the genes *IDH1* and *IDH2* occurred with a frequency of 10.8% (95%CI ± 7.5%) and mutations in *TET2* and *DNMT3A* were found with a frequency of 4.6% (95%CI ± 5.1%) each. While all aberrations in *IDH1* and *IDH2* were confined to codon 132 and 140, respectively, various mutation loci were observed in *TET2* and *DNMT3A*. All epigenetic regulator gene muta tions appeared to be mutually exclusive (Figure 1) and no mutations were identified in *EZH2* or *ASXL1*. The number of patients with a mutation was too small to allow for survival analysis. The specifics of the identified aberrations and the main clinical and genetic features of the patients carrying an epigenetic regulator gene mutation are summarized in Table 1. The epigenetic regulator gene mutations co-occurred with well-known type-I/II aberrations (Figure 1). Out of 13 patients with 10 epigenetic regulator gene mutation also carried a *FLT3-ITD* (n=6) or *RAS* mutation (n=4), and 6 of 13 patients were found to have a *CEBPA* double mutation (n=1), *NPM1* mutation (n=3), or *NUP98-NSD1* translocation (n=2). Overall, epigenetic regulator gene mutations were shown to co-exist with both a type-I and type-II driver aberration in 7 of 13 patients.

In this study of a substantial pediatric CN-AML cohort, we have identified recurrent somatic mutations in the epigenetic regulator genes *IDH1*, *IDH2*, *DNMT3A* and *TET2* in 13 of 65 cases (20%). *IDH1*/2 mutations occurred significantly more often in pediatric CN-AML than in previously reported pediatric AML cohorts with patients carrying various cytogenetic aberrations (P=0.025).[12] Although the aberration frequency of epigenetic regulator gene mutations is relatively high for a pediatric AML cohort, the prevalence is still evidently lower in pediatric CN-AML than in adult CN-AML. In addition, within adult AML, *IDH2*, *ASXL1* and *TET2* mutations are significantly more prevalent in elderly patients, suggesting an increasing prevalence of these mutations as a function of age.[3,4,6] A recent study from Welch et al. identified *DNMT3A*, *IDH1*/2, *TET2*, *ASXL1* and *NPM1* mutations as possible major initiating or driver mutations in CN-AML, rather than cooper ating events acquired at a later stage of clonal evolution.[15]

However, we found epigenetic regulator gene mutations in only 20% of our pediatric CN-AML cohort, and in addition, these mutations often co-occurred with other type-I/II aberrations thought to have leukemia driving capabilities, such as *NUP98-NSD1* and *NPM1*. This suggests that there might be other, as yet unidentified driver mutations in pediatric AML, reflecting potential differences in pathophysiology between childhood and adult AML. Future clonal evolution studies of pediatric CN-AML might lead to a better understanding of the initiating and co-operating events in this disease.

Mutations in *ASXL1* and *EZH2* were absent in our pediatric CN-AML cohort. This corresponds to literature reports that *ASXL1* and *EZH2* mutations in childhood AML are very rare. *EZH2* mutations seem to be highly uncommon in pediatric as well as in adult AML, in contrast to the high prevalence observed in other myeloid malignancies such as adult myelodysplastic syndrome.[5]

In summary, we identified epigenetic regulator gene mutations to be mutually exclusive

and recurrent, but infrequent in pediatric CN-AML. *IDH1*/2 aberrations were more frequent in pediatric CN-AML than in published series of other cytogenetic AML subgroups. The frequency of epigenetic regulator gene mutations is lower in pediatric than in adult CN-AML which underlines the difference in pathophysiology between the two. While there is currently insufficient solid rationale for routine screening of epigenetic regulator gene mutations in newly diagnosed pediatric CN-AML patients, new agents are emerging that may make screening more therapeutically appealing.

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SUMMARY & DISCUSSION

8

CHAPTER 8: SUMMARY & DISCUSSION

SUMMARY

Pediatric AML is a heterogeneous disease for which treatment decisions are mainly based on initial response to induction therapy as well as on (cyto)genetic aberrations of the leukemic cells. Pediatric patients with AML have relatively poor outcome, with 5-year overall survival rates of 60-75% and a significant relapse rate[1–8]. Although prognosis has improved significantly over the last decades, improvements in recent years are mainly attributed to better supportive care as current chemotherapeutic regimens cannot be intensified because of treatment related mortality. In addition, part of patients die early, possibly because of insufficient therapy.

Identification of deviations from the physiological hematopoiesis are important as these might provide direction for future therapy by identification of leukemogenic drivers and/or signaling pathways that can be directly targeted[9,10]. Much effort has been put in determination of the biology underlying acute myeloid leukemia, both in children and in adults. Such research has focussed on gene mutations, alterations of mRNA expression, protein expression, miRNA expression and methylation profiles. Pediatric AML differs from adult AML, most noticeably by the distribution of cytogenetic and molecular aberrations. The identification of molecular targets for the 30-40% of pediatric AML that lack classifying genetic abnormalities, so called cytogenetic normal (CN) AML is the subject of ongoing biological studies.

In the current thesis, we investigated genetic as well as epigenetic aberrations in pediatric AML. In **Chapter 2**, we show that high expression of *IGSF4* was typical for pediatric AML with t(9;11)(p22;q23) and a FAB-M5 phenotype. This was explained by the lack of methylation at the promoter site of *IGSF4*. *IGSF4* was shown to be re-expressed in AML cell lines by treatment with demethylating agents. Effects on proliferation could not be shown in our experiments, using cell supsensions. This set-up is not the optimal environment for cells to interact with each other and their physiological surroundings. As *IGSF4* is involved in cell adhesion, cell migration essays might be a more suitable experimental set-up.

Chapter 3 focuses on the occurrence of *RUNX1* mutations in AML as *RUNX1* mutations have been detected in 65% of children with congenital neutropenia who developed leukemia or MDS, and cooperated very frequently with mutations in *CSF3R*. In contrast, we found that *RUNX1* mutations were very rare in *de novo* pediatric AML (2.9%) and that *CSF3R* mutations were not present at all in *de novo* pediatric AML These results suggest a pathway of leukemogenesis specific for congenital neutropenia patients who developed leukemia that is different from patients with *de novo* AML.

