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ACUTE MYELOID LEUKAEMIA:
THE SEARCH FOR NOVEL PROGNOSTIC MARKERS

Acute myeloide leukemie: op zoek naar nieuwe prognostische merkers

Proefschrift

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
AAN DE ERASMUS UNIVERSITEIT ROTTERDAM
OP GEZAG VAN DE RECTOR MAGNIFICUS

PROF.DR. S.W.J. LAMBERTS

EN VOLGENS BESLUIT VAN HET COLLEGE VOOR PROMOTIES.

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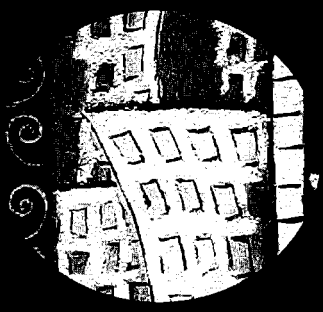
We are the flute, our music is all Thine;
We are the mountains echoing only Thee;
Pieces of chess Thou marshallest in line
And movest to defeat or victory;
Lions emblazoned high on flags unfurled -
Thy wind invisible sweeps us through the world.

Jalal ed-Din Rumi

Contents



Chapter 1

INTRODUCTION

Rudolf Ludwig Karl Virchow
(1821-1902)

Paul Ehrlich (1854-1915)

Historical overview

Identification of leukaemia

In October 1845, John Hughes Bennett (1812-1875), a lecturer in clinical medicine and pathologist at the Royal Infirmary Edinburgh, reported on a peculiar case. The blood of one of his patients had changed its color and consistency, as it was mixed with pus. When he examined it microscopically, there were in fact huge numbers of corpuscles, which he and many others were used to seeing in pus.[1] By applying acetic acid he could see the nucleus: "The nucleus was generally composed of one large granule ... but here and there two or three smaller granules".[2] Although he could find no source of inflammation, he nevertheless concluded that his patient died from "the presence of purulent matter in the blood". A few months later Rudolf Virchow, a demonstrator in pathological anatomy at the Charité Hospital in Berlin, made a similar observation, but he interpreted it differently. He remembered that the normal blood contains colourless corpuscles quite similar to those in pus and his patient's blood. The only difference was that the normal ratio of red and white corpuscles seemed reverse in this instance. Therefore he named the condition 'leukaemia' (white blood).[1] Bennett preferred the more exact title of leucocythaemia, or white cell blood.[2]

In 1868 Ernst Neumann, professor of pathological anatomy at Konigsberg established the link between blood and bone marrow and later stated that leukaemia is a disease of the marrow.[1,2]

Early classification of leukaemia

Soon after the identification of leukaemia, some differences in the manifestation of the disease were observed. Virchow divided leukaemia into two forms, one characterized by an enlargement of the lymph nodes (lymphatic leukaemia) and the other by great swelling of the spleen (splenic leukaemia). In 1878 Ernst Neumann suggested that bone marrow is the major source of splenic leukaemia and therefore the marrow derived (myeloid) leukaemia replaced the term splenic leukaemia.

In 1889 Wilhelm Ebstein, a professor of medicine in Göttingen, introduced a further subdivision of leukaemia based on the clinical progression of the disease. The 'acute' leukaemia was unresponsive to all forms of treatments and led to death, usually within weeks if not days; while the 'chronic' leukaemia could often be temporary relieved.[1]

Early treatments and prognoses

In 1893 Cabot, an American physician from Boston, studied a cohort of 34 patients with acute leukaemia which is probably the first study of its kind. The mean survival of the cohort was 4,5 weeks and the maximum survival comprised 9 weeks.[2] This is though not surprising as in those days no effective therapy was available.

Virchow's prescription for patients suffering from advanced leukaemia included nourishing diet, iron for anaemia, iodine and ointments for external use and footbaths.[1] The other therapeutic agents available at that time were opioids for diarrhea and pain, quinine for fever and arsenic. Arsenic trioxide 1% solution had already been used since

1786 for malarial fever and headaches. In 1865, Lissauer, a German physician, administered arsenic to a woman with chronic myeloid leukaemia and so induced a temporary remission.[2] This remission was described by one of Lissauer's colleagues as: "looks better, has a smoother skin and better digestion".[1] This success made Arsenic the first choice treatment for leukaemia for over 30 years. This treatment could reduce the number of white blood cells and the size of enlarged organs, such as spleen and lymph nodes. Besides, the number of red blood cells would increase and relieve anaemia. However, the major problem was that Arsenic was not effective against all kind of leukaemias. It seemed useless in acute leukaemia and was temporarily effective for the chronic forms. Therefore, when other treatments such as x-ray were discovered, Arsenic was put aside although not completely forgotten. In 1938 Forkner[3] recommended its use in certain cases like in those who had become refractory.

In the course of time, physicians learned not to think of leukaemia as a single disease. Leukaemia is a heterogeneous disease that differs in manifestation and response to therapy. Therefore it is impossible to expect a single agent like Arsenic to be effective against different forms of the disease. Recently, arsenic compounds have become a subject of renewed attention in the treatment of certain leukaemias, including acute promyelocytic leukaemia.[1,2,4,5]

Experimental haematology in progress

In 1877, the medical student Paul Ehrlich developed triacid stain, a method by which one could differentiate between types of blood cells. Using this method he differentiated between three distinct types of granulocytes. Based on their chemical affinities to aniline dyes, Ehrlich named these granulocytes acidophil (later changed to eosinophil), basophil and neutrophil. Triacid stain simplified the distinction of myeloid from lymphoid leukaemias.[2] Until then, distinctions had been made mainly on the basis of obvious pathological changes in the spleen, lymph nodes and marrow.[6]

Towards the end of the nineteenth century, the source of blood and some of its immediate precursors were identified. As leukaemia was a disease of blood, some physicians applied blood transfusions to treat patients. The first blood transfusion was carried out by George Callender in 1873 and resulted in a short relief of the patient. The second transfusion however, terminated fatally. It was not until 1900 that the most important advance in safe transfusion, the distinction between human blood groups, was discovered by Karl Landsteiner.[2]

The discovery of X-ray in 1895 by Wilhelm Röntgen brought a new treatment for leukaemia, with initially temporary results similar to those produced by arsenic.[2] However, Minot and his colleagues, in their survey of 1924, proved that acute leukaemias were resistant to X-ray therapies.[7] Later, in 1930, radiation was used as a combination therapy in RAT regimen together with arsenic and thorium-X.[8]

The advances in chemotherapy started after World War I, when mustard gas proved to induce myelosuppression. Because of the considerable toxic effects of nitrogen mustard, the search for agents with more specific actions continued.[2,6] Alkylating agents were introduced, such as Busulphan[6] and folic acid antagonists such as Aminopterin and later

methoteraxate.[2] Nonetheless, the outlook in leukaemia remained very gloomy until the 1960s,[8] when the treatment of leukaemia with methotrexate and 6-mercaptopurine was evaluated by Boggs et al.[9] In their study it was pointed out that only one in five patients benefited from this therapy. However, these results did not discourage further experiments with various chemotherapeutic regimens.[2] In the middle of the 1970s Donnall Thomas[10] presented his data on 100 patients: 54 with acute myelogenous leukaemia (AML) and 46 with acute lymphoblastic leukemia (ALL), treated with HLA-matched sibling marrow transplantation. The attitude of most haematologists in those days was: 'look what he put them through for only a few survivals'.[8] In contrast, several other haematologists who were experimenting with marrow transplantation were encouraged by these results. In due course other technologies, such as infection control, transfusion support, various kind of chemotherapies and immune suppression therapies developed and gradually improved the outcome.[8] In the past 20-25 years allogeneic bone marrow transplantation has been the most active treatment for AML with a success rate of 50-60%.[11]

This brief overview described the gradual progress in experimental haematology, which in the end can be called a success story. This reminds us of Virchow's words of 1858: 'I do not wish by any means to infer that leukaemia is incurable; I hope on the contrary that for it too remedies will at length be discovered.[2]

Acute myeloid leukaemia

Acute myeloid leukaemia (AML) is the most common type of acute leukaemia in adults with an incidence of approximately 3 per 100,000. AML affects adults of all ages but is especially common in elderly patients (median age of 55-60 years).[12,13] The disease is characterised by a maturation arrest of myeloid progenitors, which may occur at various stages of development. In normal bone marrow, myeloid progenitors develop into distinct blood cell types, i.e. erythrocytes, granulocytes, macrophages and platelets (Figure 1). The development of distinct blood cell types can be completely or partially blocked in AML as a result of acquired genetic changes in progenitor cells.[14-19] The genetic changes may involve mutations that lead to activation of proto-oncogenes, inactivation of tumour suppressor genes or generation of chimeric proteins that interfere with growth, differentiation and survival of haematopoietic progenitors.[20] As a result of such abnormalities, myeloid progenitors accumulate in blood and bone marrow and replace the normal functional blood cells. Since the differentiation arrest may occur at various stages of development, different immature cell types may accumulate and exhibit clinically and biologically distinct subtypes of AML.[21,22]

Classification by morphology

In 1976, a group of morphologists from France, the United States, and Great Britain (FAB) suggested a classification system to define distinct AML subtypes. The FAB morphologic classification distinguishes subtypes of AML according to the normal marrow cells that they most closely resemble (Figure 1). Based on this classification AML is

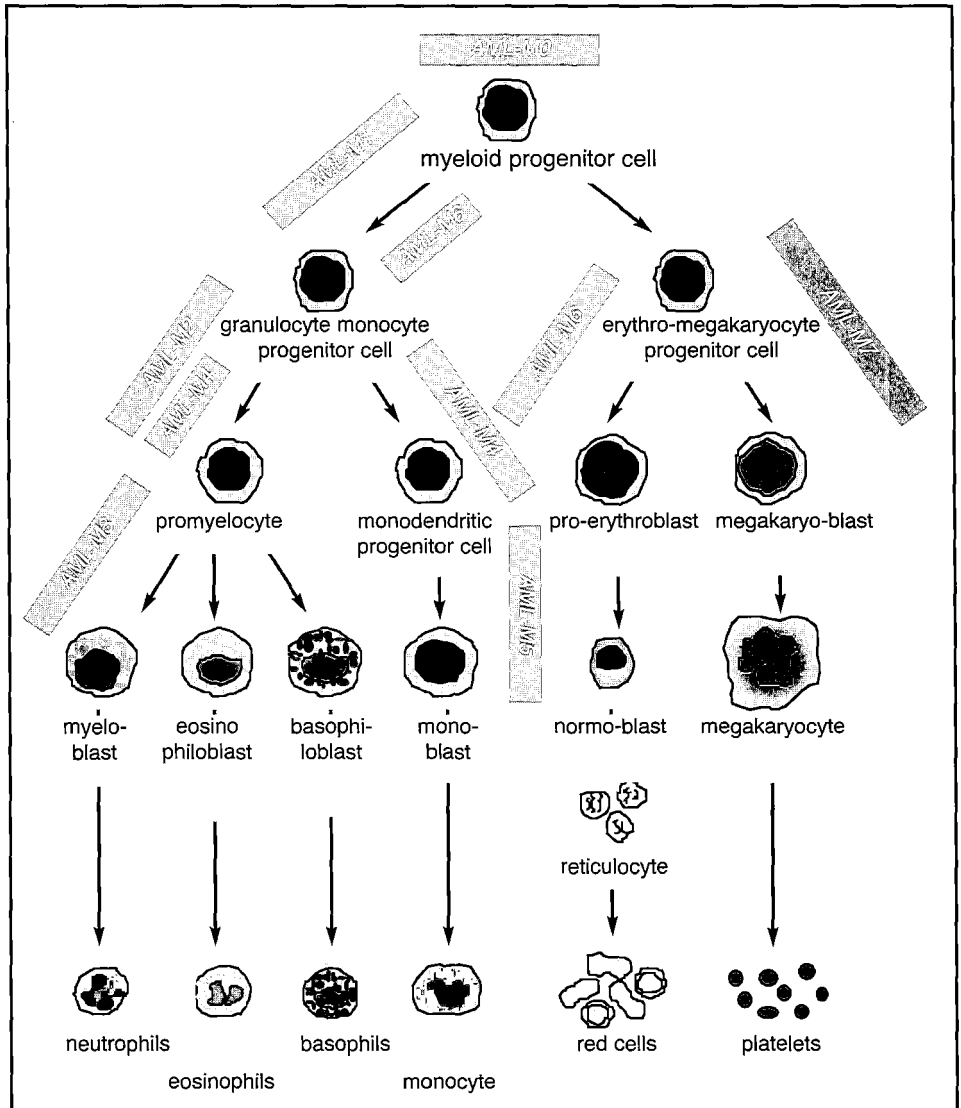


Figure 1. Schematic representation of blood cell differentiation in myeloid lineage in the context of morphologic classification of AML (FAB). The myeloid progenitors develop into distinct blood cell types, e.g. erythrocytes, granulocytes, macrophages and platelets. The FAB morphologic classification (M0-M7) shows the kind of normal marrow cells that most closely resemble the AML cells.

divided into 8 subtypes (M0-M7). AML-M0 and M1 are the most poorly differentiated AML, whereas M2 exhibits moderate myeloid maturation. AML-M3, M4 and M5 are respectively characterized by accumulation of promyelocytes, myelomonocytes and monocytes. The M6 and M7 are rare subtypes of AML that are associated with erythroid and megakaryocytic leukaemia.[23]

Cytogenetic abnormalities in AML

Cytogenetic abnormalities are observed in approximately 60% of AML patients.[24] Some of these abnormalities are recurrent. Approximately 23% of the patient group relevant to this thesis, carried a recurrent chromosomal translocation, e.g. t(8;21), t(15;17), idt(16) and 11q23 abnormalities. Some recurrent cytogenetic abnormalities are linked to FAB categories, indicating that the cell stage at which transformation occurs is closely related to the type of genetic aberration.[25-28] Table 1 shows common karyotypic abnormalities in AML in relation to FAB and prognosis of the patients. It is noteworthy that approximately 20% of the AML cases carry infrequent cytogenetic abnormalities, which are not mentioned in this table.

Table 1. Karyotypic abnormalities in AML: common karyotypic abnormalities in AML in relation to FAB and prognosis of the patients.

Cytogenetic abnormality	Genes involved	FAB	Prognosis	Percentage*
t(8;21)(q22;q22)	*AML1-ETO*	M2	Favourable	7%
idt(16)idt(16)(p11;q22)	*CBFβ-MYH11*	M4Eo	Favourable	4%
t(15;17)(q22;q11)	*PML-RARα*	M3	Favourable	7%
11q23 abnormalities	*MLL*	M5	Unfavourable	4%
-5/5q-	Unknown	Varied	Unfavourable	3%
-7/7q-	Unknown	Varied	Unfavourable	8%
t(6;9)(p23;q34)	*DEK-CAN*	M2/M4	Unfavourable	1%
t(9;22)(q34;q11)	*BCR-ABL*	Usually M1	Unfavourable	<1%
3q26 abnormalities	*EVI1*	Varied	Unfavourable	1%

Sources: *Cancer Medicine*[29] and Dash et al.[30] * The percentages refer to the cases included in this thesis. Patients with favourable prognosis studied in this thesis had a 4-year survival chance of 70% or more. The overall survival rate for the unfavourable-risk group was less than 20% at 4 years and the relapse rate was more than 70%.

Cytogenetics and risk-classification

The World Health Organization (WHO) has adapted recurrent cytogenetic abnormalities to define subgroups of AML.[31] Based on the WHO classification, AML can be subdivided into four different groups. 1- AML with recurrent translocations, 2- AML with dysplasia in two or more major lineages (erythroid, granulocyte/ monocyte and megakaryocyte), 3- therapy-related AML and 4- non-categorized AML, which accounts for half of the cases.[31] Although WHO classification in contrast to the FAB system includes cytogenetics, it offers no clear position for abnormalities such as monosomies of chromosome 5 or 7, 5q-, 7q-, t(9;11), 3q26 or complex karyotype (3 or more different cytogenetical aberrations). Consequently, using this approach approximately 50% of AML patients are not classified.[32]

In 1987, Keating et al.[33] proposed a new classification of AML based on cytogenetic abnormalities. The 4IWCL multi-centre study had already shown the value of cytogenetics in determining AML prognosis.[34] The classification method of Keating et al.[35] offered a clear stratification of disease into favourable, unfavourable and intermediate-risk AML. According to this classification, patients with translocations t(8;21), t(15;17) and idt(16) are classified in the favourable risk group. Patients with monosomies or deletions of chromosome 5 or 7, translocation t(6;9), and complex karyotypes are assigned to an unfavourable risk group and the remaining patients belong to an intermediate risk category with either a normal or an unknown-risk karyotype.[24] The prognosis of patients with 11q23 abnormalities seems to be affected by the partner genes.[36-38] Translocation t(9;11) for example, often associates with intermediate prognosis while t(11;19) is presented with an unfavourable outcome (reviewed by Bernasconi et al.).[20] Figures 2 and 3

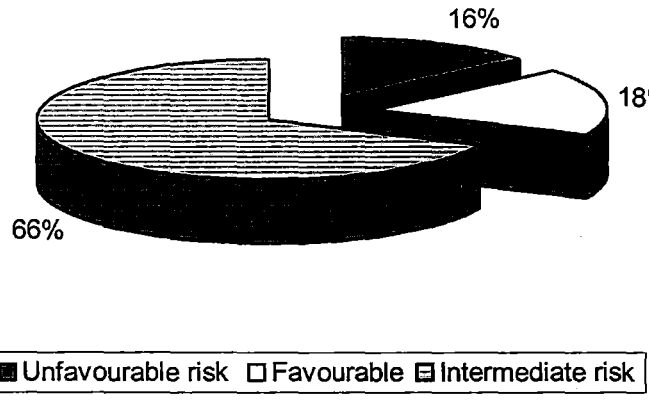
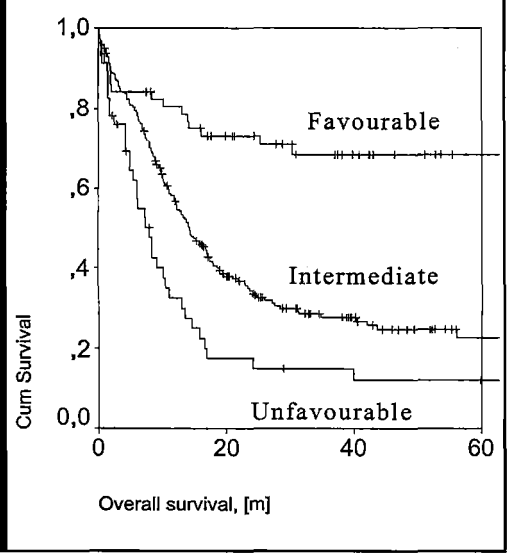


Figure 2. Distribution of AML within distinct cytogenetic risk groups. The figure is based on the data of patients included in this thesis (see chapter 2).

Figure 3. Overall survival of 319 de novo AML patients based on distinct cytogenetical risk-groups: favourable risk [t(8;21), t(15;17) and idt(16)], unfavourable risk [monosomies or deletions of chromosome 5 or 7 translocation t(6;9), 3q26 abnormalities and complex karyotype], intermediate risk [normal or an unknown-risk karyotype]. The figure is based on the data of patients included in this thesis (see chapter 2).



show the distribution and survival probabilities of distinct AML risk groups. Intermediate-risk category is probably most heterogeneous and poorly defined. Patients in this category may present with rare or complex cytogenetic abnormalities or with an apparent normal karyotype. The clinical features and prognoses of these patients differ greatly. As a result, the therapy response of a patient with an intermediate risk karyotype remains comparatively unpredictable. For this category of AML patients, more than for any other category, we require additional prognostic markers.

Molecular genetics of AML

Acute myeloid leukaemia is a heterogeneous disease, in which a large variety of genetic abnormalities can be involved.[30,39] In the past decades, an extensive number of leukaemia-associated mutations and chromosome abnormalities have been identified.[39] Chromosome abnormalities can be divided into unbalanced defects, with loss or gain of chromosome material (deletions, nonreciprocal translocations, monosomies and trisomies) and balanced defects, without loss or gain of chromosome material (translocations and inversions).[30,39,20] Classical cytogenetic analysis based on e.g. Giemsa banding is the most commonly used method for identifying aberrations in AML. However, not all the chromosomal abnormalities e.g. small or rare cryptic rearrangements can be detected in this way. Moreover, 40% of the AML patients do not show any abnormality using standard cytogenetics. Nowadays, molecular genetic techniques, such as RT-PCR, nucleotide sequencing, Southern blot analysis and Fluorescence in situ hybridisation (FISH) have made it possible to detect several leukaemia associated mutations in disease genes, e.g. *AML1*, *ETV6*, *RARα* and *MLL*.[39]

Balanced chromosomal abnormalities

A common theme in leukaemia with balanced translocations is the involvement of genes that encode transcription regulators.[30] Many of these regulators control differentiation.[40,41] Abnormalities in the structure or expression of the genes encoding transcription factors may have drastic effects on haematopoiesis (Figure 4). In this paragraph, the recurrent and the less frequent chromosomal abnormalities engaging particular transcription regulators are introduced.

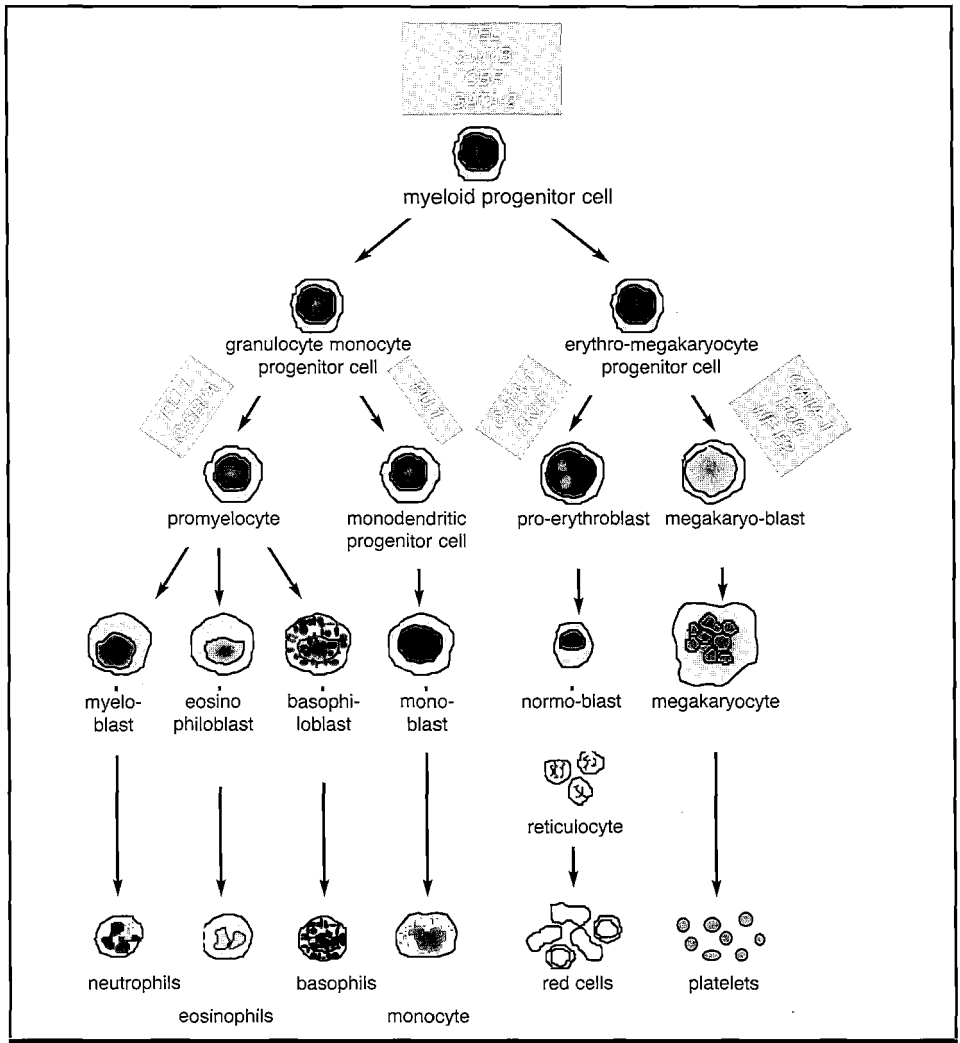


Figure 4. Schematic representation of blood cell differentiation in myeloid lineage in the context of transcriptional control system. A few prominent transcription factors that regulate myeloid lineage commitment are presented in this figure.

I. Recurrent translocations in AML: Frequent involvement of transcription regulators

Translocations t(8;21), t(15;17), inv(16)/t(16;16), as well as 11q23 abnormalities are recurrent chromosomal abnormalities in AML. Their common characteristics are the presence of transcriptional regulatory genes at one side of the breakpoints i.e. retinoic acid receptor alpha (*RARa*) in cases of translocation t(15;17), core binding factors (*CBF*s) in AML with either t(8;21) or with idt(16) and the mixed lineage leukaemia (*MLL*) gene in cases with 11q23 translocations.[20,30,42] The fusion proteins that are formed by such translocations generally interfere with the normal function of one or both of the rearranged genes.[42]

a. Translocation t(15;17) involving retinoic acid receptor alpha.

Translocations involving retinoic acid receptor alpha (*RARa*) are associated with acute promyelocytic leukaemia (APL).[43] RARa is a transcription regulator with differentiation-promoting and growth-suppressing activity in normal haematopoiesis.[44] In the absence of retinoic acid (RA), RARα takes part in a complex that represses transcription.[45-47] However, physiologic concentrations of RA normally induce a conformational change in RARα, causing release of co-repressors and recruitment of co-activator molecules, leading to activation of gene transcription (reviewed by Scandura et al.[42]). The *Rara* knock-out mice show early postnatal lethality and testis degeneration. These mice have the same characteristics as mice fed with a vitamin A-deficient diet.[48] The translocation t(15;17) gives rise to the fusion transcript *PML-RARa* and also a smaller fusion product *RARa-PML*, which is present in most but not all APL patients.[49] In *PML-RARa*, the promoter of *PML* gene on chromosome 15 stimulates expression of the *PML-RARa* fusion gene that contains the largest part of *PML* and the RAR-binding and DNA binding domains of *RARa*.[43,50-52] Grignani et al.[53] demonstrated that PML-RARα recruits the nuclear co-repressor histone deacetylase complex through the RARα CoR box. This recruitment inhibits transactivation of RARα target genes and thus suppresses the expression of genes required for promyelocytic differentiation. The maturation arrest at the promyelocyte stage can be rescued by the addition of high dose all-trans retinoic acid (ATRA).[54] ATRA binds to the RARα part of PML-RARα and releases the nuclear co-repressor complex. Subsequently the genes required for differentiation of promyelocytes to neutrophils will be expressed.[19,53] Nowadays, treatment of newly diagnosed APL patients with ATRA in combination with chemotherapy results in complete remission (CR) rates of 80 to 90%.[55]

b. Translocation t(8;21) and idt(16) involving core binding factors.

The core-binding factor (CBF) is a heterodimeric transcription factor composed of the CBFα and CBFβ subunits. CBFα2 (*AML1*) is the DNA binding element of the complex and its affinity is greatly increased in the presence of CBFβ.[56] Both Cbf subunits are critical to haematopoietic development, as mouse embryos lacking any of the genes showed no fetal liver haematopoiesis and died at approximately E12.5.[57,58] The two core binding

transcription factors, AML1 and CBFβ are frequently involved in leukaemic translocations.[30] In translocation t(8;21), the N-terminal part of *AML1* is fused to almost the entire *ETO*.[59] The leukaemogenic activity of AML1-ETO is partly due to its dominant negative activity on the wild type AML1 that is being produced by the unrearranged chromosome 21.[60] The zinc finger motifs present at the C-terminus of ETO in the fusion protein, interact with nuclear co-repressor molecules such as N-coR, HDAC1 and mSin3 and repress gene transcription.[61]

AML1 fusions with other genes such as *EVI1* in translocation t(3;21)[62] or *TEL* in translocation t(12;21)[63] are rather infrequent in AML. However, approximately 30% of childhood B-cell acute lymphoblastic cases carry a t(12;21).[64] The AML1-TEL, fusion protein interferes in a dominant negative manner with AML1-dependent transcription.[64] Besides *AML1* translocations, mutations in this gene also play a role in AML. Point mutations in *AML1* occur in less than 5% of de novo AML patients and especially in those that are poorly differentiated (M0).[27]

In AML with inv(16), *CBF*β is fused to *MYH11* (myosin heavy chain 11).[65] The CBFβ-MYH11 fusion protein binds AML1 and represses transcription through mSin3 and possibly other co-repressor molecules.[66] Like AML1 fusion proteins, the CBFβ-MYH11 fusion seems to act as a dominant negative suppressor of wild-type CBF. Both *CBF*β-*MYH11*[67] and *AML1-ETO* [68] knock-in embryos die in midgestation from haemorrhages and show a severe block in haematopoiesis, which resembles the phenotype seen in *Cbf*α and *Cbf*β knockout mice. Although this resemblance strengthens the perception that CBF fusion proteins have dominant negative effects on the wild type proteins, particular distinct characteristics of the fusion protein indicate that each may also have specific functions.[62,67,68]

c. 11q23 translocations involving mixed lineage leukaemia (*MLL*) gene.

The mixed lineage leukaemia (*MLL*) gene located on chromosome 11q23 is engaged in more than 40 different leukaemia-associated translocations. The most common translocations in AML are t(9;11)(p22;q23), and t(6;11)(q27;q23), which result in fusions between *MLL* with *AF9* and *AF6* respectively. Other translocations such as t(11;19)(q23;p13.3) and t(4;11)(q21;q23) occur less frequently in AML.[42,69] *Mll* is a positive regulator of *Hox* genes and is required for the development of multiple tissues, including skeletal, craniofacial, neural, and haematopoietic development during embryogenesis.[70,71] The knockout models have shown that *Mll* is required for normal numbers of haematopoietic progenitors and their proper differentiation, especially along the myeloid and macrophage pathways. Loss of one functional copy of *Mll* already results in defective yolk sac haematopoiesis and loss of both copies is embryonically lethal.[71] The *Mll* +/- bone marrow harbour a significantly reduced number of haematopoietic colony forming cells. Elimination of both copies of *Mll* results in an even greater reduction in the number and proliferative potential of haematopoietic progenitors.[72]

Most *MLL* translocations result in in-frame fusions between the N-terminal AT-hooks and methyltransferase domain of *MLL* and the C-terminal part of the fusion partner.[69] Thus the DNA binding and repressor domains of MLL are kept in most of fusion proteins, while the transcription activation domain of MLL is lost.[42] MLL fusion proteins

seem to transform myeloid progenitors through deregulation of *HOX* genes expression.[73] Besides translocations, rearrangements of *MLL* occur in about 5% of adult de novo acute myeloid leukaemia. Many of these leukaemias are of M4 and M5 cytological type and have a poor prognosis.[26]

II. LESS FREQUENT TRANSLOCATIONS IN AML

a. Translocations involving 3q26 abnormalities (*EVI1* gene).
EVI1 (ecotropic virus integration site-1) is involved in pathogenesis of human leukaemias carrying 3q26 abnormalities, including translocation t(3;3)(q21;q26) and inv(3)(q21q26).[74-77] Although *EVI1* translocations are infrequent in AML,[78] they are of remarkable prognostic value.[79,80] Patients with these translocations are frequently resistant to therapy.[81]

EVI1 is a DNA binding protein, localized in the nucleus, and is probably involved in transcription regulation.[82] It exists also as a longer protein that includes [188] additional amino acids at the N-terminus, named MDS1-EVI1. *MDS1-EVI1* is generated as a result of intergenic splicing between *MDS1* gene and *EVI1*. Both *EVI1* and *MDS1-EVI1* are expressed at very low levels in the normal bone marrow. Although related, the two proteins have opposite properties.[83,84] EVI1 has important roles in general proliferation and cell-specific development. Disruption of the full length Evi1 protein in homozygous mutant mice leads to embryonic death at E10.5, widespread hypocellularity and disruption in the development of the heart, somites, and neural crest-derived cells.[85] Overexpression of *EVI1* in immature haematopoietic cells interferes with erythroid and granulocytic development.[86,87] In AML with rearrangements of chromosome 3, *EVI1* gene is transcriptionally activated as a result of juxtaposition to the regulatory elements of *RPN1* gene at 3q21.[74,75] Transcriptional activation of *EVI1* is also observed in AML without rearrangements of chromosome 3, meaning that juxtaposition to RPN1 is not the only mechanism that leads to *EVI1* overexpression.[77,88-90] The frequency of *EVI1* overexpression and its impact on patients' prognosis remain to be addressed. In **Chapter 2** we aim at determining the frequencies and the prognostic value of *EVI1* and *MDS1-EVI1* overexpression in de novo AML, irrespective of 3q26 abnormalities.

