EVI1 in Acute Myeloid Leukemia

Sanne Lugthart

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EVI1 in Acute Myeloïde Leukemie

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	Prof.dr. J.A. Foekens
	Prof.dr. R. Fodde

Aan mijn ouders

EVI1 IN ACUTE MYELOID LEUKEMIA

CONTENTS

CHAPTER 1

General introduction

NORMAL HEMATOPOIESIS

Hematopoiesis comes from the greek words for blood (haima) and formation (poiesis). All blood cells are derived from haematopoietic stem cells (HSCs), which are at the basis of the adult blood cell differentiation hierarchy (Figure 1) and provide continuous hematopoeitic cell production throughout life(1).

During embryonic development blood cells are first found in the yolk sac and its vasculature. The HSCs are produced by the aorta-gonad-mesonephros region and yolk sac and placenta from where they migrate to the fetal liver, where these cells further expand. Hereafter, HSCs transfer to the bone marrow from where they reside throughout adulthood(1).

The HSCs have *self-renewal* capacity: when HSCs proliferate, at least some of their daughter cells remain as HSCs, so the pool of stem cells does not become depleted(2). The HSCs are *pluripotent*: they generate progenitor cells or other daughters of HSCs, i.e., myeloid and lymphoid progenitor cells, respectively CMPs(3) and CLPs(4), which each can commit to the distinct differentiation pathways that lead to the production of one or more specific blood cell types(5). These progenitor cells cannot self-renew, but proliferate and differentiate eventually to mature blood cells e.g., granulocytes, monocytes, platelets, B- and T-cells, which then enter the blood circulation to fulfill their function(6). Due to the short life span of the mature cells, this cell production process is continuous and tightly regulated by various growth factors(2). For instance, a growth stimulus that is important for proliferation and self-renewal of HSCs is stem cell factor (SCF). Among other factors, granulocyte-macrophage CSF (GM-CSF), granulocyte CSF (G-CSF) and macrophage CSF (M-CSF) stimulate the production of committed progenitors (Figure 1). Moreover, these latter factors are also important activators of functional blood cell formation.

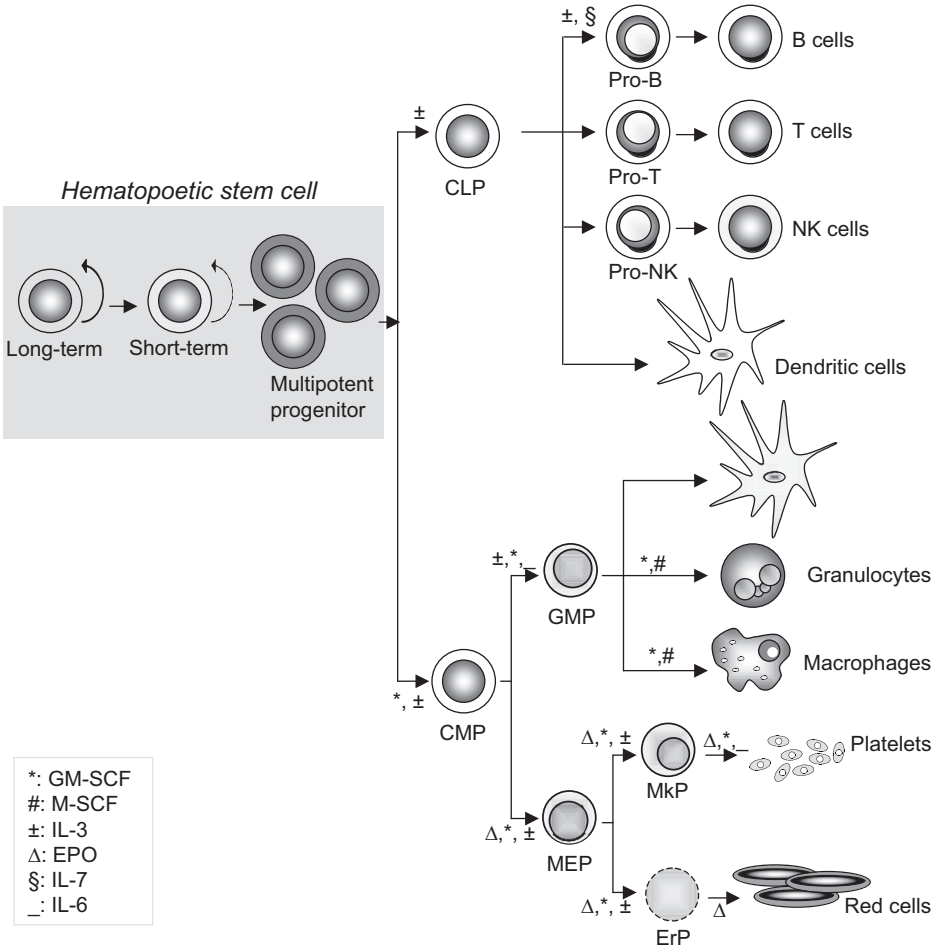

Figure 1. Hematopoeisis Diagram.
This diagram is originally derived from Reya et al.(2) It shows the development of haematopoietic stem cells (HSC). HSCs can be subdivided into long-term selfrenewing HSCs, short-term selfrenewing HSCs and multipotent progenitors. They give rise to common myeloid or lymphoid progenitor (respectively, CMP and CLP). Additionally, a selection of growth factors important for differentiation are added to each corresponding lineage. GMP; granulocytic myeloid progenitor, MEP; megakaryocyte erythrocyte precursor, ErP; erythrocyte precursor, MkP; megakaryocyte precursor, NK; natural killer, IL-3/6/7; interleukine 3/6/7, EPO; erythropoietin.

ACUTE MYELOID LEUKEMIA

AML is characterized by accumulation of immature myeloid cells, which are impaired in their ability to differentiate towards granulocytes or monocytes(7). These myeloid malignancies are heterogenous clonal disorders, with variable underlying genetic, epigenetic, molecular abnormalities and with different clinical responses to therapy.

Incidence

AML is among the most common malignant myeloid disorders in adults. The prevalence of AML is 3.8 cases per 100,000 individuals and increases with age, i.e., 17.9 cases per 100,000 adults aged 65 years and older(8). From 2002-2006, the median age at diagnosis for AML was 67 years of age. In children, AML comprises about 20% of the acute leukemias. The age specific incidence is in sharp contrast with adults and peaks with 11 cases per million at the age of 2 years, and decreases to 7 cases per million throughout the remainder of childhood and adolescence(9).

Diagnosis and Classification

The primary diagnosis of AML rests on the morphologic identification of leukemic myeloblasts in peripheral bone marrow and/or blood. At a morphologic level, the heterogeneity of AML is manifested by variability in the degree of differentiation of the cell lineage. AML has classically been categorized using the French–American–British (FAB) system(10), which is based on cytomorphology and cytochemistry. A recently updated classification model generated by the World Health Organization (WHO) in 2008, incorporates besides morphology, also cytogenetic and molecular data(11, 12). For the diagnosis of AML, a marrow blast count of over 20% is required, except for recurrent balanced chromosomal abnormalities t(15;17), inv(16) or t(16;16), t(8;21) and some cases of erythroleukemia(11). To identify lineage involvement cytochemistry and/or immunophenotyping is used. In case conventional cytogenetics is complicated, fluorescence *in situ* hybridization (FISH) and molecular diagnostic work-up may be needed to classify an AML patient into one of the five subtypes as designated by the WHO(11) (Table 1).

Table 1: WHO classification of Acute myeloid leukemia (WHO 2008)[#].

Acu
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[*]
>20% blood/marrow blasts *AND* history of myelodysplastic syndrome, or myelodysplastic/ myeloproliferative neoplasm; myelodysplasia-related cytogenetic abnormality; multilineage dysplasia; *AND* absence of both prior cytotoxic therapy for unrelated disease and recurring genetic abnormalities.
Therapy-related myeloid neoplasms
Cytotoxic agents implicated in therapy-related hematologic neoplasms: alkylating agents; ionizing radiation therapy; topoisomerase II inhibitors; others.
Acute myeloid leukemia, not otherwise specified (NOS)
Acute myeloid leukemia with minimal differentiation
Acute myeloid leukemia without maturation
Acute myeloid leukemia 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 (syn.: acute myelofibrosis; acute myelosclerosis)
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
Provisional entity: Natural killer (NK) cell lymphoblastic leukemia/lymphoma

Adapted from Swerdlow et al.(11) and Döhner et al.(13).

* Cytogenetic abnormalities sufficient to diagnose AML with myelodysplasia-related changes are:

- complex karyotype (defined as 3 or more chromosomal abnormalities)
- unbalanced changes: -7 or del(7q); -5 or del(5q); i(17q) or t(17p); -13 or del(13q); del(11q); del(12p) or t(12p); del(9q); idic(X)(q13);
- balanced changes: t(11;16)(q23;p13.3); t(3;21)(q26.2;q22.1); t(1;3)(p36.3;q21.1); t(2;11)(p21;q23); t(5;12)(q33;p12); t(5;7)(q33;q11.2); t(5;17)(q33;p13); t(5;10)(q33;q21); t(3;5)(q25;q34)

The subgroup "AML with recurrent genetic abnormalities" comprises several primary AML entities, i.e., inv(16)(p13.1q22) or t(16;16)(p13;q22), t(8;21)(q22;q22), t(15;17)(q22;q12) and t(9;11)(p22;q21). Three new cytogenetically defined entities have recently been incorporated, i.e., t(6;9)(p23;q34): *DEK-NEP214*; inv(3)(q21.q26.2) or t(3;3)(q21;q26.2): *RPN1-EVI1* and t(1;22)(p13;q13): *RBM15-MKL1*. Foremost, two new entities defined by the presence of gene mutations were added(13); nucloephosmin (*NPM1*) and CCAAT/enhancer binding protein alpha (*CEBPA*) (Table 1). These latter two subtypes are among AML patients without any recurrent chromosomal abnormality, the cytogenetically normal AML (CN-AML). The prevalence of recurrent chromosomal aberrations in AML are shown in Figure 2.

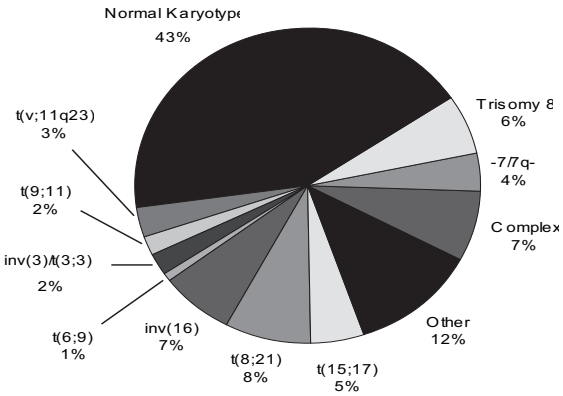

Figure 2. Distribution of Chromosomal Abnormalities in AML.
The percentage of chromosomal abnormalities of 458 AML patients younger than 60 years of age derived from various HOVON (the Haemato Oncology Foundation for Adults in the Netherlands) trails studied by Gröschel et al.(14), are shown.

Prognosis

The treatment response of a patient with AML depends on A) prognostic factors associated with treatment-related death; which can be predicted using the performing status(15) (Zubrod scale) and B) prognostic factors associated with resistance to therapy; here pre-treatment cytogenetic and molecular findings in leukemic blast are the most important predictors(8). Around 57% of the AML cases have cytogenetic abnormalities at diagnosis, leaving 43% of the leukemias with no cytogenetic abnormalities (CN-AML) (Figure 2). Together with recurrent molecular abnormalities (Table 2), response to treatment can be predicted i.e., favorable, intermediate or unfavorable risk(13) (Table 3). However, as more studies evolved many genetic abnormalities e.g., in *NPM1*(16-18), *EVI1*(19) (ecotropic viral integration site 1)

or *BAALC*(20) (brain and acute leukemia gene, cytoplasmic) were identified, each corresponding with different survival outcomes(16, 21, 22). Thus, risk classification is changing almost on a continuing basis, e.g., recently researchers identified that a monosomal karyotype (one or more monosomies and a structural abnormality) had a dismal outcome, more than complex karyotype(23). The focus of this thesis is on the AML cases in the unfavorable risk group highly expressing *EVI1* caused by chromosomal abnormalities i.e., inv(3)(q21q26.2) or t(3;3)(q21;q26.2) or caused by unknown mechanisms(19).

Table 2: Molecular abnormalities in AML.

AML subgroup	Molecular genetic defect#	Prognostic importance	Prevalence	Reference§
t(8;21)	KIT exon 8 or D816 mutation	inferior OS and EFS	2-11%	(25, 26)
	FLT3-ITD or D835 mutation	unclear	8%	(25, 26)
inv(16)/t(16;16)	KIT exon 8 or D816 mutation	inferior OS and RR	8-25%	(25-27)
	FLT3-ITD or D835 mutation	unclear	8%	(25, 27)
	N*RAS* mutation	no significant impact	18-26%	(27-29)
	K*RAS* mutation	no significant impact	9-17%	(27-29)
CN-AML	*FLT3*-TKD	no significant impact	11-14%	(30, 31)
	FLT3-ITD	inferior OS, EFS, DFS, CR rate	28-34%	(22, 31-33)
	NPM1 mutation	no effect CR rate, OS, EFS, RFS; higher CR rate, longer EFS	48-64%	(33-35)
	NPM1 mutant/*FLT3*-ITD negative	longer OS, RFS, DFS, CR rate	48-64%	(18, 30)
	CEBPA mutation	longer OS, DFS	10-15%	(22, 36)
	double *CEBPA* mutation	longer OS, EFS, DFS	10%	(37)
	single *CEBPA* mutation	no significant impact	3%	(37)
	MLL1 PTD	OS, EFS, RFS, CR rate	8-11%	(38-40)
	N*RAS* mutation	no significant impact	14%	(29)
	K*RAS* mutation	no significant impact	4%	(29)
	WT1 mutation	inferior OS and DFS	10%	(41)
	IDH1 mutation	unclear	16%	(42)
GE↑	*BAALC*	inferior OS, EFS, DFS, CR rate, CIR	†	(22, 43)
	*EVI1**	inferior OS, EFS and DFS	10%	(20)
	MN1	inferior OS, RFS, RR	†	(44, 45)
	ERG	inferior OS and CIR	†	(46, 47)

OS; overall survival, EFS; event-free survival, DFS; disease-free survival, RFS; relapse-free survival, CR; complete remission, CIR; cumulative incidence of relapse, CN-AML; normal katyotype, GE; gene overexpression.

A complete list of all abbreviations is listed at the Abbreviation Section on page 225.

§ Only a selection of articles is cited per molecular abnormality.

† Prevalence not determined, since gene expression levels were dichotomized using different criteria;

* Intermediate cytogenetic risk subgroup.

Table 3: Cytogenetic risk groups in AML*.

Favorable	
	t(8;21)(q22;q22); *RUNX1-RUNX1T1*
	inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
	Mutated *NPM1* without *FLT3-ITD* (normal karyotype)
	Mutated *CEBPA* (normal karyotype)
Intermediate	
	t(9;11)(p22;q23); *MLLT3-MLL*
	-Y; del(7q); +8; del(9q); +11; +13; del(20q); +21**
	Occurring as sole abnormality, or within a non-complex karyotype
	Mutated *NPM1* and *FLT3-ITD* (normal karyotype#)
	Wild type *NPM1* and *FLT3-ITD* (normal karyotype#)
	Wild type *NPM1* without *FLT3-ITD* (normal karyotype#)
Adverse	
	inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1*
	t(6;9)(p23;q34); *DEK-NUP214*
	t(v;11q23); *MLL* rearranged
	-5 or del(5q); -7; abnl(17p); complex karyotype‡

* Based on Dohner et al.(15); highlighted in gray are the latest molecular criteria..

** Occurring as sole abnormality, or within a non-complex karyotype

\# Includes all AMLs with normal karyotype except for those included in the favorable subgroup; most of these cases are associated with poor prognosis.

‡ Three or more chromosome abnormalities in the absence of one of the WHO designated recurring translocations or inversions.

Therapy

AML therapy consists of two phases; induction phase to achieve complete remission and consolidation phase, which aims to maintain complete remission and prevent relapse. For the past decades, the backbone of induction therapy has been the use of anthracyclines (daunorubicin and cytarabine). Patients between 18 and 60 years have three treatment options based on their risk classification, response to treatment and donor availability; an allogeneic hematopoietic stem cell transplant (allo-SCT) from an HLA-matched donor, an autologous SCT (auto-SCT) or chemotherapy(7, 8).

AML SUBTYPES STUDIED USING NOVEL TECHNIQUES

New techniques have begun to revolutionize diagnosis, prognosis and classifications of leukemias. The conventional cytogenetics e.g., G-banding is being completed by FISH analysis, allowing rapid testing for specific chromosomal translocations in metaphase and interphase cells. SKY (spectral karyotyping) uses 24 different fluorescently labeled chromosome painting probes to generate automated color display of all chromosomes. Both FISH and SKY techniques enhance accuracy and sensitivity of cytogenetic analysis(50). A more dense genome-wide technique is array comparative genomic hybridization (Array-CGH), a technique that can identify regions of genomic deletions or amplifications and subsequently disease genes at these loci. Where FISH and SKY are techniques that are being applied on a more or less routine basis, Array-CGH is a technology in progress that will most likely enter laboratory routine in the upcoming years.

Over the years, real-time quantitative PCR (Q-PCR) has been used to determine RNA expression on a gene-by-gene basis, which could have an impact on classification or prognosis of different AML subtypes. Molecular diagnosis by Q-PCR for recurring gene fusions, such as *CBFB-MYH11* (inv(16)/t(16;16)), *MLLT3-MLL* (t(9;11)) and *AML1-ETO* (t(8;21)) is being applied as a standard diagnostic work-up for AML patients by many laboratories. This method of detecting cytogenetic rearrangements is especially an option if chromosome morphology by standard cytogenetics is of poor quality, or if there is typical bone marrow morphology, but the suspected cytogenetic abnormality is not present(13). Classical examples are FAB M4 AMLs with an inv(16)/t(16;16). Another application for Q-PCR is minimal residual disease monitoring which is based on amplification and quantification of disease specific fusion genes that predict relapse(51).

Genome-wide approaches, in particular DNA microarray analysis for gene expression profiling (GEP) can delineate AML versus ALL samples based solely on patterns of gene expression(52). Within AML known and unknown subtypes with various survival outcomes could be identified based on gene expression profiles(53, 54). Molecular subtypes of AML could also be predicted using GEP(55). Furthermore, within small subtypes of AML e.g., *CEBPA* mutant AML cases, subgroups could be identified based on differences in GEPs(56). This indicates that, GEP is a valuable tool for classification, subtype discovery, and prediction of outcome(57).

More recently, epigenetic analysis by genome-wide methylation profiling using the HELP assay(58) showed that AML and ALL patients could be segregated based on differences in methylation levels of promoters of a large set of genes(59). Whether these innovative approaches will be of value for the diagnosis, classification and outcome prediction in AML requires further study and is one of the purposes of this thesis.

LEUKEMIC DISEASE MECHANISMS

One single mutation is in general believed to be insufficient to cause acute leukemia. There are different types of mutations which have been reported to cooperate in leukemogenesis(60). Particularly, in the core binding factor (CBF) leukemias, i.e., with inv(16)/t(16;16) or t(8;21) chromosomal abnormalities, class I and class II type mutations are frequently discerned. Class I mutations are the ones that cause aberrant activation of signal transduction pathways resulting in enhanced proliferation and/or survival of leukemia progenitor cells. Among these aberrations are mutations leading to activation of the receptor tyrosine kinase *FLT3* and *c-KIT* or defective RAS signaling. The second group, class II mutations, affect transcription factors or components of the transcriptional co-activation complex(8, 60). These mutations result in impaired differentiation and/or aberrant acquisition of self-renewal properties by hematopoietic progenitors, i.e., recurrent gene fusions resulting from inv(16)/t(16;16), t(8;21), t(15;17), respectively *CBFB-MYH11*, *RUNX-RUNX1T* and *PML-RARA*, as well as mutations in *CEBPA* and *MLL* and possibly *NPM1*(8). AML might develop when both mutation classes are present, which is supported by the finding that class I and II lesions occur together more commonly than each separately(8). Whether this view holds for every type of human AML is disputable.

The above mentioned AML subtypes mostly belong to the "favorable risk" leukemias, suggesting that particularly in those subtypes the two-class dogma is representative. In other AMLs, in particular the leukemias with a poor treatment response other mechanisms may be operational. For instance, the role of epigenetic factors in certain forms of cancer is evident and whether these abnormalities fit into this two class model is not clear. Various tumor suppressor genes have been reported to be hypermethylated and therefore silenced in AML. One could argue that this permanent hypermethylation is functionally equivalent to a genetic mutation(8). As an example, a group of patients has recently been identified with hypermethylation of the promoter of *CEBPA*. Based on gene expression profiling analysis it was evident that these cases were highly similar to cases that carried *CEBPA* mutations(56). Clinically however, *CEBPA*-silenced leukemias behaved differently from *CEBPA*-mutant leukemias. The latter group appeared to have a favorable response to treatment, whereas this was not evidently observed in the *CEBPA*-hypermethylated group of patients(36).

MOLECULAR TARGETS TO TACKLE AML

The increased knowledge of the genetic and molecular pathogenesis in AML has led to the development of molecular targeted therapeutic approaches, using compounds interfering with the specific function of these abnormalities.

Acute promyelocytic leukemia (APL) is associated with t(15;17)(q22;q12) giving rise to the *PML-RARA* fusion. APL is a unique leukemia according to its molecular biology and its sensitivity to all-*trans* retinoic acid (ATRA), a derivative of vitamin A(61) and arsenic trioxide (REF) . Treatment with ATRA causes differentiation of the immature leukemic promyelocytes into mature granulocytes, thereby restoring normal transcriptional differentiation programs. ATRA is typically combined with anthracycline based chemotherapy resulting in a clinical remission in approximately 90% of patients. The exquisite sensitivity of APL to ATRA is a successful example of targeted treatment of a specific molecularly defined subtype of AML (13, 61).

Risk adjusted therapeutic strategies and algorithms are also being developed. This is done according to molecular, cytogenetic and epigenetic abnormalities, mainly in AML subtypes that express prognostic significance in AML. For instance cytogenetic data have been firmly established to predicty treatment outcome. For instance core binding factor abnormalities may stratify AML as good risk or lack of cytogenetic abnormalities as intermediate risk. Various new molecular biomarkers have been discovered that add to the ability of identification of prognostic subtypes of AML. This thesis focuses on the AML subgroup highly expressing *EVI1*.

EVI1 AS A MULTIFUNTIONAL TRANSCRIPTIONAL REGULATOR IN NORMAL AND MALIGNANT HEMATOPOEISIS

Introduction to EVI1

The *EVI1* gene (ecotopic viral integration site-1) is localized on human chromosome 3q26.2 and was originally identified as a common retroviral integration site in murine myeloid tumors(64). The human gene spans about 100 kb and contains 16 exons with several alternatively spliced transcripts. As an example, a number of first exons for *EVI1* has been reported, which all splice to exon 2 of *EVI1*, i.e., 1A, -1B, -1C and 1D (Figure 3A). *EVI1* exists also as a longer form, called *MDS1/EVI1*, generated from the in-frame splicing of the small gene *m*yelo*d*ysplasia *s*yndrome *1* (*MDS1*) to the second exon of *EVI1*(65) (Figure 3A).

EVI1 is a relatively large protein with an apparent molecular mass of about 145 kDa. EVI1 contains two zinc finger domains, a proximal domain with seven zinc fingers and a distal domain with three zinc fingers. Between the two zinc finger domains a repression domain is located and a acidic region is at the C-terminus of the protein (Figure 3B)(66).

The *EVI1* gene encodes a nuclear putative transcriptional regulator and DNA interactions are coordinated through the two zinc finger domains(67). Multiple functional properties have been reported: (1) interaction with CtBP (C-terminal binding protein) and consequently acting as a repressor complex for transcription(67, 68); (2) regulation of and interference with transcription factors (GATA-2, GATA-1, Pu.1) critical for hematopoiesis and myeloid

homeostasis(69-71); (3) interaction with TGF-β-, JNK-, and PI3K-pathways(72-75); (4) and interaction with molecules implicated in genomic stability surveillance, through Sox4 by stabilization of the tumor suppressor gene *TP53*(76, 77). The EVI1 interacting proteins and their effect on cell function are shown in Figure 3B. Recent reports, show that EVI1 also interacts with histone methyltransferases, i.e., histone H3 lysine 9-specific histone methyltransferases SUV39H1(78, 79), methyl-CpG binding domain 3(80) (MBD3) and several chromatin remodeling proteins(81) such as HDAC1(82), pointing out a role for EVI1 in epigenetic regulation of gene expression.Although many functions of EVI1 remain to be elucidated, this thesis discusses the function of EVI1, its role in leukemia and new perspective mechanisms.

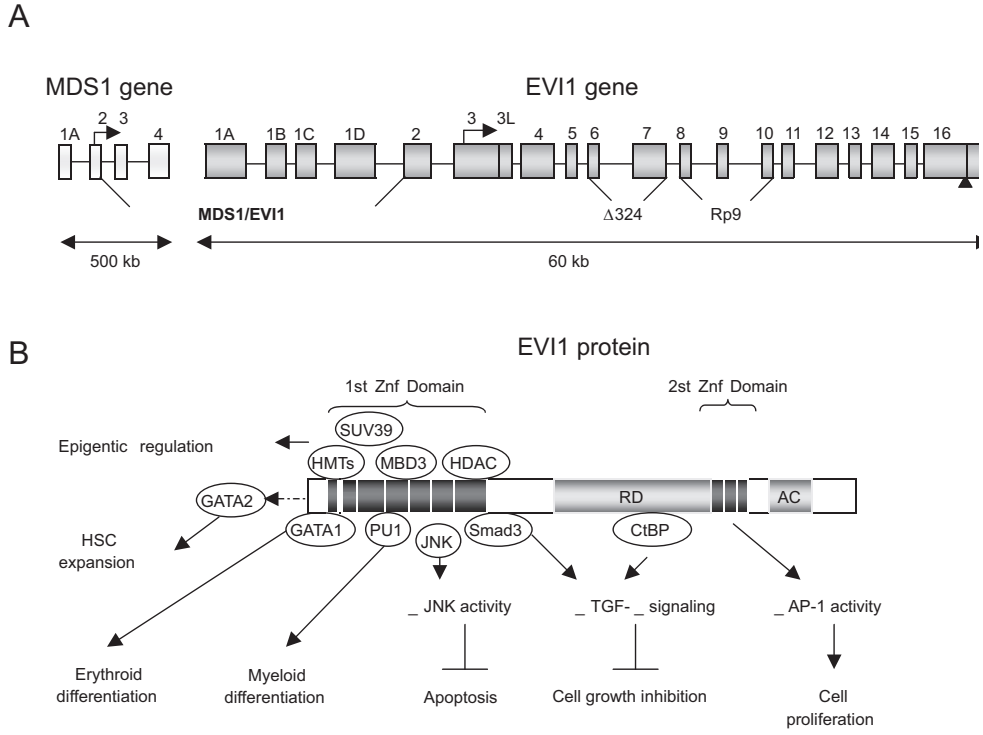

Figure 3. Genomic structure and biochemical properties of EVI1.
(A) Genomic structure of human *EVI1*, *MDS1* and *MDS1/EVI1* gene. Exons are represented by numbered boxes. The alternative splice variants are indicated by triangular lines. The picture is not drawn in scale. (B) EVI1 interacts with histone deacetylases (HDAC); HDAC1(74, 83) and HDAC2, histone methyltransferase (HMTs); H3-K9 methyltransferase SUV39H1(78, 79) (SUV39), methyl-CpG binding domain 3(80) (MBD3), thus through epigenetic regulation mediates transcriptional repression. EVI1 upregulates GATA-2 expression and promotes hematopoietic stem cell (HSC) expansion(71). EVI1 inhibits c-Jun N-terminal kinase (JNK) activity and prevents apoptosis(84). EVI1 also interacts with SMAD3(75, 85) and C-terminal binding protein(74) (CtBP) and through transforming growth factor(72) (TGF-β) blocks cell growth inhibition. Ac, acidic region; RD, repression domains, Znf; zinc-finger.

EVI1-related myeloid leukemias

The evolutionarily conserved *EVI1* gene has been implicated in a large number of human myeloid disorders. There is no question that its inappropriate expression is a dominant cause of aggressive human leukemia(66). Over-expression of *EVI1* (*EVI1*+) occurs in approximately 8% of patients with *de novo* acute myeloid leukemia (AML)(19). In AML cases carrying chromosome inv(3)(q21q26.2) or t(3;3)(q21q26.2) abnormalities, aberrant *EVI1* expression is caused by a breakpoint in or near the *EVI1* locus, i.e., 3q26.2. High levels of *EVI1* are also found in AML patients without any chromosomal rearrangements in this locus. In both groups *EVI1* predicts for adverse treatment response. However, *EVI1*+AMLs without 3q26 abnormalities represent an even larger and cytogenetically heterogeneous subset of AML(19). Accordingly, AMLs with 3q26 rearrangements i.e., inv(3)(q21q26.2) or t(3;3)(q21q26.2), often display dysplastic multilineage hemopoiesis suggestive of multipotent stem cell involvement(86) and give rise to the 3q21q26 syndrome. This syndrome is characterized by normal to elevated platelet counts at diagnosis, hyperplasia with dysplasia of megakaryocytes, poor treatment response and adverse survival outcome(87). Recently, the 3q21q26 syndrome has been associated with diabetes insipidus and T-cell antigen expression(88, 89). Other translocation partners and 3q abnormalities are not well characterized and it is unknown if the 3q21q26 syndrome is part of a more general 3q syndrome.

SCOPE OF THE THESIS

The work in this thesis focuses on the role of *EVI1* in acute myeloid leukemia. The thesis presents work, which is divided in four parts.

In the first part of the thesis (chapter 2), we studied a large cohort of AML patients with various chromosome 3q abnormalities and compared cytogenetic aberrations, molecular markers and clinical responses. We wished to investigate 1) whether AMLs with t(3;3)(q21;q26.2) or inv(3)(q21q26.2); *RPN1-EVI1* are different from AMLs with other chromosome 3q26 or 3q21 abnormalities, what the role is of *EVI1* in the different subgroups of AML and how the distinct AMLs groups with 3q aberrations responded to therapy.

In the second part, we asked what the prevalence of different *EVI1* 5' splice variants is in a cohort of AMLs. Furthermore, we wondered what the prognostic impact is in *EVI1* overexpressing AML. Cytogenetic, molecular and clinical characteristics of the subgroup of AMLs with aberrant *EVI1* expression were investigated in a large cohort of adult AML (chapter 3) as well as in pediatric AML patients (chapter 4).

The large adverse clinical impact on survival of adult *EVI1* AMLs, let us to design a new quantitative real-time PCR (Q-PCR), in which all five known *EVI1* splice variants could be measured. The aim of this diagnostic assay, which could be applied on a daily basis in the diagnostic work-up of AML patients, would be to further investigate the role of *EVI1* and its effect on prognosis. After testing this *EVI1* Q-PCR on our previously analyzed adult AML cohort, we validated the assay on a larger independent AML patient group. Combining both cohorts, we further investigated the prognostic role, possible confounders and the effect on type of treatment in this poor-risk AML group (chapter 5).

The third part of the thesis (chapter 6) centers on the elucidation of the function of *EVI1* in a subgroup of AMLs without chromosome 3q26 abnormalities, i.e., AMLs carrying an 11q23 rearrangement, generating *MLL* fusion genes. We particularly focused on human AML with a translocation t(9;11), giving rise to the *MLL-AF9* fusion gene. We hypothesize that *EVI1* is a target gene of *MLL* fusion protein. The functional correlation between 11q23 rearrangements and aberrant *EVI1* expression was investigated.

Lastly, we investigated the role of methylation in leukemogenesis in a genome-wide methylation study of a large representative cohort of AML patients. In this chapter we addressed the question if we could identify new AML subgroups that are clinically and prognostically relevant compared to recurrent molecular and cytogenetical abnormalities (chapter 7). Subsequently, we focused on *EVI1* AMLs and their methylation profiles in the following chapter. This study investigates the epigenetic role of *EVI1* AMLs and the function that *EVI1* may have in methylation is discussed (chapter 8).

Finally, the results outlined in this thesis are summarized and discussed in chapter 9.

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Clinical, Molecular, and Prognostic Significance of WHO Type Inv(3)(q21q26.2)/t(3;3)(q21;q26.2) and Various Other 3q Abnormalities in Acute Myeloid Leukemia: A Study of 6,500 Cases of AML

Sanne Lugthart[1], Stefan Gröschel[4*] H. Berna Beverloo[2], Sabine Kayser[3], Peter J.M. Valk[1], Shama L. Van Zelderen-Bhola[4], Gert-Jan Ossenkoppele[5], Edo Vellenga[6], Eva van den Berg-de Ruiter[7], Urs Schanz[8], Gregor Verhoef[9], Augustin Ferrant[10], Claus-Henning Köhne[11], Michael Pfreundschuh[12], Heinz-A. Horst[13], Elisabeth Koller[14], Marie von Lilienfeld-Toal[15], Martin Bentz[16], Arnold Ganser[17], Brigitte Schlegelberger[18], Martine Jotterand[19], Jürgen Krauter[17], Thomas Pabst[20], Matthias Theobald[21], Richard F. Schlenk[3], Ruud Delwel[1], Konstanze Döhner[3], Bob Löwenberg[1*] and Hartmut Döhner[3*]*

*These authors contributed equally to this work

[1]Department of Hematology, [2]Department of Clinical Genetics, Erasmus University Medical Center, Rotterdam (NL); [3]Department of Internal Medicine III, University Hospital Ulm, Ulm (D); [4]Department of Clinical Genetics, [5]Department of Hematology, Free University Medical Center, Amsterdam (NL); [6]Department of Hematology; [7]Department of Genetics, University Medical Center Groningen, Groningen (NL); [8]Clinic of Haematology, University Hospital Zürich, Zürich (CH); [9]University Hospital Gasthuisberg, Leuven (B); [10]Hôpital St. Luc, Brussels (B); [11]Klinik für Onkologie und Hämatologie, Städtische Kliniken Oldenburg (D); [12]Klinik für Innere Medizin I, Universitätskliniken des Saarlandes, Homburg (D); [13]III. Medizinische Klinik und Poliklinik, Universitätsklinikum Schleswig-Holstein, Kiel (D); [14]3. Medizinische Abteilung, Hanusch-Krankenhaus, Vienna (A); [15]Med. Klinik und Poliklinik III, Universitätsklinikum Bonn, Bonn (D); [16]Medizinische Klinik III, Städtisches Klinikum Karlsruhe (D); [17]Department of Hematology, Hemostasis, Oncology and Stem Cell Transplantation, [18]Department of Cell and Molecular Pathology, Hannover Medical School, Hannover (D); [19]Unité de cytogénétique du cancer, , Centre Hospitalier Universitaire Vaudois, Lausanne (CH); [20]Inselspital, Bern (CH); [21]Department of Hematology, University Medical Center of the Johannes Gutenberg, Mainz (D).

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ABSTRACT

Acute myeloid leukemia (AML) with inv(3)(q21q26.2)/t(3;3)(q21;q26.2) [inv(3)/t(3;3)] is recognized as a distinctive entity in the WHO classification. Risk assignment, clinical, and genetic characterization of AML with chromosome 3q abnormalities other than inv(3)/t(3;3) remain largely unresolved. Cytogenetics, molecular genetics, therapy response, and outcome analysis were performed in 6,515 newly diagnosed adult AML patients. Patients were treated on Dutch-Belgian-Swiss HOVON/SAKK (n=3,501) and German-Austrian AMLSG protocols (n=3,014). *EVI1* and *MDS1*/*EVI1* expression was determined by real-time quantitative PCR. 3q abnormalities were detected in 4.4% of AML cases (n=288/6,515). Four distinct groups were defined: (A) inv(3)/t(3;3) (32%); (B) balanced t(3q26) (18%); (C) balanced t(3q21) (7%); and (D) other 3q abnormalities (43%). Monosomy 7 was the most common additional aberration in groups (A) 66%, (B) 31%, and (D) 37%. N-*RAS* mutations and dissociate *EVI1* versus *MDS1/EVI1* overexpression were associated with inv(3)/t(3;3). Patients with inv(3)/t(3;3) and balanced t(3q21) at diagnosis presented with higher white blood cell and platelet counts. In multivariable analysis, only inv(3)/t(3;3), but not t(3q26) and t(3q21), predicted reduced relapse-free survival (HR=2.0, P<0.0001) and overall survival (HR=1.4, P=0.006). This adverse prognostic impact of inv(3)/t(3,3) was enhanced by additional monosomy 7. Group D 3q aberrant AML also had a very poor outcome, however, related to the co-existence of complex and/or monosomal karyotypes and cryptic inv(3)/t(3;3). Various categories of 3q abnormalities in AML can be distinguished according to their clinical, hematological, and genetic features. AML with inv(3)/t(3;3) represents a distinctive subgroup with unfavorable prognosis.

INTRODUCTION

Pretreatment cytogenetics is generally accepted as an important prognostic parameter in acute myeloid leukemia (AML) and is applied for risk stratification of the disease(1-3). In the recent World Health Organization (WHO) classification, AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1* [inv(3)/t(3;3)] has been incorporated as a new entity in the category "AML with recurrent genetic abnormalities"(4). AML with inv(3)/t(3;3) represents about 1-2% of AML(5, 6) and has been associated with characteristic morphologic features and poor outcome. Other balanced rearrangements involving bands 3q26.2 and 3q21 have been identified that occur at even much lower frequencies. Clinico-biologic features and prognostic relevance of these abnormalities remain elusive(7). Two such rearrangements, i.e., t(1;3)(p36.3;q21.1) and t(3;21)(q26.2;q22.1), are currently grouped among those abnormalities sufficient to make the diagnosis "AML with myelodysplasia (MDS)-related changes", although their clinical value remains unsettled(8-10). The same applies for other rare translocations, such as t(2;3)(p15~23;q26.2)(11) and t(3;12)(q26.2;p13)(12Furthermore, there are a number of other balanced and unbalanced 3q abnormalities. Variable risk assignments have been proposed for such cases(13). Whereas some risk classifications considered any 3q abnormality as prognostically unfavorable, in the recent AML recommendations for diagnosis and management of AML, only inv(3)/t(3;3) was specified as unfavorable abnormality(1). The lack of numerically robust studies probably explains the contradictory findings that have been reported about the prognostic value of 3q abnormalities(5-7, 14, 15).

The ecotropic viral integration site 1 (*EVI1*) gene maps to chromosomal band 3q26.2 and was first identified to be aberrantly upregulated in almost all AML with t(3;3)(q21;q26.2) (16) or inv(3)(q21q26.2)(17). Aberrant *EVI1* expression (*EVI1*+) is also found in a majority of AML with other 3q26 abnormalities(18-20). The association of *EVI1* expression and 3q abnormalities other than 3q26 remains to be elucidated. The fusion gene *MDS1/EVI1*(21) is concurrently overexpressed in many *EVI1*+AML. In a recent study, we reported a disproportionate pattern of elevated *EVI1* and non-elevated *MDS1/EVI1* expression in AML with inv(3)/t(3;3). High *EVI1* expression and complete absence of *MDS1/EVI1* expression occurred in more than half of these cases(19). The incidence and prognostic impact of other common molecular markers, e.g., *NPM1* (*nucleophosmin1*) gene mutations and *FLT3*-ITD (internal tandem duplications of the *Fms-like tyrosine-kinase-3* gene), across the spectrum of 3q aberrant AML remains to be exploredIn this study, we combined data from German-Austrian AMLSG and Dutch-Belgian-Swiss HOVON/SAKK studies to gain insight into the clinical, genetic, and prognostic features of AML with inv(3)/t(3;3), other 3q21 and 3q26 rearrangements, and remaining 3q abnormalities.

PATIENTS AND METHODS

Patient samples

A total of 6,515 newly diagnosed AML, of whom complete cytogenetics were available, were included in this study; 3,501 patients (15-79 years) enrolled between 1987 and 2008 on Dutch-Belgian Hemato-Oncology Cooperative Group/Swiss Group for Clinical Cancer Research (HOVON/SAKK) phase III trials HO04(A), -29, -42(A), and -43 (available at www.hovon.nl)(22-25) and 3.014 patients (16-85 years) recruited between 1993 and 2008 on AMLSG treatment protocols HD93(26), HD98A/B(27, 28), 06-04 (ClinicalTrials.gov Identifier NCT00151255), and 07-04 (ClinicalTrials.gov Identifier NCT00151242). Patients with acute promyelocytic leukemia were not included. All patients provided written informed consent in accordance with the Declaration of Helsinki. All trials were approved by the Institutional Review Board of Erasmus University Medical Center and University of Ulm. Cytogenetic and molecular analyses are described in the Supplementary methods.

All Supplementary methods, supplementary results, tables and figures are not included, but are available online at http://jco.ascopubs.org/.

Classification of 3q abnormalities

We defined a 3q rearrangement as any cytogenetic abnormality that involved the long arm of chromosome 3. AML with 3q abnormalities (n=288) were divided into four groups carrying (A) inv(3)(q21q26.2) or t(3;3)(q21;q26.2) (n=94), (B) other balanced 3q26 rearrangements (n=52), (C) balanced 3q21 rearrangements (n=19), and (D) remaining 3q abnormalities (n=123). Due to limitations of G-banding analysis, we included in group A also cases with inv(3)(q21~q22q26.2) (n=1) or t(3;3)(q21~q22;q26.2) (n=2), in group B cases with balanced abnormalities of bands 3q25~3q27 (n=12), and in group C cases with balanced abnormalities of bands 3q21~3q22 (n=1). We considered a chromosomal abnormality as recurrent if it was present in two or more cases of AML.

Statistics and survival analysis

Patient and cytogenetic characteristics of the 3q groups (A, B, C, D; n=288) were compared to a non-3q reference group with abnormal cytogenetics (CA) (n=2,231; excluding core binding factor leukemias and isolated losses of gonosomes) using the Wilcoxon-rank-sum test (continuous variables) and the Fisher's exact test (categorical variables). There was no significant difference in distribution of 3q aberrant AML among the clinical trials (Table S1). The definition of complete remission (CR) and overall survival (OS), event-free survival (EFS), and relapse-free survival (RFS) endpoints was based on recommended criteria(1). Survival analyses were performed in patients aged 15 to 60 years. Medium follow-up time was calculated using the method of Korn(29). Univariable survival analysis was performed

using the Kaplan-Meier survival analysis and log-rank P value. Cox proportional hazard regression models(30) with stratification to account for the two different cohorts (AMLSG versus HOVON/SAKK) were used. The proportional hazard assumption was tested(31) and no indication of non-proportionality was found for the two cohorts. Variable selection was not performed and all variables were included in the full Cox regression models, i.e., age (per 10 years); white blood count (WBC) (log10); platelet count (log10); type of AML(1) (*de novo* AML, secondary-AML [s-AML] or treatment-related AML [t-AML]), monosomy 7, complex karyotype, and monosomal karyotype(32). All statistical analyses were performed using SPSS 15.0 for Windows (SPSS Inc. Chicago, IL, USA).

RESULTS

Frequency of 3q abnormalities and relationship with age

3q abnormalities were present in 288 of 6,515 (4.4%) cases (HOVON/SAKK cohort: 146/3,501, 4.2% and AMLSG cohort: 142/3,014, 4.7%). Patients less than or equal to 60 years of age more frequently presented with 3q abnormalities (231/4,885, 4.7%) compared with patients older than 60 years of age (57/1,630, 3.5%).

Type of chromosome 3q abnormalities

The distribution of 3q abnormalities among groups A-D was as follows: Group A (n=94; 32%) included inv(3)(q21q26.2) (n=67) or t(3;3)(q21;q26.2) (n=27) (Figure 1A, Table S2); group B (n=52; 18%) included other balanced 3q26 rearrangements, such as t(2;3)(p15~23;q26.2), t(3;12)(q26.2;p13), t(3;21)(q26.2;q22.1) (Figure 1A, Table S3), or rearrangements of 3q26 with chromosome 3 bands other than 3q21 (n=10); group C (n=19; 7%) included other balanced 3q21 rearrangements, such as t(1;3)(p36.3;q21.1) and t(3;5)(q21;q31) (Figure 1A, Table S4; distribution of recurrent translocations of group B and C see Figure S1); group D (n=123; 43%) where remaining 3q abnormalities, e.g.,, add(3q), del(3q), and ins(3) were included (Figure 1A, Table S5). Individual karyotypes are shown in Tables S2-S5.

Recurrent cytogenetic abnormalities coexistent with 3q abnormalities

In AML with inv(3)/t(3;3), monosomy 7 was present in 62 of 94 (66%) cases; del(5q) (6%) and del(7q) (3%) were seen relatively infrequently, there was not a single case of monosomy 5 (Table 1). Monosomy 7 was also frequently found among groups B (31%) and D (37%), but less commonly in group C (5%) (P<0.0001). Complex karyotypes were preferentially found in association with group D type 3q abnormalities (74%) and were considerably less frequent among groups A (21%), B (17%), C (26%), and the reference CA category (30%) (Table 1). So called monosomal karyotypes(32) were found in the majority of AML of groups A (68%) and D (72%), and they were considerably less frequent among AML with balanced t(3q26) (group B; 35%), balanced t(3q21) (group C; 16%), and the reference CA group (23%) (P<0.0001).

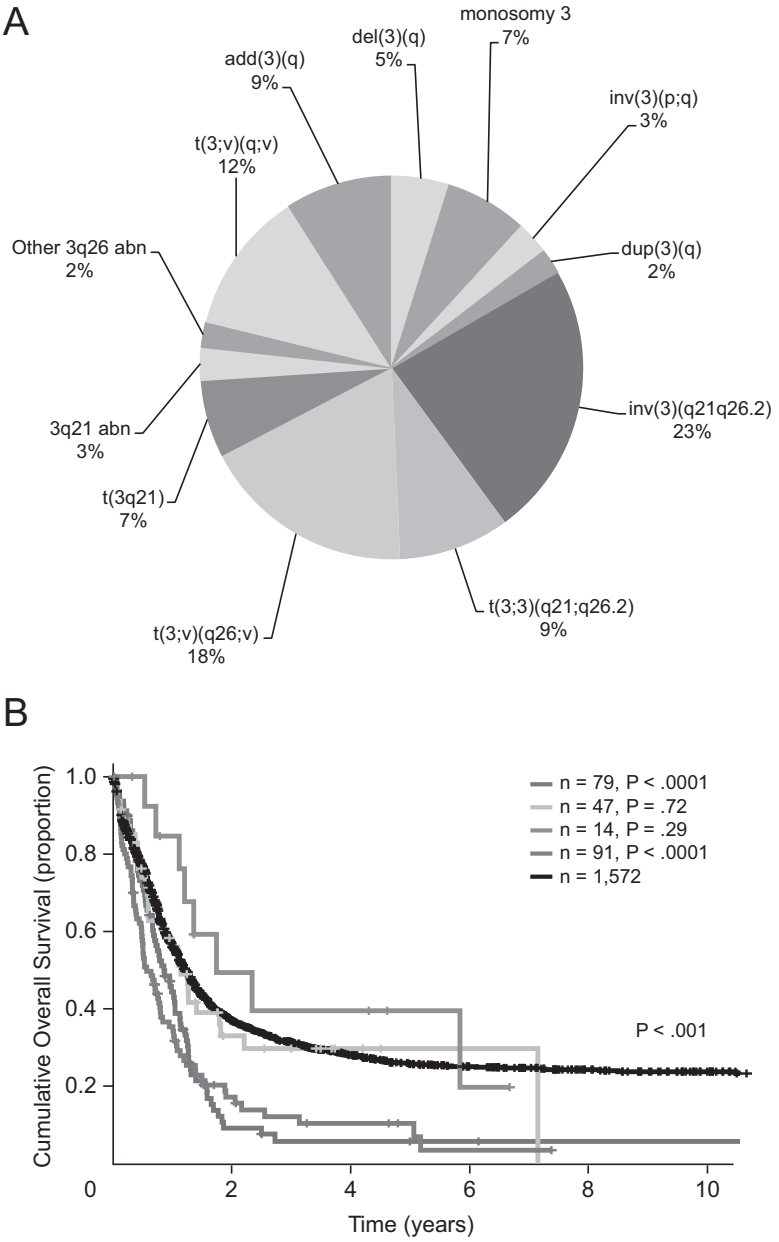

Figure 1. Distribution of cytogenetic characteristics and survival according to 3q aberrant AML groups. Distribution of 3q abnormalities (all ages, panel A) and overall survival (only 15-60 years of age, panel B) of 3q abnormal AML classified as inv(3)/t(3;3) (group A), balanced t(3q26) (group B), balanced t(3q21) (group C), and remaining 3q abnormalities (group D). A corresponding log-rank P value per comparison to the non-3q cytogenetically abnormal (CA) reference group is shown.

Table 1. Clinical characteristics and treatment effects of four 3q aberrant AML categori

	inv(3)/t(3;3) [A] n=94	P value#	t(3q26) [B] n=52	P value#	t(3q21) [C] n=19	P value#	rest 3q [D] n=123	P value#	Reference [CA] n=2231
Gender									
Male	51 (54%)	1.0	23 (44%)	0.16	14 (74%)	0.11	73 (59%)	0.81	1211 (54%)
Female	43 (46%)		29 (56%)		5 (26%)		50 (41%)		1020 (46%)
Age, years (median, range)	48 (15-75)	<0.0001	46 (19-74)	0.003	48 (21-74)	0.29	54 (24-80)	0.62	55 (15-85)
WBC, x10^9/L	14.8 (1-234)	<0.0001	7.2 (0.5-532)	0.50	14.6 (1-118)	0.54	4.9 (0.5-227)	0.06	7.2 (0.3-591)
Missing	4		1		1		7		52
Platelets, x10^9/L	144 (2-916)	<0.0001	55 (9-933)	0.78	117 (34-603)	0.001	65 (8-419)	0.16	57 (1-998)
Missing	5		2		1		8		56
Bone marrow blast, %	63 (10-100)	0.76	50 (1-95)	0.007	60 (15-95)	0.45	50 (50-96)	0.004	65 (0-100)
Missing	11		5		2		16		227
FAB classification									
M0	10 (11%)	0.92	3 (7%)	0.37	3 (19%)	0.21	7 (6%)	0.17	177 (9%)
M1	18 (21%)		9 (20%)		-		16 (15%)		391 (19%)
M2	17 (19%)		12 (27%)		7 (44%)		23 (21%)		451 (22%)
M3	-		-		-		-		12 (1%)
M4	17 (19%)		5 (11%)		3 (19%)		15 (14%)		272 (13%)
M5	9 (10%)		4 (9%)		1 (6%)		11 (10%)		344 (17%)
M6	-		1 (2%)		-		13 (12%)		82 (4%)
M7	6 (7%)		3 (7%)		-		3 (1%)		29 (2%)
Missing	6		7		3		15		200
MDS	11 (13%)		8 (18%)		2 (12%)		20 (19%)		273 (13%)

Type of AML									
de novo	82 (87%)	0.59	39 (76%)	0.17	18 (100%)	0.27	101 (84%	0.77	1852 (84%)
s-AML	10 (11%)		7 (14%)		-		11(9%)		244(11%)
t-AML	2 (2%)		5 (10%)		-		8 (7%)		105 (5%)
Missing	0		1		1		1		30
Cytogenetics*									
Monosomy 5	0 (0%)	0.006	1 (2%)	0.37	0 (0%)	0.62	35 (29%)	<0.0001	134 (6%)
Deletion 5q	6 (6%)	0.14	6 (12%)	1.0	4 (21%)	0.27	40 (33%)	<0.0001	260 (12%)
Monosomy 7	62 (66%)	<0.0001	16 (31%)	0.001	1 (5%)	0.50	45 (37%)	0.0001	298 (13%)
Deletion 7q	3 (3%)	0.11	2 (4%)	0.43	3 (16%)	0.20	21 (17%)	<0.0001	180 (8%)
Complex karyotype**	20 (21%)	0.084	9 (17%)	0.37	5 (26%)	1.0	91 (74%)	<0.0001	661 (30%)
Monosomal karyotype**	64 (68%)	<0.0001	18 (35%)	0.06	3 (16%)	0.59	88 (72%)	<0.0001	507 (23%)
	inv(3)/t(3;3) [A] n=79	**P value#**	**t(3q26) [B] n=49**	**P value#**	**t(3q21) [C] n=14**	**P value#**	**rest 3q [D] n=91**	**P value#**	**Reference [CA] n=1,572**
Clinical end points									
CR rate (%)	31%	<0.0001	44%	0.008	64%	1.0	43%	<.0001	70%
Overall survival (5-yr)	5.7%±3	<0.0001	29.7%±7	0.72	39.5%±15	0.29	10.4%±4	.0001	25.7%±1
Median survival (months)	10.3		13.7		20.9		6.7		14.6
Event-free survival (5-yr)	0%±0	<0.0001	15.8%± 6	0.002	8.2%±8	0.47	3.4%±2	<.0001	18.7%±1
Relapse-free survival (5-yr)	4.3%±4	<0.0001	45.9%±11	0.41	0%±0	0.35	9.1%±5	<.0001	28.3%±2

Abbreviations: CA, cytogenetically abnormal non-3q; WBC, white blood cell count; t-AML, therapy-related AML; s-AML, secondary AML; FAB, French-American-British classification; MDS, myelodysplastic syndrome; CR, complete remission.

P values were calculated using Wilcoxon-rank-sum test (continuous variables) and the Fisher's exact test (categorical variables), comparing each 3q category to the non-3q CA group.

* Some patients have more than one cytogenetic abnormality.

** Complex and monosomal karyotype(32) is defined as described in the Supplementary Methods section.

Molecular abnormalities in 3q abnormal AML

In a subset of cases we had access to material to assess the distribution of various recurrent gene mutations (Table S6). Interestingly, AML with inv(3)/t(3;3) (28%) and AML with other t(3q26) (25%) frequently carried N-*RAS* mutations when compared with AML of groups C (0%), D (9%), and the reference CA group (7%). *NPM1* mutations were detected at a low frequency in all 3q categories A-D between 0% and 8%. *FLT3*-ITD were observed in less than 20% of cases in any of the four 3q groups and reference CA group (16%). Compared to the non-3q CA group (6%), *FLT3*-TKD (tyrosine kinase domain) mutations were apparent at similar frequencies in 3q groups A and D (7% and 8%), absent in group B, but more frequent in group C (18%). No *MLL*-partial tandem duplication (*MLL*-PTD), c-*KIT*, and *CEBPA* mutations were detected in any of the four 3q categories.

Clinical characteristics of 3q abnormal AML

There were no differences in gender, French-American-British (FAB) classification, and type of AML (s-AML, t-AML or *de novo* AML) between the four 3q aberrant groups A-D and the non-3q reference group (Table 1). Patients in groups A and B were younger at diagnosis compared with the reference CA group. AML with inv(3)/t(3;3) (group A) as well as those with t(3q21) (group C) presented with 2-fold higher WBC (median $14.8x10^9$/L and $14.6x10^9$/L, P<0.0001, P=0.54) and higher platelet counts (median $144x10^9$/L and $117x10^9$/L, P<0.0001, P=0.001) compared with non-3q CA cases (Table 1, Figure S2).

Treatment outcome of 3q abnormal AML

Analysis of treatment response and survival was restricted to patients between 15 and 60 years of age, i.e., group A-D and reference CA included 79, 49, 14, 91, and 1,572 cases, respectively. The median follow-up time for survival was 38.7 months. CR rates were considerably lower in patients of groups A (31%, P<0.0001), B (44%, P<0.008), and D (43%, P<0.0001) compared with the reference non-3q group (70%).

Patients with inv(3)/t(3;3) (group A, n=79) had highly unfavorable 5-year survival rates (OS 5.7%±3, P<0.0001; EFS 0%, P<0.0001; RFS 4.3%±4, P<0.0001) (Figure 1B, Figure S3, Table 1). There was no difference in survival between patients with inv(3)(q21q26.2) (n=57) and t(3;3) (q21;q26.2) (n=22) (Figure 2A). OS of patients with inv(3)/t(3;3) and additional monosomy 7 was even worse compared with those not exhibiting monosomy 7 (P=0.008) (Figure 2B). Baseline clinical characteristics did not differ between the latter subgroups; of note, *EVI1* expression levels were significantly higher in cases with additional monosomy 7 (Table S7).

AML with t(3q26) (group B) and t(3q21) (group C) had intermediate survival values not differing from the reference non-3q group, i.e., 5-year OS probabilities of 29.7%±7 (group B, P=0.72) and 39.5%±15 (group C, P=0.29) (Table 1, Figure 1B). Although t(3q21) as a group had an intermediate survival rate, patients with the most frequent t(1;3)(p36.3;q21.1) had very poor outcome (5-year OS, 17.1%±16) (Figure 2C, Table S8).