We started our study of miRNAs in AML by focusing on four miRNAs; *miR-196a*, *miR-196b*, *miR-155*, and *miR-29b* (**Chapter 4)**. Selection of these miRNAs was based on results from *in vitro* and *in vivo* studies that reported subtype-specific expression in adult AML. *MiR-196a* and *miR-196b* appeared highly expressed in *MLL*-rearranged and *NPM1*-mutated AML

Figure 1: genes implicated in AML that are targeted by deregulated miRNAs

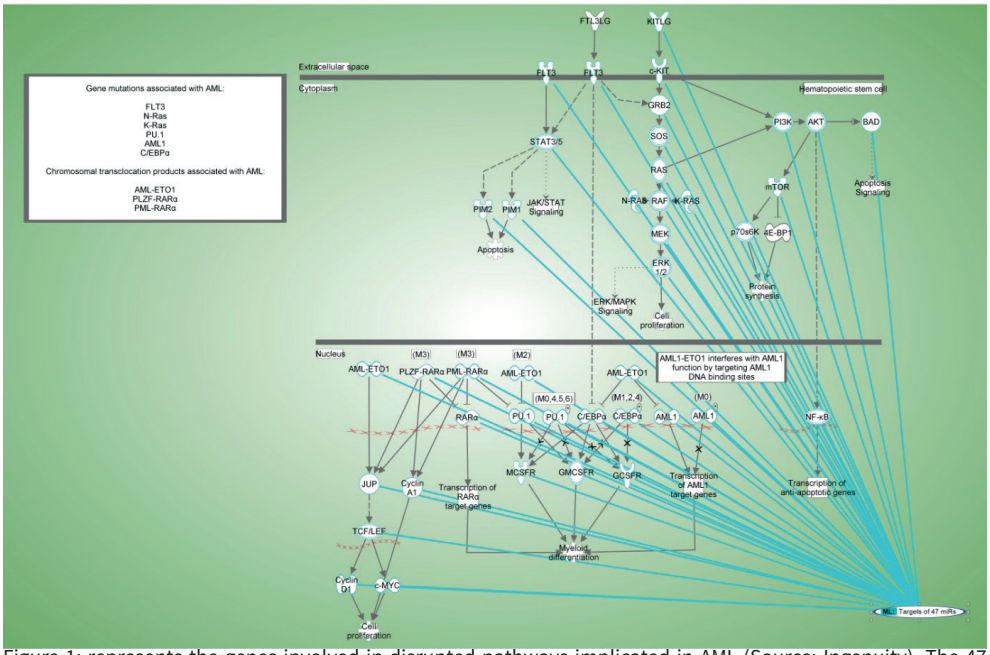

Figure 1: represents the genes involved in disrupted pathways implicated in AML (Source: Ingenuity). The 47 miRNAs used to classify pediatric AML are represented in the right hand corner, blue lines indicate possible targets of the classifying miRNAs.

and expression correlated strongly with expression of various *HOXA* and *HOXB* genes. The expression of *miR-29a* was found to be downregulated in AML with t(10;11), which are characterized by a poor outcome, while expression was upregulated in *NPM1* mutated cases, that have a better survival. However, no significant association between expression of *miR-29a* and survival was found. Expression of *miR-155* was significantly higher in AML with *FLT3*-ITD or *NPM1* mutations but was not related to cytogenetic aberrations. The results from our study were mostly consistent with results from studies of adult AML, but there were also differences. For example, we found downregulated expression of miR-29a in AML with t(10;11), while down regulation in adult AML was mostly found in t(9;11) cases[11]. These differences between pediatric and adult miRNA expression justified a large miRNA expression screening study in pediatric AML.

MiRNA expression profiles were determined by the first large scale RT-qPCR (TLDA) in 165 pediatric AML patients including all important types of pediatric AML (**Chapter 5)**. In order to identify miRNAs specific for pediatric AML types defined by cytogenetic and molecular aberrations, these miRNAs were analyzed using a double-looped classifier. This was successful for AML characterized by *MLL*-rearrangements, t(8;21), inv(16), or t(15;17). However, classification of AML with *CEBPA*-mutations or *NPM1* mutations was not possible, possibly due to patient numbers. The 47 miRNAs used for classification were involved in

several pathways, and they could potentially target almost all genes implicated in AML pathogenesis (Figure 1). The classifying miRNAs were involved in pathways that have been implicated in (amongst others) proliferation, apoptosis, and invasion of tumor cell lines, often by influencing the *RAS* and *AKT* signaling pathways. Central genes that might be targeted were *TCF4*, *MAPK1*, *KRAS*, *MAP2K1*, and *AKT2*. Twelve miRNAs used to classify pediatric AML target those genes; *miR-130a-3p, miR-139-5p, miR-181a-5p, miR-16-5p, miR-200b-3p, miR-26a-5p, let-7a, miR-203-3p, miR-133a-3p, miR-320b, miR-221-3p,* and *miR-29b-3p*. Functional studies of these miRNAs are necessary to elucidate their role in (pediatric) AML and possibilities of therapeutic use of these miRNAs. In addition, expression of *miR-99a* and *miR-486-3p* was found to be related favorable outcome. These miRNAs might prove to have an influence on leukemic cell sensitivity to therapy. Further research is needed to elucidate the clinical relevance of this finding; these miRNAs or their targets might represent new possibilities to develop novel treatment strategies. **Chapter 6** describes the role of *miR-9*, which is differentially lower expressed in t(8;21) positive pediatric AML (see also **Chapter 5**). The effects of overexpression of *miR-9* proved to be cell-context dependent: only in t(8;21) AML samples, overexpression led to growth reduction and differentiation of leukemic cells. Gene expression studies of cells transfected with *miR-9* in combination with target prediction algorithms identified *HMGA2* and *LIN28B* as targets for *miR-9* in t(8;21) positive AML. In addition, interplay with the tumor suppressive *let-7* family was confirmed. This indicates that overexpression of *miR-9* might provide a specific treatment option for t(8;21) AML.

In **Chapter 7** we studied the occurrence of mutations in epigenetic regulator genes *IDH1*, *IDH2*, *DNMT3A*, *TET2*, *EZH2* and *ASXL1* in pediatric CN-AML patients. Mutations in *IDH1* and *IDH2* were observed in 10.8% of CN-AML and mutations in *DNMT3A* and *TET2* in 4.8% of CN-AML. No mutations of *EZH2* and *ASXL1* were found. The frequency of mutations was much lower in pediatric AML than in previously reported adult AML, indicating that the increasing prevalence of these mutations are probably a function of increasing age.