The reciprocal translocation t(1;3)(p36;q21), which associates with poor prognosis, occurs in a small subset of AML and involves a gene with high homology to *EVI1*, the *MDS1-EVI1*-like-1 (*MEL1*) gene. *MEL1* is thought to be transcriptionally activated as a result of juxtaposition to the *RPN1* gene at 3q21.[91] It is not known whether *MEL1* expression is restricted to cases with this particular translocation. In **Chapter 3** we study *MEL1* expression in AML other than AML t(1;3). Furthermore, we examine whether an *EVI1* like transcript (*EL1*) can also be expressed from the same locus. The prognostic impact of *MEL1* and *EL1* mRNA level will be evaluated in the same chapter.

b. Translocations involving *TEL* gene.
The *TEL* (translocation ets leukaemia) gene also known as *ETV6* (ets translocation variant gene 6), a member of ETS family of DNA binding genes, is involved in at least 41 different translocations associated with human malignancies.[92,93] Translocation t(12;21),

which generates TEL-AML1 fusion protein is the most common genetic lesion in paediatric acute lymphoblastic leukaemia (ALL). This translocation is detected in up to 25-30% of the patients and often associates with a fully or partially deleted non-rearranged *TEL* allele.[94-96] The *TEL* translocations in AML are comparatively infrequent.[97]

The *TEL* gene encodes for a protein with transcription repressor activity, that regulates cell growth and differentiation.[98] *Tel* knockout mice are embryonic lethal and exhibit defective yolk sac angiogenesis and intra-embryonic apoptosis of mesenchymal and neural cells. Although *Tel* absence does not seem to affect haematopoiesis at the yolk sac stage,[99] it is indispensable for the development of all lineages in adult bone marrow. In chimeric mouse with *Tel* knockout ES cells, defective haematopoiesis becomes manifest within the first week after birth.[61]

The fusion partners of *TEL* have heterogeneous characteristics.[92,93] Interestingly, the contribution of TEL to the fusion oncoproteins is variable.[92] For example, the TEL-EVI1 fusion does not contain functional TEL domains, suggesting that the only part of *TEL* that is crucial in this translocation is the *TEL*-promoter. In other words, in cells with a *TEL-EVI1* fusion, the *EVI1* gene is overexpressed as the result of activation by the promoter of *TEL*.[100] In other translocations, *TEL* either provides dimerization domains in e.g. the fusion *TEL-MN1*,[101] *BTL-TEL*[102] or *TEL-ABL*,[103] resulting in constitutively activated signaling proteins. In other cases, the transforming fusion proteins contain domains that harbour transcriptional regulatory properties, i.e. the ETS DNA binding domain in e.g., TEL-PDGFRβ[104,105] and TEL-AML1.[64] As genes that are frequently involved in leukaemic translocations, e.g. *AML1*,[27] *MLL*[26,69] and *EVI1* [77,88-90] may also be affected otherwise, it is possible that *TEL* abnormalities in AML are not limited to translocations. In order to examine this hypothesis, we investigate the occurrence of *TEL* mutations and abnormal protein expression in de novo AML (**Chapter 5**).

Unbalanced chromosomal abnormalities

Unbalanced chromosome abnormalities are observed in the form of loss and/or gain of chromosomal segments, monosomies and trisomies.[39] The molecular consequences of such numerical aberrations are presently not known. Deletions and monosomies of chromosomes 5 and 7 are associated with poor prognosis. It has been proposed that loss or haploinsufficiency of tumour suppressor genes, as a result of deletions, are the transforming events in these cases.[106]

Submicroscopic mutations associated with AML

Malignant transformation is a multi-step process involving accumulation of several genetic alterations. Some of these genetic alterations are detectable by cytogenetic analysis while others are submicroscopic and are only detectable by molecular genetic techniques.[39] In this paragraph two different types of submicroscopic aberrations will be introduced: 1- mutations in haematopoietic transcription regulators (e.g. *CEBPA*, *PU.1*, *AML1* and *MLL*) that result in impaired differentiation; 2- activating point mutations in genes encoding receptor tyrosine kinases (e.g. *FLT3* and *c-KIT*) that provide a proliferative and/or survival signal to haematopoietic progenitors.[107]

I. MUTATIONS IN HAEMATOPOIETIC TRANSCRIPTION REGULATORS

As mentioned earlier, haematopoietic transcription regulators play a major role in both myeloid differentiation and leukaemic transformation.[40] Besides *AML1* mutations [27] and *MLL* rearrangements,[26] occurrence of acquired heterozygous mutations in the coding region of *CEBPA* (CCAAT/Enhancer Binding Protein α)[108] and *PU.1*[109] have recently been reported in AML. Both *PU.1* and C/EBP*a* are major regulators of gene expression during myeloid development[40,110] (Figure 4).

C/EBP*a* is a transcription factor essential for granulocytic differentiation.[111] *Cebpa* knockout mice lack mature granulocytes, while the development of other haematopoietic lineages is not affected.[112] Mutations in the C-terminus *CEBPA* (bZIP region) are in-frame insertions that result in proteins with deficient transcriptional activation and DNA binding. These proteins though do not possess dominant negative activity.[113,114] In contrast, mutations in *CEBPA* N-terminus are not in-frame and result in both a non-functional truncated protein and an increase in the production of a 30 kD protein,[113,115] which has a dominant negative effect on DNA binding and transactivation of the wild type C/EBP*a*.[113] The exact frequency of the N- and C-terminal mutations in de novo AML patient, the clinical characteristics and prognosis are presently not known. By screening a large cohort of de novo AML, we aim at assessing the frequency and prognostic value of *CEBPA* mutations (**Chapter 4**).

PU.1, a member of the ets family of transcription factors and the product of *Spi-1* oncogene, is expressed exclusively in the haematopoietic system. Mice homozygous for a disruption in the *Pu.1* DNA binding domain lack mature macrophages, neutrophils, B cells and T cells.[116] Consistent with the findings that PU.1 has a major role in myeloid development, somatic mutations in *PU.1* have been reported in 7% (9/126) of AML patients.[109] However, this finding could not be confirmed by others.[117,118] As *PU.1* mutation analyses were carried out in relatively small number of AML patients, the frequencies and prognostic significance of such mutations remain unclear. In this thesis, we investigate the occurrence of *PU.1* mutations in patients with de novo AML. The results of this study will be discussed in **Chapter 7** (General discussion and summary).

II. MUTATIONS IN RECEPTOR TYROSINE KINASES

FLT3 internal tandem duplication (ITD) is the most frequent genetic aberration in AML. It occurs in 20-30% of de novo AML patients and correlates with poor prognosis.[28,119,120] In addition to internal tandem duplications, point mutations in *FLT3* have been identified in approximately 8% of AML patients.[28] *FLT3* together with other members of receptor tyrosine kinases family, e.g. *c-ABL, c-FES, c-FMS, PDGFR*β and *c-KIT* are thought to be involved in the regulation of haematopoiesis. Phosphorylation of tyrosine residues of the receptor tyrosine kinases, following ligand binding, is the first step in the transmission of activating signals from the cell surface to the nucleus.[121] *Flt3* knockout mice show deficiencies in the primitive B lymphoid progenitors. Mice deficient for both

Flt3 and *c-Kit* exhibit a more severe phenotype characterized by overall decrease in haematopoietic cell numbers and postnatal lethality.[122] In AML, mutations in receptor tyrosine kinases usually result in constitutive activation of the receptor leading to uncontrolled cell proliferation, inhibition of apoptosis, and blockage of differentiation.[121,123] Several FLT3 inhibitors are currently in various stages of pre-clinical and clinical development to specifically treat AML with *FLT3* mutations.[124]

Murine myeloid leukaemia: a model for identification of leukaemia associated genes

A powerful and rapid approach to identify transforming genes in leukaemia is retroviral insertional mutagenesis. In fact, by applying this technology a large series of potentially transforming genes have been identified by different research groups.[125-130] The involvement of a number of genes first identified in murine leukaemia has subsequently been demonstrated in human leukaemia, e.g. *Evi1,*[75] *Pu.1*(*Spi1*),[109] *Cbf,*[56] *Myc,*[131] *YY1*[132]or *Bcl2,*[133] *Nf1,*[134] *P53*[135] and *Hoxa9.*[136]

Insertional mutagenesis in murine leukaemia usually activates transforming genes by enhancing their transcription.[137] The same mechanism is observed in patients with 3q26 abnormalities. In these cases, juxtaposition to the regulatory elements of *RPN1* gene results in *EVI1* over expression.[75] In this thesis we will investigate the mRNA expression patterns of potential transforming genes, identified in murine leukaemia, i.e. *EVI1, HOXa9, HOXa7, MEIS1, CB2, PU.1, NM23-H1, NM23-H2* and *SOX4* in a cohort of de novo AML patients. Subsequently, the expression patterns will be related to clinical characteristics and therapy outcome. The results of these studies will be discussed in **Chapter 7** (General discussion and summary).

Besides altering transcription, retroviral insertion may disrupt the coding region of genes, resulting in production of abnormal proteins. One example is *c-MYB*. Retroviral insertion within the *c-Myb* gene generates truncated N-terminus MYB protein that contributes to oncogenicity of c-MYB.[138] MYB is important for development of myeloid, lymphoid and erythroid cells and the *c-Myb* (-/-) mice lack all these lineages.[139,140] Removal of the C-terminal negative regulatory domain increases the trans-activating capacity and oncogenic activation of c-Myb.[141,142] Although an involvement of *c-MYB* in human AML has not been demonstrated, there is strong evidence that aberration in this gene results in defective haematopoiesis. Therefore, we decided to investigate the occurrence of *c-MYB* mutations in human AML. The results of this study will be discussed in **Chapter 7** (General discussion and summary).

Challenges in determining prognosis of AML

As introduced above, certain genetic abnormalities in AML are associated with prognosis. For example, translocations t(8;21), t(15;17) and idt(16) associate with favourable prognosis, whereas -5/5q- or –7/7q-, t(6;9), and complex karyotype are linked to poor

outcome.[24] Even though the two categories of favourable and unfavourable AML can be distinguished, intra-categorical variations in therapeutic response and outcome are observed,[39] suggesting the involvement of additional genetic abnormalities. For example *c-KIT* mutations, which are observed in one third of AML patients with inv(16), adversely affect the prognosis.[143]

Besides the heterogeneity of the disease, patient-related factors such as age and gender impact on the prognosis of the patients. AML patients older than 60 years have a significantly lower response rate and shorter survival as compared to the younger patients. This is not only because of comorbidity and a higher incidence of secondary AML but also because of the distinct disease biology.[24] Leith et al[144] demonstrate an increased frequency of unfavourable cytogenetics and drug resistant phenotypes in patients of older age. For instance the multidrug resistance protein MDR1 is expressed in 71% of the older patients and only in 30% in the younger cases. MDR1 is a transmembrane efflux pump that actively exports drugs, such as anthracyclines, out of the leukaemic cells and reduces their intracellular concentration.[145] The reduced intracellular concentration of anthracyclines is associated with a low response rate.[146,147]

Genetic variations affecting pharmacodynamics and pharmacokinetics of the drugs may also determine therapy response.[148] Examples of such variations are polymorphisms in Glutathion S-transferases (*GST*s). GSTs are detoxifying cytosolic enzymes that conjugate glutathione (GSH) with compounds containing an electrophilic centre.[149,150] The M1 and T1 subtype of *GST* show deletion polymorphisms, which in case of homozygosity (null genotype) leads to the absence of the enzymes.[151] The percentages of *GSTM1* and *GSTT1* null genotypes vary from 22 -90 % and 12 -38% respectively in different ethnical groups. Deletions of these genes might affect the response to several chemotherapeutic agents, such as anthracyclines. GSTs can alter the cytotoxic effect of anthracyclines by 1- decreasing its cytotoxicity through glutathione conjugation which is necessary for transportation of anthracyclines outside the cells by P-glycoprotein, MRP1 and other ABCs,[152] 2- preventing the access of anthracyclines to the nucleus following conjugation to glutathione,[153] 3- deactivating the reactive oxygen species, which are produced by anthracyclines and are partly responsible for their cytotoxic effects.[154,155]

Although several studies have focused on the relationship between *GST* null polymorphism and the failure of chemotherapy, the results of these studies remain contradictory. It should be noted that the lack of agreement might relate to differences in the study design (i.e. number and selection of patients included), chemotherapeutic regimens (type of anthracyclines and the dosage) and the ethnic background of the patients. In this thesis we aimed at determining the prognostic impact of *GSTM1* and *GSTT1* polymorphisms and expression levels in a large cohort of de novo AML patients (**Chapter 6**).

At present, a profound understanding of the individual prognostic factors is becoming of significant importance for treatment choice in AML. Treating AML patients carrying t(15;17) with ATRA is a classic example of this approach.[24] However, in more than half of the AML cases we are still not capable of pointing out a single abnormality with defined prognostic value. New parameters with enhanced prognostic significance are required to both predict the outcome and apply risk-adapted treatment.

The scope of this thesis

The main objective of this thesis is to identify novel prognostic markers in AML. We studied aberrations in important haematopoietic transcription regulators and examined the role of potential oncogenes, which had been identified by means of retroviral insertional mutagenesis in murine leukaemia. Furthermore, we considered patient-related factors that may affect pharmacodynamics or pharmacokinetics of chemotherapeutic agents. The prognostic significance of a variety of these factors was investigated by multivariable analysis in patients with newly diagnosed AML.

In chapter 2 we assessed the frequencies and prognostic impact of *EVI1* and *MDS1-EVI1* overexpression in a cohort of 319 de novo AML patients.

In chapter 3 we evaluated expression of *MDS1-EVI1* like (*MEL1*) gene in AML other than AML t(1;3). Furthermore, we examined the existence of an *EVI1* like transcript (*EL1*). The clinical characteristics of AML with *MEL1* and *EL1* mRNA expression are also presented in this chapter.

In chapter 4 we developed a rapid assay for simultaneous detection of *CEBPA* C-terminal mutations and quantification of mRNA expression levels. Furthermore, we assessed the clinical, haematological and prognostic impact of *CEBPA* mutations and variation in *CEBPA* expression levels.

In chapter 5 we investigated the involvement of TEL (*ETV6*) aberrations in the novo AML cases by nucleotide sequencing and Western blot analysis.

In chapter 6 we addressed the prognostic impact of *GSTM1* and *GSTT1* polymorphisms and expression levels in a cohort of de novo AML patients.

In chapter 7 (general discussion) we discuss the factors determining the prognosis of AML patients.

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Chapter 2

High *EVI1* expression predicts poor survival in acute myeloid leukaemia: a study in 319 de novo AML patients

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Abstract

The proto-oncogene *EVI1* encodes a DNA binding protein and is located on chromosome 3q26. The gene is aberrantly expressed in acute myeloid leukaemia (AML) patients carrying 3q26 abnormalities. Two mRNAs are transcribed from this locus, i.e. *EVI1* and a fusion of *EVI1* with *MDS1* (*MDS1-EVI1*), a gene located 5' of *EVI1*. The purpose of this study was to investigate which of the 2 gene products is involved in transformation in human AML. To discriminate between *EVI1* and *MDS1-EVI1* transcripts, distinct real-time quantitative polymerase chain reaction (PCR) assays were developed. Patients with 3q26 abnormalities often showed high *EVI1* and *MDS1-EVI1* expression. In a cohort of 319 AML patients, 4 subgroups could be distinguished: *EVI1*+ and *MDS1-EVI1*- (6 patients; Group I), *EVI1*+ and *MDS1-EVI1*+ (26 patients; Group II), *EVI1*- and *MDS1-EVI1*+ (12 patients; Group III) and *EVI1*- and *MDS1-EVI1*- (275 patients; Group IV). The only 4 patients with a 3q26 aberration belonged to Groups I and II. Interestingly, high *EVI1* and not *MDS1/EVI1* expression was associated with unfavourable karyotypes (e.g –7/7q-) or complex karyotypes. Moreover, a significant correlation was observed between *EVI1* expression and 11q23 aberrations (*MLL* involvement). Patients from Groups I and II had significantly shorter overall and event-free survival than patients in Groups III and IV. Our data demonstrate that high *EVI1* expression is an independent poor prognostic marker within the intermediate-risk karyotypic group.

Introduction

The *EVI1* proto-oncogene is located on human chromosome 3q26 and is involved in pathogenesis of human acute myeloid leukaemia (AML) or myelodysplatic syndrome (MDS) carrying 3q26 rearrangements.[1,2] Although these rearrangements are infrequent in AML, they are of remarkable prognostic value. Patients with these karyotypes often do not respond to therapy, even when the most active antileukemic therapeutic options are used.[3]

EVI1 encodes a nuclear DNA binding protein with 2 zinc finger domains, i.e. an N-terminal domain containing 7 zinc fingers and a more C-terminal domain with 3 zinc fingers. Both domains recognise and bind to specific DNA consensus sequences.[4,5] While most reports indicate that *EVI1* gene expression is not detectable in normal blood or bone marrow,[6-9] other studies suggest low but detectable expression of *EVI1* in normal bone marrow cells.[10] High expression of *EVI1* has been observed in developing oocytes and in kidney.[11] Although the exact mechanism of transformation by *EVI1* is still obscure, several studies have shown that inappropriate expression of *EVI1* in immature haematopoietic cells interferes with erythroid and granulocytic development.[12] It has previously become evident that *EVI1* may form a fusion transcript with the *MDS1* gene. *MDS1* is a 4-exon gene located upstream of *EVI1*. Splicing may occur from exon-2 of *MDS1* to the second exon of *EVI1*, to form the fusion transcript *MDS1-EVI1*. This intergenic splicing may occur in normal tissues as well as in myeloid leukaemia.[1,13] *MDS1-EVI1* encodes a longer protein containing the entire EVI1 protein but with an additional, unique N-terminal extension. Although related, the two proteins EVI1 and MDS1-EVI1 may have opposite properties.[14,15]

Previous studies showed that *EVI1* may be expressed in patients without 3q26;[8,9,16,17] however the sets of PCR primers that were chosen in these studies did not discriminate between *EVI1* and *MDS1-EVI1*. We designed different primer and probe combinations to discriminate between *EVI1* and MDS-*EVI1* and quantify the transcript levels by means of real-time PCR analysis. To provide an answer to the question, which of these transcripts are expressed in patients with 3q26 rearrangements, we first screened 7 patients carrying 3q26 abnormalities. Using the same technique we studied the expression levels of these transcripts in the bone marrow samples of healthy volunteers. To investigate how frequently *EVI1*, *MDS1-EVI1* or *MDS1* may be expressed in de novo AML, we determined expression levels of these transcripts in a cohort of 319 AML patients at diagnosis. The results were analysed in relation to haematological, cytogenetic and clinical characteristics as well as outcome of therapy. Our data demonstrate that expression of *EVI1* and not of *MDS1-EVI1* is associated with highly aggressive AML. High *EVI1* expression occurs with high frequency in patients without 3q26 abnormalities, suggesting other mechanisms of aberrant *EVI1* expression.

Material and methods

Patients and healthy volunteers

Bone marrow samples of AML patients at diagnosis and of healthy volunteers (n=9) were obtained after informed consent. Blasts from AML patients and mononucleated fractions from normal bone marrow specimens were isolated from the samples by Ficoll-Hypaque (Nygaard, Oslo, Norway) centrifugation.[18] The cells were then cryopreserved as described in Delwel et al.[19] After thawing cells were washed with Hanks Blanced Salt Solution (HBSS) and further processed for RNA isolation. AML samples treated according to this procedures usually contain more than 90% blasts after thawing.[19] Seven patients with 3q26 rearrangements (4 patients with AML, 2 with refractory anaemia with excess blasts in transformation [RAEB-t] and 1 chronic myelogenous leukaemia) were selected that had not been included in a clinical trial. A total of 319 de novo AML patients who had been referred to our institution and collaborating centres between 1987 and 2000 were chosen for analysis. Of these patients 229 patients were treated according to the HOVON-29 (Dutch-Belgian Haematology-Oncology Group) protocol, 66 according to the HOVON-4 protocol, and 13 according to the HOVON-31 protocol. These treatment protocols have been described elsewhere.[20] Eleven patients received other forms of treatment. The clinical and haematological characteristics of the 319 patients at diagnosis are shown in Table 1. AML samples were classified according to the French-American-British (FAB) nomenclature.[21]

RNA isolation, cDNA synthesis and real-time PCR

Total RNA was extracted with guanidium thiocyanate followed by centrifugation in cesium chloride solution. Then 1 ml RNA was transcribed into cDNA using Superscript (Life Technologies, Merelbeke, Belgium) and Random Hexamers in a 40 ml reaction, under standard conditions.

An aliquot of one 20th of the resulting cDNA was used for quantitative PCR amplification. Real-time PCR amplification was performed with the ABI PRISM 7700 Sequence Detector (Applied Biosystems, Nieuwekerk aan den IJssel, the Netherlands), using 50 ml mix containing 2 ml cDNA sample; 250 mM dNTPs (Amersham Pharmacia Biotech Inc, Roosendaal, the Netherlands); 15 pmol forward and reverse primer (Life Technologies); 3 mM MgCl2 (5 mM for *PBGD* reaction), 200 nM probe, labelled at the 5' end with the reporter dye molecule FAM (6-carboxy-fluorescein) for *EVI1*, *MDS1-EVI1* and *MDS1*, JOE (carboxyrhodamine) for *PBGD*, and at the 3' end with the quencher dye molecule TAMRA (6-carboxy-tetramethyl-rhodamine; Eurogentec, Maastricht, the Netherlands); 5 ml 10x buffer A; 30 ml water; and 1.25 U AmpliTaq Gold (Applied Biosystems). The thermal cycling conditions included 10 minutes at 95°C followed by 45 cycles of denaturation for 15 seconds at 95°C, annealing/extension at 60°C for 30 seconds. The primer/probe combinations were chosen such that we could discriminate between *EVI1*, *MDS1/EVI1* and *MDS1* transcripts (Figure 1). The oligonucleotide sequence of the primers and probes are shown in Table 2.

Table 1. Demographic and clinical characteristics of 319 de novo AML patients.

Sex, no.	
Male	167
Female	152
Age, median (range), y	45.1 (15.2-76.8)
Age group, no.	
Younger than 35 y	89
35-50 y	112
Older than 50 y	118
FAB, no.	
M0	10
M1	68
M2	74
M3	33
M4	56
M5	67
M6	4
Unclassified	7
Cytogenetic risk group, no.	
Favorable	57
Intermediate/unknown*	212
Unfavorable	50
WBC count, median (range), 10^9/L	23.4 (0.3-282)
Blast count, median (range), %	69 (0-98)
Platelet count, median (range), 10^9/L	49 (3-931)

FAB indicates French-American-British classification;[21] and WBC, white blood cell.
* For 6 patients of this group, no cytogenetic information was available at diagnosis.

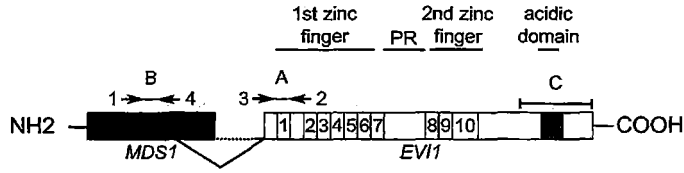

Figure 1. Schematic representation of *EVI1* and *MDS1-EVI1* and the primer and probes used for real-time PCR. Primers 1 and 2 plus probe A were used to determine *MDS1-EVI1* expression levels. *EVI1* transcript levels were determined using primers 3 and 2 plus probe A. *MDS1* expression was measured using primers 1 and 4 plus probe B. Probe C is used for northern blot analysis.

Table 2. Oligonucleotide primer and probe sequences used for quantitative real-time PCR.

	Oligonucleotide sequence (5'-3')
Primer 1	GAAAGACCCCAGTTATGGATGG
Primer 2	GTACTTGAGCCAGCTTCCAACA
Primer 3	CTTCTTGACTAAAGCCCTTGGA
Primer 4	TCTCTTCCCCAAATACAACCAAG
Probe A	TCTTAGACGAATTTTACAATGTGAAGTTCTG
Probe B	CTTCCAAGGTCTCGGAGGGTAAGCGTTC
PBGD forward primer	GGCAATGCGGCTGCAG
PBGD reverse primer	GGGTACCCACGCGAATCAC
PBGD probe	CATCTTTGGGCTGTTTTCTTCCGCC

Using seven different dilutions of a cDNA sample (equal to 0.0064-100 ng total RNA) prepared from AML cells that were positive for each of the transcripts (patient 2, Table 3), standard curves were made for *EVI1* and *MDS1-EVI1*. As the expression levels of *MDS1* transcripts were too low to measure the efficiency of amplification, *MDS1* expression was considered positive (+) when the threshold cycle (Ct) value was below 35.

To determine the expression levels in AML, all samples were tested in duplicate, and the average values were used for quantification. To quantify the relative expression of *EVI1* and *MDS1-EVI1*, the Ct values were normalised for endogenous reference ($\delta Ct = Ct_{target} - Ct_{PBGD}$) and compared with a calibrator, using the 'delta-delta Ct method' ($\delta\delta Ct = \delta Ct_{Sample} - \delta Ct_{Calibrator}$). As calibrator we used the average Ct value of *EVI1* and *MDS1-EVI1* in the 9 bone marrow samples of the healthy volunteers. We used the $\delta\delta Ct$ value to calculate relative expression ($2^{-\delta\delta Ct}$). As the $\delta\delta Ct$ method is applicable only when the amplification efficiencies of the target and the reference are essentially equal, we analysed the efficiencies for another 4 patients (patients 25, 28, 29, and 30; Table 4) using the same dilutions indicated above. The δCt values ($Ct_{target} - Ct_{PBGD}$) were plotted against the concentrations of total RNA (log). The slope of the fitted line was then determined. A slope of less than 0.1 is then indicative for equal efficiencies.

To define high *EVI1* and *MDS1-EVI1* expression, a cutoff value of 50 (relative expression $2^{-\delta\delta Ct}$) was chosen. This value was chosen to avoid the influence of particle distribution statistics, particularly in those cases with a slightly higher *EVI1* and *MDS1-EVI1* expression. To prevent bias the survival analysis was also performed at cut off points 10, 25 and 100.

Northern blotting
Northern blotting was carried out on mRNA isolated from healthy bone marrow samples as well as AML samples. A portion (20 mg) total RNA of each sample was separated on a 1% agarose, 6% formaldehyde gel, and blotted with 10x SSC onto hybond-N+ nylon membrane (Amersham). The blot was hybridised in 1N NaH2PO4-buffer containing 7% sodium dextran sulphate, and 1 mM EDTA pH 8.0. As probe, human *EVI1* (600bp HindIII-NcoI fragment) and murine *GAPDH* (777 bp HindIII-EcoRI fragment)[22] were ^{32}P labelled by random priming (Boehringer, Mannheim, Germany). The blot was hybridised at 65 °C overnight and washed for 15 minutes at 65 °C in 2x SSC/ 0.5% SDS, and for 15 minutes at 65 °C in 1x SSC/ 0.5 SDS. It was then analysed by autoradiography.

Cytogenetic analysis and stratification according to karyotype risk group
Cytogenetic analysis was carried out according to standard techniques, and the abnormalities were categorised in 3 cytogenetic groups. Patients with inv(16)/t(16;16), t(8;21) and t(15;17) abnormalities were considered as being in the favourable-risk category. The unfavourable-risk category was defined by the presence of -5/del(5q), -7del(7q), t(6;9), t(9;22), 3q26 abnormality or complex karyotype (more than 3 abnormalities). All other patients were classified as intermediate-risk. Karyotypes were described according to International System for Human Cytogenetic Nomenclature (1995).

Analysis of FLT3 internal tandem duplication mutations in AML
The internal tandem duplications in exon 11 of the human *FLT3* gene were determined as described previously.[23] Briefly, cDNA (derived form 50 ng total RNA) and genomic DNA (1 mg) were subjected to PCR using primers 11F 5'-CAATTTAGGTAT-3' and 11R 5'-CAAACTCTAAATTTTCTCT-3'. The PCR cycling conditions were as follows: 3 minutes 94°C followed by 30 cycles 1 minute 94°C, 1 minute 54°C, 1 minute 72°C; and a final step of 10 minutes 72°C. PCR products were resolved on a 2.5% agarose gel.

Statistical Analysis
Statistical analysis was performed with the Stata Statistical Software, Release 7.0. (Stata, College Station, TX). Spearman's rank correlation, Pearson's chi-square test and Kruskal-Wallis test were used to assess the association between *EVI1* and *MDS1-EVI1* expression and the clinical and haematological characteristics of patients. Actuarial probabilities of overall survival (OS; with failure death due to any cause) and event-free survival (EFS; with failure in case of no complete remission [CR] at day 1, or at relapse or at death in first CR) were estimated by the method of Kaplan and Meier. The Cox proportional hazard model was applied to determine the association of high *EVI1* expression with OS

and EFS, without and with adjustment for other factors as age, cytogenetic risk and *FLT3* internal tandem duplication (*FLT3*-ITD). All tests were done two-sided, and a P of less than 0.05 was considered statistically significant.

Results

Quantification of EVI1, MDS1-EVI1 and MDS1 by real-time PCR

EVI1, *MDS1-EVI1* and *MDS1* expression levels were analysed using real-time PCR employing specific primer/probe combinations (Figure 1; Table 2). Efficiency of the quantification method for *EVI1* and *MDS1/EVI1* was examined by standard curves made using mRNA isolated from AML samples that were positive for *EVI1* or *MDS1-EVI1*. Linear correlation between Ct values versus copy number was obtained for *EVI1* and *MDS1-EVI1* with correlation coefficients of 0.94 and 0.98 respectively. The efficiency of amplification, determined in cDNA obtained from a bone marrow sample of patient 2, was approximately 1.00 for *EVI1*, 0.95 for *MDS1-EVI1* and 0.96 for *PBGD*. To evaluate whether the δδCt method that used in our study, was indeed applicable we verified the differences in efficiencies of amplification in another 4 AML samples (samples 25, 28, 29 and 30). The slopes of the fitted lines for the mean δCt values at different mRNA concentrations were -0.089 for *EVI1* and 0.036 for *MDS1-EVI1*, indicating that the δδCt method was indeed applicable. The expression levels of *MDS1* transcripts were too low to measure the efficiency of amplification. Therefore we decided not to quantify the *MDS1* expression level but to show whether it is expressed (+) or not (-).

As calibrator we used the average expression of *EVI1* and *MDS1-EVI1* in 9 bone marrow samples of healthy volunteers. The mean Ct values of *EVI1* and *MDS1-EVI1* in these normal samples were 38.4 ± 1.5 and 38.3 ±2.6 respectively. The values obtained were normalised for the internal reference, *PBGD*. The mean *PBGD* value for normal bone marrow samples was 23.2 ± 0.9. *MDS1* expression was undetectable in bone marrow samples of healthy volunteers.

EVI1, MDS1-EVI1 and MDS1 expression in AML patients carrying 3q26 abnormalities

The relative expression of *EVI1* and *MDS1-EVI1* transcripts in patients with 3q26 abnormalities is shown in Table 3. In 3 AML patients carrying an inv(3)(q22;q26) and in 1 AML patient with a translocation t(3;3)(q22;q26), high expression of *EVI1* as well as *MDS1-EVI1* transcripts was observed. One of the 2 RAEB-t patients with t(3;12) showed high *EVI1* levels and a moderate increase in *MDS1-EVI1* expression, while in the other RAEB-t case *MDS1-EVI1* expression was high and *EVI1* expression was comparable to normal bone marrow. A CML patient with t(3;17) showed weak expression of *EVI1* and no detectable expression of *MDS1-EVI1*. Expression of *MDS1* was observed in patient 6 only.

Northern blot analysis was carried out using an *EVI1* cDNA probe on total mRNA isolated from 2 patients (patient 2 and 3) carrying inv(3)(q22q26) and a CML patient (patient 7) with a translocation t(3;17) (Figure 2). Although this probe does not

Table 3. *EVI1, MDS1-EVI1,* and *MDS1* expression in 7 AML patients with 3q26 abnormalities.

Patient	FAB	Cytogenetic abnormalities*	of *EVI1*†	of *MDS1-EVI1*†	*MDS1*¥
1	M5	inv(3)(q22q26),−7	1 618	104	−
2	M1	inv(3)(q22q26)	4 390	4 390	−
3	M0	inv(3)(q21q26)	4 771	416	−
4	M4	t(3;3)(q22;q26)	2 353	1 448	−
5	RAEB-t	t(3;12)(q26;p13)	1 951	35	−
6	RAEB-t	t(3;12)(q25a26;p12), del(7)(q22)	1	11 585	+
7	CML	t(3;17)(q26;q22),t(1;17;9;22) (p36;q12;q34;q11)/del(11)(p11.1p14)	30	1	−

* According to the ISCN. † Values represent expression levels of *EVI1* and *MDS1-EVI1* as compared with the average values determined in 9 healthy bone marrow samples 2^{-ddCt} (see "Patients, materials and methods"). ¥ MDS1 expression was considered positive (+) when the Ct value was below 35.

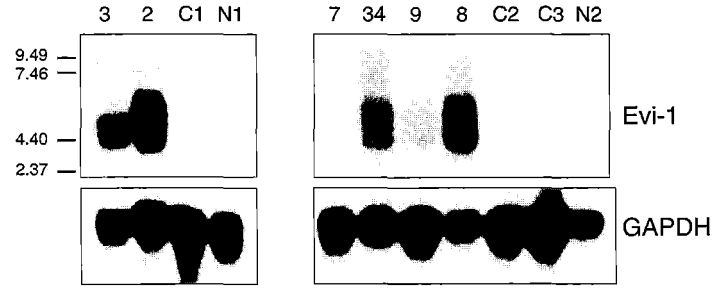

Figure 2. *EVI1* mRNA expression in AML samples as determined by Northern blotting. Human 600bp HindIII-NcoI *EVI1* probe (see Figure 1) was used, which does not discriminate between *EVI1* and *MDS1/EVI1*. Murine *GAPDH* fragment was used as control. Patient 2, 3 and 7 carry a 3q26 abnormality (Table 3), patient 8, 9 and 34 have high *EVI1* expression but no 3q26 abnormality (Table 4). C1, C2, C3 represent AML patients without *EVI1* expression. N1 and N2 represent normal bone marrow samples.

discriminate between the *EVI1* and *MDS1-EVI1* transcripts, high expression as observed by real-time PCR was confirmed. No expression was observed by Northern blot analysis in a normal bone marrow sample (N1) or in a sample from an AML patient without 3q26 abnormality (C1); these 2 samples were negative for different transcripts as determined by real-time PCR (Figure 2).