Table 2. Multivariable analysis.

	Achievement of CR				Overall Survival				Event-free Survival				Relapse-free Survival			
Prognostic markers	**OR**	**95.0% CI**		**P value[#]**	**HR**	**95.0% CI**		**P value[#]**	**HR**	**95.0% CI**		**P value[#]**	**HR**	**95.0% CI**		**P value[#]**
Age (difference of 10 years)	0.81	0.74	0.89	<0.0001	1.23	1.19	1.27	<0.0001	1.15	1.11	1.19	<0.0001	1.15	1.10	1.20	<0.0001
Log_{10}(Platelets)	0.88	0.67	1.15	0.34	0.84	0.74	0.94	0.003	0.94	0.84	1.05	0.3	0.77	0.66	0.90	0.001
Log_{10}(WBC)	0.80	0.68	0.94	0.007	1.25	1.16	1.34	<0.0001	1.16	1.09	1.24	<0.0001	1.10	1.01	1.21	0.035
Type of AML[§]																
t-AML	1.03	0.62	1.72	0.91	1.14	0.91	1.42	0.26	0.94	0.76	1.16	0.58	1.20	0.90	1.60	0.21
s-AML	0.56	0.37	0.82	0.003	1.27	1.10	1.46	0.001	1.23	1.07	1.41	0.004	1.02	0.82	1.28	0.84
Cytogenetics[*]																
Monosomy 7	0.84	0.61	1.17	0.3	1.14	0.99	1.32	0.06	1.11	0.97	1.27	0.13	1.19	0.97	1.45	0.09
Complex karyotype[**]	0.76	0.56	1.02	0.07	1.41	1.24	1.61	<0.0001	1.26	1.11	1.42	<0.0001	1.23	1.03	1.46	0.02
Monosomal karyotype[**]	0.51	0.36	0.72	<0.0001	1.78	1.53	2.07	<0.0001	1.63	1.41	1.88	<0.0001	1.57	1.27	1.94	<0.0001
3q groups																
inv(3)/t(3;3) [A]	0.29	0.17	0.51	<0.0001	1.42	1.11	1.82	0.006	2.00	1.58	2.53	<0.0001	1.99	1.35	2.92	<0.0001
balanced t(3q26) [B]	0.35	0.19	0.66	0.001	1.15	0.82	1.61	0.43	1.77	1.30	2.40	<0.0001	0.93	0.54	1.58	0.78
balanced t(3q21) [C]	0.61	0.19	1.95	0.41	0.92	0.53	1.58	0.75	1.21	0.75	1.96	0.43	1.63	0.96	2.78	0.07
rest 3q [D]	0.57	0.35	0.92	0.02	1.02	0.82	1.26	0.88	1.04	0.85	1.27	0.72	1.15	0.85	1.56	0.36

Abbreviations: OR, odds ratio; HR, hazard ratio; CI, confidence interval; WBC, white blood cell count; t-AML, therapy related AML; s-AML, secondary AML.

[#] Cox regression analysis was restricted to patients between 15 and 60 years of age and calculated as described in the Material and Methods section.

[§] Type of AML vs. *de novo* AML

[*] Monosomy 7 vs. no monosomy 7, complex karyotype vs. non complex karyotype; monosomal karyotype vs. non monosomal karyotype, 3q group vs. CA reference group.

[**] Complex and monosomal karyotype are defined as described in the Supplementary Methods section.

AML of group D with various other 3q abnormalities were highly associated with complex and monosomal karyotypes and showed very poor 5-year survival values (OS 10.4%±4, EFS 3%±2, RFS 9%±5).

In a multivariable Cox regression analysis, stratifying for the HOVON/SAKK and AMLSG cohorts, we set out to assess the prognostic impact of the four 3q categories in relation to other prognostic markers, i.e., age, WBC, platelet counts, s-AML, t-AML, complex karyotype, monosomal karyotype, and monosomy 7 (Table 2). This analysis revealed that 3q groups A, B, and D had independent negative prognostic impact on achievement of CR. With regard to survival endpoints, only inv(3)/t(3;3) was found to be an independent prognostic marker for OS (HR=1.4, P=0.006), EFS (HR=2.0, P<0.0001), and RFS (HR=2.0, P<0.0001).

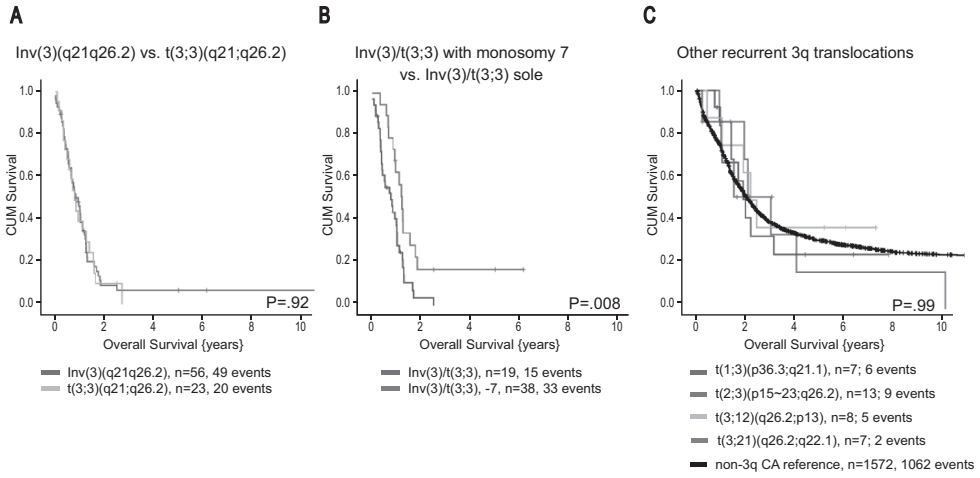

Figure 2. Survival of AML according to specific 3q26 and 3q21 rearrangements.
Overall survival (panel A) for AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2). Overall survival (panel B) for inv(3)/t(3;3) cases with or without monosomy 7. Overall survival (panel C) for other recurrent 3q26 and 3q21 translocations. A corresponding log-rank P value is shown.

EVI1 and *MDS1/EVI1* expression in AML with 3q aberrations

EVI1 and *MDS1/EVI1* expression levels are presented in Tables S2-S5, per individual patient for each 3q group.

In group A, *EVI1* was highly expressed in 95% (54/57, median expression 5.6) of cases (Table 3, Figure S5A); measurable levels of *MDS1/EVI1* expression were found in 50% (23/46) of cases (median expression 0.11) (Table 3, Figure S5B). Pairwise comparison of *EVI1* and *MDS1/EVI1* expression levels revealed that in the majority (40/46, 87%) of cases *EVI1* expression was higher than *MDS1/EVI1*, i.e., a disproportionate ratio of *EVI1* and *MDS/EVI1* transcript levels (Figure 3A).

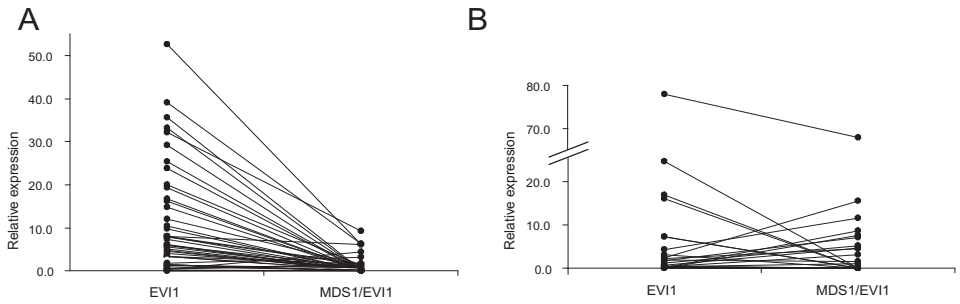

Figure 3. Pair-wise comparison of *EVI1* and *MDS1/EVI1* expression in inv(3)/t(3;3) cases and balanced 3q26 translocations.
EVI1 and *MDS1/EVI1* expression is shown for inv(3)/t(3;3) cases (panel A) and balanced t(3q26) abnormalities (panel B). Case 332_HO43 is not shown, due to high *MDS1/EVI1* levels (i.e., 154).

The relative *EVI1* and *MDS1/EVI1* expression in the balanced t(3q26) AML (group B) showed a different pattern (Figure 3B). In 85% of the cases in this group, *EVI1* was highly expressed (23/27, median expression of 1.9), and in 57% *MDS1/EVI1* expression was above threshold (13/25, median expression 0.99) (Table 3). In 12/25 cases of group B, the ratio of *EVI1* and *MDS/EVI1* was reverted with higher *MDS1/EVI1* values than *EVI1* levels. Only a small subset (32%, 8/25) of cases showed a disproportionate ratio of *EVI1* and *MDS/EVI1* transcript levels (Table S3, Figure 3B).

The balanced t(3q21) cases of group C infrequently expressed *EVI1* (20% of cases) or *MDS1/EVI1* (11% of cases) (Table 3, Figure S5), suggesting that in those patients the *RPN1* gene located at the 3q21 breakpoint had been translocated to other loci.

In group D, *EVI1* expression was found to be high in 13/44 cases (30%), and 6/37 (16%) overexpressed *MDS1/EVI1* (Table 3, Figure S5). Ten out of thirteen had disproportionate *EVI1* versus *MDS1/EVI1* ratios. Importantly, most of these *EVI1* positive cases carried an abnormality involving chromosomal band 3q26 or 3q21, i.e., add(3)(q26) (n=1), del(3)(q25), or del(3)(q21q26) (n=1), add(3)(q21) (n=4), or del(3)(q21) (n=2) (Table S5), which were not found among the cases not expressing *EVI1* and *MDS1/EVI1* in group D. The disproportionate *EVI1* versus *MDS1/EVI1* ratios suggest cryptic 3q abnormalities, thus we screened three of these group D cases [add(3)(q21), n=2; add(3)(q2?7), n=1] using the 3q FISH analysis and detected inv(3)(q21q26) in all of them.

Table 3. Molecular characteristics of the four 3q aberrant AML categories

	inv(3)/t(3;3) [A]	t(3q26) [B]	t(3q21) [C]	rest 3q [D]	Reference non-3q CA	P value[#]
EVI1						
Positive, no. (%)	54 (95%)	23 (85%)	2 (20%)	13 (30%)	87 (17%)	< 0.001
Median, Range	5.6 (0-52)	1.9 (0-78)	0.0 (0-5.4)	0.0 (0-16)	0.0 (0-43)	< 0.001
Analyzed	57	27	10	44	504	
MDS1/EVI1						
Positive, no. (%)	23 (50%)	13 (57%)	1 (11%)	6 (16%)	52 (13%)	< 0.001
Median, Range	0.11 (0-9.2)	0.99 (0-154)	0.0 (0-4.2)	0.0 (0-15)	0.0 (0-95)	< 0.001
Analyzed	46	25	9	37	398	

[#] P values were calculated using Wilcoxon-rank-sum test (continuous variables) and the Fisher's exact test (categorical variables).

DISCUSSION

In the current study, we assessed the clinical and genetic features as well as the prognostic impact of a large series of AML with chromosome 3q abnormalities (n=288) that were identified among 6,515 cases from the HOVON/SAKK and AMLSG study groups.

We provide further evidence that AML with inv(3)/t(3;3) (designated here as group A) are clinically, cytogenetically, and molecularly distinctive, supporting their incorporation as a new entity in the current WHO classification. Inv(3)/t(3;3) was associated with younger age and higher platelet and WBC counts, and they presented with notably high frequencies of monosomy 7 (66%) and N-*RAS* mutations (28%). Of note, we were able to show that AML with inv(3)/t(3;3) and additional monosomy 7 had even worse survival than cases without monosomy 7 (Figure 2B). These AML formally fulfill the definition of the notoriously unfavorable monosomal karyotype(32). Among the four 3q groups, only inv(3)/t(3;3) was found to be an independent adverse predictor of overall survival.

AML with inv(3)/t(3;3) commonly expressed high *EVI1* levels, whereas *MDS1/EVI1* transcripts were low or absent, which is in line with the observation that *MDS1/EVI1* expression is abrogated, resulting from a breakpoint between the *MDS1* and the *EVI1* gene(18-20). This uniformity in the balance of *EVI1* versus *MDS1/EVI1* mRNA expression was not apparent in the other 3q groups.

AML with other t(3q26) (group B) were also associated with younger age, but unlike cases with classical inv(3)/t(3;3) did not present with elevated platelet counts. They also had a relatively high frequency of additional monosomy 7 (31%), albeit somewhat lower compared with inv(3)/t(3;3) cases. In cases with t(3;12)(q26.2;p13) and t(3;21)(q26.2;q22.1), the *EVI1* locus is translocated to *ETV6* and *RUNX1*, respectively(7, 20, 33). The high *MDS1*/*EVI1* levels in five t(3;21) cases, but the complete absence of *MDS1*/*EVI1* in two other t(3;21) indicate that *RUNX1-MDS1*/*EVI1*, as well as *RUNX1-EVI1* may be formed, depending on the location of the 3q26.2 breakpoint. Thus, *RUNX1* or *ETV6* may translocate 5' of *MDS1* or 5' of *EVI1*. Whether the expression of the distinct fusion types is associated with differences in treatment response cannot be answered and requires an even larger patient cohort. Although in the t(3q26) group the CR rate was significantly lower compared with the reference group, there was no impact on survival (Figure 1B). Also, in multivariable analysis, t(3q26) had no impact, indicating that in terms of outcome these AML are different from classical inv(3)/t(3;3). Although the number of abnormalities remained small, we did carry out an exploratory subgroup analysis for the most frequent recurrent t(3q26) cases, i.e., t(2;3)(p15~23;q26.2), t(3;12)(q26.2;p13), and t(3;21)(q26.2;q22.1) (Figure 2C, Table S8, and Supplementary results).

AML with balanced t(3q21) (group C) represented a small subset. Similar to cases with inv(3)/t(3;3), these AML presented with high platelet counts. In contrast to AML with inv(3)/t(3;3) and other t(3q26), there was only a single case with additional monosomy 7. Although prognosis of t(3q21) as a group was indistinguishable from the reference group

(Figure 1B), the most frequently found t(1;3)(p36.3;q21.1) had a poor outcome (Figure 2C, Table S8). AML with t(1;3)(p36.3;q21.1) have been studied molecularly, i.e., the *RPN1* locus at 3q21, the translocation partner of *EVI1* in inv(3)/t(3;3) AML, translocates to *PRDM16* (*MEL1*; *MDS1/EVI1*-like-1) at 1p36, that is highly homologous to *EVI1* (*PRDM3*)(8). The aberrant expression of *PRDM16,* as well as *EVI1* in AML, frequently involves the *RPN1* locus, which points to novel directions of expression regulation of *PRDM* family members via *RPN1*. Few cases were found with translocations of 3q21 to chromosome bands other than 1p36.3. Interestingly, in one patient 3q21 was translocated to band 11p15 that harbors *PRDM11* (Figure S1C). In group D, one case was observed with a putative translocation between chromosomes 3q21 and 6q21, the locus that harbors *PRDM1* (*BLIMP*). Together, these data suggest that *EVI1* homologues might play a role in transforming myeloid progenitors, in particular when they come under the control regulatory elements in the *RPN1* gene.

Group D constituted a very heterogeneous cohort comprising various other mostly unbalanced 3q abnormalities without any specific presenting clinical and molecular characteristics. This group was associated with a very poor outcome similar to that of patients with inv(3)/t(3;3). In a large proportion, these abnormalities were part of a complex and/or a monosomal karyotype. In addition, we were able to detect cryptic inv(3) by FISH analysis in cases with high *EVI1*, but low or no *MDS1/EVI1* transcript levels, in which enough material was available. These cases frequently had del(3)(q21) or add(3)(q21), highly suggestive of the existence of a cryptic inversion/translocation involving *EVI1* and *RPN1*. Further molecular studies will be necessary to identify biologic subsets of AML among these cases. These observations indicate the importance of introducing *EVI1* and *MDS1/EVI1* expression analysis as well as FISH analysis as a routine to support cytogenetic practice(34).

In conclusion, the incidence of 3q abnormalities in AML is 4.4%. The inv(3)/t(3;3) abnormalities are frequently associated with monosomy 7 and N-*RAS* mutations and confer independent prognostic value as regards treatment response and survival. AML with diverse other 3q abnormalities, i.e., unbalanced 3q26 or unbalanced 3q21 abnormalities, present with monsomal karyotypes and complex karyotypes in the great majority of cases and show dismal survival as well. AMLs with cryptic 3q rearrangements, identified according disproportionate *EVI1* and *MDS1/EVI1* expression quotients, in the future, should be included in the WHO entity inv(3)/t(3;3).

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CHAPTER 3

High *EVI1* Levels Predict Adverse Outcome in Acute Myeloid Leukemia: Prevalence of *EVI1* Overexpression and Chromosome 3q26 Abnormalities Underestimated

Sanne Lugthart[1], Ellen van Drunen[2], Yvette van Norden[3], Antoinette van Hoven[1], Claudia A.J. Erpelinck[1], Peter J. M. Valk[1], H. Berna Beverloo[2], Bob Löwenberg[1] and Ruud Delwel[1]

[1]Department of Hematology; [2]Department of Clinical Genetics; and [3]Department of Trials and Statistics, Erasmus University Medical Center, Rotterdam, The Netherlands

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ABSTRACT

Inappropriate expression of *EVI1* (ecotropic virus integration-1), in particular splice-form *EVI1-1D*, through chromosome 3q26 lesions or other mechanisms has been implicated in the development of high-risk acute myeloid leukemia (AML). To validate the clinical relevance of *EVI1-1D,* as well as of the other *EVI1* splice-forms and the related *MDS1/EVI1 (ME)* gene, real-time RQ-PCR was performed in 534 untreated adults with de novo AML. *EVI1-1D* was highly expressed in 6% of cases (n=32) whereas 7.8% were *EVI1* positive (n=41) for all splice variants. High *EVI1* predicted for a distinctly worse event free survival (HR=1.9; P=0.002) and disease free survival (HR=2.1, P=0.006) following multivariate analysis. Importantly, we distinguished a subset of *EVI1* positive cases that lacked expression of *ME* (*EVI1+ME-*; n=17) from cases that were *ME* positive (*EVI1+ME+*; n=24). The atypical *EVI1+ME-* expression pattern exhibited cytogenetically detectable chromosomal 3q26 breakpoints in eight cases. Fluorescence *in situ* hybridization revealed seven more *EVI1+ME-* cases that carried cryptic 3q26 breakpoints, which were not found in the *EVI1+ME+* group. *EVI1+ME-* expression predicts for an extremely poor prognosis distinguishable from the general *EVI1+* AML patients (OS; P<0.001 and EFS; P=0.002). We argue that *EVI1/ME* quantitative expression analysis should be implemented in the molecular diagnostic procedures of AML.

INTRODUCTION

Inappropriate expression of *EVI1*, through chromosome 3q26 lesions, e.g. t(3;3)(q21;q26) or inv3(q21q26) has been implicated in the development or progression of high-risk acute myeloid leukemia (AML)(1, 2) Importantly, *EVI1* is also highly expressed in a subgroup of AML without 3q26 rearrangements(3, 4). High expression of *EVI1*, i.e. splice form *EVI1-1D*, is an independent negative prognostic indicator of survival in AML irrespective of the presence of 3q26 rearrangements(3). At least four additional splice variants of *EVI1* were recently identified, i.e. *EVI1-1A*, *-1B*, *-1C*, and *-3L*(5), mainly differing in their 5' untranslated regions. Since, we previously only determined the relative expression of *EVI1-1D*(3), it is feasible that *EVI1* positive AML cases have been underestimated. The prognostic value of *EVI1* in AML, taking into account the distinct *EVI1* splice forms has not been evaluated yet.

Myeloid cells may also express *MDS1/EVI1 (ME)*, an *EVI1* fusion variant generated through intergenic splicing with *MDS* (6), a gene located ~140 kb upstream of *EVI1* with a currently unknown function. Among *EVI1* positive AML patients, leukemias with selective *ME* expression (*ME*+) can be distinguished from those being *ME* negative (*ME*-)(3, 4). Currently, it is unclear whether *EVI1*+*ME*- and *EVI1*+*ME*+ leukemias are clinically and biologically different. Normal $CD34^+$ bone marrow cells express *EVI1* as well as *ME* (7, 8), suggesting that an *EVI1*+*ME*- expression pattern in AML is abnormal. In fact, chromosomal breaks in 3q26 may occur between *MDS1* and *EVI1*(9) thereby preventing *ME* fusion but instigating transcriptional activation of *EVI1* alone. How frequently *ME* negativity in *EVI1*+ leukemias (*EVI1*+*ME*- genotype) is the result of genetic alterations in this locus is unknoIt has also remained unexplained why certain leukemias express high levels of *EVI1* without carrying a 3q26 abnormality. It is conceivable that these AML cells represent normal marrow $CD34^+$ precursors, which have been shown to express *EVI1* as well as *ME*(7, 8). Another explanation could be that *EVI1* and *ME* expression is the result of defects in other genes, which function upstream and cause high *EVI1* and *ME* levels by elevating their transcription. In this study we examined another possibility, i.e. whether hidden 3q26 lesions exist in *EVI1*+ AML cases without cytogenetically detectable aberrations in this locus.

We demonstrate in a cohort of 534 AML cases that high *EVI1* expression, considering the various currently known *EVI1* splice variants, is an independent predictor of poor survival. Of the *EVI1*+ AMLs a considerable number of patients could only be identified by RQ-PCR detecting alternative *EVI1* splice forms, but not *EVI1-1D*. The *EVI1*+*ME*- subgroups of AMLs often carry chromosome 3q26 lesions, some cryptic and only recognizable by fluorescence *in situ* hybridization. Importantly, among the *EVI1* positive AMLs, the *EVI1*+*ME*- leukemia subtype showed an extremely poor treatment outcome. Finally, the results reveal a positive correlation between *EVI1*+*ME*+ overexpression and 11q23 chromosomal abnormalities, suggesting a possible role for MLL fusion proteins in the regulation of *EVI1* and *ME* expression.

PATIENTS AND METHODS

Patients and Molecular analyses

Leukemic blast cells were isolated from bone marrow or blood of 534 patients with AML, enrolled in the HOVON-04(10-12), -29, -32, -42 or -43 protocols (available at www.hovon.nl). The control group contained seven healthy bone marrow specimens. Blasts and mononuclear cells form healthy bone marrow specimens and AML samples were purified as previously reported (13). RT-PCR and sequence analyses for mutations in *FLT3*-ITD, *FLT3*-TKD, *NPM1*, N-*RAS*, K-*RAS* and *CEBPA* were performed as described previously(14-17). All subjects provided written informed consent. This research has been approved by the Institutional Review Board of Erasmus University Medical Center.

Real-time quantitative PCR and Northern blot analyses

RNA isolation, cDNA synthesis and real-time quantitative RT-PCR (RQ-PCR) were performed as described(3, 5, 13). *EVI1-1D* splice form and *ME* expression levels were determined using probes(3), whereas the other *EVI1* splice variants(5) (*-1A*, *-1B*, *-1C* and *-3L*) were analyzed using SYBR green (Applied Biosystems, Foster City, CA). A systematic overview of the *EVI1* splice forms and the primer/probe localisations are shown in Figure 1. Primer and probe sequences are shown in Table S1. *EVI1* expression levels were determined using the ΔΔCt method(18). The average expression of each *EVI1* splice variant and *ME* in 7 bone marrow samples from healthy volunteers was used as calibrator. The mean Ct values in the normal bone marrow samples were 29.6± 1.1 for *EVI1-1A*, 29.1 ± 0.82 for *EVI1-1B*, 37.2± 1.7 for *EVI1-1C*, 38.6± 1.2 for *EVI1-1D*, 32.8± 0.92 *EVI1-3L* and 35.9± 1.9 for *ME*. The Ct values obtained were normalized for the internal reference(3), porphobilinogen deaminase (*PBGD*). The mean *PBGD* Ct value for normal bone marrow samples was 27.8 ± 1.0. For the ΔΔCt calculation to be valid(18), the absolute value of the slope in the plot of the log cDNA dilution versus ΔCt was determined for all primer combinations and was close to zero. A sample was considered *EVI1* positive if the relative expression was above 30 for one or more *EVI1* splice variants. All samples were tested in duplicate and the average values were used for quantification. The amplification efficiency of each primer combination using 5 different dilutions (equal to 1.25 ng to 20 ng total RNA) was determined using mRNA isolated from four *EVI1* positive samples. The mean amplification efficiencies of *EVI1-1A*, *-1B*, *-1C*, *-1D*, *-3L* and *ME* were respectively, 1.00, 0.99, 0.90, 1.00, 0.94 and 0.95. Northern blot analyses for *EVI1* expression was carried out as described previously(3).

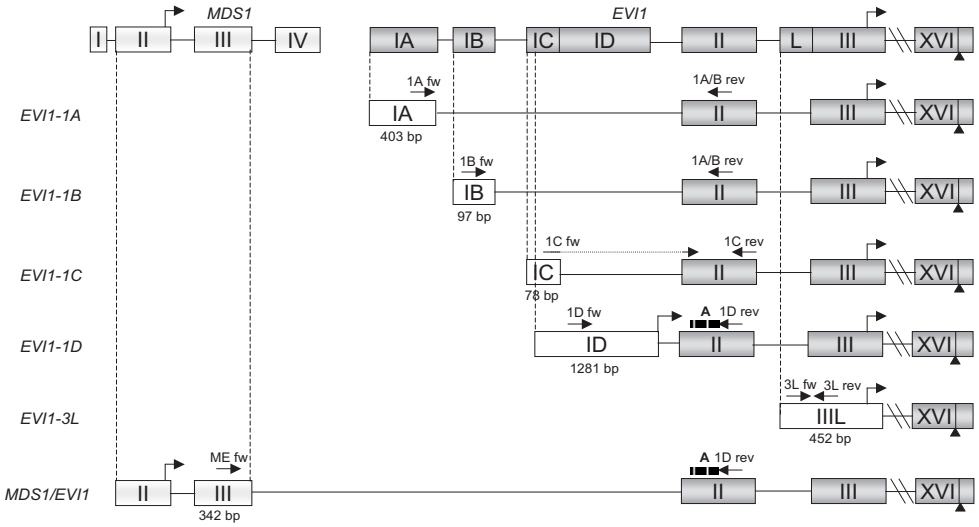

Figure 1. Gene structure and primer/probe locations of *EVI1* splice variants *-1A*, *-1B*, *-1C*, *-1D*, *-3L* and *MDS1/EVI1 (ME).*

The exons, introns and translational starts are depicted in boxes, connective lines and stand-up arrows respectively. The first exon's size in base pairs (bp), primers (arrows) and probes (bold line) are shown. Nucleotide sequences of primer/probe are presented in Table S1.

Fluorescence in situ hybridization

Dual color fluorescence *in situ* hybridization (FISH) was performed with BAC clones located on chromosome 3q26, the EVI1 and/or the MDS1 locus, RP11-82C9 (EVI1) RP11-672P8 (EVI1), RP11-141C22 (MDS1) and RP11-250A4 (3q26; MDS1), Furthermore BAC clones RP11-456K4 and RP11-912D21 located on chromosome 3q21, the ribophorin I (RPN1) locus and RP1-196F4 located on 3q telomere were used. Clone isolation and labeling were performed using biotin-16-dUTP and digoxigenin-11-dUTP (Roche Diagnostics Belgium, Vilvoorde, Belgium) according to the manufacturer's protocol. The FISH analysis was performed as previously described(19). Each sample was analyzed by two different hybridizations. The evaluation of the hybridization pattern and signal intensity served as a reciprocal internal control. A minimum of 100 interphase cells and 10 metaphases were analyzed for each probe combination. Threshold values for true positivity were calculated from the average percentages, plus three times the standard deviations of nuclei falsely positive for each of the aberrant hybridizations patterns in the control group. The control group consists of four healthy control and fourteen *EVI1+ME+* samples who yielded more than 90% normal methaphases, i.e two pairs of red and green fusion signals in both hybridizations with BAC clones RP11-82C9 plus RP11-141C22 and RP11-672P8 plus RP11-250A4.

Statistical analysis

Statistical analysis was performed with Stata Statistical Software, Release 9.2 (Stata, College Station, TX). Spearman rank test, Fisher-exact test, Chi-square test and Student's t-test were calculated using Excel software (Microsoft, Redmond, WA). All patients received induction therapy and were included in the survival analysis. Actuarial probabilities of overall survival (OS, with death due to any cause), event-free survival (EFS, with failure in case of no complete remission at day 1 (CR1) or relapse or death) and disease-free survival (DFS; with death in CR1 or relapse) were estimated by the method of Kaplan and Meier (20). The Cox proportional hazards analysis(21) was applied to determine the association of high total *EVI1* expression (as a binary variable) with OS, EFS, and DFS without and with adjustment for age, cytogenetic risk (i.e favorable, intermediate or unfavorable(22)) and *FLT3* internal tandem duplication (*FLT3* ITD) together with known important poor prognostic AML markers i.e monosomy 7 and MLL translocations. All tests were two-tailed and a P value of less than 0.05 was considered statistically significant.

RESULTS

The predictive value of EVI1-1D validated in an independent cohort of 272 AML patients

We have previously demonstrated in patients with newly diagnosed AML that high *EVI1* mRNA levels, i.e. splice-form 1D (*EVI1-1D*) (Figure 1) significantly predict for poor survival (3). Here, we show in an independent cohort of 272 cases of newly diagnosed AML (cohort A; Table S2) high *EVI1-1D* levels in 6.2% (n=17) patients (Table 1). Importantly, high *EVI1-1D* expression again correlated with significantly reduced event free survival (EFS) and overall survival (OS) (P<0.001, Figure S1). In the following experiments we combined cohort A with samples from the previously investigated patient cohort (3) (cohort B (n=262); Table S2). Only patients were included that were treated according to the HOVON cooperative group protocols. In cohort B, 5.8% of cases (n=15) were *EVI1-1D* positive (Table 1). Hence, among the combined 534 AMLs 6.0% (n=32) of cases were *EVI1-1D* positive.

EVI1 positive cases identified by expression analysis of alternative EVI1 splice forms

Four other *EVI1* splice forms have been reported, which mainly differ in their 5'-untranslated region, i.e. *EVI1-1A, -1B, -1C* and *-3L* (Figure 1). To investigate for the frequency of expression of each of these *EVI1* splice variants in AML and to verify whether *EVI1-1D* negative AMLs might express other *EVI1* splice forms, we determined the relative expression by splice form specific RQ-PCRs (Figure 1). A sample was considered *EVI1* positive if the relative expression was above 30 for one or more *EVI1* splice variants. Different cut-off points i.e. 50, 30, 20 and 10 were tested based on event-free survival and showed minor differences in survival

and in *EVI1* positive patients respectively 40, 41, 52 and 57. Differences in survival between *EVI1* positive versus *EVI1* negative cases appeared to be significant in each situation (data not shown). Any cut-off is arbitrary, but in order to prevent including false positive patients we chose the cut-off level of 30 for further analysis and calculations. Although Spearman correlation coefficients comparing expression levels of distinct splice forms were high (Figure S2), indicative of frequent co-expression, nine *EVI1-1D* negative cases were identified that were positive for one or more other *EVI1* splice forms (Table 1). The total *EVI1* positive fraction (*EVI1*+) was increased to 7.8% (n=41 cases). *EVI1-1A* or *EVI1-3L* were found to be most frequently expressed in those cases. Northern blot analysis performed on samples from selected patients that expressed distinct *EVI1* splice variants (Figure 2), revealed bands of the expected size, confirming the true identity of *EVI1*.

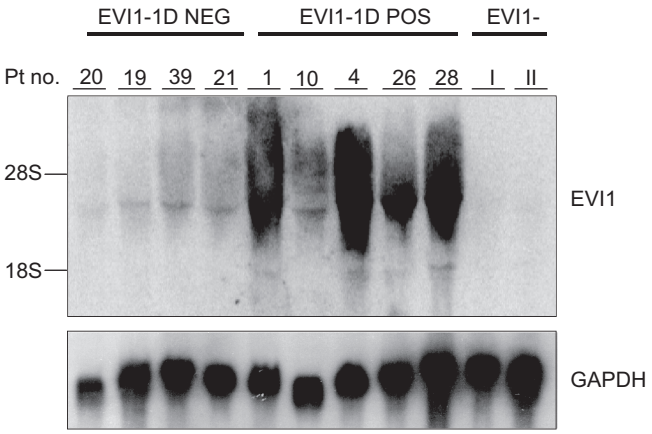

Figure 2. *EVI1* mRNA expression levels in *EVI1-1D*+ and *EVI1-1D*- AML samples in cohort A and B determined by Northern blot.
Human 600 bp *EVI1* probe and as control a murine *GAPDH* fragment was used. Patient I and II represent AML samples without *EVI1* expression. The patient numbers correspond to those in Table 1.

EVI1 expression and clinical characteristics

No differences in age and sex distributions, FAB classifications, pre-treatment white blood cell counts (WBC) or percentages of bone marrow blasts were observed between patients with *EVI1*+ and *EVI1*- *de novo* AML. Clinical characteristics of *EVI1*+ versus *EVI1*- patients within the cohort of 534 AML patients are depicted in Table 2. Chromosomal aberrations in the *EVI1* locus, i.e. 3q26 abnormalities, were seen in 8 of the 41 (20%) *EVI1*+ cases, whereas only 2/493 (0.4%) *EVI1*- AMLs showed a 3q26 abnormality (Tables 1 and 2). Possibly, in those latter two cases another gene present in the 3q26 locus may have been affected. Other cytogenetic lesions that are frequently seen in association with *EVI1* positivity are -7/7q- deletions and translocations involving 11q23. Deletions -7/7q- were found in 13/41 (38%) *EVI1*+ leukemias and 34/493 (7%) *EVI1*- cases. Translocations involving 11q23 were observed in 8/41 (20%) *EVI1*+ versus 8/493 *EVI1*- (1.6%) AMLs. Furthermore, an inverse correlation was seen between *EVI1*+ patients and *NPM1* mutations (P<0.001).

Table 1. Relative expression of EVI1 splice variants (-1D, -1A, -1B, -1C, -3L) and MDS1/EVI1 (ME), a priori karyotype and FISH results in EVI1+ patients.

No	1D	1A	1B	1C	3L	ME	Karyotype (ISCN 2005) COHORT A	FISH
1	850	1634	199	80	819	0,1	45,XY,inv(3)(p12p2?4),-7[16]/46,XY[8]	inv(3)
2	540	2159	75	73	157	0,2	45,XY,t(3;3)(q21;q26),-7[20]	ND
3	254	1473	42	6	479	0,1	46,XY,t(3;3)(q21;q26)[51]	ND
4	265	1405	67	58	76	0,0	45,XX,-7[24]	inv(3)
5	352	88	33	4	65	0,5	47,XX,del(5)(q23q34),+del(21)(q21q22)[4]/47,idem, t(2;3)(p2?2;q2?7)[20]/46,XX[3]	t(2;3)
6	796	943	160	225	131	3	45,XY,inv(3)(q21q26.2),-7[13]	ND
7	68	74	47	5	43	8	45,XY,-7[16]	inv(3)
8	166	177	42	5	43	25	46,XY[14]	NA
9	50	84	21	3	137	89	48,XY,+9,+21[9]/49,idem,+21[14]	NA
10	686	408	209	57	361	95	47,XX,del(3)(q25) or del(3)(q21q26),+mar	NA
11	54	163	50	1	212	145	46,XX,t(6;11)(q27;q23)[36]	NN
12	217	174	73	10	327	224	53,XY,+6,+8,+9,t(11;16)(q23;p13),+13,+14,+19,+21[15]	NN
13	227	694	88	3	855	269	46,XX	NA
14	120	380	85	6	527	289	46,XX,t(6;11)(q27;q23)[28]	NN
15	94	494	119	4	680	437	46,XY[30]	NA
16	183	1013	136	30	628	934	47,XY,+?8(9%)/46,XY	NA
17	54	3	2	5	2185	1048	46,XY,t(3;21)(q26;q22),del(12)(p12p13)[20]	ND
18	13	132	7	0,0	21	0,2	46,XY[38]	inv(3)
19	21	89	11	0,5	90	24	46,XY,?der(11)(q2?)[3]/46,XY[18]	NN
20	14	67	16	0,5	21	32	46,XY,inv(3)(q21q26)[32]	inv(3)
21	26	99	27	1	135	86	46,XY,t(11;19)(q23;p13.1)[15]/46,XY[5]	NN
22	7	27	9	2	77	216	NA	NA
No	**1D**	**1A**	**1B**	**1C**	**3L**	**ME**	**Karyotype (ISCN 2005) COHORT B**	**FISH**
23	1624	1256	363	59	834	19	45,XX,inv(3)(q22q26),-7[29]/46,XX[1]	ND
24	*584*	*417*	*116*	*74*	*37*	4	45,XY,inv(3)(q22q26),-7[25]	ND
25	506	845	*119*	*37*	*350*	0,2	45,XY,inv(3)(q12q26.2),-7[20]	ND
26	*700*	*1121*	*161*	*76*	*244*	0,0	45,XX,-7[27]/46,XX[3]	inv(3)
27	214	*1074*	*44*	*9*	*541*	0,1	NA	NN
28	*173*	*172*	*28*	*10*	*46*	0,0	46,XX[68]	inv(3)
29	*196*	*239*	*69*	*6*	*167*	0,0	46,XX,t(1;6)(p32;q24~25),del(2)(q34)[33]/46,XX[1]	inv(3)
30	829	1258	132	68	333	*63*	NA	NA
31	*745*	*2354*	*147*	*23*	*1309*	*286*	45,XY,-7,t(9;11)(p21;q23)[33]	NN
32	*315*	*641*	*174*	*9*	*2532*	*532*	46,XX,t(11;19)(q23;p13)[21]	NN
33	*207*	*186*	*118*	*5*	*373*	*168*	46,XY,t(9;22)(q34;q11)[22]	NN
34	*192*	897	*112*	*8*	*3266*	*539*	46,XX,t(2;9;11)(p13;p22;q23)[20]	NA
35	*83*	*306*	*43*	*3*	*517*	*448*	46,XY[40]	NN
36	*79*	*67*	*36*	*3*	*204*	*1065*	46,XX,del(7)(q22)[41]/46,XX[1]	NN
37	*52*	*157*	*27*	*0,9*	*605*	*429*	46,XY,t(6;11)(q25;q23)[22]	NN
38	*14*	*7*	*6*	*0,1*	*95*	*139*	47,XY,+13[11]/46,XY[28]	NN
39	*16*	*39*	*9*	*0,9*	*33*	*223*	45,XY,-7,t(7;8)(q22;p11)[21]	NN
40	*11*	*6*	*6*	*1,7*	*144*	*397*	47,XX,+13[27]/46,XX[1]	NN
41	*14*	*13*	*5*	2,8	*234*	*1830*	46,XY,-7,add(12)(p12),+mar[46]/46,XY[54]	NN

Relative expression of each of the distinct *EVI1* transcripts was determined as explained in the Patients and Methods section. A positive *EVI1* or *ME* signal, i.e. >30 is indicated in red. Abbreviations: *ME*, *MDS1/EVI1*; FISH, fluorescent *in situ* hybridization; NN, normal 3q21, 3q26 and 3q telomere locus; inv(3), inversion of chromosomal band 3q21 and 3q26; NA, not available; ND, not determined.

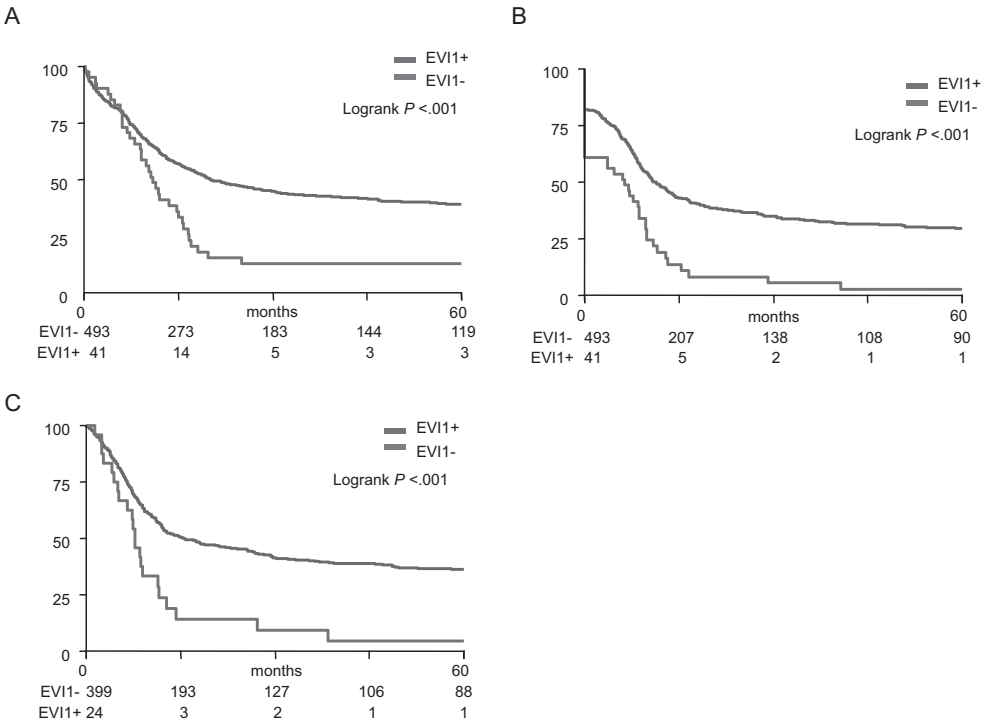

Figure 3. High *EVI1* expression associates with poor survival outcome in AML.
Kaplan Meier analysis of (A) overall survival (OS), (B) event-free survival (EFS) and (C) disease-free survival (DFS) shows an inferior outcome for *EVI1*+ patients in comparison to patients without *EVI1* overexpression in a total cohort of 534 AML patients.

AML cases analyzed were obtained from six different HOVON studies, i.e. HOVON-04, 04A, 29, 32, 42 and 43. As can be seen from Table 2, *EVI1* expressing cases were equally distributed among the distinct treatment groups (Table 2 and data not shown).

Table 2. Clinical characteristics of *EVI1* positive patients in relation to clinical parameters, morphology, cytogenetics and molecular characteristics of 534 patients with newly diagnosed AML.

	No. of *EVI1* negative patients (%)	No. of *EVI1* positive patients (%)	P value
Sex[‡]			0.19
Male	242	25 (11)	
Female	251	16 (17)	
Age (years) [‡]			0.22
Less than 35	117	14 (11)	
Between 35 and 50	166	12 (15)	
Older than 50	210	15 (15)	
HOVON protocol[‡]			
04A	49	3 (6)	> 0.99
04	40	8 (17)	0.50
29	203	12 (6)	0.32
32	5	0 (0)	> 0.99
42	140	11 (7)	0.85
43	55	7 (11)	0.54
FAB*			
M0	16	2 (11)	0.64
M1	104	6 (5)	0.42
M2	123	6 (5)	0.18
M3	24	1 (4)	0.71
M4	82	13 (14)	0.11
M5	104	10 (9)	0.85
M6	7	1 (12)	0.47
Mx	33	2 (6)	> 0.99
Cytogenetic abnormalities[§,‡]			
-5/5q-	16	3 (16)	0.23
-7/7q-	21	13 (38)	< 0.001
3q26	2	8 (80)	< 0.001
t(9;22)(q34;q11)	1	1 (50)	0.17
t(11q23)	8	8 (50)	< 0.001
t(15;17)(q22;q21)	21	1 (5)	0.71
t(8;21)(q22;q22)	39	0 (0)	0.039
inv(16)/t(16;16)	37	0 (0)	0.065
+8	22	2 (8)	0.71
+21	3	2 (40)	0.05
t(6;9)(p23;q34)	6	0 (0)	> 0.99
Complex	20	1 (5)	> 0.99
Other	65	6 (8)	> 0.99
Normal	218	6 (3)	< 0.001
ND	20	2 (9)	> 0.99
Cytogenetic risk			

Favorable	89	1 (1)	0.23
Intermediate	347	17 (5)	< 0.001
Unfavorable	57	23 (29)	< 0.001
Molecular abnormalities‡			
FLT3-ITD	135	5 (4)	0.027
FLT3-TKD	43	1 (2)	0.23
K-RAS	5	0 (0)	> 0.99
N-RAS	40	5 (11)	0.57
CEBPA	43	1 (2)	0.23
NPM1	158	2 (1)	< 0.001
WBC x 10^9/L‡‡			0.18
Mean; SD	52; 63	45; 49	
Platelets x 10^9/L‡‡			0.01
Mean; SD	72; 101	165; 241	
Blast% in BM‡‡			0.38
Mean; SD	61; 27	61; 24	

Abbreviations: FAB, French-American-British classification; BM, bone marrow; *FLT3*-ITD, internal tandem duplication of the *FLT3* gene; *FLT3*-TKD, a mutation in tyrosine kinase domain of the *FLT3* gene; Mx, FAB not available; ND, not determined; SD, standard deviation.
§All patients with a specific abnormality were considered irrespective of the presence of additional abnormalities.
‡ *P* values were calculated using the 2-tailed Chi-square test.
‡‡ *P* values were calculated using 2-tailed t-test.

High EVI1 expression is an independent prognostic marker in AML

We next investigated the prognostic impact of *EVI1* positivity (i.e. *EVI1-1A, -1B, -1C, -1D* or *-3L* positive) in the total cohort of 534 patients with AML. Patients with *EVI1*+ AML less often attained a complete remission (61% *v* 82%; Chi2 P=0.001) and the probability of relapse was considerably higher compared to *EVI1*- AMLs (51% *v* 41%; Chi2 P=0.04). Survival analysis revealed a severe disadvantage for *EVI1*+ AML patients regarding the 5 years overall survival (OS) (13%±5% *v* 39%±2%; Figure 3A), event-free survival (EFS) (3%±3% *v* 29%±2%; Figure 3B) and disease-free survival (DFS; probability of relapse) (5%±4 *v* 32%±3%; Figure 3C). Table 3 describes the increased hazard ratios for *EVI1*+ AML for OS, EFS and DFS following univariate analysis and multivariate analysis.

Multivariate analysis established high *EVI1* expression as an independent prognostic marker in relation to *FLT3*-ITD mutations and other prognostic cytogenetic abnormalities with hazard ratios for EFS and DFS of 1.88 (95% CI, 1.29-2.76; P=0.002) and 2.14 (95% CI, 1.29-3.54; P=0.006) (Table 3), whereas for OS a hazard ratio with a decreasing trend of 1.47 (95% CI, 0.98-2.21, P=0.073) was established.

Table 3. Univariate and multivariate analysis of high *EVI1* expression as prognostic factor for survival.

	EFS			DFS			OS		
	HR	(95% CI)	P value	HR	(95% CI)	P value	HR	(95% CI)	P value
Univariable analysis									
High *EVI1* expression[#]	2.17	(1.55-3.03)	<0.001	2.26	(1.46-3.50)	<0.001	1.91	(1.34-2.72)	<0.001
Multivariable analysis									
Intermediate[§]	1.62	(1.17-2.26)	0.004	1.91	(1.30-2.82)	0.001	1.84	(1.27-2.68)	0.001
Unfavorable[§]	2.90	(1.88-4.49)	<0.001	3.8	(2.27-6.36)	<0.001	3.46	(2.14-5.60)	<0.001
Age 35-50 year	1.03	(0.78-1.35)	0.84	1.10	(0.81-1.52)	0.53	1.22	(0.90-1.65)	0.20
>50 year	1.23	(0.94-1.60)	0.13	1.17	(0.86-1.60)	0.33	1.48	(1.11-1.98)	0.007
FLT3-ITD[‡]	1.40	(1.11-1.77)	0.005	1.25	(0.93-1.67)	0.13	1.55	(1.21-1.99)	<0.001
Monosomy 7[*]	1.37	(0.89-2.11)	0.15	1.05	(0.58-1.89)	0.87	1.35	(0.86-2.13)	0.20
MLL translocation[**]	0.68	(0.35-1.29)	0.24	0.67	(0.32-1.39)	0.28	0.79	(0.39-1.56)	0.49
High *EVI1* expression[#]	1.88	(1.29-2.76)	0.002	2.14	(1.29-3.54)	0.006	1.47	(0.98-2.21)	0.072

P values were calculated using the Cox regression model. Abbreviations: HR, hazard ratio with high *EVI1* expression; CI, confidence interval; EFS, event free survival; DFS, disease-free survival; OS, overall survival; *FLT3* ITD, internal tandem duplication of the *FLT3* gene.

[#] High *EVI1* expression versus no *EVI1* expression.

[§] Cytogenetic risk versus favorable cytogenetic risk.

[‡] *FLT3* ITD versus no *FLT3* ITD.

[*] Monosomy 7 versus no monosomy 7.

[**] *MLL* translocation versus no *MLL* translocation.

We performed a Cox regression analysis with expression of *EVI1-1A, 1B, 1D* and *3L* as a continuous variable. The data were log-transformed to guarantee an equal distribution. The results shown in Table S3 demonstrate that the relative expression of variants *EVI1-1A, -1B* and *-3L,* but not *EVI1-1D* was of significant influence in the EFS, DFS and OS of the AMLs studied. We also performed a Cox regression analysis where *EVI1* expression as a time dependent covariate was added to the original multivariate model for OS. High *EVI1* expression as a time dependent covariant was not of significant influence in this model (HR=1.0, 95%CI; 0.98-1.04, P=0.54).

EVI1 positivity and MDS1/EVI1 negativity associate with 3q26 lesions

Since *EVI1* is normally co-expressed with the intergenic splice form *MDS1/EVI1* (*ME*) (Figure 1), we argued that the atypical dissociated expression pattern *EVI1+ME-* that is noted in a proportion of cases of AML*(3)*, might be caused by chromosome 3q26 lesions disrupting the *MDS1/EVI1* locus. We estimated *ME* levels in the *EVI1+* AML subgroup and observed absence of *ME* expression in 17/41 cases. Six of these (6/17, 36%) carried a cytogenetically detectable 3q26 abnormality, while the remaining leukemias carried other cytogenetic aberrations or had a normal karyotype (Table 1). *ME* was co-expressed with *EVI1* in 24/41 cases. Only 2/24 (8%) *EVI1+ME+* cases showed a 3q26 lesion. In fact, in one case (#20) *ME* expression levels were border line (Table 1) while in the other case (#17) the translocation t(3;21) was apparent, which is known to result in an *AML1/MDS1/EVI1* fusions(20, 23).

Frequent hidden 3q26 lesions in *EVI1+ME-* AML patients

The positive association between *EVI1+ME-* expression and cytogenetically detectable 3q26 abnormalities prompted us to investigate whether hidden 3q26 chromosomal lesions might be present in the *EVI1+ME-* subgroup of AML. Dual-color FISH analyses using BAC clones covering *EVI1* and *MDS1* (Figure 4A) were carried out on metaphase spreads of 10 cases of *EVI1+ME-* AML without any a priori cytogenetically detectable 3q26 abnormality. In 8/10 patients (#1, 4, 5, 7, 18, 26, 28 and 29) split signals were observed in metaphase and interphase nuclei, indicating the presence of hidden 3q26 lesions (Figure 4B, Table 1 and Figure S3A-C). Additionally, the FISH analysis redefined the breakpoint of the cytogenetically identified t(2;3)(p2?;q2?7) into t(2;3)(p2?;q26) (#5; Figure S3D-E). Next, FISH analysis using BAC clones covering *RPN1* (3q21) and *EVI1* revealed involvement of the *RPN1* locus in the remaining 7 patients (Figure 4C and Table 1). The complete FISH results for each patient analyzed are shown in Table 1. FISH experiments using the same BAC clones covering *EVI1*, *MDS1* and *RPN1* were carried out in *EVI1+ME+* leukemias but did not demonstrate the existence of inv3(q21q26) or t(3;3)(q21;q26) in any of the 14 cases studied. Other chromosome 3 translocations were excluded in these samples by FISH using a BAC clone located telomeric on 3q (Figure S3F).

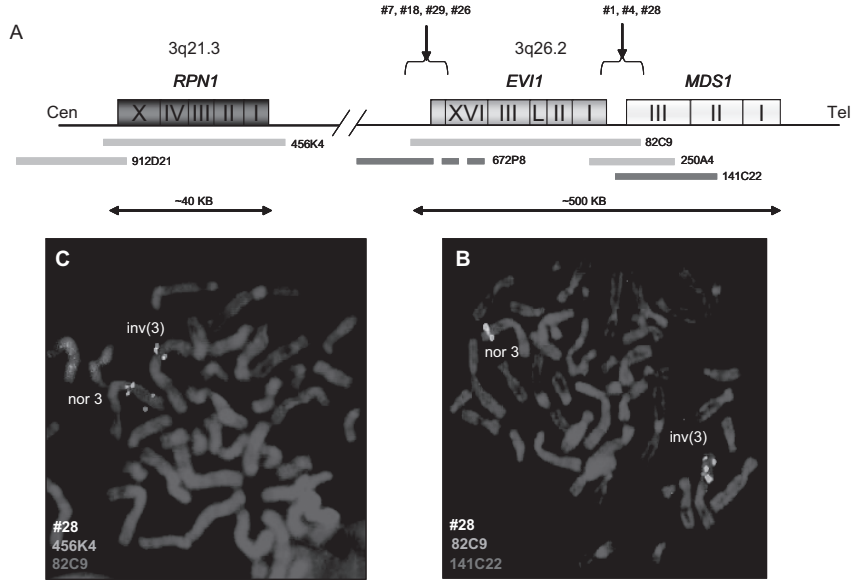

Figure 4. Fluorescent *in situ* hybridisation (FISH) of chromosome 3q26 and 3q21 loci reveal hidden 3q26 aberrations.

BAC clone localization from centromere (Cen) to telomere (Tel) (A). A metaphase from *EVI1+* patient #28 revealed a cryptic inv(3)(q21q26) (inv3) and a normal chromosome 3 (nor3) using *EVI1* (RP11-82C9) and *MDS1* (RP11-141C22) (B) and *RPN1* (RP11-456K4) BAC clones (C).

EVI1+ME- and EVI1+ME+ AMLs are cytogenetically and clinically different

No significant differences in sex and age distributions, FAB classification, WBC counts, bone marrow blast percentages, karyotype risk classification and molecular abnormalities were observed between the *EVI1+ME-* (n=17) and the *EVI1+ME+* subsets of patients with AML (n=24) (Table S4). Besides the frequent occurrence of 3q26 lesions, patients with *EVI1+ME-* AML also frequently presented a –7/7q- deletion (n=9; P=0.11). Interestingly, the platelet count, which was significantly elevated in the total *EVI1+* group compared to the *EVI1-* AML patients (Table 1), was only elevated in the *EVI1+ME-* subgroup but not in the *EVI1+ME+* subgroup (P=0.024; Table S4). *EVI1+ME+* cases frequently carried 11q23 abnormalities (n=8, P=0.013). While *EVI1+ME+* and *EVI1+ME-* subgroups both showed a poor treatment outcome (Figure 5), *EVI1+ME-* cases showed an even worse prognosis. This was evident from the fact that they less often achieved complete remission than patients with *EVI1+ME+* AML (18% *v* 92%; Chi2 P=0.001) and showed a significantly reduced OS (9%±3% *v* 24%±6%; Figure 5A) and EFS (3%±3% *v* 29%±2%; Figure 5B) at 1.5 years. In fact, a significantly worse OS, EFS and DFS (OS; HR=2.45, P=0.002, EFS; HR=3.21, P<0.001, DFS; HR=2.44, P=0.13) for *EVI1+ME-* cases as compared to *EVI1+ME+* AML patients was also evident from multivariate analysis, although one should keep in mind that patients numbers were relatively small for such subdividing and requires further validation in other cohorts.

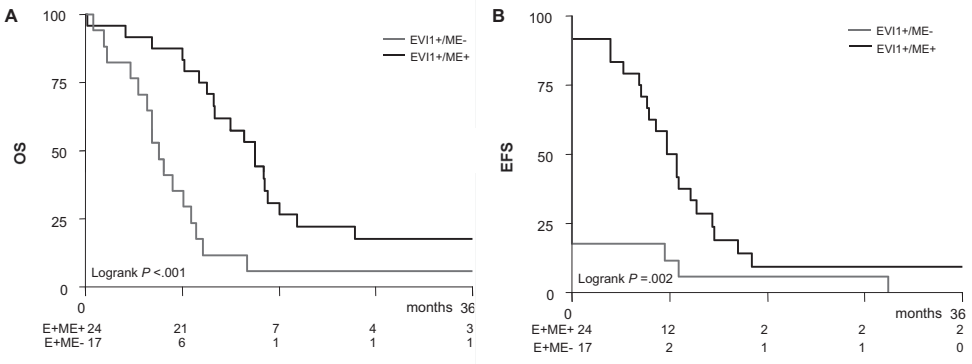

Figure 5. Inferior outcome for *EVI1+ME-* patients in comparison to *EVI1+ME+* patients.
Kaplan Meier analysis of (A) overall survival (OS) and (B) event-free survival (EFS) shows an inferior outcome for *EVI1+ME-* patients in comparison to *EVI1+ME+* patients in the cohort of 41 *EVI1+* AML patients.