In conclusion, this thesis describes differential gene expression in specific types of pediatric AML characterized by cytogenetic aberrations. Low *IGSF4* expression was typical for AML patients with *MLL*-rearrangements and a FAB-M5 phenotype. AML with *MLL*-rearrangements, t(15;17), t(8;21), and inv(16) have specific miRNA expression profiles and *miR-9* appeared to be a tumor suppressor only in a t(8;21) AML background. In contrast, *RUNX1* mutations were not typical for any cytogenetic aberration. Mutations in epigenetic regulators are rare in pediatric AML and occur much less frequent in pediatric CN-AML compared to adult CN-AML. The described results are useful to give direction in further investigations towards developing stratification as well as targeted therapy for specific groups of pediatric AML characterized by cytogenetic aberrations.

DISCUSSION

In our efforts to molecularly unravel pediatric AML subtypes, we found that high *IGSF4* mRNA and protein expression in monoblastic pediatric AML with t(9;11) *MLL*-rearrangements is associated with a hypomethylated promoter region of *IGSF4*, but that downregulation of *IGSF4* did not affect proliferation (Chapter 2).

Loss of *IGSF4* expression in *MLL*-rearranged AML resulting from promoter hypermethylation has also been reported in adult AML[13]. To date, the exact role of *IGSF4* expression in hematopoiesis and leukemogenesis remains to be elucidated. A role in cell-cell adhesion has been described but seems unlikely to be important in leukemia. Loss of *IGSF4* expression is known to result in *AKT*-pathway stimulation leading to increased cell migration[14]. In solid tumors, IGSF4 expression on the cell surface triggers cytotoxicity mediated by natural-killer cells and CD8+ T-cells[15]. As normal leukocytes do not express high levels of *IGSF4*, AML cells expressing high levels of *IGSF4* might be more likely to be recognized by the immune system[16]. AML cells with low *IGSF4* expression can evade the immune system by this mechanism. Theoretically, patients with low *IGSF4* expressing leukemia might benefit from re-expression of *IGSF4*, for example by use of demethylating agents but this is an unspecific approach (Chapter 2).

Further studies on *IGSF4* in AML may focus on cell-cell adhesion and evading the immune system.

A well known gene involved in pediatric and adult AML is *RUNX1*. Co-occurring mutations in *RUNX1* and *CSF3R* were found in a large portion of patients with secondary AML that evolved from congenital neutropenia but not in patients with *de novo* pediatric AML(Chapter 3). The co-occurrence of *RUNX1* and *CSF3R* mutations in the same leukemic cell clone suggests that these mutations cooperate in leukemogenesis in congenital neutropenia. However, this appeared not to be the case in *de novo* pediatric AML. *RUNX1* mutations have also been described in familial platelet disorder-AML and Fanconi anemia patients, two disorders with a high risk of developing secondary AML[17,18]. *RUNX1* mutation screening during follow up of patients with bone marrow failure syndromes might be considered to improve monitoring of possible leukemic transformation. Moreover, in patients with congenital neutropenia and both *RUNX1* and *CSF3R* mutations, the high risk of rapid evolution to leukemia may be taken into account when considering stem cell transplantation. However, we only studied the co-occurrence of these mutations in patients with congenital neutropenia that developed AML. Therefore such a change in clinical decision making must be preceded by a study that reports co-occurence of these mutations in congenital neutropenia patients that do not develop AML.

Aside from genetic aberrations, epigenetic regulation is known to be important in AML. These epigenetic mechanisms include methylation, acetylation as well as miRNA-targeted repression of target mRNAs. As miRNAs regulate gene expression not only via degradation

of mRNAs but also through translational inhibition, they are likely to play an important role in AML leukemogenesis. By use of an RT-qPCR based method, we found differential expression of *miR-29a*, *miR-155*, *miR-196a*, and *miR-196b* in clinically relevant genetic entities of pediatric AML that was, in part, concordant to findings in adult AML (Chapter 4). The specific association of miRNA expression to genetically defined patient groups of pediatric AML suggests that these miRNAs may be involved in the biology of pediatric AML.

MiRNA expression profiles characterize AML patient groups by cytogenetic aberration, despite the fact that the miRNAs overlap between groups. This indicates a general effect of deregulated miRNAs in AML. Although classification of AML based on miRNA expression was successful, using miRNA expression profiles does not add to current classification methods that are mainly based on conventional cytogenetics[9,19]. The value of the identified patient clusters based on miRNA expression that did not have any other currently known denominator in common (like cytogenetics, FAB-type, or molecular aberrations) remains to be elucidated. These are the patients of whom the underlying biology remains unclear, and further research into these groups seems warranted.

The 47 identified miRNAs used for classification were involved in several pathways, and they could potentially target a large variety of genes implicated in AML pathogenesis (Figure 1). Further research of these miRNAs should focus on the genes that are targeted by the deregulated miRNAs and the functional effects of restoring the normal expression of identified miRNAs in the leukemic

This may give an indication of clinical applicability of these findings, for instance whether these miRNAs or their target genes represent future therapeutic targets. Of course, clinical applicability will only be feasible if the effect of miRNA regulation is obtained in the appropriate cell type and the leukemic cell does not apply another (escape) mechanism to evade differentiation or cell death.

We identified *miR-9* deregulation as an important denominator of leukemogenesis in pediatric AML with t(8;21)(Chapter 6). We discovered that *miR-9* suppresses leukemic growth while inducing monocytic differentiation in t(8;21) AML *in vitro* and *in vivo*, and that at least part of this effect of *miR-9* is accomplished in cooperation with *let-7* by repressing the oncogenic *LIN28B*/*HMGA2* axis.

The release of differentiation block by *miR-9* was previously described in the context of oncogenic *EVI-1* expression[20]. This implicates clinical relevance as *miR-9* attenuation might be used to release the differentiation block of leukemic cells[21,22]. As *miR-9* is only expressed in macrophages during normal hematopoietic differentiation, elevating the expression of *miR-9* could provide a specific treatment option for t(8;21) AMLs. Further *in vivo* experiments are needed to shed light on the feasibility of this approach, and should mainly address questions regarding delivery of the miRNA to the right cell and effects of this overexpression on other cells. A recent study that used a nanoparticle based delivery

system for synthetic *miR-29b* in immunocompetent mice engrafted with leukemic cell line MV4-11 showed prolonged survival times for the treated mice with no significant organ impairment, body weight changes, hemoglobin levels, white blood cell counts, or platelet counts[23]. Interestingly, overexpression of *miR-9* in *MLL*-rearranged AML has oncogenic effects. This contradictory effect was also reported in a study focusing on adult AML[24]. Apparently, the *MLL*-fusion activates *miR-9* expression and down regulates two tumor suppressive genes in *MLL*-rearranged AML: *RYBP* and *RHOH*. These results emphasize that caution should be taken when translating laboratory findings to the patients' bedside[24,25].