EVI1, MDS1-EVI1 and MDS1 expression in a cohort of 319 de novo AML patients

The expression levels of *EVI1*, *MDS1-EVI1* and *MDS1* were next investigated by real-time PCR in bone marrow samples of 319 newly diagnosed patients with AML. This cohort did not include the 7 patient from Table 3. Of these 319 patients, 44 expressed *EVI1*, MDS-*EVI1* or both (Table 4): 6 expressed *EVI1* only (Group I), 26 expressed *EVI1* as well as *MDS1-EVI1* (Group II) and 12 expressed *MDS1-EVI1* only (Group III). In the remaining 275 cases (Group IV) neither *EVI1* nor *MDS1-EVI1* was expressed. In 15 patients *MDS1*

Table 4. Cytogenetic characteristics and FAB classifications of 44 de novo AML patients with high *EVI1* and or *MDS1-EVI1* expression.

Patient no. and group	Karyotype*	FAB†	Relative expression of *EVI1*‡	Relative expression of *MDS1-EVI1*‡	*MDS1* expression§
Group I, *EVI1*$^+$ and *MDS1-EVI1*$^-$					
8	46,XX,r(2)(p?q?),add(5)(q1?3),der(11)t(11;12)(q1?4;p13), der(12)t(11;12)(q1?4;p13)del(12)(p13p13)[23]/45,idem, −7[3]/46,XX[6]	Mx	1 176	1	−
9	46,XX[68]	M4	3 821	1	−
10	46,XX,t(1;6)(p32;q24or25),del(2)(q34)[33]/46,XX[1]	M0	4 420	1	−
11	43-45,XY,−3[19],−5[4],der(5)t(5;17)(q1?3;q2?1)[15], −7[19],i(8)(q10)[11], −11[13],−17[19],ider(19)(q10)add (19)(q13)[18],add(2)(q1?3)[13]/46,XY[2]	M6	4 705	1	−
12	45,XY,Inv(3)(q12q26.2),−7[20]	M0	19 552	1	−
13	46,XX,−7[27]/46XX[3]	M4	24 920	1	−
Group II, *EVI1*$^+$ and *MDS1-EVI1*$^+$					
14	46,XY[23]	M5	53	220	−
15	46,XX,der(6)t(6;11)(p12;q23),der(11)t(6;11)add(11)(p15)[4]/46,XX[6]	M4	55	685	−
16	47,XY,+8[4]/46,XY[16]	M5	93	220	−
17	47,XX,+13[58]/46,XX[1]	M5	141	12 810	−
18	46,XX[34]	M1	149	340	−
19	46,XY,−7,add(12)(p12)[13]/46,XY[15]	M1	179	95 950	+
20	45,XY,−7,t(7;8)(q22;p11)[25]	M5	308	12 766	+
21	46,XX,t(9;22)(q34;q11)[10]	M2	389	15 936	+
22	46,XX,t(9;11)(q34;q23)[5]	M1	578	15 771	−
23	46,XY,t(3;3)(q2?3;q26),−7,t(10;20)(p13;q11),+r.ishr(7)(cen7+)[5]/46,idem,i(21)(q10)[8]/46,idem,ins(12;?)(q175;?) [2]/46,XY[4]	Mx	699	25 532	+
24	45,XY,−7[5]/46XY[4]	M2	690	1 716	+
25	46,XY,t(9;22)(q34;q11)[22]	M2	2 436	14 067	−
26	46,XX,del(7)(q22)[41],46,XX[1]	M2	2 513	231 395	+
27	46,XX[10]	M5	2 947	32 996	+
28	46,XY,t(6;11)(q25;q23)[38]	M1	3 083	156 956	+
29	46,XY[40]	M5	3 456	151 085	+
30	45,XY,−7,t(9;11)(p21;q23)[33]	M4	3 916	29 944	−
31	46,XY,t(11;19)(q23;p13)[11]	M4	4 513	31 542	+
32	45,XX,t(2;9;11)(p13;p22;q23)[20]	M4	4 804	21 174	+
33	46,XX,t(9;11)(p21;q23)[10]	M5	7 383	25 268	+
34	45,XY,−7[33]/46,XY[8]	M3	9 541	15 286	+
35	45,XY,inv(3)(q22q26),−7[25]	M5	12 119	551	−
36	Failure	M5	16 845	5 349	+
37	46,XY,t(6;11;18)(q26;q23;q23)[23]	M5	17 560	150 562	+
38	46,XY,der(3)del(3)(p1?4q2?4)add(3)(q24),del(18)(q22q24)[5]/46,idem, del(6)(q11q27)[2],del(7)(q2?2q36)[cp3]/45, idem,−7[9]/46,XY[10]	M0	19 082	28 725	−
39	45,XX,inv(3)(q22q26),−7[31]/46,XX[1]	M5	27 554	3 040	−
Group III, *EVI1*$^-$ and *MDS1-EVI1*$^+$					
40	46,XX[31]	M5	1	53	−
41	46,XY[32].	M2	1	67	−
42	46,XX,inv(16)(p13q22)[8]/47,XX,idem,+22[47]	M4	1	70	−
43	46,XY,del(12)(q11q21),t(15;17)(q22;q11)[65]	M3	1	73	−
44	46,XX[37]	M2	1	88	−
45	47,XX,+21[22]/46,XX[7]	M2	1	129	−
46	46,XY,del(6)(q14q16),t(10;17)(p15;q21),?der(11)[7]/46,XY[3]	M1	1	151	−
47	45,X,−Y,t(8;21)(q22;q22),−13[27]/46,XY[1]	M2	1	165	−
48	44,XX,add(2),−5,−7[2]/45,XX,dic(5;7)(p1;p1)del(5)(q3?1q3?3)[2]/37−42,XX,der(1)t(1;4)(q1?;p1?),−4,dic(5;7)(p1;p1)del(5)(q3?1q3?3), del(8)(p?21;p2?2),inv(10)(p12q?23),−15,add(15)(q2?3),dic(17;?)(p11;?),−18,del(20)(q11q13)/46,XX[13]	M3	1	209	−
49	46,XX,Inv(11)(p15q13),t(15;17)(q22;q12)[28]/46,XX[1]	M3	1	209	−
50	46,XY[52]	M3	1	296	−
51	46,XX[26]	M1	1	28 725	−

Group IV (*EVI1*$^-$ and *MDS1-EVI1*$^-$) contained 275 of the 319 patients analyzed.

*According to the ISCN.[22]

†Mx indicates FAB not defined.

‡Values represent expression levels of *EVI1* and *MDS1-EVI1* as compared with the average values determined in 9 healthy bone marrow samples $2^{-\delta\delta Ct}$ (see "Patients, materials, and methods").

§*MDS1* expression was considered positive (+) when the Ct value was below 35.

gene (+) was detectable. *MDS1* expression was always associated with high *EVI1* plus *MDS1-EVI1* levels (Table 4).

To confirm the results obtained by real-time PCR, Northern blot analysis was carried out in 6 cases. Patients who appeared to be highly positive for *EVI1* and/or *MDS1-EVI1* by real-time PCR (patients 8, 9 and 34) also showed the proper sized transcripts by Northern blotting analysis (Figure 2). Two patients who were negative by real-time PCR (C2 and C3) and a normal bone marrow sample (N2) showed no transcripts by Northern blot analysis.

3q26 abnormalities in de novo AML samples expressing EVI1 or MDS1-EVI1

Cytogenetical analysis among the 44 patients who were positive for *EVI1* and/or *MDS1-EVI1*, revealed that only 4 AML patients (patients 12, 23, 35, 39) carried a 3q26 abnormality (Table 4). None of the patients within group IV (*EVI1*- and *MDS1-EVI1*-) carried 3q26 aberrations.

EVI1 expression correlates with unfavourable karyotypes

3q26 defects in AML are frequently accompanied by additional unfavourable cytogenetic abnormalities (e.g. –7/7q-). In fact, in 2 cases in Table 3 (patients 1 and 6) and all 4 cases with a 3q26 aberration shown in Table 4 (patients 12, 23, 35, 39), chromosome 7 abnormalities were observed. We next investigated whether *EVI1* and/or *MDS1-EVI1* expression in de novo AML without a 3q26 abnormality also correlated with the presence of poor-risk karyotypes (Tables 4 and 5). In 67% cases (4 of 6) from group I (*EVI1* only) and 42% of patients (11 of 26) from group II (*EVI1*+ and *MDS1-EVI1*+), unfavourable karyotypic abnormalities, i.e. –7/7q-, -5/5q-, t(9;22), t(6;9) or complex karyotypes (> 3 abnormalities) were present. In contrast, only 8% (1 of 12) of the patients from group III (*EVI1*- and *MDS1-EVI1*+) and 12% (33 of 275) of the patients from group IV (*EVI1*- and *MDS1-EVI1*-) carried unfavourable karyotypes. Thus *EVI1* expression (Groups I and II) correlated with unfavourable karyotypes ($p< 0.0001$), whereas the presence of *MDS1-EVI1* alone (group III) did not. Moreover, favourable-risk karyotypes, i.e. t(8;21), t(15;17) or inv(16) were not noted in any of the *EVI1*- expressing AML patients from Group I or Group II. In contrast, 33% (4 of 12) of the patients in group III (*EVI1*- and *MDS1-EVI1*+) and 19% (53 of 275) of the patients in group IV (*EVI1*- and *MDS1-EVI1*-) exhibited favourable-risk karyotypes (Table 5).

Among the 212 cases that belong to the leukaemias with intermediate-risk karyotype, 14 showed an 11q23 translocation (*MLL* rearrangement). Interestingly, 8 of these 14

Table 5. Distribution of different karyotypic risk categories and *FlT3*-ITD mutations among groups of AML patients.

	n	Flt3-ITD, no.	Risk karyotype, no.		
			Favorable*	Unfavorable†	Intermediate/unknown‡
Group I, *EVI1*+ and *MDS1-EVI1*-	6	1	0	4	2
Group II, *EVI1*+ and *MDS1-EVI1*+	26	1	0	12	14
Group III, *EVI1*- and *MDS1-EVI1*+	12	1	4	1	7
Group IV, *EVI1*- and *MDS1-EVI1*-	275	82	53	33	189

*t(8;21), t(15;17) or inv(16).
†–7/7q-, –5/5q-, t(9;22), t(6;9) or complex karyotype (>3 abnormalities).
‡Other cytogenetic aberrations or normal karyotype.

patients (57%) were found in Group II, that is, patients expressing *EVI1*+ and *MDS1-EVI1*+ (Table 4). These data suggest a strong correlation between *EVI1* expression and the presence of *MLL* rearrangements.

Correlation of EVI1 and MDS1/EVI1 expression with other prognostic indicators
We next investigated whether *EVI1* and/or *MDS1/EVI1* expression showed any correlation with other known prognostic indicators. Internal tandem duplication in the *FLT3* receptor tyrosine kinase gene has been observed in approximately 20-30% of AML. Moreover, this mutation appears to confer a poor prognosis in many studies carried out in the past 5 years.[24,25] In 85 (27%) of the 319 cases investigated a *FlT3*-ITD was found. As shown in Table 5, high *EVI1* and *MDS1-EVI1* expression rarely coincided with *FlT3*-ITD mutation. Almost all the patients with an *FlT3*-ITD mutation belonged to group IV, and 82% (70 of 85) carried an intermediate-risk karyotype. No significant correlation was found between *EVI1* and/or *MDS1-EVI1* expression and sex, age, WBC count, platelet count, blast counts in blood or bone marrow or FAB classification (data not shown).

EVI1 an independent unfavourable prognostic marker in the intermediate-risk karyotypic group
All patients received induction therapy and were included in the survival analysis. Clinical outcome was investigated in the distinct groups of patients based on their *EVI1* and *MDS1-EVI1* expression. Survival analysis was performed using a cut off value of 50. The remission rates for patients in Groups I, II, III and IV were 50%, 77%, 67% and 79% respectively (Table 6). All patients in group I died within 12 months and 25 of 26 patients of group II (*EVI1*+ and *MDS1-EVI1*+) died within 30 months (Table 6; Figure 3A and B).

Table 6. Therapy response and actuarial survival probability of survival at 60 months in relation to *EVI1* and *MDS1-EVI1* expression.

	n	Complete remission, no. (%)	Actuarial probability of survival at 60 mo, %		Relapse, no. (%)
			EFS	OS	
Group I, *EVI1*$^+$ and *MDS1-EVI1*$^-$	6	3 (50)	0	0	1 (33)
Group II, *EVI1*$^+$ and *MDS1-EVI1*$^+$	26	20 (77)	4	8	15 (75)
Group III, *EVI1*$^-$ and *MDS1-EVI1*$^+$	12	8 (67)	25	33	3 (38)
Group IV, *EVI1*$^-$ and *MDS1-EVI1*$^-$	275	218 (79)	27	33	100 (46)

The actuarial survival probabilities at 60 months were 33% in Groups III and IV and only 0% for group I and 8% for group II (Table 6; Figure 3A and B). Thus patients in Groups I and II had a significantly shorter overall survival (P= 0.002). The event-free survival probabilities at 60 months for groups I and II (0% and 4% respectively) were much lower than those for Groups III and IV (25% and 27% respectively). A significant difference (P= 0.009) was also observed between EFS in patient with high *EVI1* expression (Groups I and II) and that in AML patients without *EVI1* expression (Groups III and IV). We also analysed survival using alternative cutoff values (i.e.10, 25 and 100). Although the

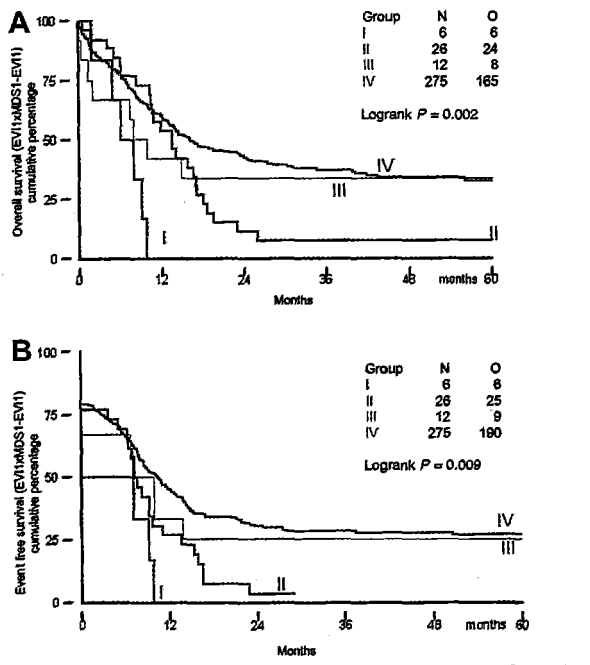

Figure 3. Overall survival and event-free survival in AML patients based on *EVI1* and *MDS1-EVI1* expression.
(A) overall survival; (B) event-free survival.

numbers of patients within the 4 different subgroups were slightly different, changing the cutoff values did not alter the conclusions drawn from the analysis at cutoff value 50 (data not shown).

Cox regression analysis was applied to assess the prognostic significance of high *EVI1* expression for OS and EFS (Table 7). High *EVI1* expression was associated with an increased hazard ratio for death (OS; HR=1.85) or failure (EFS; no CR, death in CR or relapse, HR=1.82), which was statistically significant in univariable analysis. After adjustment for karyotypic risk factors, age and *FLT3*-ITD in a multivariable analysis high *EVI1* expression still associated with an increased hazard ratio (P= 0.09). As high *EVI1* expression is often associated with unfavourable karyotypes, its prognostic value seemed to be overshadowed. To exclude the effect of unfavourable cytogenetics, we decided to investigate the prognostic value of *EVI1* in intermediate-risk group.

We investigated whether high *EVI1* expression would be of prognostic value for patients carrying intermediate-risk karyotypes, as half of the patients with *EVI1* expression (group I and II) belonged to this risk group. As shown in Figure 4A-B, intermediate-risk patients with high *EVI1* expression (n=16) have a significantly shorter OS and EFS (P= 0.05 and P=0.03) than their *EVI1* negative counterparts (n= 196). Furthermore, the disease-free survival was significantly shorter in patients with high *EVI1* expression (P= 0.007). None of the *EVI1*-overexpressing patients carried an *FLT3*-ITD, whereas *FLT3*-ITD was observed

Table 7. Univariable and multivariable analysis of high *EVI1* expression as prognostic factors for survival.

	EFS		OS	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Univariable analysis				
High *EVI1* expression	1.82 (1.25-2.67)	.002	1.85 (1.25-2.73)	.002
Multivariable analysis				
Cytogenetics risk		<.0001		<.0001
Favorable	1 (—)		1 (—)	
Intermediate	2.29 (1.47-3.57)		2.85 (1.71-4.77)	
Unfavorable	3.10 (1.78-5.41)		4.20 (2.26-7.83)	
Age, y		.64		.56
Younger than 35	1 (—)		1 (—)	
35-50	0.98 (0.69-1.38)		1.12 (0.78-1.61)	
Older than 50	1.12 (0.81-1.57)		1.21 (0.85-1.73)	
FLT3 mutation vs no mutation	1.48 (1.11-1.98)	.01	1.53 (1.12-2.09)	.009
High *EVI1* expression	1.47 (0.96-2.25)	.09	1.48 (0.96-2.29)	.09

— indicates not applicable.

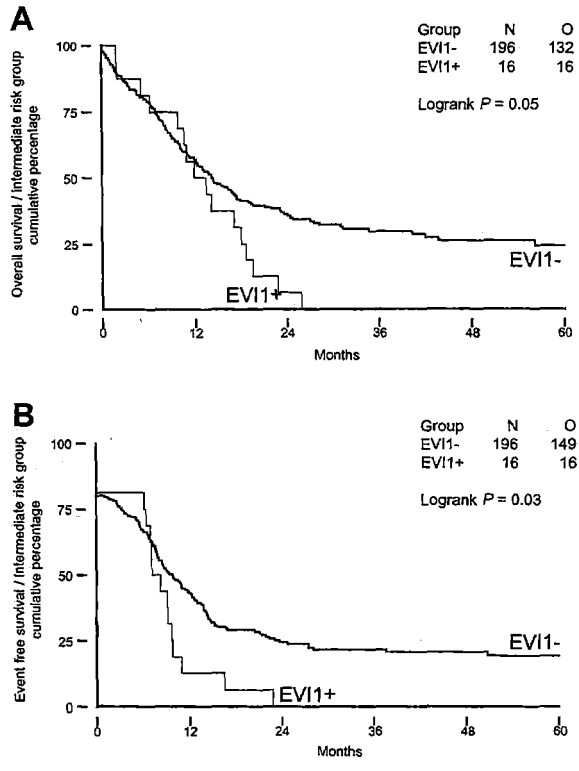

Figure 4. Overall survival and event-free survival in AML patients with intermediate-risk karyotype based on *EVI1* expression. (A) overall survival; (B) event-free survival.

in 36% (70/196) of the patients without high *EVI1* expression. Univariable and multivariable analysis revealed that high *EVI1* expression serves as an independent prognostic marker for EFS and OS in the intermediate-risk group (Table 8).

	EFS		OS	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Univariable analysis				
High *EVI1* expression	1.76 (1.05-2.97)	.05	1.69 (1.01-2.87)	.06
Multivariable analysis				
Age, y		.61		.91
Younger than 35	1 (—)		1 (—)	
35-50	0.84 (0.56-1.26)		0.97 (0.64-1.49)	
Older than 50	0.99 (0.67-1.47)		1.05 (0.69-1.61)	
Flt3 mutation vs no mutation	1.71 (1.22-2.38)	.002	1.62 (1.14-2.30)	.009
High *EVI1* expression	2.09 (1.22-3.60)	.01	2.01 (1.16-3.47)	.02

— indicates not applicable.

Table 8. Univariable and multivariable analysis of high *EVI1* expression as prognostic factor for survival in intermediate-risk karyotype.

Discussion

We developed a sensitive method to quantify *EVI1* and its fusion transcript *MDS1-EVI1* in AML. Our data demonstrate that *EVI1* rather than *MDS1-EVI1* is a strong indicator for poor treatment response and survival. *MDS1* expression was seen in 5% (15 of 319) of the AML patients and was always associated with high *EVI1* and *MDS1-EVI1* expression. High *EVI1* mRNA expression was observed in 10% (32 of 319) of the patients with newly diagnosed AML. Poor clinical outcome of patients with 3q26 abnormality has previously been reported.[3,26-28] As we demonstrated in this study, patients with 3q26 abnormality represent a minor subgroup of patients with high *EVI1* expression. In fact only 12.5% (4 of 32) of the patients with high *EVI1* expression carried a 3q26 abnormality. High *EVI1* expression was significantly correlated with the presence of unfavourable cytogenetic abnormalities. Favourable-risk karyotypes were not present among the *EVI1*-expressing groups.

Evi1 was first discovered as a proto-oncogene in retrovirally induced myeloid leukaemias in the mouse. Retroviral insertions in the *Evi1* locus in those tumours mostly occurred 5' in close vicinity of the first two or three exons of *Evi1*, causing *Evi1* overexpression.[29] These data underline that *EVI1* rather than *MDS1-EVI1* is the transforming gene in AML. This conclusion is further strengthened by the fact that in the present study, *EVI1* and not *MDS1-EVI1* expression correlated with unfavourable-risk leukaemias.

In contrast to what has been published previously, we demonstrate that *EVI1* expression in de novo AML may be an important parameter to define a subgroup of poor-risk AML. Langabeer et al[30] studied 197 de novo AML patients but did not find any prognostic value for high *EVI1* expression. This is not surprising, however, as the investigators

did not discriminate between *EVI1* and *MDS1-EVI1* expression. A number of *EVI1*-positive patients in this study carried a favourable-risk karyotype, indicating that these patients are most likely expressing *MDS1-EVI1* rather than *EVI1*. Furthermore, previous studies were carried out using classical RT-PCR, while we performed quantitative real-time PCR to be able to determine high *EVI1* expression levels based on a defined cutoff value.

Cytogenetic analysis provides a powerful approach to discriminate between favourable-risk and unfavourable-risk groups among AML patients. However, using karyotyping, only 30% to 40% of the AML patients can be classified within these 2 subgroups. In other words, a majority of AML patients belong to intermediate or unknown karyotypic risk groups. A major challenge will be discriminating favourable-risk from unfavourable-risk patients within this heterogeneous group of patients by means of molecular biological approaches. Among the cases with intermediate-risk karyotype studied, 33% (70 of 212) harboured an *FLT3*-ITD mutation that predicts poor prognosis. Sixteen (8%) of the 212 patients had high *EVI1* expression and showed very poor survival. Interestingly, non-of the *EVI1*-positive cases harboured an *FLT3*-ITD, indicating that the *EVI1*-expressing group represents a distinct subclass of poor-responsive leukaemias. Within the same group with intermediate-risk karyotype a subpopulation of poor responders has been defined with very low mRNA levels of the *CEBPA* gene (this thesis). Again no overlap was found with the other 2 molecularly defined classes. Moreover, mutation analysis revealed another subset of intermediate-risk AML cases that harboured 3' mutations within the *CEBPA* gene. These cases could be categorised as leukaemias with a good prognosis (this thesis). These observations encourage additional gene expression studies and mutation analyses to further unravel different classes of AML, particularly within the intermediate-risk karyotypic subgroup of AML.

In 8 of 14 patients with an 11q23 abnormality, we observed high *EVI1* and *MDS1-EVI1* expression. In previous studies, the correlation between *EVI1* with 11q23 has been overlooked, as the cohorts were not large enough and the methods used did not discriminate between *EVI1* and *MDS1-EVI1*. Two of 16 *EVI1*-positive patients screened by Ohyashiki et al [17] and 1 out of 29 *EVI1*-positive patients found by Langabeer et al,[31] carried an 11q23 abnormality. Translocations involving chromosome band 11q23 disrupt the *MLL* gene. MLL is a putative transcription regulator that may form complexes with other transcription factors. It contains both a strong activation domain and a repression domain.[32] In de novo leukaemia, 75% of breakpoints in *MLL* are mapped to the centromeric half of the breakpoint cluster region (BCR),[33] which is located in the repression domain. Disruption of the repression domain in these particular 11q23 translocations might lead to an alteration of the repressor function of MLL, leading to an upregulation of downstream target genes. A possible explanation for high *EVI1* and *MDS1-EVI1* expression in a large proportion of patients with 11q23 defects could therefore be that transcription of those genes is under the control of MLL. We hypothesise that MLL normally repress *EVI1* and *MDS1-EVI1* expression. This repression might then be disrupted as a result of a chimeric protein generated by 11q23 translocation, as it has been suggested for *HOX* genes.[5] Cloning and nucleotide sequencing analysis of the *MLL* fusion

genes in *EVI1*-positive versus *EVI1*-negative patients may provide critical information on this issue.

One of the goals of this study was to investigate whether *EVI1*, *MDS1-EVI1* or both transcripts were expressed in AML patients with 3q26 aberrations. All of the 8 AML patients with a classical t(3;3) or an inv(3) (Tables 3 and 4) showed high expression of *EVI1*. High *EVI1* expression in 7 patients was associated with high *MDS1-EVI1*. Thus, although our data suggest that *EVI1* rather than *MDS1-EVI1* is the critical gene involved in transformation of myeloid precursors, it is noteworthy that *MDS1-EVI1* is also frequently expressed. Since the 3q26 breakpoints are often located between the *MDS1* and *EVI1* loci, the normal allele is responsible for *MDS1-EVI1* expression. *MDS1-EVI1* expression might be directly or indirectly up-regulated by *EVI1* expression. Another possible explanation is that aberrantly expressed EVI1 as a result of 3q26 aberration mainly transforms progenitor cells that normally have high *EVI1* and/or *MDS1-EVI1* levels. Fractionated CD34+ progenitor cell populations indeed show high *EVI1*, *MDS1-EVI1* and *MDS1* expression (our unpublished data). Previous studies[10,34] also confirmed *EVI1* expression in early CD34+ progenitor cells. In fact, transformation in these progenitors may be a result of a disturbance in the tightly controlled balance between *EVI1* and its fusion transcript.

The mechanism by which *EVI1* is expressed in cases without 3q26 is unknown. It is conceivable that defects in the *EVI1* promoter or in *EVI1*-regulatory genes affect expression. It is also possible that CD34+ progenitor cells naturally expressing *EVI1* and/or *MDS1/EVI1* are transformed by other mechanisms and arrested at that particular stage of differentiation. It should be noted that high *EVI1* expression is often associated with the presence of poor-risk abnormalities. For example, complex karyotypes are seen twice as often in patients with high *EVI1* expression than in patients without *EVI1* expression. Our data point to a critical role that *EVI1* may play in genomic instability in AML patients expressing this gene.

The data presented here demonstrate frequent *EVI1* overexpression in AML patients and its correlation with poor treatment outcome. We propose that *EVI1* gene expression measurements with real-time PCR should be incorporated into the diagnostic procedures for the novo AML patients, especially in the subpopulation with intermediate- or unknown-risk karyotypes. Determination of *EVI1* expression levels in this particular category of AML patients will be useful in distinguishing a subgroup of patients with poor prognosis. Identification and classification of subtypes of AML with specific molecular defects will be of great value in designing unique stratified treatment approaches.

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Chapter 3

Low expression of *MDS1-EVI1*-like-1 *(MEL1)* and EVI1-like-1 *(EL1)* genes in favourable-risk acute myeloid leukaemia

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Abstract

The expression of an *MDS1-EVI1*-like-1 (*MEL1*) gene is reported in acute myeloid leukaemia (AML) and myelodysplastic syndrome (MDS) with translocation t(1;3)(p36;q21). *MEL1* (at chromosome band 1p36.3) is thought to be transcriptionally activated as a result of juxtaposition to the *RPN1* gene at 3q21. It is not known whether *MEL1* expression is restricted to patients with this particular translocation. Using real-time PCR, we measured *MEL1* expression levels in 162 de novo AML patients, normal bone marrow and distinct blood cell fractions. We also investigated the existence of an *EVI1*-like gene (*EL1*) by applying the same method. The existence of these transcripts was confirmed by northern blot analysis. *MEL1* expression was detected in 87% (141 of 162) of de novo AML patients. The *EL1* (*EVI1*-like) transcript was also detected in the majority of the patients. *MEL1* and *EL1* showed variable expression levels in AML. However, *EL1* expression levels highly correlated with *MEL1* expression levels. All the patients with favourable-risk karyotypes (i.e. t(15;17), t(8;21) or inv(16)), showed low *MEL1* and *EL1* expression levels. Expression analysis of *MEL1/EL1* compared with *MDS1-EVI1/EVI1* in normal marrow or distinct blood cell fractions revealed that 1) all four gene-products are expressed in CD34+ progenitor cell fractions, 2) Both *MEL1* and *EVI1* are turned down in neutrophils and monocytes/macrophages; while 3) *MDS1-EVI1* and *EL1* remain expressed in mature blood cell fractions. Our data suggest that simultaneous low *MEL1/EL1* expression in AML is abnormal and that favourable disease highly associates with this abnormal phenotype.

Introduction

The *MDS1-EVI1*-like-1 (*MEL1*) gene is located at chromosome band 1p36.3. and is highly homologous with the *MDS1-EVI1* fusion transcript. The N-terminal PR domain of MEL1 is 52% identical to MDS1 protein. Furthermore, the C- and N-terminal DNA-binding domains of MEL1 show 74% and 96% homology with the two respective domains in EVI1.[1] *MEL1* expression has been shown in acute myeloid leukaemia (AML) and myelodysplastic syndrome (MDS) patients carrying a reciprocal translocation t(1;3)(p36;q21).[1] Likewise, high *MDS1-EVI1* expression has been demonstrated in patients with a translocation t(3;3)(q26;q21). It is noteworthy that high *MDS1-EVI1* expression is not restricted to patients with 3q26 abnormalities; a subgroup of patients without such aberrations do also show high *MDS1-EVI1* expression.[2] *MEL1* expression, however, has not been reported in AML or MDS cells without a translocation t(1;3)(p36;q21).[1]

MDS1-EVI1 transcript is an intergenic splicing product of *MDS1* and *EVI1*.[3] AML cells may express *MDS1-EVI1*, *EVI1* or both transcripts.[2] Whether *MEL1* also has an *EVI1* like (*EL1*) counterpart in primary AML or healthy bone marrow cells has not been documented. Northern blot analysis, using a *MEL1*-specific probe, on mRNA isolated from AML blasts carrying a translocation t(1;3)(p36;q21) revealed the existence of several transcripts.[1,4] Mochizuki et al.[1] demonstrated the presence of 2 different-sized MEL1 proteins by in-vitro transcription and translation experiments. Although the exact nature

of the distinct *MEL1* transcripts and proteins has not been fully resolved yet, it seems that one of these products represents an EVI1-like (EL1) protein, that is, MEL1 without the N-terminal MDS1-like part. The existence of the EL1 protein might be important in the diagnosis of human AML, as our previous study demonstrated that *EVI1* rather than *MDS1-EVI1* is a strong indicator for poor treatment response in AML.[2]

In this study, we investigated the expression of *MEL1* transcripts in a cohort of 162 de novo AML patients. We also studied the existence of the *EL1* mRNA in human AML. We demonstrate that *MEL1* expression is not restricted to AML with translocation t(1;3)(p36;q21). In fact, *MEL1* transcripts are present in about 87% of the patients. Besides, we demonstrate the existence of an *EL1* transcript, which is normally transcribed from the *MEL1* locus (Figure 1A-B). *MEL1* and *EL1* expression in AML are highly correlated. Although expression levels of *MEL1* and *EL1* varies extensively amongst AML patients, the expression of both genes are always low in the karyotypically defined 'favourable-risk' group, i.e., AML with translocations t(15;17), t(8;21) or inv(16). Finally, we investigated the expression pattern of *MEL1/EL1* compared with that of *MDS1-EVI1/EVI1* in CD34 positive normal marrow precursors and in blood neutrophils and monocytes/macrophages. Our data suggest a tightly controlled expression regulation of these four transcripts during granulopoiesis and monopoiesis.