DISCUSSION

Molecular markers are of utmost importance in resolving the genetic heterogeneity of AML and deriving discriminative prognostic algorithms. Recently, markers such as expression levels of *EVI1*(3), *BAALC*(24) and *ERG*(25) have been demonstrated to be powerful indicators of prognostically distinct AML subsets. High expression of *EVI1,* i.e. in particular splice variant *EVI1-1D,* has been shown to be a strong negative indicator for treatment response in AML(3). Here, we confirmed the adverse prognostic significance of *EVI1-1D* in an independent cohort of 272 AML patients. In addition, we demonstrate in a large cohort of 534 adults with pre-treatment AML that 41 (7.8%) cases expressed high levels of *EVI1* mRNA, which significantly predicted for an unfavorable prognosis. Importantly, 22% of these cases (9/41) were *EVI1-1D* negative, but could be designated as *EVI1+* when the expression of the other recently identified *EVI1* splice forms(5) was determined. Our findings inquire for the development of a reproducible assay, which discriminates *EVI1+* from *EVI1-* AML for diagnostic purposes.

A diagnostic assay should be rapid and quantitative and it should take into account each of the different *EVI1* splice forms. A multiplex PCR assay, by which the expression levels of each of the different *EVI1* splice variants(26, 27) will be determined separately, is feasible but may be complicated to interpret. It may also be possible to develop primer probe combinations, which recognize all splice forms in one single *EVI1* specific Q-PCR. Such an assay has the advantage that "high" versus "low" *EVI1* cases may be determined in one easy-to-use test and it may provide the possibility to study *EVI1* expression level as a continuous- rather than a categorical variable. This would allow us to study further whether a correlation exists between actual *EVI1* expression levels and survival time of AML patients.

Although, a correlation analysis as discussed above would be helpful to further substantiate that high *EVI1* levels associate with poor treatment response, a diagnostic assay should provide binary results, which allows a reliable discrimination between *EVI1* positive and negative cases. To avoid the inclusion of false positive cases, we scored patients *EVI1* positive with expression levels of 30 or higher compared to 6 normal bone marrow controls. Consequently, it may be possible that, by choosing such a high cut-off level, *EVI1* positive cases have been missed. Since important decisions will be made based on the outcome of a diagnostic *EVI1* assay, it will be very important to include proper reproducible positive and negative controls, and develop algorithms by which *EVI1* positive cases will be indisputably identified.

AML with 3q26 lesions are among the most aggressive forms of human leukemia (3)(28). An important message of this study is that a large fraction of AML cases that carried a 3q26 aberration were missed by standard karyotyping but was found positive by FISH. The abnormal pattern of elevated *EVI1* levels but absence of *MDS1/EVI1* expression (*EVI1+ME-*) provided the clue to these cryptic 3q26 abnormalities. Since *EVI1* and *ME* are normally co-expressed, we hypothesized that a cryptic break within the 3q locus between these two closely related genes might cause their dissociated expression. *EVI1+ME-* leukemias that we recognized represent the most unfavorable subgroup of AML and they include most of the patients that

carry 3q26 lesions. This provides another argument to implement an *EVI1/ME* multiplex RQ-PCR in the molecular diagnostic procedures of AML. Applying *EVI1/ME* RQ-PCR and karyotyping in combination with *MDS1/EVI1* and *RPN1* specific FISH on selected cases will disclose AMLs that belong to this distinctly unfavorable subgroup.

In only two *EVI1+ME-* cases we did not find 3q26 lesions by FISH, suggesting another mechanism for aberrant *EVI1* expression. *BLIMP,* an *EVI1* homologue, which is a frequent target for chromosomal breaks (1p36) in B-cell lymphomas, has been shown to carry *BLIMP* point mutations in certain cases without 1p36 translocations(29), giving rise to short *BLIMP* (like *EVI1*) forms in favor of the long (like *ME*) product. Whether within the small *EVI1+ME-* group without major 3q26 lesions mutations at the molecular level have occurred in the *MDS1/EVI1* locus, remains to be investigated.

Using FISH we did not detect hidden 3q26 aberrations in *EVI1+ME+* AMLs. We carried out nucleotide sequencing in all *EVI1+ME+* AML patients available but have not found any lesions at the molecular level in *EVI1* or *ME* (data not shown). It is possible, that excessive *EVI1* and *ME* levels observed in those AML samples are caused by genetic defects in disease genes acting upstream of *ME* and *EVI1*. Interestingly, in a significant number of *EVI1+ME+* leukemias, 11q23 chromosomal alterations were found. Kumar and colleagues have reported that hematopoietic stem cells from *MLL-AF9* knock-in mice express high levels of *EVI1*(30), which may suggest that enforced expression of *MLL*-fusion genes directly or indirectly affect *EVI1* and *ME* transcription. Transduction experiments in hematopoietic precursor cells may shed light on a putative effect of *MLL*-fusion proteins on transcription of *ME* and *EVI1*. Another subset of *EVI1+ME+* leukemias did not carry cytogenetically identifiable 11q23 lesions. We ruled out hidden 11q23 translocations in those patients using Southern blot analyses and *MLL*-specific FISH (data not shown). Neither did we observe the existence of *MLL*-PTD by applying genomic PCR on those patients(31) (data not shown), so that for the time being there is no suggestive clue as regards possible mechanisms for the excessively high levels of *EVI1* and *ME* in this particular patient group.

Assessment of *EVI1/ME* does not only provide a useful diagnostic and prognostic marker but, at least in '*EVI1+ME-*' AML with co-existent 3q26 chromosomal lesions, it pinpoints AML cases in which *EVI1* may be the major disease gene playing a critical role in leukemic transformation. It remains a major challenge to unravel the mechanisms of transformation by *EVI1* protein. *EVI1* encodes a nuclear protein, which interacts with several proteins important in transcriptional control, e.g. CtBp1(32), HDAC(33), SMAD3(34), P/CAF(32) and GATA1(35). Studies unraveling how these interactions exactly take place and as to whether those associations are crucial in leukemic transformation may provide insight that could be valuable for developing tools to specifically target *EVI1+* leukemia cells.

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CHAPTER 4

EVI1 Overexpression in Distinct Subtypes of Pediatric Acute Myeloid Leukemia

Brian V. Balgobind[1], Sanne Lugthart[2], Iris H. Hollink[1], Susan T.J.C.M Arentsen-Peters[1], Elisabeth R. van Wering[3], Siebold S.N. de Graaf[3,4], Dirk Reinhardt[5], U. Creutzig[6], Gertjan J.L. Kaspers[7], Eveline S.J.M. de Bont[8], Jan Stary[9], Jan Trka[9], Martin Zimmermann[5], H. Berna Beverloo[10], Rob Pieters[1], Ruud Delwel[2], C. Michel Zwaan[1], Marry M. van den Heuvel-Eibrink[1]

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

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ABSTRACT

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

INTRODUCTION

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

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

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

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

PATIENTS AND METHODS

Patients

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

Cytogenetic and molecular analysis

Leukemic samples were routinely investigated for cytogenetic abnormalities by standard chromosome-banding analysis, and screened for recurrent non-random genetic abnormalities characteristic for AML, including t(15;17), inv(16), t(8;21) and *MLL*-rearrangements, using either RT-PCR and/or fluorescent in-situ hybridization (FISH) by each study group. *NPM1*, *CEBPα*, *MLL*-PTD, *N-RAS*, *K-RAS*, *PTPN11*, *C-KIT*, *FLT3* mutational screening was performed as previously described(21-26).

Microarray-based gene expression profiling

Integrity of total RNA was checked using the Agilent 2100 Bio-analyzer (Agilent, Santa Clara, USA). cDNA and biotinylated cRNA was synthesized hybridized and processed on the Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, USA) according to the manufacturer's guidelines. Data-acquisition was performed using expresso (Bioconductor package Affy(27)) and probe-set intensities were normalized using the variance stabilization normalization (Bioconductor package VSN(28)) in the statistical data analysis environment R, version 2.2.0(29).

Identification of EVI1 overexpression with gene expression profiling.

Four probe sets are positioned within the *EVI1* gene (Figure S1). Normalized intensities of these probe sets were extracted from the complete Supplementary dataset and further clustering analysis was performed with Genemaths XT (Applied Maths, Austin, USA). Of each probe set, the standard deviation using the median as cut off was calculated for all patients. Since, the probe sets *243277_x_at* and *215851_at* were located in introns of the *EVI1* gene, only the probe sets *221884_at*, and *226420_at* were used for hierarchical clustering using the Euclidean distance. Samples were considered to have an abnormal *EVI1* expression (*EVI1*+) based on the hierarchical clustering dendrogram.

Gene expression signatures for EVI1+ cases.

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

Real-time quantitative PCR and Fluorescence in situ hybridization (FISH).

In 179 samples, including 22 samples with *EVI1* overexpression based on microarray analysis, the RNA expression could be validated by real-time quantitative RT-PCR (RQ-PCR). For the other 49 samples no additional RNA was available to perform RQ-PCR. The relative expression of the *EVI1* transcripts (*EVI1*-1A, -1B, -1D, and -3L) and *MDS1*/*EVI1* transcript was calculated using the comparative cycle time (ΔCt) method, with *GAPDH* as the housekeeping gene(32). Primer en probe sequences are shown in Supplementary Table S1. A sample was considered *EVI1*+ with RQ-PCR if the cumulative relative expression *EVI1*-1A, -1B and -3L to *GAPDH* was above 1.5%. This showed the highest correlation with *EVI1*+ cases based on gene expression profiling and all normal bone marrow samples were below this threshold. Interphase cytospins of cases with a high *EVI1* expression were screened with

FISH for cryptic 3q26 abnormalities with the Poseidon™ Repeat Free™ EVI1 t(3;3), inv(3) Break probe (Kreatech, Amsterdam, The Netherlands) *a*ccording to manufacture protocol.

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

RESULTS

EVI1 overexpression in pediatric AML as determined by gene expression profiling
Four probe sets on the Affymetrix Human Genome U133 Plus 2.0 Array were present within the *EVI1* gene (Figure S1). These probe sets were located in regions common for all isoforms of *EVI1*. However, *243277_x_at* was found in intron 2-3 and *215851_at* was found partly in intron 15-16. Since these short probe sets also showed more random variation of expression, they were not included in the hierarchical clustering analysis. By means of hierarchical clustering of the gene expression profiling data, 3 separate clusters could be identified. One cluster with high expression of *EVI1* (cluster 1), one cluster with an intermediate to high expression of *EVI1* (cluster 2) and one large cluster with low expression of *EVI1*. Therefore, 24 cases in the clusters with intermediate and high expression were considered *EVI1+* based on the dendrogram (Figure 1). These cases included 19/213 (9%) patients with *de novo* AML, 4/8 (50%) patients with secondary AML, and one patient of whom only relapse material was available.
Hierarchical clustering of 228 pediatric AML samples with probe sets *221884_at* and *226420_at* representing the *EVI1* gene. Red represents high expression; black intermediate expression; and green low expression for the specific probe set.

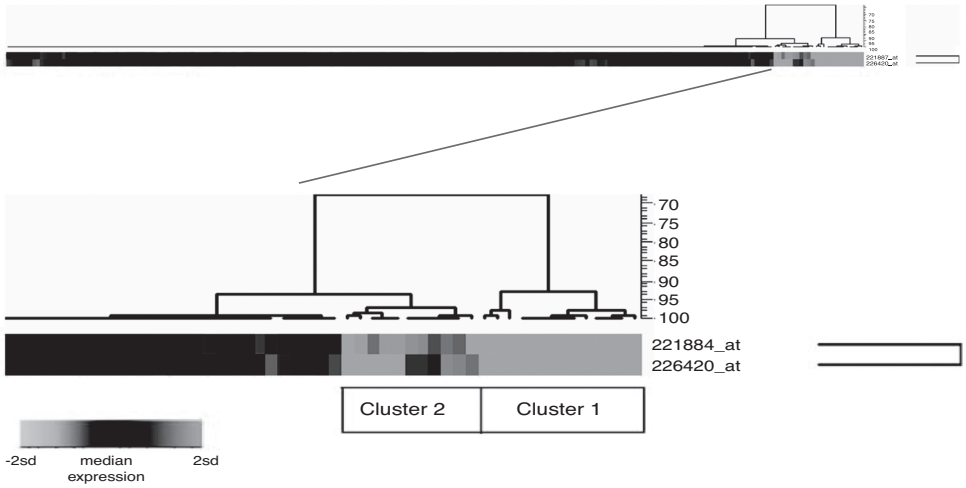

Figure 1. Hierarchical clustering using the gene expression of the 3 probe sets representing EVI1 in 228 pediatric AML samples reveals a subclustering of 24 EVI1+ cases.

Validation of EVI1 expression and 3q26-aberrations by real-time quantitative PCR and FISH

Of the 24 patients with a high *EVI1* expression, 22 could be investigated for the various *EVI1* and the *MDS1/EVI1* transcripts using RQ-PCR. Of 2 patients the amount of available mRNA was not sufficient. In addition, RQ-PCR was also performed on 150 of the 197 remaining pediatric AML samples for which additional mRNA material was available (Figure 2). Normal bone marrow samples of 8 individuals (6 adults and 2 pediatric) were used as a control for normal expression of the different transcripts.

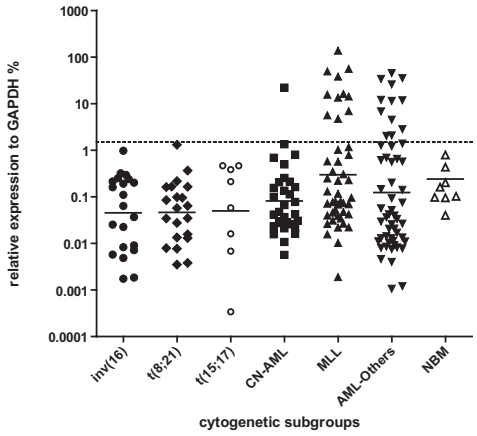

Figure 2: *EVI1* expression in different cytogenetic subgroups.
Cumulative mRNA expression levels of the *EVI1-1A*, *-1B*, *-3L*, relative to *GAPDH* (%) for 179 samples in different cytogenetic subgroups in pediatric AML and in 8 normal bone marrow samples (NBM). A cumulative relative expression of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

Twenty-one of the 22 patients identified by gene expression profiling had a cumulative relative expression to GAPDH of at least 1.5% for one of the *EVI1* transcripts (Table 1). The other patient (#20), whom lacked a cumulative relative expression of at least 1.5%, did show overexpression of *EVI1*-1D and *MDS1/EVI1*, and was considered *EVI1*+. In addition, one patient with FAB-M7 (#24) whom showed low expression of *EVI1* on gene expression profiling, did show abnormal expression of *EVI1* with RQ-PCR. All remaining samples did not show *EVI1* overexpression (Figure 2). Although only 22/23 *EVI1*+ cases could be identified with gene expression profiling in comparison with RQ-PCR, still RQ-PCR for these four *EVI1* isoforms showed high correlation with the gene expression data. Spearman correlation coefficients were 0.68 for *EVI1*-1A, 0.63 for *EVI1*-1B, 0.52 for *EVI1*-3L and 0.78 for *EVI1*-1D. Moreover, microarray analysis showed a sensitivity of 95%, a specificity of 100%, a positive predictive value of 100% and a negative predictive value of 99%.

Based on conventional cytogenetics, none of the patients harbored a 3q26 aberration. In addition, only three *EVI1*+ patients lacked the *MDS1/EVI1* transcript, which has previously shown to be a marker to detect cryptic 3q26 aberrations(13). However, in all *EVI1*+ cases, cryptic 3q26-rearrangements were not detected by FISH (Table 1). Thus, combining data of the gene expression profiling and RQ-PCR, 25 *EVI1*+ cases were identified and none of them harbored a 3q26-aberration.

Clinical characteristics of EVI1+ in pediatric AML

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

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

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

ID	*AML* type	Karyotype	FAB	*MLL* FISH	RQ-PCR[1] *EVI1* %					inv(3) FISH
					-1A	-1B	-3L	-1D	ME	
1	s-AML	46,XX,t(9;11)(p22;q23)[41]/47,XX, idem,+8[2]	M5	yes	3.44	1.7	0.48	0.02	0.00	neg
2	p-AML	46,X,t(X;6)(p1?2;q2?1)	M6	no	3.65	2.46	0.66	0.07	0.25	neg
3	r-AML	NA	M1	no	1.89	0.76	0.16	0.00	0.00	neg
4	p-AML	46,XY,t(6;11)(q27;q23)	M5	yes	35	7.29	13.5	0.07	0.23	neg
5	p-AML	47,XY,+21	M5	no	15.3	7.81	2.73	0.18	0.09	neg
6	p-AML	46,XY,t(9;11)(p22;q23)	M7	yes	76.4	48.3	11.9	1.00	1.29	neg
7	p-AML	46,XY,t(9;11)(p22;q23)/48, idem,+8,+mar	M5	yes	6.94	4.24	3.12	0.03	0.15	neg
8	p-AML	46,XX,t(11;20)(p15;q1?2)[20]	M4	no	31.6	7.26	10.3	0.20	0.03	neg
9	p-AML	46,XX,t(6;11)(q27;q23)	M4	yes	21.3	8.49	8.42	0.09	1.28	neg
10	p-AML	46,XX,t(11;19)(q23;q11)	M2	yes	2.45	1.41	0.84	0.01	0.03	neg
11	s-AML	46,XX,t(11;17;?)(q23;q?21;?)	M4	no	6.75	3.57	1.66	0.08	0.00	neg
12	p-AML	46,XX,inv(9)(p11q13),t(11;17)(q23;q12)	M5	yes	11.6	3.11	0.89	0.05	0.03	neg
13	s-AML	46,XX,t(9;11)(p22;q23)[4]/46,XX[7]	M5	yes	11.6	1.96	2.65	0.04	0.07	neg
14	p-AML	42~44,XY,t(6;11)(q27;q23)[cp2]/ 51, idem,+X, +der(6)t(6;11) (q27;q23),+8,+19,+21[5]	M5	yes	31.6	7.26	10.3	0.20	0.03	neg
15	p-AML	46,XX	M5	no	9.35	10.7	2.15	0.07	0.05	neg
16	p-AML	NA	M7	no	2.9	5.55	2.99	0.05	0.09	neg
17	p-AML	46,XY,add(11)(q23),inc	M1	yes	5.13	0.87	0.92	0.01	0.10	neg
18	p-AML	NA	M6	no	2.26	1.99	0.24	0.02	0.00	neg
19	p-AML	46,XX,t(8;13)(q22;q1?4) [8]/48,idem,+6,+mar[4]/46,XX[8]	M7	no	9.23	8.1	16.5	0.02	0.34	neg
20	s-AML	45,XY,-7[8]/49,XY,-7,+9,+10,+14, +21[12]	M2	no	0.17	0.2	0.19	0.01	0.13	neg
21	p-AML	46,XX,t(9;11)(p22;q23)[5]	M4	yes	NA	NA	NA	NA	NA	neg
22	p-AML	46,XY[20]	M5	yes	NA	NA	NA	NA	NA	neg
23	p-AML	45, XX,inv(2)(p24q14),-7	M4	no	0.8	0.85	0.43	0.01	0.01	neg
24	p-AML	NA	M7	no	1.01	2.47	8.35	0.00	0.02	neg
25	p-AML	46,XY,t(6;11)(q27;q23)[15]	M1	yes	7.26	3.48	2.7	0.03	0.53	neg
-		normal bone marrow (median expression)	-	-	0.04	0.09	0.03	0.00	0.00	neg

[1]Relative expression to *GAPDH*, [2]no material available; NA, not available; neg, negative; ND, not determined; p-AML, primary-AML; s-AML, secondary-AML; r-AML, relapsed-AML.

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

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

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

Gene expression signature differences within EVI1+ cases

In order to get insight into the biology of *EVI1*+, we analyzed our dataset to identify a specific gene expression signature for *EVI1*+ pediatric AML. Using an empirical Bayes linear regression model (30), 2103 discriminative probe sets for *EVI1*+ were identified. However, within the *MLL*-rearranged AML subtype a different gene expression signature was observed compared to the *EVI1*+ cases in other AML subtypes (Figure S2). After applying correction

for cytogenetic subtype, the amount of discriminating probe sets decreased drastically from 2103 to 253 and only 88 of these 253 probe sets were detected to be strongly significant in both groups, i.e., *MLL*-rearranged AML and other AML subtypes. Therefore, these 88 probe sets were considered to be highly discriminative for *EVI1*+ (FDR-corrected P<0.001) independent of their cytogenetic background. The top 4 probe sets represented overexpression of the *EVI1* and *MDS1* gene themselves. Interestingly, some other probe sets represented genes that have been reported to play a role in hematopoiesis and/or the development of leukemias, e.g., *PBX1* and *RUNX2*(33, 34) (Table S2).

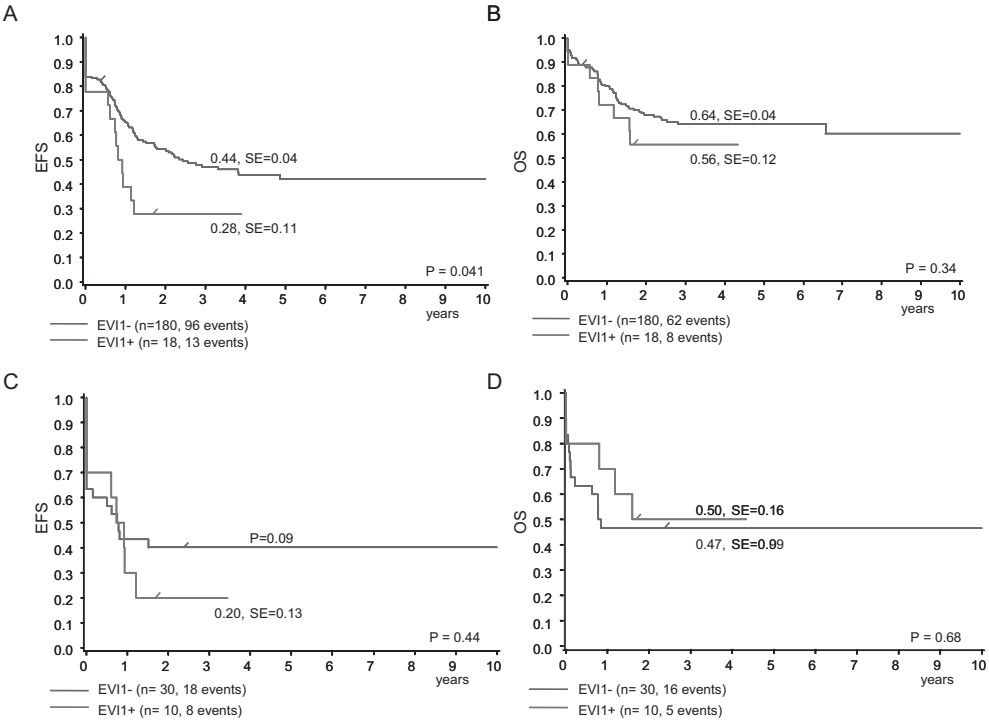

Figure 3. Survival outcome for high *EVI1* expression in pediatric AML.
(A) Kaplan-Meier estimates for pEFS in the total cohort between *EVI1*+ and *EVI1*- patients. (B) Kaplan-Meier estimates for pOS in the total cohort between *EVI1*+ and *EVI1*- patients. (C) Kaplan-Meier estimates for pEFS in the cohort of *MLL*-rearranged AML between *EVI1*+ and *EVI1*- patients. (D) Kaplan-Meier estimates for pOS in the cohort of *MLL*-rearranged AML between *EVI1*+ and *EVI1*- patients. Per plot a Log-rank P value is depicted.

Survival analysis and prognosis of EVI1+ in pediatric AML

Survival data were available for 198 patients, including 18 *EVI1*+ cases. Patients with *EVI1* overexpression had a significantly worse 4 years pEFS (28%±11% *v* 44%±4%, P=0.04) as compared to patients without *EVI1* overexpression. However, the overall survival was not significantly different between both groups (56%±12% *v* 64%±4%, P=0.34) (Figure 3A and

3B). Within the *MLL*-rearranged AML group (n=40) no significant difference for 4 years pEFS (20%±13% *v* 40%±9%, P=0.44) nor for 4 years pOS (50%±16% *v* 47%±9%, P=0.68) between *EVI1*+ and *EVI1*- patient was found (Figure 3C and 3D). The fact that *EVI1* overexpression did not influence outcome in pediatric AML was confirmed with multivariate analysis, including favorable karyotype, age and WBC, and showed that *EVI1*+ lacked independent prognostic significance for pEFS (HR 1.2, P=0.67) and for pOS (HR 1.0, P=0.97) (Table 3).

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

	EFS			OS		
	HR	(95% CI)	P value	HR	(95% CI)	P value
EVI1+	1.2	(0.6-2.2)	0.67	1.0	(0.5-2.1)	0.97
Favorable karyotype*	0.4	(0.2-0.6)	<0.001	0.2	(0.1-0.5)	<0.001
WBC > 50 x 10^9/L	1.0	(0.7-1.5)	0.97	1.3	(0.8-2.1)	0.36
Age older than 10 years	1.0	(0.6-1.4)	0.87	0.8	(0.5-1.3)	0..34

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

DISCUSSION

Evi1 was detected by Morishita *et al.* as a common integration site by retroviral insertion in a murine model system, leading to *Evi1* overexpression and leukemia, which suggests a role for *EVI1* as an oncogene(5). Subsequently, the human *EVI1* gene was detected in the breakpoint region of chromosome 3q26-rearrangements in different myeloid malignancies(1). Additional evidence that *EVI1* may act as on oncogene comes from studies by Laricchia-Robbio *et al.* and Kilbey *et al.* who showed that aberrant overexpression of *EVI1* results in loss of cell-cycle control and increased self-renewal(35, 36). Moreover, adult AML patients with high *EVI1* expression, irrespective of harboring a 3q26 abnormality, have a poor prognosis(13). Until now, no information was available on the role of the *EVI1* gene in pediatric AML, in which cytogenetically detectable 3q26 abnormalities do not seem to occur frequently(14). In the present study cohort, without cytogenetically detectable 3q26 abnormalities, we discovered *EVI1* overexpression in 20/213 (9%) of the children with *de novo* AML, and in 4/8 (50%) patients with secondary-AML. Moreover, we showed a strong association between *EVI1* overexpression and specific genetic and morphologic subtypes of AML. In contrast to adult AML, we did not find any evidence for chromosome 3q26 aberrations, nor for cryptic 3q26 rearrangements in *EVI1*+ cases. However, we did identify *EVI1*+ in FAB-M6/M7 cases, which has not been reported in adults so far. In addition, we identified *EVI1*+ in subgroups that are considered to have an intermediate or poor prognosis, i.e., pediatric AML with *MLL*-rearrangements and monosomy 7, which included 3 of the 4 secondary-AML cases. Overexpression of *EVI1* was also identified in *MLL*-rearrangements and monosomy 7 in adult AML(14, 37, 38). Interestingly, *EVI1* expression was mutually exclusive with CBF-AML, t(15;17) and *NPM1* mutations which represent favorable types of pediatric AML.

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

Several findings, however, may support a role for *EVI1*+ in leukemogenesis in these specific cases. For instance, all *MLL-AF6* and a significant proportion of monosomy 7 cases are clearly associated with *EVI1*+, not only in pediatric but also in adult AML(13). Moreover, *in vivo* studies with an *MLL-AF9* mouse-model showed overexpression of *Evi1* after the leukemic

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

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

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

Although these data strongly suggest differences in biology between subgroups of *EVI1*+, still probe sets were identified to be discriminative for *EVI1*+, independent of their cytogenetic or morphologic background after multivariate analysis. This could indicate a specific role for *EVI1* overexpression in the development of leukemia in these cases, especially since some of these probe sets included genes that have been previously been reported to play a role in hematopoiesis and/or the development of leukemias, e.g., *PBX1* and *RUNX2*(33, 34). Moreover, recent analysis of the Pbx1 promoter region in mice revealed that Evi1 upregulates Pbx1 transcription(50). This emphasizes that *PBX1* is a possible target gene of *EVI1* involved in the leukemogenesis of *EVI1*+ patients.

Although differences in event-free and overall survival for *EVI1*+ and *EVI1*- pediatric AML were observed in our study, we showed that *EVI1*+ has no independent prognostic value for pediatric AML, which is in contrast to adult AML(12, 13). The latter may mainly be caused

by differences in frequency of 3q26 abnormalities, but also by differences in therapy and prognosis between adults and children.

In this first study on the relevance of *EVI1* overexpression in pediatric AML, we conclude that *EVI1*+ is found in 9% of *de novo* pediatric AML. *EVI1* is overexpressed in specific cytogenetic (*MLL*-rearrangements and monosomy 7) and morphologic (FAB-M6/7) subtypes. However, the typical *EVI1*+ associated 3q26 aberrations reported in adult AML were not identified, indicating that there may be a difference for the role of *EVI1*+ in adult AML as compared to pediatric AML. Although *EVI1*+ was not an independent prognostic factor, it was predominantly found in types of pediatric AML that are related with an intermediate to unfavorable prognosis, e.g., *MLL-AF6* and monosomy 7. This underscores the need for further studies to identify the biological role of *EVI1* in the pathogenesis of childhood leukemia.

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CHAPTER 5

High *EVI1* Expression Predicts Outcome in Younger Adult Patients with Acute Myeloid Leukemia and Is Associated with Distinct Cytogenetic Abnormalities

Stefan Gröschel[1], Sanne Lugthart[2]*, Richard F. Schlenk[1], Peter J.M. Valk[2], Karina Eiwen[1], Chantal Goudswaard[2], Wim J.L. van Putten[3], Sabine Kayser[1], Leo F. Verdonck[4,5], Michael Lübbert[6], Gert-Jan Ossenkoppele[7], Ulrich Germing[8], Ingo Schmidt-Wolf[9], Brigitte Schlegelberger[10], Jürgen Krauter[11], Arnold Ganser[11], Hartmut Döhner[1], Bob Löwenberg[2], Konstanze Döhner[1]*, and Ruud Delwel[2]**

*These authors contributed equally to this work

[1]Internal Medicine III, University of Ulm, Ulm, Germany; [2]Hematology, Erasmus University Medical Center, Rotterdam, Netherlands; [3]Department of Trials and Statistics, Erasmus University Medical Center, Rotterdam, Netherlands; [4]Haematology, University Medical Center, Utrecht, Netherlands; [5]Department of Internal Medicine, Isala Clinics, Zwolle, The Netherlands; [6]University of Freiburg Medical Center, Freiburg, Germany; [7]Hematology, VU University Medical Center, Amsterdam, Netherlands; [8]Universitätsklinik Düsseldorf, Düsseldorf, Germany; [9]Medizinische Klinik und Poliklinik III, University of Bonn, Bonn, Germany; [10]Department of Cell and Molecular Pathology, Hannover Medical School, Hannover, Germany; [11]Department of Hematology, Hemostasis, Oncology and Stem Cell Transplantation, Hannover Medical School, Hannover, Germany

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ABSTRACT

The aim of this study is to investigate frequency and prognostic significance of high *EVI1* expression in acute myeloid leukemia (AML). A diagnostic assay detecting multiple *EVI1* splice variants was developed to determine the relative *EVI1* expression by single real-time quantitative PCR in 1,382 newly diagnosed adult AML patients younger than 60 years. Patients were treated on four Dutch-Belgian HOVON (n=458) and two German-Austrian AMLSG protocols (n=924). The *EVI1* assay was tested in the HOVON-cohort and validated in the AMLSG-cohort. High *EVI1* levels (*EVI1*+) were found with similar frequencies, in both cohorts combined with a 10.7% incidence (148/1,382). *EVI1*+ independently predicted low CR rate (OR; 0.54, P=0.002), adverse relapse-free (RFS; HR, 1.32, P=0.05), and event-free survival (EFS; HR, 1.46, P=0.0003). This adverse prognostic impact was more pronounced in the intermediate cytogenetic risk group (EFS; HR, 1.64, P=0.0006; and RFS; HR, 1.55, P=0.02), and was also apparent in cytogenetically normal AML (EFS; HR, 1.67, P=0.008). Besides inv(3)/t(3;3), *EVI1*+ was significantly associated with chromosome abnormalities monosomy 7 and t(11q23), conferring prognostic impact within these two cytogenetic subsets. *EVI1*+ was virtually absent in favorable risk AML and AML with *NPM1* mutations. *EVI1*+ AML patients (n=28) who received allogeneic stem cell transplantation in first CR had significantly better 5-year RFS (33%±10% versus 0%). *EVI1* expression in AML is unequally distributed in cytogenetic subtypes. It predicts poor outcome, particularly among intermediate cytogenetic risk AML. Patients with *EVI1*+ AML may benefit from allogeneic transplantation in first CR. Pretreatment *EVI1* screening should be included in risk stratification.

INTRODUCTION

Acute myeloid leukemia (AML) represents a heterogeneous group of neoplasms with variable clinical behavior and different responsiveness to treatment, which can be classified based on unique genetic abnormalities(1, 2). Although biological insight of AML has increased in the past decade(3), the discovery and validation of novel discriminative biomarkers remains of utmost value to improve outcome prediction.

Ecotropic viral integration site 1 (*EVI1*) was first identified as a common retroviral insertion in murine myeloid leukemias(4). Several mouse studies have shown that *EVI1* positive mice display a myeloid dysplastic condition, including hyperproliferation of bone marrow and progressive pancytopenia(5, 6). Clinically, high *EVI1* expression (*EVI1*+) occurs in approximately 8% of patients with *de novo* AML.(7) In AML carrying the chromosome abnormalities inv(3)(q21q26.2) or t(3;3)(q21;q26.2), aberrant *EVI1* expression is caused by a breakpoint in or near the *EVI1* locus at band 3q26.2. High *EVI1* levels are also found in AML without chromosome rearrangements in this locus(7). Both groups are prognostically important due to poor treatment response. However, *EVI1*+ AML without 3q26.2 abnormalities represent an even larger and cytogenetically heterogeneous subset of AML(8).

Recently, *EVI1* has been implicated in a prognostic multi-marker model for cytogenetically normal AML (CN-AML) cases in a relatively small cohort(9). The prognostic value of *EVI1*+ could not be assessed in particular cytogenetic risk categories or genotypic subsets of AML due to numerical limitations of previously reported series(8-11). In addition, various splice variants of *EVI1* have been identified(10). Some have suggested that *EVI1*+ may not be associated with inferior treatment response in any subsets without 3q26.2 abnormalities and hence questioned the need of *EVI1* screening in routine genetic analysis(12). Due to the presence of numerous 5' *EVI1* splice variants, *EVI1* screening could only be performed using different specific 5' real-time quantitative polymerase chain reaction (RQ-PCR) assays, which are time-consuming and thus hamper efforts to implement *EVI1* screening in an efficient diagnostic setting(8, 10, 11). The clinical impact of *EVI1*+ on long-term outcome in other AML subtypes is also less clear. To date, no study exists in which a large homogeneous patient cohort younger than 60 years of age has been explored for *EVI1* expression, allowing for identification of *EVI1*+ AML subsets and straightforward risk assessment.We established an *EVI1* RQ-PCR assay, covering the various *EVI1* splice variants. This diagnostic assay was tested in one AML cohort, and the prognostic significance of *EVI1*+ was independently validated in a second AML cohort. Joint analysis revealed independent significance of *EVI1*+ as a prognostic marker in this large cohort of adult AML patients younger than 60 years of age. Furthermore, subgroup analyses identified *EVI1*+ as an important prognostic factor for AML with intermediate cytogenetic risk and separately in AML with 11q23 translocations.

PATIENTS AND METHODS

Patients and treatment

All patients for this study were recruited within two major leukemia cohorts. AML patients from Cohort I (n=458) were enrolled in the Dutch-Belgian Hemato-Oncology Cooperative Group (HOVON) -04(A), -29, and -42 trials (available at www.hovon.nl) (13-15). Cohort II (n=924) comprised patients who were enrolled in the AML Study Group (AMLSG) trials AML HD98A(16) and AMLSG 07/04 (ClinicalTrials.gov Identifier: NCT00151242). Details of the treatment protocols are shown in Figure S1.

The proportions of analyzed to recruited patients within the prospective treatment trials were: AMLSG trials, 60% (924/1,538); HOVON trials, 17% (458/2,780). No significant differences for the endpoints OS (P =.20), EFS (P =.15), and RFS (P=0.36) were found comparing the 1,382 analyzed and 2,936 not analyzed patients.

All adult patients younger than 60 years of age and availability of diagnostic blood or bone marrow samples were included. Patient characteristics of both cohorts are shown in Table S1. Cytogenetics and molecular analyses were performed as described in the Supplement

All patients provided written informed consent in accordance with the Declaration of Helsinki. All trials were approved by the Institutional Review Board of Erasmus University Medical Center and University of Ulm.

All Supplementary methods, supplementary results, tables and figures are not included, but are available online at http://jco.ascopubs.org/.

EVI1 real-time quantitative PCR

RNA isolation technique and conditions used for the *EVI1* RQ-PCR are described in the Appendix. The *EVI1* and *PBGD* primer/probe sequences and location are shown in respectively, Table S2 and Figure S2A.

The 3q26 amplified cell line SKOV3 overexpressing *EVI1*(17) served as a calibrator for quantification. Only standard curves established by serial dilutions of SKOV3 cDNA aliquots with correlation coefficients larger than 0.9 were taken into account. Equal amplification efficiencies of target and reference genes both in *EVI1*+ samples and SKOV3 at different cDNA concentrations were seen. The relative *EVI1* expression was calculated using the ddCT method.(18)

An overview of the *EVI1* diagnostic assay validation is shown in Figure S2B. Based on a separate cut-off analysis (Supplementary methods), *EVI1* expression levels were dichotomized based on a cut-off of 0.1 relative to SKOV3, i.e., values higher than 0.1 were defined as *EVI1*+ (Figure S3).

Statistical analysis

The definition of complete remission (CR) and survival endpoints such as overall survival (OS), event-free survival (EFS), and relapse-free survival (RFS) were based on the recommended consensus criteria.(19). OS endpoints were death (failure) and alive at last follow-up (censored), as measured from entry onto trial. EFS endpoints were remission induction failure, disease relapse, or death from any cause, measured from entry onto trial. RFS endpoints, measured from the date of first documented CR, were relapse (failure), death in CR (failure), and alive in CR at last follow-up (censored). The method of Korn was used for assessment of the median follow-up for survival.(20) The follow-up time of surviving patients ranged from 0.1 to 18.7 years, including 88% (533/605) with a follow-up time of two years.

Patient characteristics were compared by the Wilcoxon rank sum test (continuous variables) and the Fisher's exact test (categorical variables). Distribution estimations and survival distributions of OS, EFS, and RFS were calculated by respectively, the Kaplan-Meier method and the log-rank test. 95% confidence intervals (CI) were computed according to the cumulative hazard function using Greenwood's formula for the standard error (SE) estimation (21).

To determine the prognostic value of *EVI1*+, Cox proportional hazard regression models(22) with stratification to account for the two different cohorts were used. The proportional hazard assumption was tested(23) and no indication of non-proportionality was found. A variable selection was not performed and all variables were included in the final Cox regression models. Besides *EVI1*+, the prognostic variables used were age (per 10 years); white blood count (WBC) (log); platelet count (log); type of AML(19) (*de novo* AML, secondary AML [s-AML] or treatment-related AML [t-AML]); *NPM1* mutant/*FLT3*-internal tandem duplication negative status ($NPM1^{mut}$/*FLT3*-ITD^{neg}); and cytogenetic risk. The cytogenetic risk was categorized in three groups, i.e., *favorable risk*, t(15;17), t(8;21), inv(16)/t(16;16); *unfavorable risk*, inv(3)/t(3;3), t(6;9), t(*v*;11q23) other than t(9;11), -5/del(5q), -7, abn(17p), complex karyotype (three or more abnormalities in the absence of a WHO(2) designated recurring chromosome abnormality); and *intermediate risk*, all chromosome abnormalities not classified as favorable or unfavorable. Missing data of covariates were estimated using 50 multiple imputations by chained equations utilizing predictive mean matching. All statistical analyses were performed using the statistical software environment R, version 2.4.1, with the R package Design, version 2.0-12.

RESULTS

EVI1 diagnostic assay validation

An *EVI1* diagnostic RQ-PCR assay was designed (Figure S2) and applied on 458 AML samples from Cohort I, on which an *EVI1* cut-off level was determined as described in the Supplement. All AML cases previously demonstrated to be *EVI1*+, using a combination

of five different RQ-PCR assays(8), were positive with this single test. *EVI1*+ was found in 9.6% (95%-CI 7.2-12.7%) of the 458 AML of Cohort I. The *EVI1*+ incidence was comparable (P=0.41) in Cohort II of 924 AML, i.e., 11.3% (95%-CI 9.4-13.5%). In univariable Cox regression models, the impact of *EVI1*+ in both cohorts on the major clinical endpoints CR rate (Cohort I OR, 0.47, 95%-CI 0.23-0.97; Cohort II OR, 0.31, 95%-CI 0.20-0.48), EFS (Cohort I HR, 1.98, 95%-CI 1.42-2.76; Cohort II HR, 2.43, 95%-CI 1.95-3.02), RFS (Cohort I HR, 2.10, 95%-CI 1.39-3.16; Cohort II HR, 1.75, 95%-CI 1.32-2.34), and OS (Cohort I HR, 1.84, 95%-CI 1.30-2.61; Cohort II HR, 1.86, 95%-CI 1.46-2.38) was comparable as well. Based on these results, both AML cohorts were combined for further analyses.

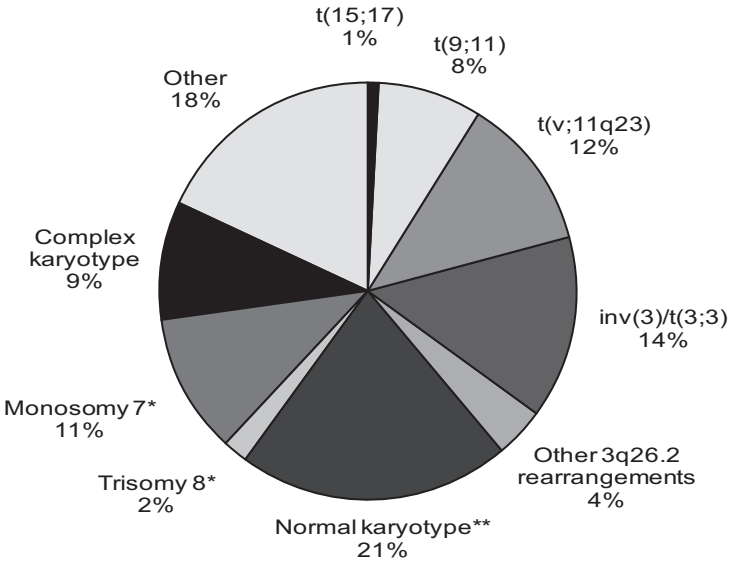

Figure 1. Distribution of cytogenetic abnormalities among *EVI1*+ AML (n=148).
*Occurring in a non-complex karyotype and not associated with inv(3)/t(3;3). **Among the *EVI1*+ with a normal karyotype, 66% of this group carried the *NPM1*^{wt}/*FLT3*-ITD^{neg}/*CEBPA*^{wt} genotype.

Clinical and genetic patient characteristics

The clinical features of the *EVI1*+ (n=148) compared with *EVI1*- (n=1,234) AML patients are summarized in Table 1. *EVI1*+ was associated with type of AML, i.e., was found more frequently in s-AML and t-AML; and *EVI1*+ AML tended to have higher platelet counts compared with *EVI1*- AML (P=0.06). No significant differences in age, gender, WBC, and bone marrow blast percentages were noted between *EVI1*+ and *EVI1*- AML.

Expression levels of *EVI1* were high in 21 of 23 AML with inv(3)/t(3;3) (P<0.0001). Other cytogenetic abnormalities overrepresented among *EVI1*+ cases included t(9;11) and other t(*v*;11q23), as well as monosomy 7 occurring within a non-complex karyotype and in absence of inv(3)/t(3;3) (Table 1 and Figure 1). On the other hand, *EVI1*+ and favorable risk cyto-

genetics [t(8;21), inv(16), t(15;17)] were almost exclusive. CN-AML were underrepresented among *EVI1*+ cases (*P*<0.0001).

Molecular marker analysis revealed an inverse correlation of *EVI1*+ with *NPM1* mutations (P<.0001) and *FLT3*-ITD (P=0.002). In CN-AML, *FLT3* tyrosine kinase domain and *CEBPA* mutations were not differently distributed among *EVI1*+ cases. The CN-AML genotype *NPM1*^{wt}/*FLT3*-ITD^{neg}/*CEBPA*^{wt} was significantly overrepresented and accounted for 66% of the *EVI1*+ CN-AML patients (P<0.0001).

EVI1 as prognostic marker in AML

The median follow-up time for survival was 58.9 months. Patients with *EVI1*+ AML had a lower CR rate compared with patients with *EVI1*- AML (53% *v* 77%; P<0.0001). Multivariable logistic regression analysis revealed *EVI1*+ as an independent negative prognostic marker for achievement of CR (OR, 0.54, P=0.002; Table 2). Survival analyses revealed a significantly inferior OS (P<0.0001), EFS (P<0.0001), and RFS (P<0.0001) for patients with *EVI1*+ AML compared with *EVI1*- AML patients (Figure 2A-C). In multivariable Cox regression models, *EVI1*+ significantly affected the endpoints EFS (HR, 1.46, P=0.0003), RFS (HR, 1.32, P=0.05), but not OS (HR, 1.17, P=0.18) (Table 2).

To evaluate the impact of allogeneic hematopoietic stem cell transplantation (HSCT) in patients with *EVI1*+ AML, patients were categorized on an as-treated basis. Univariable analysis revealed a significant benefit in OS (P=0.05) and RFS (P=0.001) for *EVI1*+ patients (n=28) who received allogeneic HSCT in first CR compared with intensive chemotherapy or autologous HSCT (Figure 3).

Beside a trend towards a younger age between *EVI1*+ patients who received an allogeneic HSCT and those who did not (median age 41 years and 46 years, respectively, P=0.06), no significant difference in cytogenetic risk, type of AML, age and WBC counts was found.

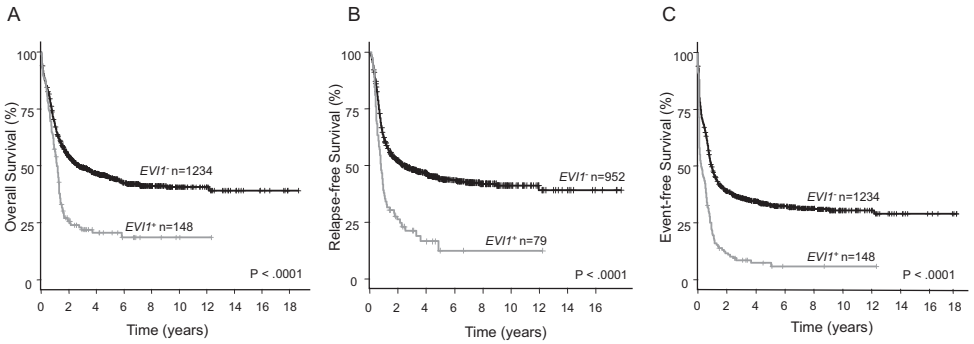

Figure 2. Survival analysis of 1,382 patients with AML according to their *EVI1* expression status for overall survival (A), relapse-free survival (B), and event-free survival (C).
For each Kaplan Meier plot a corresponding log-rank *P* value is shown.

Table 1. Clinical and genetic characteristics according to EVI1 status

Characteristics	*EVI1* negative (n = 1234)	*EVI1* positive (n = 148)	P value
Age, years			0.91
Median (range)	46 (15 – 60)	46 (15 - 60)	
Sex, no. (%)			0.26
Male	629 (51)	68 (46)	
Female	605 (49)	80 (54)	
WBC, × 10^9/L			0.73
Median (range)	21.4 (0.2 – 427)	19.3 (0.5 – 532)	
missing	n=18	n=2	
Platelets, × 10^9/L			0.06
Median (range)	51 (2 – 933)	64 (4 – 998)	
missing	n=21	n=2	
Bone marrow blasts, %			0.31
Median (range)	73 (0 – 100)	69.5 (12 – 100)	
missing	n=83	n=10	
Type of AML, no. (%)			0.02
de novo AML	1152 (94)	129 (87)	
M0	60 (5.5)	13 (11)	
M1	190 (17.5)	24 (20)	
M2	257 (23.5)	26 (22)	
M3	82 (7.5)	2 (2)	
M4	267 (24)	29 (24)	
M5	180 (16.5)	16 (13)	
M6	23 (2)	0 (0)	
M7	4 (0.5)	1 (1)	
Unclassified	31 (3)	9 (7)	
missing	n=58	n=9	
s-AML	21 (2)	6 (4)	
t-AML	54 (4)	13 (9)	
missing	n=7	n=0	
Cytogenetic characteristics, no. (%)*			
t(8;21)	82 (7.0)	0 (0)	0.0001
inv(16)/t(16;16)	104 (8.9)	0 (0)	<0.0001
t(15;17)	77 (6.6)	1 (0.7)	0.003
t(6;9)	11 (1.0)	0 (0)	0.61
t(9;11)	18 (1.6)	12 (8.8)	<0.0001
t(*v*;11q23)	15 (1.3)	18 (13.2)	<0.0001
inv(3)/t(3;3)	2 (0.2)	21 (15.4)	<0.0001
Normal karyotype	559 (48.1)	31 (22.3)	<0.0001
-7 within non-complex karyotype	5 (0.5)	33 (27.5)	<0.0001
+8 within non-complex karyotype	66 (6.2)	3 (2.5)	0.011
Complex karyotype	97 (8.3)	18 (13)	0.08
Other	128 (10.4)	26 (17.6)	0.02

Cytogenetic risk, no. (%)**			0.001
Favorable	262 (23)	1 (1)	
Intermediate	774 (67)	62 (46)	
Unfavorable	118 (10)	73 (53)	
missing	n=80	n=12	
Molecular abnormalities, no. (%)			
FLT3-ITD	325 (27)	22 (15)	0.002
missing	n=18	n=2	
FLT3-TKD	125 (11)	9 (6)	0.14
missing	n=49	n=7	
NPM1-mutated	386 (32)	4 (3)	<0.0001
missing	n=19	n=6	
NPM1^{mut}/*FLT3*-ITD^{neg}	199 (14)	2(1)	<0.0001
missing	n=16	n=5	
CEBPA-mutated CN-AML	76 (14)	2 (7)	0.41
missing	n=19	n=2	
NPM1^{wt}/*FLT3*-ITD^{neg}/*CEBPA*^{wt} CN-AML	132 (22)	19(66)	<0.0001
missing	n=22	n=2	

Abbreviations: AML, acute myeloid leukemia; WBC: white blood cell count; *FLT3*-ITD, *FLT3* internal tandem duplication; *FLT3*-TKD, *FLT3* tyrosine kinase domain; *CEBPA*, CCAAT/enhancer binding protein alpha; *NPM1*, nucleophosmin 1; Subheadings under "*de novo* AML" refer to French American British classification subtypes; wt, wild-type; neg, negative.

* Patients may be counted more than once owing to the coexistence of more than one cytogenetic abnormality in the leukemic clone.

** *Favorable risk*, t(15;17), t(8;21), inv(16)/t(16;16); *unfavorable risk*, inv(3) or t(3;3), t(6;9), t(*v*;11q23) other than t(9;11), -5 or del(5q), -7, abn(17p), complex karyotype (three or more abnormalities in the absence of a WHO(2) designated recurring chromosome abnormality); and *intermediate risk*, all chromosome abnormalities not classified as favorable or unfavorable.

Table 2. Multivariable analysis of *EVI1* as a prognostic marker for survival

Achievement of CR	OR	95%-CI	P value
EVI1+	0.54	(0.36-0.80)	0.002
NPM1^{mut}/*FLT3*-ITD^{neg}	2.35	(1.52-3.65)	0.0001
Cytogenetic unfavorable risk	0.49	(0.34-0.71)	0.0001
Cytogenetic favorable risk	1.62	(1.07-2.44)	0.02
Age (difference of 10 years)	0.79	(0.70-0.89)	0.0007
Log_{10}(WBC)	0.63	(0.52-0.78)	<0.0001
Overall Survival	**HR**	**95%-CI**	**P value**
EVI1+	1.17	(0.93-1.46)	0.18
NPM1^{mut}/*FLT3*-ITD^{neg}	0.52	(0.41-0.65)	<0.0001
Cytogenetic unfavorable risk	1.96	(1.60-2.40)	<0.0001
Cytogenetic favorable risk	0.49	(0.39-0.62)	<0.0001
Age (difference of 10 years)	1.40	(1.27-1.54)	<0.0001
Log_{10}(WBC)	1.52	(1.35-1.70)	<0.0001
Event-free Survival	**HR**	**95%-CI**	**P value**
EVI1+	1.46	1.19-1.87)	0.0003
NPM1^{mut}/*FLT3*-ITD^{neg}	0.43	(0.35-0.54)	<0.0001
Cytogenetic unfavorable risk	1.68	(1.38-2.04)	<0.0001
Cytogenetic favorable risk	0.48	(0.39-0.59)	<0.0001
Age (difference of 10 years)	1.12	(1.05-1.19)	0.0007
Log_{10}(WBC)	1.35	(1.22-1.50)	<0.0001
Relapse-free Survival	**HR**	**95%-CI**	**P value**
EVI1+	1.32	(0.99-1.76)	0.05
NPM1^{mut}/*FLT3*-ITD^{neg}	0.47	(0.37-0.61)	<0.0001
Cytogenetic unfavorable risk	1.67	(1.29-2.17)	<0.0001
Cytogenetic favorable risk	0.48	(0.37-0.61)	<0.0001
Age (difference of 10 years)	1.12	(1.03-1.21)	0.005
Log_{10}(WBC)	1.43	(1.25-1.64)	<0.0001

Abbreviations: WBC, white blood cell count; *FLT3*-ITD; *FLT3* internal tandem duplication; *FLT3*-TKD, *FLT3* tyrosine kinase domain; *NPM1*, nucleophosmin 1. Cytogenetic risk, unfavorable and favorable risk defined as described in Patients and Methods section. *The non-significant prognostic markers used in the Cox model, i.e. type of AML and platelet count are not shown.

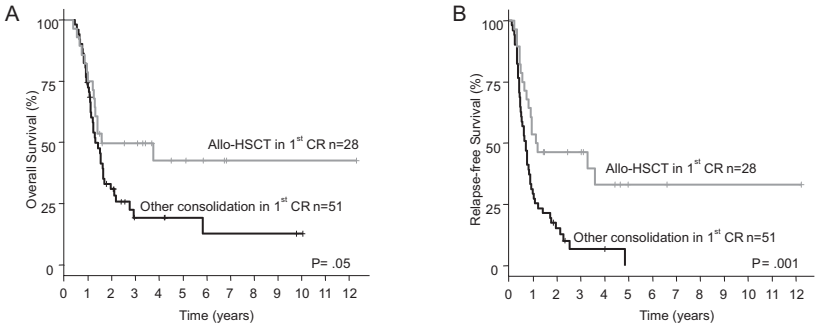

Figure 3. Overall survival (A) and relapse-free survival (B) of *EVI1*+ patients after allogeneic hematopoietic stem cell transplantation (HSCT) or after chemotherapy or autologous HSCT in first complete remission (CR). For each survival plot a corresponding log-rank P value is shown.

EVI1 expression in relation to cytogenetic risk categories

Given the extremely low frequency of *EVI1*+ in favorable cytogenetic risk AML, *EVI1*+ does not have a role in prognostication in this AML subset. Forty-six percent of *EVI1*+ AML had intermediate risk cytogenetics (Table 1). Patients with *EVI1*+ AML and intermediate risk cytogenetics had a slightly lower CR rate compared with *EVI1*- AML patients in this risk group (71% *v* 78%; P<0.27). In univariable analysis, *EVI1*+ predicted inferior EFS (P<0.0001), RFS (P=0.006), and OS (P=0.05) (Figure 4A and Figure S4; top panel). In multivariable models, *EVI1*+ also was an independent adverse factor for EFS (HR, 1.64, P=0.0006), RFS (HR, 1.55, P=0.02), and in trend OS (HR, 1.34, P=0.07). A separate subset analysis focusing on CN-AML is presented in the Supplementary Results and Figure S5, showing that *EVI1* was an independent adverse prognostic marker for EFS (HR, 1.67, P=0.008).