Results of this thesis illustrate the heterogeneity of (pediatric) AML. In addition, findings were often cell type specific. Other studies also suggested the importance of miRNAs in AML. For example, attenuation of *miR-126* expression in CD34+CD38- stem/progenitor cells in AML led to reduction of clonogenic capacity and elimination of leukemic cells *in vitro* but not of normal bone marrow cells[25]. MiR-based therapeutics in other diseases are even more encouraging. Examples are down regulation of *miR-122* with an antimiR in chronic hepatitis C virus infection, which is studied in clinical Phase-I and Phase-II studies and a clinical phase-I study of upregulation of *miR-34* by a mimic in unresectable primary liver cancer[26,27].

Disruption of epigenetic regulator genes seems to be another mechanism contributing to leukemogenesis. We showed that mutations in epigenetic regulator genes are infrequent, mutually exclusive and recurrent events in pediatric AML with normal cytogenetics (Chapter 7). Mutations in these genes occur much more frequently in adult cytogenetically normal AML. This reflects the difference in pathophysiology between children and adults with AML and indicates that the increasing prevalence of these mutations are probably a function of increasing age[28]. Although reports on prognostic significance of mutations in epigenetic regulator genes are ambiguous, patients with mutations in *IDH1* or *IDH2* are found to have a hypermethylation phenotype. Others have reported favorable responses to treatment with the demethylating agent decitabine for elderly AML patients with *DNMT3A* mutations[29]. Our results showing a very low frequency of epigenetic regulator mutations in pediatric AML indicate that there is no need to screen for these mutations at diagnosis in children with cytogenetically normal AML. However, as the therapeutic use of demethylating agent decitabine is tested in Phase II trials, screening for patients that might benefit from it becomes more and more appealing.

All approaches described in this thesis added to the knowledge about pediatric AML. However, integration of the results of these different approaches might be even more important. At the moment, results from this type of data-integration have not been described yet but will become more and more important in an era of high-throughput platforms and methods. This requires a closer cooperation between clinicians, preclinical researchers, and bio-informaticians and strong (inter)national cooperation for rare diseases as pediatric AML, especially for specific groups with poor outcome.

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9

NEDERLANDSE SAMENVATTING

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Acute myeloïde leukemie (AML) in kinderen is een heterogene ziekte waarbij beslissingen omtrent behandeling voornamelijk gebaseerd worden op de respons op inductietherapie en de (cyto)genetische afwijkingen van de leukemische cellen. Kinderen met AML hebben een relatief slechte prognose: de 5-jaarsoverleving ligt rond 60-75%. Daarnaast komt de AML bij een significant aantal patiënten terug[1–8]. Hoewel de prognose van deze patiënten sterk verbeterd is in de laatste decennia, zijn de verbeteringen in het recente verleden voornamelijk het resultaat van betere ondersteunende zorg. Dit komt voornamelijk doordat de huidige behandeling met chemotherapie niet geïntensiveerd kan worden omdat dit een hogere mortaliteit met zich mee brengt. Daarnaast overlijdt een aantal patiënten vroeg in de behandeling, wat mogelijk wijst op therapie-insufficiëntie.

Het is belangrijk te identificeren waarin leukemische cellen afwijken van fysiologische processen omdat dit richting kan geven aan toekomstige therapieën. Bijvoorbeeld door de identificatie van leukemogene genen en/of signaaltransductiepaden die gericht door behandeling beïnvloed kunnen worden[9,10]. Zowel in kinder- als volwassen-AML is er veel onderzoek gedaan naar de onderliggende biologie die belangrijk is in de leukemische cel. Te denken valt aan genmutaties, veranderingen van mRNA-expressie, veranderingen van eiwitexpressie, verandering van miRNA-expressie en verandering van methylatieprofielen. Kinder-AML verschilt van volwassen-AML: het meest opvallend is de andere frequentie van voorkomen van cytogenetische en moleculaire afwijkingen. Momenteel is nog bij 30-40% van de kinder-AML niet duidelijk wat de onderliggende afwijking is, wat ervoor zorgt dat identificatie van moleculaire aangrijpingspunten in deze groep een onderwerp is dat veel bestudeerd wordt.

Het onderzoek omschreven in dit proefschrift was gericht op zowel genetische als epigenetische veranderingen in kinder-AML. In **hoofdstuk 2** beschrijven we dat hoge *IGSF4*-expressie typisch is voor AML met t(9;11)(p22;q23) en een FAB-M5-fenotype. Deze hoge expressie werd verklaard door verminderde methylatie van de promotorregio van *IGSF4*. Daarnaast kon *IGSF4*-expressie in AML-cellijnen waarin *IGSF4*-expressie laag was teruggebracht worden door behandeling met demethylerende middelen. Effecten op celproliferatie werden in onze studie, gebaseerd op celsuspensieculturen, niet gezien. Gelet op het feit dat *IGSF4* betrokken is in celadhesie was een celmigratie-essay mogelijk een gunstiger onderzoeksopzet geweest voor de bestudering van de effecten van *IGSF4*.

In **hoofdstuk 3** ligt de focus op *RUNX1*-mutaties in AML. Dit werd gedaan omdat deze mutaties voorkomen in 65% van de patiënten met congenitale neutropenie die daarna leukemie of MDS ontwikkelen. *RUNX1*-mutaties werden in deze patiënten vaak gevonden in combinatie met mutaties in *CSF3R*. In kinder-AML bleken *RUNX1*-mutaties weinig voor te komen: in 2.9% van de patiënten. Mutaties in *CSF3R* werden helemaal niet gevonden in *de novo* kinder-AML. Deze resultaten suggereren dat deze samenwerkende mutaties een leukemogeen mechanisme vormen dat van belang is in congenitale neutropenie maar

niet in patiënten met *de novo* AML.