Material and methods

Patients, healthy volunteers and different cell fractions

Bone marrow samples of AML patients at diagnosis and healthy volunteers (n=6) were obtained after informed consent. Blasts from AML patients and mononucleated fractions from healthy bone marrow specimens were isolated from the samples by Ficoll-Hypaque (Nygaard, Oslo, Norway) centrifugation.[5] The cells were then cryopreserved as described by Delwel et al.[6] After thawing cells were washed with Hanks Balanced Salt Solution (HBSS) and further processed for RNA isolation. AML samples treated according to this procedures usually contain greater than 90% blasts after thawing.[6] A total of 162 de novo AML patients that had been referred to our institution between 1987 and 2000 were chosen for analysis. Of these patients, 108 were treated according to the HOVON-29 (Dutch-Belgian Haematology-Oncology Group) protocol and 54 according to the HOVON-4. These treatment protocols have been described previously.[7] AML samples were classified according to the French-American-British nomenclature.[8]

Total RNA was also isolated from peripheral blood granulocytes and monocytes/macrophages using the same procedures. Peripheral blood granulocytes were obtained from the cell pellet after Ficoll-Hypaque centrifugation.[5] Monocytes/macrophages were obtained from the interphase following Ficoll-Hypaque centrifugation and subsequent plastic adherence as described previously.[9]

RNA isolation, cDNA synthesis and Real-time PCR

Total RNA was extracted with guanidium thiocyanate followed by centrifugation in cesium chloride solution. Then 1 ml RNA was transcribed into cDNA using Superscript (Life

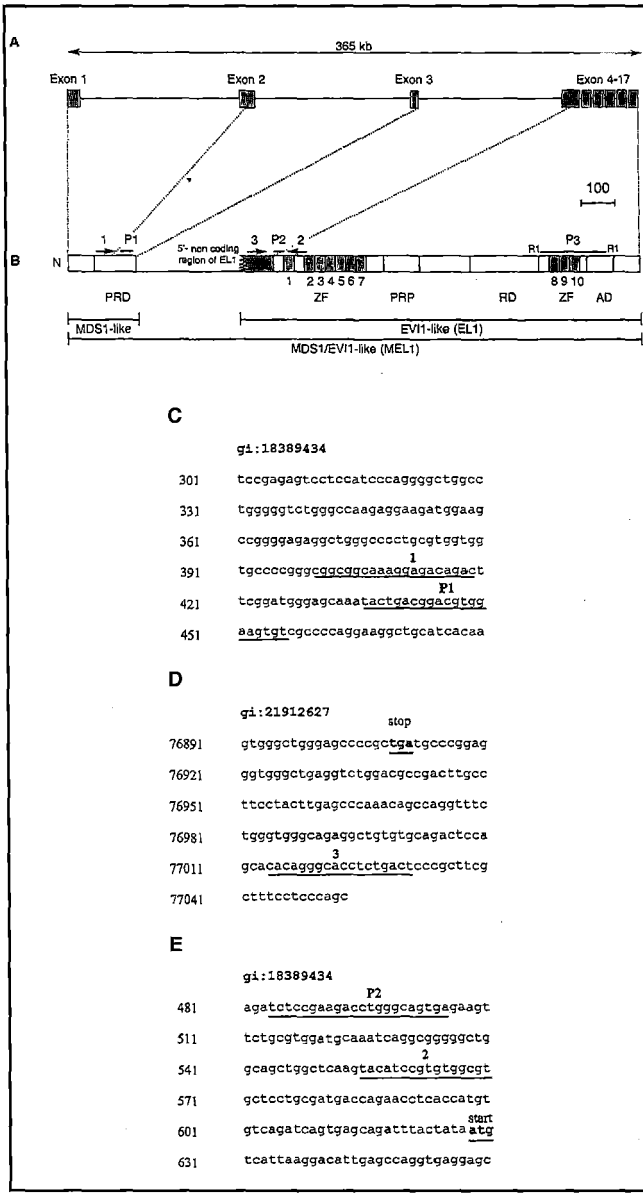


Figure 1. Representation of *MEL1* and *EL1* and the primer and probes used for the real-time PCR. (A) Genomic structure of *MEL1* (B) Schematic representation of *EL1/MEL1* structure: different domains are indicated as: PRD (PR domain) ,ZF (zinc fingers), PRR (proline-rich domain), RD (repression domain) and AD (acidic domain). Primers 1 and 2 plus probe P1 were used to determine *MEL1* expression levels. *EL1* transcript levels were determined using primers 3 and 2 plus probe P2. Probe P3 was used for Northern blot analysis. (C) Nucleotide sequence containing primer 1 and probe P1 at 5' of *MEL1*, (D) nucleotide sequence containing primer 3 at 5' non-coding region of *EL1*, (E) nucleotide sequence containing primer 2 and probe P2. The nucleotide sequences in Figures C (gi:18389434) , D (gi:21912672) and E (gi:18389434) are from the NCBI database.

Technologies, Merelbeke, Belgium) and Random Hexamers in a 40 ml reaction, under standard conditions. An aliquot of 1/20 th of the resulting cDNA was used for quantitative PCR amplification. Real-time PCR amplification was performed with the ABI PRISM 7700 Sequence Detector (PE Biosystems, Nieuwekerk a/d IJssel, the Netherlands), using 50 ml mix containing 2 ml cDNA sample; 250 mM deoxyribonucleoide triphosphates

(dNTPs Amersham Pharmacia Biotech Inc, Roosendaal, the Netherlands); 15 pmol forward and reverse primer (Life Technologies); 3 mM MgCl2; 200 nM probe; labelled at the 5' end with the reporter dye molecule FAM (6-carboxy-fluorescein) for *EL1* and at the 3' end with the quencher dye molecule TAMRA (6-carboxy-tetramethyl-rhodamine) (Eurogentec, Maastricht, the Netherlands); 5 ml 10x buffer A; 30 ml water; and 1.25 U AmpliTaq Gold (PE Applied Biosystems). The thermal cycling conditions included 10 minutes at 95°C followed by 45 cycles of denaturation for 15 seconds at 95°C, annealing/extension at 60°C for 30 seconds. The primer/probe combinations were chosen such that we could discriminate between *EL1* and *MEL1* transcripts (Figure 1B). The oligonucleotide sequences of the primers and probes are shown in Figure1C-D. The Real-time PCR conditions and the oligonucleotide sequences of the primers/probe used for quantifying *PBGD*, *EVI1* and *MDS1-EVI1* are described elsewhere.[2]

To determine the expression levels, all samples were tested in duplicate, and the average values were used for quantification. To quantify the relative expression of *EL1* and *MEL1*, the Ct (threshold cycle) values were normalized for endogenous reference ($\delta Ct = Ct_{target} - Ct_{PBGD}$) and compared with a calibrator, using the 'δδCt' method ($\delta\delta Ct = \delta Ct_{Sample} - \delta Ct_{Calibrator}$). As the calibrator we used the average *MEL1* or *EL1* Ct value in the six bone marrow samples from healthy volunteers. Using the δδCt value, relative expression was calculated ($2^{-\delta\delta Ct}$). As the δδCt method is only applicable when the amplification efficiencies of the target and the reference are essentially equal, we measured the efficiency of *MEL1*, *EL1* and *PBGD* using 6 different dilutions of a cDNA sample (equal to 0.0005-50 ng total RNA). The mean δct values were plotted against the concentration of total RNA (log). The slope of less than 0.1 was indicative of equal efficiencies.

Northern blotting

Northern blotting was carried out on mRNA isolated from 1 healthy bone marrow and 9 AML samples. Twenty micrograms of total RNA from each sample was separated on a 1% agarose/6% formaldehyde gel, and blotted with 10x SSC (sodium chloride/sodium citrate; Amersham) onto hybond-N+ nylon membrane (Amersham). The blot was hybridized in 1N NaH2PO4-buffer containing 7% sodium dextran sulfate, and 1 mM EDTA pH 8.0. As probe, human *MEL1* (639bp EcoR1-EcoR1 fragment) and murine *GAPDH* (777 bp HindIII-EcoRI fragment) 10 were ^{32}P labeled by random priming (Boehringer, Mannheim, Germany). The blot was hybridized at 65 °C overnight and washed for 15 minutes at 65 °C in 2x SSC/ 0.5% SDS and for 15 minutes at 65 °C in 1x SSC/ 0.5 SDS. It was then analyzed by autoradiography.

Cytogenetic analysis and stratification according to karyotype risk group

Cytogenetic analysis was carried out according to standard techniques, and the abnormalities were categorized in 3 cytogenetic groups. Patients with inv(16)/t(16;16), t(8;21) and t(15;17) abnormalities were considered as favourable risk. The unfavourable-risk category was defined by the presence of -5/del(5q), -7del(7q), t(6;9), t(9;22), 3q26 abnormality or complex karyotype (more than 3 abnormalities). All other patients were classified as intermediate risk. Karyotypes were described according to ISCN nomenclature (1995).

Statistical Analysis

Statistical analysis was performed with the SPSS Statistical Software 10. Spearman's rank correlation, Fisher's exact test, Kruskal-Wallis test and Mann-Whitney U-test were used to assess the association between *EL1* and *MEL1* expression and the clinical and haematological characteristics of the patients. All tests were done two-sided. P-values <0.05 were considered statistically significant.

Results

MEL1 expression is constant in normal bone marrow but variable in AML samples

MEL1 expression levels were measured in 6 ficoll fractionated normal bone marrow samples by real-time PCR. The mean Ct values of *MEL1* in these normal samples was 29.9 ± 0.5. The values obtained were normalized for the internal reference, (*PBGD*). The mean *PBGD* value for normal bone marrow samples was 25.3 ± 0.7. The small variation in mean δCt value (4.8 ± 0.6) indicates the rather constant expression level of *MEL1* gene in normal bone marrow.

MEL1 expression levels were subsequently measured in the bone marrow of 162 de novo AML patients using the same technique. The slope of the fitted line for the mean δCt value of *MEL1* at different mRNA concentration was less than 0.1, indicating that the δδCt method was applicable (data not shown). The values obtained were normalized for the internal reference (*PBGD*) and compared with the average expression in normal bone marrow (calibrator). *MEL1* expression had an extensive variability among the AML patients, ranging from below detection level to 309 times more than the average expression in normal bone marrow (Figure 2). *MEL1* transcripts were found in 141(87%) of 162 patients with AML. None of the patients in our cohort carried a 1p36.3 aberration.

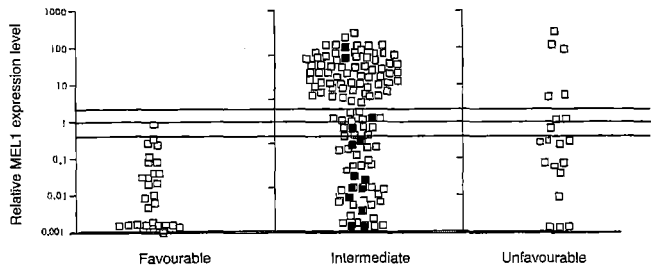


Figure 2. Relative expression of *MEL1* in different karyotypic risk groups.
MEL1 expression levels in different karyotypic risk groups, compared with the average expression in normal bone marrow. The average expression in normal bone marrow is equal to 1 (confidence interval: 0.4-2.3). Each box indicates a patient. Cases with a *CEBPA* mutation are indicated as black boxes.

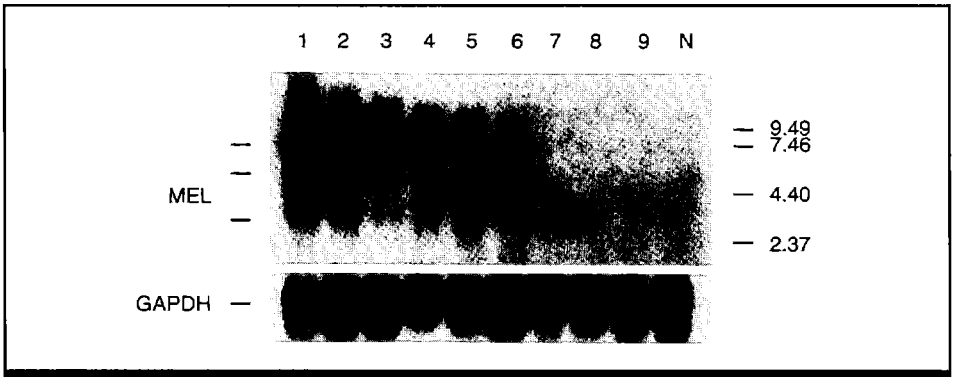


Figure 3. Expression of *MEL1* gene in mRNA isolated from 9 AML patients and a healthy volunteer. Cases 1-6 had relatively high *MEL1* expression levels (52, 24, 64, 42, 247 and 56 respectively) as quantified by Real-time PCR. In addition to the full lengths *MEL1* (8 kb), two other bands (5 kb and 4 kb) are observed in these cases. *MEL1* expression levels in cases 7-9 were 0.2, 0.9 and 0.001 (not detectable). N indicated a normal bone marrow sample.

MEL1 transcripts of different sizes are present in AML

Northern blot analysis was carried out using *MEL1* cDNA probe P3 (Figure1B) to confirm the real-time PCR results. Total mRNA samples were selected from 6 patients with high *MEL1* expression, 3 patients with low *MEL1* levels and from one normal bone marrow (Figure 3). High *MEL1* expression in AML patients 1 through 6 was confirmed by Northern blot analysis. In addition to the 8 kb *MEL1* transcript, 2 other bands (5 and 4 kb) were detected, which is in line with observations of Mochizuki et al[1]. No *MEL1* mRNA was found by Northern blot analysis in normal bone marrow (N) or in samples from patients 7 through 9, which showed low or non-detectable transcript levels as determined by real-time PCR.

Favourable-risk karyotype is associated with low MEL1 expression

AML patients were divided into 3 different groups based on *MEL1* expression levels. Patients with an expression level in the range of 95% confidence interval (0.4-2.3), defined for the average *MEL1* expression in normal bone marrow, were classified in the 'intermediate' *MEL1* expressing group (19 patients). The other patients were divided equally between the 'high' (range 2.5- 309) and 'low' (range 0.001-0.41) *MEL1* expressing categories (72 and 71 cases, respectively). Characteristics of the different *MEL1* expressing groups are shown in Table 1. Low *MEL1* expression was observed in 96% (26 of 27) of the patients with a favourable-risk karyotype (Table 1; Figure 2). In contrast to the favourable risk, the other AML risk groups did not reveal any association with distinct *MEL1* expressing groups. Recently, an additional subgroup of AML with favourable prognosis was characterized by mutations in the *CEBPA* gene.[10] *CEBPA*-mutated AML is

distinguished as a favourable-risk subset among AML with intermediate-risk cytogenetics. Here we show that *CEBPA* mutation is also significantly associated with low *MEL1* expression (Fisher's Exact test: P=0.046; Figure 2). In fact, 10 (71%) of 14 patients with *CEBPA* mutation expressed low levels of *MEL1* mRNA. Furthermore no significant correlation between *MEL1* expression and other variables was observed (Table 1).

Table 1. Clinical characteristics of 162 de novo AML patients based on the *MEL1* expression level

Characteristics		Low MEL1 (n = 1)	Intermediate MEL1 (n = 19)	High MEL1 (n = 2)
Gender	Male	36	10	34
	Female	35	9	38
Age	Median (range)	40 (16–59)	38 (19–61)	45 (16–60)
FAB	M0	2	0	2
	M1	17	6	18
	M2	16	6	14
	M3	12	0	0
	M4	15	1	11
	M5	8	6	22
	M6	0	0	2
	Unclassified	1	0	3
Cytogenetic risk group	Favorable	26	1	0
	Intermediate	33	15	67
	Unfavorable	12	3	5
CEBPA mutation +		10	2	2
WBC count (10^9/L)	Median (range)	20 (0.3–220)	9 (0.7–108)	38 (0.7–263)
Blast count (%)	Median (range)	63 (0–96)	68 (3–98)	73 (21–97)
Platelet count (10^9/L)	Median (range)	40 (7–339)	36 (8–265)	60 (6–931)

AML, acute myeloid leukemia; FAB, French-American-British classification [8]; WBC, white blood cells.

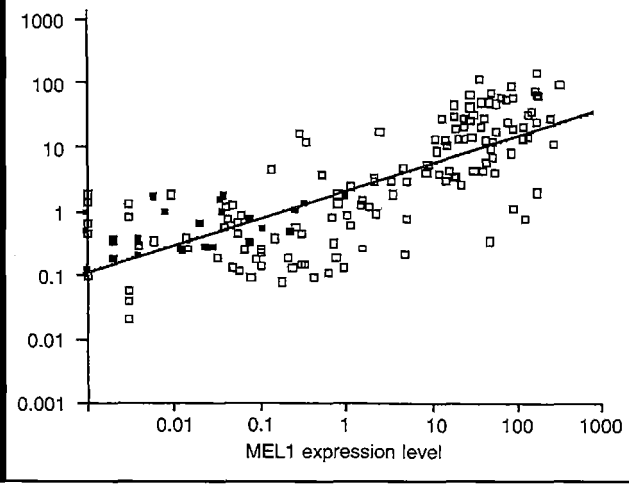
Cox regression analysis was applied to assess the prognostic value of *MEL1* expression levels for overall survival (OS) and event-free survival (EFS). Low *MEL1* expression level was associated with a significant decrease in hazard ratio (HR) for death (OS; HR= 0.65, P= 0.03) and failure (EFS; no CR, death in CR or relapse, HR= 0.72, P= 0.04). After adjustment for karyotypic risk factors, age and *FLT3*-ITD in a multivariable analysis low *MEL1* expression seemed to have no significant impact on the survival of AML patients within the karyotypically defined intermediate- or unfavourable-risk group (data not shown).

EL1 gene is expressed in normal bone marrow and in AML

Because *MEL1* is highly homologous to *MDS1-EVI1*, we decided to investigate the presence of an *EVI1* like gene (*EL1*). By searching the database, we came across a potentially unique *EL1* transcript. To study the existence of this transcript, we designed specific primers to amplify this potential mRNA by RT-PCR (Figure 1B). *EL1* transcripts were amplified in normal bone marrow as well as in AML samples. Subsequent sequence analysis of the PCR products confirmed the existence of this alternative transcript. *EL1* expression levels were measured by real-time PCR in the same 6 normal bone marrow samples described earlier. The mean Ct values of *EL1* in these normal samples

was 32.9 ± 1.3. *EL1* expression levels were subsequently determined in the cohort of AML patients. The values obtained were normalized for the internal reference (*PBGD*) and compared with the average *EL1* expression in normal bone marrow (calibrator). *EL1* expression levels in AML cases varied from 0.001 (below detection level) to 146 times more than the average expression in normal bone marrow. Remarkably, *EL1* expression levels highly correlated with *MEL1* expression levels (Spearman's rho: correlation coefficient=0.76, P<0.0001; Figure 4). Accordingly, *EL1* expression was also significantly lower in patients with favourable risk karyotype (Figure 4).

Figure 4. *EL1* and *MEL1* expression mRNA levels highly correlate: Scatter plot demonstrate the correlation between *EL1* and *MEL1* expression levels (Spearman's rho: Correlation coefficient= 0.76, P<0.0001). The black boxes indicate cases with favorable risk karyotype.



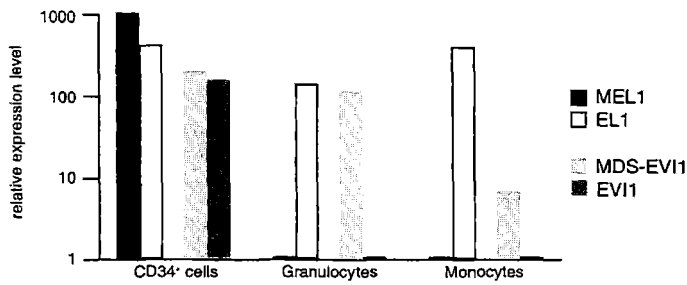


Figure 5. *EL1/MEL1* and *EVI1/MDS1-EVI1* expression levels in different cell fractions. Expression levels of these genes are measured in normal CD34+ sample and distinct cell fractions isolated from peripheral blood (granulocytes and monocytes/macrophages). The expression levels are compared with a Ct of 40, which corresponds to the detection limit. Accordingly, each bar demonstrates the extent of expression over the detection limit.

MEL/EL and MDS1-EVI/EVI expression in normal haematopoietic cells

MEL1, *EL1*, *EVI1* and *MDS1-EVI1* expression levels were determined by real-time PCR in a normal CD34+ sample and distinct cell fractions isolated from peripheral blood, i.e., granulocytes and monocytes/macrophages (Figure 5). *MEL1/EL1* as well as *MDS1-EVI1/ EVI1* were highly expressed in CD34+ cells. The expression of *EL1* was relatively high in granulocytes and monocytes/macrophages, whereas *MEL1* expression was reduced in those cells. On the other hand, *MDS1-EVI1* was expressed in the differentiated blood cells, whereas *EVI1* was below the detection level.

Discussion

MEL1 gene was recently identified at the breakpoint cluster region of chromosome band 1p36.3 in cells from MDS/AML patients carrying a translocation t(1;3)(p36;q21).[1,4] Previous studies concluded that *MEL1* is transcriptionally activated in haematopoietic cells as a result of the juxtaposition of *MEL1* at the 1p36.3 locus to the *RPN1* gene at 3q21.[1,4] It should be noted that in previous studies *MEL1* expression analysis was only carried out in selected groups of AML patients carrying translocation t(1;3)(p36;q21). In those studies, AML cell lines without this particular translocation served as control cells. In the present study, we applied a sensitive assay to measure *MEL1* expression in 162 patients with AML. This cohort did not include patients with translocation t(1;3)(p36;q21). We demonstrate that *MEL1* expression is not restricted to leukaemia cells with chromosome 1p36 aberrations. In 87% of the AML patients, *MEL1* transcripts were detectable by real-time PCR. We also demonstrated high *MEL1* expression levels by Northern blot analysis in several AML samples. Based on these results we conclude that *MEL1* is frequently expressed in AML and that expression of *MEL1* does not require juxtaposition of the 3q21 locus.

This study also establishes for the first time the existence of an alternative variant of the *MEL1* transcript in AML and normal bone marrow, which encodes for an EVI1 like protein (EL1). The expression levels of *EL1* in AML patients highly correlate with *MEL1* transcript levels. In fact, the existence of two different MEL1 protein products of 170-kDa and 150-kDa in AML and in cells transfected with cDNA constructs has been demonstrated by Morishita and Nishikata.[11] The shorter protein lacks the N-terminal PR domain of MEL1 and appears to be initiated from an internal initiation codon ATG597. Whether one of the bands observed on the Northern blot (Figure 3) truly represent the *EL1* variant is still unclear, as a suitable cDNA fragment that could discriminate between *MEL1* and *EL1* has not yet been identified.

It previously was suggested that *MEL1* was not present in normal bone marrow samples.[1,4] *MEL1* transcripts are not detectable in normal marrow by Northern blot analysis. However, real-time PCR data clearly demonstrated that significant levels of *MEL1* as well as *EL1* transcripts are present in the marrow cells of healthy individuals. These data suggest that *MEL1/EL1* expression is, in principle, not the effect of a genetic alteration.

The fact that *MEL1* and *EL1* expression is not restricted to cases with a translocation t(1;3)(p36;q21) does not implicate that this particular aberration has no effect on *MEL1*

expression. In fact, a similar situation has been documented for *EVI1*. *EVI1* is abnormally expressed as the result of translocations involving chromosome band 3q26, where the gene resides.[12] However, *EVI1* gene is also expressed in approximately 10 % of AML cases without 3q26 abnormalities.[2] *MEL1* and *EL1* are expressed in the majority of AML cases and in CD34+ purified marrow cells. In contrast, in neutrophils and monocyte/macrophage fractions, the *EL1* levels were high whereas *MEL1* levels were undetectable. This suggests that the relative expression between those two transcripts changes during differentiation. Most of the 1p36.3 breakpoints cluster in a region 5' of the *MEL1* gene.[4] Translocation t(1;3)(p36;q21) may disrupt the tight control of transcriptional regulation. *MEL1* and *EL1* are then constitutively turned on under the control of the regulatory sequences of *RPN1*, located on chromosome 3q21. In this model, *MEL1*, which normally extinguishes during differentiation, remains transcriptionally active. In cases with 3q26 abnormalities, the breakpoints frequently occur 5' end of *EVI1* and 3'of *MDS1*.[12] As a result, *EVI1*, which normally is switched off during differentiation (Figure 5), may remain transcriptionally active. Our data suggest that the homologous MEL1/EL1 and MDS1-EVI1/EVI1 proteins may cooperate under tight control in the same pathway. Disruption of this control mechanism by chromosomal translocations could seriously alter the balanced expression of these genes and thereby disturb a critical pathway required for differentiation. It will be interesting to investigate the interaction and the function of these proteins in normal haematopoiesis as well as in leukaemic transformation.

Favourable-risk AML patients, i.e., patients with inv(16)/t(16;16), t(8;21) or t(15;17) showed consistently low *MEL1* and *EL1* expression levels. Furthermore, we also demonstrated low *MEL1/EL1* levels in another recently molecularly defined favourable-risk group, i.e., cases with *CEBPA* mutations.[2,13] An altered common pathway in favourable-risk patients may result in down-regulation of *MEL1/EL1*. High *EL1* and *MEL1* expression levels in about half of the AML cases probably represent high numbers of non-differentiated haematopoietic precursor cells. However, low expression of both *MEL1* and *EL1* in the patients with a favourable prognosis is unusual, because even in normally differentiated cells (e.g. neutrophils) *EL1* is still highly expressed. Therefore, it seems that low expression of both genes is an aberrant phenotype associated with favourable disease. Obviously this phenotype is observed in all the patients with chromosomal translocations involving critical transcription factors, e.g. CBF (Core Binding factors), C/EBPα or RARα. The modified proteins produced as a result of mutations or translocations may act as transcription repressors for *MEL1* and *EL1*. In fact, several potential binding domains for PML-RARα or AML-ETO were identified upstream of *MEL1* (data not shown). It would be interesting to investigate whether these modified gene-products affect *MEL1* promoter activity. Altogether, the association with favourable risk AML may suggest a relation between severity of the disease and *MEL1/EL1* expression in this particular group of leukaemia.

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Chapter 4

Biallelic mutations in the *CEBPA* gene and low *CEBPA* expression levels as prognostic markers in intermediate-risk AML

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Abstract
The CCAAT/Enhancer Binding Protein α is an essential transcription factor for granulocytic differentiation. Recent studies reported N-terminal and C-terminal *CEBPA* mutations in approximately 7% of acute myeloid leukaemia (AML) patients. C-terminal mutations are usually in-frame and occur in the Basic-leucine zipper (bZIP) domain, resulting in deficient DNA binding. Using a rapid PCR approach, we screened for bZIP mutations and determined the prognostic value of these mutations in a cohort of 277 de novo AMLs. In addition we set out to quantify *CEBPA* mRNA levels by 'real-time' PCR using TaqMan® technology. In-frame insertions were observed in 12 (4.3%) cases. All cases with mutations carried an intermediate-risk karyotype and all but one belonged to M1 or M2 FAB class. Further sequence analysis revealed that *CEBPA* C-terminal mutations are associated with frameshift mutations in the N-terminus of *CEBPA*. These two mutations were always found in different alleles. Event free survival (EFS) and overall-survival (OS) of patients with *CEBPA* mutations were significantly increased (P= 0.02 and P= 0.03, respectively) in comparison to the patients lacking these mutations. Mutations were associated with a significantly reduced hazard ratio for death (OS, HR=0.35, P=0.04) and failure (EFS: no CR, death in CR or relapse, HR=0.37, P=0.03). This favourable hazard ratio was maintained after adjustment for cytogenetic-risk, *FLT3*-ITD and *CEBPA* expression levels in multivariable analysis. In contrast, low *CEBPA* expression in AML with intermediate-risk karyotype (n=6) seemed to be associated with poor prognosis (not significant). By including this newly developed PCR assay, we define a subgroup of good-risk patients within the heterogeneous intermediate-risk group of AML.

Introduction
The CCAAT/Enhancer Binding Protein α (C/EBPα) is a transcription factor essential for granulocytic differentiation. The expression of C/EBPα initiates at the time of commitment of stem cells to myeloid lineage, is specifically up-regulated in granulocytes and is down-regulated in peripheral blood monocytes.[1] The *Cebpa* knockout mice lack mature granulocytes, while the development of other haematopoietic lineages is not affected.[2] Using conditional expression constructs, it was demonstrated that expression of C/EBPα in U937 and HL-60 cells is sufficient to induce granulocytic differentiation. Moreover, conditional expression of C/EBPα in U937 cells blocks the monocytic differentiation program. Thus, it seems that C/EBPα is able to induce a switch in differentiation towards neutrophils, thereby prohibiting monocytic development.[1]

C/EBPα specifically binds DNA through a domain that is rich in basic amino acids (basic region) and dimerises through two amphipathic α-helices (leucine zipper). Both the basic region and the leucine zipper are located in the C-terminus of the C/EBPα protein.[3] DNA binding depends on a stringently fixed three-dimensional relation between basic region and leucine zipper. If the register between these two regions (fork) is altered by either insertion or deletion, then the sequence-specific recognition of DNA is eliminated.[4] In contrast to the C-terminus of C/EBPα, which is highly conserved amongst

CEBPA family members and a number of oncogenes,[5-7] the N-terminus is more diverse. This region contains an attenuator domain that is sandwiched by two distinct transactivation domains. Although some studies report that the transactivation domains play no obvious role in the capacity of C/EBPα to bind to its DNA substrate,[8-10] others suggest that N-terminal C/EBPα does influence DNA binding activity.[11]

Recently, C-terminal mutations of *CEBPA* in acute myeloid leukaemia (AML) with intermediate-risk karyotype have been identified.[11-13] These mutations are frequently in-frame insertions within the bZIP region of *CEBPA* and result in proteins with deficient transcriptional activation and DNA binding. These proteins though do not possess dominant negative activity.[11,12] Besides the C-terminal mutations, N-terminal *CEBPA* mutations have also been identified in AML. These mutations usually result in a nonfunctional truncated protein and an increase in the production of a 30 kD protein that is generated by use of an alternative initiation codon within the same reading frame.[11,14] This 30 kD protein exerts a dominant negative effect on DNA binding and transactivation of the wild type C/EBPα.[11] Apart from mutations in *CEBPA*, differences in *CEBPA* mRNA expression levels have also been observed in AML.[15]

As the bZIP region of C/EBPα is highly conserved and is crucial for DNA binding and dimerisation, we decided to design a rapid assay to detect mutations in this region. Since the mutations in this region often involve insertions or deletions, a rapid PCR/EtBr electrophoreses strategy was applied to identify these abnormalities in a cohort of 277 de novo AMLs. Subsequent sequence analysis of the entire gene was carried out in 35 patients to investigate the presence of other mutations in *CEBPA*. Furthermore real-time PCR was performed to investigate the differences in *CEBPA* expression levels in the same cohort. Using these approaches we assessed the clinical, haematological and prognostic impact of *CEBPA* mutations and variation in *CEBPA* expression levels.

Material and methods

Patients and healthy volunteers

Bone marrow samples of AML patients at diagnosis and of healthy volunteers (n=6) were obtained after informed consent. Blasts from AML patients and mononucleated fractions from normal bone marrow specimens were isolated from the samples by Ficoll-Hypaque (Nygaard, Oslo, Norway) centrifugation.[16] The cells were then cryopreserved as described. After thawing cells were washed with Hanks Blanced Salt Solution (HBSS) and further processed for RNA isolation. AML samples treated according to this procedures usually containmore than 90% blasts after thawing.[17] A total of 277 de novo AML patients that had been referred to our institution and our collaborating centres between 1987 and 2000 were collected for analysis. In all, 211 patients were treated according to the HOVON-29 (Dutch-Belgian Hematology-Oncology Co-operative Group) protocol and 66 patients according to the HOVON-4. These treatment protocols have been described elsewhere.[18] Table 1 shows the characteristics of 277 patients at diagnosis. AML samples were classified according to the French-American-British nomenclature.[19]

Table 1. Clinical characteristics of 277 de novo AML patients.

Characteristics	*No.*
Gender	
Male	144
Female	133
Age	
Median (range)	43.4 (15.2–60.8)
Age groups	
Younger than 35	85
35–50	104
50 and older	88
FAB	
M0	10
M1	62
M2	64
M3	22
M4	50
M5	61
M6	3
Unclassified	5
Cytogenetic risk group	
Favourable	55
Intermediate	187
Unfavourable	35
WBC count (10^9/l)	
Median (range)	25.5 (0.3–282)
Blast count (%)	
Median (range)	68 (0–98)
Platelet count (10^9/l)	
Median (range)	49 (3–931)

RNA isolation, cDNA synthesis, real-time PCR

Total RNA was extracted with guanidium thiocyanate followed by centrifugation in cesium chloride solution. RNA concentration, quality and purity were analysed applying the RNA 6000 Nano Assay (Agilent Technologies, Amstelveen, the Netherlands). All samples included in the study showed neither RNA degradation (28S/18S ration was ≥2) nor DNA contamination. The 1 mg RNA was transcribed into cDNA using Superscript (Life Technologies, Merelbeke, Belgium) and Random Hexamers in a 40 ml reaction, under standard conditions.

An aliquot of one 20th of the resulting cDNA was used for quantitative PCR amplification. Real-time PCR amplification was performed with the ABI PRISM 7700 Sequence Detector (Appleid Biosystems, Nieuwekerk a/d IJssel, the Netherlands), using 50 ml mix containing 2 ml cDNA sample; 250 mM dNTPs (Amersham Pharmacia Biotech Inc, Roosendaal, the Netherlands); 15 pmol T-forward and T-reverse primer (Table 2, Figure 1) (Life Technologies); 3 mM MgCl2 for *CEBPA* and 5 mM for *PBGD* (Porphobilinogen deaminase) reaction; 200 nM probe; 5 ml 10x buffer A; 30 ml water; and 1.25 U AmpliTaq Gold (Applied Biosystems). The thermal cycling conditions included 10 minutes at 95°C followed by 45 cycles of denaturation for 15 seconds at 95°C, annealing/extension at 60°C for 30 seconds. TaqMan probe was labelled at the 5'

end with the reporter dye molecule FAM (6-carboxy-fluorescein), and at the 3' end with the quencher dye molecule TAMRA (6-carboxy-tetramethyl-rhodamine) (Eurogentec, Maastricht, the Netherlands). To check the PCR mix for any kind of contamination, we took a negative control (2 ml water instead of cDNA) with every TaqMan plate.

To determine the expression levels in AML, all samples were tested in duplicate, and the average values were used for quantification. To quantify the relative expression of *CEBPA*, the Ct (threshold cycle) values were normalised for endogenous reference (δCt = Ct target- Ct *PBGD*) and compared with a calibrator, using the 'δδCt method' (δδCt= $\delta Ct_{Sample} - \delta Ct_{Calibrator}$). As calibrator we used the average *CEBPA* Ct value in the 6 bone marrow samples of healthy volunteers. Using the δδCt value, relative expression was calculated ($2^{-\delta\delta Ct}$). As the δδCt method is only applicable when the amplification efficiencies of the target and the reference are essentially equal, we analysed the efficiency of *CEBPA* and *PBGD* using 6 different dilutions of a cDNA sample (equal to 0.0005-50 ng total RNA). The mean δCt values (δCt = Ct target- Ct *PBGD*) were plotted against the concentration of total RNA (log). The slope of the fitted line was then determined. A slope of less than 0.1 was indicative for equal efficiencies.