Relationship of EVI1 expression with t(11q23) and -7 chromosome abnormalities

EVI1+ was associated with specific recurrent chromosome abnormalities, including inv(3)/t(3;3), monosomy 7 (occurring as sole abnormality or within a non-complex karyotype), and 11q23 translocations.

Of 64 cases with 11q23 translocations, 30 were *EVI1*+; 12 of the 30 cases carried a t(9;11), including 7 *de novo* and 5 t-AML. Subtype analysis in the AML cases carrying 11q23 translocation revealed that *EVI1*+ patients showed an adverse survival rate with significant differences in RFS (P=0.0006), but not OS (P=0.20) (Figure 4B and Figure S4; middle panel)

Thirty-eight AML cases had monosomy 7 (-7) occurring either as sole abnormality (n=8) or within a non-complex karyotype (n=30). Of these 38 cases, 33 (87%) were *EVI1*+. The vast majority (31/33) of patients with -7/*EVI1*+ AML failed to achieve CR after first induction, and 31 patients died after a median of 8.6 months (95%-CI 5.3-14.4 months), the remaining 2 patients with -7/*EVI1*+ AML survived after 3 years.

In the -7/*EVI1*+ subset, 18 of 33 cases carried an inv(3)/t(3;3), 4 of 33 had another 3q26.2 chromosome rearrangement, whereas in 8 of the 11 remaining cases monosomy 7 was the

sole chromosome abnormality. Importantly, all patients with -7/*EVI1*+ AML had a dismal survival (2-year RFS 0%; 2-year OS 0%), irrespective of the presence or absence of inv(3)/t(3;3) (Figure 4C and Figure S4; lower panel).

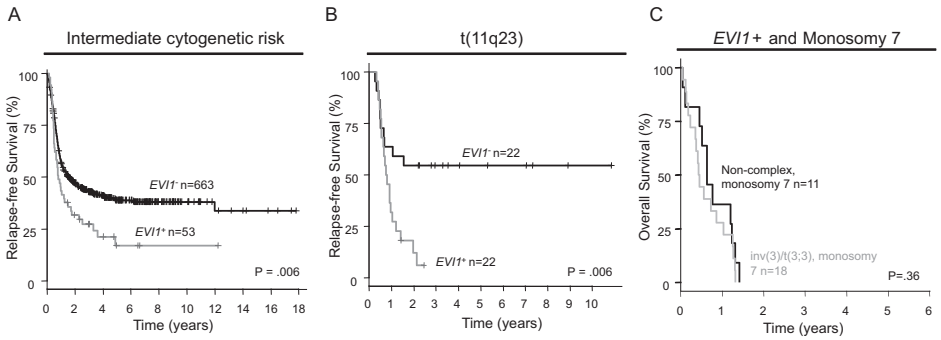

Figure 4. Relapse-free survival of patients with intermediate cytogenetic risk AML (A) and AML with t(11q23) (B) according to *EVI1* status.
Panel C shows the overall survival of AML cases with *EVI1*+ and monosomy 7, according to the presence (n=18) or absence (n=11) of inv(3)/t(3;3).

DISCUSSION

High *EVI1* mRNA expression has initially been proposed as a negative prognostic marker in a study led by investigators of this report, and, more recently, in a multimarker model of CN-AML(7, 9). To our knowledge, these results have not yet been validated in an independent cohort. Furthermore, due to limitations in sample size of these studies, identification of other *EVI1*+ AML subsets remained unresolved. Here we assessed the incidence and prognostic impact of *EVI1*+ in a cohort of 924 adult AML patients less than 60 years of age treated within prospective multi-center trials of the German-Austrian AMLSG. Joint analysis with combined trial cohorts of the Dutch-Belgian HOVON Study Group and German-Austrian AML Study Group allowed us to assess the prognostic value of *EVI1*+ in a total of 1,382 patients and identified novel subgroups of *EVI1*+ AML.

Previously described *EVI1* assays necessitate multiple PCR reactions to account for different 5' splice variants, rendering *EVI1* screening hardly feasible in a routine setting(10). We show that by using a single RQ-PCR assay, screening for *EVI1* expression is feasible in a routine diagnostic work-up. *EVI1* overexpression was identified in 148 of 1,382 (10.7%) AML patients, consistent with previous results(7, 8). Within the *EVI1*+ AML cohort, there was overrepresentation of specific chromosome abnormalities including inv(3)/t(3;3), monosomy 7, t(11q23), and among CN-AML there was overrepresentation of the triple negative genotype (*NPM1*^{wt}/*FLT3*-ITD^{neg}/*CEBPA*^{wt}). AML with inv(3)/t(3;3) in fact represent a subgroup within the *EVI1*+ AML, as opposed to *EVI1*+ being a mere surrogate marker for this specific chromosome alteration. Furthermore, *EVI1*+ was virtually absent in favorable cytogenetic risk AML and also in AML with *NPM1* mutations.

EVI1+ independently predicted a reduced CR rate (53%), EFS, and RFS, but not OS. In view of the poor response to induction therapy and the reduced remission duration, alternative consolidation therapies need to be investigated. Due to differences in treatment protocols among trials and the long recruitment phase, this analysis had to be carried out retrospectively and on an as-treated basis. Nonetheless, our data suggest that allogeneic HSCT from a matched related or unrelated donor in first CR may be beneficial for patients with *EVI1*+ AML with regards to OS and RFS. Allogeneic HSCT may represent a viable treatment option while targeted therapies are not yet available for this patient cohort.

The high number of patients in this study enabled us to evaluate the prognostic impact of *EVI1*+ in cytogenetic risk categories, in particular the intermediate risk group and also the subset of CN-AML. In multivariable analysis, *EVI1*+ predicted inferior EFS, RFS, and in trend also OS in intermediate risk AML, and inferior EFS in CN-AML. Molecular genotyping showed that *NPM1* mutations were highly underrepresented in *EVI1*+ CN-AML, especially the favorable genotype *NPM1*^{mut}/*FLT3-ITD*^{neg}(24). This observation, together with the fact that *EVI1*+ predominantly associates with high risk cytogenetics, might explain why OS was not significantly affected by *EVI1*+ in multivariable analysis in the total cohort and the

CN-AML subset. In case of relapse, chances of achievement of a durable second CR are very low for both groups independently of other molecular or clinical features(24-26). Therefore, the effect of *EVI1*+ as a surrogate for both groups is outweighed when adjusting for these genotypic features in multivariable models.

Another interesting aspect relates to the finding of the association of *EVI1*+ with monosomy 7. On the one hand, monosomy 7 is the most frequent secondary chromosome change in AML with inv(3)/t(3;3) (found in ~50% of cases), and virtually all these cases show deregulated *EVI1* expression by chromosomal rearrangement of the *EVI1* locus at 3q26.2. On the other hand, of the 16 monosomy 7 cases without 3q26.2 rearrangement in our study, 11 were also *EVI1*+. Of note, outcome of patients with -7/*EVI1*+ AML was dismal irrespective of whether inv(3)/t(3;3) was present or not (Figure 4C), thus pointing to an alternative mechanism of *EVI1* deregulation in monosomy 7 AML. How *EVI1* overexpression contributes to an aggressive course or chemotherapy unresponsiveness remains speculative. Notwithstanding, the interaction of *EVI1* with several epigenetic regulators, such as methytransferases(27), could also define *EVI1* as a new target for treatment with hypomethylating agents of *EVI1*+ AML associated with monosomy 7. It has been reported that myelodysplastic syndromes (MDS) with monosomy 7 potentially benefit from therapy with hypomethylating agents(28). Whether *EVI1*+ AML patients may benefit from treatment with hypomethylating agents needs to be evaluated in future trials, which may open a new therapeutic door with regards to the biologic role of *EVI1*+ in MDS or AML(5, 29). More functional studies elucidating the biological role of *EVI1* are needed to determine whether in human AML the deregulation of PU.1 through disruption of the c-Jun interaction impairs myelopoiesis(30).

Another subgroup in this study negatively affected by high *EVI1* expression was AML with t(11q23). Recently, prognostic factors for t(11q23) i.e., *MLL*-rearranged AML were described, upon which a risk stratification model was presented(31). These mainly included clinical parameters and the presence or absence of t(9;11) and t(6;11). Here, we show that by rapid testing for *EVI1*+, a new molecular screening target for 11q23 rearranged AML conducive for an accurate risk assessment becomes available, which has not been reported before.

This study shows that aberrant *EVI1* expression is a strong prognostic marker for therapy response and survival in patients with AML. Pretreatment screening for *EVI1*+ should therefore be considered in newly diagnosed AML patients to better guide risk assessment and therapeutic approaches. In patients with *EVI1*+ AML who achieve CR, it seems justified to prospectively evaluate the impact of allogeneic HSCT from a matched related or unrelated donor.

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CHAPTER 6

EVI1 Positive and *EVI1* Negative *MLL-AF9* Rearranged Acute Myeloid Leukemias Differ Clinically, Molecularly, Phenotypically and Mechanistically

Sanne Lugthart[1], Marije Havermans[1], Jurgen R. Haanstra[1], Elwin Rombouts[1], Claudia Erpelinck[1],Chantal Goudswaard[1], H. Berna Beverloo[2], Chi Wai E. So[3], John H. Kersey[4,5], Hartmut Döhner[6], Bob Löwenberg[1], Ashish Kumar[4,5], Ruud Delwel[1] and Eric Bindels[1]

[1]Department of Hematology, Erasmus University Medical Center, Rotterdam, The Netherlands; [2]Department of Clinical Genetics, Erasmus University Medical Center, The Netherlands; [3]Haemato-Oncology Section, The Institute of Cancer Research, Sutton, United Kingdom; [4]Masonic Cancer Center, University of Minnesota, Minneapolis, MN, USA; [5]Pediatric Hematology/Oncology/Blood and Marrow Transplantation, University of Minnesota, Minneapolis, USA; [6]Internal Medicine III, University of Ulm, Ulm, Germany.

Work in Progress.

ABSTRACT

Aberrant expression of *EVI1* occurs in acute myeloid leukemia (AML) with chromosome 3q26 abnormalities, the locus where the gene resides, but may also occur in AMLs with *MLL* rearrangements caused by 11q23 translocations. Applying *EVI1* quantitative PCR, we observed *EVI1* over-expression in 39/83 *MLL*-rearranged AMLs. High *EVI1* levels were observed in 11/13 *MLL-AF6*, 17/42 *MLL-AF9* and 7/14 *MLL-ENL* AML patients. In the present study, we investigated the relationship between *EVI1* expression and *MLL-AF9* in 11q23 rearranged leukemias. *MLL-AF9* leukemias expressing *EVI1* showed a significant adverse outcome compared to *MLL-AF9 EVI1* non-expressors, which were mainly of the FAB-M5 subtype. *MLL-AF9* transfection into murine bone marrow (mBM) cells resulted in high *Evi1* levels in pooled primary colonies. Upon replating, 17% of the single colonies showed high *Evi1* levels (*Evi1*$^{+}$). More than 60% were *Evi1*$^{-}$. We compared *Evi1*$^{+}$ with *Evi1*$^{-}$ transformed clones and found that in the *Evi1*$^{+}$ clones the immature common myeloid progenitors (CMPs) were present. *Evi1*$^{-}$ *MLL-AF9* transformed cells only contained the more mature granulocyte-macrophage progenitors (GMPs). In *Evi1*$^{+}$ transformed clones we observed immunophenotypically mature cells that aberrantly expressed *Evi1*. These data are in line with sorting experiments of *MLL*-rearranged AMLs in which aberrantly high *EVI1* levels were found in CD34-/CD38+ or CD34-/CD38- cells. In normal marrow these cells are *EVI1*-. *Evi1* knockdown in *Evi1*$^{+}$ MLL-AF9 mouse bone marrow cells resulted in a significant decrease in colony growth after the first replating. This study identifies a new AML *MLL-AF9* subtype over-expressing *EVI1* and demonstrates a role for *EVI1* in initiation of *MLL-AF9* leukemia.

INTRODUCTION

Aberrant expression of *EVI1* (*ecotropic viral integration site-1*) occurs in approximately 6-8% of human acute myeloid leukemias (AML) and has been shown to be associated with poor treatment outcome(1-3). The *EVI1* gene is located on chromosome 3 band q26 and is particularly highly expressed in patients with chromosome translocations involving the 3q26 region(4). *EVI1* encodes a nuclear protein with two zinc finger domains, each capable of binding DNA in a nucleotide specific manner(5, 6). In addition, it has been demonstrated that the protein can interact with a number of transcriptional and epigenetic regulators, such as CtBPs(7), HDACs(8), MBD3(9) or histone-methyltranferases(10). Different effects of aberrant *EVI1* expression on cellular responses have been reported(11, 12). Aberrant expression of *EVI1* associates with myelodysplatic syndrome (MDS), both *in vitro* and *in vivo*(13-15). *EVI1* blocks myeloid differentiation when over-expressed in transformed myeloid progenitors and it may provide proliferative advantage under certain circumstances(16-18). Although it is at present unclear why different effects may be evoked by *EVI1*, its role in leukemia development is indisputable and the particular interest of the present studyHigh expression of *EVI1* was also observed in both adult and pediatric AMLs without chromosomal abnormalities within the *EVI1* locus, especially in AMLs with *MLL*-gene rearrangements(1, 2, 19, 20). The prevalence within specific *MLL*-gene rearrangements remains unknown. Since multiple genetic events are required to obtain AML, both in humans and in murine leukemia models(21), it is possible that the observed *Evi1* up-regulation in *MLL*-rearranged leukemia is a secondary phenomenon, acquired via additional genetic or epigenetic changes rather than a direct effect of the *MLL*-fusion gene induced transformation. High expression of *Evi1* was found in the pre-leukemic stem and progenitor cells of knock-in *MLL-AF9* mice compared to their corresponding wild type cells(22). The knock-in *MLL-AF9* mice recapitulate the human disease by developing AML. Importantly, among the various *MLL-AF9* hematopoietic stem and progenitor cells a direct correlation was observed between the level of *Evi1* expression and the level of transformation, with the highest *Evi1* expressing $Lin^-/Sca1^+/c\text{-}Kit^+$ (LSK) cells inducing leukemia with the highest efficiency in transplant recipients(22). These results suggest that at least in the murine *MLL-AF9* model, *Evi1* up-regulation is a downstream effect of the fusion oncogene rather than a random, acquired secondary effect. A putative role for EVI1 in myeloid transformation has recently been reported for *MLL-ENL* fusion oncogene(23).

In the present study, we sought to define the relationship between *EVI1* and the various *MLL*-fusion genes by studying a large cohort (n=83) of 11q23 rearranged leukemias. We demonstrate that *EVI1* expression is associated with *MLL-AF6*, *MLL-AF9* and *MLL-ENL* transformed human AMLs. However, in both subgroups a significant number of AML cases are *EVI1* negative. Based on clinical, morphological, immunological, *in vitro* proliferation and gene expression profiling data the *EVI1*+ cases are clearly different from the *EVI1*-

AMLs. Experiments with *MLL-AF9* transduced mouse bone marrow cells demonstrate that the intermittent expression of *EVI1* in human *MLL-AF9* AMLs can be reproduced in murine models. Furthermore, we show that the *EVI1* expression pattern in human AML as well as in *MLL-AF9* transduced murine bone marrow cells is aberrant, since *Evi1* is highly expressed in cells that are normally *Evi1* negative. Knockdown of *Evi1* in *MLL-AF9* transformed cells *in vitro* point to a role of *EVI1* in the pathogenesis of the *EVI1* expressing *MLL-AF9* leukemias.

PATIENTS AND METHODS

Patient samples

Leukemic blast cells isolated at initial diagnosis from bone marrow or blood of 83 patients with 11q23 rearranged (*MLL*-rearranged) acute myeloid leukemia (AML) were purified as previously reported(24, 25). All patients provided written informed consent in accordance with the Declaration of Helsinki. The study have been approved by the Institutional Review Board of Erasmus University Medical Center and Ulm University.

Real-time quantitative PCR human EVI1

RNA isolation and cDNA synthesis were performed as previously described(24). Real-time quantitative PCR (Q-PCR) to determine relative expression of *EVI1* was performed as described(25). In summary, using human *EVI1* forward primer 5'-AGTGCCCTGGAGATGAGTTG-3', *EVI1* reverse primer 5'-TTTGAGGCTATCTGTGAAGTGC-3' and *EVI1* probe FAM- CCCCAGTGAGGTATAAAGAGGA using the ΔΔCt method(1, 2) with the human *EVI1* over-expressing SKOV3 cell line(26) as reference and the *PBGD* gene (porphobilinogen deaminase) as calibrator (Ct values > 30.5 were discarded), the *EVI1* relative expression was calculated. The patient samples with *EVI1* relative expression above 0.1xSKOV3 were dichotomized as *EVI1*+ and cases below this threshold were *EVI1*-(25).

Statistical analysis

Fisher's Exact test was performed to determine the distribution of French-American-British classification (FAB) among the MLL-rearrranged AMLs. Survival analysis for overall survival (OS), event-free survival (EFS) and relapse-free survival (RFS) were performed according to recommended guidelines(27). Kaplan Meier analysis and log-rank P values were calculated for AML patients younger than 60 years of age with a t(9;11)(p22;q23) (*MLL-AF9*) based on *EVI1* status, i.e. *EVI1*+ versus *EVI1*-.

Clustering analysis of the gene expression profiles (Gene Expression Omnibus GSE6891) from 12 *EVI1- MLL-AF9* and 8 *EVI1+ MLL-AF9* cases was performed using Omniviz software as previously described(24, 28).

Real-time quantitative PCR of murine genes

RNA isolation of the cells obtained from the colony assays and cDNA synthesized was carried out as previously described(24, 29). Q-PCR for murine *Evi1* was performed using forward primer 5'-CCAATCTTGACAGACACCTTGAA-3' and reverse primer 5'- GGTT-GCTGTTCCCGATGAAATT-3' using SYBR Green (Applied Biosystems) according to manufacture's protocol. The reference gene *Hprt* (hypoxanthine-guanine phosphoribosyl-transferase), with forward primer 5'-AGCCTAAGATGAGCGCAAGT-3' and reverse primer 5'-GGGTACCCACGCGAATCAC-3', was used with the empty vector (EV) as a calibrator *Meis1* Q-PCR was performed as previously described(22, 30).

Retroviral transduction

pMSCV vectors containing *MLL-AF9* puromycin and *E2A-PBX* puromycin were published previously(31, 32). A pMSCV-*eGFP* vector was used as a negative control (EV). 293T cells were co-transfected with each construct separately and pCL-Eco using FUGENE6 transfection reagent (Roche, Mannheim, Germany) according to manufacture's protocol. Viral supernatants were collected 48 hours after transfection, filtered, and used for transduction. One milliliter viral supernatant was added to 12 μg/ml Retronectin (Lonza, Basel, Switzerland) coated tissue culture dishes and incubated for four hours. Next, $2x10^6$ C57BL/6 mouse bone marrow cells separated by Ficoll (Axis Shield, Rodelokka, Oslo, Norway), were added to the Retronectin coated dishes. The viral transduction procedure was repeated after twenty-four hours. Forty-eight hours post-infection, cells were harvested and placed in colony assays.
Colony assays, in vitro cultures (Greiner Bio-One) and flow cytometry analysis were performed as described previously(22).

Western blot analysis

In human AML samples nuclear extracts were generated according to manufacture's protocol (NePer, Thermo Scientific, Rockford, USA). In 40 μg nuclear protein lysate human EVI1 protein levels were determined using an anti-human EVI1 antibody (Cell Signaling, Danvers, MA).
Murine cells were lysed using a buffer containing 20 mM Tris-HCL, 137 mM NaCl, 10 mM EDTA, 50 mM NaF, 1% Triton, 10% glycerol and protease inhibitors. EVI1 protein level was determined in 40 μg protein lysate from transfected mouse bone marrow cells with EVI1 antibodies directed against the N-terminal part(5) of the protein.
For the western blot analysis performed on the *MLL-AF9* leukemia cell line 4166, 30 μg protein lysate was used with antibodies against respectively, EVI1 (Santa Cruz Biotech., Santa Cruz, CA), Caspase-3, -8, -9, -12, PARP (Cell Signaling, Danvers, MA), HSP90 (BD Biosciences, San Jose, CA) or Actin (Sigma-Aldrich, St. Louis, MO).

Knock down experiments using lentiviral shRNAs

Lentiviral short hairpin RNA (shRNA) clones were obtained from Open-Biosystems (Huntsville, AL). The shRNAs include a hairpin with a 21 base-pair sense and antisense stem and a 6 base-pair loop and were cloned into the pLKO.1 lentiviral vector that carried a blasticidine or neomycine resistance marker. A total of three *EVI1* shRNAs were screened for effectiveness of *Evi1* knockdown. ShRNAs E95 (Clone ID: TRCN0000096095) and E97 (clone ID: TRCN0000096097) were found most effective. Lentivirus was produced by co-transfecting lentivirus expression vectors with the packaging plasmids pMDG and pCMVR 8.91 into 293T cells using LT1 transfection reagent according to the manufacturer's protocol (Roche, Indianapolis, IN). Culture supernatants containing lentivirus were harvested 48 to 72 hours post-transfection. Viral titers were determined by transducing NIH3T3 cells using diluted culture supernatants and determining the number of viable cells after five days of culturing in the presence or absence of puromycin (1.5 μg/ml). To transduce target cells, lentivirus containing culture supernatants were filtered through a 0.45 μ filter (Millipore Bedford, MA) and concentrated by ultracentrifugationm 12,000 x g) for 2 hours. The pellets were resuspended in serum-free IMDM. Unless specified, a multiplicity of infection (MOI) of 10-20 was used in subsequent experiments by spin transduction..

RESULTS

EVI1 is frequently expressed in MLL-rearranged human AML

Expression of *EVI1* transcripts was studied in 83 AML patient samples with *MLL*-rearrangements using a 3' specific *EVI1* Q-PCR(25). High *EVI1* levels (*EVI1*+) were found in 39/83 of the *MLL*-rearranged cases (47%). Clinical characteristics of the *MLL*-rearranged AMLs and their *EVI1* relative expression values are shown in Table S1. *EVI1* expression was found in 11/13 *MLL-AF6* cases (85%), 17/42 *MLL-AF9* (41%) and 7/14 *MLL-ENL* cases (50%) (Figure 1A). EVI1 western blot analysis revealed the presence of EVI1 protein in three selected AML samples with *MLL-AF6*, *MLL-AF9* or *MLL-ENL* rearrangements and with high *EVI1* mRNA levels (Figure 1B). As expected, in a control AML sample with a 3q rearrangement, high EVI1 protein levels were detected, whereas EVI1 protein was absent in an AML that did not express *EVI1* mRNA (*EVI1*-) (Figure 1B).

Abnormal EVI1 expression pattern in EVI1+ MLL-rearranged AMLs

In normal bone marrow samples *EVI1* is expressed in primitive CD34+/CD38- fractions (Figure 2A). The levels of *EVI1* transcripts decrease strongly with normal differentiation, i.e. markedly lower expression is found in CD34+/CD38+, CD34+/CD38++ or CD34- cells compared to cells from the CD34+/CD38- fraction (Figure 2A). To address the question whether *EVI1* expression pattern in immunophenotypically defined subfractions of *MLL*-rearranged

AML was abnormal, we determined *EVI1* levels in sorted fractions of two *MLL*-rearranged (*MLL-AF6*) AML samples. In one case (#5351) comparable numbers of cells were present in CD34+/CD38-, CD34+/CD38+, CD34-/CD38+ and CD34-/CD38- fractions, which were all equally *EVI1* positive (Figure 2B, left panel). Patient cells from case #2207 were mainly CD34+/CD38+ or CD34-/CD38+. High levels of *EVI1* were found in both fractions (Figure 2B, right panel). Thus, our data point to lineage infidelity of *EVI1* expression in *EVI1*+ *MLL*-rearranged AMLs..

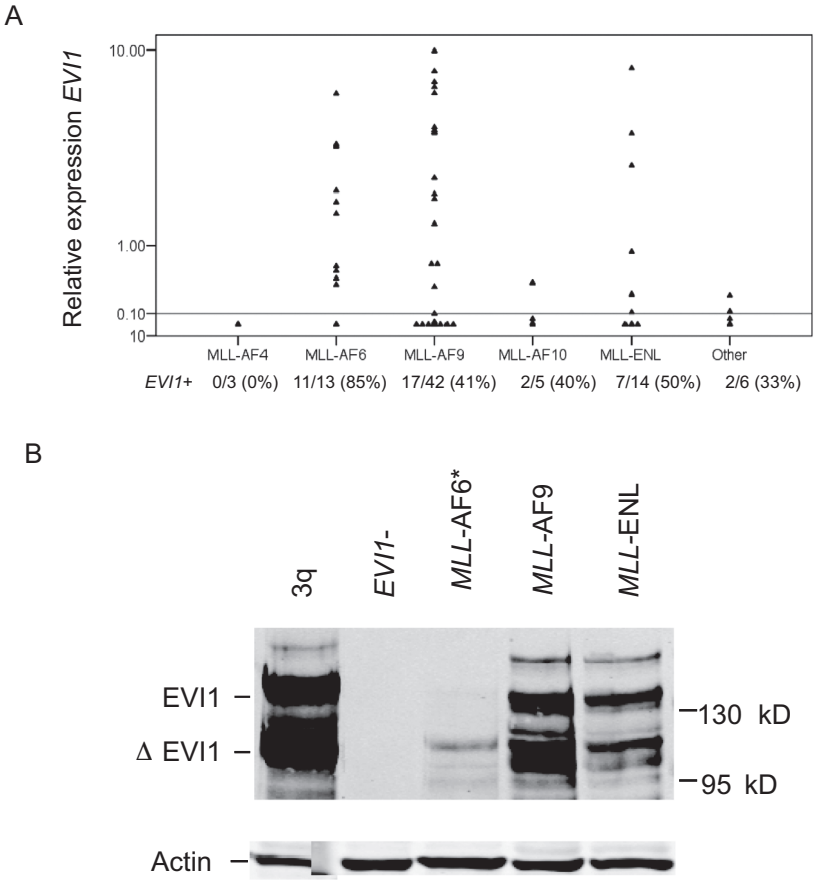

Figure 1. *EVI1* is frequently expressed in *MLL*-rearranged AMLs. (A) The relative expression of *EVI1* is shown for patients with different 11q23 translocations corresponding to *MLL*-fusion genes, i.e. *MLL-AF4*, *MLL-AF6*, *MLL-AF9*, *MLL-AF10*, *MLL-ENL* and other *MLL* fusions. Per *MLL*-fusion patient group the percentage of *EVI1* positive (*EVI1+*) patients iwnindicated. (B) An EVI1 western blot analysis of three *MLL*-rearranged *EVI1+* cases, one *EVI1* negative (*EVI1-*) case and one 3q26-rearranged case is shown. High EVI1 protein levels were seen in three *MLL*-rearranged cases. Western blot staining using an actin antibody was applied to show comparable protein loading. *EVI1 protein (140 kD) was detected using increased exposure to the higher mass band (not shown).

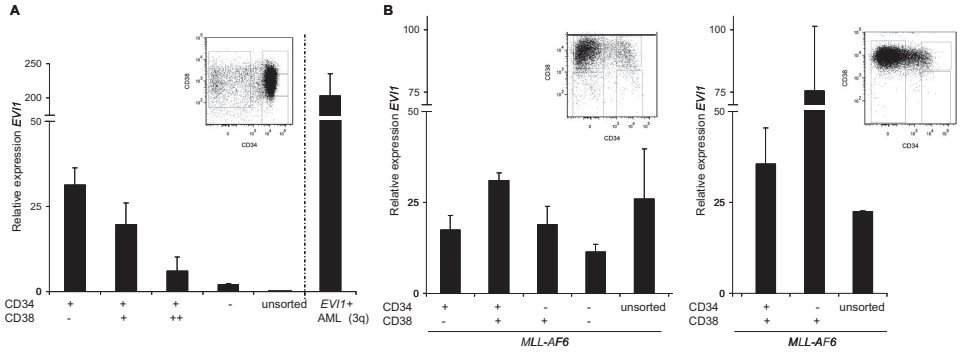

Figure 2. *EVI1* expression in CD34 and CD38 fractions in normal bone marrow (A) and 11q23 rearranged AML (B).

Bone marrow cells from a healthy individual and from two MLL-rearranged AML patients were stained with CD34 and CD38 and different fractions were isolated by flowcytometric sorting. Marrow cells from the second MLL-AF6 AML patient only containd CD34+/CD38+ and CD34-/CD38+ cells. Relative expression of *EVI1* was calculated using *PBGD* as reference gene. Each measurement was carried out in triplicate and standard deviation is shown per measurement. *EVI1* expression was determined in different fractions and unsorted bone marrow.

EVI1+ and EVI1- MLL-rearranged AMLs differ clinically and molecularly

MLL-rearranged *EVI1*+ versus *EVI1*- cases showed a significant adverse survival outcome for OS, EFS and RFS of *EVI1*+ cases(25). Two other data sets revealed that *MLL*-rearranged AMLs can be subdivided into two distinct subtypes that are respectively, *EVI1*+ and *EVI1*-. A correlation view of an unsupervised gene expression profiling analysis of a subset of *MLL*-rearranged AMLs revealed that samples that were *EVI1*- clustered separately from the *EVI1*+ samples (Figure S1A). Secondly, *EVI1- MLL-AF6, MLL-AF9* and *MLL-ENL* patients were almost exclusively of the morphologic subclass FAB-M5 (26/30), whereas *EVI1*+ cases with the same fusions were found among all FAB-categories (Fisher's Exact test P<0.0001) (Table S1). We next wondered whether in the *EVI1- MLL*-rearranged FAB-M5 cases, mainly consisting of monoblasts, the minor fraction of CD34+ cells did express *EVI1*. Since *MLL-AF9* cases formed the major fraction of *MLL*-rearranged AMLs (51%), we focused for the remaining of our study on this subtype only. Survival analysis within the *MLL-AF9* population showed the same significant adverse survival outcome for OS, EFS and RFS of *EVI1*+ cases (Figure S2). *EVI1- MLL-AF9* AMLs were also mainly of the FAB-M5 (21/25) morphological class as well (Fisher's Exact test P<0.0001). The percentages of sorted CD34+/CD38- or CD34+/CD38+ cells in 9 samples as indicated in Table S2 were low and importantly these cells did not express *EVI1* (Figure S3). Thus these *MLL-AF9* AMLs are really *EVI1*-.

High Evi1 expression in MLL fusion gene transduced mouse marrow cells

We next investigated whether there is a causal relationship between the presence of *MLL-AF9* fusion genes in mouse bone marrow cells and *Evi1* expression (Figure 3A). Ficoll separated mouse bone marrow cells were transduced with *MLL-AF9*, *E2A-PBX* or empty vector under

puromycin selection. Comparable primary colony numbers were found at day 7 in methylcellulose colony assays for each construct (Figure 3B). Upon replating, secondary and tertiary colonies were formed with the *MLL-AF9* and *E2A-PBX* but not with empty vector transduced marrow cells (Figure 3B). High levels of *Evi1* and control *Meis1* transcripts were observed in collected primary colonies of *MLL-AF9* transformed marrow cells, but not in primary colonies of vector control or *E2A-PBX* transduced cells (Figure 3C, day 7 panel). After the second replating, *Evi1* and *Meis1* mRNA levels increased in *MLL-AF9* transformed colony cells (Figure 3C, day 14 panel). In accordance with the mRNA expression data, western blotting of the cell lysates of collected colonies revealed expression of EVI1 protein in *MLL-AF9* at day 7 and day 14, but not in the *E2A-PBX* transformed cells (Figure 3D).

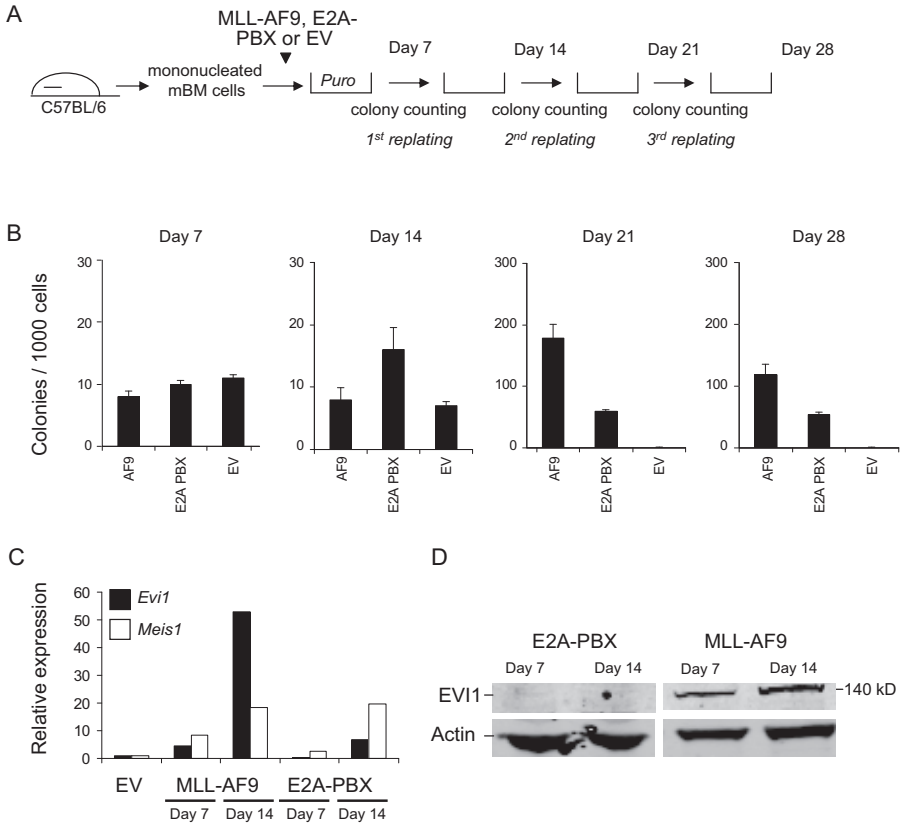

Figure 3. High *Evi1* expression in *MLL-AF9* fusion transformed murine bone marrow progenitors.
(A) Schematic illustration of the experimental approach to study the *in vitro* effects of *MLL-AF9*-fusion introduced in normal murine bone marrow cells and the correlation of *Evi1* expression to the leukemogenic potential of *MLL-AF9*. (B) Colony formation of murine mononucleated hematopoietic cells was observed after viral transduction of *MLL-AF9*. *E2A-PBX* and empty vector (EV). Note that, colony numbers at day 7 are calculated per number of plated retrovirus exposed cells. As puromycin selection occurred during this first week of culture, these colony numbers represent CFUs relative to virus infected and virus non-infected cells. Colonies formed after replating represent numbers of CFUs per virus infected cells. All constructs, except the empty vector derived coloniet were replatable. (C) *Evi1* and *Meis1* relative expression was determined compared to EV from mRNA obtained from colonies at day 7 or day 14, i.e. after the first replating. (D) EVI1 protein was detected by Western blot analysis. Lysates were derived from pooled colonies at day 7 and 14. Three experiments were performed, one representative experiment is shown.

MLL-AF9 transformed bone marrow cells generate Evi1+ and Evi1- colonies

The experiments from Figure 3 demonstrate that *Evi1* expression is high in pooled myeloid progenitors when transduced with *MLL-AF9*, but does not answer the question whether *Evi1* expression is activated in every *MLL-AF9* transformed progenitor cell. To address this question we picked 100 single primary *MLL-AF9* transduced colonies that were subsequently replated (Figure S4A). Seventy five of those picked colonies formed new colonies and remained indefinitely replatabley Messenger RNA was isolated from those 75 replated cultures and analyzed for *Evi1* mRNA expression. In 13/75 (17%) clones *Evi1* was highly expressed (Figure S4B). In another 13 replates, *Evi1* was expressed at intermediate levels and i4952/75 secundary colonies *Evi1* was negative, i.e. relative expression of *Evi1* less than 1 (Figure S4B). *Evi1*$^+$ versus *Evi1*$^-$ transformed cells showed equal replating capacity (Figure S5A) and in liquid cultures the growth rates werl similar as well (Figure S5A). In subsequent replatings, *Evi1*$^+$ colonies remained positive, whereas *Evi1*$^-$ colonies remained negative (Figure S5B). There was no clear difference in cell type distribution and growth rate between *Evi1*$^+$ and *Evi1*$^-$ colonies (Figure S5C and D). Together these experiments demonstrate that, the intermittent expression of *EVI1* in human *MLL-AF9* AMLs, can be reproduced in murine bone marrow transformation assays.

Evi1 expression in MLL-AF9 transformed murine bone marrow cells is abnormal

Detailed flowcytometric analysis of *MLL-AF9* transformed marrow cells revealed a clear difference between *Evi1*$^+$ versus *Evi1*$^-$ *MLL-AF9* transformed clones. In *Evi1*$^-$ *MLL-AF9* transformed cell fractions only granulocyte/macrophage progenitors (GMPs) were detectable, whereas GMPs as well as the more immature common myeloid progenitors (CMPs) were found in *Evi1*$^+$ *MLL-AF9* cells (Figure 4A and B). No Lin$^-$/Sca1$^+$/c-Kit$^+$ (LSK) cells, representing hematopoietic stem cells, were present in clones frin both groups. This is remarkable, since Q-PCR on mRNA obtained from sorted normal mouse bone marrow progenitor fractions revealed high *Evi1* levels particularly in LSK fractions, whereas in normal CMP, GMP or MEP (megakaryocytic/erythroid progenitors) compartments *Evi1* expression is lower or absent (Figure 4C). We next addressed the question whether *Evi1* was expressed in mature cells of *Evi1*$^+$ clones. Q-PCR on lineage positive cells sorted from *Evi1*$^+$ *MLL-AF9* transformed clones showed high *Evi1* levels (Figure 4D). Thus, *Evi1* expression pattern in *MLL-AF9* transformed clones is abnormal and mirrors the lineage infidelity observed in human *EVI1*+ *MLL*-rearranged AMLs.

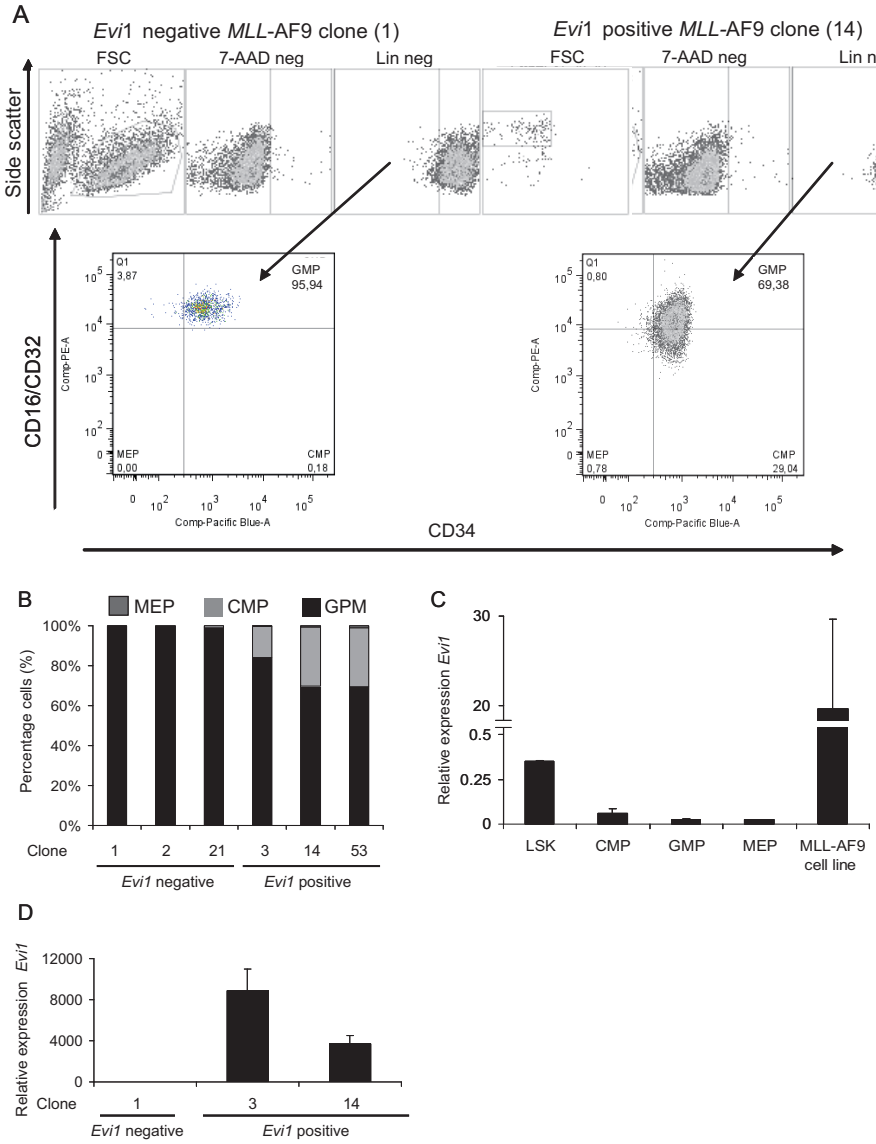

Figure 4. Enrichment of common myeloid progenitors in *Evi1* positive single *MLL-AF9* clones.
(A) Strategy of flow cytometrical analysis performed on *Evi1* positive and *Evi1* negative single *MLL-AF9* clones. Forward scatter (FSC) and side scatter were used for cell size and shape, 7-AAD for separating living cells and lineage marker negativity (Lin neg) was used to select immature cells. Monoclonal antibodies for CD16, CD32, and CD34 were used to differentiate between granulocytic myeloid progenitor (GMP), megakaryocyte erythrocyte precursor (MEP) and common myeloid progenitors (CMP). (B) The percentages of CMP, GMP and MEP relative to the total numbers of progenitors are shown for each clone. (C) *Evi1* expression in different cell fractions of normal bone marrow. Relative expression of *Evi1* was determined in different cell fractions, LSK, CMP, GMP and MEP, as positive control the *MLL-AF9* cell line 4166 was used. Three experiments were carried out and the standard deviation is depicted. (D) Relative *Evi1* levels determined in lineage positive subfraction of *MLL-AF9* clones. The expression of *Evi1* was calculated relative to the lineage negative fraction of *MLL-AF9* clone #1. Three experiments were carried out and the standard deviation is depicted.

Evi1 knock down inhibits proliferation of MLL-AF9 transformed marrow cells in vitro

To study the role of *Evi1* in *MLL-AF9* transformation we carried out shRNA knock down experiments in *Evi1*$^{+}$ and *Evi1*$^{-}$ *MLL-AF9* clones. In cells treated with *Evi1* specific shRNA, *Evi1* expression declined (Figure 5A) and the number of colonies at day 7 decreased significantly after replating (Figure 5B). Control shRNA did not affect *Evi1* expression levels nor did colonies numbers decline. No effect on replating ability with any of the shRNAs was observed in *Evi1*$^{-}$ *MLL-AF9* transformed cells (data not shown).

Recently, a *MLL-AF9* knock-in leukemia cell line 4166, was generated(30). This knock-in model of *MLL-AF9* closely mimics the conditions in human disease, because each cell contains only one copy of *MLL-AF9*, expressed under the control of the endogenous *MLL* promoter(33). This leukemia cell line model highly expresses *Evi1*(22) mRNA and EVI1 protein. *Evi1* mRNA levels as well as EVI1 protein were prominently reduced by the shRNA construct E95 and E96 but not by E94 (Figure 6A and 6D). *Evi1* knockdown (E95) in 4166 cells showed a significant reduction of colony growth (Figure 6C). Based on these knock down experiments in transduced bone marrow cells and the 4166 cell line, we conclude that *Evi1* plays a critical role in *MLL-AF9* transformation when over express

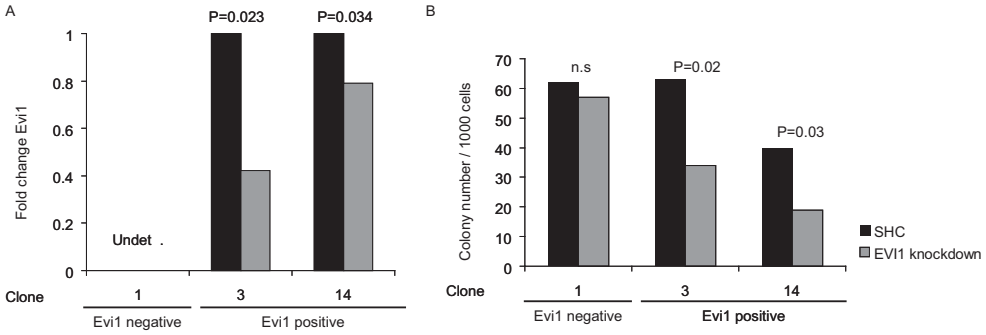

Figure 5. *Evi1* knockdown in *MLL-AF9* clones results in decreased colony formation.
(A) *Evi1* shRNA E95 and short hairpin control (SHC) were transduced in two *MLL-AF9 Evi1* positive clones and one *Evi1* negative clone. *Evi1* fold change was determined by comparing the relative *Evi1* expression of the SHC to the relative expression of the *Evi1* knockdown per clone. (B) Colony assays show that *Evi1* knockdown results in decreased colony formation compared to SHC. One experiment was performed in duplo.

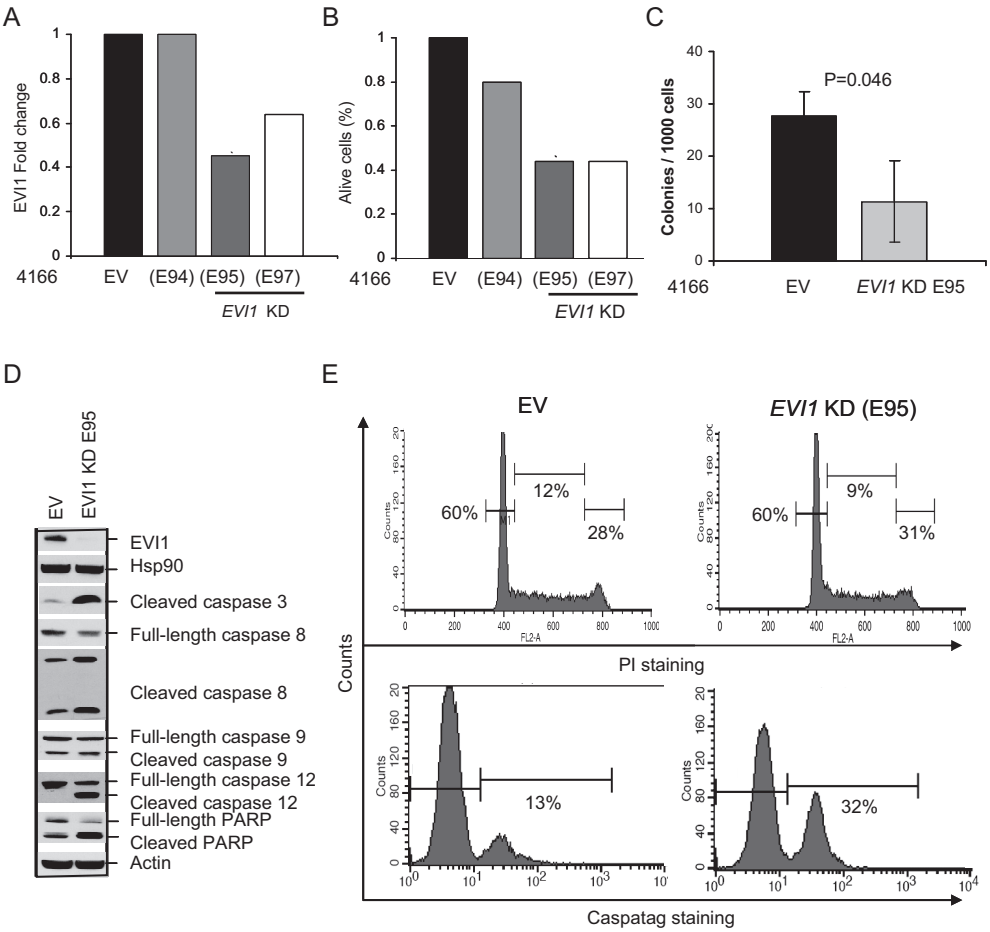

Figure 6. Lentiviral shRNA mediated *Evi1* knockdown in *MLL-AF9* cell-line 4166 inhibits cell growth, reduces cell self-renewal and induces apoptosis.

4166 cells were transduced with three lentiviral constructs or a control virus and cultured for 5 days under puromycin selection. (A) Relative expression of *Evi1* in shRNA transduced 4166 cells. *Gapdh* was used as a reference gene and the expression was calculated relative to the virus control. The vector control is set to 100%. One representative experiment out of three is shown. (B) Percentage of alive cells, i.e., tryptan blue negative cells 5 days after shRNA transduction is depicted. One representative experiment is shown. (C) Methylcellulose colony assay of transduced 4166 cells with virus control and *Evi1* shRNA (EVI1 KD E95) under puromycine selection showed significant reduction of colony formation in the *Evi1* knockdown 4166 cells. The error bars represent 2SD (2 times standard deviation), the t-test P value is shown. (D) Western blot analysis shows knockdown of *Evi1* using EVI1 KD E95 construct. Antibodies against actin and Hsp90 were used to show, comparable protein loading and cell viability, respectively. The effect on apoptosis on *Evi1* knockdown is shown by the protein levels of several full length and cleaved caspases and PARP. (E) The 4166 cells were transduced with EVI1 KD E95 or control virus and cultured for 48 hours without puromycine. Nuclei were isolated and stained with PI (top panel). Analysis of DNA content by flow cytometry showed no increase in the proportion of G0/G1 nuclei (left peak) in the KD-transduced cells compared with control virus. Increased uptake of the pan activated caspase maker CaspaTag in the KD-transduced cells indicate increased apoptosis in these *Evi1* knockdown 4166 cells (bottom panel).

Loss of Evi1 induces apoptosis without affecting cell cycle distribution

To discern by which mechanism *Evi1* knockdown leads to reduced cell growth we performed cell cycle and apoptosis analysis. Flow cytometric analysis of PI-stained nuclei showed that *Evi1* knockdown did not lead to drastic changes in the proportions of cells in G0/G1, S or G2/M phase of the cell cycle (Figure 6E, upper panel). Trypan blue staining of the transduced cells revealed that *Evi1* knockdown resulted in increased cell death (Figure 6B). Moreover, *Evi1* knockdown did result in increased apoptosis compared to the control group, evidenced by the increase in activated caspatag staining in E95-transduced cells as compared to vector control treated 4166 cells, respectively 32% versus 13% (Figure 6E, lower panel). Western blotting also revealed the presence of a strong increase of cleaved forms of Caspase -3, -8 and -12 as well as cleaved PARP in 4166 cells in which *Evi1* expression was reduced (Figure 6D). These experiments demonstrate that interfering with *Evi1* expression in *MLL-AF9* transformed cells causes inhibition of proliferation through induction of apoptosis without affecting the cell cycle.

DISCUSSION

This study included a large cohort of AML patients carrying several different 11q23 (*MLL*) translocations. We showed high *EVI1* expression in approximately 40% of *MLL*-rearranged leukemias. Although the level of *EVI1* expression was in general lower than that seen in AMLs with 3q26-rearrangements(25), the expression was significant and *MLL*-rearranged leukemias were the only AMLs with recurrent translocations analyzed showing *EVI1* expression. We did not observe any clear correlation between the *MLL*-fusion partner and *EVI1* expression. The preponderance of FAB M5 AMLs in the *EVI1*$^-$negative cases suggests that the *EVI1* expression might be influenced by the phenotype of the leukemic cell. Our sorting experiments in these patients showed that the minor fraction of CD34+/CD38- or CD34+/CD38+ cells are also *EVI1* negative. Thus *MLL*-rearranged AML can truly be subdivided into *EVI1*- versus *EVI1*+ patients. *EVI1*+ *MLL-AF9* AMLs show a worse response to therapy than the *EVI1*- cases. Transplantation experiments that we are currently conducting might give us insight into whether *Evi1*$^+$ *MLL-AF9* transformed marrow cells are indeed more aggressive *in vivo*. Although we particularly focused on *MLL-AF9*, which is the most frequently occurring *MLL*-rearrangement in human AML, we hypothesize that our conclusions drawn from this study may be equally valid to the other *EVI1*+ *MLL*-fusion transformed AMLs (Figure 1, Figure S1).

It is unclear, why certain *MLL-AF9* leukemias are *EVI1*+, whereas others with the same translocation do not express this oncogene. One explanation could be that in these leukemias, subsequent to the *MLL*-rearrangements additional mutations occur, which lead to aberrant *EVI1* expression. It is also possible that the cell of transformation differs among patients,

such that the *EVI1*+ leukemias could have originated in cells that were *EVI1*+ to begin with. This second explanation would fit with the finding that in normal marrow precursors *EVI1* positivity is particularly found in the most immature fraction, i.e. CD34+/CD38- in humans and LSKs in mice. Although this may indeed explain our findings, this does not explain the aberrant *EVI1* expression pattern, i.e. in both human and in experimental mouse bone marrow studies, *EVI1* expression was observed in immunologically defined mature cell subsets, that are normally *EVI1*-. We hypothesize that in the *EVI1*+ cases, *MLL-AF9* (co-)activates *EVI1* mRNA transcription in cells that were initially already *EVI1*+. In cells that were *EVI1*-, *MLL-AF9* and other *MLL*-fusions were not capable of activating transcription of this gene. Chromatin immunoprecipitation (ChIP) followed by promoter chip hybridization (ChIP-Chip) or deep sequencing (ChIP-Seq), combined with studies using reporter assays, should clarify whether *EVI1* is indeed a selective downstream target for *MLL-AF9*.

Loss of *Evi1* expression led to a decrease in colony formation of *MLL-AF9* leukemia cells *in vitro*, suggesting that *Evi1* plays a role in the maintenance of *MLL-AF9* leukemia. We hypothesize that in *EVI1- MLL-AF9* AMLs, another mutated or aberrantly expressed oncogene is responsible for leukemic maintenance. We found that the growth inhibitory effects of *Evi1* knockdown were mediated by increased apoptosis without any effect on cell cycling. In the same 4166 cell line, these results are in contrast to the effects of *Meis1* knockdown, a known down-stream target of MLL-fusion proteins(30). In this cell line, it was found that *Meis1* inhibition caused 4166 cells to arrest at the G0/G1 phase of the cell cycle. Additionally, while both *Meis1* and *Evi1* knockdown resulted in reduced colony growth in methylcellulose colony assays, *Meis1* inhibition led to an increase in the proportion of the more differentiated type II and type III colonies, while the undifferentiated type I colony proportion was reduced(30). These results suggest that while both *MEIS1* and *EVI1* are required for growth of certain *MLL-AF9* leukemias, they are not part of the same biologic pathway, but rather complement each other. In a recent report, Jin et al. showed that *Evi1* accelerated leukemia caused by overexpression of *Hoxa9* and *Meis1*(14). Thus, *EVI1* and *MEIS1,* both activated by *MLL*-fusion proteins, might cooperate in the pathogenesis of leukemia. An important question to be addressed is, whether knocking down *Evi1* in *MLL-AF9* transformed cells interferes with *in vivo* leukemia growth. Our *in vitro Evi1* knock down experiments and studies previously reported by Goyama et al.(23) showing that MLL-ENL induced tumor formation is greatly reduced in *Evi1-/-* bone marrow cells, suggest a critical role for EVI1 in *MLL*-fusion induced leukemia development. Targeting the EVI1 function might be considered as therapeutic option in the poor responding *MLL*-rearranged AMLs

SUPPLEMENTARY TABLES

Table S1. Patient characteristics of 83 *MLL*-rearranged AML patients. Per patient, age, sex, FAB type, karyotype, *MLL*-rearranged fusion gene, relative expression (RE) of *EVI1* and *EVI1* over-expression (+) or no *EVI1* expression (-) are shown. The karyotyping was performed according to the International System for Human Cytogenetic Nomenclature (2008) for each patient.