Ons onderzoek naar epigenetische regulatie van genexpressie door miRNA-interactie begon met gericht onderzoek naar expressie van vier miRNA's: *miR-196a*, *miR-196b*, *miR-155*, en *miR-29b* (**Hoofdstuk 4**). Deze miRNA's werden geselecteerd gebaseerd op resultaten van *in vitro* en *in vivo* onderzoek en de gerapporteerde subtype-specifieke expressie in volwassen-AML. *MiR-196a* en *miR-196b* kwamen hoog tot expressie in AML met *MLL*-herschikkingen of *NPM1*-mutaties. Expressie van deze miRNA's had een nauwe correlatie met expressie van verschillende *HOXA*- en *HOXB*-genen. *MiR-29a*-expressie was verlaagd in AML met t(10;11), wat gerelateerd wordt aan slechte uitkomst, terwijl *miR-29a*-expressie verhoogd was in gevallen met een *NPM1*-mutatie, die een betere uitkomst hebben. Toch kon er geen significante relatie tussen *miR-29a*-expressie en overleving aangetoond worden. Expressie van *miR-155* was significant hoger in AML met *FLT3-ITD*- of *NPM1*-mutaties en niet gerelateerd aan cytogenetische afwijkingen. Veel van de resultaten uit deze studie komen overeen met onderzoek uitgevoerd in volwassen-AML, maar er waren wel wat verschillen. In ons onderzoek werd verlaagde *miR-29a*-expressie gevonden in AML met t(10;11) terwijl dit in volwassen-AML voornamelijk in AML met t(9;11) gevonden werd[11]. De gevonden verschillen tussen kinder- en volwassen-AML rechtvaardigden een grote screening van miRNA-expressie in kinder-AML.

MiRNA-expressieprofielen werden vervaardigd van 165 kinder-AML-monsters waarbij alle belangrijke typen kinder-AML vertegenwoordigd waren (**Hoofdstuk 5**). Om miRNA-expressie specifiek voor cytogenetische en moleculaire subgroepen te bepalen werd een analyse gedaan met behulp van een *double-looped classifier*. Dit was succesvol voor kinder-AML gekarakteriseerd door *MLL*-herschikkingen, t(8;21), inv(16), of t(15;17). Classificatie van AML met *CEBPA*-mutaties of *NPM1*-mutaties bleek niet accuraat, mogelijk doordat er te weinig patiënten geïncludeerd waren. De 47 miRNA's die gebruikt werden voor de classificatie spelen een rol in verschillende signaaltransductiepaden, bijvoorbeeld in proliferatie, apoptose en invasie van tumorcellijnen. Daarnaast kunnen ze mogelijk bijna alle genen die gerelateerd zijn aan AML-pathogenese beïnvloeden (Figuur 1). In de meeste gevallen verloopt deze beïnvloeding via de RAS- en AKT-signaaltransductiepaden. Genen die mogelijk een centrale rol spelen hierin waren *TCF4*, *MAPK1*, *KRAS*, *MAP2K1*, en *AKT2*. Deze kunnen beïnvloed worden door 12 van de 47 miRNA's die gebruikt werden om AML te classificeren; *miR-130a-3p, miR-139-5p, miR-181a-5p, miR-16-5p, miR-200b-3p, miR-26a-5p, let-7a, miR-203-3p, miR-133a-3p, miR-320b, miR-221-3p,* and *miR-29b-3p*. Om hun rol in (kinder-)AML en therapeutisch potentieel te doorgronden zijn functionele studies nodig.

Hoofdstuk 6 beschrijft de rol van *miR-9*, dat significant lager tot expressie komt in kinder-AML gekarakteriseerd door t(8;21). De effecten van verhoogde *miR-9*-expressie bleken celcontextspecifiek: overexpressie in t(8;21) leidde tot verminderde groei en stimuleerde differentiatie van leukemiecellen. Genexpressieonderzoek van cellen getransfecteerd met

miR-9 werd gecombineerd met voorspelling van gendoelen, wat leidde tot identificatie van *HMGA2* en *LIN28B* als doelen voor *miR-9* in AML met t(8;21). Daarnaast kon interactie met tumorsuppressor *let-7*-familie aangetoond worden. Dit impliceert dat overexpressie van *miR-9* een specifieke behandelmogelijkheid zou kunnen zijn voor t(8;21) AML.

We bestudeerden ook de rol van mutaties in epigenetische regulatorgenen in kinder-AML, aangezien dit op dat moment nog niet beschreven was in kinderen maar wel een belangrijk oncogeenmechanisme leek te zijn in volwassen-AML[12]. In **hoofdstuk 7** beschrijven we het voorkomen van mutaties in epigenetische regulatorgenen *IDH1*, *IDH2*, *DNMT3A*, *TET2*, *EZH2* en *ASXL1* in kinder-AML zonder cytogenetische afwijkingen (n=65). Mutaties in *IDH1* en *IDH2* werden het vaakst gevonden (10.8%), gevolgd door mutaties in *DNMT3A* en *TET2* (4.8% elk). In deze patiënten werden geen mutaties in *EZH2* en *ASXL1* gevonden. De door ons gevonden mutatiefrequentie was veel lager dan wat beschreven was voor volwassen-AML, wat erop wijst dat de stijgende prevalentie van deze mutaties waarschijnlijk gerelateerd is aan toenemende leeftijd.

Dit proefschrift beschrijft verschillen in genexpressie tussen specifieke types kinder-AML gekarakteriseerd door type II-mutaties. Lage *IGSF4*-expressie was typisch voor AML met *MLL*-genherschikkingen en FAB-M5-fenotype. We vonden ook dat AML met *MLL*-herschikkingen, t(15;17), t(8;21), of inv(16) een specifiek miRNA-expressieprofiel hebben en dat *miR-9* een tumorsuppressor lijkt in cellen met t(8;21) AML. Daartegenover staat dat mutaties in *RUNX1* niet typisch waren voor enig type II-afwijking. Mutaties in epigenetische regulatorgenen werden veel minder vaak gezien in kinder-AML zonder cytogenetische afwijkingen dan in volwassen-AML. De resultaten beschreven in dit proefschrift kunnen richting geven aan verder onderzoek naar nieuwe stratificatiemethoden en gerichte therapie voor specifieke groepen kinderen met AML gekarakteriseerd door type II-afwijkingen.