Polymerase chain reaction on N- and C-terminus and entire CEBPA

The 3' coding region of *CEBPA* gene was amplified using forward primer 3 and reverse primer 8. The N-terminus was amplified by forward primer 1 and reverse primer 5 (Figure 1, Table 2). The PCR mix contained 2 ml cDNA sample; 1mM dNTPs (Amersham Pharmacia Biotech Inc, Roosendaal, the Netherlands); 20 pmol forward and reverse primer (Life Technologies); 2.5 mM MgCl2; 5 ml expand 10 x PCR buffer; 5 ml DMSO; 30 ml water; and 5 U Taq DNA Polymerase (Amersham Pharmacia Biotech Inc). The thermal cycling conditions included 5 minutes at 95°C followed by 30 cycles of 1 minute denaturation at 95°C, 1 minute annealing at 62°C, 1 minute extension at 72°C and a final step of

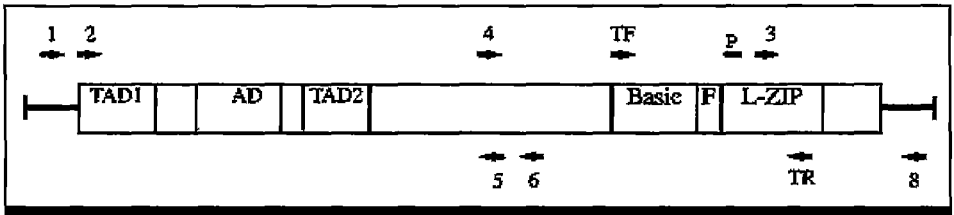

Figure 1. Schematic representation of *CEBPA* and the primers and probe used in this study. Primer TF and TR were used to detect *CEBPA* bZIP mutations and measure the mRNA expression level at the same time (using probe P). Primer 3 and 8 were applied to screen the remaining 3'coding region of *CEBPA*. Primer 1 and 5 were used to amplify the N-terminal *CEBPA*. The amplified N-terminus was subsequently sequenced with primer 2. The entire *CEBPA* was amplified using primers that are described elsewhere.[12] Primers 4 and 6 were applied to sequence the entire coding region of *CEBPA*. Abbreviations: TAD, transactivation domain; AD, attenuator domain; F, Fork; L-ZIP, Leucine zipper.

Table 2. Nucleotide sequences of primers and probe.

Oligonucleotides	*Nucleotide sequence*
TF	5′-TCGGTGGACAAGAACAG-3′
TR	5′-GCAGGCGGTCATTG-3′
1	5′-CGCCATGCCGGGAGAACTCT-3′
2	5′-TGGAGTCGGCCGACTTCTA-3′
3	5′-GTGCTGGAGCTGACCAGTGAC-3′
4	5′-GCGGCCGCTGTGACTAAG-3′
5	5′-GGAAGAGGCCGGCCAGCG-3′
6	5′-GGCGGCTGGTAAGGGAAGAGG-3′
8	5′-CCCAGGGCGGTCCCACAGC-3′
Probe P	5′-TGGAGACGCAGCAGAAGGTG-3′

The names or numbers of the oligonucleotides correspond to those indicated in Figure 1.

10 minutes 72°C. The primers used for amplifying the entire coding region of *CEBPA* and the PCR condition are described elsewhere.[12] The fragments were gel-purified and cloned in a TA-cloning vector (Invitrogen, Life Technologies, Merelbeke, Belgium).

Nucleotide sequence analysis

The real-time PCR products were electophoresed on an ethidium bromide 3 % agarose gel. Samples with a shifted/double band were cloned in to TA-cloning vector (Invitrogen), and sequenced in both directions using ABI Prism BigDye Terminator (Applied Biosystems) and an ABI Prism 3100 Genetic Analyser. The N-terminal *CEBPA* was sequenced with primer 2 using the same technique. The entire *CEBPA* that was cloned in TA-cloning vector was sequenced with primer 4 and primer 6.

Cytogenetic analysis and stratification according to karyotypic risk group

Cytogenetic analysis was carried out according to standard techniques, and the abnormalities were categorised in 3 cytogenetic groups. Patients with inv(16)/t(16;16), t(8;21) and t(15;17) abnormalities were considered as favourable-risk. The unfavourable-risk category was defined by the presence of -5/del(5q), -7del(7q), t(6;9), t(9;22), 3q26 abnormality or complex karyotype (more than 3 distinct clonal abnormalities). All other patients were classified as intermediate-risk. Karyotypes were described according to ISCN nomenclature (1995).

Analysis of FLT3 internal tandem duplication mutations in AML

The internal tandem duplications (ITD) in exon 11 of the human *FLT3* gene were determined as described previously.[20] Briefly, cDNA (derived form 50 ng total RNA) and genomic DNA (1 mg) were subjected to PCR using primers 11F 5'-CAATTTAGGTAT-3' and 11R 5'-CAAACTCTAAATTTTCTCT-3'. The PCR cycling conditions were as follows: 3 minutes 94°C followed by 30 cycles 1 minute 94°C, 1 minute 54°C, 1 minute 72°C and a final step of 10 minutes 72°C. PCR products were resolved on a 2.5% agarose gel.

Statistical Analysis

Statistical analysis was performed with the Stata Statistical Software Release 7.0. (Stata 2001, College Station, TX). Spearman's rank correlation, Pearson's chi-square test and Kruskal-Wallis test were used to assess the association between *CEBPA* expression and the clinical and haematological characteristics of patients. Actuarial probabilities of overall survival (OS; with failure as death due to any cause) and event-free survival (EFS; with failure at day 1 in case of not attaining a CR, or at relapse or at death in first CR) were estimated by the method of Kaplan and Meier. The Cox proportional hazard model was applied to determine the relationship between *CEBPA* expression levels and mutations with OS and EFS, without and with adjustment for other factors as age, cyto-genetic risk and *FLT3*-ITD. All tests were done two-sided. P-values <0.05 were considered statistically significant.

Results

CEBPA C-terminal in-frame insertions occur in 12 of 277 de novo AML patients and associate with *CEBPA* N-terminal mutations

The primers chosen for the rapid PCR assay to detect bZIP mutation (T-forward and T-reverse) covered the most conserved domain of *CEBPA* (Figure 1). Analysis of the PCR products using ethidium bromide agarose gel electrophoreses revealed double bands in 12 cases (Figure 2). Cloning and subsequent nucleotide sequence analysis revealed in-frame insertions that often consisted of repeats (Figure 3). One patient had

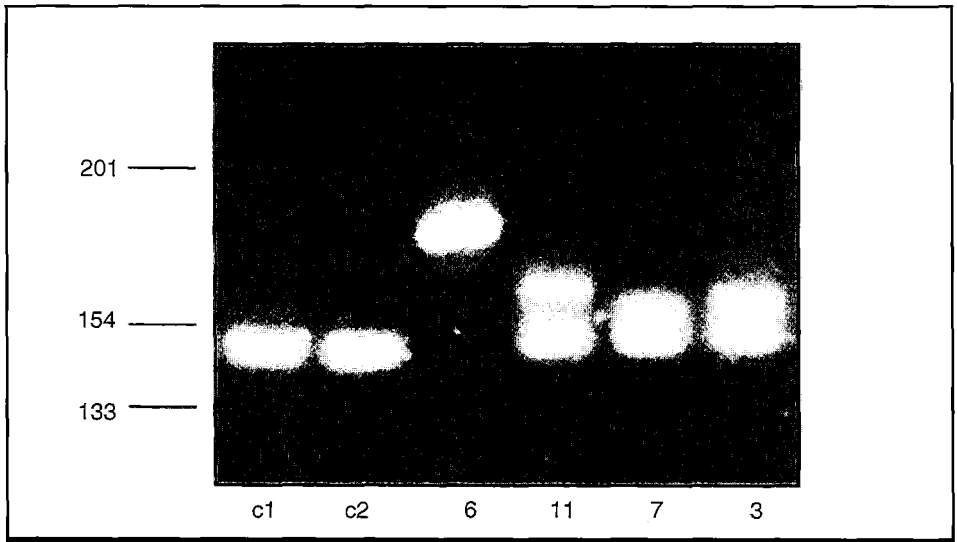

Figure 2. PCR products of 4 patients with mutated (6, 11, 7 and 3) and two patients with wild type bZIP *CEBPA* (c1 and c2), analysed by EtBr agarose-gel electrophoresis. The size of the wild-type PCR fragment is 141 base pairs.

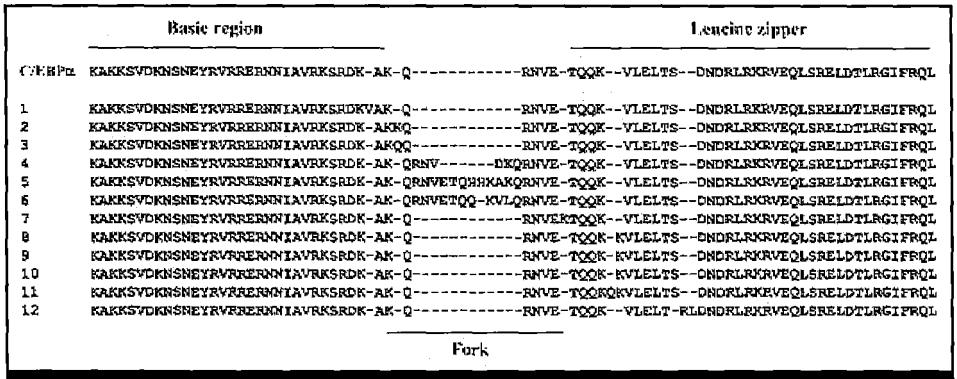

Figure 3. Comparison of amino acid sequence of wild type *CEBPA* with 12 AML cases carrying insertion-mutations in bZIP region.

an insertion in the basic region, 6 patients carried insertions in the fork region and in 5 patients insertions were apparent in the leucine zipper. In addition we carried out PCR analysis on the distal end of the 3' coding region of *CEBPA* gene in 150 patients using primers 3 and 8 (See Figure 1). No additional insertions or deletions were found following the latter analysis. We next investigated whether N-terminal mutations in *CEBPA* were present in the 12 cases with C-terminal insertions as well as in 23 other patients. Nucleotide sequence analysis revealed N-terminal frameshifts in 11 out of 12 cases with *CEBPA* bZIP mutations. The only patient without an N-terminal mutation (patient 6) appeared homozygous for the bZIP mutation (Figure 2, 3; Table 3). No N-terminal mutations were found in the 23 cases without a C-terminal abnormality. Cloning of full-length *CEBPA* cDNA and subsequent nucleotide sequencing of 8 of the 11 cases with both types of mutations revealed that the distinct mutations never occurred in one transcript. This indicates the involvement of both *CEBPA* alleles.

Patient number	*Mutation*	*Amino-acid change*
1	425insA	K92fsX107
2	395insGG	L82fsX159
3	252delCCGGGGCGCG	P34fsX156
4	213delC	S21fsX159
5	213delC	S21fsX159
6	—[a]	
7	333delC	S61fsX159
8	343insA	K65fsX107
9	437delG	A96fsX159
10	302delCGCTGGGCGGCATCTG	P51fsX154
11	395insTGTT	F82fsX108
12	358delC	R70fsX159

Table 3. Additional N-terminal mutations detected in patients with *CEBPA* bZIP mutations .

[a] This patient is homozygous for bZIP mutation. Nucleotide numbering is based on Genbank sequence # XM_009180 [gi:13632311].

Mutations in CEBPA associate with intermediate risk karyotype and favourable prognosis

The characteristics of patients with *CEBPA* mutations are shown in Table 4. The patients carried an intermediate-risk karyotype, expressed relatively high levels of *CEBPA* mRNA levels (See next paragraph) and all but one belonged to the M1 or M2 FAB-class. Only one patient with a *CEBPA* mutation harboured an *FLT3*-ITD. There was no significant difference in age, sex, blast count, WBC count, platelet count between the group with and without mutation. The distribution of patients within the randomisation arms of the study was equal. A total of 33% of the patients with mutation received stem cell transplantation of whom 1 autologous (AuSCT) and 3 allogenic (AlloSCT); Likewise 29% (38 AuSCT, 38 AlloSCT) of the patients without mutation received stem cell transplantation. Clinical outcome was investigated in the patients based on the presence or absence of *CEBPA* mutations. EFS and OS of patients with *CEBPA* mutations were significantly increased (P= 0.02 and P= 0.03, respectively) in comparison to the patients lacking these mutations (Figure 4a and b). The actuarial overall survival probabilities at 60 months were 63% in patients with *CEBPA* mutations and only 29% for patients lacking these mutations. Besides, the EFS probabilities at 60 months were also higher in patients with *CEBPA* mutation than in patients without these abnormalities (57% and 23% respectively) (Table 5). The survival probabilities for patients with and without mutations at 24 months showed the same pattern (OS: 57% versus 27% and EFS: 73% versus 38%, respectively).

Table 4. Characteristics of patients with *CEBPA* mutation

No.	*Cytogenetics*	*FLT3-ITD*	*Age (years)*	*FAB*	*Blast (%)*	*WBC (10^9/l)*	*Platelet (10^9/l)*	*CEBPA expression*[a]
1	46,XX	—	48	M4	51	92	20	28.1
2	46,XY	—	45	M1	75	8	8	118.2
3	46,XX,del(9)(q1?1q3?1)	—	54	M1	65	8	98	5.5
4	46,XX,del(12)(p12.3p13.1)	—	58	M2	47	2	207	16.5
5	46,XY	—	43	M2	54	20	55	34.7
6	46,XY	—	41	M1	93	174	39	66.3
7	46,XY	—	48	M1	91	95	43	13.6
8	46,XX	—	31	M2	60	11	69	11.9
9	46,XY	—	45	M1	64	87	63	8.6
10	46,XY,del(9)(q3?1)	—	35	M1	72	34	25	69.3
11	46,XY,del(11)(q14q25)	—	38	M2	53	35	23	111.0
12	46,XY	+	19	M1	79	118	38	30.7

[a] For details see text.

Table 5. Therapy response and actuarial survival probability at 60 months based on *CEBPA* mutation.

CEBPA mutation	*Total number of patients*	*Complete remission n (%)*	*Actuarial probability at 60 months*		*Relapse n (%)*
			EFS	*OS*	
+	12	11 (92%)	57%	63%	4 (36%)
−	265	210 (79%)	23%	29%	104 (50%)

EFS: Event-free survival. OS: Overall survival.

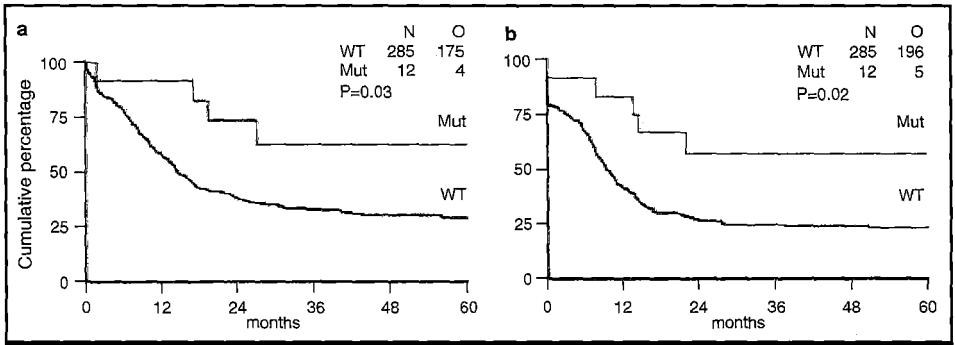

Figure 4. Overall survival (a) and event-free survival (b) of 187 AML patients with intermediate risk karyotype based on *CEBPA* mutation. Mut: *CEBPA* mutations (n=12). WT: Wild type (n=175).

Variable expression levels of CEBPA in AML

CEBPA expression levels in AML blasts were analysed using real-time PCR employing specific primer/probe combinations (Figure 1). The slope of the fitted line for the mean δCt value at different mRNA concentration was -0.08, indicating that the δδCt method was applicable. As calibrator we used the average expression of *CEBPA* in 6 bone marrow samples of healthy volunteers. The mean Ct value of *PBGD* and *CEBPA* in these normal samples were 25.4 ± 0.7 (SD) and 27.01± 0.91(SD), respectively. *CEBPA* expression was measured in 277 de novo AML patients using the same technique. The values obtained were normalised for the internal reference (*PBGD*) and compared with the calibrator. An expression level in the range of 99% confidence interval (0.2-5), defined for the average *CEBPA* expression level in the healthy population, was considered 'intermediate'. The majority of patients (141 of 277) had an intermediate *CEBPA* expression level. In all, 129 of 277 patients showed high expression levels (range 5.06-154) while only 9 cases showed an expression level below the intermediate range (range 0.19-0.001). Characteristics of different *CEBPA* expressing groups are shown in Table 6. Although the number of patients with low *CEBPA* expression was small, they appeared to present distinct characteristics, i.e. a relative lower WBC and platelet counts. Furthermore one-third (3 of 9) of the patients with low *CEBPA* expression carried a t(8;21); while the other two-thirds carried a normal or intermediate-risk karyotype.

Low CEBPA expression within the group with intermediate-risk karyotype

A total of 277 patients received induction therapy and were included in the survival analysis. The percentage of patients receiving stem cell transplantation in the low, intermediate and high *CEBPA* expressing group was 44% (4 AuSCT), 30% (19 AuSCT, 23 AlloSCT) and 27% (16 AuSCT, 18 AlloSCT) respectively. Clinical outcome was investigated based on *CEBPA* expression levels. The actuarial probabilities of OS and EFS in patients with low, intermediate and high *CEBPA* expression at 60 months were essentially equal

Table 6. Clinical characteristics of 277 de novo AML patients based on the *CEBPA* expression level.

Characteristics	*Low (<0.2)*	*Intermediate (0.2–5)*	*High (>5)*
Gender			
Male	3	84	57
Female	6	57	70
Age			
Median (range)	44 (29–57)	43 (15–60)	44 (17–61)
Age groups			
Younger than 35	3	44	38
35–50	3	56	45
50 and older	3	41	44
Fab			
M0	2	6	2
M1	2	28	32
M2	2	33	29
M3	0	6	16
M4	3	25	22
M5	0	37	24
M6	0	3	0
Unclassified	0	3	2
Cytogenetic risk group			
Favourable[a]	3	28	24
Intermediate	6	94	87
Unfavourable	0	19	16
FLT3-ITD	1	35	37
WBC count (10^9/l)			
Median (range)	3 (0.9–27)	23 (0.5–282)	33 (0.3–263)
Blast count (%)			
Median (range)	71 (35–99)	67 (0–98)	68 (0–98)
Platelet count (10^9/l)			
Median (range)	29 (3–181)	53 (4–931)	47 (8–494)

[a] The number of patients with t(8;21) in the low, intermediate and high *CEBPA* expressing groups were 3, 13 and 6, respectively.

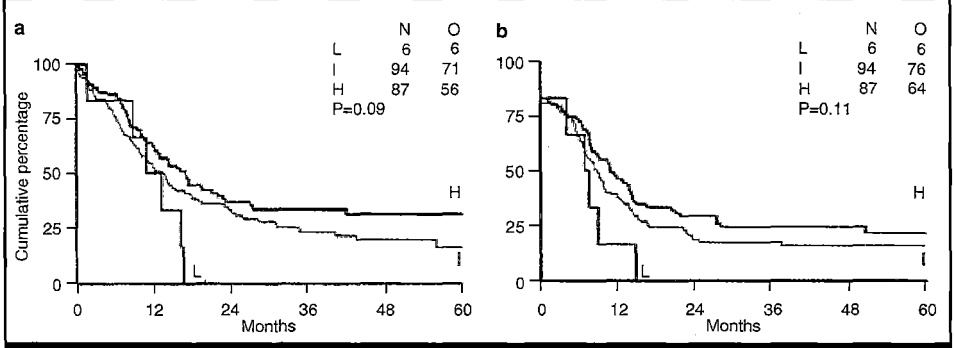

Figure 5. Overall survival (a) and event-free survival (b) of 187 AML patients with intermediate risk karyotype based on *CEBPA* expression levels. H: High expression (n=87), I: Intermediate expression (n=94). L: Low expression (n=6).

(OS: 22 versus 25 versus 25% and EFS: 22 versus 28 versus 34%, respectively). No significant differences in EFS and OS among patients with different *CEBPA* expression levels were apparent.

We then investigated whether *CEBPA* expression levels had prognostic value within the distinct cytogenetic-risk groups. The prognosis of patients within the intermediate

Table 7. Univariable and multivariable analysis of *CEBPA* mutations and expression levels as prognostic factors for event-free survival and overall survival in 277 AML patients.

	Overall survival			*Event-free survival*		
	Hazard ratio	*95% CI*	*P-value*	*Hazard ratio*	*95% CI*	*P-value*
Univariable analysis CEBPA						
Mutations	0.35	0.13–0.94	0.04	0.37	0.15–0.91	0.03
Expression			0.65			0.87
Intermediate	1			1		
High	0.91	0.67–1.22		0.95	0.72–1.27	
Low	1.24	0.58–2.68		1.16	0.54–2.49	
Multivariable analysis						
Age			0.45			0.94
35–50 years	1			1		
⩽35 years	0.87	0.60–1.26		0.99	0.70–1.39	
> 50 years	1.37	0.97–1.93		1.36	0.98–1.89	
Cytogenetic-risk			<0.0001			<0.0001
Intermediate	1			1		
Good	0.32	0.19–0.53		0.40	0.26–0.62	
Poor	1.68	1.11–2.54		1.59	1.07–2.36	
Flt3 mutation						
Versus no mutation	1.61	1.16–2.23	0.004	1.54	1.13–2.11	0.006
CEBPA mutations	0.35	0.13–0.96	0.04	0.36	0.14–0.89	0.03
CEBPA expression level			0.46			0.53
Intermediate	1			1		
High	0.95	0.70–1.29		0.99	0.74–1.32	
Low	1.62	0.75–3.51		1.60	0.74–3.47	

risk group seemed to be influenced by different *CEBPA* expression levels (Figure 5a and b). Particularly patients with low *CEBPA* expression (n=6) seemed to have a relatively poor OS and EFS (not significant). Variations in *CEBPA* expression levels had no impact on the survival of patients with other risk karyotypes (data not shown).

CEBPA mutation, an independent marker for good prognosis in AML

Cox regression analysis was applied to assess the prognostic significance of *CEBPA* mutations and expression levels for OS and EFS in relation to other prognostic variables (Table 7). *CEBPA* mutations were associated with a significantly reduced hazard ratio for death (OS; HR 0.35 P=0.04) and failure (EFS; no CR, death in CR or relapse, HR 0.37 P=0.03). This favourable hazard ratio was maintained after adjustment for age, cytogenetic-risk, *FLT3*-ITD and *CEBPA* expression levels in multivariable analysis.

Discussion

This study introduces a rapid PCR-based assay to identify in-frame insertions within the bZIP region of *CEBPA*. In-frame insertions in *CEBPA* occur in AML cases with intermediate-risk karyotype and are associated with N-terminal mutations in the other allele. We report that *CEBPA* mutations are independent prognostic markers, which appears to correlate with enhanced EFS and OS. Besides the mutations, *CEBPA* mRNA expression levels seem to influence the prognosis of patients with intermediate-risk karyotype. However, a larger cohort of AML patients is required to establish the prognostic value of *CEBPA* expression levels.

Our study is in agreement with the data reported by Gombart et al.,[12] Pabst et al.[11] and Preudhomme et al;[13] that is, *CEBPA* mutations often occur in patients with intermediate-risk karyotype and FAB-classes M1 or M2. The fact that *CEBPA* mutations frequently

occur in certain subgroups suggests that this genetic alteration is a transforming event, affecting particular haematopoietic precursor cells arrested at specific stages of differentiation.

CEBPA mutations are reported in 10 out of 137 AML patients (7.3%) analysed by Pabst et al, 7 out of 78 cases (9%) screened by Gombart et al and in 15 out of 135 patients (11%) screened by Preudhomme et al. These studies report on both N-terminal and C-terminal mutations.[11-13] However the exact distribution and frequencies of C-terminal versus N-terminal mutations in *CEBPA* remains unclear. In this study, we demonstrated that C-terminal mutations in de novo AML occur in 4.3% of the cases and are associated with N-terminal mutations in the other allele. Such biallelic mutations were also observed in 7 out of 15 patients with *CEBPA* mutation detected by Preudhomme et al.[13] The involvement of both *CEBPA* alleles seems to be a common event, as the only patient without N-terminal abnormality was homozygous for bZIP mutation. In this study, we analysed only 23 cases without a C-terminal abnormality for N-terminal mutations. Although none of those 23 cases appeared to carry mutations in this gene, monoallelic *CEBPA* mutations in the N-terminus are expected in a small group among the 242 cases that are currently under investigation. As our study is carried out in a large number of patients, we may as well be able to define whether cases with monoallelic versus biallelic *CEBPA* mutations belong to separate subgroups of AML.

Cytogenetic analysis provides a powerful approach to discriminate between favourable and unfavourable-risk AML. However, only 30-40% of AML cases can be classified by cytogenetic analysis. One major challenge is to classify the remaining 60%-70% of the cases that are currently included in the intermediate-risk group. In our study 33% of the patients with intermediate-risk karyotype harboured an *FLT3*-ITD mutation that is generally considered a poor risk indicator.[21-23] Furthermore, we recently identified, in the same cohort of intermediate-risk AML, a sub-group (8%) with high *EVI1* expression and poor survival (this thesis). In this study, we describe a new subgroup (6.4%) within the heterogeneous intermediate-risk group that carries *CEBPA* mutations and has favourable prognosis. As these different subgroups hardly show any overlap, based on these molecular analyses, we have been capable of providing prognostic information for approximately half of AML cases within the intermediate-risk group. Taken together, these observations encourage additional gene expression studies and mutation analyses to further unveil specific subgroups of AML.

Using real-time PCR we were capable of defining three subgroups, i.e. high, intermediate and low *CEBPA* mRNA expressing patients. All low *CEBPA* expressing patients with intermediate-risk karyotype died within 18 months. As this subgroup only contained 6 cases, *CEBPA* expression analyses should be carried out on larger cohort of AML patients to verify the reproducibility of these observations. Compiling data from for instance gene-array studies in the coming years may reveal the impact of low *CEBPA* expression on clinical outcome of AML.

In this study we developed a rapid PCR assay to detect bZIP mutations and distinguish more than 6.4% of AML patients for whom no valuable prognostic marker was available. A PCR/agarose gel electrophoresis assay to identify *FLT3*-ITDs in human

AML, in particular in the group of cases with intermediate-risk karyotype, has been validated[20] and routinely used. Although, sequencing of the entire coding region of *CEBPA* in AML samples is required to identify both N-terminal and C-terminal mutations, a validated PCR/agarose gel electrophoreses for *CEBPA* will be an easy and rapid assay that can be implemented in the molecular diagnosis of AML.

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Chapter 5

Somatic heterozygous mutations in the functional domains of *TEL* (*ETV6*) and frequent absence of TEL protein in Acute Myeloid Leukaemia

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Oncogene, in press

Abstract

The transcription repressor gene *TEL* (*ETV6*) is involved in different translocations associated with human malignancies. These translocations often associate with deletion or inactivation of the non-rearranged *TEL* allele, which appears to be a critical secondary event for leukaemogenesis. Although *TEL*-translocations are relatively infrequent in AML, structural mutations or abnormal expression of TEL may contribute to the pathogenesis of the disease. In order to investigate the involvement of *TEL* in AML, we screened 300 newly diagnosed patients for mutations in the coding region of the gene by direct sequencing. Furthermore, we studied TEL protein expression in 77 patients using two specific antibodies. Five somatic heterozygous mutations were detected, which affected either the homodimerization- or DNA-binding domain of TEL. The proteins translated from the cDNAs of these mutants were unable to repress transcription and showed dominant negative potentials. Moreover, we demonstrated that one third of AML patients have deficient TEL protein expression, which is not related to *TEL* mRNA expression levels. In this study we demonstrate that *TEL* abnormalities are not restricted to translocations and occur more frequently in AML than previously thought. Additional comprehensive studies are required to define the clinical consequence of TEL loss of function in AML.

Introduction

The *TEL* (translocation ets leukaemia) gene also known as *ETV6* (ets translocation variant gene 6) is located on the short arm of chromosome 12 and is known to be involved in different translocations associated with human malignancies.[1,2] TEL is a transcription repressor with two functional domains; an ETS DNA binding domain in the C-terminus[3,4] that recognizes the ETS binding sites (EBS) in promoter regions of target genes[5,6] and an N-terminal pointed (PNT) homo-dimerization domain.[7] Dimerization of TEL through the PNT domain is indispensable for EBS-driven transcription repression.[3]

The transcription repressor activity of TEL seems to be important in the regulation of cell growth and differentiation. Fenrick et al.[5] demonstrated that expression of TEL in Ras-transformed NIH 3T3 cells inhibits cell growth through repression of target genes, such as stromelysin-1. Moreover, TEL has been shown to stimulate erythroid differentiation of the murine erythroid leukaemia (MEL) cell line.[8] *Tel* knockout mice are embryonic lethal at day 10.5-11.5 and exhibit yolk sac angiogenesis defect and intra-embryonic apoptosis of mesanchymal and neural cells. Although *Tel* absence seems not to affect haematopoiesis at the yolk sac stage,[9] it seems indispensable for the development of all lineages in adult bone marrow. Chimeric mice with *Tel* knockout embryonic stem cells present defective hematopoiesis within the first week after birth.[10]

The fusion partners of *TEL* in translocations have heterogeneous characteristics. Furthermore, the contribution of *TEL* to the oncogenic fusion-proteins is variable. For example, in translocation t(3;12)(q26;p13), the *TEL* promoter which is situated in the vicinity of the *EVI1* gene results in *EVI1* over-expression.[11] In several translocations, *TEL* provides dimerization domains, e.g. in *TEL-MN1*,[12] *BTL-TEL*[13] or *TEL-ABL*,[14] resulting

in constitutively activated signalling proteins. In other cases, *TEL* provides transcriptional properties via the ETS DNA binding site, e.g. in *TEL-PDGFRb*[15,16] and *TEL-AML1*.[17] *TEL-AML1*, which is generated as a result of translocation t(12;21)(p13;q22) is the most common genetic lesion in paediatric acute lymphoblastic leukaemia (ALL). This translocation is detected in up to 25-30% of the patients and often associates with a fully or partially deleted non-rearranged *TEL* allele.[18-20] Deletion of the wt allele in these cases results in loss of functional TEL expression, which might contribute to the pathogenesis of the disease. A few studies have demonstrated that deletion of the wt *TEL* allele in cases with t(12;21)(p13;q22) is not the only mechanism that leads to the loss of functional TEL.[21,22] Small structural abnormalities or epigenetic modification may also lead to the loss of wt TEL expression.[21] Accordingly, *TEL* abnormalities in ALL seem not to be restricted to translocations or deletions. Approximately 23% of ALL patients that do not carry a t(12;21) show an abnormal expression pattern of TEL isoforms.[23] Although *TEL* translocations are infrequent in acute myeloid leukaemia (AML),[24] abnormal expression patterns or mutations in *TEL* might contribute to the pathogenesis of the disease. Kibel et al [25,26] have demonstrated *TEL* mutations in 2 out of 19 patients with prostate carcinoma and in 3 of 25 xenografts and cell lines. The mutations in *TEL* often associated with loss of heterozygosity,[26] which fits well with Knudsen's 'two-hit' hypothesis. In order to investigate the involvement of *TEL* in AML, we analyzed 300 newly diagnosed patients for mutation in the coding region of the gene. We identified 5 cases of AML with nonsence/frameshift mutations or in frame insertions in *TEL* and investigated the consequential properties of such mutations. Furthermore, we studied TEL protein expression in 77 newly diagnosed AML patients using two highly specific antibodies. We demonstrate that despite considerable *TEL* mRNA expression, the protein is frequently absent in AML blasts.

Material and methods

Patient material

Bone marrow samples of a total of 300 de novo AML patients at diagnosis were obtained after informed consent. Patients were treated as described previously.[27] Bone marrow blast cells were purified and further processed for DNA and RNA isolation. cDNA synthesis was performed according to standard protocols.[28] The DNA samples, isolated from non-haematopoietic tissues (skin or intestinal) of 3 patients with TEL mutations were provided by the Department of Pathology at Erasmus MC Rotterdam, the Netherlands.

Polymerase chain reaction and direct sequencing

The entire coding region of *TEL* was amplified using 20 pmol forward primer (FW: 5'-ttctgggttggggagag-3') and 20pmol reverse primer (REV: 5'-cgctgaggtggactgttg-3'). In brief, the reaction was performed using 1.7 U Expand High Fidelity enzyme, 5 ml PCR buffer, 2 mM MgCl2 (Roche diagnostics GmbH, Mannheim, Germany), 1mM dNTPs (Amersham Pharmacia Biotech Inc, Roosendaal, The Netherlands) and cDNA. The thermal cycling conditions included 5 minutes at 95°C followed by 30 cycles of 1 minute at

95°C, 1 minute at 53°C, 3 minutes at 72°C and a final step of 10 minutes at 72°C. PCR products were purified on Montage PCR96 filter plates (Millipore Corporation) and sequenced in both directions using ABI Prism 3100 Genetic Analyser (PE Applied Biosystems) and 10 pmol primer (FW, FW mid: 5'-tcccaccattgaactgt-3', REV, REV mid: 5'-cggtgatttgtcgtgatag-3'). Similar PCR and sequencing conditions were used to confirm the mutations on genomic DNA using specific primers for exon 3 (FW 5'-ggctcttgagat-gtggaga-3', REV 5'-catcccttccttgtgatga-3') exon 4 (FW: 5'-caggtgcgctccaattgta-3', REV: 5'-acacgaagaagaccagctta-3') exon 6 and 7 (FW: 5'-tcttctggttagtgcctcaa-3' REV: 5'-caggtagagatcttaacagt-3').