Sample	Age	Sex	FAB	G-banding	*MLL*-fusion	RE *EVI1*	*EVI1*+
642	48	M	M1	46,XY,t(10;11)(q22;q23)	*MLL-AF10*	0.44	+
114	30	M	M4	46,XY,t(10;11)(q11;q23)[8]/45,XY,add(1)(p36), t(10;11)(q11;q23),der(12)t(12;18)(p11;q11),-18[11]	*MLL-AF10*	0.45	+
292	50	F		47,XX,add(7)(p22),inv(8)(p23p13),+8,del(10) (p11), der(11)t(11;10;7)(pter->11q23::?->?::q23->pter),del(19)(q13)	*MLL-AF10*	0.0	-
336	37	M	M4	46,XY,ins(10;11)(p13;q23q13)[12]/48,XY,+8, ins(10;11)(p13;q23q13),+19[7]/46,XY[1]	*MLL-AF10*	0.05	-
582	31	M	M5	46,XY,ins(10;11)(p13;q13q23)	*MLL-AF10*	0.01	-
2255	45	M	M1	46,XY,t(4;11)(q21;q23)[18]	*MLL-AF4*	0.0	-
524	38	F		46,XX,t(4;11)(q21;q23),del(20)(q13) [7]/46,XX,t(4;11)(q21;q23), del(5)(q31q33),del(12) (p11),del(20)(q13)[19]	*MLL-AF4*	0.0	-
15017	34	M	M4	46,XY,t(4;11)(q21;q23)[21]	*MLL-AF4*	0.0	-
818	60	F	M0	48,XX,t(6;11)(q27;q23),+21,+21[9]	*MLL-AF6*	2.22	+
590	59	F	M1	46,XX,t(6;11)(q27;q23)	*MLL-AF6*	0.67	+
675	58	F	M4	46,XX,t(6;11)(q27;q23)	*MLL-AF6*	3.75	+
18	41	F	M1	46,XX,t(6;11)(q26;q23)	*MLL-AF6*	1.92	+
964	34	F	M4	46,XX,t(6;11)(q27;q23)	*MLL-AF6*	0.5	+
2207	31	M	M1	46,XY,t(6;11)(q25;q23)	*MLL-AF6*	0.7	+
6238	30	F	M4	46,XX,t(6;11)(q27;q23)[28]	*MLL-AF6*	1.1	+
15018	30	M	M4	46,XY,t(6;11)(q27;q23)	*MLL-AF6*	6.5	+
549	28	F	M0	46,XX,t(6;11)(q27;q23)	*MLL-AF6*	0.42	+
14294	19	M	M5	46,XY,t(6;11)(q26;q22)	*MLL-AF6*	0.6	+
5351	67	F	M4	46,XX,t(6;11)(q27;q23)[36]	*MLL-AF6*	0.39	+
15015	40	M	M5	46,XY[35],46,XY,t(6;11)(q26orq27;q23)[6]	*MLL-AF6*	0.0	-
889	22	F	M5	45,XX,t(6;11)(q27;q23),-8,+2x i(8)(q10)	*MLL-AF6*	0.0	-
14454	72	F		45,XX,add(1)(p?),der(2)t(2;6)(p16;q13)t(2;6) (q32;p12), add(5)(q21),der(5)t(5;18)(q31;?),?inv(7) (q31q35), der(9)t(9;11)(q22~31;q13),del(12) (q13q23),-17,-18,+mar	*MLL-AF9*	0.1	+
14290	62	F	M1	46,XX,t(9;11)(p22;q23)[16]	*MLL-AF9*	0.7	+
14456	61	F	Sec	46,XX,t(9;11)(p22;q23)[21]	*MLL-AF9*	2.6	+
7072	61	M	M4	46,XY,der(11)(q2?)[3]/46,XY[18] .ish t(9;11) (p22;q23)	*MLL-AF9*	0.1	+
464	60	F		46,XX,t(9;11)(p22;q23)	*MLL-AF9*	4.38	+
2682	57	F	M4	46,XX,t(2;9;11)(p13;p22;q23)	*MLL-AF9*	1.0	+
14457	51	M		46,XY,t(9;11)(p22;q23)[18]	*MLL-AF9*	0.7	+
709	51	M	M4	46,XY,t(4;11;9)(q27;q23;p22)	*MLL-AF9*	4.6	+
1055	46	M	M4	46,XY,t(9;11)(p22;q23)	*MLL-AF9*	8.15	+

Sample	Age	Sex	FAB	G-banding	*MLL*-fusion	RE *EVI1*	*EVI1+*
485	45	F		46,XX,t(9;11)(p22;q23)[13]/47,XX,t(9;11)(p22;q23),+19[2]	*MLL-AF9*	6.52	+
716	43	F	M0	46,XX,t(9;11)(p22;q23)	*MLL-AF9*	4.46	+
587	34	M	M4	46,XY,t(9;11)(p22;q23)	*MLL-AF9*	4.32	+
589	32	F	M5	46,XX,t(9;11)(p22;q23)[3]/47,XX,t(9;11)(p22;q23),+13[8]	*MLL-AF9*	2.13	+
2288	31	M	M4	45,XY,-7,t(9;11)(p21;q23)[33]	*MLL-AF9*	1.7	+
375	23	M	M5	46,XY,t(9;11)(p22;q23)	*MLL-AF9*	1.42	+
261	23	F	M5	46,XX,t(9;11)(p22;q23)	*MLL-AF9*	6.95	+
15014	17	F		47,XX,t(9;11)(p22;q23),+der(9)t(9;11)(p22;q23)	*MLL-AF9*	2.0	+
14293	66	F	M5	51-52,XX,+4[2],+8,t(9;11)(p22;q23)[3],+13,+16[2],+21[2], +22[2][cp4]/46,XX[3]	*MLL-AF9*	0.0	-
14289	59	M	M5	46,X,-Y,+8,t(9;11)(p21;q23),t(18;20)(q21;p12)[13]	*MLL-AF9*	0.0	-
2541	57	F	M5	45,XX,-7[3]/45,idem,t(9;11)(p21;q23)[20]/46,XX[3]	*MLL-AF9*	0.0	-
799	56	M	M5	47,XY,+8,t(9;11)(p22;q23),inv(16)(p13q22),del(17)(p11)	*MLL-AF9*	0.0	-
210	53	F	M5	47,XX,+8,t(9;11)(p22;q23)	*MLL-AF9*	0.0	-
649	52	F	M5	46,XX,t(9;11)(p22;q23)[10]/47,XX,t(9;11)(p22;q23),+8[4]	*MLL-AF9*	0.01	-
944	50	M	M5	46,XY,t(9;11)(p22;q23)	*MLL-AF9*	0.0	-
6364	49	M	M5	46,XY,t(9;11)(p22;q23)[19]	*MLL-AF9*	0.0	-
65	48	M	M5	46,XY,t(9;11)(p22;q23)	*MLL-AF9*	0.0	-
274	48	F	M5	46,XX,t(9;11)(p21;q23)	*MLL-AF9*	0.0	-
2694	46	M	M5	46,XY,t(9;11)(p22;q23)	*MLL-AF9*	0.0	-
7166	44	F	M5	46,XX,t(9;11)(q22;q23)[11]/46,XX[4]	*MLL-AF9*	0.0	-
3316	44	F	M5	46,XX,t(9;11)(p22;q23),t(10;12)(q21;q24)[22]	*MLL-AF9*	0.0	-
5358	40	F	M5	46,XX,add(6)(q2?5),t(9;11)(p22;q23)[20]/	*MLL-AF9*	0.0	-
350	37	F	M5	46,XX,t(9;11)(p22;q23)	*MLL-AF9*	0.0	-
413	36	F		47,XX,t(9;11)(p21;q23),+21	*MLL-AF9*	0.01	-
14288	35	M	M5	46,XY,inv(2)(q1?1q35),t(9;11)(p22;q23)[38]	*MLL-AF9*	0.0	-
1083	35	F		46,XX,t(9;11)(p21;q23)	*MLL-AF9*	0.01	-
348	34	F	M5	47,XX,+8,t(9;11)(p22;q23)[9]/46,XX[2]	*MLL-AF9*	0.0	-
14295	30	M	M5	47,XY,+8,t(9;11)(p22;q23)[22]	*MLL-AF9*	0.0	-
15013	30	M	M3	47,XY,+8,der(9)t(9;11)(q;q),t(15;17)(q22;q21)	*MLL-AF9*	0.0	-
15019	29	M	M5	50,XY,+5,+6,t(9;11)(p21;q23),+19,+22[4]	*MLL-AF9*	0.0	-
691	28	M	M5	46,XY,t(9;11)(p22;q23)[10]/47,XY,+8,t(9;11)(p22;q23)[5]	*MLL-AF9*	0.02	-
14453	28	M		46,XY,der(9)ins(9;?)(q12;?)del(9)(q12;q2?2),t(9;11)(p22;q23)[16]	*MLL-AF9*	0.0	-
2285	18	F	M5	46,XX,t(9;11)(p21~22;q23)[57]	*MLL-AF9*	0.0	-
15016	64	M	NA	46,XY,t(11;19)(q23;p13)	*MLL-ENL*	4.3	+
14460	57	F	NA	46,XX,t(11;19)(q23;p13)[29]	*MLL-ENL*	0.3	+
360	55	F		46,XX,t(11;19)(q23;p13)	*MLL-ENL*	0.11	+
60	52	F		46,XX,t(11;19)(q23;p13)	*MLL-ENL*	0.31	+
7306	51	M	M5	46,XY,t(11;19)(q23;p13.1)[15]/46,XY[5]	*MLL-ENL*	0.3	+

Sample	Age	Sex	FAB	G-banding	*MLL*-fusion	RE *EVI1*	*EVI1+*
126	48	F	M4	46,XX,t(11;19)(q23;p13)[9]/46,XX[1]	*MLL-ENL*	3.02	+
3328	41	F	M5	46,XX,t(11;19)(q23;p13)[21]	*MLL-ENL*	0.8	+
14459	75	F	M4	46,XX,t(11;19)(q23;p13)[5]	*MLL-ENL*	0.0	-
465	45	M	M5	46,XY,t(11;19)(q23;p13)	*MLL-ENL*	0.0	-
572	39	M	M5	45,X,-Y,t(11;19)(q23;p13)	*MLL-ENL*	0.0	-
143	39	M	M1	46,XY,t(2;8)(p12;q24),add(7)(q32),?t(10;11;19)(p13;q23;p13)	*MLL-ENL*	0.0	-
14291	38	F		47,XX,+X,t(11;19)(q23;p13)[23]	*MLL-ENL*	0.0	-
3322	35	F	M5	47,XX,+8,t(11;19)(q23;p13)[25]	*MLL-ENL*	0.0	-
14462	34	F	M5	47,XX,+8,t(11;19)(q23;p13)[25]	*MLL-ENL*	0.0	-
374	49	M	M4	46,XY,t(11;17)(q23;q21)	*Other*	0.12	+
247	47	F	M2	46,XX,t(7;11)(p11;q23)	*Other*	0.29	+
363	56	F	M4	46,XX,t(11;22)(q23;q13)[18]/46,XX[3]	*Other*	0.0	-
1102	55	M		46,XY,t(11;21)(q23;q11)	*Other*	0.0	-
613	47	F	M4	46,XX,t(1;11)(q21;q23),del(20)(q11)[4]/46,XX[2]	*Other*	0.05	-
7307	40	F	M1	46,X,ins(X;11)(q13;q23q22)[1]/48,idem,+6,+19[33]/46,XX[7]	*Other*	0.0	-

Table S2. Percentages of the CD14 negative population and the percentages of the CD34+CD38+ and CD34+CD38- populations within the CD14 negative population.

Sample	CD14- (%)	CD34+CD38+ (%)	CD34+CD38- (%)
2747	14.7	79.3	10.97
2275	22.1	9.09	2.97
2220	26.5	0.43	0.16
3221	10.5	0.13	0.47
2261	6.5	0.52	0.48
14288	73.5	5.11	0.85
2285	50.1	0.20	0.01
5358	21.8	0.02	0.00
14293	28.5	23.33	56.88

SUPPLEMENTARY FIGURES

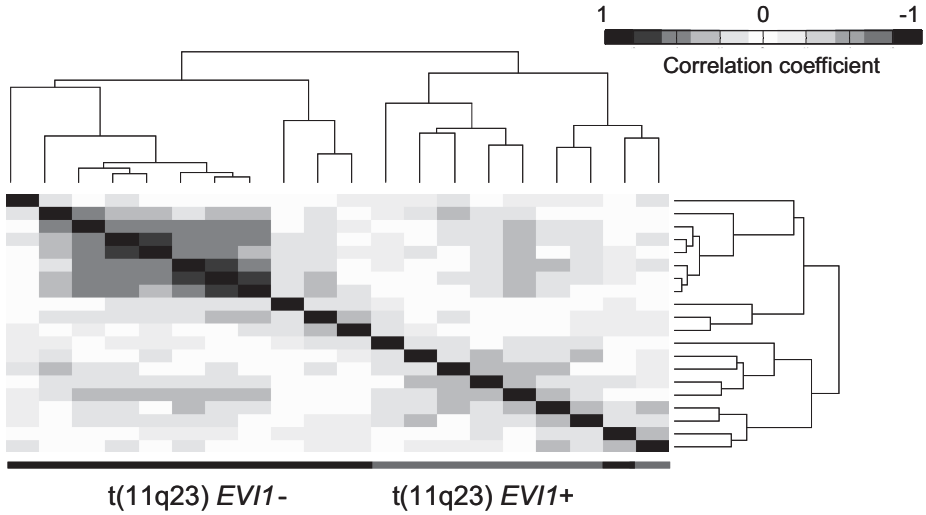

Figure S1. Acute myeloid leukemia patients carrying an 11q23 translocation expressing *EVI1* (*EVI1*+) and not expressing *EVI1* (*EVI1*-) show a distinct mRNA gene expression pattern. In the Pearson's correlation clustering 1455 probe sets (standard deviation > 4) where used. The colors correspond to high correlation (red) and low correlation (blue) between mRNA expression of genes in patient samples.

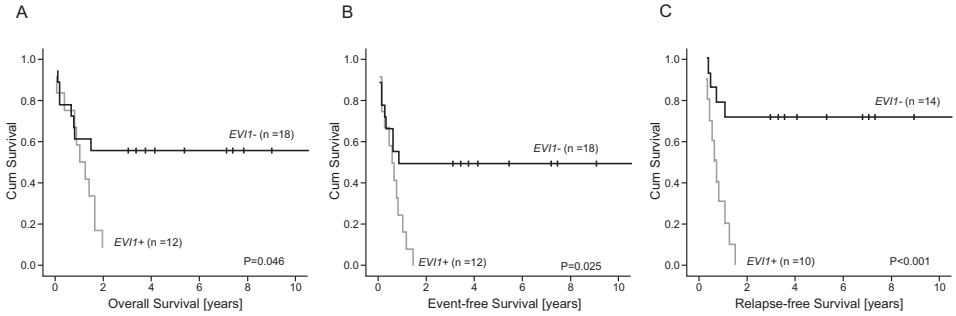

Figure S2. *MLL-AF9* leukemias with *EVI1* expression show an adverse outcome compared to *MLL-AF9* leukemias not expressing *EVI1*. Overall survival (A), event-free survival (B) and relapse-free survival (C) for *MLL-AF9* cases with *EVI1* expression (*EVI1*+) and cases not expressing *EVI1* (*EVI1*-) were compared. Only patients between 15 and 60 years of age were included. Per Kaplan-Meier analysis a corresponding log-rank P value is shown.

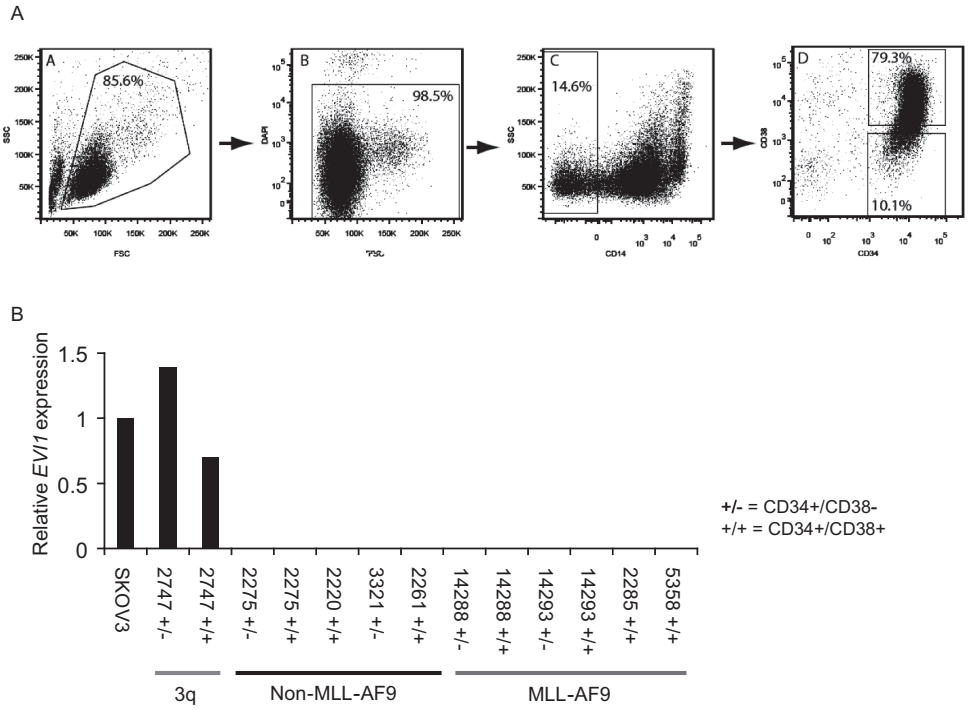

Figure S3. The hematopoietic stem and progenitor cells in FAB M5 leukemia are not expressing *EVI1*. (A) Gating strategy for sorting. The leukemic cells of AML samples (FAB M5) were sorted for CD34+/CD38- (corresponding to immature stem cells) and CD34+/CD38+ (corresponding progenitor cells). As a initial step, dead cell and debris were excluded based of forward- and side scatter properties (A panel), any residual dead cells were removed in by exlusion of DAPI positive cells (B panel) after which the CD14 negative cells were selected (C panel). From these CD14-negative cells the CD34+CD38+ and CD34+CD38low/- cells were sorted according to the gates depicted in the D panel. (B) The *EVI1* expression of samples carrying an *MLL-AF9* fusion was compared to cases that do not carry this fusion. An AML sample carrying a 3q26 abnormality was used as positive control. The relative expression of *EVI1* was measured in duplo and relative to the SKOV3 cell line.

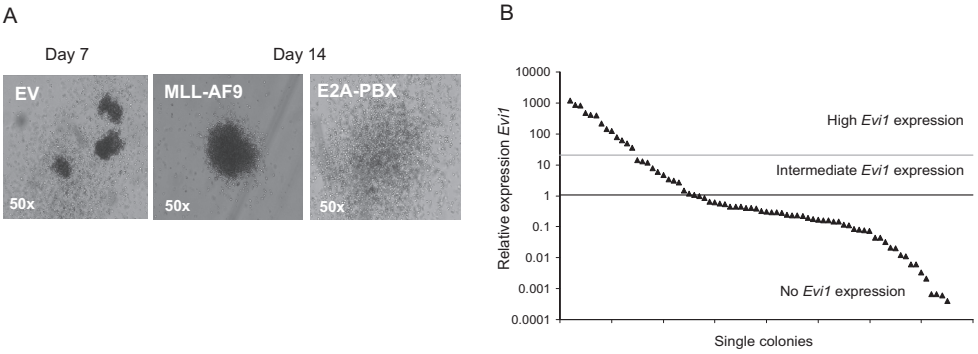

Figure S4. Nearly 20% of the single colonies picked from *MLL-AF9* transduced murine bone marrow cells after the first replating (day 14) show high *Evi1* expression. (A) Representative colonies after first replating (day 14) of *MLL-AF9* and *E2A-PBX* transduced murine bone marrow cells As a reference, representative colonies of murine bone marrow cells transfected with empty vector (EV) were taken at day 7. All pictures were taken using a 50x magnification. (B) *Evi1* expression was determined in 72 single colonies. High *Evi1* expression was defined as larger than 35, intermediate expression between 35 and 1, low or no expression of *Evi1* with levels smaller than 1. A relative expression *Evi1* of 1 represents equal *Evi1* levels compared to the reference murine bone marrow transfected with empty vector.

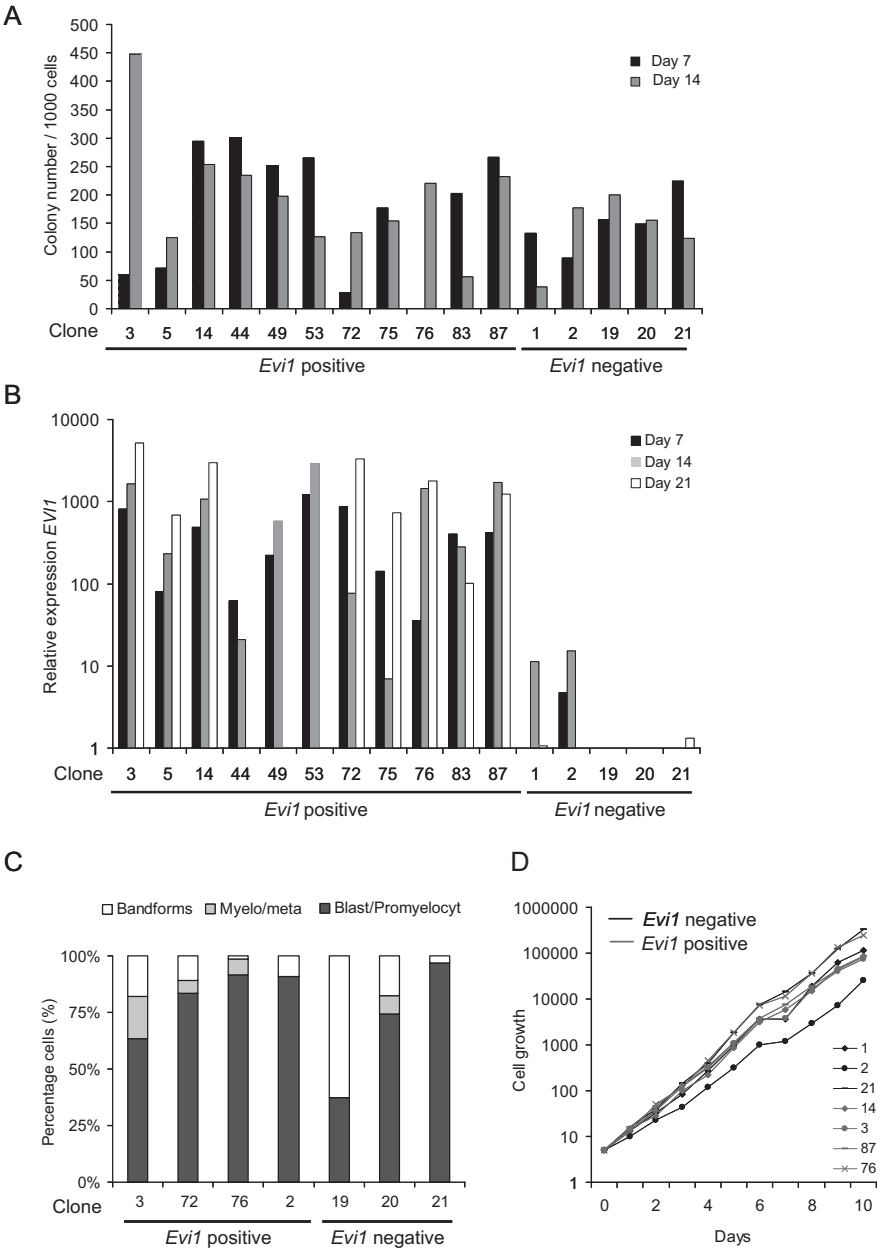

Figure S5. *MLL-AF9* single colonies with *Evi1* expression show no difference in self-renewal, morphology and growth compared to *Evi1* negative *MLL-AF9* colonies. (**A**) Colony numbers of *MLL-AF9* single colonies (*Evi1* positive and *Evi1* negative) after first and second replating (respectively, day 7 and 14) in methylcellulose assays. Experiment was carried out in duplo, average colony numbers are shown. (**B**) Relative expression of *Evi1* per single colony after 7, 14 and 21 days in methylcellulose assays. Experiment was carried out in duplo, average relative expression of *Evi1* is shown. (**C**) Cell morphology of *MLL-AF9* single colonies. 100 cells were scored twice, the average percentage of bandforms, myeloblasts or metablasts and blasts or promyelocytes is shown. (**D**) A 10-day growth curve of *Evi1* positive and *Evi1* negative *MLL-AF9* single colonies.

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DNA Methylation Signatures Identify Biologically Distinct Subtypes in Acute Myeloid Leukemia

Maria E. Figueroa[1], Sanne Lugthart[2], Yushan Li[1], Claudia Erpelinck-Verschueren[2], Xutao Deng[3], Paul J. Christos[4], Elizabeth Schifano[5], James Booth[5], Wim van Putten[7], Lucy Skrabanek[3,6], Fabien Campagne[3,6], Madhu Mazumdar[4], John M. Greally[8], Peter J.M. Valk[2], Bob Löwenberg[2], Ruud Delwel[2], and Ari Melnick[1]

[1]Department of Medicine (Hematology Oncology Division), Weill Cornell Medical College, New York, USA. [2]Department of Hematology, Erasmus University Medical Center, Rotterdam, The Netherlands. [3]HRH Prince Alwaleed Bin Talal Bin Abdulaziz Alsaud Institute for Computational Biomedicine, Weill Cornell Medical College, New York, USA. [4]Dept of Biostatistics & Epidemiology, Weill Cornell Medical College, New York, USA. [5]Biological Statistics and Computational Biology, Cornell University, Ithaca, New York, USA. [6]Department of Physiology and Biophysics, Weill Cornell Medical College, New York, USA. [7]Department of Trials and Statistics, Erasmus University Medical Center, Rotterdam, The Netherlands. [8]Department of Genetics, Albert Einstein College of Medicine, New York, USA.

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ABSTRACT

We hypothesized that DNA methylation distributes into specific patterns in cancer cells, which reflect critical biological differences. We therefore examined the methylation profiles of 344 patients with acute myeloid leukemia (AML). Clustering of these patients by methylation data segregated patients into 16 groups. Five of these groups defined new AML subtypes that shared no other known feature. In addition, DNA methylation profiles segregated patients with *CEBPA* aberrations from other subtypes of leukemia, defined four epigenetically distinct forms of AML with *NPM1* mutations, and showed that established *AML1-ETO*, *CBFB-MYH11* and *PML-RARA* leukemia entities are associated with specific methylation profiles. We report a 15-gene methylation classifier predictive of overall survival in an independent patient cohort (P<0.001, adjusted for known covariates).

INTRODUCTION

Acute myeloid leukemia (AML) is a highly heterogeneous disease from the biological and clinical standpoint. This remains a significant barrier towards the development of accurate clinical classification, risk stratification and targeted therapy of this disease. Epigenetic control of gene expression has been suggested to play a pivotal role in determining the biological behavior of cells. One such epigenetic mechanism is DNA cytosine methylation, which can alter gene expression by creating new binding sites for methylation dependent repressor proteins(1, 2), or by disrupting the ability of transcription factors to bind to their target sequences(3, 4). In normal development the proper distribution of DNA methylation plays a critical role in tissue differentiation and homeostasis(5, 6). Disruption of normal DNA methylation distribution is a hallmark of cancer and can play critical roles in initiation, progression and maintenance of the malignant phenotype. For example, aberrant hypermethylation and silencing of certain tumor suppressor genes such as $p15^{CDKN2B}$ has been widely reported in leukemias and other myeloid neoplasms(7-10). We recently showed that hypermethylation and silencing of the master regulatory transcription factor *CEBPA* was associated with a leukemia entity with T-cell/myeloid features, hypermethylation of a number of additional transcriptional regulators, and distinctive biological features(11, 12).
Based on these data we hypothesized that DNA methylation distributes into specific patterns in cancer, and that these methylation profiles impose and reflect critical biological differences with practical clinical and therapeutic implications. In order to test this hypothesis we performed a comprehensive exploration of DNA patterning in human a disease, focusing on a well-characterized cohort of 344 patients with AML.

PATIENTS AND METHODS

Patient samples

We made use of 344 AML cases collected at Erasmus University Medical Center (Rotterdam) between 1990-2008 for which sufficient patient material was available(13, 14). Patients had been treated on study protocols of the Dutch-Belgian Hemato-Oncology Cooperative Group (HOVON) (available at http://www.hovon.nl). Patients in the HO04/A, HO29 and HO42 trials received standard backbone AML treatment and no significant survival difference has been found between these slightly different treatments. The HO43 therapy protocol included patients over 60 years of age and showed a more adverse outcome with increasing age as a prognostic confounder and for this reason we have included age as a covariable in our statistical analyses. Samples were processed as previously described(13, 14). 165 of the patients in this study were included in the 285-patient cohort studied by gene expression by Valk et al(13), and the methylation status of 16 patients was previously reported in a publication by

our group(12). Median follow-up time based on survivors was 71 months (range: 7 months, 215 months). Table 1 summarizes patients' characteristics, Table S1 shows detailed information for each patient, and Table S2 summarizes treatment information for each cluster. Eight normal bone marrow CD34+ cell specimens were obtained from the Translational Trials Development and Support Laboratory, Cincinnati Children's Hospital (Cincinnati, OH) and Allcells (Emeryville, CA). This research was approved by the institutional review boards at Weill Cornell Medical College and Erasmus University Medical Center, and written donor informed consent was obtained in accordance with the Declaration of Helsinki.

DNA methylation microarrays

High-molecular-weight DNA was isolated from mononuclear cell fractions consisting of >90% blasts using a standard high salt procedure. The HELP (HpaII tiny fragment enrichment by ligation-mediated PCR) assay was carried out as previously described(15),(16) and samples were hybridized onto a custom human promoter array covering 25,626 HpaII amplifiable fragments (>50,000 CpGs), annotated to ~14,000 genes (Roche NimbleGen, Design name: 2006-10-26_HG17_HELP_Promoter, Design ID: 4802). HpaII amplifiable fragments (HAF) are defined as genomic regions contained between two flanking HpaII restriction sites that are found between 200 and 2000 bp apart. HAF were first re-aligned to the HG18 build of the human genome and then annotated to the nearest transcription start site (TSS), allowing for a maximum distance of 5 kb from the TSS. Hybridization and normalization steps are described as Supplementary methods. All microarray data are available from the GEO repository(17) (accession number GSE18700).

Gene expression microarrays

Gene expression data for these patients had been previously published by Verhaak et al.(14) (GEO accession number: GSE6891). Briefly, gene expression data were obtained using Affymetrix Human Genome 133 Plus2.0 GeneChips. mRNA isolation, labeling, hybridization and quality control were carried out as described previously(13). Raw data were processed using the GC-RMA package (version 2.16.0) from BioConductor(18).

Microarray data analysis

Statistical analysis was performed using R 2.8.1(19) and BioConductor(20). Unsupervised hierarchical clustering of HELP data was performed using the subset of probe sets (n=3745) with standard deviation > 1 across all cases. We used 1- Pearson correlation distance, followed by a Lingoes transformation of the distance matrix to a Euclidean one(21) and subsequent clustering using Ward's method. Clusters were considered to be representative of a given molecular or cytogenetic finding when > 50% of cases were positive and a two-sided Fisher's test was significant at $p < 0.05$ after adjusting for multiple testing using the Bonferroni method. Identification of the aberrant DNA methylation signature for each cluster

was performed using an ANOVA test, with correction for multiple testing according to the Benjamini-Hochberg method, followed by Dunnett's post hoc test using the normal CD34+ samples as the reference group(22). Only genes with adjusted P values < 0.05 and an absolute difference in $\log_2$(HpaII/MspI) ratios > 2 (which corresponds to at least 35% difference in DNA methylation) were selected for each cluster.

Quantitative DNA methylation sequencing by MassARRAY EpiTYPER

Validation of HELP data was performed by MALDI-TOF mass spectrometry using EpiTYPER by MassARRAY (Sequenom, San Diego, CA) on bisulfite-converted DNA as previously described(23). MassARRAY primers were designed as previously described(12) (See Supplementary methods).

Pathway analysis

Ingenuity Pathway Analysis software (Redwood City, CA) was used to perform pathway analysis of relevant gene signatures. The top scoring networks were identified for the gene expression signatures of the epigenetically defined clusters. A comparative analysis of the canonical pathways deregulated in each of the clusters, as captured by the integration of the DNA methylation and gene expression signatures was also performed. Enrichment for specific pathways was determined relative to the ingenuity knowledge database using a Benjamini-Hochberg adjusted Fisher's test, at a significance level of adjusted P value <0.05.

Survival analysis

Kaplan-Meier survival analysis for overall survival was performed to compare survival differences between different groups of clusters. A multivariate Cox proportional hazards regression model was constructed for the including age, cytogenetic risk, *NPM1* mutation status, *FLT3*-ITD mutation status and cluster membership as the variables to be tested. Detailed description of the model is found as Supplementary methods. All survival analyses were performed in SAS Version 9.2 (SAS Institute, Inc., Cary, NC, and Stata Version 10.0 (StataCorp, College Station, TX).

Development of an epigenetic predictive model for overall survival in AML

In order to develop a prognostic biomarker model predictive of AML overall survival we used the supervised principal components (SuperPC) algorithm developed by Bair and Tibshirani(24). The data set was randomly divided into three groups: a training set (n=200), a test set (n=95) and the remaining 49-patient cohort to be used as the independent validation set. Table S6 summarizes the patient characteristics for each of the three groups. Table S8 shows the clinical outcome for each patient in the cohort. A detailed description of the model training, testing and independent validation procedures, as well as the R script used can be found as Supplementary methods.

RESULTS

AML is composed of epigenetically distinct diseases

Since the molecular heterogeneity of AML remains only partially resolved, the first goal of our study was to determine whether DNA methylation profiling could identify new clinically and biologically relevant disease subtypes. For that purpose, blast cells of 344 newly diagnosed AML patients were subjected to DNA methylation profiling of over 50,000 CpG dinucleotides contained within ~14,000 unique gene loci using the HELP (HpaII tiny fragment enrichment by ligation-mediated PCR) method(15, 16). Table 1 summarizes patients' characteristics. DNA methylation measured by HELP was highly concordant with a quantitative single locus DNA methylation validation assay (correlation coefficient r= -0.88) in these AML patients (Figure S1A). An unsupervised analysis using hierarchical clustering (1 - Pearson correlation distance and Ward's clustering method. showed that leukemias could be distinctly grouped according to their methylation profiles. A cut-off of 16 clusters was selected for further analysis since this segregation most accurately overlapped with the currently known molecular subtypes of AML while at the same time revealing the existence of additional epigenetic differences among the remaining patients. The stability of these clusters was verified by performing comparison of multiple cluster analyses using a decreasing number of probe sets (based on alternative cutoffs of across-patient standard deviation, Figures S1B-S1E). Table 2 shows the clinical, cytogenetic and molecular features of each of the 16 clusters. Three of these patient clusters correspond to AML subtypes defined by the WHO classification(25)(Figure 1), another eight clusters were enriched for cases harboring specific genetic or epigenetic lesions, and the remaining five clusters could not be explained by any known morphologic, cytogenetic or molecular feature.

Each of these DNA methylation-defined AML subtypes displayed a unique epigenetic signature when compared to normal bone marrow CD34+ cells (Figure 2 and Tables S3A-3P). Taken together these data indicate that DNA methylation is not randomly distributed in AML blasts but rather is organized into highly coordinated and well-defined patterns. In most cases the AML subgroups showed a very strong hypermethylation signature as compared to normal marrow CD34+ cells. In contrast, a few of the clusters were hypomethylated in comparison to normal controls. This distinctive patterning is highly suggestive of a biologically significant role for altered DNA methylation in these different AML subtypes. The data also suggest that the most prevalent tumor-associated abnormality in gene promoter DNA methylation abundance is not always hypermethylation but can also be hypomethylation.

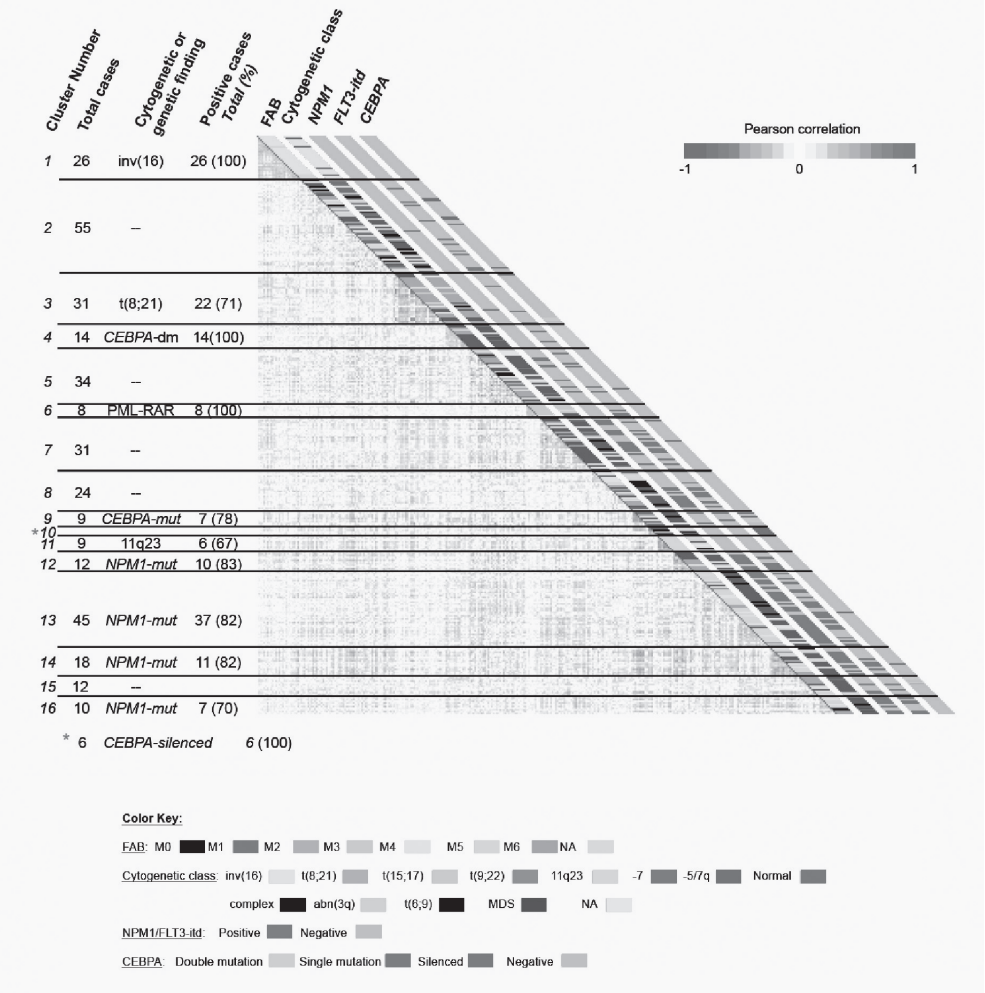


Figure 1. DNA methylation segregates AML patients into 16 groups.
Heatmap representation of a correlation matrix in which each patient's DNA methylation profile is correlated with that of the other patients in the dataset. Patients are ordered according to the unsupervised analysis (hierarchical clustering) results, so that highly correlated patients are located next to each other. Parallel bars on the right of the heatmap have been used to indicate the principal cytogenetic and molecular findings for each patient. Cluster membership and cluster feature summaries are described on the left of the heatmap.

Table 1: Patient characteristics.

Characteristic	Group	Total (%)
Gender	Male	188 (54)
	Female	156 (46)
Age	< 60 years	294 (85%)
	> 60 years	50 (15%)
	Median years	48 (15-77)
FAB	M0	12 (3.5%)
	M1	75 (21.8%)
	M2	82 (23.8%)
	M3	9 (2.6%)
	M4	65 (18.9%)
	M5	70 (20.3%)
	M6	3 (0.87%)
	NA*	28 (8.1%)
Cytogenetics	inv(16)/t(16;16)	30 (9%)
	t(8;21)	24 (7%)
	t(15;17)	10 (3%)
	t(9;22)	2 (0.6%)
	t(6;9)	3 (0.9%)
	t(*v*;11q23)	13 (3.8%)
	3q abnormalities	2 (0.6%)
	del5(q)/del7(q)	19 (5.5%)
	Trisomy 8	14 (4%)
	del9q	8 (2.3%)
	Complex	8 (2.3%)
	Normal	152 (44%)
	Other	43 (12.5%)
	NA*/Failure	13 (3.8%)
Cytogenetic risk	Favorable	53 (15%)
	Intermediate	231 (67%)
	Unfavorable	47 (14%)
	NA*	14 (4%)
CEBPA	Double mutation	24 (7%)
	Single mutation	11 (3.1%)
	Silenced	8 (2.4%)
NPM1		105 (30.5%)
***FLT3*-ITD**		96 (28%)
EVI1		27 (8%)

*NA = not available

Cytogenetically defined AML subtypes have unique epigenetic signatures

The WHO classification of AML defines cases with t(8;21), inv(16) and t(15;17) translocations or the presence of the relevant fusion genes as separate entities indicative of a favorable clinical prognosis(26-28). All three of these AML subtypes presented with a unique methylation profile. Methylation cluster 1 (n=26) consisted entirely of cases carrying either inv(16) or t(16;16) (22/26 cases), or the *CBFB-MYH11* fusion gene (4/26). Methylation cluster 3 was significantly enriched for cases positive for t(8;21) (22/31 cases, Fisher's exact test P value <1.85e-25), and all cases in methylation cluster 6 carried the t(15;17) or the *PML-RARA* fusion gene (8/8 cases). Patients in the two core binding factor clusters did not further segregate according to c-*KIT* mutation status, indicating that the presence of this mutation does not result in a specific DNA methylation pattern. Supervised analysis comparing each of these clusters to a cohort of normal CD34+ cells from healthy donors revealed that they all exhibited a unique signature, with a strong shift towards genes being methylated in the AML subtypes compared to CD34+ normal marrow blasts. (Figure 2 and Table S3). The data are consistent with a scenario whereby each of these fusion oncoproteins can drive epigenetic patterning in hematopoietic cells, and/or cooperate to drive leukemogenesis when specific sets of complementary genes are deregulated through aberrant DNA methylation.

Cluster 3 included nine cases that did not present with the t(8;21) or *AML1-ETO* fusion gene, yet the survival curves of these patients were indistinguishable from the 22 t(8;21) positive patients in cluster 3 (log-rank test, P value =0.83). This finding reflects the ability of DNA methylation profiles to identify a subset of patients with comparable risk and epigenetic patterning to that of t(8;21) patients despite their lack of the aberrant *AML1-ETO* fusion gene. Even though the number of patients is small, the robustness of this common epigenetic profile is reflected in the fact that these patients all continue to cluster together even when different numbers of probe sets are used in the analysis (Figure S1B-1E). Furthermore, unsupervised analysis of these patients using gene expression data failed to segregate them according to the presence or absence of the t(8;21). (Figure S1F)

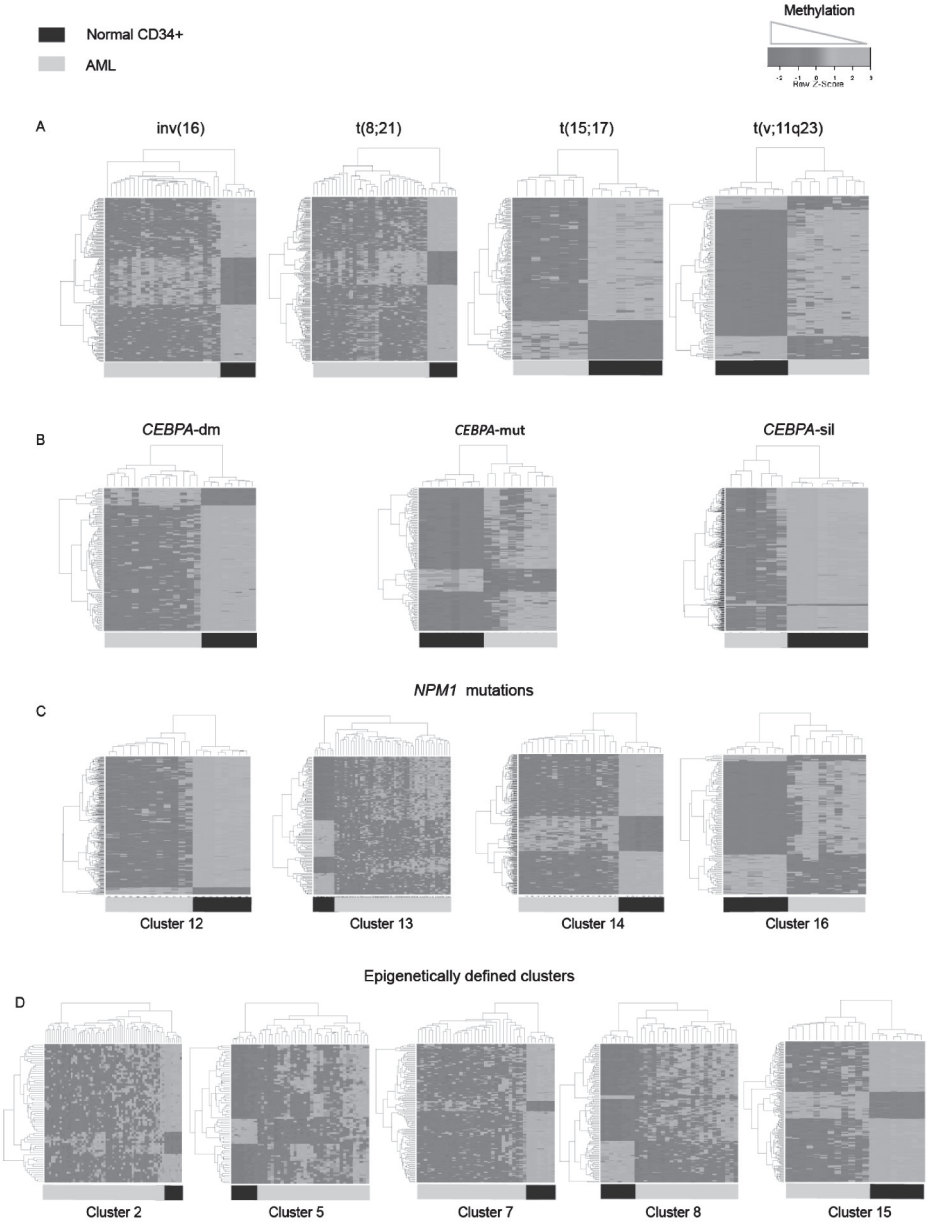


Figure 2. Distinct DNA methylation signatures define each of the 16 clusters.
Heatmap representation of the aberrant DNA methylation signatures of specific clusters compared to a cohort of normal CD34+ hematopoietic cells obtained from healthy donors. Each row of the heatmap represents one probe set of the HELP array, and each column represents an AML patient (denoted by light brown bars) or a healthy donor (denoted by dark brown bars). (A) DNA methylation signatures for clusters with recurrent translocations, (B) DNA methylation signatures associated with abnormalities of *CEBPA*, (C) DNA methylation signatures for clusters presenting *NPM1* mutations, (D) DNA methylation signatures for the 5 epigenetically defined clusters.

Table 2. Summary of clinical, cytogenetic and molecular features of the 16 DNA methylation clusters.
For complete cytogenetic and molecular information for each patient see Tables S1 and S2. Also, see Figure S2 for CEBPA methylation status of case 5630, which is in cluster 10.

CLUSTER #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Total
N	26	55	31	14	34	8	31	24	9	6	9	12	45	18	12	10	344
Clinical markers																	
Gender																	
Male	12	36	22	8	19	4	17	12	5	4	5	6	25	6	4	3	188
Female	14	19	9	6	15	4	14	12	4	2	4	6	20	12	8	7	156
Age																	
<60 yr	24	37	29	13	27	8	25	20	8	6	9	11	43	16	10	8	294
>60 yr	2	18	2	1	7	0	6	4	1	0	0	1	2	2	2	2	50
FAB																	
0	0	6	0	0	3	0	0	0	0	2	0	0	0	0	0	1	12
1	0	9	6	10	6	0	21	4	2	3	0	5	2	4	2	1	75
2	0	13	20	3	11	0	5	6	2	0	0	6	6	3	5	2	82
3	0	0	1	0	1	7	0	0	0	0	0	0	0	0	0	0	9
4	23	8	3	1	2	1	1	7	1	0	0	0	8	4	3	3	65
5	3	6	1	0	7	0	4	3	0	0	9	1	26	6	2	2	70
6	0	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0	3
NA	0	12	0	0	4	0	0	4	4	1	0	0	2	0	0	1	28
Cytogenetic markers																	
Cytogenetic class																	
inv(16)/t(16;16)	26*	2	1	0	1	0	0	0	0	0	0	0	0	0	0	0	30
t(8;21)	0	0	22	0	0	0	1	0	0	0	0	0	0	1	0	0	24
t(15;17)	0	0	1	0	1	8#	0	0	0	0	0	0	0	0	0	0	10
t(9;22)	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	2
t(6;9)	0	0	0	0	0	0	0	2	0	0	0	0	1	0	0	0	3
t(*v*;11q23)	0	0	0	0	0	0	0	5	0	0	6	1	0	1	0	0	13
3q	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	2
del5(q)/del7(q)	0	12	1	0	1	0	1	1	0	1	0	0	0	0	1	1	19
tri8	0	1	0	0	0	0	2	2	1	0	1	0	5	1	0	1	14
del9q	0	1	0	3	2	0	1	0	1	0	0	0	0	0	0	0	8
Complex	0	3	1	0	1	0	0	1	0	2	0	0	0	0	0	0	8
Normal	0	17	4	10	19	0	17	10	6	1	1	7	33	11	8	8	155
Other	0	13	0	1	5	0	7	3	0	2	0	3	2	4	3	0	43
NA/Failure	0	0	1	0	3	0	1	1	1	0	1	1	4	0	0	0	13

CLUSTER #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Total
Cytogenetic risk																	
Favorable	20	1	24	0	2	5	1	0	0	0	0	0	0	0	0	0	53
Intermediate	6	32	4	14	25	3	27	15	8	3	6	10	41	17	11	9	231
Unfavorable	0	22	2	0	3	0	2	8	0	3	2	1	1	1	1	1	47
Molecular Markers																	
CEBPA																	
Double mutant	0	0	2	14	0	0	0	0	5	0	0	0	0	0	3	0	24
Single mutant	0	3	1	0	1	0	2	0	2	0	0	0	1	1	0	0	11
Silenced	0	3	0	0	0	0	0	0	0	6	0	0	0	0	0	0	9
Wild-type	26	49	28	0	33	8	29	24	2	0	9	12	44	17	9	10	300
NPM1																	
Wild-type	26	54	30	14	26	8	17	13	7	6	9	2	8	7	9	3	239
Mutated	0	1	1	0	8	0	14	11	2	0	0	10	37	11	3	7	105
***FLT3*-ITD**																	
Negative	26	45	28	12	28	4	18	15	7	6	9	4	21	11	9	5	248
Positive	0	10	3	2	6	4	13	9	2	0	0	8	24	7	3	5	96
EVI1																	
Negative	26	42	31	14	31	8	30	17	9	6	8	12	45	16	12	10	317
Positive	0	13	0	0	3	0	1	7	0	0	1	0	0	2	0	0	27

Cytogenetic class is defined in order of importance according to the 1999 WHO classification(34): t(8;21), inv(16), t(15;17), Complex (>3 clonal abnormalities), del5(q)/del7(q), 3q= any abnormality involving 3q, t(6;9), t(9;22), 11q23= any abnormality involving 11q23, tri8=trisomy 8, del9q, NN= no abnormalities in karyotype, Other= does not classify in any other group. NA= not available.

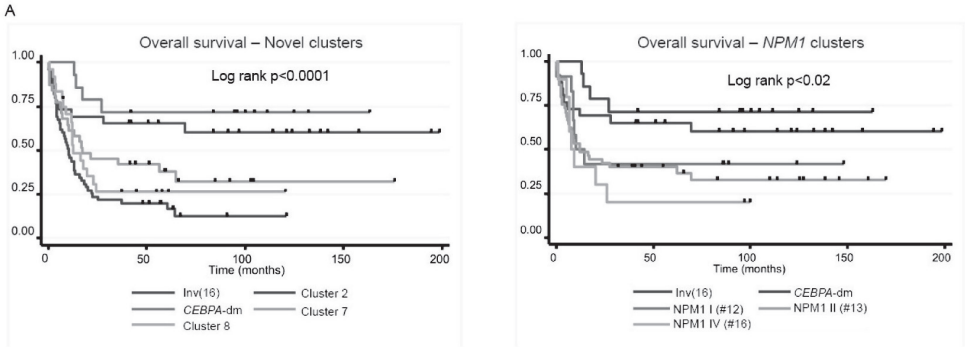
* including four inv(16) cases detected by *CBFB-MYH11* fusion gene PCR,

including three t(15;17) cases detected by *PML-RARA* fusion gene PCR.

Epigenetic differences define NPM1-mutated, CEBPA-mutant and CEBPA-silenced AMLs

Methylation profiling defined 13 additional AML subtypes. Four of those methylation clusters (clusters #12, 13, 14 and 16) were all significantly enriched for cases carrying *NPM1* mutations (Bonferroni adjusted Fisher's exact test P values: <0.0008, <9.4e-14, <0.02 and <0.048, respectively). Mutations in exon 12 of the *NPM1* gene which result in aberrant cytoplasmic localization of the protein constitute an independent favorable prognostic marker in AML(29). However, when this mutation occurs in the context of an associated *FLT3*-ITD, then this favorable prognostic impact is lost(30). The *NPM1*-mutant clusters 12 and 13 were enriched for characteristic morphological subtypes, i.e., FAB M1/M2 (11/12) and M4/M5 (34/45), respectively. The variety of *NPM1* methylation clusters could not be explained solely by the presence or absence of concurrent *FLT3*-ITD (Table 2). While the four clusters were all enriched for *NPM1* mutations, they still presented enough unique characteristics to separate into 4 methylation clusters, each of them with a specific aberrant DNA methylation signature (Figure 2 and Table S3). Differential methylation in cluster 12 consisted almost entirely of

hypermethylated genes, while DNA methylation in the remaining *NPM1* clusters was more evenly distributed when compared to normal controls. These data support the notion that *NPM1* mutations play a dominant role in defining AML biology, but can be modified to a significant extent by additional alterations in epigenetic or unidentified genetic factors. A significant difference in overall survival was observed for the *NPM1* clusters 12, 13, 14, and 16 (log-rank test, P value =0.02), when compared to clusters 1, 3 and 4, which contained patients with inv(16), t(8;21) and *CEBPA* double mutations (*CEBPA*-dm) respectively (Figure 3A). These differences in survival remained significant after adjustment for age, cytogenetic risk, *NPM1* mutation and *FLT3*-ITD mutation status following multivariate analysis (Figure 3B).



Variables in Model	P-Value	Hazard Ratio (HR)	95.0% CI for HR	
			Lower	Upper
cluster #2	0.013	3.178	1.271	7.947
cluster #3 (t(8;21))	0.821	1.100	0.482	2.512
cluster #4 (CEBPA-dm)	0.626	0.720	0.192	2.700
cluster #5	0.155	2.035	0.765	5.414
cluster #7	0.050	2.640	1.001	6.961
cluster #8	0.026	3.156	1.146	8.691
cluster #12 (NPM1 I)	0.081	2.998	0.872	10.305
cluster #13 (NPM1 II)	0.002	4.665	1.721	12.642
cluster #14 (NPM1 III)	0.006	4.349	1.527	12.387
cluster #15	0.020	3.598	1.226	10.560
cluster #16 (NPM1 IV)	0.004	5.442	1.743	16.993
Age > 60 years	0.024	1.543	1.059	2.248
Intermediate cytogenetic risk	0.424	0.738	0.351	1.552
High cytogenetic risk	0.915	1.044	0.475	2.294
NPM1 mutation	<0.0001	0.426	0.277	0.655
FLT3-ITD mutation	0.001	1.778	1.255	2.520

Figure 3. DNA methylation captures clinically significant differences among AML patients.
(A) Left: Kaplan-Meier curves for overall survival for the favorable risk clusters 1 (inv(16)) and 4 (*CEBPA*-dm), and the novel epigenetically defined clusters. For plotting simplicity curves for clusters 3 (t(8;21)), cluster 5 and cluster 15 were not included in the plot. Figure S3 shows a Kaplan-Meier plot including all the clusters in the overall survival analysis. Right: Kaplan-Meier curves for overall survival for the favorable risk clusters 1 (inv(16)) and 4 (*CEBPA*-dm), and the *NPM1* clusters. For plotting simplicity curves for clusters 3 (t(8;21)), and *NPM1* cluster 14 were not included in the plot. Figure S3 shows a Kaplan-Meier plot including all the clusters in the overall survival analysis. (B) Kaplan-Meier curves for overall survival (left) for the five novel clusters. On the right: Table summarizing the multivariate Cox proportional hazards regression model, using cluster 1 (inv(16)) as the referent cluster. Additional Kaplan Meier plots are shown in Figure S3.