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ABOUT THE AUTHOR

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CHAPTER 10: CURRICULUM VITAE

CURRICULUM VITAE

Jenny Esther Katsman-Kuipers werd op 3 mei 1986 geboren te Leiden. Zij haalde in 2004 haar VWO-diploma aan het Scala College te Alphen aan den Rijn. Haar profielwerkstuk had als onderwerp 'Het Humaan Genoom project'. In september 2004 startte zij met haar studie Geneeskunde aan de Universiteit Leiden. Ter afronding van de doctoraalfase van haar studie verrichtte zij een wetenschapsstage bij het laboratorium Kinderoncologie van het Erasmus MC - Sophia Kinderziekenhuis te Rotterdam met als onderwerp 'Genexpressieprofielen in kinder-AML met *MLL*-gen herschikkingen'. Zij haalde in februari 2010 haar doctoraaldiploma aan de Universiteit Leiden. De wetenschapsstage had haar interesse in de onderliggende genetische factoren van kinder-AML aangewakkerd, zodoende begon zij in maart 2009 aan haar promotieonderzoek in de kinder-AML-onderzoeksgroep van het Erasmus MC – Sophia Kinderziekenhuis. Dit onderzoek deed zij onder begeleiding van dr. Van den Heuvel-Eibrink, dr. Zwaan en prof. dr. Pieters. De resultaten van het promotieonderzoek zijn beschreven in dit proefschrift. In maart 2013 hervatte Jenny haar studie Geneeskunde aan de Universiteit Leiden en begon zij aan haar co-schappen. Zij deed een oudste coschap Gynaecologie en Verloskunde in het Westeinde Ziekenhuis in Den Haag. In februari 2015 behaalde zij haar artsexamen. Momenteel woont zij in Den Haag met haar echtgenoot, Maarten Katsman.

CHAPTER 10: LIST OF PUBLICATIONS

LIST OF PUBLICATIONS

Kuipers JE & Coenen EA, Balgobind B V, Stary J, Baruchel A, de Haas V, de Bont ESJM, Reinhardt D, Kaspers GJL, Cloos J, Danen-van Oorschot AA, den Boer ML, Marschalek R, Meyer C, Pieters R, Zwaan CM, van den Heuvel-Eibrink MM. *High IGSF4 expression in pediatric M5 acute myeloid leukemia with t(9;11)(p22;q23).* ***Blood.*** 2011 Jan 20;117(3):928–35.

Skokowa J & Steinemann D, **Katsman-Kuipers JE**, Zeidler C, Klimenkova O, Klimiankou M, Unalan M, Kandabarau S, Makaryan V, Beekman R, Behrens K, Stocking C, Obenauer J, Schnittger S, Kohlmann A, Valkhof MG, Hoogenboezem R, Göhring G, Reinhardt D, Schlegelberger B, Stanulla M, Vandenberghe P, Donadieu J, Zwaan CM, Touw IP, van den Heuvel-Eibrink MM, Dale DC, Welte K. *Cooperativity of RUNX1 and CSF3R mutations in severe congenital neutropenia: a unique pathway in myeloid leukemogenesis.* ***Blood.*** 2014 Apr 3;123(14):2229–37.

Danen-van Oorschot AA & **Kuipers JE**, Arentsen-Peters S, Schotte D, de Haas V, Trka J, Baruchel A, Reinhardt D, Pieters R, Zwaan CM, van den Heuvel-Eibrink MM. *Differentially Expressed miRNAs in Cytogenetic and Molecular Subtypes of Pediatric Acute Myeloid Leukemia.* ***Pediatr Blood Cancer.*** 2012;58:715–21.

Emmrich S & **Katsman-Kuipers JE**, Henke K, El Khatib M, Jammal R, Engeland F, Dasci F, Zwaan CM, den Boer ML, Verboon LJ, Stary J, Baruchel A, de Haas V, Danen-van Oorschot AA, Fornerod M, Pieters R, Reinhardt D, Klusmann J-HH, van den Heuvel-Eibrink MM. *MiR-9 is a tumor suppressor in pediatric AML with t(8;21).* ***Leukemia.*** Nature Publishing Group; 2014 May;28:1022–32.

Valerio DG, **Katsman-Kuipers JE**, Jansen JH, Verboon LJ, de Haas V, Stary J, Baruchel A, Zimmermann M, Pieters R, Reinhardt D, van den Heuvel-Eibrink MM, Zwaan CM. *Mapping epigenetic regulator gene mutations in cytogenetically normal pediatric acute myeloid leukemia.* ***Haematologica.*** 2014 Aug;99(8):e130–2.

Hollink IHIM, van den Heuvel-Eibrink MM, Arentsen-Peters STCJM, Pratcorona M, Abbas S, **Kuipers JE**, van Galen JF, Beverloo HB, Sonneveld E, Kaspers G-JJL, Trka J, Baruchel A, Zimmermann M, Creutzig U, Reinhardt D, Pieters R, Valk PJM, Zwaan CM. *NUP98/NSD1 characterizes a novel poor prognostic group in acute myeloid leukemia with a distinct HOX gene expression pattern.* ***Blood.*** 2011 Aug 2;118(13):3645–56.

CHAPTER 10: PHD PORTFOLIO

PHD PORTFOLIO

Summary of PhD training and teaching	
Name PhD student:	Jenny E. Katsman - Kuipers
Erasmus MC Department:	Pediatric Oncology
Research School:	MolMed
PhD period:	1st March 2009-28th February 2013
Promotor(s):	Prof.dr. R. Pieters
Supervisor:	Dr. M.M. van den Heuvel - Eibrink

1. PhD. Training		
General courses	Year	Workload
Classical Methods for Data Analysis (CC02)(NIHES)	2009	5.7 ECTS
Research Management for PhDs	2009	1.0 ECTS
Biomedical English Writing and Communication	2010	4.0 ECTS
Succesful presentations (NWO)	2010	0.3 ECTS

Specific courses (e.g. Research school, Medical Training)	Year	Workload
Analysis of microarray gene expression data (MGC)	2009	2.0 ECTS
Basic course on 'R' (MolMed)	2010	1.4 ECTS
Bioconductor Course (MolMed)	2010	1.0 ECTS

Seminars and workshops	Year	Workload
Photoshop CS3 (MolMed)	2010	0.25 ECTS
COST workshop on bioinformatics (EuGESMA)	2010	
Illustrator CS3 (MolMed)	2010	0.25 ECTS
Indesign Workshop (MolMed)	2011	0.15 ECTS

Presentations	Year	Workload
Six 30-minute presentations at weekly meetings of department of Pediatric Oncology	2009-2013	4.0 ECTS