Constructs

N- and C-terminal constructs of *TEL* were generated by PCR with primer combination FW- REVmid and FWmid- REV respectively. All PCR products together with the wt allele and the mutants (#2290, 2236, 2649) were cloned into TA vector (Promega) followed by sub-cloning into Eco-RI sites of mammalian expression vector pCMV, which is derived from the pEGFP-C1 vector (Clonetech lab. Palo Alto, Ca) from which the EGFP fragment was removed following Nhe1 and Xho1 digestion and subsequent religation. Single point mutations were generated in the methionine 208, 240, 315 and 319 of the #2290 construct cloned into pCMV, using QuikChange Site-Directed Mutagenesis kit (Stratagene, CA, USA). The oligonucleotide sequence of the sense and antisense primers are available on request.

Immunoblotting

Phoenix cells were maintained in Dulbecco's modified medium with 10% FCS(Life Technologies, Breda, the Netherlands). 24 hours prior transfection 2x 10^6 cells were plated onto 10 cm dishes. Cells were transfected with 20 μg of corresponding *TEL* construct. 48 hours post transfection cells were harvested and lysed in lysis buffer (Tris-HCL pH 8.0 20mM, NaCl 137mM, EDTA 10mM, NaF 100mM, NP40 1% and Glycerol 10%) with a cocktail of protease inhibitors. The protein extracts were fractionated on SDS-12% polyacrylamide gels and transferred to nitrocellulose membranes by electroblotting. The TEL protein was detected by rabbit polyclonal antibodies (1:1000) directed either to the first 54 amino acid or to the C-terminal half of TEL protein (serum #68 and #71 provided by J. Ghysdael, Universite Pierre et Marie Curie, Paris, France). Polyclonal Swine Anti-rabbit Immunoglobulines/HRP (DakoCytomation, Glostrup, Denmark) was applied as secondary antibody.

Luciferase assays

Luciferase assays were performed as described previously.[29] In short, $8x10^4$ phoenix cells were seeded in 24-well dishes and transfected after 24 hours by calcium phosphate precipitation method with corresponding plasmids. A volume of 100 μl calcium phosphate precipitate was used for each well with in total 2 μg of plasmid DNA. Cells were transduced with 1 μg of luciferase construct pGL2-754TR (provided by S.W. Hiebert, Vanderbilt University School of Medicine, TN, USA) containing EBS sites. Variable

amounts (15-100 ng) of TEL-expressing plasmids plus 0.2 μg of LacZ supplemented with empty pCMV. The luciferase assay was carried out 48 hours post transfection. After two days cells were lysed and assayed for luciferase activity using steady-Glo reagents (Promega, Madison, WI). In parallel, the transfection efficiency was determined using lacZ staining. Luciferase activity levels were corrected for transfection efficiency using β-galactosidase expression levels. All experiments were carried out in triplicate.

Results

Somatic heterozygous TEL mutations in de novo AML

Nucleotide sequence analysis on the cDNA samples, prepared from leukaemic blast cells of 300 AML patients, revealed 7 distinct novel heterozygous mutations in the coding region of *TEL* (Table 1). In addition to these mutations, we observed 2 previously described polymorphism at nucleotide 532 (G–>A, Thr–>Thr) and 876 (T–>C, Leu–>Pro) in 17% and 5% of the patients respectively. Furthermore, we detected a rare polymorphism at nucleotide 654 (G–>A, Arg–>Gln) in 4 patients. This mutation was described earlier in CWR31 xenograft.[25]

Table 1. Mutations in the *TEL* coding region in AML patients.

Patient	age	FAB	Karyotype	Base pair change	Amino acid change
2290	42	M2	46,XX,t(X;21;4)(p11.2;q11;q32 ~33)[28]	500G→ T	E76X
2210	37	M2	45,X,-Y,t(8;21)(q22;q22),-13[27]/46,XY[1]	509insAGGA	S78X
2236	37	M1	46,XX[31]	655delATTCTT insGG	R127fsX207
2228	41	M4	46,XX[68]	1159C→ T	D295D
2649	NA	NA	NA	1307-1308insGGG	Y344-345insG
2188	51	M2	46,XY[18]/47,XY,+8[2]	1349C→ A	R359R
2296	39	M5	46,XY[20]		
3102	68	M2	45,XY,-7[33]/46,XY[8]	1423delAAGAA	H383fsX389

Two silent (# 2228, 2188 and 2296), 2 nonsense (#2290 and 2210), 2 frame-shift (#2236 and 3102) and 1 in-frame mutation were detected in the coding region of *TEL*. Sequence numbering is according to NCBI: BC043399 [gi:27695075] FAB: classification according to the nomenclature of the French-American-British Cooperative Group.[43]

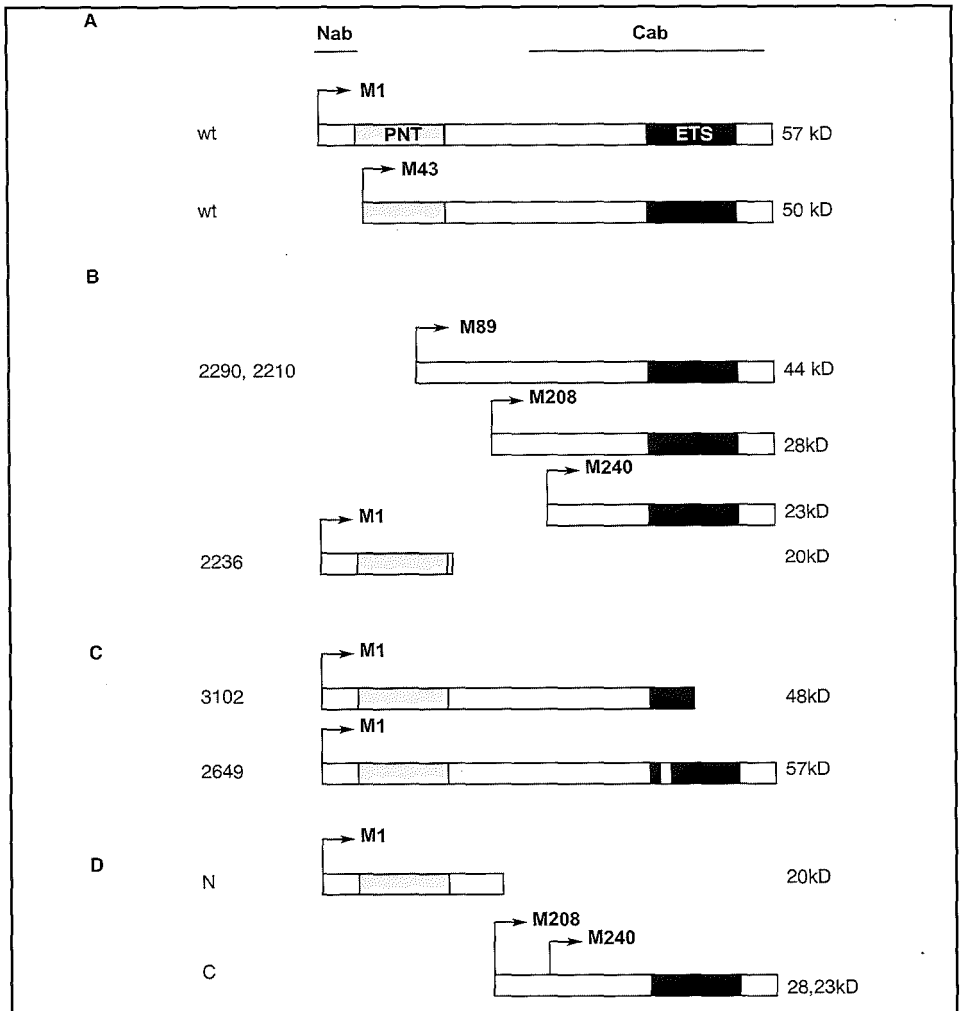

Figure 1. Schematic representation of proteins encoded by wild-type (wt) *TEL* and the mutants. (A) The 57 and 50 kD isoforms of TEL which are initiated by methionine M1 and M43 respectively. The regions of TEL protein that are recognized by the N- and C-terminal antibodies (Nab and Cab) are indicated in this figure. (B) The N-terminal mutants: 2290, 2210 and 2236. Mutations in the PNT domain give rise to the production of several short C-terminal fragments (44, 28, 23 kD) which are generated by translation initiation from methionine M89, M208 and M240. (C) The C-terminal TEL mutants: #3102 has a frame-shift mutation whereas the mutation in #2649 is in-frame. In both cases the ETS DNA binding domain is affected. (D) The N and C constructs used for luciferase assay; N expresses the N-terminal half of TEL while C expresses the two C-terminal fragments of 28 and 23 kD.

From the 7 novel mutations identified, 2 were silent point mutations at nucleotide 1159 (C–>T) and 1349 (C–>A) while in the other 5 cases (# 2210, 2290, 2236, 2649 and 3102) the predicted amino acid sequence and consequently the structure of the protein were affected (Table1 and Figure 1). The presence of these mutations was confirmed by nucleotide-sequencing analysis on genomic DNA isolated from leukaemic blasts of the same patients. In all 5 cases, wt non-mutated *TEL* was observed at equal levels as the mutated forms. Nucleotide-sequence analysis on genomic DNA extracted from non-haematopoietic tissues (skin or intestine) of patients # 2210, 2290 and 2236, revealed the absence of mutations, indicating that the *TEL* mutations were somatic and confined to the leukaemia clone. Interestingly, 4 of the 5 mutations that affected TEL protein structure occurred in the well-characterized functional domains of *TEL*; i.e. in 2 cases (#2210 and 2290) in the pointed (PNT) domain and in another 2 cases (#2649 and 3102) in the DNA-binding ETS domain (Figure 1). Mutation #2236 that did not localize within the functional domains was situated at 3 amino acid downstream of the PNT domain. The mutations in cases #2210, 2290, 2236 and #3102 resulted in an early stop whereas the in-frame mutation (GGG) in-patient #2649, introduced an extra glycine between amino acids 344 and 345. The clinical and cytogenetic characteristics of the patients are given in Table 1. The functional mutations in *TEL* were found in AML of FAB subtypes M1 and M2.

Table 2. Clinical characteristics of 77 de novo AML patients and the frequency of TEL protein deficiency

Characteristics		Total nr of patients	TEL -
Gender	Male	47	14 (30%)
	Female	30	10 (33%)
FAB	M0	2	0
	M1	25	6
	M2	10	6
	M3	1	0
	M4	17	8
	M5	18	2
	M6	2	2
	Unclassified	2	0
Cytogenetic risk group	Favorable	7	6 (86%)
	Intermediate	62	18 (29%)
	Unfavorable	8	0 (0%)

TEL- indicates undetectable full-length TEL protein expression by Western blot analysis. Favourable-risk karyotypes are t(8;21), t(15;17) or inv(16). Unfavourable risk karyotypes are –7/7q-, -5/5q-, t(9;22), t(6;9) or complex karyotype (> 3 anomalies). Patients carrying other cytogenetic aberrations or with a normal karyotype were included in the intermediate-risk group.

Abnormal TEL proteins translated from the cDNA of the mutants

We next studied the effects of the structural mutations on TEL protein expression using *TEL* cDNA. Mutant *TEL* cDNAs obtained from four patients (#2290, 2236, 3102 and 2649) were cloned into pCMV and transfected into phoenix cells. Subsequently, the expression of *TEL* constructs was studied by Western blot analysis using specific anti-TEL antibodies. As expected, the wt construct produced two isoforms (50 and 57 kDa), generated by the first two in frame ATGs (codon 1 and 43).[4] These isoforms were detected by the antibody directed to the C-terminal part of TEL (Figure 1). The antibody directed to the first 54aa of TEL (the N-terminal antibody) only recognizes the 57 kDa wt-protein (Figure 1).

N-terminal mutants. Western blot analysis of cells transfected with *TEL*-mutant #2290 showed no TEL bands when stained with N-terminal TEL antibody (Figure 2A). However, staining with C-terminal TEL antibody revealed three short fragments (44, 28 and 23 kDa) (Figure 2B). These bands were not observed when wt-*TEL* was introduced into these cells (Figure 2B). To investigate whether these short fragments are generated by translation initiation from alternative ATGs, we mutated putative in-frame ATG-start codons in cDNA #2290 into ACG. Mutation of the ATGs representing Methionine M89, M208 or M240, resulted in disappearance of the 44, 28 and 23 kD proteins respectively

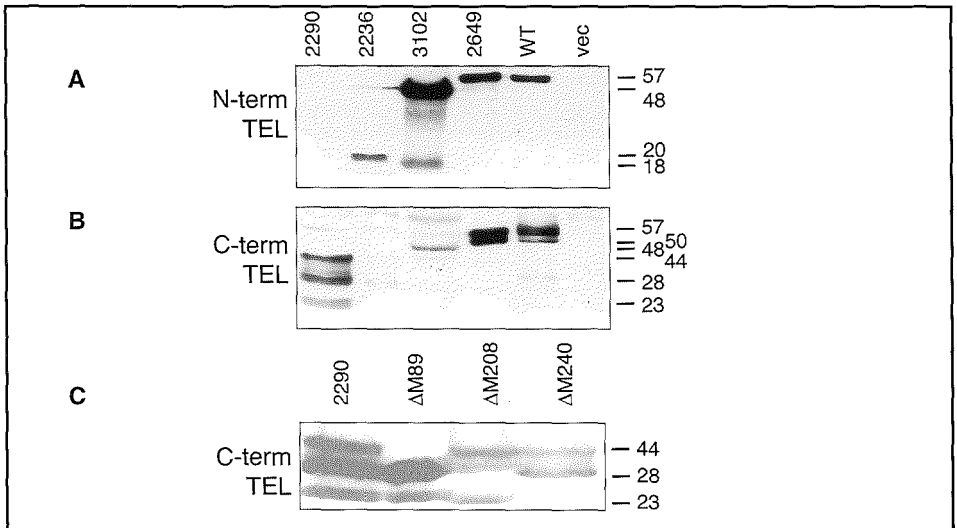

Figure 2. Western blot analysis on the lysates of phoenix cells transfected with different TEL constructs. The proteins generated by the WT construct or the mutants are stained with (A) the N-terminal TEL antibody (Nab, Figure1A) and (B) the C-terminal TEL antibody (Cab, Figure1A). (C) Using the C-terminal TEL antibody, we demonstrate that the 44, 28 and 23 kD fragments are generated by methionine M89, M208 and M240 respectively. In each construct an ATG is mutated into ACG (ΔM89, ΔM208 and ΔM240).

(Figure 2C). However, mutating the other ATGs (Methionine M315 or M319) had no effects on the distinct protein bands. Using the N-terminal TEL antibody, we demonstrated the production of an expected short 20kDa TEL protein in phoenix cells transfected with cDNA #2236 (Figure 2A). The expression of the short C-terminal TEL fragments was significantly reduced as compared to the mutant # 2290 (Figure 2B).
C-terminal mutants. The frame-shift mutation in #3102 presented an expected 48kDa band, which could be detected by both C- and N- terminal TEL antibodies. However, the presence of an 18 kDa fragment in #3102, detected by N-terminal antibody, was unexpected (Figure 2A). As expected, the in-frame mutation in patient #2649 did not visibly alter the TEL protein size.

Mutant TEL in AML samples

AML blasts from 3 patients with *TEL* mutations were available for Western blot analysis:

N-terminal mutants. Western blot analysis on the protein lysate, obtained from AML blasts of patient # 2290, revealed complete absence of the 57kDa and 50kDa TEL proteins, using N- and C-terminal TEL antibodies (Figure 3A). The same was observed in cells from patient #3, which did not harbour mutations in *TEL*. Using the C-terminal TEL antibody, multiple smaller fragments could be identified. These smaller bands were also observed in CD34+ bone marrow cells and in the other AML blasts samples, irrespective of *TEL* mutation (Figure 3A). Western blot analysis comparing patient samples with phoenix cells transfected with *TEL* construct # 2290 revealed that the bands correspond to the sizes of C-terminal TEL isoforms (44, 28 and 23 kD) (Figure 3B). Taken together, these data suggest that TEL has normally several short C-terminal isoforms, which lack the N-terminal homo-dimerization domain. Certain AML cases, such as patient #3 and #2290 express only these short isoforms of TEL.

Western blot analysis using N-terminal TEL antibodies, failed to show the short N-terminal protein encoded by mutant #2236. The normal sized isoforms (57 and 55 kD) produced by the non-mutated allele and the short C-terminal TEL fragments could be detected by C-terminal TEL antibody (Figure 3A).

C-terminal mutant. Neither N- nor C-terminal specific antibodies were able to detect the expected 48kD TEL protein in case #3102. The normal sized isoforms (57 and 55 kD) produced by the non-mutated allele and the short C-terminal TEL fragments could be detected in this case (Figure 3A).

Deficit TEL protein expression in one third of the patients

Western blot analysis on 3 patients with intact *TEL* alleles revealed the lack of TEL protein expression in 1 case (patient #3, Figure 3A). To investigate the relevance of this finding and determine the frequency of TEL protein loss, we studied TEL protein expression pattern in 77 AML patients that had already been screened for *TEL* mutations. The coding sequence of *TEL* was intact in all these patients. The presence of TEL protein was studied by Western blot analysis using the C-terminal TEL antibody. Immunostaining with ERK1 antibody demonstrated equal loading of the samples on the Western blot. Surprisingly, 24 cases lacked the 57 and 50kD TEL proteins (Figure 3C).

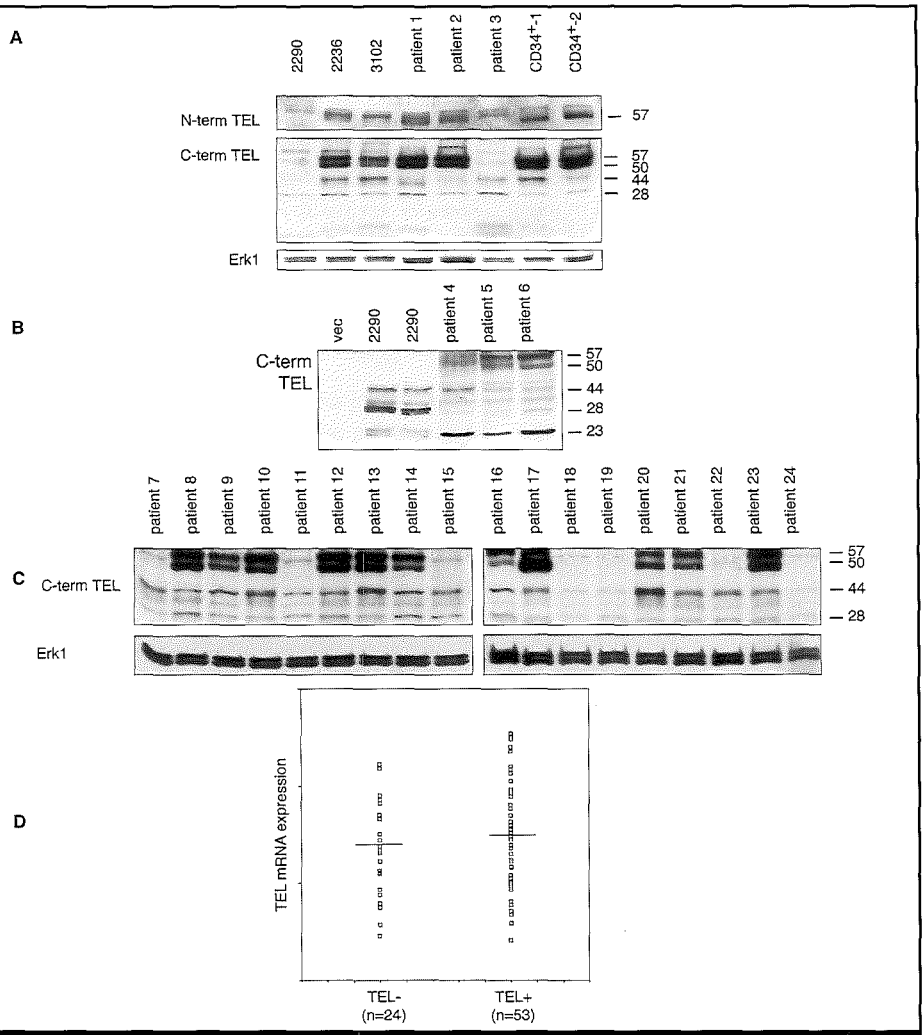

Figure 3. Western blot analysis on the protein lysate, obtained from AML blasts of patients (A) The protein lysates obtained from 3 patients with *TEL* mutations (#2290 #2236 and 3102), 2 AML patients with normal *TEL* alleles (patient #1– 3) and 2 normal CD34+ samples (CD34+1 and CD34+2) are stained with the N-and C-terminal TEL antibodies. (B) Western blot analysis using the C-terminal TEL antibody, comparing the size of short TEL fragments (44, 28 and 23 kD) in patients with those in phoenix cells transfected with *TEL* construct # 2290. (C) Western blot analysis on 18 representative AML cases (patient #7- 24) with normal *TEL* alleles using C-terminal TEL antibody. (D) Comparison of *TEL* mRNA expression levels in 77 AML patients in relation to the absence or presence of TEL protein expression. The *TEL* mRNA expression level is determined by GeneChip.[27]

However, all the patients showed expression of shorter bands that corresponded to the sizes of the C-terminal short isoforms of TEL detected in-patient #2290 (44, 28 and 23 kD). The clinical characteristics of the 77 AML cases studied by Western blot analysis are demonstrated in Table 2. The distribution of TEL negative cases varies between distinct karyotypic risk groups. Remarkably all of the unfavourable risk patients expressed TEL protein, whereas the majority of favourable-risk patients showed no expression.

To investigate whether the absence of TEL protein was the consequence of altered transcription, we investigated the levels of *TEL* mRNA in these AML cases. Previously we had determined gene expression profiles of 285 AML cases,[27] which included the 77 cases that were investigated in the present study. Analysis of mRNA hybridization to the *TEL*-probe set on the gene-chip revealed various levels of *TEL* mRNA expression among these cases. However, we did not observe any relation between *TEL* mRNA transcript levels and the loss of TEL protein (Figure 3D). To validate the gene-chip data, we carried out quantitative real-time PCR analysis on *TEL* transcripts using cDNA of 3 AML cases with TEL and 3 cases in whom the TEL protein expression was lost. In all these cases, comparable levels of *TEL* mRNA were detected irrespective of absence or presence of TEL protein (data not shown).

TEL mutants and the short C-terminal isoforms of TEL lack transcription repression potentials

Using a luciferase assay applying the pGL2-754TR reporter construct, which contains an ETS binding site (EBS), we determined the transactivation repressing potentials of distinct TEL mutants including those that generate short C-terminal TEL isoforms (#2290 and C, Figure1B and D). Figure 4A shows significant repression of luciferase activity by the wt TEL. The mutants expressing C-terminal TEL isoforms (#2290 and C) as well other mutants of TEL (#2236, 3102 and 2649) were unable to repress luciferase activity. Remarkably, the luciferase activity of the mutants was even higher than the basal levels observed with the empty vector control. This effect might be caused by a dominant negative effect of the mutants on a possible low endogenous repression activity in phoenix cells.

TEL Mutants act as dominant negative in a luciferase repression assay

In order to investigate whether mutant *TEL* constructs harbour dominant negative activity, wt *TEL* (15 ng) was co-transfected with 100ng of the *TEL* mutants (Figure 4B).

N-terminal mutant. Both N-terminal TEL mutants (#2290 and #2236) abolished the transactivation repression potential of the wt TEL. However, the inhibition by #2236 was much stronger than #2290 that only expresses short C-terminal isoforms. Similar to #2290, the C-terminal construct (C), which expresses two C-terminal TEL isoforms (28 and 23 kD) showed weak dominant negative effect on the wt.

C-terminal mutant. The two C-terminal mutants (#3102 and #2649) had strong dominant negative potentials and completely abolished the TEL-induced repression (Figure 4B). The dominant negative effect of truncated TEL proteins seems to be strong in the presence of an intact homo-dimerization domain. Accordingly, the constructs encoding the N-terminal half of TEL (N) had a strong dominant negative effect on the wt TEL.

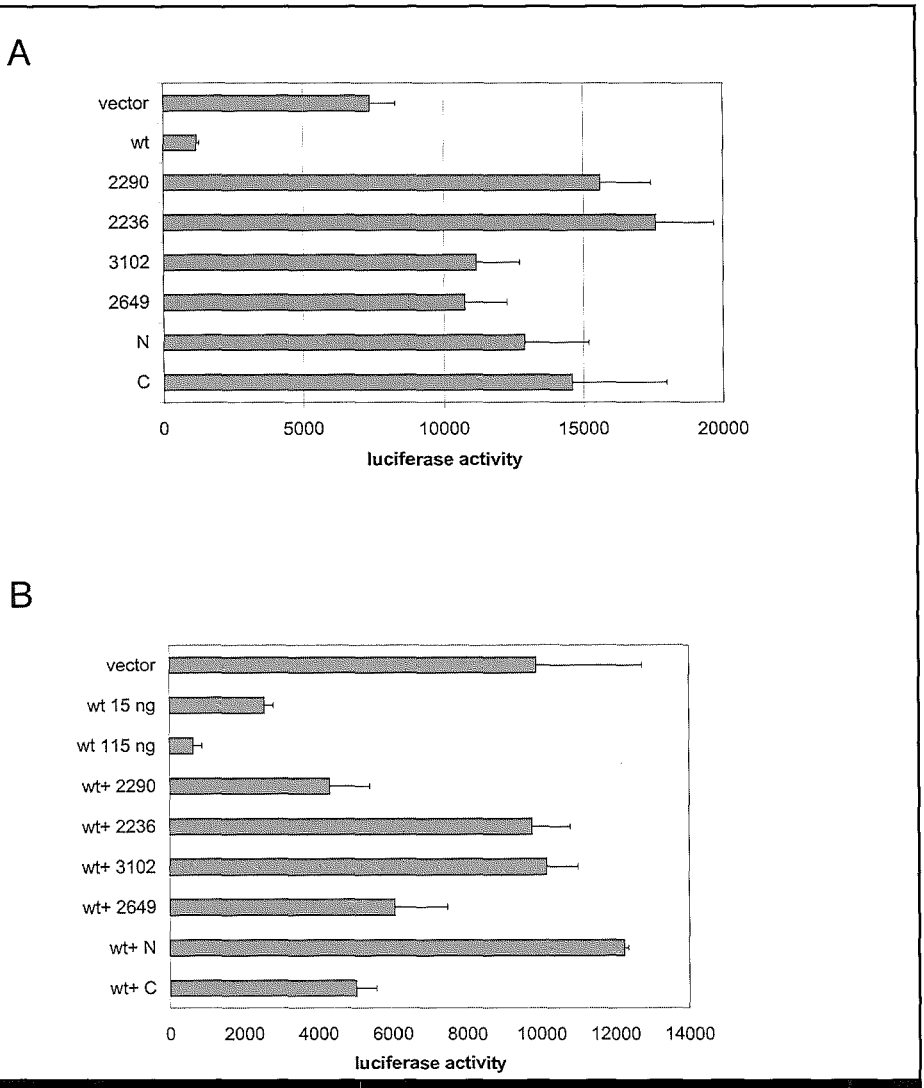

Figure 4. Determining transactivation repressing potential by luciferase assay. (A) Shows the repressing ability of the wt versus the truncated N- and C-terminal TEL and the mutants. Phoenix cells were transiently transfected with 100 ng of each TEL-expressing plasmids (PCMV) and 1μg of luciferase promoter with EBS (pGL2-754TR). The values are normalized for transfection efficiency by measuring b-galactosidase activity from the co-transfected CMV-LacZ construct. The bars present mean values of three repetitions and the error bars denote standard deviation. (B) Shows the inhibition of wt transcriptional repression (15 ng) by 100 ng of the mutants. The mutants exhibit a dominant negative effect on the wt.

Discussion

In this study we report the occurrence of somatic heterozygous mutations of the *TEL* gene in de novo AML patients. Furthermore, we demonstrate that one third of AML patients have deficient TEL protein expression. Several studies have demonstrated the involvement of *TEL* in distinct chromosomal translocations associated with haematological malignancies.[11-17] However, somatic *TEL* mutations in human AML have to our knowledge not been reported before. The functional *TEL* mutations identified in this study affected either the PNT domain in the N-terminus or the C-terminal DNA binding ETS domain. The three mutations in the N-terminus resulted in premature termination of translation by either single point mutation or out-of-frame insertion/deletion. Accordingly, translation of the cDNAs of these mutants demonstrated the loss of wt TEL isoforms. However, the downstream ATGs managed to initiate translation and generated 44, 28 and 23 kD C-terminal TEL fragments. The C-terminal *TEL* mutations were either in-frame or resulted in truncation of the ETS domain by out-of-frame mutation. In concordance with earlier studies,[8,30] the truncated TEL proteins were impaired in repressing transcription in a TEL-specific luciferase assay. Moreover, co-expression of the mutants with an intact homo-dimerization domain (# 2236, 3102, 2649 and N) completely abolished the transcription repression by wt TEL. This is in accordance with the fact that dimerization of TEL through the PNT domain is indispensable for EBS-driven transcription potentials.[3]

Many forms of cancer involve nonsense or frame-shift mutations that generate premature termination codons.[31-36] However the predominant consequence of such mutations is not always the synthesis of truncated proteins.[37] Accordingly, Western blot analysis on the protein extracts obtained from AML blasts of 2 mutants (#2236 and #3102) failed to show the expression of truncated TEL proteins. Generally, the absence of abnormal protein is justified by the possible degradation of mutant transcripts through a nonsense-mediated mRNA decay pathway.[37-39] Nucleotide sequence analysis on patient #2236 demonstrated that the mutant transcript level was decreased to only one-third of the wt level (data not shown). All the other patients with mutations presented no evidence of nonsense-mediated decay, as the mutants' transcript levels in these cases were as abundant as the wt *TEL*. The fact that despite the equal mutant/ wt transcription levels (data not shown), no abnormal protein could be detected is intriguing. It should be noted that only a minority of the AML cells, the AML colony forming cells (AML-CFU), proliferate and give rise to undifferentiated AML progeny.[40] It is possible that the expression of truncated TEL proteins is restricted to AML-CFUs. As demonstrated in the luciferase assay, truncated TEL proteins interfere with the wt function and may in this way disrupt the development of the myeloid precursor cells. Thus, the fact that truncated TEL proteins are undetectable in the bulk of the AML cells does not exclude their potential involvement in AML development.

Western blot analysis on the AML blast of patients and CD34+ cells suggested the expression of short C-terminal TEL fragments. These fragments, which were generated by downstream initiation codons lacked the wt TEL transactivation potentials. In the normal situation, the C-terminal TEL isoforms may regulate the repressing potentials of

TEL. Accordingly the abnormal ratio of different TEL isoforms may relate to leukaemia. In this study we demonstrated that the wt TEL isoforms (57 and 50 kD) are absent in one third of AML cases. The absence of TEL expression is also observed in ALL cases with t(12;21)[21,22]. Loss of TEL expression in these cases seems to be a critical secondary event for leukaemogenesis, as expression of the TEL/AML1 fusion protein alone is not sufficient for induction of malignant haematological disorder in transgenic mice.[41] In ALL cases without t(12;21), the wt TEL protein is present, however the expression level and the ratio of the two 57 and 50 kD TEL isoforms can be altered.[23] It is presently unknown why TEL protein is absent in some AML cases. The absence of protein in the presence of significant levels of mRNA suggests that TEL is post-transcriptionally regulated. Joosten et al.[42] have demonstrated that *Nm23M2* mRNA is normally non-polysome bound and therefore not translated. However, upon stimulation of growth factors such as Epo, SCF, IL3 or G-CSF, *Nm23M2* mRNA relocates to the polysomes. It would be interesting to investigate the ratio of polysome-bound *TEL* to the non-translatable free mRNA fraction in distinct AML samples. Furthermore, nucleotide sequencing analysis of the 5' and 3' untranslated regions of *TEL* mRNA might reveal mutations or polymorphisms, which could explain the TEL protein loss. Other explanations for the TEL protein loss are alterations in the pathways that regulate translation or accelerated TEL protein degradation.

The data presented here demonstrate that *TEL* abnormalities are not restricted to translocations and occur more frequently in AML than previously thought, i.e. either by somatic mutations or by the absence of wt TEL protein through a currently unknown mechanism. A comprehensive study in a large series of AML patients is needed to define the clinical consequence of TEL loss of function.

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Chapter 6

GSTT1 mRNA expression as an independent prognostic marker for AML

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Submitted

Abstract

Purpose: Homozygous deletion-polymorphism of *GSTM1* and *GSTT1* leads to the absence of major detoxifying enzymes and might affect the cytotoxicity of chemotherapeutic agents, such as anthracyclines. The present study was conducted to examine whether GSTs modify the prognosis of AML patients.

Patients and Methods: We developed a Q-PCR assay to measure *GSTM1* and *GSTT1* expression in a cohort of 269 AML patients.

Results: This assay proved to be not only as efficient as the genomic-PCR, which detects the *GST*-deletions but also capable in detecting cases that are negative for *GSTT1* mRNA expression (*GSTT1*-null). *GSTT1*-null associated with a significantly improved event-free (P= 0.003) and overall survival (P= 0.006), particularly in favourable-risk patients with t(8;21) and t(15;17). The presence of *GSTT1* expression proved to be an independent prognostic factor for event-free survival. In contrast to *GSTT1*, *GSTM1* had no prognostic value.

Conclusion: We conclude that *GSTT1* is a determinant of response in AML with t(8;21) and t(15;17) and that these patients may benefit from an adapted treatment protocol.