The CEBPA transcription factor is a critical mediator of hematopoietic cell differentiation(31), and *CEBPA*-dm AMLs are associated with a favorable clinical prognosis(32). These cases split

into two distinct subtypes with different methylation signatures. Methylation cluster 4 displayed a markedly hypermethylated profile and consisted entirely of *CEBPA*-dm cases (n=14; Fisher's exact test P value <6.88 e-19). The clinical outcome of cluster 4 patients was even better than the known favorable risk core-binding factor leukemias, i.e. t(18;21) and inv(16) (2-year overall survival ± standard error [SE]; 78.6%±11.0%) (Figure 3A). DNA methylation cluster 9 was also significantly enriched for *CEBPA* mutant cases (n=7/9, Fisher's exact test P value <0.000009), most of which (5/7) harbored *CEBPA* double mutations. However, the cluster 9 signature was predominantly hypomethylated vs. controls, suggesting that these *CEBPA*-related leukemias are biologically distinct from the *CEBPA*-dm cluster 4. Cluster 9 contained insufficient numbers to allow for a comparative survival estimate.
Five out of the six patients in cluster 10 had previously been shown to display a phenotype featuring *CEBPA* hypermethylation and silencing (*CEBPA^{sil}*), a hypermethylated gene profile, but with hypomethylation of certain T-cell genes, T-cell lineage infidelity, and poor clinical outcome(11, 12). The remaining patient in this cluster 10 had not previously been recognized as a *CEBPA^{sil}* leukemia (case 5360), but was demonstrated upon further investigation in this study to indeed display all the characteristic features of *CEBPA^{sil}* leukemias (Figure S2 and data not shown). Mutations or silencing of *CEBPA* thus appear to result in or to be associated with three epigenetically distinct forms of leukemia.

Unique epigenetic differences independent of (cyto)genetically defined AML subtypes
Methylation clusters 2, 5, 7, 8, and 15 were defined solely by their DNA methylation profile and could not be explained by the enrichment of any currently known recurrent cytogenetic, molecular or clinical feature (Table 2). Each of these AML subtypes displays a unique and significant epigenetic signature vs. normal CD34+ controls (Table S3). Normal cytogenetics AML cases were distributed among all 5 clusters, and although 5/24 cases in cluster 8 harbored 11q23 abnormalities this was not a defining feature since it represented only 20.8% of the cases. Gene expression profiles of each of these epigenetically defined clusters were obtained in a supervised analysis comparing them to a set of normal CD34+ controls. Each of the 5 clusters presented with a distinct gene expression profile. Figure 4A shows the top scoring networks associated with each of these expression signatures.

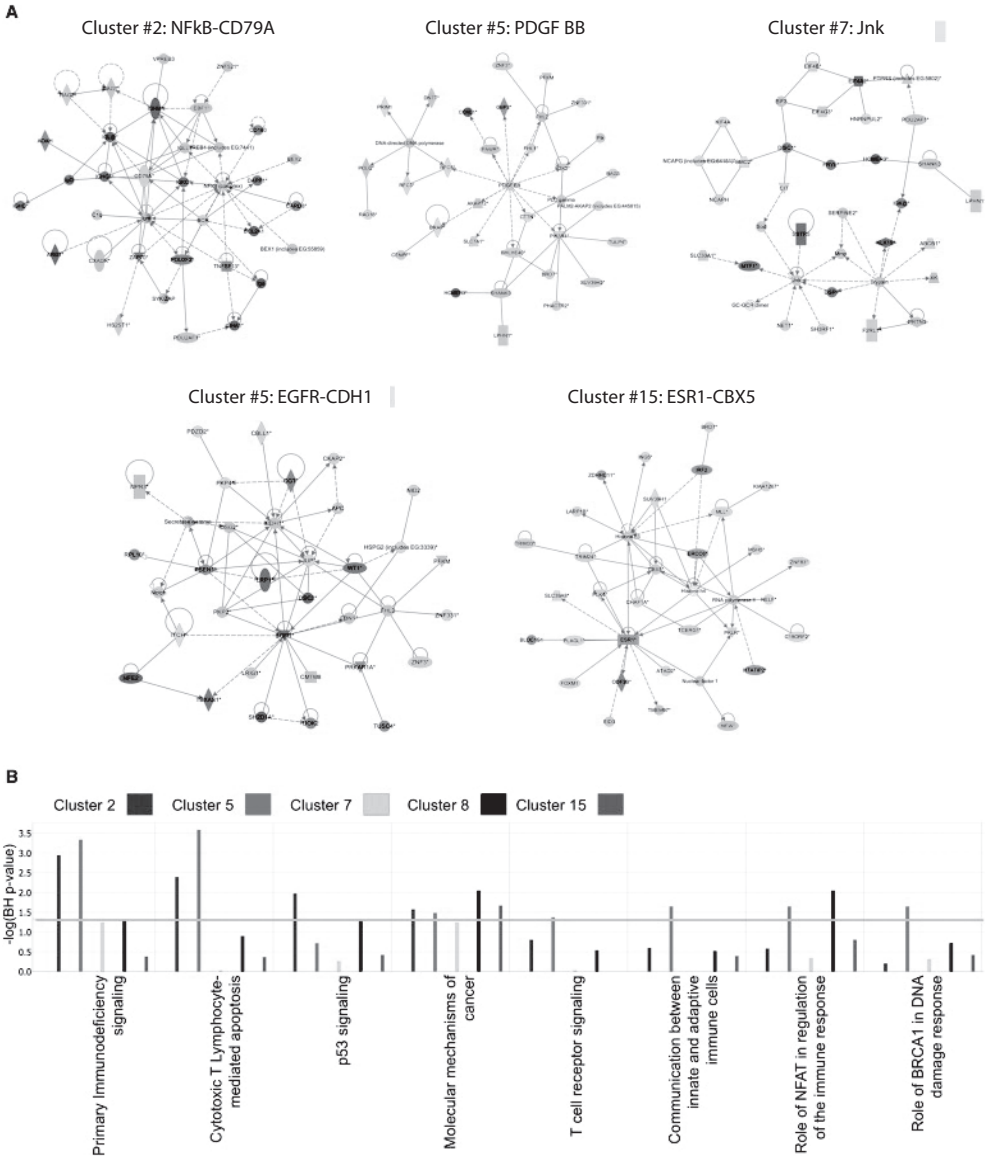


Figure 4. Pathway analysis for the epigenetically defined clusters.
(A) Top scoring aberrantly expressed gene networks for each of the 5 epigenetically defined clusters. Genes over-expressed compared to normal CD34+ cells are colored in red, while downregulated genes appear in green. (B) Comparative analysis of the most significantly deregulated canonical pathways of the 5 epigenetically defined clusters as captured by an integration of the aberrant epigenetic and gene expression signatures.

Aberrantly expressed genes far exceeded and only partially overlapped with the aberrantly methylated genes in each cluster, which suggests that even relatively small changes in epigenetic patterns can have a significant biological impact in the cell. In order to determine the biological impact of this epigenetic deregulation, we performed an integrative pathway

analysis of the combined aberrantly methylated and aberrantly expressed genes. This analysis revealed that each of these clusters resulted in deregulation of different canonical pathways. Cluster 5 showed deregulation of immunity related pathways, involving immunodeficiency signaling, cytotoxic T-cell mediated apoptosis and T cell receptor signaling. Cluster 2, on the other hand, was the only one that significantly deregulated p53-signaling. Clusters 8 and 15 showed predominant deregulation of pathways involved in molecular mechanisms of cancer, deregulating genes in the DNA damage repair mechanism such as *ATM, CHK1, MDM2* and *FANCD2,* genes involved in cell cycle regulation such as *CDK4,* and *CYCLIN D,* as well as genes from the AKT signaling pathway (Figure 4B). Most notably, a significant difference in survival was observed between these novel AML subtypes. For instance, clusters 5 and 7 correlated with an evidently better outcome (2-year overall survival ± SE; 58.8%±8.4% and 45.2%±8.9% for clusters 5 and 7, respectively, vs. 23.6%±5.7%, 26.4%±9.2% and 33.3%±13.6%, for clusters 2, 8 and 15, respectively) (log-rank test, P value=0.04). After adjustment for age, cytogenetic risk, *NPM1* mutation, and *FLT3*-ITD mutation status in a multivariate Cox proportional hazards regression model including all the clusters with at least 10 patients, 4 of the 5 novel clusters presented a statistically significant increased hazard ratio with respect to the favorable risk inv(16) cluster, while cluster 5 did not reach statistical significance (Figure 3B). Epigenetic profiling thus identified a clinically relevant and significant difference among AML subtypes not captured by other methodologies.

AMLs present a common epigenetic signature of consistently aberrantly methylated genes

While the above studies were geared towards finding the unique signatures of epigenetically defined AML subtypes, we also wondered whether a set of genes could be defined whose DNA methylation was consistently deregulated across all the AML subtypes. We indeed identified a common aberrant DNA methylation signature consisting of 45 genes, most of them hypermethylated, that was consistently detected in at least 10 of the 16 clusters' methylation signatures and affecting at least 70% of the cases studied (Figure 5A). Genes in this signature are likely to be part of a common epigenetic pathway involved in leukemic transformation of hematopoietic cells. Among these genes we found the tumor suppressor *PDZD2*, transcriptional regulators (*ZNF667, ZNF582, PIAS2, CDK8*), nuclear import receptors (*TNPO3, IPO8*), and *CSDA*, a repressor of *GM-CSF*. A complete list of the genes in this common signature is found in Table S4.

We next looked at the gene expression levels of these genes on Affymetrix HGU133 Plus 2.0 microarrays performed on the same patients(14), and compared them to those of a cohort of normal CD34+ bone marrow cells. 8/45 genes had to be excluded from the analysis due to failure of the gene expression probe sets (n=6) or because of lack of representation of the transcript of interest on the expression arrays (n=2). For the remaining 37 genes, in all but 5 we found either complete silencing or downregulation of the corresponding transcript. Eighteen of these showed the expected differential gene expression when compared to normal CD34+

cells (ANOVA followed by Dunnett's test P value < 0.05) (Figure 5B). Four bidirectional promoters showed silencing of one transcript with high expression of the transcript from the opposite strand. The remaining genes were silenced in both the AMLs and the normal CD34+ cells. The latter might be explained, as we have previously shown, by the relative insensitivity of gene expression microarrays to detect differential gene expression of low abundance transcripts, which can be overcome by looking at the more sensitive epigenetic marks(33). Alternatively, this finding could reflect the establishment of a more irreversible state of silencing of these genes in the AML blasts compared to the normal CD34+ cells.

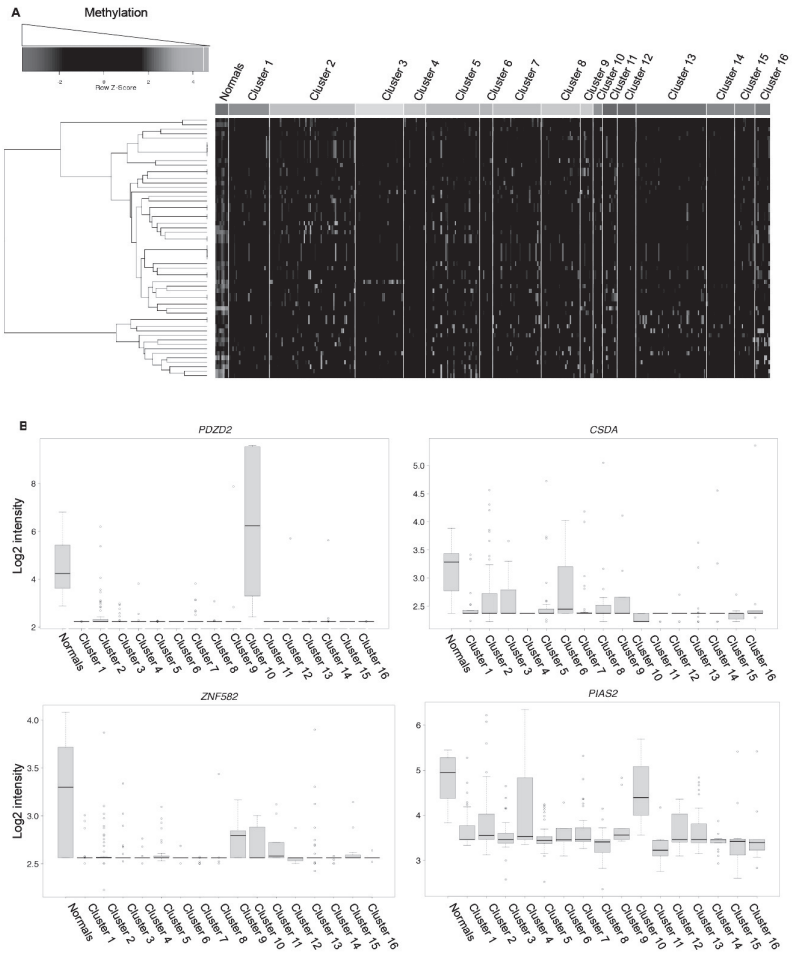
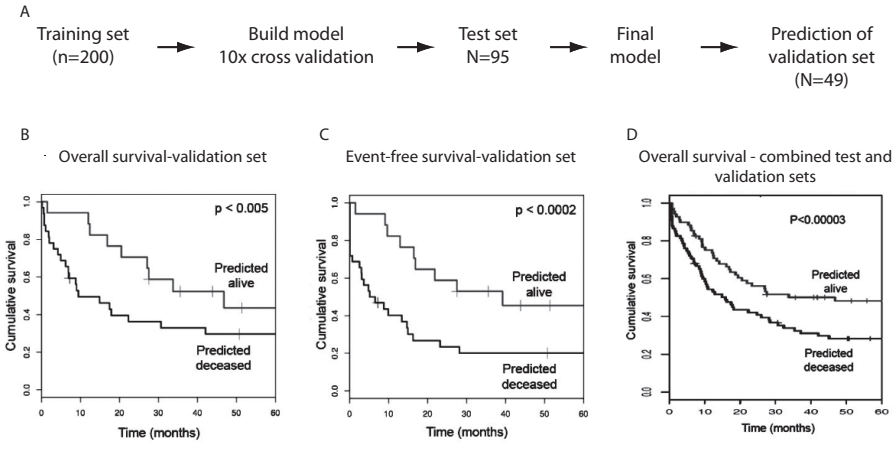


Figure 5. 45 genes are consistently aberrantly methylated in AML.
(A) Heatmap representation of the common 45-gene signature consistently aberrantly methylated in AML. Each row represents a probe set from the HELP microarray and each column represents a sample. (B) Boxplots of gene expression levels in 4 representative genes from the 45-gene common epigenetic signature demonstrating downregulation of expression in the AML samples compared to normal CD34+ cells The list of genes is shown in Table S4.

A DNA methylation classifier predicts clinical outcome in AML

The fact that aberrant DNA methylation of gene promoters represents an epigenetic modification that is stably transmitted among leukemic blasts and that this is done in an organized pattern that correlates with disease subtypes led us to explore its potential as predictor of important clinical features. Moreover, since DNA is relatively stable in clinical samples and DNA methylation is easy to measure, it is very likely that small sets of methylated genes could readily be harnessed as clinically useful biomarkers. Therefore, in order to determine whether we could identify and validate methylation biomarkers of independent prognostic value in AML we applied a three-step approach of model development and validation. The complete patient cohort was randomly divided into a training set (n=200), a test set (n=95) and an independent validation set (n=49). Cluster membership was not taken into consideration for this part of the analysis. Using the supervised principal components (SuperPC) method of Bair and Tibshirani(24), a Cox proportional hazards regression model for overall survival was trained with data in the training set (see Supplementary methods). Parameters of the model were chosen so that they maximized performance, as estimated by 10 fold cross-validation on the training set. The model resulting from the maximum cross-validation performance estimate was tested on the test set, found predictive, and used to predict survival status on the independent validation set (Figure 6A). This model included 18 probe sets, corresponding to 15 genes. The predictor model included transcription factors (*E2F1, ZFP161, BTBD3*), genes related to protein metabolism (*USP50, SRR, PRMT7, GALNT5*), regulation of telomeres (*SMG6*) and signaling (*CXCR5, LCK*) (see Table S5 for the complete list of features used in this model). The predictive performance of this model was validated on the 49-patient independent validation set both for overall survival (Hazard ratio: 1.39, 95% CI = 1.10-1.75; P value <0.005; SuperPC score range= -5 to 5) (Figure 6B) and event-free survival (Hazard ratio: 1.53, 95% CI = 1.21-1.93; P value <0.0002; SuperPC score range= -5 to 5) (Figure 6C). After controlling for clinical and other known predictors, i.e., age, cytogenetic risk, *CEBPA* status, *NPM1* mutations and *FLT3*-ITD; the model was still found informative (multivariate Cox proportional hazards model, Hazard ratio: 1.29, 95% CI: 1.11-1.49; P value < 0.001) (Figure 6D and 6E). In order to confirm the robustness of DNA methylation markers as predictors of clinical outcome, we performed 30 additional random splits of the data set into a training set of 200 patients and a test set of 144 and ran the SuperPC algorithm with a common set of parameters for all 30 runs. Under these stringent conditions in which the parameters were not individually selected for the optimal threshold in each run, 26 out of the 30 runs validated with a significant P value of <0.05 in a Cox proportional hazards regression model (Table S7). These results demonstrate that DNA methylation status of individual patients can help predict the future survival of the AML patient, and suggest that DNA methylation biomarkers should be evaluated alongside other predictors in future model development and evaluation studies.



E

Variables	P-Value	Adjusted Hazard Ratio	95.0% CI for HR	
			Lower	Upper
Methylation score	0.001	1.29	1.11	1.49
Age	0.002	1.02	1.01	1.04
NPM1	0.016	0.47	0.26	0.87
FLT3-ITD	0.020	1.84	1.10	3.08
CEBPA	0.758	0.89	0.42	1.87
Cytogenetic risk: Intermediate	0.735	0.89	0.46	1.74
Cytogenetic risk: High	0.755	1.14	0.51	2.54

Figure 6. A DNA methylation classifier predicts clinical outcome in AML patients.
(A) Outline describing the steps for building the DNA methylation classifier. In a first step, 200 randomly selected patients were used to identify HELP probe sets that best predicted survival. The model was then tested on a different cohort of 95 patients (test set). Once the final model was selected, its performance in predicting survival was tested in an independent validation set consisting of 49 randomly selected cases. (B) Kaplan-Meier curves for overall survival for the predicted groups in the independent validation set. Overall survival was compared between patients in an independent validation set that were predicted either alive or deceased by the DNA methylation classifier. (Cox Proportional hazards P value <0.005, hazard ratio= 1.39, 95% CI =1.10, 1.75) (C) Kaplan-Meier curves for even-free survival for the predicted groups in the independent validation set. Event-free survival was compared between patients in an independent validation set that were predicted either alive or deceased by the DNA methylation classifier (Cox Proportional hazards P value <0.0002, Hazard ratio: 1.53, 95% CI =1.21, 1.93) (D) Kaplan-Meier curves for overall survival for the predicted groups in the combined test and independent validation sets. Overall survival was compared between patients in the combined test and independent validation sets that were predicted either alive or deceased by the DNA methylation classifier. (Cox Proportional hazards P value <0.000003, Hazard ratio: 1.34, 95% CI =1.18, 1.51). (E) Multivariate Cox proportional hazards regression model for the DNA methylation predictor, age, cytogenetic risk, *NPM1* mutation, *FLT3*-ITD and *CEBPA* mutations. For additional information please see Tables S5, S6, S7 and S8, as well as Supplementary R scripts.

DISCUSSION

This comprehensive and large-scale study of DNA methylation profiles associated with ~14.000 genes in a human disease demonstrates that epigenetic patterning distributes into signatures of biological and clinical significance and that DNA methylation classifiers can be derived from population studies with clinical predictive power. From the biological standpoint these data offer an opportunity to better understand the mechanisms through which hematopoietic cells undergo leukemogenesis. Much effort has been invested in identifying genetic lesions that cooperate with known recurrent translocations such as t(8;21), t(15;17) and inv(16) or in patients with normal karyotype leukemia. While this effort has led to the identification of *bona fide* leukemogenic mutations such as those in *CEBPA, FLT3* and *NPM1*, it now appears that recurrent genetic lesions insufficiently explain the biological diversity of clinical AML. In contrast, our data show that epigenetic lesions are abundant and common, raising the possibility that a number of the oncogenic lesions in AML could be epigenetic in nature. Thus, further research exploring the contribution of genes affected by aberrant DNA methylation seems warranted.

The clinical significance of DNA methylation profiles is underlined by the fact that it contributes to identifying groups of patients that share a common clinical outcome, in some cases even beyond what their cytogenetic class is, such as the case of cluster 3 leukemias. This cluster, which was enriched for t(8;21) patients, included others without this cytogenetic marker, however, there was no difference in survival between the two subgroups. While some of the patients negative for the t(8;21) presented other cytogenetic and molecular indicators of favorable risk, this finding reflects the existence of a common DNA methylation profile for these patients. This epigenetic signature aggregated these leukemias together beyond the presence of other molecular and cytogenetic markers, and in addition identified additional cases that did not present with any favorable risk indicator. Similarly, a hypermethylated gene signature defines a subset of leukemias with *CEBPA* silencing due to hypermethylation, T-cell lineage infidelity, resistance to myeloid growth factors and a poor prognosis(12). These cases formed cluster 10 in this cohort. AMLs with mutations on both *CEBPA* alleles or with homozygous mutations were recently shown to have a highly favorable prognosis(32) and these cases also presented with a defining DNA methylation profile. Taken together, these data warrant considering both of these subtypes as distinct leukemia diseases that should be assigned to risk stratified therapy regimens and explored for the development of specific targeted therapy.

NPM1 mutations distributed to four related but slightly distinct signatures. These epigenetic variations cannot be explained by the presence or absence of a concurrent *FLT3*-ITD, suggesting that other as yet unrecognized mechanisms might be at play in determining these different epigenetic groups. We were unable to identify a DNA methylation signature associated

with *FLT3* lesions, indicating that mutations of this gene do not exert their effects in AML by imposing an aberrant epigenetic pattern.

One of the notable findings of this study was the identification of five methylation signatures with no other common morphologic or molecular features, but with distinct clinical outcomes, suggesting that these too are unique forms of AML with their own biological characteristics. It is particularly significant that these AML subtypes cannot be identified by any available diagnostic method, underlining that epigenetic signatures provide a critical layer of additional information. The fact that these cases included both normal karyotype leukemias as well as those with cytogenetic lesions and across multiple FAB subtypes supports a move away from definitions rooted in standard karyotyping, rather towards a more functional classification of AML. Future studies will be required to explore the biological basis of these epigenetically defined subtypes in the effort to develop risk-adapted and molecular targeted clinical trials that more accurately reflect inter-individual differences among leukemia patients. However, the presence of a strong hypermethylated signature in some of these clusters (clusters 2, 7 and 15) (Figure 2D). along with their unfavorable prognosis leads us to speculate that these patients, as well as those in the *CEBPA*-silenced cluster, might benefit from the inclusion of hypomethylating agents as part of their therapeutic regime.

Furthermore, in this study we identified the presence of a common DNA methylation signature that is detected in the vast majority of cases. The nature of the genes found in this common epigenetic signature, which included tumor suppressors, putative and well-described transcription factors, nuclear import proteins, apoptosis-related proteins, and a regulator of myeloid cytokines, is highly suggestive of a role in leukemic transformation. In addition, we found that this aberrant methylation was accompanied by significant downregulation of these genes. The fact that these genes are affected in a broad fashion, across multiple different subtypes of AML leads us to believe that deregulation of these genes is most likely a necessary, though probably not sufficient, event during the malignant transformation process of hematopoietic cells.

Finally, the study identified a robust 15-gene methylation classifier that was predictive of overall survival, which was generated in an unbiased manner using a large enough data set to perform training, testing and independent validation. The methylation predictor was further validated as an independent risk factor in a multivariate analysis. Since DNA is stable and readily obtained from clinical specimens, we believe that this DNA methylation classifier could serve as a clinically useful biomarker used for decision-making in future clinical trials.

In conclusion, while epigenetic deregulation has been recognized as a hallmark of cancer for some time, the use of epigenomics to further expand our understanding of the biology of these diseases has only more recently become feasible in the clinical context. Here we show that DNA methylation profiling is a powerful tool for the clinical stratification of AML and to further explore and define the biology of this disease.

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CHAPTER 8

Aberrant DNA Hypermethylation Signature in Acute Myeloid Leukemia directed by EVI1

Sanne Lugthart[1], Maria E. Figueroa[2], Eric Bindels[1], Lucy Skrabanek[3], Peter J.M. Valk[1], Yushan Li[2], Stefan Meyer[5], Claudia Erpelinck-Verschueren[1], John Greally[4,6], Bob Löwenberg[1], Ari Melnick2 and Ruud Delwel[1]**

[1]Department of Hematology, Erasmus University Medical Center, Rotterdam, The Netherlands. [2]Department of Medicine (Hematology Oncology Division), Weill Cornell Medical College, New York, United States of America. [3]Department of Physiology and Biophysics and HRH Prince Alwaleed Bin Talal Bin Abdulaziz Alsaud Institute for Computational Biomedicine, Weill Cornell Medical College, New York, United States of America. [4]Department of Molecular Genetics, Albert Einstein College of Medicine New York, United States of America. [5]Academic Unit of Paediatric Oncology, Christie Hospital Trust, University of Manchester, Manchester, UK. [6]Department of Medicine, Albert Einstein College of Medicine, New York, United States of America., Mainz (D).

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ABSTRACT

DNA methylation patterns are frequently dysregulated in cancer, although little is known of the mechanisms through which specific gene sets become aberrantly methylated. The *EVI1* locus encodes a DNA binding zinc-finger transcription factor that is aberrantly expressed in a subset of AML patients (AMLs) with poor outcome. We find that the promoter DNA methylation signature of *EVI1* AML blast cells differs from those of normal $CD34^+$ bone marrow cells and other AMLs. This signature contained 294 differentially methylated genes, of which 238 (81%) were coordinately hypermethylated. An unbiased motif analysis revealed that EVI1 binding sites were overrepresented among these aberrantly hypermethylated loci. EVI1 was capable of binding to these promoters in two different *EVI1* expressing cell-lines, whereas no binding was observed in an *EVI1* negative cell-line. Furthermore, EVI1 was observed to interact with DNMT3A and not with DNMT3B or DNMT1. Among the *EVI1* AML cases, two subgroups were recognized of which one contained AMLs with many more methylated genes, which was associated with significantly higher levels of *EVI1* than in the cases of the other subgroup. Our data point to a role for EVI1 in directing aberrant promoter DNA methylation patterning in *EVI1* AMLs.

INTRODUCTION

Patterning of DNA methylation plays a critical role in epigenetic gene regulation during normal development(1). Aberrant cytosine methylation of gene promoters occurs frequently in many forms of cancer, including acute myeloid leukemias (AML)(2). Several tumor suppressor genes, e.g., *CDKN2B* and *CEBPA* are found to be abnormally methylated and silenced in AML patients(3, 4). Moreover, aberrant distribution of promoter DNA methylation occuring in specific and distinct patterns has been shown to be a universal feature occurring in all AML patients. (5). However, the mechanisms that mediate these aberrant methylcytosine patterns have not been defined.

Abnormal expression of the *EVI1* (*ecotropic viral integration-1*) gene, as the result of inv(3)(q21q26.2)/t(3;3)(q21;q26.2) or through other unknown mechanisms, is associated with unfavorable AML outcome(6, 7). *EVI1* encodes a C2H2 zinc finger transcription factor, binding DNA in a sequence-specific manner and functions as a repressor(8-10). Retroviral insertion mutagenesis studies suggest that EVI1 deregulation plays a role in leukemogenesis(11). The mechanism through which EVI1 mediates these effects is unknown. Given the function of EVI1 as a transcriptional repressor we wondered whether EVI1 might be associated with aberrant epigenetic programming in AML patients. In order to test this hypothesis, we conducted a large-scale DNA methylation profiling study in human *EVI1* AMLs. A specific promoter DNA methylation signature is uncovered in *EVI1* AMLs and evidence is provided that EVI1 contributes to aberrant promoter DNA methylation patterning in those leukemias.

MATERIAL AND METHODS

Patient samples

Diagnostic material from 26 AML patients over-expressing *EVI1* (*EVI1* AML), determined using *EVI1* real-time quantitative PCR(7), were included based on material availability. All patients were enrolled in Dutch-Belgian Hemato-Oncology Cooperative Group trials (available at http://www.hovon.nl), and provided written informed consent in accordance with the declaration of Helsinki. IRB approval was obtained at the Erasmus University Medical Centre. Normal $CD34^+$ progenitor cells ($CD34^+$ NBM) were purified from bone marrow specimens from 8 healthy donors: 4 acquired from the Translational Trials Development and Support Laboratory, Cincinnati Children's Hospital and 4 purchased from Allcells (Emerville, CA, USA).

Genome wide DNA methylation by the HELP assay

Blasts and mononuclear cells from AML samples at diagnosis were purified as previously reported(12). The HELP assay was carried out in the *EVI1* AML and $CD34^+$ normal bone marrow (NBM) samples as previously published(5). Based on the density of the HpaII/MspI

ratios from the 25,626 probe sets in the 26 *EVI1* AMLs and 8 $CD34^+$ NBM samples; hypermethylation was defined as a $\log_2$ (HpaII/MspI) < 1 and hypomethylation $\log_2$ (HpaII/MspI) of a probe set was > 1. The HELP data have been submitted to the NCBI Gene Expression Omnibus (GEO) repository (GSE18700).

DNA methylation data analysis

Unsupervised clustering of the HELP data by hierarchical clustering using Pearson correlation distance with Ward clustering method and principal component analysis was performed using R.2.8.1(13) and BioConductor(14) using the package MADE4(15). Supervised analysis was carried out using a moderated T-test with a significance level of P-value <0.05 after correcting for multiple testing using the Benjamini-Hochberg (BH) approach. An absolute difference in methylation >1.5 between the means of the two populations ($mean^{EVI1}$ log(HpaII/MspI) – $mean^{NBM}$ log(HpaII/MspI)) was required to increase the likelihood of detection of biologically significant changes in methylation levels.

Quantitative DNA methylation analysis by MassARRAY EpiTyping

Technical validation of the HELP data was performed by MALDI-TOF mass spectrometry using EpiTyper by MassARRAY (Sequenom, CA) in 13 randomly selected *EVI1* AML samples on bisulfite-converted DNA using a panel of 15 genes using MassARRAY primers as previously described(4, 5, 16, 17). The correlation between the MassARRAY results and the HELP methylation data was calculated using the r value of the regression line

CpG/CG enrichment, gene ontology and regulatory element analysis

Enrichment of probe sets overlapping with CpG islands or CG clusters(18) in the gene signatures vs. all probe sets of the HELP assay was calculated using the Fisher's exact test using R 2.8.1. Gene ontology (GO) analysis was performed using DAVID(19), with the entire HELP microarray as the background reference against which enrichment of level 5 GO categories was determined. FIRE (Finding Informative Regulatory Elements) was used as described(20), to detect motifs in promoter regions i.e., sequences up to 2,000 bp upstream of the TSS defined by the UCSC browser 2008(21) , that were able to distinguish between *EVI1* AML and $CD34^+$ NBM signature genes and a group of 5,000 control sequences.

Chromatin immunoprecipitation

The chromatin immunoprecipitation (ChIP) was carried out simultaneously in an *EVI1* overexpressing AML SB1690CB cell-line(22, 23), K562 a cell-line with intermediate *EVI1* levels, and an *EVI1* negative MOLM13 cell-line according to manufacturer's protocol (SimpleChIP Enzymatic Chromatin IP Kit, Magnetic Beads, Cell Signaling, Bioke, CA, USA, Catalog #9003) using anti-EVI1 (Cell Signaling, #2593) or an equal amount of IgG isotype as negative control (Cell Signaling, #2729) or anti-Histone H3 (Cell Signaling, #2650) as positive control.

Three independent experiments were carried out and according to manufacturer's protocol, in each experiment over 1% of the input from the positive control RPL30 (Histone H3 binding target) was precipitated (mean 2%, standard deviation 0.78%). The amount of immunoprecipitated DNA in each experiment is represented as signal relative to the amount of input and was calculated with the quantitative real-time PCR results using primers directed to promoter regions of *FAM83b*, *IL11RA*, *MORF4L1*, *CRHBP*, *CASP2* and *VPREB3* containing respectively 7, 8, 5, 5, 4, and 5 bp of the first EVI1 binding domain. The ChIP primer sequences are shown in the Supplement.

EVI1 and DNMT co-immunoprecipitation assays

The expression constructs pcDNA-HA-DNMT1, pSVK-HA-DNMT3A and pSVK-HA-DNMT3B were a kind gift of Dr. Stephen Baylin and the latter two constructs were subcloned into pcDNA3.1 to generate pcDNA-HA-DNMT3A and pcDNA-HA-DNMT3B. pCMV-empty and pCMV-FLAG-EVI1 have been described before(24). We also generated pcDNA-FLAG-DNMT3A and pCMV-HA-EVI1 to carry out the reciprocal Co- =immunoprecipitation (Co-IP) experiments. For Co-IP experiments, HEK293T cells were transfected with 8μg of total DNA using FuGENE6 transfection reagents (Roche, Mannheim, Germany). Forty-eight hours after transfection, FLAG-immunoprecipitations on nuclear extracts were performed according to the Nuclear Complex Co-IP kit protocol (Active Motif, Carlsbad, Ca, USA), and bound proteins were detected by Western blotting with anti-Flag (Sigma-Aldrich, St. Louis, Mo, USA) or anti-HA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies. Co-IPs were carried out in SB1690CB cells with anti-DNMT3A (Abcam, Cambridge, MA) and anti-EVI1 (Cell Signaling Technology, Boston, MA).

RESULTS

EVI1 AML blasts display a specific aberrant promoter DNA methylation signature

We compared and contrasted the abundance of cytosine methylation at 14,000 gene promoters using the HELP assay in 26 AML patients overexpressing *EVI1* (*EVI1* AMLs) and in 8 $CD34^+$ normal bone marrow controls ($CD34^+$ NBM). The accuracy of the HELP assay in detecting variance in DNA methylation was validated by single locus quantitative EpiTyping and the correlation between the two assays was r=0.83 (Figure S1).

Unsupervised analysis using hierarchical clustering (Figure 1A) and principal component analysis (data not shown) revealed clear segregation of the 26 *EVI1* AMLs from the 8 $CD34^+$ NBM controls. Hierarchical clustering in a previously published cohort of 344 AML patients(5), revealed that the *EVI1* AMLs were almost all contained within two cohorts of patients defined solely based on their epigenetic signatures, but not having any other common known cytogenetic or molecular abnormality (Figure S2). Thus, the *EVI1* AMLs clustered separately from any of the epigenetic clusters with well-defined cytogenetic or molecular abnormalities, i.e., inv(16), t(8;21), t(15;17), *CEBPA*-mutant AML, *CEBPA*-silenced leukemias or *NMP1* mutant cases (Figure S2). *EVI1* AML leukemia cells thus present with a DNA hypermethylation signature that differs entirely from that of normal bone marrow progenitors, as well as from those of other genetically well-defined AMLs

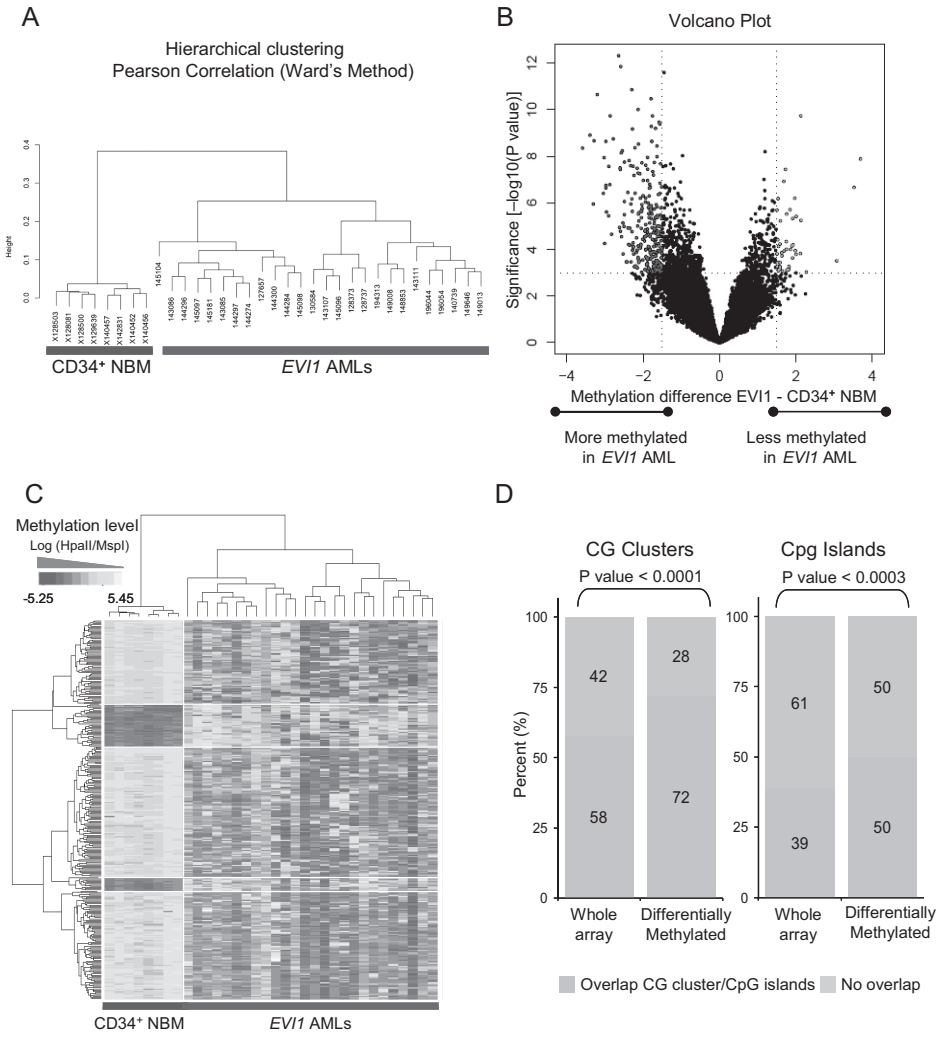

Figure 1. *EVI1* acute myeloid leukemia patients (*EVI1* AMLs) have a unique genome wide methylation profile compared to CD34⁺ normal bone marrow samples (CD34⁺ NBM).

(A) The dendrogram represents a hierarchical clustering in 8 CD34+ NBM blasts and 26 *EVI1* AMLs. (B) The volcano plot shows the methylation difference comparing the 26 *EVI1* AMLs to 8 CD34⁺ NBM samples with corresponding moderated T-test P-value. Probe sets significantly hypermethylated (P<0.001 and methylation difference less than -1.5) are shown in red, probe sets significantly hypomethylated (P<0.001 and methylation difference larger than 1.5) are shown in green. Significant probe sets that did not have an absolute methylation difference larger than 1.5 are depicted in blue. (C) The heatmap shows the methylation levels (Log (HpaII/MspI)) of differentially methylated genes (rows) in *EVI1* AMLs and CD34⁺ NBM cases (columns). (D) The histograms show the percentages of genes containing (green) CG clusters and CpG islands and those not overlapping (grey) in all genes on the HELP array and in the *EVI1* AML differentially methylated genes. A Chi-square test P-value is shown per panel.

A supervised analysis (moderated T-test) was next performed in order to more precisely define the nature of aberrant epigenetic programming in *EVI1* AMLs vs. CD34⁺ NBM controls. 303 differentially methylated probe sets, corresponding to 294 unique genes (Figure 1B) were identified as differentially methylated in *EVI1* AMLs with P<0.001 (BH corrected P<0.05) and methylation log ratio difference >1.5 (corresponding to >25% differences in DNA methylation level). 81% (238/294) of the genes were coordinately hypermethylated in *EVI1* AML cases, whereas only 19% (56/294) were hypomethylated, compared to CD34⁺ NBM controls (Figure 1C).

A significantly greater than expected percent of these probe sets were associated with CpG islands (P<0.0001) and CG clusters (P<0.0003) as compared to the distribution of all probe sets on the HELP array (Figure 1D). Of note, 28% and 50% of the differentially methylated genes did not contain CG clusters or CpG islands, respectively, suggesting that DNA methylation beyond these areas may remain important as well(25).

Unique biological and molecular features of genes metylated in EVI1 AMLs

From the functional standpoint the *EVI1* DNA methylation signature was enriched in genes associated with transcription regulation and RNA biosynthetic process (Table S3). Three of the aberrantly methylated genes in the *EVI1* signature (i.e. *TOPORS*(26), *PCDH16*(27) and *CTCFL*(28, 29)) have been reported to function as tumor suppressors. Thus, *EVI1* AML associates with a unique set of functionally related and coordinately hypermethylated genes.

To better understand the mechanisms that might contribute to aberrant methylation in *EVI1* AMLs, we used an unbiased motif analysis algorithm (FIRE(20)), to examine the promoter regions in the signature for DNA sequences of potential functional significance. This approach yielded three motifs significantly over-represented ($P<0.05$ after Bonferroni correction) in the 294 differentially methylated genes in *EVI1* AML compared to a set of 5,000 randomly selected non-differentially methylated promoters (Figure 2A). Two of the three over-represented motifs in the *EVI1* AML methylation signature contained sequences, that overlap with the TGACAAGATAA consensus sequence, that is bound by the first zinc finger domain of EVI1 (Figure 2A, Figure 2C). A subsequent FIRE motif analysis focusing, separately on the 238 hypermethylated and 56 hypomethylated genes revealed two motifs, that were highly enriched among the hypermethylated genes and each of these contained respectively, 5 bp and 4 bp of the first and second EVI1 zinc finger domain binding sequence (Figure 2B).

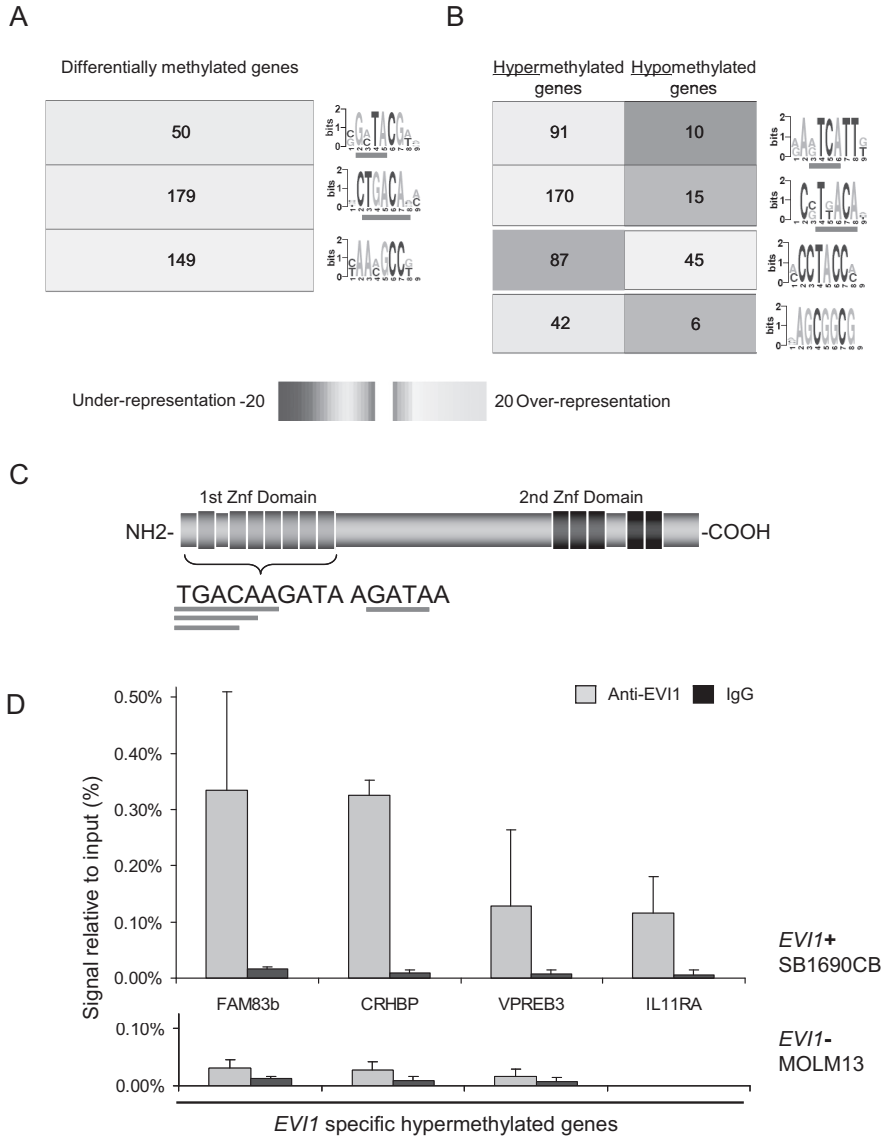

Figure 2. EVI1 binding sites are over-represented in the hypermethylated promoter regions of *EVI1* AMLs and EVI1 binds these hypermethylated promoters in vivo.

(A) Motif analysis of the in *EVI1* AML differentially methylated genes showed a significant overrepresentation (yellow in the heat map color key) of three 7 bp motifs. Per bar each 7 bp optimized motif is shown. The unlined sequences overlap with the first or second EVI1 binding domain. The number of genes that harbored the representative motifs in their promoter sequences are depicted per bar. (B) Further analysis of the hypermethylated and hypomethylated genes reveals two overrepresented motifs in the promoter regions of the hypermethylated genes and one overrepresented in hypomethylated genes. (C) A schematic representation of the EVI1 nuclear zinc-finger protein, with the binding sequence of the first and second EVI1 zinc finger domains. The overlapping motifs over-represented in hypermethylated genes are underlined in respectively blue and green. (D) Quantitative PCR of chromatin immunoprecipitation in the *EVI1* positive (*EVI1*+) SB1960CB cell line and the *EVI1* negative (*EVI1*-) MOLM13 cell line using EVI1 and IgG antibody. Percentage of amount of input material is shown. The mean and standard deviation of three independent experiments is shown.

A role for EVI1 in promoter hypermethylation in EVI1 AML

In order to determine whether the presence of putative EVI1 binding sites indicated that these were EVI1 target genes, we first performed HELP analysis on the recently derived human AML SB1690CB cell line, carrying a chromosomal 3q26 aberration and overexpressing *EVI1*(23). A highly significant overlap of hypermethylated genes (89%) was found in the SB1690CB cell line compared to the hypermethylated genes identified in the *EVI1* AML patient samples (211/238 genes) (Table S5). Secondly, we performed chromatin immunoprecipitation (ChIP) assays on six randomly chosen hypermethylated genes, containing the above mentioned EVI1 binding sequences. As compared to control antisera, EVI1 antibodies enriched all six genes, indicating that EVI1 was indeed bound to these genes (Figure 2D, Figure S3A). Similarly, in another cell line expressing *EVI1* (K562), ChIP revealed EVI1 binding to the four loci studied (Figure S3B). In contrast, these promoters could not be immunoprecipitated in the *EVI1* negative myeloid MOLM13 leukemia cell line (Figure 2D, Figure S3).

EVI1 interacts with DNMT3A

The results implicate EVI1 in promoter methylation. We therefore examined whether EVI1 could interact with DNA methyltransferases (DNMTs). Co-immunoprecipitation experiments were carried out using FLAG-tagged DNMT1, -3A or -3B and HA-tagged EVI1 transduced into 293T cells. FLAG-DNMT3A interacted with HA-EVI1 (Figure 3A, left panel), whereas no interaction was observed between HA-EVI1 and FLAG-DNTM1 or -3B (data not shown). Similarly, HA-DNMT3A co-precipitated with FLAG-EVI1 in the reverse experiment (Figure 3A, right panel). EVI1 is frequently found to be present in nuclear speckles. Confocal microscopy revealed that HA-tagged EVI1 co-localized with FLAG-tagged DNMT3A within the nuclei of transfected 293T cells, but not with FLAG-tagged DNMT3B (Figure 3B). Most importantly, in SB1690CB cells, endogenously expressed EVI1 protein co-immunoprecipitated with DNMT3A (Figure 3C). Moreover, DNMT3A was found to be highly expressed in primary *EVI1* AMLs as compared to other AML patients (Figure S4).

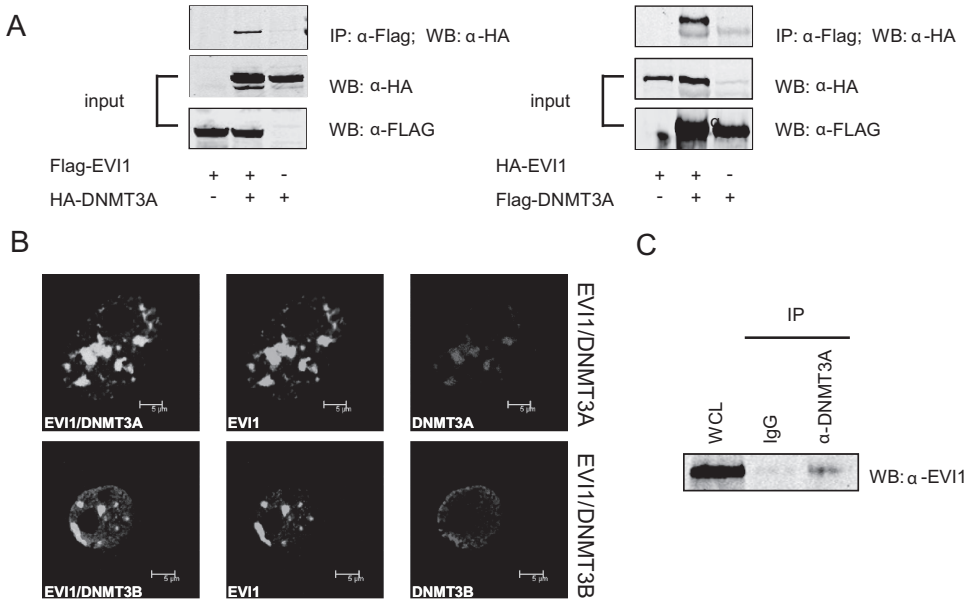

Figure 3. EVI1 interacts with DNMT3A.
(A) Western blot analysis using anti-FLAG antibodies shows the input of the immunoprecipitation of transfected 293T cells and the pulldown using anti-HA. (B) Confocal microscopy of 293T cells transfected with HA-tagged EVI1 (green) and FLAG-tagged DNMT3A and -3B (red). (C) Western blot for EVI1 on lysates from SB1960CB cell-line. The left lane shows the input band; the second and third lane show EVI1 staining following immunoprecipitation with IgG control (second lane) or anti-DNMT3 (third lane).

Levels of EVI1 correlated with levels of methylation in EVI1 AMLs

Detailed analysis of the hierarchical clustering (Figure 1A and 1C) revealed that the 26 *EVI1* AML cases could be divided into two distinct subclusters (A and B) each with identifiable methylation profiles (Figure 4A). The robustness of both subclusters was determined by 10,000 bootstrap re-sampling. The P-values (arbitrary unit: AU values) were more significant (<95%) for subcluster B than subcluster A, indicating that methylation profiles in subcluster B are more homogeneous (Figure S5). Strikingly, subcluster A contained 7/8 *EVI1* AMLs that carried 11q23 rearrangements (Fisher's Exact test P-value 0.009). Most *EVI1* AMLs that carried a 3q26 abnormality (7/8) were contained within subcluster B (Fisher's Exact test P-value 0.03). This cluster was also enriched for cases with -7/7q- chromosomal lesions (8/11), which are frequently associated with 3q26 abnormalities (Figure 4A).

Of note *EVI1* was expressed at higher levels in subcluster B than in subcluster A (Moderated T-test P-value=0.01, Figure 4B). Supervised analysis revealed 122 significantly differentially methylated genes between those two groups (Moderated T-test P<0.001, BH corrected P<0.05, and absolute methylation difference >1.5). 117 of those 122 (96%) were exclusively hypermethylated in the subcluster B (Figure 4C and 4D).

In summary, these data demonstrate that, although *EVI1* AMLs share a methylation profile that discriminates them from normal marrow blasts, two subgroups can be identified, which show a positive correlation between *EVI1* transcript levels and methylation levels. The fact that subcluster B cases frequently carry 3q26 lesions and monosomy 7(30), may be a critical determinant in the increased number of methylated genes in those AMLs.

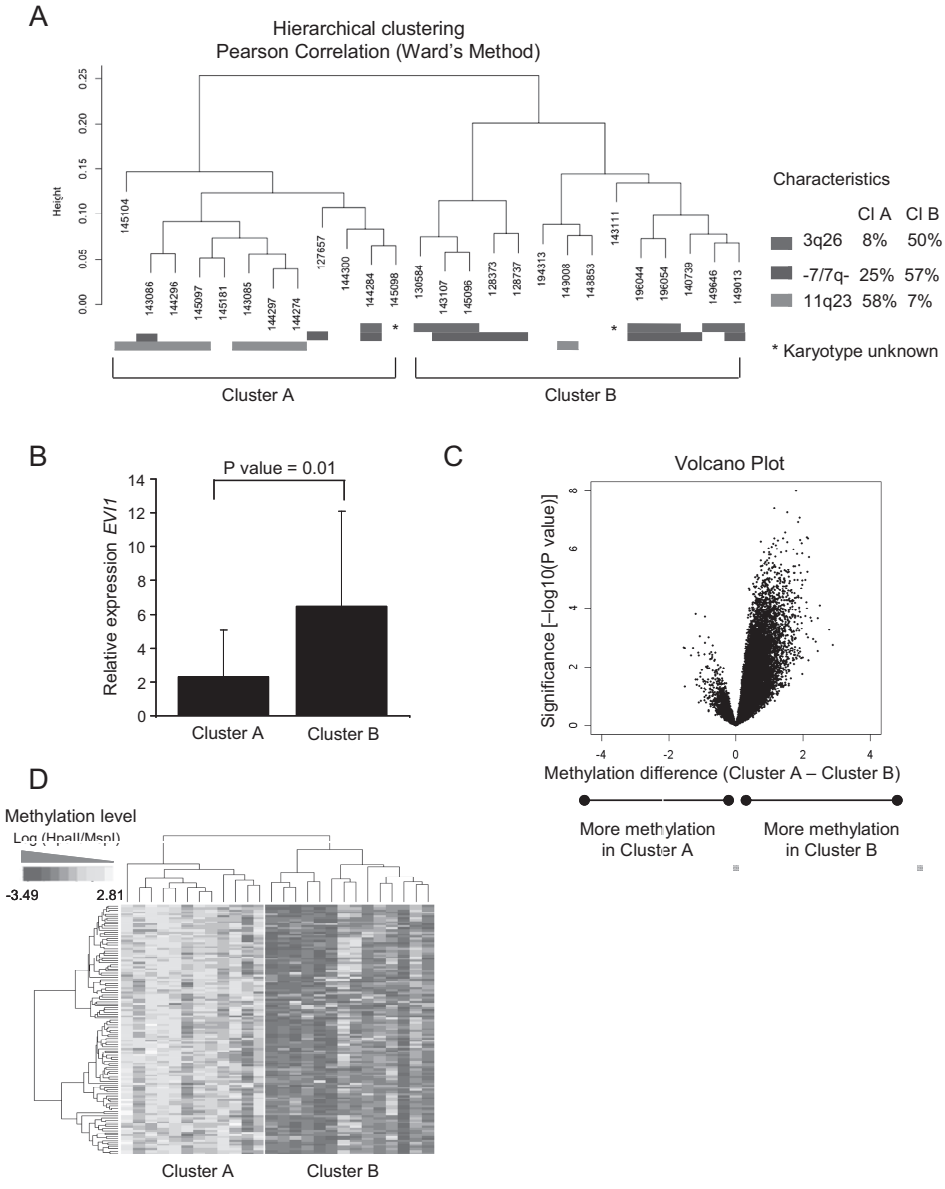

Figure 4. Unsupervised analysis identified two epigenetically distinct *EVI1* AML subgroups correlating with *EVI1* relative expression.

(A) Unsupervised hierarchical clustering with Pearson's correlation using Ward's method revealed two *EVI1* subclusters i.e., A and B. The cytogenetic characteristics are shown per patient; chromosome 3q26 abnormalities (red), monosomy 7 or deletion 7q (-7/7q-) (blue) and 11q23 rearrangements (grey). The percentages of each characteristic are shown per cluster. (B) Median *EVI1* relative expression levels and 2SD are shown per subcluster. P-value was calculated using a moderated T-test. (C) The volcano plot shows the methylation difference of all probe sets (n=25,626) (x-axis) comparing the methylation levels of cases in subcluster A with the cases in subcluster B with corresponding P-value ($-\log_{10}$ P-value moderated T-test) on the y-axis. (D) The heatmap shows the 122 probe sets (110 unique genes) differentially methylated in subcuster B, when both cluster were compared to each other using a moderated T-test (P<0.001 and absolute methylation difference >1.5). All genes are hypermethylated in *EVI1* AMLs from subcluster B.