(Inter)national conferences	Year	Workload
51st ASH annual meeting, New Orleans, USA (poster presentation)	2009	2.0 ECTS
3rd AML-BFM Research Symposium, Hannover, Germany	2010	1.0 ECTS
Eugesma Cost Meeting Barcelona, Spain (oral presentation)	2010	2.0 ECTS
SIOP 2010 Boston (oral presentation)	2010	2.0 ECTS
Keystone meeting "MicroRNAs and Non-Coding RNAs and Cancer" (poster presentation)	2011	2.0 ECTS
4th AML-BFM Research Symposium, Hannover, Germany (oral presentation)	2012	2.0 ECTS
16th Working group meeting Childhood Leukemia, Santiago, Chili (oral presentation)	2012	2.0 ECTS
8th Biennial Childhood Leukemia Symposium, Santiago, Chili	2012	1.5 ECTS

Other	Year	Workload
MolMed Day	2009-2012	1.2 ECTS

2. Teaching		
Supervising practicals and excursions	Year	Workload
Practical during Medicine course 'pediatric oncology'	2010-2012	1 ECTS

CHAPTER 10: AUTHOR AFFILIATIONS

AUTHOR AFFILIATIONS

Alexander Kohlmann	Munich Leukemia Laboratory, Munich, Germany
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CHAPTER 10: DANKWOORD

DANKWOORD

Dit proefschrift is tot stand gekomen met hulp van vele kanten. Ongelooflijk veel dank ben ik verschuldigd aan de patiënten en hun ouders. Bedankt voor de toestemming die jullie gegeven hebben voor deelname aan onderzoek, zonder jullie was het onderzoek beschreven in dit proefschrift niet mogelijk geweest.

"*Education is the most powerful weapon which you can use to change the world.*" ~Nelson Mandela

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"*Coming together is a beginning. Keeping together is progress. Working together is success.*" ~Henry Ford

Many thanks go to the international collaborators that I have worked with over the past years. The research each of you performs makes a significant contribution to the unravelling of pediatric leukemia. In particular I would like to thank prof.dr. **Dirk Reinhardt, dr. Jan-Henning Klusmann, dr. Stephan Emmrich, and Kristen Henken**. Thank you for the hospitality you showed by inviting me to your lab. Our cooperation has been the start of a promising line of research towards miRNA function in AML.

Dank ben ik ook verschuldigd aan de andere werkgroepleiders op het gebied van kinderleukemie op het lab, **Ronald**, **Monique**, en **Jules**. De gedachtewisselingen over mijn projecten op de woensdagochtend waren een mooie manier om even op een andere manier naar mijn eigen onderzoek te kunnen kijken.

"Alone we can do so little, together we can do so much." ~Helen Keller

Van levensbelang tijdens een promotieonderzoek zijn goede collega's. De meest directe collega's zijn dan die van de AML-groep. Wat prettig om geholpen te worden door geweldige analisten; **Susan**, **Nicola**, **Linda**, **Dyonne**, en **Lonneke**. Voor inhoudelijke ondersteuning was het prettig terug te kunnen vallen op postdocs **Astrid**, **Maarten** en **Eva B**. De input van de andere AIO's, **Brian**, **Iris, Andrica**, **Marjolein**, **Trudy**, **Eva**, **Anna**, **Malou**, **Jasmijn** en **Daria** was zeer waardevol, onze samenwerking heeft mooie resultaten opgeleverd. Ook dank aan geneeskundestudent **Danique** voor haar bijdrage aan ons onderzoek. Ondertussen zijn er ook weer nieuwe collega's begonnen in de AML-groep, fijn dat jullie het onderzoek voortzetten!

Onderzoek doe je nooit alleen, en op het lab kinderoncologie in Rotterdam kunnen we zeker niet zonder onze analisten. **Jessica**, **Wilco**, **Clarissa**, **Paulien**, **Lydia**, **Merel**, **Sandra**, **Mathilde**, **Ellen**, **Marieke**, en **Pieter**: dank voor alles wat jullie mij geleerd hebben en het geduld met al mijn vragen. Door jullie inzet kan het onderzoek met patiëntenmateriaal steeds doorgaan.

"A problem shared is a problem halved."~ Engels gezegde

De andere AIO's op het lab waren er altijd voor een goed gesprek, of dit nu werkgerelateerd was of niet. Bedankt **Diana**, **Farhad**, **Arian**, **Stephanie**, **Ingrid**, **João**, **Lieneke**, **Bob**, **Irene**, **Linda**, **Maartje, Laura, Rui, Dominique**, **Jill**, **Marieke, Emma, Floor, Imbritt**. Voor advies over experimenten kon ik altijd terecht bij de postdocs van de kinderoncologie: **Mirjam, Renee**, **Judith**, **Eddy**, **Patricia**, en anderen. Ik wil **Ingrid** en **Marcel** bedanken voor hun hulp bij allerlei organisatorische en praktische zaken. Ook de secretaresses van de kinderoncologie zijn altijd een enorme hulp geweest, **Anita, Jeanine** en **Jeanny** stonden altijd voor mij klaar. **Jacqueline**, heel erg bedankt voor al je hulp, voornamelijk bij het laatste stuk van mijn promotieonderzoek. Geen probleem waar jij geen oplossing voor kunt bedenken!

"I get by with a little help from my friends." ~The Beatles

Wat is het leuk om contact te houden met de meiden waarmee ik Geneeskunde studeerde, **Dorien**, **Lisanne**, **Gianta**, **Esther**, **Rianne** en **Marloes**. De afgelopen jaren zagen we elkaar soms meer en dan weer minder. We zijn op zeer uiteenlopende plekken aan het werk (geweest) en wonen lang niet meer allemaal in Leiden. Het contact met jullie zorgt altijd weer voor een bredere blik op de dagelijkse beroepspraktijk en geeft perspectief aan de zaken waar een beginnende arts tegenaan loopt. Fijn dat jullie er nog steeds voor mij zijn!

Susan, ons contact begon gelijk innig: de avond van de dag waarop we elkaar voor het

eerst ontmoetten sliepen we al samen in één bed. Sindsdien kwam ik op kraambezoek, kregen we (veel) nieuwe collega's, dronken vele koppen koffie, organiseerden het beste kerstfeest *ever*, aten allerlei gerechten van jullie barbecue en vierden we een heel aantal feestjes. Je vond het zelfs goed dat ik ten huwelijk gevraagd werd op jouw verjaardagsfeest! Dank je voor alles wat je me geleerd hebt, je eerlijkheid wanneer ik naar je mening vroeg en de steun je die altijd gegeven hebt. Ik hoop dat we nog heel veel leuke dingen mee maken met jou, **Alan**, **Katia,** en **Iris**.