Introduction

Cytogenetic analysis is the major tool for risk-classification of acute myeloid leukaemia (AML). Although favourable and unfavourable-risk AML appear to be well defined by cytogenetics, notable differences in therapeutic response and outcome are still observed, implying the involvement of genetic variations or abnormalities within particular risk-groups. Glutathion S-transferases (GST) are detoxifying cytosolic enzymes that conjugate glutathione with compounds containing an electrophilic center.[1,2] In humans, at least 20 GST isoenzymes exist, subdivided in four major subfamilies, i.e. GSTα, GSTπ, GSTμ, and GSTθ. Within the GSTμ and GSTθ subfamilies, *GSTM1* and *GSTT1* respectively exhibit deletion polymorphisms, which in case of homozygosity lead to the absence of the enzymes. The percentages of homozygous deletions in different ethnical groups varies between 22 to 90 % for *GSTM1* and 12 -38% for *GSTT1*.[3] Deletions of these genes may alter the cytotoxic effect of several chemotherapeutic agents, such as anthracyclines, used in the treatment of AML.[3-5] Although several studies have focused on the relationship between *GST*-null polymorphism and the failure of chemotherapy, the results of these studies remain contradictory. The main reasons for these controversial results are the small cohort sizes and the variety in chemotherapeutic regimens used. We developed a Quantitative PCR (Q-PCR) assay to assess the prognostic impact of *GSTM1* and *GSTT1* polymorphisms and expression levels in a cohort of 269 cases with AML.

Material and Methods

Bone marrow samples of 269 AML patients at diagnosis were obtained after informed consent. Patients were treated as described previously.[6] Blasts were isolated and further

processed for DNA and RNA, and cDNA synthesis as described.[7] The multiplex PCR was carried out using 10 pmol of each forward and reverse primers for *GSTT1*, *GSTM1*[8] and albumin (5'-tgaaacatacgttcccaaagag-3' and 5'-ctctccttctcagaaagtgtgca-3') in a 50 all mixture containing 200 ng DNA, 2 mM MgCl2, 5 ml 10x PCR buffer, 2 U Taq DNA-polymerase (Amersham Pharmacia Biotech Inc, Roosendaal, the Netherlands), 250 mM deoxyribonucleoide triphosphates (dNTPs) (Amersham Pharmacia). PCR conditions were: 10' 95°C, followed by 1' at 95°C, 1' at 55°C and 2' at 72°C (40 cycles). Products were analyzed on a 2% EtBr-agarose gel.

GSTT1, *GSTM1* and *PBGD* (porphobilinogen deaminase) mRNA expression levels were measured separately by Q-PCR (ABI PRISM 7700 Sequence Detector, PE Biosystems, Nieuwekerk a/d IJssel, The Netherlands), using 50 ml mix containing 2 ml cDNA sample, 250 mM dNTPs, 15 pmol forward and reverse primer (same as with multiplex-PCR), 3 mM MgCl2, 200 nM probe, 5 ml 10x buffer A and 1.25 U AmpliTaq Gold (PE Applied Biosystems). Q-PCR conditions: 10' at 95°C, followed by 15" at 95°C and 30" at 60°C (40 cycles). Probes sequences were VIC-aggccgacccaagctggccaca-TAMRA (PE Biosystems) for *GSTT1* and FAM-agcggccatggtttgcaggaaac-TAMRA (Eurogentec, Seraing, Belgium) for *GSTM1*. The Q-PCR conditions and primers/probe combination used for quantifying *PBGD* have been described elsewhere.[7]
Statistical analysis was performed two-sided using Stata/SE 8.2. P-values <0.05 were considered statistically significant.

Results and Discussion

GSTM1 and *GSTT1* deletion-polymorphisms were detected by multiplex-PCR in 50% (66/131) and 22% (29/131) of the AML patients respectively. Q-PCR confirmed that cases with a *GSTT1*-null or *GSTM1*-null genotype did not express the respective transcripts (Table1A). All cases that were *GSTM1* positive on DNA level showed *GSTM1* mRNA expression. However, sixteen AML patients without *GSTT1* deletions appeared mRNA negative, suggesting an alternative mechanism leading to the absence of *GSTT1* transcripts. Thus, Q-PCR is superior to multiplex-PCR in detecting *GSTT1* negative cases.

We expanded the cohort of AML patients (Table1B) and determined *GSTT1*/*GSTM1* mRNA levels. *GSTM1* or *GSTT1* were absent in 48% (128/269) and 35% (93/269) of the patients respectively. In accordance with a previous report[9] we observed a significantly higher frequency of *GSTT1*-null expression in patients with t(15;17) (P=0.01) and t(8;21) (P=0.06) (Table1B). The absence of *GSTT1* detoxifying activity in these patients might have resulted in accumulation of genotoxic agents, which could play a role in the aetiology of translocations.

In concordance with previous studies[10-12] *GSTM1* mRNA expression did not have an impact on complete remission (CR) rate, relapse and survival of the patients (Table1B). Remission and relapse rates for *GSTT1*-null cases were 86% and 37% versus 76% and 46% for *GSTT1*-positive AML. The OS and EFS probabilities for *GSTT1*-null patients at 60 months were 44% and 36% respectively as compared to 26% and 18% in the *GSTT1*-

Table 1. *GSTT1* and *GSTM1*-null expression: (A) the concordance between genomic PCR and Q-PCR techniques in detecting the *GSTT1* and *GSTM1* negative cases (n=131). (B) Clinical characteristics, therapy outcome and hazard ratio for survival of 269 de novo AML patients.

A

		Genomic PCR on *GSTT1*		Total
		Null	Positive	
Q-PCR on *GSTT1**	Null expression	29	16	45
	Positive	0	86	86
Total		29	102	131

		Genomic PCR on *GSTM1*		Total
		Null	Positive	
Q-PCR on *GSTM1**	Null expression	66	0	66
	Positive	0	65	65
Total		66	65	131

B

Characteristics		Total nr of patients	*GSTT1*-null	*GSTM1*-null
Gender	Male	138	49 (36%)	63 (46%)
	Female	131	44 (34%)	65 (50%)
Age (years)	Median (range)	44 (15-65)	40 (16-61)	41 (16-63)
FAB	M0	9	4	4
	M1	57	24	33
	M2	64	24	29
	M3	21	13	7
	M4	48	11	29
	M5	62	17	25
	M6	3	0	1
	Unclassified	5	0	0
Cytogenetic risk group	Favourable	56	29 (52%)	29 (52%)
	inv (16)	13	5 (38%)	10 (77%)
	t(15;17)	20	12 (60%)	6 (30%)
	t(8;21)	23	12 (52%)	13 (56%)
	Intermediate	179	51 (28%)	85 (47%)
	Unfavourable	34	13 (38%)	14 (41%)

Complete remission %	86% of *GSTT1*-null (versus 76% GSTT1+)	81% of *GSTM1*-null (versus 78% GSTM1+)
Relapse %	37% of *GSTT1*-null (versus 46% GSTT1+)	43% of *GSTM1*-null (versus 43% GSTM1+)

HR♣ for OS	0.64 (CI: 0.47-0.88, P=0.005)	0.94 (CI: 0.70-1.27)
HR for OS adjusted for cytogenetic-risk Favourable-risk: HR=1 Intermediate-risk: HR=2.68, CI: 1.66- 4.33 Unfavourable-risk: HR= 4.56, CI: 2.57- 8.11	0.74 (CI: 0.53-1.02, P=0.06)	0.99 (CI: 0.74- 1.33)
HR for EFS	0.64 (CI: 0.47- 0.87, P=0.003)	0.95 (CI: 0.72-1.27)
HR for EFS adjusted for cytogenetic-risk Favourable-risk: HR=1 Intermediate-risk: HR=2.02, CI: 1.32- 3.08 Unfavourable-risk: HR= 3.55, CI: 2.09- 6.00	0.70 (CI: 0.52-0.96, P= 0.025)	0.99 (CI: 0.74-1.30)

* Cases that showed no amplification product after 40 Q-PCR cycles were considered negative. The Ct values of *GSTT1* and *GSTM1* positive patients varied from 30-38 and 22-38 respectively. All samples were *PBGD* positive (Ct values: 25-29). The amplified *GSTT1* fragment was 255 bp and GSTM1 was 133 bp. ♣ hazard ratio of *GST*-null (negative by Q-PCR) versus *GST*-positive (*GST*+).

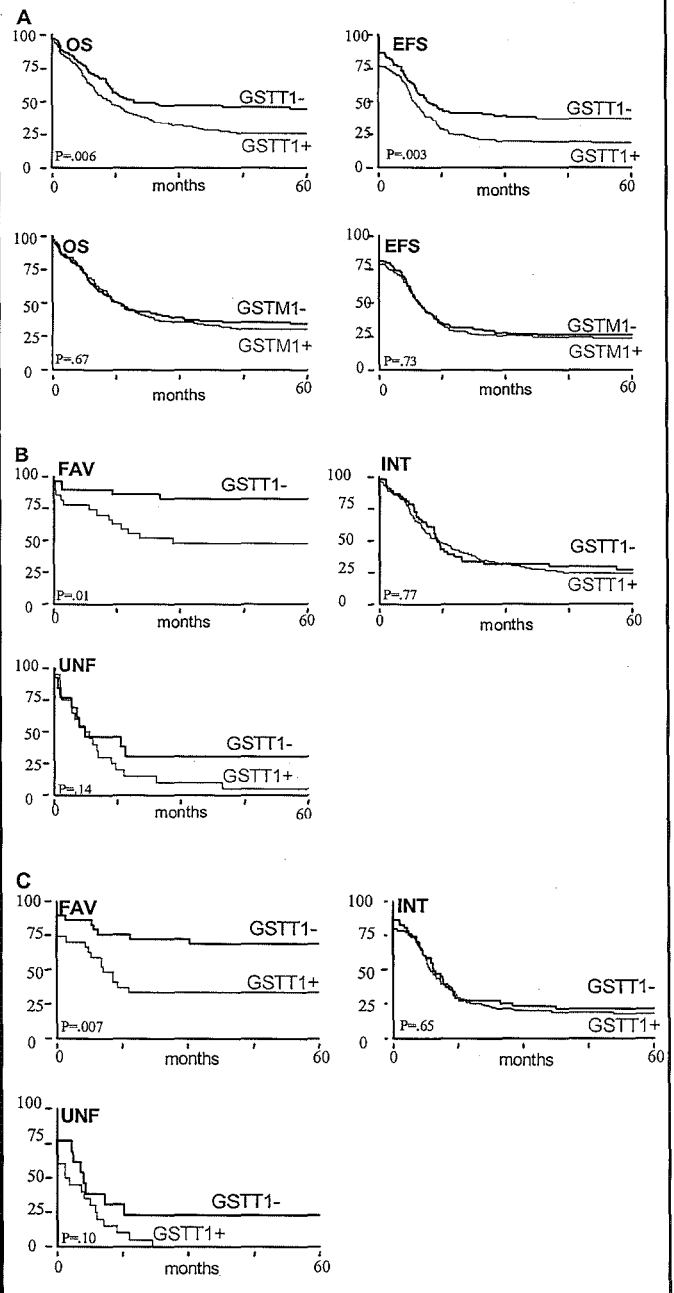

Figure 1. Overall and Event-free survival of AML patients based on the presence or absence of *GST* expression. (A) Event-free survival (EFS) and overall survival (OS) in the total cohort of patient (n=269) (B) OS in different karyotypically-defined risk groups: FAV [favorable risk: t(8;21), t(15;17) and inv(16)], UNF [unfavorable risk: 5/del(5q), -7del(7q), t(6;9), t(9;22), 3q26 abnormality or complex karyotype] and INT [Intermediate risk: other cytogenetic subgroups and patients with a normal karyotype]. (C) EFS in different karyotypically-defined risk groups. At the vertical axes the cumulative survival is depicted. P: indicates the P-value determined in Log-rank test.

positive group. Thus, *GSTT1*-null correlated with a higher event-free (P= 0.003) and improved overall survival (P= 0.006) (Table1B, Figure 1A). Accordingly, the hazard ratio (HR) for death (OS, HR 0.64, P=0.005, CI: 0.47-0.88) and failure (EFS: no CR, death in CR or relapse, HR 0.64, P=0.003, CI: 0.47-0.87) was significantly lower in *GSTT1*-null AML. Bone marrow transplantation had no effect on the survival analysis, as the percentage of transplanted patients was similar in both *GSTT1*-null and positive group (29% and 28% respectively). After adjustment for karyotypic risk factors in a multivariable analysis, *GSTT1*-null expression remained an independent prognostic marker for EFS (P= 0.025) but no longer for OS (P= 0.06) (Table1B). An association between *GSTT1*-null genotype and favourable prognosis has previously been demonstrated in de novo myelodysplastic syndrome[11] and childhood acute lymphoblastic leukemia,[13] consistent with the hypothesis that GSTT1 detoxifies chemotherapeutic agents and reduces the effective dose of drug within the cell. In accordance with these findings, Koberda et al.[14] reported that AML patients who achieved CR had the lowest value of GST enzyme activity.

The effect of *GSTT1* within each of the three karyotypically defined risk groups (Legends Figure 1), was different (test for interaction: P=0.048 for OS and P=0.035 for EFS). *GSTT1* expression showed no impact on the EFS and OS of intermediate-risk patients (Figure 1B). *GSTT1*-positivity in the favourable risk patients was associated with a significant decrease in EFS (P= 0.007) and OS (P= 0.01). In fact, the effect of *GSTT1* within the favorable-risk group was only apparent within the t(15;17) and t(8;21) subgroups and not in the patients with an inv(16) (test for interaction: P=0.04 for OS and P=0.07 for EFS). *GSTT1* expression in patients with a poor-risk karyotype was associated with a low, though not significant, EFS and OS. It is at present unclear why *GSTT1* expression has an effect on the outcome of therapy in particular cytogenetic risk-groups and not in others.

A few studies reported on increased toxic deaths in patients with a *GSTT1*-null genotype.[10,12] However, in agreement with findings reported by Voso et al.,[8] we did not observe such an association. From the 6 cases that died from toxicity, only one was *GSTT1*-null and two were *GSTM1*-null. A possible explanation for this discrepancy may be the distinct treatment protocols that were applied in different studies.[12]

Several studies have shown that the sensitivity of cells to the cytotoxic effect of anthracycline is inversely related to the cellular glutathione level.[4,15] Conjugation of anthracyclines with glutathione prevents their access to the nucleus and decreases their cytotoxicity.[4] Likewise, glutathione depletion of APL cells has been shown to be effective in overcoming resistance to arsenic trioxide.[16] Accordingly, we observed that glutathione and glutathione S-transferases are determinants of response in subsets of AML, particularly in cases with t(8;21) or t(15;17). The *GSTT1*-positive patients might benefit from adapted treatment protocols in which either the GSTT1 is inhibited or glutathione is depleted.

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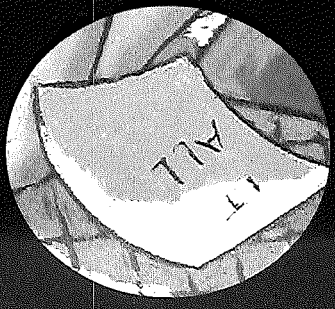
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Chapter 7

General discussion and summary: The prognosis triangle

Introduction

Acute myeloid leukaemia (AML), with an incidence of approximately 3 per 100.000, is the most common type of acute leukaemia in adults.[1] Treatment aims at eradicating leukaemia. Intensive chemotherapy may first lead the patient into a complete remission.[2] After a complete remission has been accomplished, consolidation therapy is applied to prevent the relapse of the disease. Consolidation therapy might involve additional chemotherapy or high-dose therapy in conjunction with autologous or allogeneic stem cell transplantation (SCT). Chemotherapy alone is associated with the highest risk of relapse, but it has the lowest treatment-related mortality (TRM). Autologous SCT has an intermediate risk of relapse and TRM, whereas allogeneic SCT has the lowest risk of relapse with the highest TRM. The choice for allogeneic SCT is based upon the eligibility criteria such as age, cytogenetics and the availability of a matched donor.[3,4]

Standard chemotherapy induces complete remission in 60-80% of patients.[2,4] Unfortunately, some patients are resistant to therapy or die in the induction period. Those who initially enter a remission, may experience a relapse of leukaemia after some time.[2] This means that a large number of patients are exposed to chemotherapy while they do not benefit from it. Therefore, it would be useful to apply prognostic markers to distinguish good- from poor responders.

The determinants of response in AML can be divided in three major categories: 1) factors related to the characteristics of the leukaemic cells e.g. cell type, cytogenetic and molecular aberrations, 2) the patient-related factors, e.g. age, sex and genetic variations affecting pharmacodynamics and pharmacokinetics of the drugs and 3) the drug arsenal available (Figure 1). Obviously, these three factors are inter-related and their interactions determine the treatment outcome in each individual. In this chapter, the disease or patient-related markers that can be used in determining prognosis of AML will be discussed

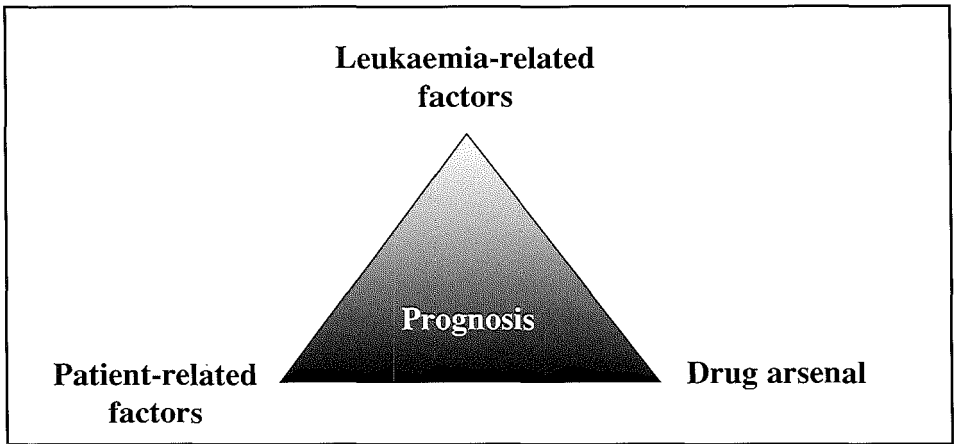

Figure 1. Prognosis triangle: the treatment outcome in each AML patient is determined by interactions between the nature of the therapy and the disease- and patient-related factors.

1- The molecular characteristics of the leukaemic cells as determinants of prognosis

At present, cytogenetics provides the best validated prognostic information available on the outcome of therapy.[5] Patients with translocations t(15;17), t(8;21) and inv(16) have a relatively favourable prognosis with a 4-year survival probability of 70% or more. In contrast, monosomies or deletions of the long arms of chromosomes 5 and 7, inversions and translocations of chromosome 3, translocation t(6;9), translocation t(9;22) and abnormalities of chromosome 11q23 have been associated with poor response to chemotherapy. The overall survival rate in this group is less than 20% at 4 years and the relapse rate is more than 70%.[6] Strikingly, the majority of patients with AML have either a normal karyotype or infrequent abnormalities with unknown prognostic value. These patients are currently classified in the standard or intermediate-risk category.[7,8] The intermediate-risk group, which is by number the largest category, is heterogeneous and is assumed to consist of mixed clinical and prognostic entities. At present *FLT3* internal tandem duplications (ITD), which occur in 20-30% of de novo AML patients, offer molecular markers for recognising poor-risk patients within the intermediate-risk group.[9-11] The major goal in this thesis was to pursue molecular biological approaches for defining subgroups of favourable- and unfavourable-risk AML, currently hidden within this heterogeneous group of patients.

The search for molecular aberrations in AML

We pursued two approaches in searching for molecular aberrations, which determine patients' prognosis. Firstly, we examined whether abnormal expression of genes that had been identified in the vicinity of common virus integration sites (cVIS) in murine myeloid leukaemia were involved in AML and predicted disease outcome. In **Chapter 2** we demonstrated abnormal mRNA expression of *EVI1*, a cVIS in murine leukaemia, in almost 10% of newly diagnosed AML patients. High *EVI1* expression was associated with an increased hazard ratio for death (OS; HR=1.85, CI: 1.25-2.73) and failure (EFS; HR=1.82, CI: 1.25-2.67).

Secondly, we studied aberrations in haematopoietic transcription factors with an established critical role in myeloid development. Transcription factors regulate the expression of genes that induce cell differentiation. Naturally, any aberration in the structure or expression of genes encoding transcription factors may have drastic effects on haematopoiesis. In fact, the involvement of transcription factors has been demonstrated in several leukaemic translocations, including t(8;21), t(15;17), idt(16) and 11q23 abnormalities.[12-14] Moreover, many transcription factors have been identified as cVIS in murine leukaemia, e.g. *HOXa9, HOXa7, MEIS1, PU.1*, and *c-MYB*.[15-20] In **Chapter 4** we demonstrated that mutations in the gene encoding transcription factor C/EBPα provide independent prognostic information with respect to enhanced survival. *CEBPA* mutations were associated with a decreased hazard ratio for death (OS; HR=0.35: 0.13-0.94) and failure (EFS; HR=0.37, CI: 0.15-0.91). Besides the mutations, *CEBPA* mRNA expression levels seemed to influence the prognosis of patients with intermediate-risk karyotype. A preliminary study has revealed that methylation of the CpG island in the

promotor region of *CEBPA* associates with transcriptional silencing of this gene in a subset of AML patients (C. Erpelinck and R. Delwel, Personal communication, 2004). In **Chapter 4** we demonstrate that low transcription levels of *CEBPA* predict poor prognosis.

Interestingly, we observed hardly any overlap between patients with low *CEBPA* or high *EVI1* expression and cases carrying *CEBPA* mutations or *FLT3*-ITD. This observation was further confirmed in a gene expression study using Affymetrix U133A GeneChips. Alike the major leukaemic translocations, e.g. t(8;21), t(15;17) and inv(16), both AML with *CEBPA* mutations and AML with high *EVI1* expression represented distinct subclasses of leukaemia characterized by a unique composite expression signature (Figure 2).[21]

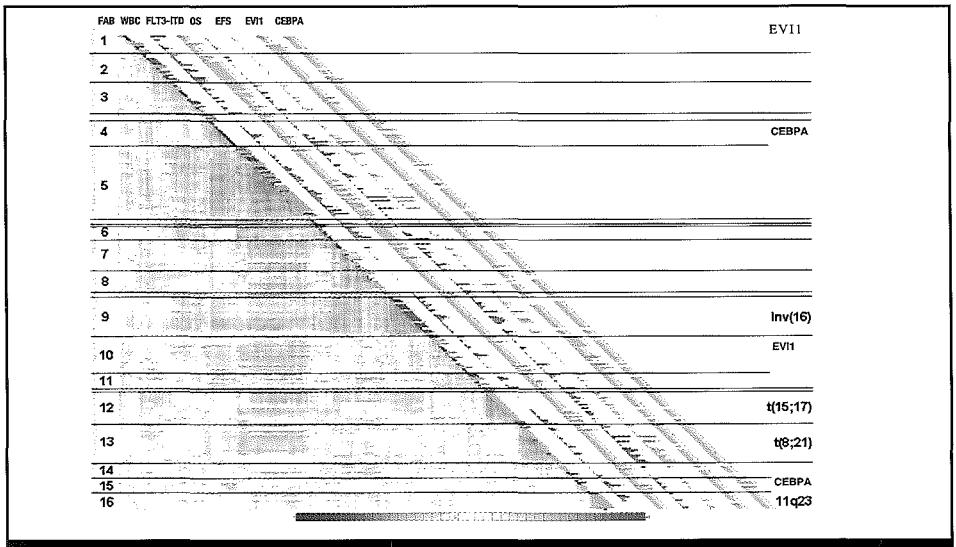

Figure 2. Pairwise correlation between gene expression profile of 285 AML patients using 2856 probe sets.[21] The colors of the cells relate to Pearson's correlation coefficient values. Red indicates positive and blue negative correlation. Negative correlation means that genes that have high expression in one sample have low level of expression in the other sample. The FAB classification is shown in the diagonal column: M0 = black, M1 = green, M2 = purple, M3 = orange, M4 = yellow, M5 = blue and M6 = gray. The 3 other diagonal columns show *FLT3*-ITD, *CEBPA* mutations and high *EVI1* expression. red presents the presence of a given abnormality and green its absence. Based on this gene expression correlation view 16 clusters of AML are identified. Some clusters were related to the cell morphology, e.g. cluster 5 with FAB M4 or M5 and the others were related to the genetic abnormalities present, e.g. t(8;21), t(15;17), inv(16) and 11q23 abnormalities. Furthermore, the molecular aberration proposed as valuable prognostic markers in this thesis had their own gene expression patterns. Cases with *CEBPA* mutations were categorized in cluster 4 and 15, while cluster 1 and 10 consisted mainly of cases with high EVI1 expression.

Stratification of intermediate-risk group based on molecular aberrations and gene-expression signatures

We analysed a series of 187 AML patients with cytogenetically defined intermediate risk for the presence of molecular aberrations with independent prognostic significance. Thirty-one percent harboured *FLT3*-ITD mutations; 8% had high *EVI1* mRNA expression and 3% had low *CEBPA* mRNA levels. These molecular features correlated with poor prognosis. In contrast, mutations in *CEBPA*, detected in 8% of the intermediate-risk patients, correlated with favourable prognosis. These molecular markers allowed us to distinguish 50% of the intermediate-risk group as poor and good responders (Figure 3). These results encouraged us to further unravel different classes of AML through additional gene expression studies and mutation analyses.

We studied mRNA expression patterns of potentially transforming genes, identified in murine leukaemia, i.e. *Hoxa9, Hoxa7, Meis1, Cb2, Pu.1, Nm23* and *Sox4* in patients with AML (data not shown). All patients with favourable cytogenetics showed reduced levels of *HOXa9, HOXa7* and *MEIS1* transcripts. In fact, their expression patterns were very similar to those of *MEL1* and *EL1*, which is demonstrated in **Chapter 3**. An altered common pathway in favourable risk patients may result in down regulation of transcription in these genes. The transcript levels of *PU.1, NM23-H1, NM23-H2* and *SOX4* did not correlate to any cytogenetic category and had no prognostic value. Although mRNA expression levels of *CB2* in AML were comparable to those in normal bone marrow, abnormal levels of CB2 protein might be involved in leukaemia. In fact, Rayman et al observed variable protein expression of CB2 in AML patients, with possible prognostic significance (manuscript in preparation).

Retroviral insertion may disrupt the coding region of genes, resulting in production of abnormal proteins. Therefore we searched for possible mutations in the coding region of two transcription factors, which are also targeted by virus integration, *c-MYB* and *PU.1* (data not shown). Mutational analysis of the coding region of *c-MYB* in a series of patients with AML did not reveal any functional mutations. In *PU.1*, we detected silent point mutations in two cases only. Mueller et al.[22] had reported the presence of *PU.1* somatic mutations in 7% (9/126) of patients with AML. However, these findings could not be confirmed by others.[23-25] Our study, which is the largest study on *PU.1* mutations, was based on the same methodology as Mueller et al, i.e. direct sequencing of the cDNA. In this way we could detect the large deletion mutations that were described earlier. However, it seems that differences in methodology or clinical characteristics can not explain these controversial findings. As suggested,[24,25] ethnical differences might explain the discrepancy, as the patient cohort studied by Mueller et al. mainly consisted of Japanese patients with AML.

In **Chapter 5** we demonstrated heterozygous somatic mutations in the *TEL* gene. Several studies have shown the frequent involvement of *TEL* in chromosomal translocations associated with haematological malignancies.[26-31] In addition to mutations in *TEL*, we demonstrated that the TEL protein is absent in one third of AML patients. Western blot analysis on 77 AML patients revealed loss of TEL protein in 24 cases. Remarkably, loss of TEL protein within the intermediate-risk category, was associated with an

increased hazard ratio for death (OS; HR=2,06, CI: 1.04-4,09) and failure (EFS; HR=1.98, CI: 1.05-3,76).

Subsequently, we studied the heterogeneity within the unclassified half of the intermediate-risk group, using microarray data.[21] Within the intermediate-risk group, 70 patients did not carry any known molecular aberrations, including *FLT3*-ITD, *CEBPA* mutations and high *EVI1* expression. Figure 4 shows pairwise correlation between gene expression profiles of these 70 AML patients. Except the few clear clusters, which relate to the cell morphology (M1, M2 and M5), the expression profiles of the rest of the patients hardly show any homology. One explanation could be that these cases present leukaemias with extremely infrequent aberrations. In that case, we might be able to define novel AML clusters by screening a larger cohort of patients. In addition, application of other approaches, such as proteomics might improve the classification of AML.

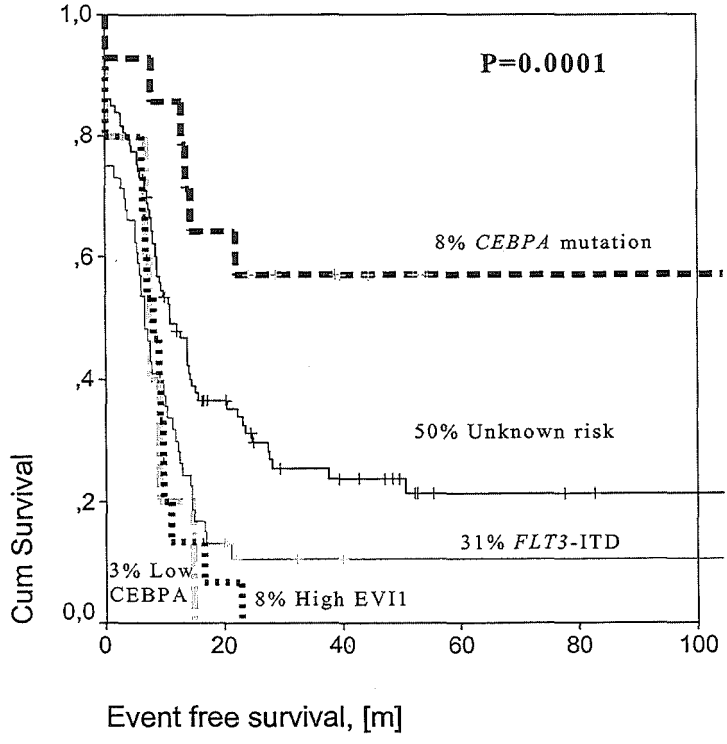

Figure 3. Stratifying AML patients (n=187) with intermediate risk karyotype by means of the known prognostic markers.

2- Patient-related factors affecting prognosis of patients

The genetic aberrations of the leukaemic cells provide valuable information about the outcome of the disease. In addition, patient-related factors might modify prognosis. Polymorphisms in genes encoding drug metabolising enzymes are considered as constitutional factors of therapy response.[32] For example cellular resistance to antracyclines in AML is related to the activity of a family of phase II metabolising enzymes, the Glutathion S-transferases.[33] Glutathion S-transferase theta 1 (*GSTT1*), a member of this family, shows homozygous deletion-polymorphism in approximately 12-38% of the population[34], which results in the absence of a major detoxifying enzyme. In **Chapter 6** we demonstrated that *GSTT1* expression is an independent prognostic factor for a subset of AML cases. As GSTT1 normally detoxifies chemotherapeutic agents and reduces their effective dose within the cell, the absence of the enzyme may associate with increased drug availability and more effective treatment. Based on this assumption, the treatment protocols for *GSTT1* positive patients might require dose-adjustment for allowing effective intracellular drug concentration. This study shows how individualized dosing based upon genotype or phenotype of relevant drug metabolising enzymes might be relevant for improving the therapy outcome of AML patients.

3- AML drug arsenal

The remission induction therapy in AML consists of a combination of anthracyclines (idarubicin and daunorubicin) and cytarabine, sometimes supplemented with a third drug (e.g. etoposide, thioguanine).[4] Moreover, sensitisation of leukemic cells with granulocyte colony-stimulating factor (G-CSF) factor might reduce relapse and improve overall and disease-free survival of AML patients, especially within the intermediate-risk group.[4] However, the heterogeneous characteristics of AML make it difficult to design an effective uniform treatment protocol for treating all the patients. Until now more than 200 different mutations and chromosomal translocations have been described in AML. Acute promyelocytic leukaemia (APL) which associates with translocation t(15;17), can specifically be treated with all trans retinoic acid (ATRA). ATRA plus chemotherapy has improved the cure rate of APL patients from less than 40% to over 70%.[35,36] Nevertheless, with the exception of acute promyelocytic leukaemia (APL), we have not yet succeeded in developing effective targeted therapies for other subsets of AML.