DISCUSSION

AML patient samples can be classified based on unique DNA methylation signatures(5), but the mechanisms that direct coordinated methylation in the different genetically well-defined AML subsets are unknown. Recruitment of DNMTs to target promoters and subsequent promoter hypermethylation has been proposed as being mediated by the oncogenic transcription factor PML-RARA(31) in acute promyelocytic leukemia and AML1-ETO(32) in favorable risk AML using cell-line models. However, EVI1 is to our knowledge the first example of a transcription factor that may direct a unique recurrent DNA methylation signature in human disease.

EVI1 AMLs express a methylation signature that discriminates them from normal marrow $CD34^+$ blasts and from other AMLs. Within this differential signature ~80% of the genes were hypermethylated and ~20% hypomethylated in the *EVI1* AMLs as compared to $CD34^+$ NBM controls. Moreover, an even stronger hypermethylation signature was observed in the subcluster with the highest *EVI1* expression (Figure 4). EVI1 binding sites were overrepresented in hypermethylated promoters in *EVI1* AMLs. These hypermethylated genes are apparently *bona fide* target genes since EVI1 was shown to bind to these promoters. EVI1 could also form a complex with DNMT3A, but not with other DNMT family members. Together, these results suggest a role for EVI1 in directing *de novo* DNA hypermethylation in human AMLs that overexpress this transforming nuclear protein.

Knock down of *EVI1* in AML models would be an attractive approach to study whether genes might become demethylated in the absence of EVI1. However, we observed that knock down of *EVI1* resulted in an almost complete cell cycle arrest of SB1690CB as well as K562, whereas no growth inhibition was observed in the *EVI1* negative myeloid cell line MOLM-13 (data not shown). This observation may be of importance for future therapy of this AML type, but it does not provide the optimal condition to answer our question, since cell cycle is a requirement in order to gradually lose methylation. The *EVI1* methylation profile was not affected following knock down (data not shown). Since *EVI1* expression in AML appears so tightly regulated by regulatory loci on chromosome 3q21 in case of inv(3)(q21q26.2)/t(3;3)(q21;q26.2) or by *MLL*-fusion genes as the result of a chromosome 11q23 aberration, proper animal models should be generated to study mechanisms of methylation by EVI1 in a reliable manner.

We previously reported on another AML subtype, i.e., *CEBPA*-silenced leukemias(4), in which the majority of loci were predominantly methylated in the AMLs and not in the $CD34^+$ NBMs. One might argue that this is not surprising as aberrant promoter hypermethylation is a general event found in many tumors. However, this is not a priori true for AML. *CEBPA*-mutant AMLs carry more hypomethylated loci when compared to *CEBPA*-silenced leukemias or to $CD34^+$ normal samples(4, 5). Moreover, *CEBPA*-silenced leukemias show a very strong methylation signature, but it is completely different from that of *EVI1* AMLs. In the unsuper-

vised clustering analysis the *CEBPA*-silenced cases are grouping together completely separate from the *EVI1* AMLs (Figure S2). Thus, in *EVI1* AMLs a different set of genes is methylated, again pointing to a specific role for EVI1 in these AMLs. We show that EVI1, which has been reported to also interact with histone deacetylases(33) (HDACs), as well as with C-terminal binding proteins(34), histone methyl transferases(35) and MBD3/NuRD complex(24), is also capable to bind DNMT3A. These findings, together with the observation that EVI1 is capable to bind DNA in a sequence specific manner and that EVI1 binding motifs are highly enriched in hypermethylated loci, support the hypothesis that EVI1 integrates functions in chromatin remodeling complexes and DNA methylation to mediate transcriptional repression.

Treatment with both DNMT and HDAC inhibitors has been shown to reverse aberrant epigenetic silencing and induce cell death in various cancer types(31, 36). The combination of DNMT and HDAC inhibitors has been proposed for therapeutic purposes, although it is currently not possible to identify a priori patients likely to respond to this treatment(37). The fact that EVI1 appears to mediate its gene silencing effects both through recruitment of HDAC complexes and DNMT3A, suggests that combination therapy with DNMT and HDAC inhibitors could be active in these AML cases.

SUPPLEMENTARY FIGURES

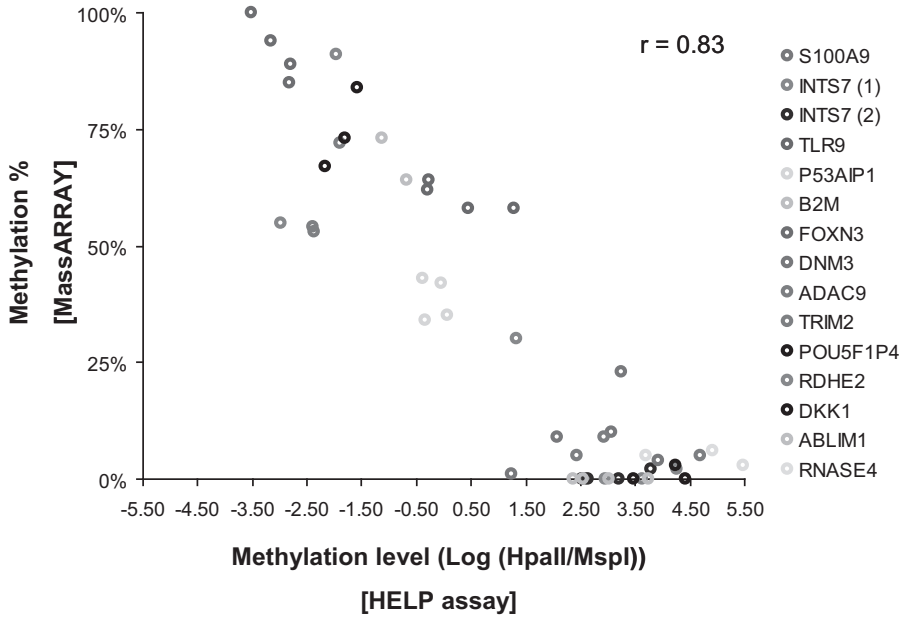

Figure S1. Validation of HELP methylation levels by MassARRAY Epityper. Dot plot showing correlation between $\log_2$ (HpaII/MspI) ratios (x-axis) and percentage of methylation as determined by MassARRAY Epityper (y-axis) for 15 probe sets in 13 randomly selected *EVI1* AML patients. The correlation coefficient is shown.

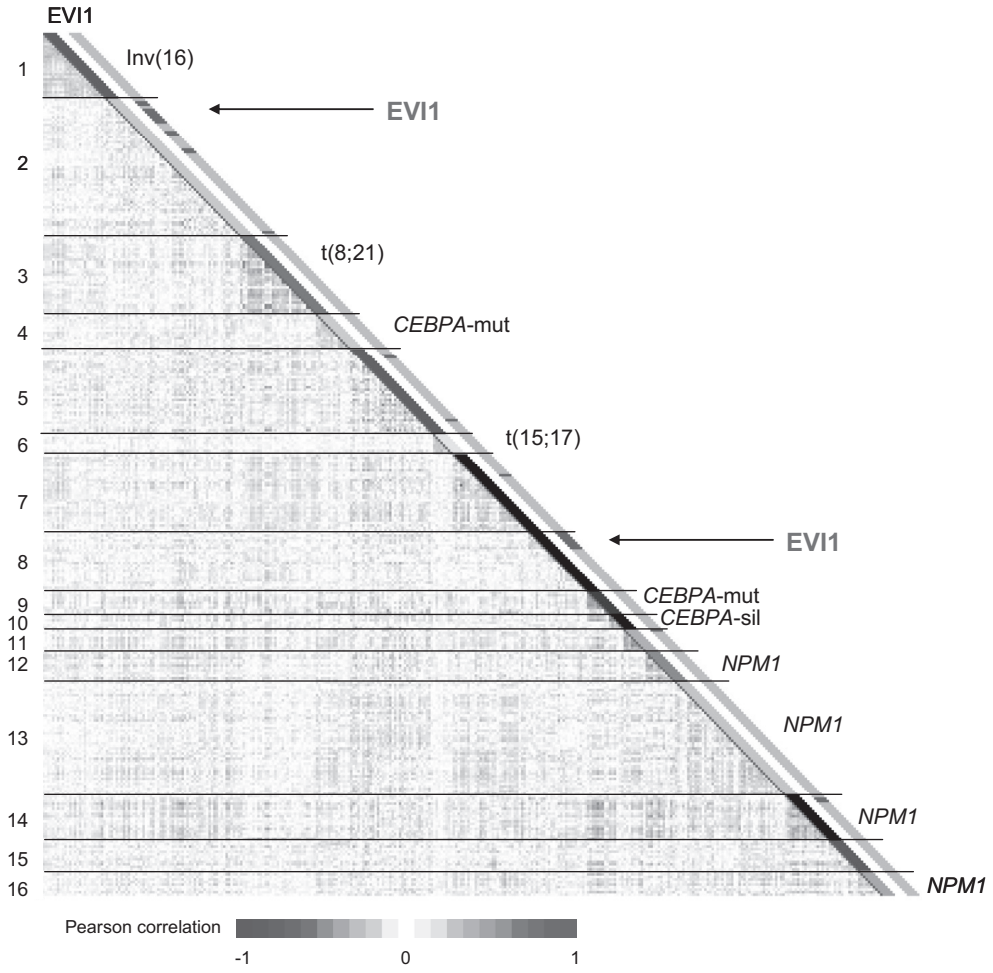

Figure S2. *EVI1* AML patients have a distinct genome-wide methylation profile compared to other recurrent molecular and cytogenetic AML cases. The correlation plot shows the Pearson correlation of the high variance probe set methylation levels between 344 AML patients. The 16 clusters that were defined by Figueroa et al[1], are labeled accordingly and the most recurrent cytogenetic or molecular abnormalities, are shown. Clusters #2 and #8, have been identified as AML subgroups with unique methylation signatures, without common genetic or cytogenetic abnormalities and contain the majority of *EVI1*+ patients (labeled red for *EVI1*+ and green for *EVI1*-).

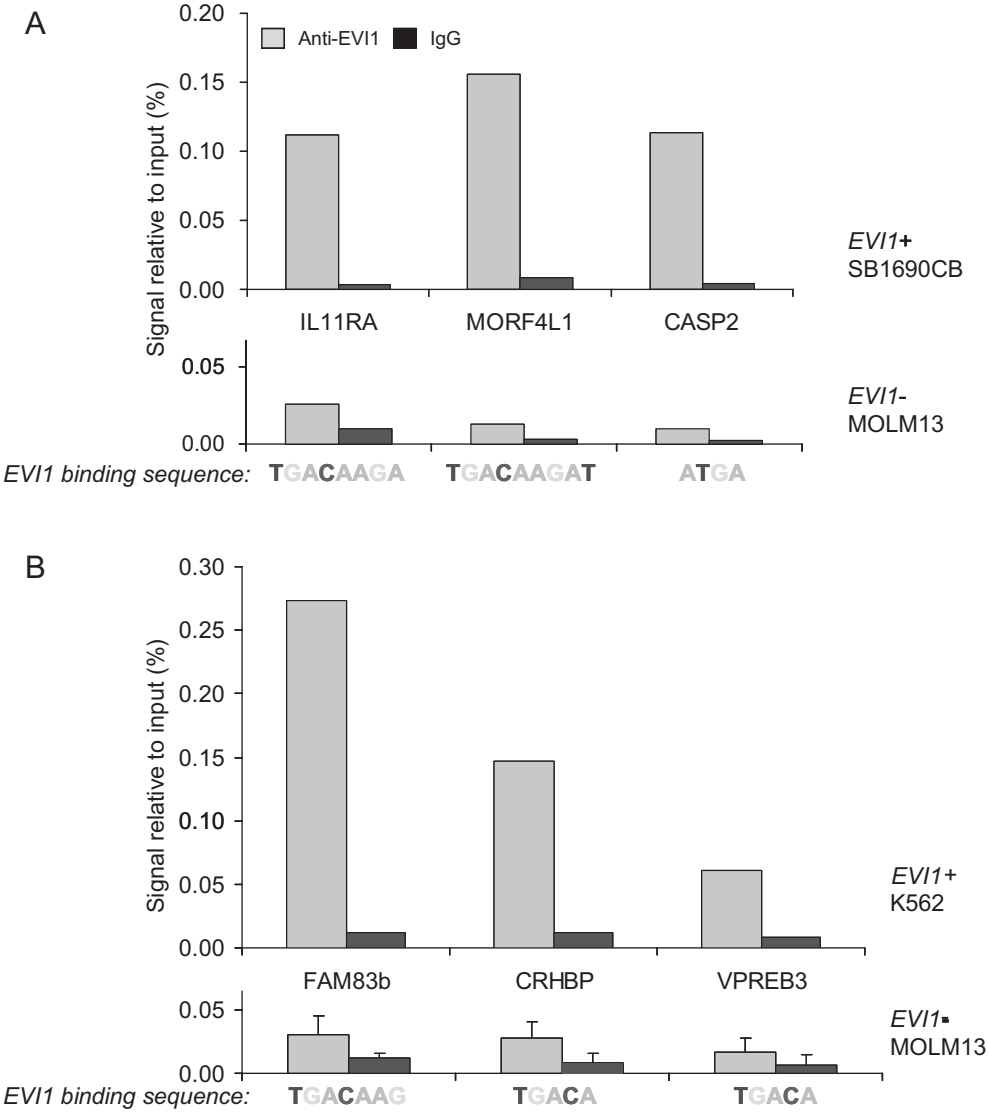

Figure S3. EVI1 binds hypermethylated genes containing EVI1 binding sequences. Quantitative PCR of chromatin immunoprecipitation in the *EVI1* positive (*EVI1*+) SB1960CB cell line (A) and the K562 cell line (B) and the *EVI1* negative (*EVI1*-) MOLM13 cell line using EVI1 and IgG antibody. Percentage of amount of input material is shown. The mean of two independent experiments is shown. The EVI1 binding sequence present in the promoter region of the hypermethylated genes is depicted.

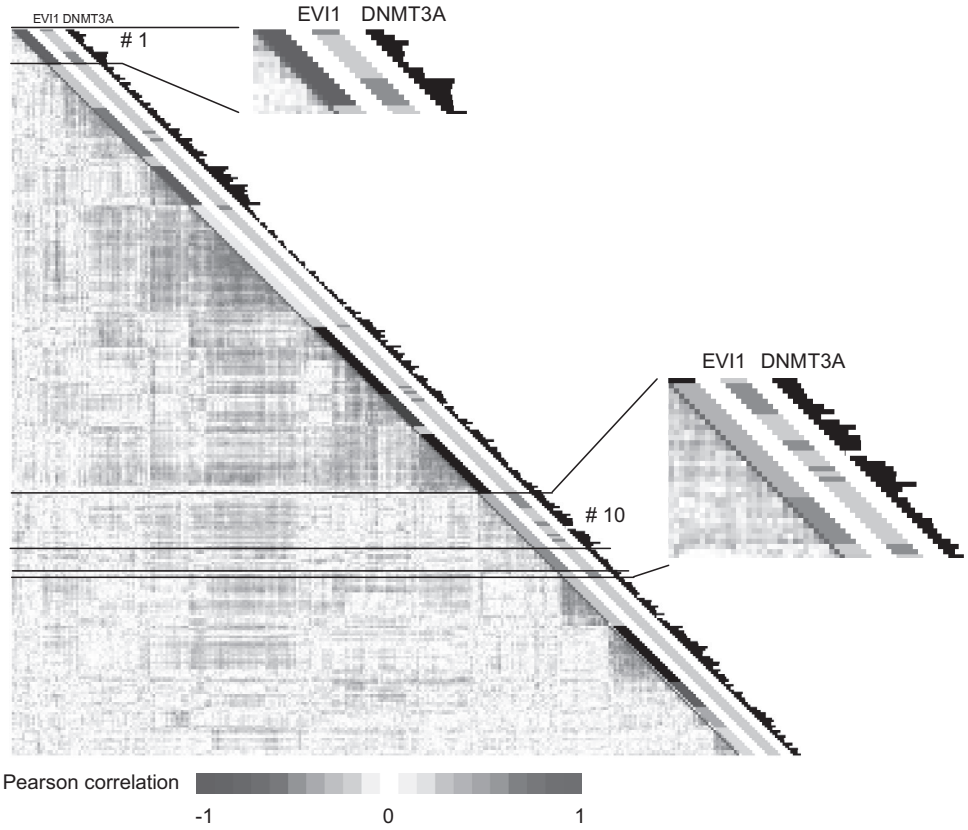

Figure S4. *DNMT3A* overexpressed in *EVI1* AMLs. A gene expression correlation view of 285 AMLs as previously described[2] shows the relative expression of *DNMT3A* in bars per patient. Focusing on the clusters highly over-represented with *EVI1* AMLs a trend for *DNTM3A* overexpression is seen.

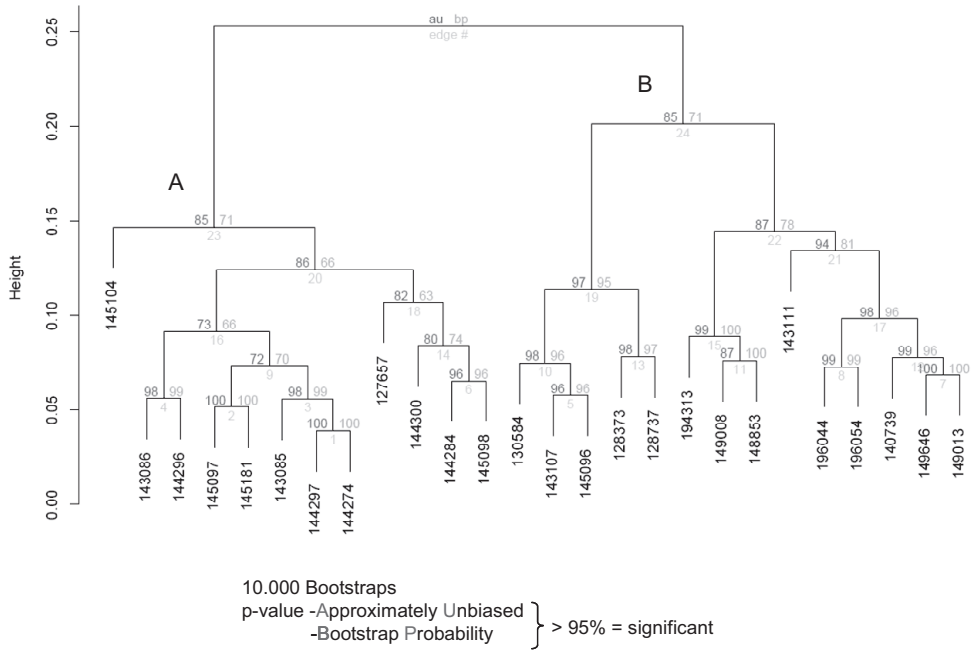

Figure S5. Unsupervised hierarchical clustering using genome wide methylation levels in *EVI1* AMLs revealed two main clusters. Unsupervised cluster analysis using Pearson's correlation with Ward's method revealed two distinct *EVI1* subclusters; A and B. Using 10.000 bootstraps the Approximately Unbiased (AU) and Bootstrap Probability (BP) percentages were calculated per tree in the hierarchical clustering to show the tightness per subcluster, i.e. higher percentage means tighter cluster.

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Summary and General Discussion

SUMMARY

To diagnose patients with acute myeloid leukemia (AML) in an optimal manner, the combined application of conventional and modern cytogenetics with state-of-the-art molecular diagnostics is a requirement. Although at present, the WHO accurately classifies an array of (2008) of human AML patients based on karyotyping combined with molecular diagnostic procedures, insight into the molecular defects of human AML is still increasing. As a result of that, the classification of AML will be approved in the upcoming years. The focus of this thesis was to increase our understanding of specific subtypes of human leukemia. We focused on AMLs with chromosome 3q rearrangements, in particular on patients with an inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1* (shortly: (inv(3)/t(3;3)), frequently associated with aberrant expression of *EVI1*, a gene that resides on 3q26.2. Secondly, we wished to elucidate why certain human AMLs showed high *EVI1* expression without having chromosome 3q26 abnormalities. We investigated the importance high *EVI1* levels in those leukemias and studied the potential to include *EVI1* expression analysis in the diagnosis of AML. The data obtained from the last three chapters increase our understanding of the molecular and biological effects of *EVI1* when aberrantly expressed in AML.

In chapter 2, we compared clinical, cytogenetic and molecular features of AML patients with different chromosome 3q rearrangements. We investigated whether AML cases with a 3q abnormality respond poor to treatment, whether the new WHO classification group inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1* is indeed a specific entity separate from the cases with different 3q abnormalities involving either the 3q26 or the 3q21 locus. We elucidated whether *EVI1* expression analysis could be of help to classify the distinct AML subtypes with distinct 3q rearrangements. Four groups with 3q abnormalities were identified, i.e. patients with (A) inv(3)/t(3;3), (B) balanced translocations involving t(3q26), (C) balanced t(3q21) translocations and (D) other 3q abnormalities. The inv(3)/t(3;3) cases could be recognized as a distinct subgroup; i) they frequently carried a monosomy 7, ii) they often carry N-*RAS* mutations, iii) they demonstrated discordant *EVI1* and *MDS1/EVI1* expression and iv) this karyotype was found to be an independent prognostic marker for survival and complete remission. The cases in the group of patients with remaining 3q abnormalities (D), showed an adverse survival outcome as well, but in this set of patients complex and monosomal karyotypes were highly frequent. Certain cases in group D showed discordant *EVI1* and *MDS1/EVI1* expression, very much alike inv(3)/t(3;3) AMLs. These leukemias showed frequently the rare karyotypes, add(3q21), del(3q21), add(3q26) or del(3q26). In fact, detailed FISH analysis in these cases revealed the existence of cryptic inv(3)/t(3;3) in the cases that were analyzed.

In chapter 3, we investigated whether *EVI1* has a prognostic impact on AML and what the relation of *MDS1*/*EVI1* is with high *EVI1* expression (*EVI1*+). The expression levels of five different *EVI1* 5' splice variants were determined. Beside the splice variant *EVI1-1C*, all variants were highly expressed in *EVI1*+ patients. Cases that expressed both, short form *EVI1* and

the long form *MDS1/EVI1* were correlated with 11q23 rearrangements and showed a slightly better survival compared to cases with high *EVI1*, which did not express *MDS1/EVI1*. We uncovered a subset of patients with high *EVI1* and low or no *MDS1/EVI1* expression without karyotopypically recognizable 3q26 abnormalities in which cryptic 3q rearrangements were detected using FISH analysis. *EVI1* was found to be of prognostic value independently from cytogenetic risk, age, monosomy 7 and t(11q23). From this study, we concluded that a combined *EVI1* and *MDS1/EVI1* Q-PCR should be added to the arsenal of molecular assays to classify human AMLs.

In chapter 4, a comparable study was carried out in pediatric AML patients, in which *EVI1*+ cases could be identified using gene expression profiling and the quantitative *EVI1* specific PCR assay (Q-PCR). The positive correlation between the presence of t(11q23) and aberrant *EVI1* expression was also found in these pediatric AML patients, in which the frequency of the t(11q23) cases is even higher than in adults. The prognostic impact of *EVI1* in pediatric AML was adverse, though not independent from other prognostic markers in pediatric AML. Remarkably, no 3q rearrangements were identified in this pediatric AML cohort.

A novel single Q-PCR approach was established (chapter 5), to measure all the different 5' *EVI1* splice variants in one assay. In a large cohort of nearly 1,000 AML cases *EVI1* was identified as independent prognostic marker for achievement of CR, EFS and RFS. *EVI1*+ patients with intermediate cytogenetic risk, as well as *EVI1*+ cases that carried t(11q23), showed adverse survival response compared to the *EVI1*- patients in both groups, underlining that *EVI1* screening should be taken into account for routine AML diagnostic work-up. The importance hereof is demonstrated by the finding that *EVI1*+ patients who received an allogeneic SCT showed a significantly better survival compared those receiving autologous SCT or chemotherapy alone.

The studies from chapter 6 were carried out to understand the clinical, molecular and mechanistic differences between *EVI1*+ and *EVI1*- AMLs with 11q23 rearrangements. In nearly 40% of the t(11q23) cases screened (n=83), *EVI1* was highly expressed. The *EVI1*+ t(11q23) cases showed to represent a distinct group that could be separated from the *EVI1*- t(11q23) cases: i) they expressed a different gene expression profiles leading to separated clustering of *EVI1*+ t(11q23) AMLs from the *EVI1*- cases; ii) morphologically they were different from each other; and iii) *EVI1*+ t(11q23) cases showed a significantly inferior response to therapy than *EVI1*- t(11q23) cases. *In vitro* transformation of murine bone marrow cells by *MLL-AF9* lead to the generation of *Evi1*$^{+}$ and *Evi1*$^{-}$ transformed clones. The progenitors from the *Evi1*$^{+}$ clones appeared to carry GMP, as well as immature CMP markers, whereas the *Evi1*$^{-}$ clones were more mature, i.e. only GMPs were found. Cell sorting experiments demonstrated that in *EVI1*+ *MLL-AF9* transformed human AML, as well as in transformed mouse bone marrow cells, *EVI1* expression was aberrant, i.e. the gene was expressed in mature cells, which are normally *EVI1* negative. *In vitro* self-renewal of *Evi1*$^{+}$ *MLL-AF9* transformed myeloid

progenitors was inhibited upon *EVI1* knockdown, indicating a fundamental role of EVI1 in transformation in a subset of *MLL-AF9* transformed AMLs.

In chapter 7 and 8, the research focus was on genome wide methylation levels in AML and on the mechanisms of how methylation may be regulated in AML (chapter 7), in particular in *EVI1* AMLs (chapter 8). We identified in a group of 344 AMLs, unique AML subtypes that were characterized by distinctive aberrant DNA methylation signatures. Cases with particular cytogenetic or genetic aberrations, such as cases with translocations t(8;21) or t(15;17), but also leukemias with mutations in *CEBPA* carried unique DNA methylation profiles. Five unique AML subsets without a common molecular or cytogenetic abnormality, but with discrete DNA methylation signatures were identified. In addition, a 15-gene methylation classifier was recognized and found independently predictive for survival in AML. In chapter 8, we focused on *EVI1* AML, which show an aberrant methylation profile compared to CD34+ normal bone marrow. The promoters of the aberrantly methylated genes overrepresented putative EVI1 binding sites, and EVI1 binding to the promoters of these genes was confirmed in an AML cell line model that carried a 3q26 abnormality. An interaction that was observed between DNMT3A and EVI1 made us hypothesize that EVI1 recruits DNMT3A to those promoter areas leading to *de novo* methylation of these regions.

GENERAL DISCUSSION AND FUTURE DIRECTIONS

I – 3q26 rearrangements and high EVI1 transcript levels in the diagnosis of AML

Human AMLs with the specific 3q26 rearrangements, inv(3)(q21q26.2)/t(3;3)(q21;q26.2) (inv(3)/t(3;3)) represent a unique AML subtype, which show a very poor response to treatment. The identification of *EVI1+ MDS1/EVI1-* cases with cryptic inv(3)/t(3;3), in the group of remaining 3q abnormalities, i.e. excluding the inv(3)/t(3;3) or the balanced translocations t(3q26) and t(3q21) groups (chapter 2), is a finding that stresses that AML patients at diagnosis should be analyzed for the presence of this unique abnormality. We propose that besides standard cytogenetic analysis using G-banding, the expression of *EVI1* should be determined in each newly diagnosed AML patient. In the majority of cases this combination of assays should be sufficient to uncover an AML with an inv(3)/t(3;3) and high *EVI1*. A Q-PCR to determine *MDS1/EVI1* expression levels, which are usually low or even absent in these AMLs, may confirm that a patient is indeed of this particular AML subtype. When a patient is *EVI1* positive and G-banding does not point to the existence of an inv(3)/t(3;3), a *MDS1/EVI1* Q-PCR combined with FISH analysis should be carried out to investigate whether such an AML is *EVI1+ MDS1/EVI1-* and carries a cryptic inv(3)/t(3;3).

II – Monosomy 7 in EVI1 expressing AMLs with inv(3)/t(3;3)

More than 60% of human AMLs with an inv(3)/t(3;3) carry a monosomy 7 (chapter 2, 3 and 5). In fact, no other AML subtype shows such a high correlation with monosomy 7, suggesting a causal relation with high *EVI1* expression. The order of events in primary AML, i.e. first aberrant *EVI1* expression followed by monosomy 7, or first a monosomy 7 followed by aberrant *EVI1* expression, is not fully solved yet. However, recent studies of a gene therapy trial for granulomatous disease provided new insights into the putative mechanism of EVI1 mediated disease progression(1). It was demonstrated that as a result of retroviral insertion in the 3q26 locus *EVI1* was overexpressed, which associated with abnormal centrosome duplication, linking *EVI1* activation to the development of genomic instability, monosomy 7 and clonal progression towards myelodysplasia(1, 2). If aberrant *EVI1* expression indeed causes monosomy 7 to occur, one would predict that with higher *EVI1* levels, the chances to obtain a monosomy 7 would increase. Indeed, we found that *EVI1* transcript levels are much higher in inv(3)/t(3;3) cases with a monosomy 7 than in cases without (Figure 1).

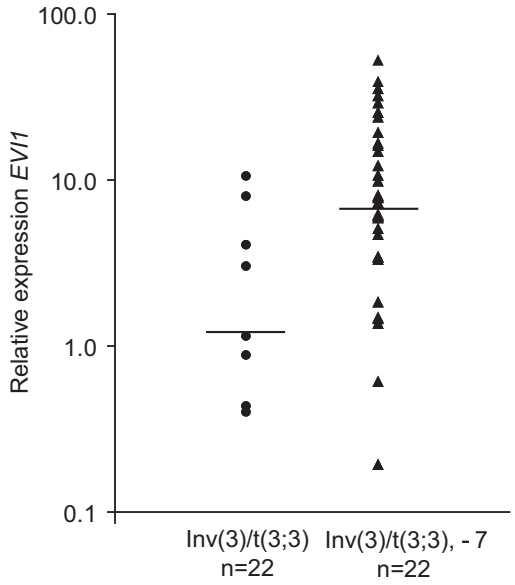

Figure 1. Higher EVI1 expression in inv(3)/t(3;3) and monosomy 7 cases compared to inv(3)/t(3;3) as sole abnormality.

The question remains, why chromosome 7 and not another chromosome is so frequently lost in inv(3)/t(3;3) AMLs. The most likely explanation would be that loss of chromosome 7 provides a proliferative advantage to *EVI1* over-expressing cells. This could mean that, one or more critical tumor suppressor genes are located on chromosome 7. In case the expression of these genes is diminished, the transforming activity by EVI1 is further increased. Interestingly, in view of this hypothesis, investigators were able to pinpoint two small regions on chromosome 7, that were shown to be selectively deleted in inv(3)/t(3;3) AML cell lines without a monosomy 7(3). Possibly one or more genes in those loci may act as tumor suppressor gene with overexpressed *EVI1*. Array-CGH, deep-sequencing and selective analysis of gene expression data of the many inv(3)/t(3;3) *EVI1* expressing AML samples available should provide insight into this enigma.

III – Mechanism of aberrant EVI1 transcription in AML with 3q26 rearrangement

How do chromosome 3q26 aberrations lead to high *EVI1* expression levels? In case of balanced translocations involving 3q26 that involve other chromosome translocation partners, such as in t(3;21) or t(3;12), high *EVI1* levels are easily explained by the regulatory control of the partner fusion gene. Fusion genes that arise from these translocations are *AML1/EVI1*(4) or *AML1/MDS1/EVI1*(5) in case of a translocations t(3;21) or *ETV6/EVI1*(6) or *ETV6/MDS1/EVI1*(7) when a translocation t(3;12) is apparent. *AML1* and *ETV6* are normally expressed in myeloid progenitors and consequently, the newly generated fusion genes are expressed under the control of the *AML1* or *ETV6* promoter sequences. *EVI1* is highly expressed in the

majority of those cases, but depending on the chromosomal breakpoint, i.e. 5' of *EVI1* or 5' of *MDS1/EVI1* the fusion partner is either *EVI1* or *MDS1/EVI1* (chapter 2)

The expression of *EVI1* and the frequent absence of *MDS1/EVI1* in AML with an inv(3)/t(3;3) is more complicated. The location of different 3q breakpoints in inv(3)/t(3;3) cases have been reported previously(8-13). Suzukawa(12) et al., first described that t(3;3)(q21;q26.2) and inv(3)(q21q26.2) cases have different breakpoints i.e. 5' and 3' of *EVI1* respectively. Poppe(11) et al., described an even more variable pattern of breakpoints for the t(3;3)(q21;q26.2) cases, i.e. breaks could occur 5' and 3' of *EVI1* or even 5' of *MDS1*. We performed a detailed FISH analysis in a couple of cases (see chapter 2 and chapter 3), showing breakpoints 5' and 3' of *EVI1* as well. We extended this series using multiple probes overlapping *EVI1* and *MDS1*, covering the 5' and 3' *EVI1* region (Figure 2). In 5/9 inv(3)(q21q26.2) cases we found that the breakpoints were located either 5' within the *EVI1* locus (#9118), 5' in between *EVI1* and *MDS1* (#2276, #5288, #6982) or even in the *MDS1* gene (#6357) (Figure 2). The sole t(3;3)(q21;q26.2) case (#3q76G), as well as 4 other AMLs with an inv(3) (#6367, #2228, #2542, #9212) showed breakpoints, that were 3' of *EVI1*. In all cases the expression of *EVI1* appears to be controlled by regulatory sequences of the *RPN1* gene, located on 3q21. High *EVI1* expression in those AMLs seems to result from a "classical" enhancer type of aberrant gene activation, since the insertions of the *RPN1* regulatory regions occur either 3' or 5'of the *EVI1* gene.

Why do the chromosomal aberrations inv(3)/t(3;3) almost always lead to the dissociative pattern of *EVI1* and *MDS1/EVI1* expression, i.e. high *EVI1* expression and no or low *MDS1/EVI1* expression? The majority of the inv(3)(q21q26.2) cases have a 5' *EVI1* breakpoint causing a dissociation of *EVI1* or *MDS1,* which easily explains why only *EVI1* is upregulated and not *MDS1/EVI1*. Why *MDS1/EVI1* expression is frequently low or even absent in AMLs with inv(3)/t(3;3) at the 3'end of *EVI1* is unclear. It is possible that the *RPN1* regulatory regions can only act as an enhancer to the *EVI1* promoter and not to the *MDS1* promoter. This lack of activity could be caused by the absence of the required combinations of transcription binding motifs in the *MDS1* promoter and *RPN1* enhancer. Another theory could be, that the *MDS1* promoter is simply to far from the 3' region of *EVI1* (~1 Mbps). However, it has now been well established that by looping of the DNA promoters can be co-regulated, even when they are separated over a fairly long distance. Therefore, this latter explanation does probably not hold. Array-CGH on a patient sample with a proven 3' breakpoint showed evidence of copy number loss of part of the *MDS1* locus (data not shown). As this was a single case, we propose that, more AML cases 3' *EVI1* breakpoints need to be analyzed using array-CGH to study whether *MDS1* is indeed frequently deleted in those samples. Analysis of patient samples using tiling arrays that cover the *MDS1, EVI1* and *RPN1* loci that we have generated should help to solve this issue in detail.

The fact that in inv(3)/t(3;3) AMLs with aberrant *EVI1* expression, the *RPN1* locus is always involved points to an important role for this particular locus to activate *EVI1* expression. We assume that, when *EVI1* translocates to the *RPN1* locus or the other way around, this *RPN1* locus contains the right enhancer at the right time and place in the right cell. This is underlined by the fact that in human AML with a chromosome 1p36 translocation, the *PRDM16* gene, which is highly homologous to *EVI1* (*PRDM3*), translocates to *RPN1* on 3q21 as well. In fact, as reported in chapter 2, we found three other very rare translocations involving chromosome 3q21, in which the translocated locus is known to contain a *PRDM/EVI1* homologue, i.e. chromosomes 4q21, 6q21 and 11p15, harbouring *PRDM8, 1* and *11* respectively. FISH and Q-PCR should reveal whether in those situations these *PRDM* genes were involved in the translocation and whether their expression has indeed been altered, which subsequently would make detection of these rare translocations by Q-PCR fairly easy.

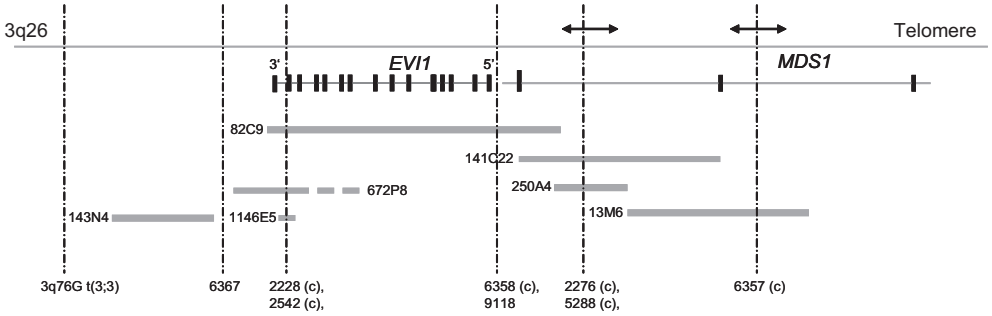

Figure 2. FISH analysis of 3q26 rearrangements.
FISH analysis using RP11 probes depicted aside from each probe. The identified 3q breakpoint location is shown per case depicted with corresponding patient number. All cases carry an inv(3), identified by FISH, i.e. cryptic (c) or by conventional cytogenetic analysis, except 3q76G and 6358, which carry respectively t(3;3) and t(2;3).

IV – Dissociation of MDS1 from EVI1 in inv(3)/t(3;3) AML; transformation by EVI1 and not MDS1-EVI1

The dissociation that occurs between the *MDS1* and the *EVI1* loci in AMLs with inv(3)/t(3;3) causing an overexpression of *EVI1* suggests that *MDS1/EVI1* does not have the same transforming ability as *EVI1*. A previously reported RAT-1 fibroblast *in vitro* soft agar assay has been developed and applied, to study the transforming ability of *EVI1*(14). Using this assay, we addressed the question whether *EVI1* and *MDS1/EVI1* were equally capable to transform RAT-1 cells. Preliminary results from our laboratory showed that in the RAT-1 fibroblast cell system, the transformation capacity of *EVI1* was larger compared to *MDS1/EVI1*. MDS1/EVI1 protein was shown to be as highly as EVI1 was in RAT-1 transduced clones. The numbers of colonies in *EVI1* and *MDS1/EVI1* transfected RAT-1 fibroblast after 14 days plated in soft agar are shown (Figure 3). Others reported that *Evi1* increased the proliferation of immature hematopoietic cells(15-20), and disruption of *Evi1*'s locus impaired proliferation

and enhanced spontaneous apoptosis of murine hematopoietic stem and progenitor cells through *GATA-2* expression(21). Furthermore, EVI1 inhibited programmed cell death in response to a variety of apoptotic stimuli(17, 22, 23), and its ectopic expression interfered with erythroid and granulocytic differentiation(16). The role of *Mds1/Evi1* was not evaluated in those studies. *Evi1* accelerated the cell cycle of murine embryonic stem cells (ES)(15), whereas *Mds1/Evi1* lowered ES cell proliferation(15). Thus, *Evi1* and *Mds1/Evi1* exhibited opposite biological activities as proposed in several reports(15, 24-26). These observations underscore that, not only the activation of *EVI1* expression, but also the dissociation of the *MDS1* locus from the *EVI1* region is required for transformation of AML with inv(3)/t(3;3).

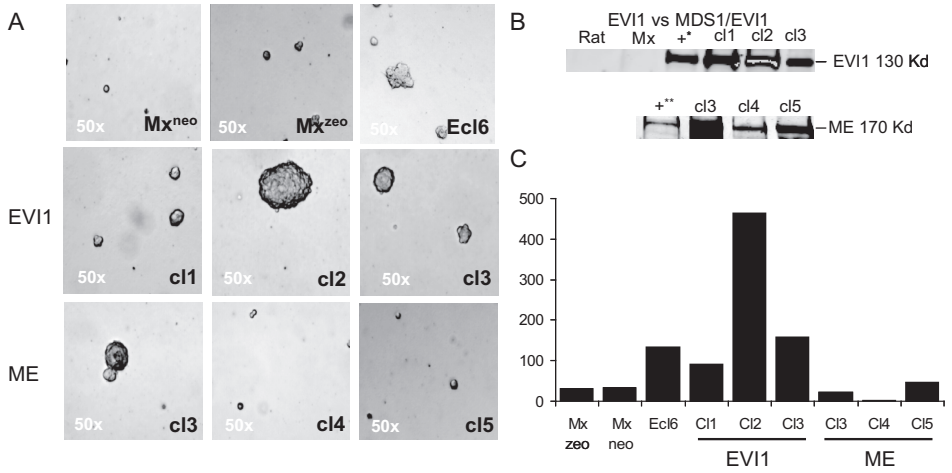

Figure 3. *EVI1* has increased colony formation capacity compared to *MDS1/EVI1* transfected RAT-1 fibroblast single clones. (A) Colonies formed in the *EVI1* and *MDS1/EVI1* (*ME*) transfected single colonies. RAT-1 fibroblasts transfected with P50Mx neomycine and P50Mx zeomycine were used as negative control and the *EVI1*+ and transforming RAT-1 fibroblast clone 6 (Ecl6) as positive control. Western blot analysis (B) of the clones used for the soft agar assay (C). The mean number of colonies of one representative duplo experiment are depicted on the x-axis. *positive control RAT-1 FL(14), a full length *EVI1* transfected RAT-1 clone. **positive control transfected *ME* clone.

V – EVI1 and MDS1/EVI1 expression in AML with 11q23 rearrangements

In a number of AML patients we observed high *EVI1* levels, which were not associated with aberrations in the 3q26 locus, but frequently with 11q23 rearrangements. Moreover, in those cases, besides high *EVI1* levels, high *MDS1/EVI1* transcript levels were observed as well. Since the expression of *EVI1* and *MDS1/EVI1* is not caused by 3q26 defects, the presence of high transcript levels has to be the result of altered transcription regulation, possibly by MLL-fusion proteins that are the result of 11q23 rearrangements.

To understand how the expression of *EVI1* and *MDS1/EVI1* are regulated in those leukemias, insight should be obtained about the two promoter regions, i.e. what are critical motifs in the promoters, how is expression of *EVI1* and *MDS1/EVI1* regulated and what could be the role of MLL-fusion proteins? We have strong evidence that the *MDS1* and *EVI1* promoters, which are ~1Mbps separated from each other, are co-regulated. In leukemia without a 3q26 abnormality either both transcripts are present or both are absent. In normal bone

marrow CD34+ cells both transcripts are detectable and they decline coordinately with differentiation. In a small number of leukemias where we found that the *MDS1* promoter was methylated, we observed the *EVI1* promoter was methylated as well (data not shown). Finally, in CD34/CD38 sorted fractions of *EVI1* expressing *MLL*-rearranged leukemias we found co-expression of *MDS1/EVI1* in each fraction, i.e. in CD34+/CD38-, CD34+/CD38+, CD34-/CD38+ and CD34-/CD38- cells (data not shown). We hypothesize that the promoters may be controlled by the same combinations of transcription factor complex(es) and that advanced motif analysis, genome alignments across species and 3C/4C experiments will be valuable to understand this complicated regulation of transcription.

The question remains why *MDS1/EVI1* and *EVI1* are so highly expressed in certain *MLL*-fusion gene transformed AMLs and not in other AMLs (chapter 2 and 3). In some leukemias it may simply be a reflection of the expression pattern in normal hematopoietic progenitors. A low percentage of normal marrow hematopoietic stem and progenitor cells express *EVI1* and *MDS1/EVI1* (*ME*). It is possible that in certain cases these *EVI1+/ME+* cells are transformed by *MLL*-fusion genes. In that case, *EVI1+/ME+* expression in these AMLs is not abnormal. However, as we demonstrate in chapter 6, *EVI1* (also *MDS1/EVI1*; data not shown) remains high in more mature cells, which are normally *EVI1* negative. We hypothesize that MLL-fusion protein may transform myeloid precursors, that are initially *EVI1+/ME+* or *EVI1-/ME-*. We hypothesize that in case a cell was already *EVI1+/ME+* at the time of transformation, the MLL-fusion proteins can maintain this high expression, even when a cell partially differentiates into a cell that should normally become $EVI1^{-}/ME^{-}$. To prove this hypothesis, mouse bone marrow transfection experiments should be conducted in which prior to *MLL-AF9* transduction, the cells are fractionated by sorting into either *EVI1+* (LSKs) or $EVI1^{-\text{ or }low}$ (CMP, GMP) precursor fractions. We hypothesize that the *MLL-AF9* transformed *EVI1+/ME+* clones will be generated from the LSK fractions and will maintain positive even when the cells partially differentiate. *MLL-AF9* transformed cells from the other sorts will mostly be negative for *EVI1* and *MDS1/EVI1*. Another issue to be addressed is, how *EVI1* and *MDS1/EVI1* expression is maintained so high in those transformed cells and what the role of MLL-fusion oncoprotein is herein? MLL-AF9 chromatin immunoprecipitation (ChIP) followed by promoter Chip hybridizations or deep-sequencing in combination with *EVI1* and *MDS1* promoter-reporter assays should reveal whether there is evidence for a direct role of MLL-fusion transforming protein in *EVI1* and *MDS1/EVI1* transcriptional control in these cells.

VI – Functional difference between EVI1 and MDS1/EVI1

We demonstrated that in inv(3)/t(3;3) AML EVI1 and not MDS1/EVI1 is critical for transformation. High expression of both genes have been reported in *MLL*-fusion gene transformed cells, as well as in experimental models (chapter 6). Replating abilities as determined in an *in vitro* self-renewal assay of cancer stem cells, could be inhibited by knocking down EVI1 and MDS1/EVI1. Thus, the role of EVI1 and in particular MDS1/EVI1 in MLL-rearranged leuke-

mic transformation has to be, at least partially, different from transformation in inv(3)/t(3;3) AML.

The two proteins differ from each other by the absence (EVI1) or presence (MDS1/EVI1) of an N-terminal PR domain(27). The PR domain is highly similar to the SET domain, a domain that is characteristic for the histon methyltransferase superfamily of proteins(28). This family consists of many different members including e.g. SUV39 or G9a (histone H3K9 methyltransferase), proteins which have been demonstrated to interact with EVI1. Interestingly, another protein with a SET domain is MLL itself, which has H3K4 histone methyltransferase activity(28-30).

MLL possesses histone methyltransferase activity (H3K4) through the SET domain(31), forming a complex that associates with the promoter of target genes such as *HOXA9*. The SET domain is lost in *MLL* fusions, emerging another mechanism the acquisition of alternative unique histone methyltransferase activity by partner proteins(32). It is possible that in t(11q23) AML cases expressing *EVI1* and *MDS1/EVI1*, the PR alias SET domain of the latter one is taking over the function of the MLL-SET domain, thereby maintaining gene activation through H3K4. ChIP-chip or ChIP-seq experiments should reveal whether *MLL* target genes are also bound by MDS1/EVI1 and whether the presence of MDS1/EVI1 affects histon modification such as H3K4 methylation on putative target genes, e.g. *HOXA9* or *MEIS1*. Protein interaction studies should be carried out to investigate whether MLL-fusion proteins form an active complex with MDS1/EVI1.

VII – Methylation mechanisms in EVI1 AML

In this thesis, genome wide methylation profiling was carried out on a large cohort of AML patient samples. Ideally, this would give insight into the genes that are aberrantly methylated in specific AML subtypes. What is the definition of aberrant? In chapter 7 we compared the methylation signatures of CD34+ normal bone marrow control samples to those of the complete cohort of AML patient samples. We identified a set of 45 genes that was almost always differentially methylated in any AML. Many of those appeared to be silenced and may thus function as tumor suppressor genes in AML. The question is, how to proceed and study the role of these genes in myeloid development? Which genes should we focus on? The fact that, we only identified 45 genes methylated, that in essence may represent tumor suppressor genes, should make it possible to rapidly screen the effect of knocking down each of those in appropriate hematopietic models *in vitro*.

Besides these 45 genes identified, the relevance of genes selectively methylated per AML subgroup may be of interest as well. AML groups with recurrent abnormalities such as translocations t(8;21), inv(16)/t(16;16) or t(15;17), but also leukemias with *CEBPA* double mutations, each carried a unique methylation signature. These findings suggest that certain methylated genes, particularly associate with certain AMLs with unique molecular abnormalities. It may

also point to unique mechanisms of DNA methylation in the different molecularly defined AMLs. In this thesis we therefore we focused on *EVI1* AMLs solely (chapter 8).

We particular studied the methylation patterns in these *EVI1* AMLs, and wondered how methylation of specific sets of genes may occur in these AML. We showed that *EVI1* AMLs express a unique methylation signature, that separates them from other AMLs and from normal marrow CD34+ cells. Since DNMT3A can bind to EVI1 and EVI1 binding motifs highly enriched in the methylated genes, we hypothesize that DNMT3A may be recruited to putative EVI1 target genes. DNA methylation at the dinucleotide CpG in regulatory regions is a hallmark of stable transcriptional silencing(33). Major epigenetic modifications also include other processes, such as histone de-acetylation and methylation which are often closely coupled to DNA methylation(34) and recruitment of transcription factor repressor complexes. EVI1 has been shown to interact with histone de-acetylases (HDACs), as well as to histone methyl transferases, SUV39H1 or G9a. Moreover, it has been demonstrated that. EVI1 binds to MBD3 a member of the histone deacetylation complex (NuRD) and to CtBP1, an interaction that leads to strong transcriptional repression of target genes. Thus, our data point to a central role for EVI1 in epigenetic silencing.

EVI1 expressing AMLs are very difficult to cure and therefore these patients may benefit from novel tailored forms of treatment. Knockdown of *EVI1* in cell line models showed that proliferation of those leukemias could be abolished almost completely (chapter 6). These findings point to a critical role of EVI1 in proliferative behavior of those tumors. Improving our knowledge of the function of EVI1 and how it interacts with each of the earlier mentioned partner proteins is invaluable for the development EVI1 specific drugs.

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NEDERLANDSE SAMENVATTING

Leukemie of bloedkanker is een ziekte van het beenmerg, de plaats waar bloedcellen worden aangemaakt. Er zijn veel verschillende typen bloedcellen, welke allemaal een specifieke functie in het lichaam hebben. Bij leukemie heeft één cel type de overhand genomen en de andere (gezonde) cellen verdrongen. In dit proefschrift is onderzoek gedaan naar een bepaalde vorm van leukemie, namelijk acute myeloïde leukemie (AML). Cellen die zich eigenlijk zouden moeten ontwikkelen tot granulocyten kunnen dat niet meer, vermenigvuldigen zich ongeremd en overwoekeren het beenmerg en bloed. Eigenlijk is AML niet één ziekte maar een verzameling van aandoeningen. Wanneer we naar de cellen kijken onder de microscoop ziet de ziekte er vaak wel hetzelfde uit, maar op basis van andere eigenschappen blijken er grote verschillen te zijn. Hierdoor zijn sommige patiënten beter te behandelen dan anderen. AML wordt onder andere veroorzaakt door fouten in het DNA (mutaties). In de afgelopen twee decennia is er veel onderzoek gedaan naar deze genetisch afwijkingen in AML. Wij weten nu dat sommige mutaties geassocieerd zijn met een goede prognose, terwijl bij andere afwijkingen de vooruitzichten minder goed gesteld zijn. Het is daarom voor de behandeling van AML belangrijk om de verschillende vormen van deze ziekte goed te kunnen onderscheiden. In het verleden werd AML voornamelijk onderverdeeld op basis van morfologisch eigenschappen (microscopisch onderzoek) en chromosomale afwijkingen (cytogenetisch onderzoek) van de leukemie cellen In de nieuwe classificatie van de "World Health Organization" (WHO) worden zowel cytogenetische als moleculaire methoden (gen mutatie en gen expressie onderzoek) succesvol gecombineerd. Echter het einde is nog lang niet in zicht en de kennis over de genetische afwijkingen in AML neemt nog steeds toe. De indeling van AML patiënten zal dus ook in de nabije toekomst steeds verder verbeteren.

Het doel van dit proefschrift was om de moleculaire inzichten van bepaalde groepen AML patiënten verder te vergroten. De nadruk van het onderzoek lag bij patiënten met afwijkingen op de lange arm van chromosoom 3 (3q); zoals AML patiënten met een inv(3)(q21q26.2) of t(3;3)(q21;q26.2); *RPN1/EVI1* afwijking (afgekort inv(3)/t(3;3)). Deze afwijkingen zijn gecorreleerd met hoge expressie van *EVI1*, een oncogen dat gelokaliseerd is op chromosoom 3q26.2. Daarnaast, hebben we onderzoek gedaan naar de AMLs die geen cytogenetisch detecteerbare chromosoom 3q26 afwijkingen bezaten, maar wel een hoge *EVI1* expressie (*EVI1*+ AMLs) hadden. *EVI1* expressie niveaus werd bepaald in grote series patiënten met AML. Het belang om de *EVI1* expressie analyse te includeren in de diagnostische routine van AML patiënten werd onderzocht in de hoofdstukken 2 tot en met 5. In de laatste drie experimentele hoofdstukken (6 tot en met 8) wordt ingegaan op de vraag welke rol EVI1 heeft in AML.

In hoofdstuk 2, hebben we de klinische, cytogenetische en moleculaire karakteristieken van patiënten met verschillende chromosoom 3q afwijkingen vergeleken. Vier groepen van AML patiënten met 3q afwijkingen werden bestudeerd; patiënten met (A) inv(3)/t(3;3), (B)

gebalanceerde 3q26 afwijkingen, (C) gebalanceerde 3q21 afwijkingen, en (D) alle andere 3q afwijkingen. Er werd onderzocht hoe deze verschillende patiënten reageerden op behandeling. Daarnaast werd onderzocht of de nieuw gedefinieerde WHO groep van AML patiënten met een inv(3)/t(3;3) zich onderscheidt van de AML groepen met andere 3q afwijkingen. De *EVI1* expressie waarden van de verschillende 3q groepen werden onderling vergeleken. De inv(3)/t(3;3) patiënten behoren tot een unieke groep, omdat ze; i) vaak één chromsoom 7 hebben verloren, ii) frequent N-*RAS* mutaties hebben en iii) een hoog *EVI1* en laag *MDS1/EVI1* expressie patroon vertonen. *MDS1/EVI1* is een fusie van de twee genen *MDS1* en *EVI1*, die aan elkaar grenzen op chromosoom 3q26. De aanwezigheid van een inv(3)/t(3;3) was een significant onafhankelijke prognostische parameter, en voorspelt een lage kans op complete remissie en een verminderde overlevingskans. Ook de groep patiënten met AML met resterende 3q afwijkingen (groep D) toonde een ongunstige overleving. De slechte prognose van deze laatste groep kon deels verklaard worden door de aanwezigheid van andere chromosomale afwijkingen, vooral het zogenaamde complexe karyotype, waarvan bekend is dat het een slechte respons op therapie voorspelt. In deze groep werden ook regelmatig verborgen inv(3)/t(3;3) afwijkingen gevonden. Deze afwijkingen konden worden aangetoond door middel van chromosomale fluorescentie *in situ* hybridisatie (FISH), waarmee specifiek *EVI1* op chromosoom band 3q26, *RPN1* op chromosoom band 3q21 en veranderingen in het patroon van deze twee genen zichtbaar kunnen worden gemaakt.

In hoofdstuk 3 onderzochten we de prognostische waarde van de expressie van *EVI1* en *MDS1/EVI1* mRNA transcripten in AML. De expressie van vijf verschillende *EVI1* varianten werd bepaald. Behalve variant *EVI1-1C*, kwamen alle varianten hoog tot expressie bij *EVI1*+ AML. AML patiënten met zowel hoge *EVI1* als *MDS1/EVI1* expressie, bleken vaak een chromosoom 11q23 translocatie te hebben. Bij deze translocaties wordt het *MLL* gen, dat op chromosoom 11q23 ligt, gefuseerd is met het gen van het getransloceerde chromosoom. AML patiënten met hoge *EVI1* en *MDS1/EVI1*, toonden een enigszins gunstigere overleving dan de patiënten met hoog *EVI1* en laag *MDS1/EVI1* expressie patroon. Deze laatste patiënten behoorden meestal tot de inv(3)/t(3;3) groep. Bij een aantal patiënten zonder cytogenetisch zichtbaar chromosoom 3q26 defect, maar met hoge *EVI1* en lage *MDS1/EVI1* expressie, identificeerden wij verborgen inv(3)/t(3;3) afwijkingen, door middel van *EVI1* specifieke FISH. Hoge *EVI1* expressie manifesteerde zich als een onafhankelijke prognostische factor voor ongunstige overleving in deze groep AML patiënten.