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Jasmijn, Daria, en Ingrid, een tijdje konden we het lab onveilig maken als de vier musketiers. Wat een lol hebben we gehad! **Ingrid**, af en toe kon ik het niet weerstaan om je op de kast te jagen als het ging om Limburg, *ut spit miech*. Je bent een enorm goede wetenschapper, en nu je ook internationale ervaring hebt zul je, wanneer je terug komt, zeer gewild zijn. Ik ben benieuwd waar dat je zal brengen. Dank je voor alle support die je geboden hebt tijdens het onderzoek, zowel werkgerelateerd als privé. **Daria**, hoewel in afstand ver weg ben je voor mijn gevoel nog altijd dichtbij. Je kwam als laatste AIO bij de AML-groep, je positieve instelling was op dat moment de perfecte aanvulling voor ons team. Supertrots ben ik dat je het aandurfde om je leven om te gooien om onderzoek te gaan doen in de VS. Je bent een zeer betrokken mens, en ik hoop dat we nog lang op elkaar zullen steunen.

"*A friend is one that knows you as you are, understands where you have been, accepts what you have become, and still, gently allows you to grow*."~ William Shakespeare

Lieve paranimfen, Linda en Jasmijn, heel erg fijn vind ik het dat jullie me op deze belangrijke dag bij willen staan als paranimf.

Linda, hoeveel kilometers zullen we samen afgelegd hebben de afgelopen jaren? Dagelijkse treinreizen leidden al snel tot innige gesprekken over alles wat ons bezig hield. Gek genoeg hadden we dan zelfs tijdens de koffiepauzes iets om over te kletsen, ons hele leven hebben we gedeeld. Dat was wennen toen je weg ging! Ik wens nog heel lang lief en leed te delen met jou, **Mark**, **Tamo** en jullie nieuwe gezinslid.

Jasmijn, onze vriendschap begon met een uniek moment; het passen van mijn bruidsjurk. Sindsdien hebben we veel andere leuke dingen gedaan, we hebben samen een roadtrip

gemaakt naar Kiel, zijn gaan shoppen in Antwerpen en kunnen altijd gezellig samen of met **Chris** en Maarten ergens iets eten of drinken. Ook kunnen we altijd onze projecten met elkaar bediscussiëren. Leuk dat we samen kunnen gaan groeien in onze carrières als arts, ik ben benieuwd waar ons dat zal brengen!

"*Above all, children need our unconditional love, whether they succeed or make mistakes, when life is easy and when life is hard.*" ~ Barack Obama

Vrije vertaling: "*Het belangrijkste wat kinderen nodig hebben is onvoorwaardelijke liefde, of ze nu slagen in het leven of fouten maken, wanneer het leven makkelijk is en wanneer het leven moeilijk is.*"

Lieve **pap** en **mam**, ik denk dat deze uitspraak van president Obama precies weergeeft wat ik van jullie meegekregen heb. Jullie liefde heeft ervoor gezorgd dat ik sterk in mijn schoenen sta, wat er ook gebeurt. In het verlengde hiervan ligt jullie onvoorwaardelijke steun, meest treffend weergegeven door de volgende uitspraak: "Ik ben trots op je, wat je ook wilt worden later, als je maar je uiterste best doet". Dank jullie voor deze fundamentele zekerheid. **Gonny** en **Alex**, dank jullie voor de acceptatie van ons, de kinderen. Ik was en ben altijd welkom, dat is iets om dankbaar voor te zijn.

Lieve **Ko** en **Sarie**, wat vond ik het als 16-jarige fijn dat mijn nieuwe vriendje zo'n leuke familie had. De steun die van jullie uitgaat is bijzonder. Dank jullie wel dat ik gelijk welkom was, ik voelde me vanaf het begin deel van jullie gezin.

Opa en **oma Rouw**, wat vind ik het leuk deel uit te maken van jullie familie. Ik hoop dat we nog lang mogen geniet van jullie oprechte interesse en liefde!

"*Siblings: children of the same parents, each of whom is perfectly normal until they get together.*" ~ Sam Levenson

Lieve **Lisette** en **Paul**, ik heb even getwijfeld of ik bovenstaande quote wel kon gebruiken maar ik ga ervan uit dat jullie hem wel kunnen waarderen. Mijn kleine zusje en broertje zijn zus en broer geworden, we zorgen nu voor elkaar. De band die wij samen hebben is zo bijzonder, daar kan niets en niemand tussen komen. Bijzonder dat **Anton** en **Thirza** zo moeiteloos in ons gezin geïntegreerd zijn, dat zij erbij zijn maakt het alleen maar gezelliger. Ik ben trots op jullie. Dank jullie dat jullie er altijd voor mij zijn.

Lieve **Arno**, wat een geluk heb ik met zo'n leuke zwager. Of we nu naar een festival gaan, een familiefeest hebben of een weekend weg gaan, we hebben het altijd goed naar onze zin. Dank voor je niet-aflatende interesse in mijn onderzoek, zelfs voor de inhoudelijke details schrik jij niet terug!

"If you live to be 100, I hope I live to be 100 minus 1 day, so I never have to live without you."
~ A.A. Milne, *Winnie-the-Pooh*

Lieve **Maarten**, op het moment dat jij, al die jaren geleden, op het balkon van je ouders mijn hand vastpakte, veranderde mijn leven. Je was niet alleen mijn nieuwe liefde maar ook al snel mijn beste vriend. Samen hebben we veel mooie maar ook zeker veel heftige dingen meegemaakt. Dat we het gered hebben ondanks de tegenslagen is voor mij een bevestiging dat het tussen ons wel goed zit. De afgelopen jaren heb je me onvoorwaardelijk gesteund, eerst met mijn beslissing om een promotieonderzoek te doen voor ik mijn studie Geneeskunde had afgerond, en later met het doorlopen van de coschappen. Dat is ook voor jou lang niet altijd makkelijk geweest. Ik realiseer me sterk dat dit zonder jou veel lastiger geweest zou zijn. We gaan een spannende tijd tegemoet met het verwerven van een opleidingsplaats en onze aanstaande gezinsuitbreiding. Ik zie onze toekomst vol vertrouwen tegemoet omdat ik weet dat jij naast me zult staan. Ik houd van jou.