Through molecular genetic studies on leukaemic populations a number of genetic abnormalities and aberrant pathways associated with the disease have been identified. The ultimate goal of these studies is to disclose novel therapy targets in leukaemia. In this thesis, we have demonstrated the prognostic value of high *EVI1* expression and *CEBPA* mutation in AML patients. Whether these molecular markers will also offer valuable targets for therapy, remains to be seen. In order to answer this question elaborate in-vitro and in-vivo studies are required to examine whether high *EVI1* expression or *CEBPA* mutations provide critical transforming signals in leukaemic cells and whether these molecular targets can be inhibited. In fact, circumstantial evidence does suggest that high *EVI1* expression or *CEBPA* mutation provide critical transforming signal. High

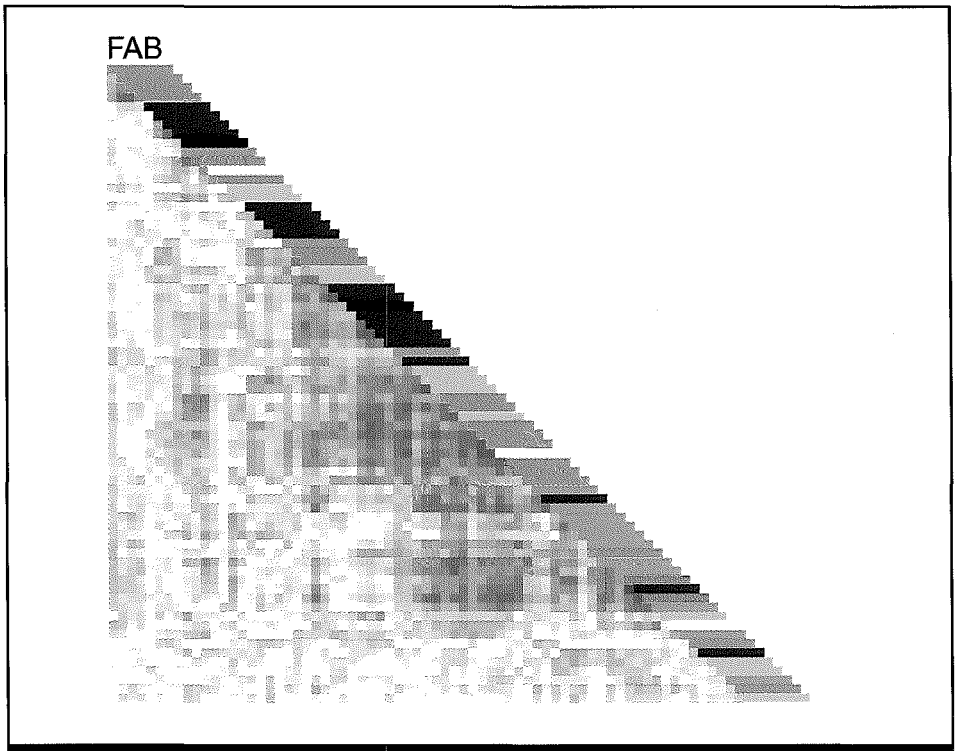

Figure 4. Pairwise correlation between gene expression profile of 70 AML patients with an intermediate/unknown-risk karyotype using 2856 probe sets. The intermediate-risk patients with high *EVI1* expression, *FLT3*-ITD or *CEBPA* mutations are excluded. The colors of the cells relate to Pearson's correlation coefficient value. Red indicates positive and blue negative correlation. Negative correlation means that genes that have high expression in one sample have low level of expression in the other sample. The FAB classification is shown in the diagonal column: M0 = black, M1 = green, M2 = purple, M3 = orange, M4 = yellow, M5 = blue and M6 = gray.

expression of *EVI1* in cell-lines and bone marrow is known to impair terminal differentiation of progenitors into erythroid cells[37] and granulocytes.[38] Furthermore, conditional overexpression of *EVI1* in mice erythroid cells seems to induce dysplasia, as observed in patients with 3q26 abnormalities (E. van de Akker and D. Spensberger, 2004 personal communication). In theory, inhibition of EVI1 function through for instance small molecules that interfere with EVI1 complex formation might recover the EVI1-induced differentiation block. Unlike *EVI1* overexpression, *CEBPA* mutations seem to block differentiation only in man but not in mice. Schwieger et al[39] have demonstrated that the expression of an N-terminal *CEBPA* mutant in primary human progenitors blocks differentiation of both erythroid and myeloid lineages, supporting the role of such mutations

in leukaemic transformation. The N-terminal mutations in *CEBPA* result in the production of a dominant negative protein, which blocks the DNA binding and transactivation potentials of the wt C/EBPα.[40] Interestingly, microarray analysis shows that the gene expression signature of *CEBPA* mutants is similar to that of cases with low *CEBPA* expression (R. Delwel, 2004, personal communication). By interfering with the function of mutant C/EBPα protein or with critical downstream targets, we might be able to rescue the differentiation blockade induced by *CEBPA* aberrations. Interestingly, Zheng et al[41] have recently demonstrated that *FLT3*-ITD blocks myeloid differentiation through suppression of *CEBPA* mRNA expression. In the past years, pharmaceutical companies have shown interest in developing kinase inhibitors that abrogate *FLT3*-ITD activity. Several kinase inhibitors such as SU11248, PKC412, CEP-701 and CT53518 are currently in phase I-II of clinical development.[4,36] The critical role of C/EBPα in leukaemic transformation suggests that treatments based on modulation of C/EBPα activity may provide a new approach in the therapy of a subset of AML patients.

Conclusions

At present, a profound understanding of the individual prognostic factors is becoming of significant importance for treatment choice in AML. A classic example of this approach is treatment of patients with acute promyelocytic leukaemia with oral retinoids.[4,7] Unfortunately, in most AML cases, we are still not capable of pointing out a dominant abnormality with defined prognostic value that also offers a target for drug intervention. This study represents a series of efforts to identify novel molecular prognostic markers in AML. We demonstrated the prognostic impact of aberrations in two major haematopoietic transcription factors, C/EBPα and EVI1. Both aberrations defined distinct subclasses of leukaemia with unique gene expression signatures.[21] Aberrations in another transcription factor, TEL, were frequently demonstrated in AML and were shown to alter patients' prognosis. Furthermore, we demonstrated that patient-related constitutional genetic variations, such as *GSTT1* deletion-polymorphisms may impact on disease prognosis. We anticipate that in the near future, additional discoveries of prognostic markers will enable a progressively precise prediction of the outcome and thus be of significant clinical value for developing and applying risk-adapted treatment in AML.

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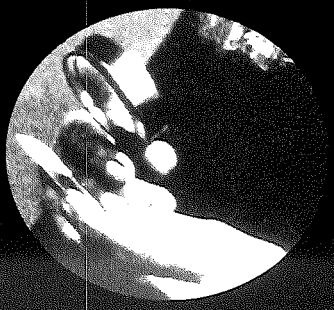

Hoofdstuk 7

Algemene discussie en samenvatting: De prognose driehoek

Introductie

Acute myeloide leukemie (AML) is, met een frequentie van ongeveer 3 per 100.000, de meest voorkomende vorm van acute leukemie bij volwassenen.[1] De behandeling van patiënten met AML is gericht op de uitroeiing van de leukemie. Met intensieve chemotherapie kan bij de patiënt een complete remissie worden bereikt.[2] Na het bereiken van de complete remissie wordt aanvullende therapie toegepast om de terugkeer van de ziekte te voorkomen. Deze therapie kan bestaan uit een extra kuur van chemotherapie of een hoge-dosis chemotherapie en/of radiotherapie in combinatie met autologe of allogene stamceltransplantatie (SCT). Chemotherapie zonder SCT betekent een hogere kans op terugkeer van de ziekte, maar anderzijds is de therapie-gerelateerde mortaliteit (TRM) geringer. Allogene SCT impliceerde laagste kans op recidief van de leukemie, maar de meeste morbiditeit en mortaliteit ten gevolg van de behandeling. Autologe SCT neemt een tussenpositie in ten aanzien van terugkeer van leukemie en behandelings mortaliteit. Allogene SCT is dus de meest krachtige anti-leukemische behandeling bij volwassenen met AML in remissie. De keuze voor allogene SCT wordt gemaakt op basis van geschiktheidcriteria zoals leeftijd, leukemie-prognose (cytogenetische kenmerken) en de beschikbaarheid van een donor.[3,4]

Chemotherapie leidt in ongeveer 60-80% van de patiënten naar complete remissie.[2,4] Bij patiënten die een remissie bereiken kan na enige tijd de leukemie terugkeren.[2] Het is daarom noodzakelijk om prognostische merkers te identificeren, die ons in staat stellen om voor de aanvang van de therapie de goede 'responders' te onderscheiden van de slechte 'responders'.

De factoren die de response van AML patiënten bepalen kunnen worden onderverdeeld in drie categorieën: 1) factoren die gerelateerd zijn aan de karakteristiek van leukemische cellen e.g. cel type, cytogenetica en moleculaire afwijkingen, 2) de patiëntgerelateerde factoren, e.g. leeftijd, geslacht en genetische variaties die effect hebben op de farmacodynamiek en farmacokinetiek van geneesmiddelen en 3) het arsenaal aan geneesmiddelen (Figuur 1, pagina 106). Deze drie factoren zijn bovendien sterk met elkaar verbonden en hun interactie bepaalt de uitkomst van de behandeling bij elke patiënt. In dit proefschrift beschrijven we ziekte- of patiëntgerelateerde merkers die kunnen worden gebruikt bij het vaststellen van de prognose.

1- De moleculaire karakteristieken van de leukemische cellen als determinanten van de prognose

Tegenwoordig verschaft cytogenetica belangrijke informatie om zowel de uitkomst van de remissie-inductie als het postremissie verloop te voorspellen.[5] Chromosoom translocaties t(15;17), t(8;21) en inv(16) worden als gunstige afwijkingen beschouwd. Patiënten met deze afwijkingen hebben een kans van 70% of meer op een overleving van 4 jaar. Daarentegen wordt een inferieure response op chemotherapie veelal waargenomen bij patiënten met afwijkingen aan de lange arm of compleet verlies van één chromosoom 5 of 7, translocaties of inversies van het chromosoom 3, t(6;9), t(9;22), of afwijkingen aan chromosoom 11q23. De kans op overleving na 4 jaar bij deze laatste groep van patiënten

is minder dan 20% en de kans op een terugkeer van de ziekte boven 70%.[6] Een beperking van de cytogenetische risico-classificatie is dat een aanmerkelijke deel van de patiënten geen informatieve cytogenetische afwijkingen hebben. Dit maakt het moeilijk om de prognose bij een grote groep van patiënten vast te stellen. Deze patiënten worden geclassificeerd als standaard of behorend tot de intermediaire risicogroep.[7,8] De intermediaire risicogroep is niet scherp gedefinieerd en bestaat uit leukemien met verschillende klinische en prognostische entiteiten. Op dit ogenblik is *FLT3* internal tandem duplicatie (ITD), die bij 20-30% van de novo AML voorkomt, een aanvullende moleculaire merker die wordt gebruikt om patiënten met een slechte prognose te herkennen binnen de intermediaire risico groep.[9-11] Het hoofddoel van het in dit proefschrift beschreven onderzoek was nieuwe moleculaire merkers te identificeren om subgroepen te definiëren binnen de heterogene intermediaire risicogroep.

De zoektocht naar moleculaire afwijkingen in AML

Voor de zoektocht naar de moleculaire afwijkingen die de prognose bepalen, is gebruik gemaakt van twee verschillende methoden van aanpak. Bij de eerste aanpak wordt onderzocht of transformerende genen die zijn opgespoord in leukemie bij de muis, ook een rol spelen bij menselijke leukemie. Deze genen werden in muizen met leukemie geïdentificeerd door middel van retrovirale insertionele mutagenese. In **hoofdstuk 2** tonen we abnormale mRNA expressie van één van deze genen, *EVI1* aan bij ongeveer 10% van de patiënten met leukemie. Hoge expressie van *EVI1*, een gen dat oorspronkelijk als een cVIS gevonden is, gaat gepaard met een slechtere overlevingskans (OS: HR=1.85, CI:1.25-2.73) ten gevolg van een hogere kans op recidief van leukemie (EFS; HR=1.82, CI: 1.25-2.67).

De tweede benadering die werd gevolgd, betreft de opsporing van afwijkingen in transcriptiefactoren, die kritische functies hebben bij de myeloide ontwikkeling. Het gaat in dit verband om transcriptiefactoren die de expressie van genen reguleren die een belangrijke rol spelen in de differentiatie van hematopoietische voorlopercellen. Eerder is de betrokkenheid van transcriptiefactoren bij de ontwikkeling van leukemie al aangetoond bij de verschillende chromosoom translocaties bij AML, zoals t(8;21), t(15;17), inv(16) en 11q23 afwijkingen.[12-14] Bovendien zijn er bij muizeleukemie veel transcriptiefactoren geïdentificeerd als cVIS, bijvoorbeeld Hoxa9, Hoxa7, Meis1, Pu.1, of c-Myb.[15-20] Vanzelfsprekend kan iedere afwijking in de nucleotide volgorde of expressie van één van de genen een drastisch effect hebben op de bloedcelvorming. In **hoofdstuk 4** tonen wij aan we dat mutaties in het gen dat voor de transcriptiefactor C/EBPα codeert, een onafhankelijke prognostische merker is. Patiënten met *CEBPA* mutaties hebben een betere overleving (OS: HR=0.35, CI:0.13-0.94) en een lagere kans op recidief van leukemie (EFS; HR=0.37, CI: 0.15-0.91). Verder blijkt dat de hoeveelheid aan *CEBPA* mRNA transcripten in een cel ook een voorspellende waarde heeft bij patiënten met AML die behoren tot de intermediaire risico groep, een lage *CEBPA* expressie voorspelde, namelijk een slechte prognose (hoofdstuk 4). Voorlopige studies hebben aangetoond dat methylatie van de CpG eilanden in de promotorregio van *CEBPA* in de meeste gevallen de oorzaak is van de transcriptionele 'silencing' van dit gen bij deze subgroep van AML patiënten (C. Erpelinck and R. Delwel, Persoonlijke communicatie, 2004).

Het valt op dat er nauwelijks sprake is van overlap tussen patiënten met een lage *CEBPA* expressie, met *CEBPA* mutaties, hoge *EVI1* expressie of *FLT3*-ITD mutaties. Deze observatie werd bevestigd door een genexpressie-studie met behulp van biochips (Affymetrix U133A GeneChips). Net zoals AML samples met de bekende leukemische translocaties, namelijk t(8;21), t(15;17) en inv(16), hebben AML cellen met *CEBPA* of *EVI1* afwijkingen een kenmerkend genexpressie-patroon. Dit onderstreept dat we hier te maken hebben met specifieke subgroepen van leukemie (Figuur 2, pagina 108).[21]

Stratificatie van de intermediaire risicogroep, gebaseerd op moleculaire afwijkingen en het genexpressie patroon

In totaal werden 187 AML monsters van patiënten met intermediair risico geanalyseerd op de aanwezigheid van de hierboven genoemde moleculaire afwijkingen. Bij 31% van deze patiënten werden *FLT3*-ITD mutaties gevonden; 8% van de patiënten toonde een hoge *EVI1* expressie en 3% had een laag *CEBPA* mRNA niveau. Deze moleculaire afwijkingen correleerden elk met een slechte prognose. Mutaties in *CEBPA* werden gevonden bij 8% van de patiënten met intermediair risico en correleerde daarentegen met een goede prognose (Figuur 3, pagina 110). Met behulp van deze serie moleculaire merkers waren we in staat om 50% van de patiënten binnen de intermediaire risico groep te onderscheiden als patiënten met een goede of slechte prognose. Deze kennis heeft ons aangemoedigd door middel van aanvullende genexpressie-studies en verdere mutatie analyses de andere subgroepen van AML te analyseren.

We bestudeerden het mRNA-expressiepatroon van potentiële oncogenen bij AML patiënten. De betrokkenheid van deze genen was al aangetoond bij muizeleukemie. Deze onderzochte genen waren onder andere *HOXa9, HOXa7, MEIS1, CB2, PU.1, NM23-H1, NM23-H2* en *SOX4*. Alle patiënten met cytogenetische afwijkingen die correleren met een gunstige ziektebeloop, bijvoorbeeld t(8;21) of t(15;17), vertoonden een laag expressieniveau van *HOXa9, HOXa7* en *MEIS1* transcripten (data niet gepresenteerd). In feite was het expressieniveau patroon van deze genen vergelijkbaar aan die van *MEL1* en *EL1* (Zie **hoofdstuk 3**). De verlaagde expressie van deze transcriptiefactoren bij patiënten met een relatief gunstige prognose kan het gevolg zijn van een verandering in een gemeenschappelijke intra-cellulaire signaalroute. De mRNA-expressieniveaus van *PU.1, NM23-H1, NM23-H2* en *SOX4* hielden geen verband met bepaalde cytogenetische afwijkingen en bleken geen prognostische waarde te hebben. Alhoewel het mRNA-expressieniveau van het oncogen *CB2* bij AML vergelijkbaar was met dat in normaal beenmerg zou een afwijkend aantal *CB2* receptoren op AML cellen wel degelijk geassocieerd kunnen zijn met verschillende subgroepen van leukemie, maar dit vereist nader onderzoek. Afgezien van de inductie van veranderingen in het transcriptieniveau, kan retrovirale insertie ook de coderende regio's van genen verstoren. Dit kan vervolgens leiden tot de productie van eiwitten met een afwijkende structuur. Daarom is tevens onderzoek gedaan naar mogelijke mutaties in *c-MYB* and *PU.1*, twee genen die ook zijn geïdentificeerd als leukemie-genen door middel van retrovirale insertionele mutagenese. Nucleotide sequentie analyse van de coderende regio's van *c-MYB* heeft geen structurele mutaties aangetoond bij patiënten met AML. Mueller et al.[22] hebben gerapporteerd dat

somatische mutaties van *PU.1* voorkomen bij 7% (9/126) van patiënten met AML. Deze bevinding kon echter niet door anderen worden bevestigd.[23-25] Gebruik makend van dezelfde methodologie als Mueller et al,[22] i.e. 'direct sequencing' van *PU.1* cDNA, hebben wij celmonsters van meer dan 300 patiënten geanalyseerd op de aanwezigheid van mutaties. Op deze manier konden we in principe ook de grote deletie-mutaties, die door Mueller et al[22] gevonden waren, aantonen. In *PU.1* hebben wij slechts twee 'silent' puntmutaties gevonden. Het lijkt niet dat de methodologie of verschillende klinische kenmerken van patiënten deze controversiële bevindingen kunnen verklaren. Zoals eerder werd gesuggereerd[24,25] zouden etnische verschillen deze tegenstrijdigheid mischien wel kunnen verklaren: de groep bestudeerd door Mueller et al. bestaat voornamelijk uit Japanse patiënten.

In **hoofdstuk 5** laten we heterozygote somatische mutaties zien in het *TEL*-gen. Vele studies hebben aangetoond dat *TEL* vaak betrokken is bij chromosomale translocaties, die voorkomen bij hematologische maligniteiten.[26-31] Afgezien van de *TEL*-mutaties bij AML laten we zien dat bij een derde van het aantal van AML-patiënten het TEL-eiwit ontbreekt. Western blot analyse bij 77 AML patiënten toonde het verlies van de TEL-eiwit expressie in 24 gevallen. Het is opmerkelijk dat binnen de intermediaire risicogroep, het verlies van het TEL-eiwit geassocieerd was met een ongunstiger overleving (OS: HR=2.06, CI:1.04-4.09) en een hogere kans op terugkeer van leukemie (EFS; HR=1.98, CI: 1.05-3.76).

Met behulp van microarray analyse[21] hebben wij getracht de heterogeniteit binnen de intermediaire risicogroep verder op te helderen. Binnen de intermediaire risicogroep bleken 70 patiënten geen van de bekende moleculaire afwijkingen met een prognostische waarde te hebben, zoals *FLT3*-ITD, *CEBPA* mutaties en hoge *EVI1* expressie. De genexpressieprofielen van deze patiënten toonden nauwelijks overeenkomst (Figuur 4, pagina 112). De clusters met onderscheiden expressie-profielen waren gerelateerd aan de celmorfologie (M1, M2 and M5). De aanwezigheid van gevarieerde en uiterst zeldzame afwijkingen in deze groep van patiënten en de betrokkenheid van uiteenlopende signaalroutes kan een verklaring zijn voor de opmerkelijke verscheidenheid van de genexpressie profielen. De analyse van grotere aantallen patiënten kan wellicht leiden tot de herkenning van nieuwe AML clusters. Daarnaast kan wellicht, door toepassing van andere methoden, zoals het analyseren van eiwitexpressie, de classificatie van AML verder worden ontwikkeld.

2- Patiënt-gerelateerde factoren die de prognose beïnvloeden

De genetische afwijkingen van de leukemiecellen verschaffen waardevolle informatie over het beloop van de ziekte en de uitkomst van therapie. Verschillende andere factoren zoals het voorkomen van gelijktijdige niet-hematologische aandoeningen en andere patiëntgerelateerde factoren (bijvoorbeeld leeftijd) kunnen de prognose eveneens beïnvloeden. Polymorfismen in genen, die voor geneesmiddel-metaboliserende enzymen coderen, worden als een belangrijke erfelijke basis voor verschillen in therapierespons beschouwd.[32] Zo kan bijvoorbeeld cellulaire resistentie tegen antracyclinen bij AML worden gerelateerd aan de activiteit van een familie van fase II-metaboliserende enzymen, de

Glutathion S-transferases.[33] Glutathion S-transferase theta 1 (*GSTT1*), een lid van deze familie, vertoont homozygote deletie-polymorfismes bij ongeveer 12-38% van de populatie,[34] hetgeen een afwezigheid van dit belangrijke detoxificerende enzym tot gevolg heeft. In **hoofdstuk 6** tonen wij aan dat de *GSTT1*-expressie een onafhankelijke prognostische factor is. De afwezigheid van het GSTT1-enzym zou kunnen leiden tot een verhoogde beschikbaarheid van chemotherapeutica en een effectievere behandeling. Op basis van deze theorie zou de behandeling van *GSTT1*-positieve patiënten wellicht aangepast moeten worden, opdat de concentratie van de chemotherapeutica binnen de cel, ook bij de subgroep met *GSTT1* deletie, het beoogde niveau kan bereiken. Deze studie suggereert dat een geïndividualiseerde dosering gebaseerd op het genotype of fenotype van een relevant metaboliserend enzym de uitkomst van de therapie zou kunnen verbeteren.

3- Het arsenaal aan geneesmiddelen

De remissie-inductie therapie in AML bestaat uit een combinatie van anthracyclinen (idarubicin en daunorubicin) en cytarabine en in sommige gevallen etoposide.[4] Bovendien verbetert sensitisatie van leukemische cellen met granulocyte colony-stimulating factor (G-CSF) de kans op een ziektevrije overleving van AML patiënten, vooral van die binnen de intermediaire risicogroep.[4] Vanwege de heterogene kenmerken van leukemie is toepassing van een uniform en effectief behandelingsprotocol voor alle patiënten onwaarschijnlijk. Tot nu toe zijn er meer dan 200 verschillende mutaties en chromosomale translocaties in AML beschreven. Acute promyelocyten leukemie (APL), is geassocieerd met de translocatie t(15;17) en wordt behandeld met All Trans Retinoic Acid (ATRA). ATRA gecombineerd met chemotherapie heeft de kans op genezing in APL-patiënten verbeterd van globaal 40% tot meer dan 70%.[35,36] Tot heden is het buiten de behandeling van APL nog niet gelukt om effectieve doelgerichte therapieën te ontwikkelen voor patiënten met AML.

Door middel van moleculaire genetische studies op leukemische populaties zijn een aantal genetische afwijkingen en abnormale signaalroutes in leukemiecellen geïdentificeerd. Het ultieme doel van dergelijke studies is om nieuwe cellulaire doelwitten op te sporen voor de behandeling van leukemie. In dit proefschrift hebben we de prognostische waarde van een hoge *EVI1*-expressie en van *CEBPA*-mutatie bij AML aangetoond. Dit roept de vraag op of deze moleculaire merkers als doelwitten voor therapie-benaderingen zouden kunnen dienen. Om deze vraag te kunnen beantwoorden, dienen functionele in-vitro en in-vivo studies verricht te worden. Daarbij zou moeten worden nagegaan of en indien in hoeverre een hoge *EVI1*-expressie en *CEBPA*-mutatie voor kritische transformerende signalen zorgen en of deze signalen kunnen worden onderbroken. In feite ondersteunen verschillende studies de hypothese dat een hoge *EVI1*-expressie of *CEBPA*-mutatie kritische transformerende signalen leveren. Het is bekend dat een hoge *EVI1*-expressie in cel-lijnen en beenmerg de terminale differentiatie van voorlopercellen tot erythroide cellen[37] en granulocyten[38] verhindert. Conditionele overexpressie van *EVI1* in erythroide cellen van muizen schijnt bovendien dysplasie te induceren, die ook bij patiënten met een 3q26 afwijking gezien wordt (E. van de Akker and D. Spensberger,

2004 persoonlijke communicatie). Theoretisch gezien is het denkbaar dat remming van de EVI1-functie door bijvoorbeeld 'small molecules', die met het EVI1-complex kunnen interfereren, het EVI1-geinduceerde differentiatieblok zou kunnen herstellen. In tegenstelling tot hoge *EVI1* expressie schijnen *CEBPA*-mutaties alleen in de mens een differentiatieblok te kunnen induceren en niet in de muis. Schwieger et al[39] hebben aangetoond dat de expressie van een N-terminale mutant van *CEBPA* in primaire voorlopercellen van de mens, de differentiatie blokkeert van zowel erythroide als myeloide cellen, hetgeen de betekenis van deze mutaties voor leukemische transformatie bevestigt. Als gevolg van de N-terminale mutaties van *CEBPA* wordt een dominant negatief eiwit geproduceerd, dat de DNA-binding en het transactivatie potentieel van het wild type eiwit teniet doet.[40] In dit verband is het interessant dat de microarray analyse laat zien dat het genexpressiepatroon van de *CEBPA*-mutanten vergelijkbaar is met genexpressiepatronen van patiënten met een lage *CEBPA*-expressie (R. Delwel, 2004, persoonlijke communicatie). Door regulatie van het gemuteerde C/EBPα of daaraan gerelateerde eiwitten zouden we het C/EBPα geïnduceerde differentiatieblok kunnen herstellen. Zheng et al[41] hebben recentelijk laten zien dat het *FLT3*-ITD geïnduceerde differentiatieblok van myeloide cellen mede veroorzaakt is door de onderdrukking van *CEBPA* mRNA-expressie. Sinds enige jaren hebben farmaceutische industrieën actief interesse getoond in de ontwikkeling van FLT3-inhibitoren, onder andere vanwege de hoge frequentie van deze mutaties in AML. Verschillende FLT3-inhibitoren, zoals SU11248, PKC412, CEP-701 en CT53518, zijn op dit ogenblik in klinisch onderzoek.[4,36] De kritische rol van C/EBPα bij leukemische transformatie suggereert dat therapieën gebaseerd op modulatie van de C/EBPα-activiteit nieuwe mogelijkheden zouden kunnen verschaffen voor de behandeling van een subgroep van AML.

Conclusie

Specifieke kennis van individuele prognostische factoren wordt steeds belangrijker voor het kiezen van een therapie in AML. Een inmiddels klassiek voorbeeld van deze aanpak is de behandeling van patiënten met acute promyelocyten leukemie met retinoïden.[4,7] In de hier gerapporteerde onderzoekingen zijn nieuwe genetische prognostische merkers gevonden bij AML. Het betreft afwijkingen in twee belangrijke hematopoietische transcriptiefactoren (C/EBPα en EVI1). Beide afwijkingen vertegenwoordigen twee subklassen van leukemie met unieke genexpressie patronen.[21] Frequente afwijkingen in een andere transcriptie factor (TEL) werden ook aangetoond. Voorlopige studies doen suggereren dat patiënten waarbij het TEL-eiwit afwezig is, een relatief slechte prognose hebben. Verder is in deze studies aangetoond dat patiënt-gerelateerde factoren, zoals constitutionele *GSTT1* deletie-polymorphismen, de prognose kunnen beïnvloeden. Wij verwachten dat in de nabije toekomst de identificatie van nieuwe prognostische merkers een aangepaste, op risico gebaseerde behandeling van AML mogelijk zal maken.

Voor **noten**: zie bladzijden 114-115.

List of Abbreviations

ABC	ATP-binding cassette
ABL1	abelson murine leukaemia viral oncogene homolog 1
ALL	acute lymphoblastic leukaemia
AML	acute myeloid leukaemia
APL	acute promyelocytic leukaemia
ATRA	all-trans retinoic acid
BCL2	B-cell CLL/lymphoma 2
BCR	breakpoint cluster region
BTL	BRX-like gene translocated in leukaemia
bZIP	basic-leucine zipper
BCR	breakpoint cluster region
CAN	NUP214, cain gene
CB2	peripheral cannabinoid receptor
CBF	core binding factor
cDNA	copy DNA
C/EBP	CCAAT/enhancer binding protein
cFES	feline sarcoma oncogene
CFU	colony forming unit
CI	confidence interval
cKIT	mast cell growth factor receptor
CML	chronic myeloid leukaemia
CR	complete remission
Ct	threshold cycle
cVIS	common virus integration site
DEC	deleted in endometrial carcinoma
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
EBS	ETS binding sites
EFS	event-free survival
EL1	EVI1-like1
Epo	erythropoietin
ES	embryonic stem
ETV6	ets translocation variant gene 6
EVI	ecotropic virus integration site
FAB	French-American-British
FISH	fluorescence in situ hybridisation
FLT3	fms like tyrosine kinase 3
FMS	macrophage colony stimulating factor I receptor precursor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
G-CSF	granulocyte colony-stimulating factor
GSH	glutathione

GST	glutathion s-transferases
HBSS	hanks balanced salt solution
HDAC	histone deacetylase
HOVON	Dutch-Belgian hemato-oncology cooperative study group
HOX	homeobox
HR	hazard ratio
IL	interleukin
ISCN	International System for Human Cytogenetic Nomenclature
ITD	internal tandem duplication
IWCL	International Workshop on Chromosomes in Leukemia
MDR	multidrug resistance
MDS	myelodysplastic syndrome
MEIS1	homeobox protein Meis1
MEL1	MDS1-EVI1-like 1
MEL	murine erythroid leukaemia
MLL	mixed lineage leukaemia
MN	superoxide dismutase
mRNA	messenger RNA
MRP	multidrug resistance-associated protein
MYB	avian myeloblastosis viral oncogene homolog
Myc	avian myelocytomatosis viral oncogene homolog
MYH	myosin heavy chain
N-coR	nuclear receptor co-repressor
NF1	neurofibromatosis Type 1
NM23	nonmetastatic protein 23
OS	overall survival
P53	transformation related protein 53
PBGD	porphobilinogen deaminase
PCR	polymerase chain reaction
PDGFR	platelet-derived growth factor receptor
PML	promyelocytic leukaemia
PNT	pointed
PU.1	spleen focus forming virus (SFFV) proviral integration oncogene spi1
Q-PCR	quantitative polymerase chain reaction
RA	retinoic acid
RAEB-t	refractory anaemia with excess of blasts in transformation
RAR	retinoic acid receptor
RNA	ribonucleic acid
RPN1	ribophorin 1
RT-PCR	reverse transcriptase PCR
SCF	stem cell factor
SCT	stem cell transplantation
SDS	sodium dodecyl sulfate

SOX4	SRY-box 4
Spi-1	spleen focus forming virus (SFFV) proviral integration 1
SSC	saline sodium-citrate
TEL	translocation ets leukaemia
TRM	treatment-related mortality
YY1	Yin Yang 1
WBC	white blood cell
WHO	World Health Organization
wt	wild type

Curriculum Vitae

Sahar Khosrovani was born in Tehran, Iran (Persia), August 24 1972 in a family with a great passion for art. Her father, Mohammad Reza Khosrovani, is an architect and her mother, Fereshteh Pishvaee, a film producer and a graphic designer. As a child, she and her sister Sara spent much of their free time running after their mother in the film studios. Sometimes they had roles in the films for children, which were made by their mother's colleagues. At high school, two great teachers, Mrs. Farid, and Mrs. Olang, infested her with an interest in chemistry and biology. In 1990, she embarked on the study of pharmacy: the first 3 months at the Jondi-Shapour Medical University in Ahwaz and the following 5½ years at the Tehran Medical University. As part of her graduation research and thesis she studied the medicinal effects of allin and allicin under supervision of Dr. Mohammad Reza Niakan. At that time she also published *A Guide to the Management of Common Illnesses*. At the end of her study, her aunt Dr. Parvin Khosrovani invited her to teach a course on 'using medicine during pregnancy' at the Tehran Azad University. Preparing that course, she noticed how intriguing the study of gene-drug interaction could be.

In November 1995, she met Leo (Ferydoun) Barjesteh van Waalwijk van Doorn, brother of her friend Lisa, who was visiting his family in Iran. It was love at very first sight. In 1996, she graduated and started working as a pharmacist in the North Tehran Health Centre. Soon afterwards though, she married and moved to Rotterdam. Her first year in the Netherlands was filled with Dutch language lessons and a Dutch integration course that mainly consisted of how to use public transport and how not to be surprised being offered only one cookie when visiting a Dutch friend!!! In 1997, a dear relative, Dr. Jelle T. Braaksma, then chairman of the board of the hospital, introduced Sahar to the Pharmacy of the Dijkzigt Hospital, where Dr. Arnold G. Vulto made her familiar with Dutch Hospital Pharmacy. In June 1997, Leo and Sahar were blessed with the birth of a lovely son, Shayan. Later she passed her Dutch NT2 state examination and started the post graduate study of pharmacy at the University of Utrecht. By then Sahar was determined to do a PhD research in the field of genetics. Through Arnold Vulto and Professor Pieter Sonneveld she met Dr. Ruud Delwel. His Revolving Fund project 'Studying prognostic markers in acute myeloid leukemia' was a great challenge. As a result, in June 2000, when she had just finished the post graduate study in Utrecht, she started working in the lab of Ruud Delwel and Professor Bob Löwenberg. Four years of research, in which a tremendous lot was learned about genetic techniques and molecular biology. The results are partly presented in this book. In September 2004, Professor Frederik-Jan van Schooten en Professor Jos Kleinjans offered her the opportunity to combine her knowledge of pharmacy with genetics by conducting pharmacogenetic/toxicogenetic studies. She was appointed to the post of assistant professor of genetic toxicology at the University of Maastricht, after which the family moved to Gronsveld.

Sahar Khosrovani is a member of the Royal Dutch Society for the Promotion of Pharmacology (KNMP), the Dutch Society for Gene Therapy (NVGT) and The Pharmacogenetics Study Group (University of Utrecht). Since the founding in 2000, she

has been an active member of the International Qajar Studies Association and has helped organizing conferences at the University of Leiden, the Kantonal Hospital in Geneva and at Santa Barbara City College. She has also been made honorary member of the Kadjar Family Association. Since 2004, she is a member of the founding and administrative committees of the Archives and Collections of the Imperial Kadjar (Qajar) Family, the Qalèh Kiab Family Foundation and the Shahab Khosrovani Memorial Trust.

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10. Barjesteh van Waalwijk van Doorn-Khosrovani S, Löwenberg B, Delwel R. GSTT1 mRNA expression as an independent prognostic marker for AML, submitted.

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