Een zelfde onderzoeksbenadering werd in hoofdstuk 4 gevolgd bij kinderen met AML. In deze studie werd zowel gebruik gemaakt van een genoom brede gen expressie analyse als moleculaire methoden om *EVI1* expressie te meten. De positieve correlatie tussen hoge *EVI1* en *MDS1/EVI1* expressie met 11q23 chromosomale afwijkingen en de trend van ongunstige overleving werd ook bij kinderen met AML gevonden. Bij kinderen met *EVI1*+ AML werden geen (cryptische) chromosomale 3q26 afwijkingen gevonden.

In hoofdstuk 5 hebben we een nieuwe moleculaire test ontwikkeld om de expressie van alle *EVI1* gen varianten in één experiment bij AML te kunnen bepalen. In een groep van ~1.000 AML patiënten werd *EVI1* expressie bepaald. Hoge *EVI1* expressie werd geïdentificeerd als prognostische marker, voor het bereiken van een complete remissie, het voorspellen van de response op therapie en het krijgen van een recidief. Binnen de groepen met een intermediair cytogenetisch risico profiel of met 11q23 chromosomale afwijkingen, hadden patiënten met *EVI1*+ AML een slechtere overleving dan *EVI1*- patiënten. Verder bleken de *EVI1*+ patiënten, die tijdens hun behandeling een allogene stam cel transplantatie hebben ondergaan, een significant betere overlevingskans te hebben dan de patiënten die een autologe stam cel transplantatie of alleen chemotherapie kregen. Deze bevindingen onderstrepen het nut om *EVI1* bepaling op te nemen in de diagnostiek van AML.

In hoofdstuk 6 onderzochten we de klinische, moleculaire, phenotypische en mechanistische verschillen tussen *EVI1*+ en *EVI1*- AML patiënten met een chromosoom 11q23 translocatie (t(11q23)). Bij 40% van de t(11q23) gevallen bleek dat de *EVI1* expressie hoog was. De *EVI1*+ t(11q23) AML patiënten kunnen op basis van de volgende bevindingen worden onderscheiden van de *EVI1*- t(11q23) patiënten: i) ze hebben een verschillend gen expressie profiel, ii) de morfologie van de twee groepen is verschillend, en iii) *EVI1*+ t(11q23) patiënten hebben een ongunstiger ziektebeloop vergeleken met *EVI1*- t(11q23). *MLL-AF9*, het fusie gen dat ontstaat bij translocatie t(9;11), bleek na transfectie beenmerg cellen uit een muis te transformeren tot cellen die zich in kweek (*in vitro*) ongeremd vermenigvuldigen. Hierbij ontstonden zowel *Evi1*$^{+}$ als *Evi1*$^{-}$ getransformeerde klonen. In de *Evi1*$^{+}$ klonen was een kleine maar significante populatie van onrijpe voorloper cellen detecteerbaar, de zogenaamde "common myeloid progenitors". De voorloper cellen van de *Evi1*$^{-}$ klonen waren juist rijper. In de *Evi1*$^{+}$ klonen, was *Evi1* expressie ook aantoonbaar in de meer uitgerijpte cellen. Dit abnormale *Evi1* expressie patroon werd ook waargenomen in cellen van AML patiënten met een *MLL* fusie gen, maar niet in gezonde rijpe beenmerg cellen van een muis of mens. Wanneer *Evi1* werd uitgeschakeld in *Evi1*$^{+}$ *MLL-AF9* cellen, met een "knockdown" techniek, werd de vermenigvuldiging van de getransformeerde cellen weer teruggedrongen. *EVI1* speelt dus een belangrijke rol in de transformatie van een subset van *MLL-AF9* AMLs.

In hoofdstuk 7 en 8 werd onderzoek gedaan naar genoom brede methylering in AML. DNA methylering kan leiden tot de uitschakeling van genen. In kanker cellen kan de expressie van zogenaamde "tumor supressor" genen door methylering worden onderdrukt. In 344 AML patiënten (hoofdstuk 7) werden unieke DNA methylerings profielen gevonden waarmee verschillende AML subgroepen konden worden herkend. AML met bijvoorbeeld *CEBPA* mutaties of een translocatie t(8;21) vertonen ieder een eigen methylerings profiel. Er werden ook vijf AML patiënten groepen onderscheiden die elk een uniek methylerings profiel hadden, maar waar geen bekende overeenkomstige cytogenetische of moleculaire afwijking kon worden aangetoond. Daarnaast, werd een set van 15 gemethyleerde genen onderscheiden, welke in combinatie een voorspellende waarde hadden op de overleving van AML. We con-

cluderen dat DNA methylatie optreedt in een specifieke set van genen in AML. Daarnaast kunnen unieke methylerings patronen worden gedefinieerd bij verschillende AML groepen. Wat de rol is van deze verschillende genen bij leukemie ontwikkeling moet verder worden onderzocht. In hoofdstuk 8 werd aangetoond dat de *EVI1* AML patiënten ook een specifiek methylerings profiel hebben. Dit profiel verschilt sterk van het profiel in vergelijkbare gezonde onrijpe beenmerg cellen. In de promotor regio's van deze afwijkend gemethyleerde genen werden sequenties gevonden waarvan bekend is dat EVI1 daar specifiek aan kan binden. In zogenaamde chromatine immunoprecipitatie experimenten werd de interactie van EVI1 met deze promotoren aangetoond. De complex vorming tussen EVI1 en de DNA methyl transferase DNMT3A die wij vervolgens aantoonden suggereert dat, EVI1 het enzym DNMT3A rekruteert naar deze specifieke promotor regio's. We veronderstellen op basis van deze bevindingen dat deze interactie kan leiden tot methylering van deze promotoren.
In de afsluitende discussie in hoofdstuk 9 worden de belangrijke bevindingen uit het proefschrift in een bredere context behandeld en in perspectief geplaatst.

DANKWOORD

Allereerst wil ik mijn promotor Bob Löwenberg bedanken. Ik weet nog goed, dat ik in de Daniel den Hoed op gesprek ging en jij met vertelde dat ik maar eens moest gaan praten met Ruud Delwel, aangezien hij ook in Memphis was geweest. Vanaf dat moment is de bal gaan rollen en ben ik begonnen als AIO in het lab. Bedankt voor je steun, betrokkenheid, visie en samenwerking in alle projecten. Het is een waar genoegen om op afdeling hematologie onder jouw leiding te hebben gewerkt.

Dan kwam er op het nippertje nog een promotor bij; Ruud Delwel. Ruud, ik had me geen betere begeleider kunnen wensen, samenwerken met jou was top! Ik liep vaak stormend je kantoor binnen, en jij had altijd tijd. Bedankt voor je gezelligheid, je enthousiasme, je drive en je schrijf input. Ik hoop in de toekomst nog veel van je te horen. Straks bij jouw oratie zit ik op de eerste rij, bijkletsen met Ferdi, dat wordt een mooi feestje.

De leden van de kleine commissie; prof.dr. Riccardo Fodde, prof.dr. John Foekens en prof.dr. Rob Pieters, dank voor het completeren. Rob, dankzij jouw contacten met Bill Evans, heb je me de mogelijkheid gegeven om een jaar als student naar Memphis te gaan. Zoals je toen zei, "Memphis stelt niet veel voor"; ik had het voor geen goud willen missen. St. Jude Children's Research Hospital heeft een speciale plek gekregen in mijn herinneringen, dank hiervoor.

Prof.dr. Ivo Touw en prof.dr. Hartmut Döhner, Berna Beverloo en Peter Valk; "Vielen Dank", het is een waar genoegen om jullie in de grote commissie te hebben.

Berna, de FISH analyses waren niet zo goed gelukt als ik niet via jou, samen met Ellen van Drunen achter de microscoop was gekropen. Mijn dank is groot, ook voor de goede samenwerking op het 3q project in H2, ik heb met plezier veel over de cytogenetica geleerd. Ellen, ik geloof dat ik zeker een keer langs rij bij je in Frankrijk voor een wijntje.

Peter Valk, de IDH's vliegen nog om mijn oren, dank voor het samenwerken op dit project en voor je feedback tijdens werkbesprekingen. De dames van de diagnostiek, in het bijzonder Chantal Goudswaard, bedankt voor het inzetten van de EVI1 Q-PCRs.

De ideale nauwkeurige analist, die zelfs met EVI1 Chip-on-ChIP overweg kan, Claudia Erpelinck, dank voor al je pipetteer werk en de vele PCRs voor het EVI1 methylatie project en MLL project, je bent een goede steun voor de Delwel groep, daar Ruud altijd zegt; "vraag maar aan Claudia, die weet dat wel".

Eric Bindels, de andere vraag-baak van het lab, ik wens je het beste toe met al het EVI1 onderzoek, maar met Marije Havermans erbij moet dit zeker gaan lukken. Dank voor al je advies, tips en feedback. De anderen in de groep van Ruud Delwel; Irene Louwers, Lucila Solerno, Erdogan Taskesen, bedankt voor jullie gezelligheid.

Our collaborators from Cornell Medical College in New York City; Ari Melnick, thank you for giving me the opportunity to work in your laboratory and see how methylation research is done overseas. I enjoyed my time in NYC and gained experience in methylation research and bioinformatics. Kena Figueroa, I still remember our time in the lab, always on R project

on the so beloved Apple computers. Your determination and enthusiasm kept me going. I am positive you'll be a good PI. The remaining Melnick lab; Leandro Cerchietti and Rita Shaknovich, thanks for the laugh and good fun during Halloween in the lab.
De fellow Ph-D studenten; Saman Abbas, Su Ming Sun, Sophie Corthals, Annemarie Meenhuis, Karishma Palande, Elnaz Farahbakhshian, Renee Beekman, Eric Vroegindeweij, Simone Talens, Mathijs Sanders, Rastislav Horos, Kerim Hoorweg, allen veel succes met het afronden van jullie promotie. Bedankt voor de leuke tijd in het lab.
Resterende (ex-) collega's van de 13e kan ik natuurlijk niet vergeten om te bedanken voor hun hulp, praatje en plezier; Menno Dijkstra en Onno Roovers; lab-dag rules, Marieke von Lindern, Stefan Erkeland, Mojca Jongen-Lavrencic, Annemiek Broyl en Jurgen Haanstra.
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De secretariële ondersteuning op de afdeling hematologie was altijd behulpzaam en ook voor mijn staven snel; Ans Mannens en Monique Mes, we Skypen!, Jeanne Vlasveld en Nathalie van Dommelen. Egied Simons verrichtte mooi werk door van 9 Word bestanden en veel Powerpoint presentaties een geheel proefschrift te maken, voor u ligt het resultaat.
Mijn twee paranimfen, Marije Havermans en Manon van der Velde, bedankt dat jullie samen met mij deze dag tot een succes willen maken. Marije, ik wil jouw speciaal bedanken voor al je werk en vele colony assays, muizen proeven, en je altijd enthousiaste humeur, ook wanneer er wel eens niet iets goed ging, wat zeer zelden het geval was. Je bent een slimme meid en ik weet zeker dat je het EVI1 onderzoek samen met Eric een stap verder zal brengen! Manon, ik vind je een schat! Wie had ooit kunnen denken dat we als huisgenoten, totaal vreemden van elkaar, 5 jaar later zulke goede vriendinnen zijn!
Na het leven als onderzoeker achter een computer in een 'grijs' lab, hetgeen ik zeker mis, werk ik nu met een leuke groep arts-assistenten in het IJsselland Ziekenhuis, ook hen wil ik bedanken, daar zij mij door mijn eerste periode als onwettende arts-assistent hebben heen gesleurd, en me veel praktische kennis hebben aangeleerd.
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CURRICULUM VITAE

Sanne Lugthart werd geboren op 25 oktober 1980 in Rotterdam. In september 1999, na het afronden van het Voorbereidend Wetenschappelijk Onderwijs aan het Merewade College in Gorinchem, startte ze met de studie Geneeskunde aan de Erasmus Universiteit (Erasmus MC) te Rotterdam. Als doctoraal afstudeeronderzoek deed zij via prof. dr. R. Pieters 12 maanden onderzoek naar effect van chemotherapie resistentie bij kinderen met acute lymfatische leukemie op gen expressie en overleving, onder de supervisie van dr. William E. Evans aan het St. Jude Children's Research Hospital in Memphis (VS). In mei 2006 na het behalen van het artsexamen begon zij als promovendus in de groep van prof. dr. H.R. Delwel op de afdeling Hematologie van het Erasmus MC (promotoren prof. dr. B. Löwenberg en prof. dr. H.R. Delwel). Aldaar vond het onderzoek beschreven in dit proefschrift plaats. Tijdens haar promotieonderzoek in 2008 heeft Sanne reeds zes maanden in New York (VS) te Cornell Medical College onderzoek gedaan naar methylatie profielen in patiënten met acute myeloïde leukemie in de groep van dr. A. Melnick. In mei 2010 begon zij aan het Erasmus MC de opleiding tot internist (prof. dr. J.L.C.M van Saase).

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ABBREVIATIONS

AML	Acute myeloid leukemia
ALL	Acute lymphoblastic leukemia
ATRA	All-*trans* retinoic acid
BAALC	Brain and acute leukemia gene, cytoplasmic
BAC	Bacterial artificial chromosome
BMT	Bone marrow transplantation
CBF	Core binding factor
CBFB	Core binding factor beta gene
CEBPA	CAAT/enhancer binding protein alpha gene
CGH	Comparative genomic hybridization
CLP	Common lymfoid progenitors
CML	Chronic myeloid leukemia
CMP	Common myeloid progenitor
CtBP	C-terminal binding protein
CR	Complete remission
DFS	Disease-free survival
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
EFS	Event-free survival
ERG	v-Ets erythroblastosis virus E26 oncogene homolog gene
EVI1	Ecotrpoic viral integration site 1
FAB	French-American Britisch
FISH	Fluorescent in situ hybridization
FLT3-ITD	FMS-like tyrosin kinase 3 (gene) internal tandem duplication
FLT3-TKD	FLT3-tyrosine kinase domain
GEP	Gene expression profiling
GMP	Granulocytic myeloid progenitor
HDAC	Histone deacetylase
HELP	HpaII Enriched Ligated mediate PCR assay
HOVON	'Stichting Hemato-Oncologie voor Volwassenen Nederland'
HSC	Hematopoetic stem cells
IDH1	Isocitrate dehydrogenase 1
KRAS	v-ki ras2 Kirsten rat sarcoma viral oncogene homolog
MBD3	Methyl-CpG binding domain 3
MDS1	Myeloid dysplastic syndrome gene 1
MEP	Megakaryocyte erythrocyte precursor
MLL	Myeloid/lymfoid or mixed lineage leukemia
MLL-PTD	MLL partial tandem duplication
MN1	Meningioma 1 gene
MP	Methylation profiling
MYH11	Myosin, heavy chain 11 gene
NPM1	Nuceophsomin 1
NRAS	Neuroblostoma RAS viral (v-ras) oncogene homolog
OS	Overall survival
PCR	Polymerase chain reaction
PR domain	Prositive regulatory domain
PML	Promyelocytic leukemia
Q-PCR	Quantitative real-time reverse transcription PCR
RARA	Retinoic acid receptor, alpha gene
RPN1	Ribophorin 1 gene
RNA	Ribonucleic acid
RFS	Relapse-free survival
SKY	Spectral karyotyping
SNP	Single nucleotide polymorphism
SMAD3	Similar mothers against decapentaplegic homolog 3 gene
SUV39H1	Histone H3 lysine 9-specific histone methyltransferases H1
WBC	White blood cell count
WT1	Wilm's tumor 1
WHO	World heath organization

PHD PORTFOLIO

Name PhD student: Sanne Lugthart Erasmus MC Department: Hematology Research School: Molecular Medicine (MM)	PhD period: May 2006 – May 2010 Promotors and Supervisors: Prof. B. Lowenberg, Prof. R. Delwel		
1. PhD training			
	Year	**Workload**	
		Hours	**ECTS**
General courses			
- Statistics, Access Course	2007	20	
Specific courses			
- Annual Course Molecular Medicine (MM)	2006		10
- Course Basic and Translational Oncology (MM)	2006		10
- Biomedical Research Techniques (MM)	2006	10	
- The Course SNPs and Human Diseases (MM)	2007		10
- Analysis of microarray gene expression data (MM)	2007		10
- Bioinformatic Analysis, Tools and Services (MM)	2008		10
Seminars and workshops			
- The Workshop Applied Bioinformatics (MM)	2007	10	
- The Workshop 'Browsing Genes and Genomes with Ensembl' (MM)	2007 2009	10 30	
- The 8th International Workshop on Myeloid Stem Cell Development and Leukemia			
Presentations			
- 8 Hematology Presentations	2006-2009	40	
- 3 Journal Club Presentations	2007-2010	30	
- CTMM presentation	2009	10	
(Inter)national conferences			
- Dutch Hematology Congress (oral presentation)	2007	20	
- European Hematology Conference (oral presentation)	2007	30	
- FASEB Hematopoietic Malignancies (poster presentation)	2007	30	
- European Hematology Conference (Fellowship award)	2008	20	
- 50th American Association of Hematology Annual Meeting (oral presentation)	2008 2009	50 20	
- Dutch Hematology Congress (oral presentation)	2009	30	
- European Hematology Conference (oral presentation)	2010	20	
- 51th American Association of Hematology Annual Meeting (oral presentation)			
2. Teaching activities			
- **Lecturing:** Hematology Microscopy Course Medicine Students	2007	20	
- **Other:** Invited Speaker Lunch Hematology , Writing	2007-2008	20	
- Writing Application EHA Fellowship and Application ZON MW AGIKO	2007	40	
Total		460	50

COLOR SECTION

Chapter 1

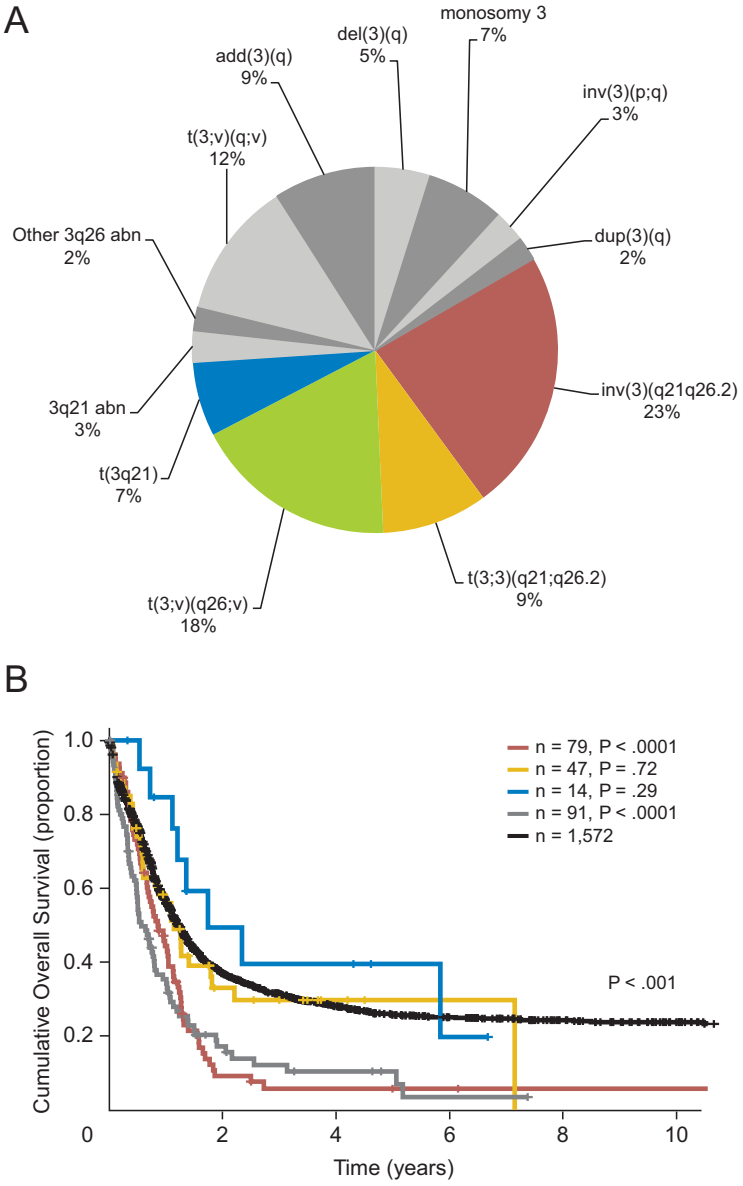


Figure 1. Distribution of cytogenetic characteristics and survival according to 3q aberrant AML groups.
Distribution of 3q abnormalities (all ages, panel A) and overall survival (only 15-60 years of age, panel B) of 3q abnormal AML classified as inv(3)/t(3;3) (group A), balanced t(3q26) (group B), balanced t(3q21) (group C), and remaining 3q abnormalities (group D). A corresponding log-rank P value per comparison to the non-3q cytogenetically abnormal (CA) reference group is shown.remaining 3q abnormalities (group D). A corresponding log-rank P value per comparison to the non-3q cytogenetically abnormal (CA) reference group is shown.

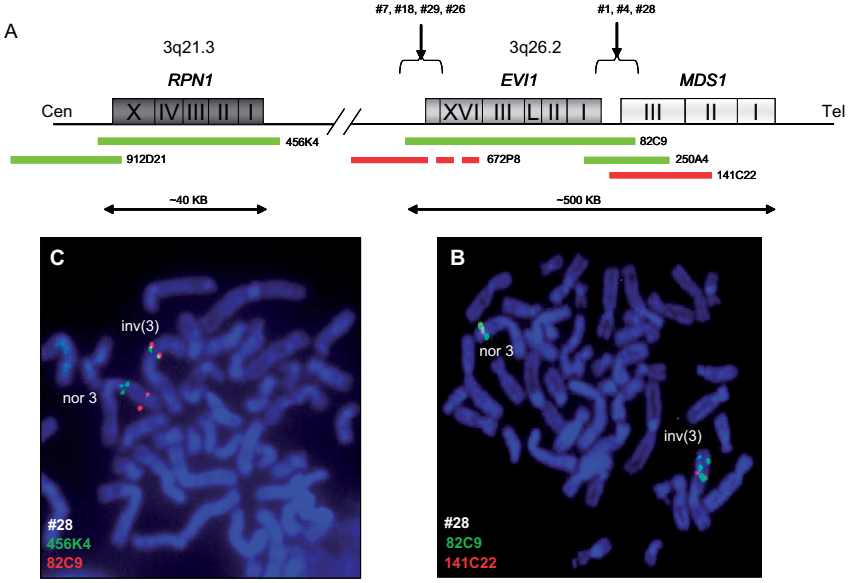


Figure 4. Fluorescent *in situ* hybridisation (FISH) of chromosome 3q26 and 3q21 loci reveal hidden 3q26 aberrations.

BAC clone localization from centromere (Cen) to telomere (Tel) (A). A metaphase from *EVI1*+ patient #28 revealed a cryptic inv(3)(q21q26) (inv3) and a normal chromosome 3 (nor3) using *EVI1* (RP11-82C9) and *MDS1* (RP11-141C22) (B) and *RPN1* (RP11-456K4) BAC clones (C).

Chapter 4

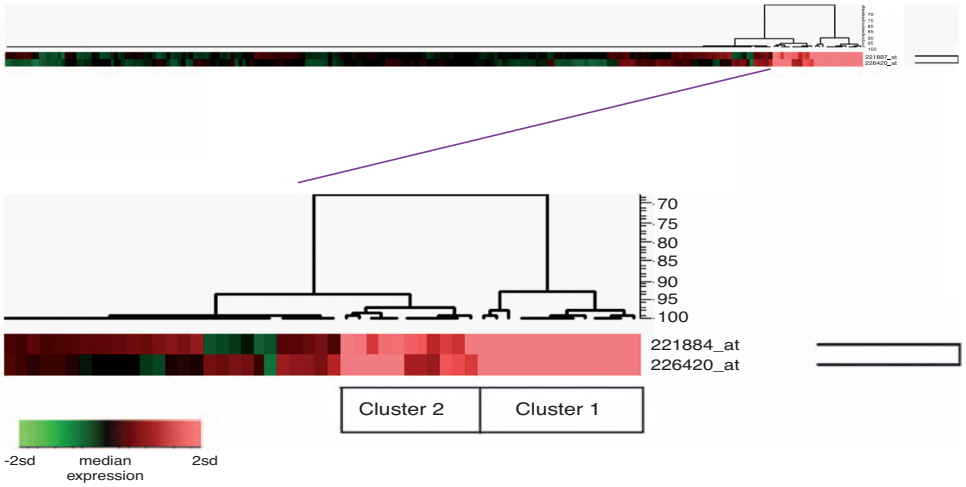


Figure 1. Hierarchical clustering using the gene expression of the 3 probe sets representing EVI1 in 228 pediatric AML samples reveals a subclustering of 24 EVI1+ cases.
Chapter5

Chapter 5

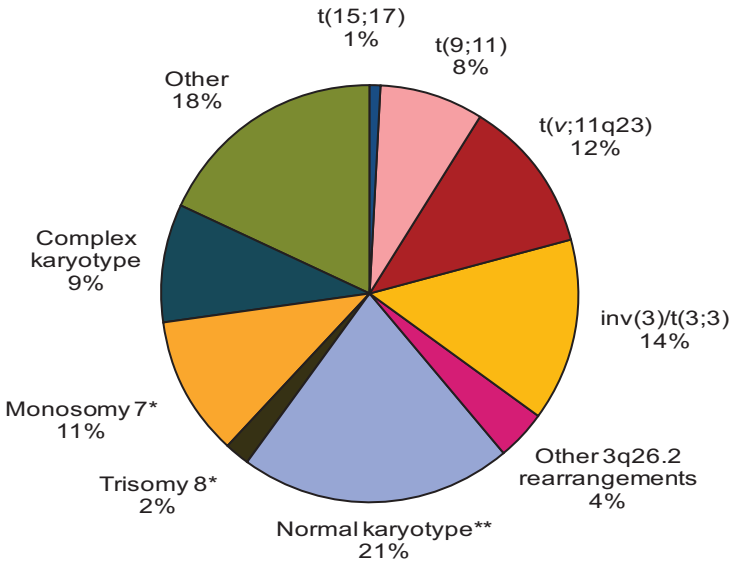
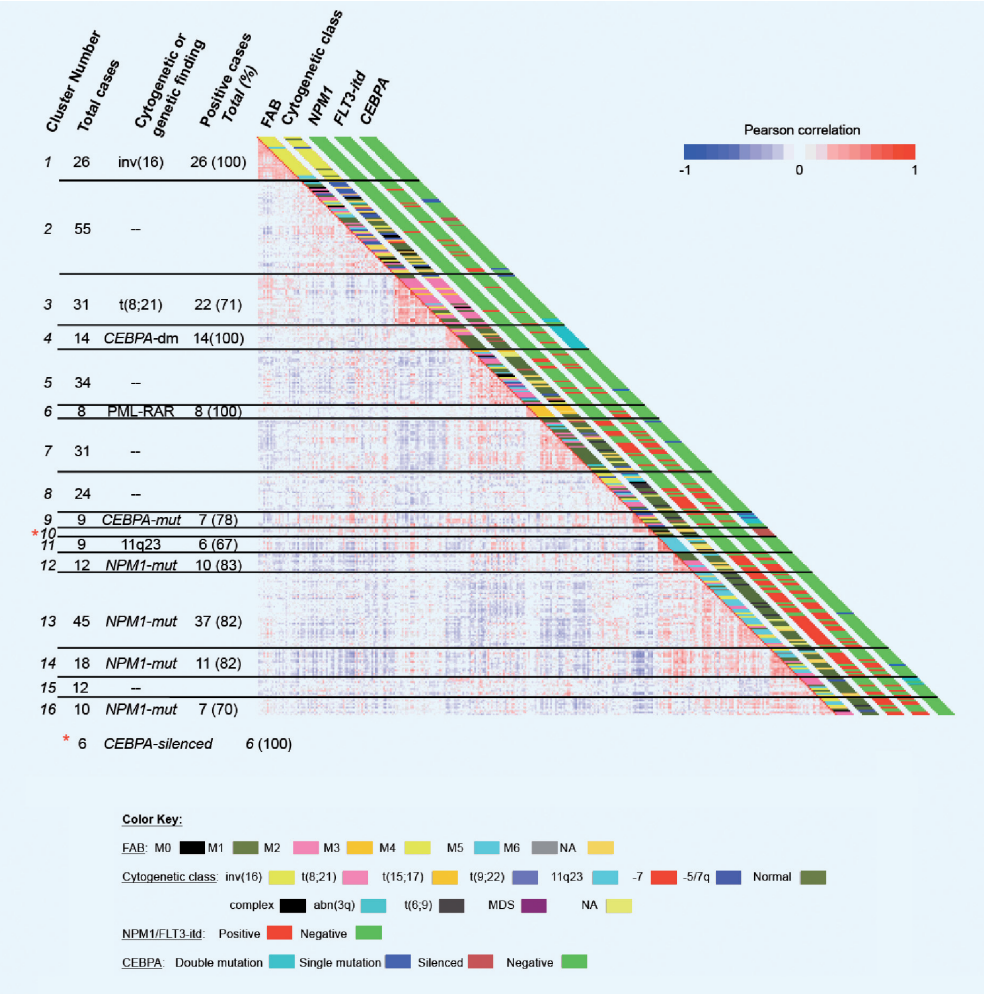


Figure 1. Distribution of cytogenetic abnormalities among *EVI1*+ AML (n=148).
*Occurring in a non-complex karyotype and not associated with inv(3)/t(3;3). **Among the *EVI1*+ with a normal karyotype, 66% of this group carried the *NPM1*^{wt}/*FLT3*-ITD^{neg}/*CEBPA*^{wt} genotype

Chapter 7

Figure 1. DNA methylation segregates AML patients into 16 groups.
Heatmap representation of a correlation matrix in which each patient's DNA methylation profile is correlated with that of the other patients in the dataset. Patients are ordered according to the unsupervised analysis (hierarchical clustering) results, so that highly correlated patients are located next to each other. Parallel bars on the right of the heatmap have been used to indicate the principal cytogenetic and molecular findings for each patient. Cluster membership and cluster feature summaries are described on the left of the heatmap.



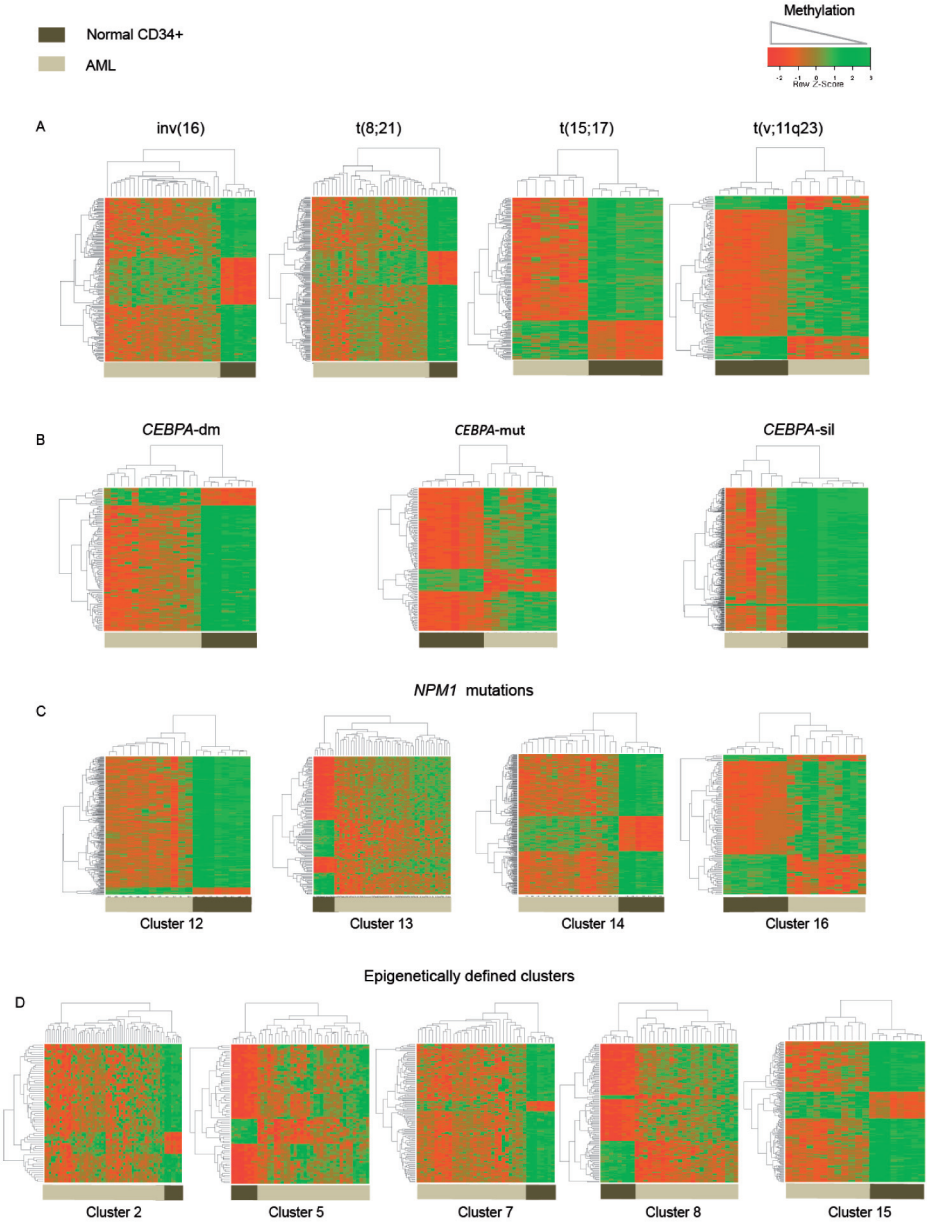
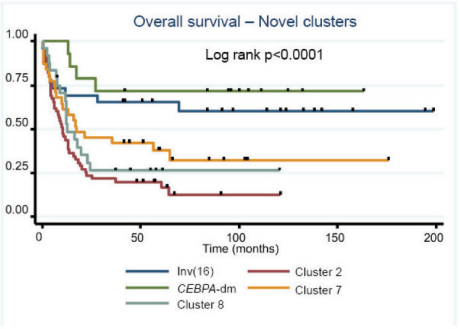
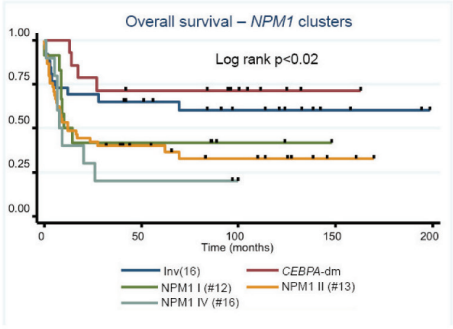
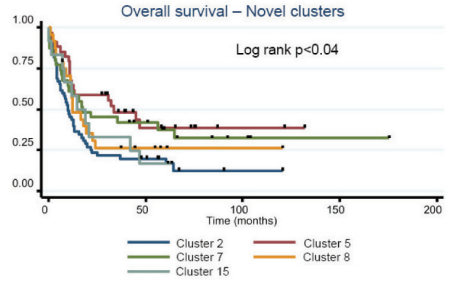


Figure 2. Distinct DNA methylation signatures define each of the 16 clusters.
Heatmap representation of the aberrant DNA methylation signatures of specific clusters compared to a cohort of normal CD34+ hematopoietic cells obtained from healthy donors. Each row of the heatmap represents one probe set of the HELP array, and each column represents an AML patient (denoted by light brown bars) or a healthy donor (denoted by dark brown bars). (A) DNA methylation signatures for clusters with recurrent translocations, (B) DNA methylation signatures associated with abnormalities of *CEBPA*, (C) DNA methylation signatures for clusters presenting *NPM1* mutations, (D) DNA methylation signatures for the 5 epigenetically defined clusters.





B



Variables in Model	P-Value	Hazard Ratio (HR)	95.0% CI for HR	
			Lower	Upper
cluster #2	0.013	3.178	1.271	7.947
cluster #3 (t(8;21))	0.821	1.100	0.482	2.512
cluster #4 (CEBPA-dm)	0.626	0.720	0.192	2.700
cluster #5	0.155	2.035	0.765	5.414
cluster #7	0.050	2.640	1.001	6.961
cluster #8	0.026	3.156	1.146	8.691
cluster #12 (NPM1 I)	0.081	2.998	0.872	10.305
cluster #13 (NPM1 II)	0.002	4.665	1.721	12.642
cluster #14 (NPM1 III)	0.006	4.349	1.527	12.387
cluster #15	0.020	3.598	1.226	10.560
cluster #16 (NPM1 IV)	0.004	5.442	1.743	16.993
Age > 60 years	0.024	1.543	1.059	2.248
Intermediate cytogenetic risk	0.424	0.738	0.351	1.552
High cytogenetic risk	0.915	1.044	0.475	2.294
NPM1 mutation	<0.0001	0.426	0.277	0.655
FLT3-ITD mutation	0.001	1.778	1.255	2.520

Figure 3. DNA methylation captures clinically significant differences among AML patients.
(A) Left: Kaplan-Meier curves for overall survival for the favorable risk clusters 1 (inv(16)) and 4 (*CEBPA*-dm), and the novel epigenetically defined clusters. For plotting simplicity curves for clusters 3 (t(8;21)), cluster 5 and cluster 15 were not included in the plot. Figure S3 shows a Kaplan-Meier plot including all the clusters in the overall survival analysis. Right: Kaplan-Meier curves for overall survival for the favorable risk clusters 1 (inv(16)) and 4 (*CEBPA*-dm), and the *NPM1* clusters. For plotting simplicity curves for clusters 3 (t(8;21)), and *NPM1* cluster 14 were not included in the plot. Figure S3 shows a Kaplan-Meier plot including all the clusters in the overall survival analysis. (B) Kaplan-Meier curves for overall survival (left) for the five novel clusters. On the right: Table summarizing the multivariate Cox proportional hazards regression model, using cluster 1 (inv(16)) as the referent cluster. Additional Kaplan Meier plots are shown in Figure S3.

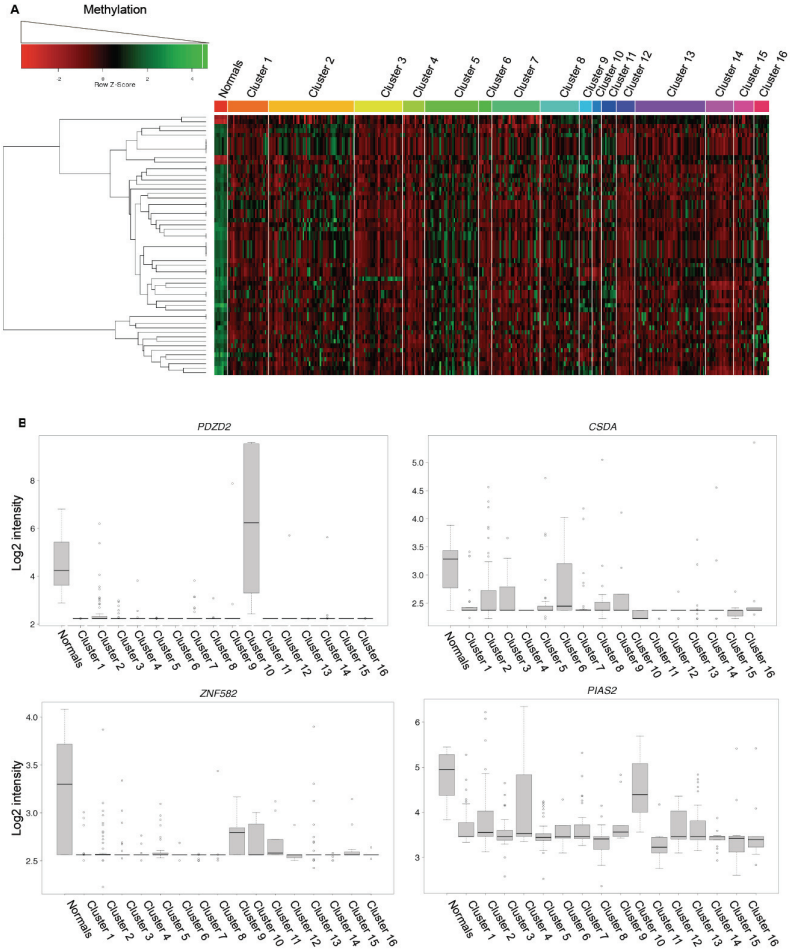


Figure 5. 45 genes are consistently aberrantly methylated in AML.
(A) Heatmap representation of the common 45-gene signature consistently aberrantly methylated in AML. Each row represents a probe set from the HELP microarray and each column represents a sample. (B) Boxplots of gene expression levels in 4 representative genes from the 45-gene common epigenetic signature demonstrating downregulation of expression in the AML samples compared to normal CD34+ cells The list of genes is shown in Table S4.

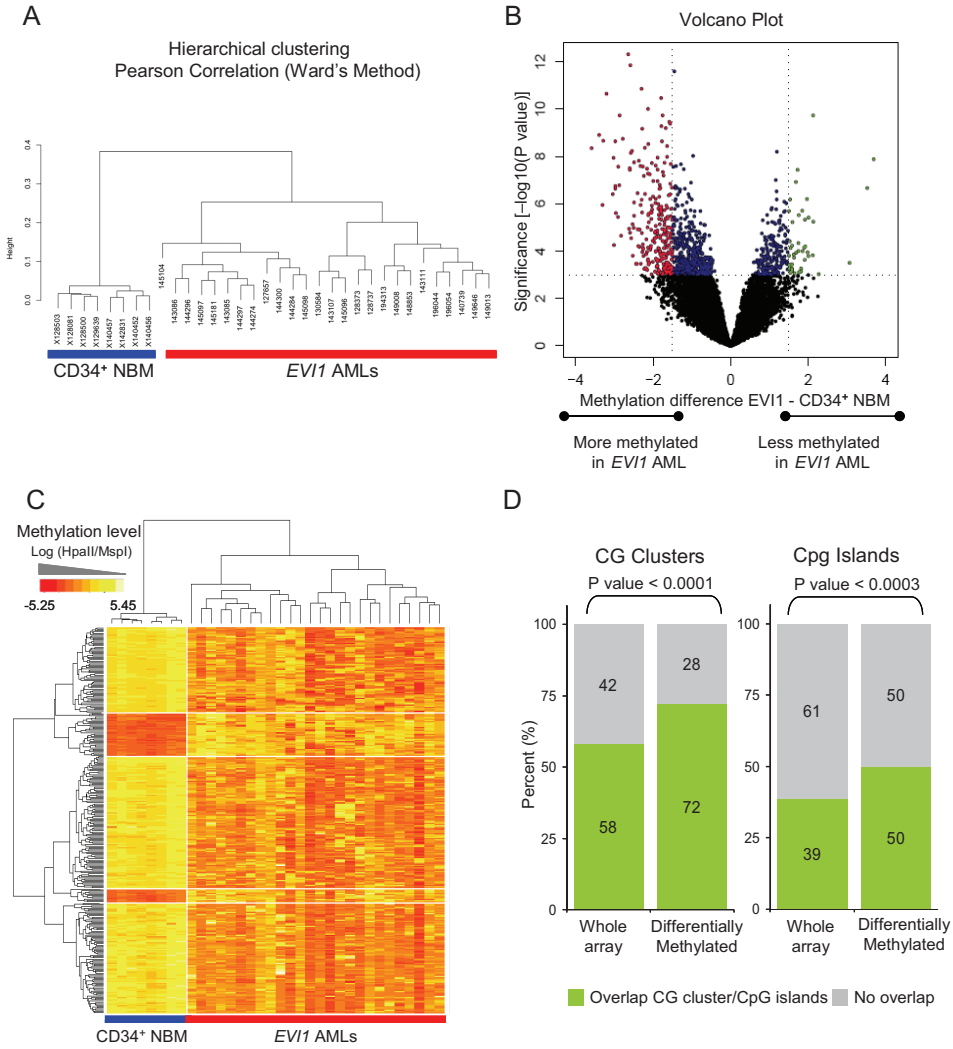


Figure 1. *EVI1* acute myeloid leukemia patients (*EVI1* AMLs) have a unique genome wide methylation profile compared to CD34⁺ normal bone marrow samples (CD34⁺ NBM).

(A) The dendrogram represents a hierarchical clustering (i) and a principal component analysis (ii) in 8 CD34+ NBM blasts and 26 *EVI1* AMLs. (B) The volcano plot shows the methylation difference comparing the 26 *EVI1* AMLs to 8 CD34⁺ NBM samples with corresponding moderated T-test P-value. Probe sets significantly hypermethylated (P<0.001 and methylation difference less than -1.5) are shown in red, probe sets significantly hypomethylated (P<0.001 and methylation difference larger than 1.5) are shown in green. Significant probe sets that did not have an absolute methylation difference larger than 1.5 are depicted in blue. (C) The heatmap shows the methylation levels (Log (HpaII/MspI)) of differentially methylated genes (rows) in *EVI1* AMLs and CD34⁺ NBM cases (columns). (D) The histograms show the percentages of genes containing (green) CG clusters and CpG islands and those not overlap

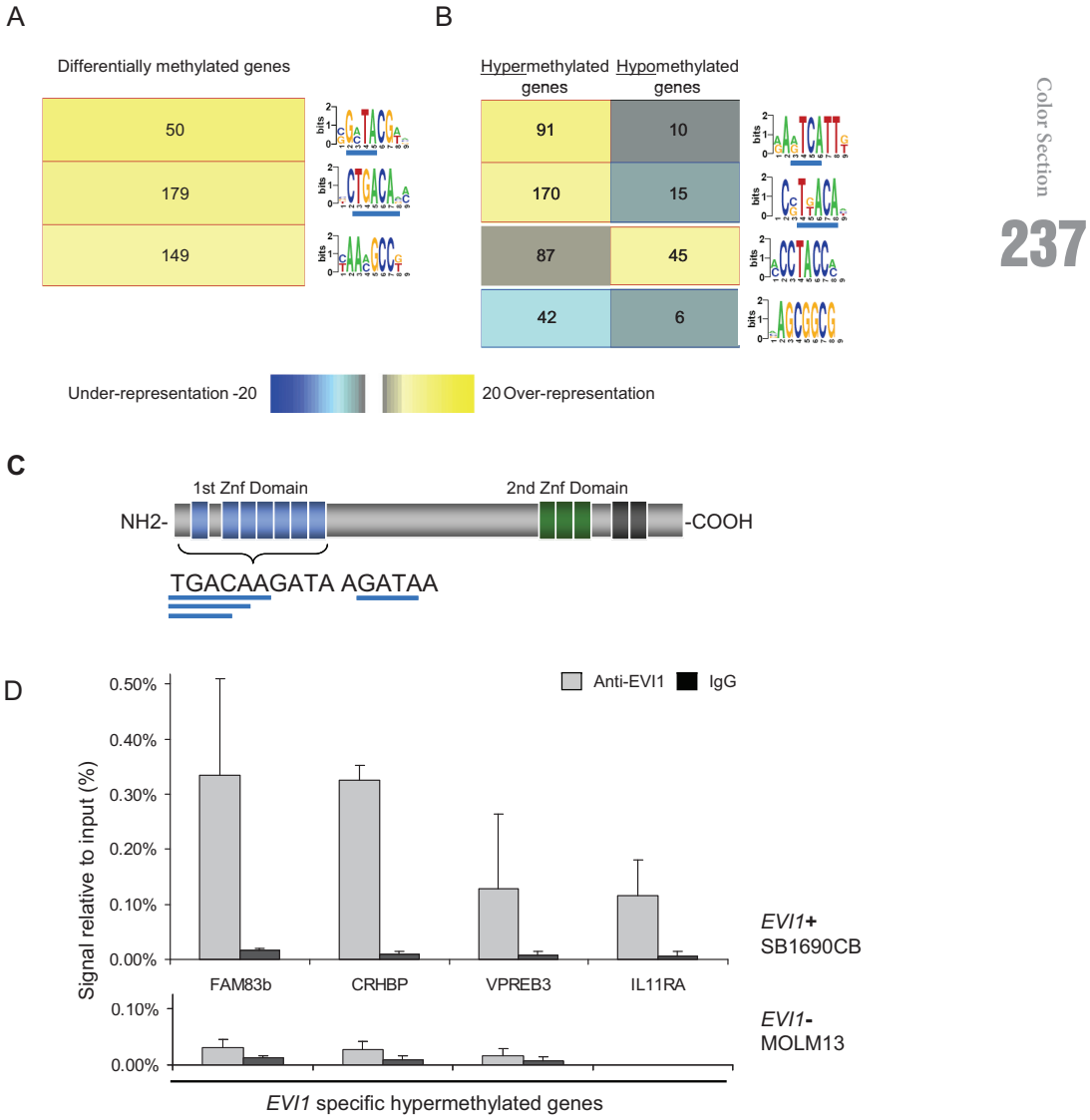


Figure 2. EVI1 binding sites are over-represented in the hypermethylated promoter regions of *EVI1* AMLs and EVI1 binds these hypermethylated promoters in vivo.

(A) Motif analysis of the in *EVI1* AML differentially methylated genes showed a significant overrepresentation (yellow in the heat map color key) of three 7 bp motifs. Per bar each 7 bp optimized motif is shown. The unlined sequences overlap with the first or second EVI1 binding domain. The number of genes that harbored the representative motifs in their promoter sequences are depicted per bar. (B) Further analysis of the hypermethylated and hypomethylated genes reveals two overrepresented motifs in the promoter regions of the hypermethylated genes and one overrepresented in hypomethylated genes. (C) A schematic representation of the EVI1 nuclear zinc-finger protein, with the binding sequence of the first and second EVI1 zinc finger domains. The overlapping motifs over-represented in hypermethylated genes are underlined in respectively blue and green. (D) Quantitative PCR of chromatin immunoprecipitation in the *EVI1* positive (*EVI1*+) SB1960CB cell line and the *EVI1* negative (*EVI1*-) MOLM13 cell line using EVI1 and IgG antibody. Percentage of amount of input material is shown. The mean and standard deviation of three independent experiments is shown.

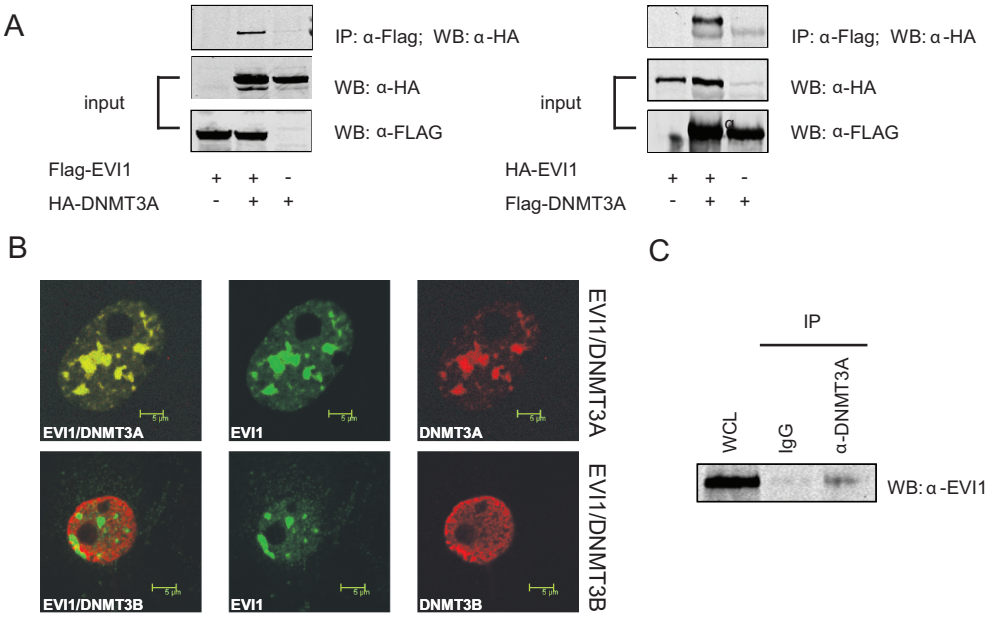


Figure 3. EVI1 interacts with DNMT3A.
(A) Western blot analysis using anti-FLAG antibodies shows the input of the immunoprecipitation of transfected 293T cells and the pulldown using anti-HA. (B) Confocal microscopy of 293T cells transfected with HA-tagged EVI1 (green) and FLAG-tagged DNMT3A and -3B (red). (C) Western blot for EVI1 on lysates from SB1960CB cell-line. The left lane shows the input band; the second and third lane show EVI1 staining following immunoprecipitation with IgG control (second lane) or anti-DNMT3 (third lane).

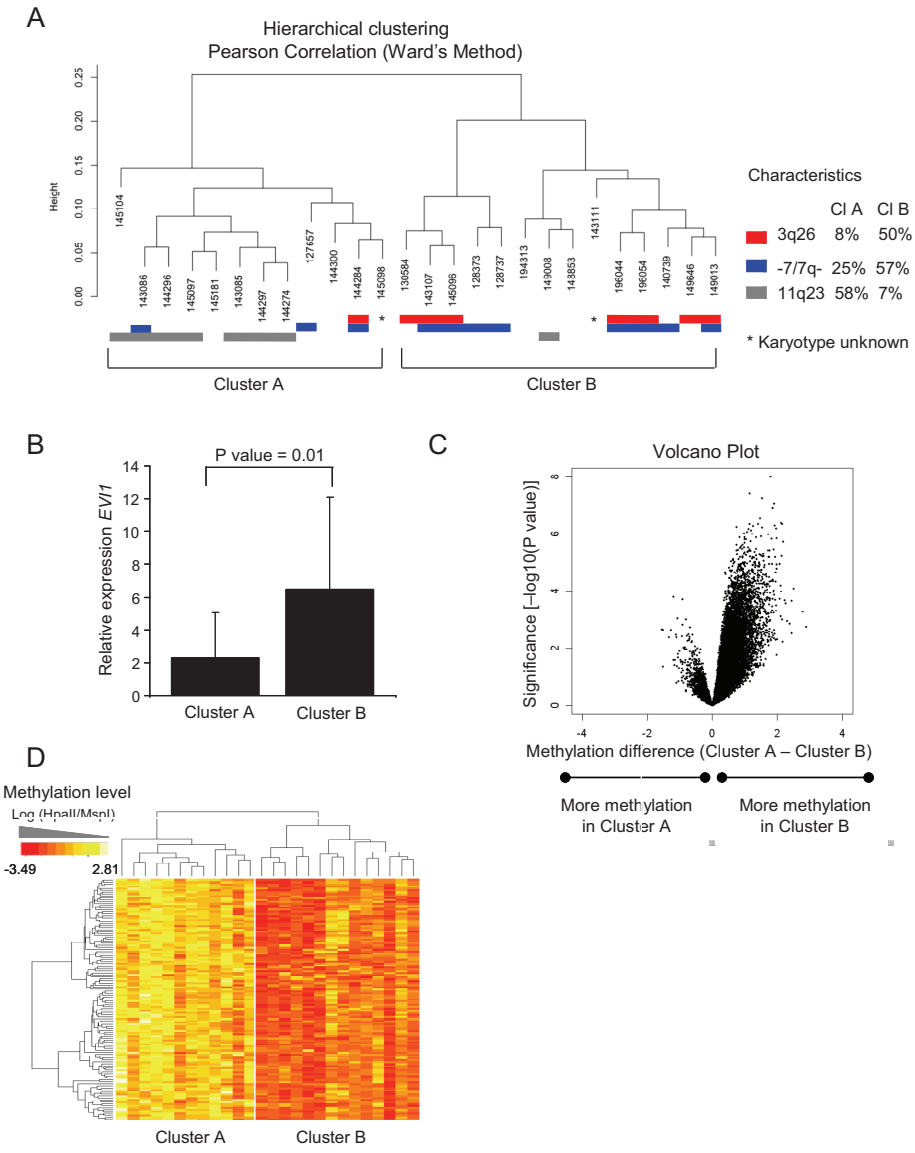


Figure 4. Unsupervised analysis identified two epigenetically distinct *EVI1* AML subgroups correlating with *EVI1* relative expression.

(A) Unsupervised hierarchical clustering with Pearson's correlation using Ward's method revealed two *EVI1* subclusters i.e., A and B. The cytogenetic characteristics are shown per patient; chromosome 3q26 abnormalities (red), monosomy 7 or deletion 7q (-7/7q-) (blue) and 11q23 rearrangements (grey). The percentages of each characteristic are shown per cluster. (B) Median *EVI1* relative expression levels and 2SD are shown per subcluster. P-value was calculated using a moderated T-test. (C) The volcano plot shows the methylation difference of all probe sets (n=25,626) (x-axis) comparing the methylation levels of cases in subcluster A with the cases in subcluster B with corresponding P-value (-$\log_{10}$ P-value moderated T-test) on the y-axis. (D) The heatmap shows the 122 probe sets (110 unique genes) differentially methylated in subcuster B, when both cluster were compared to each other using a moderated T-test (P<0.001 and absolute methylation difference >1.5). All genes are hypermethylated in *EVI1* AMLs from subcluster B.