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

Sahar Barjesteh van Waalwijk van Doorn-Khosrovani, Claudia Erpelinck, Wim L. J. van Putten, Peter J. M. Valk, Sonja van der Poel-van de Luytgaarde, Ronald Hack, Rosalyn Slater, Elisabeth M. E. Smit, H. Berna Beverloo, Gregor Verhoef, Leo F. Verdonck, Gert J. Ossenkoppele, Pieter Sonneveld, Georgine E. de Greef, Bob Löwenberg, and Ruud Delwel

**The proto-oncogene *EVI1* encodes a DNA binding protein and is located on chromosome 3q26. The gene is aberrantly expressed in acute myeloid leukemia (AML) patients carrying 3q26 abnormalities. Two mRNAs are transcribed from this locus: *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 unfavorable karyotypes (eg, −7/7q−) or complex karyotypes. Moreover, a significant correlation was observed between *EVI1* expression and 11q23 aberrations (mixed lineage leukemia [MLL] gene 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. (Blood. 2003;101: 837-845)**



## Introduction

The *EVI1* proto-oncogene is located on human chromosome 3q26 and is involved in pathogenesis of human acute myeloid leukemia (AML) or myelodysplastic 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, an N-terminal domain containing 7 zinc fingers and a more C-terminal domain with 3 zinc fingers. Both domains recognize 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 the kidney.[11] Although the exact mechanism of transformation by *EVI1* is still obscure, several studies have shown that inappropriate expression of *EVI1* in immature hematopoietic cells interferes with erythroid and granulocytic development.[12] It has 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 leukemia.[1,13] *MDS1-EVI1* encodes a longer protein containing the entire EVI1 protein but with an additional, unique N-terminal extension. Although related, the 2 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 polymerase chain reaction (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 analyzed in relation to hematologic, 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

From the Institute of Hematology, Erasmus Medical Centre, Rotterdam, Netherlands; the Department of Cell Biology and Genetics, Erasmus Medical Centre, Rotterdam, Netherlands; the Department of Clinical Genetics, Erasmus Medical Centre, Rotterdam, Netherlands; the Department of Hematology, Vrije Universiteit Medical Centre, Amsterdam, Netherlands; the Department of Hematology, University Medical Centre, Utrecht, Netherlands; and the Department of Hematology, University Hospitals, Katholieke Universiteit Leuven, Leuven, Belgium.

Submitted May 20, 2002; accepted September 6, 2002. Prepublished online as *Blood* First Edition Paper, October 3, 2002; DOI 10.1182/blood-2002-05-1459.

Supported by grants from the Dutch Cancer Society (Koningin Wilhelmina Fonds) and the Erasmus University Medical Centre (Revolving Fund).

**Reprints:** Ruud Delwel, Institute of Hematology, Erasmus Medical Centre, PO Box 1738, 3000 DR Rotterdam, Netherlands; e-mail: delwel@hema.fgg.eur.nl.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked ''advertisement'' in accordance with 18 U.S.C. section 1734.

occurs with high frequency in patients without 3q26 abnormalities, suggesting other mechanisms of aberrant *EVI1* expression.

## Patients, materials, and methods

### Patients and healthy volunteers

Bone marrow samples of AML patients at diagnosis and healthy volunteers (n = 9) 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.[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 these procedures usually contain more than 90% blasts after thawing.[19] Seven patients with 3q26 rearrangements (4 patients with AML, 2 with refractory anemia with excess blasts in transformation [RAEB-t], and 1 with chronic myelogenous leukemia) 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 centers between 1987 and 2000 were chosen for analysis. Of these patients, 229 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 hematologic characteristics of the 319 patients at diagnosis are shown in Table 1. AML samples were classified according to 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 μL RNA was transcribed into cDNA using Superscript (Life Technologies, Merelbeke, Belgium) and random hexamers in a 40-μL 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, Nieuwerkerk aan den IJssel, Netherlands), using 50 μL mix containing 2 μL cDNA sample; 250 μM deoxyribonucleoside triphosphates (dNTPs; Amersham Pharmacia Biotech, Roosendaal, Netherlands); 15 pmol forward and reverse primer (Life Technologies); 3 mM $MgCl_2$ (5 mM for porphobilinogen deaminase [PBGD] reaction); 200 nM probe, labeled at the 5′ end with the reporter dye molecule FAM (6-carboxy-fluorescein) for *EVI1*, *MDS1-EVI1*, and *MDS1* or with JOE (carboxyrhodamine) for PBGD and at the 3′ end with the quencher dye molecule TAMRA (6-carboxy-tetramethyl-rhodamine; Eurogentec, Maastricht, Netherlands); 5 μL 10 × buffer A; 30 μL 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 and 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 sequences of the primers and probes are shown in Table 2.

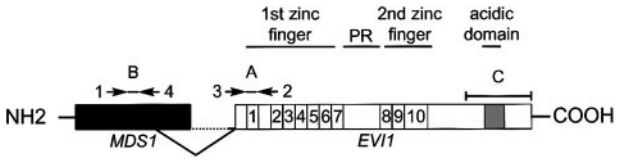


**Figure 1. Schematic representation of** *EVI1* and *MDS1-EVI1* and the primer and probes used for real-time PCR. Primers 1 and 2 plus probe A were used to determine *MDS1-EVI1* expression levels. *EVI1* transcript levels were determined using primers 3 and 2 plus probe A. *MDS1* expression was measured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

Using 7 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 normalized for endogenous reference ($\delta Ct = Ct_{target} - Ct_{PBGD}$) and compared with a calibrator, using the δδ 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 δδCt value to calculate relative expression ($2^{-\delta\delta\ Ct}$). As the δδCt method is applicable only when the amplification efficiencies of the target and the reference are essentially equal, we analyzed the efficiencies for another 4 patients (patients 25, 28, 29, and 30; Table 4), using the same dilutions indicated above. The mean 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 indicative of 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 cutoff points of 10, 25, and 100.

**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.

**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 | TCTTAGACGAATTTTACAATGTGAAGTTCTGCATAGA.TG |
| Probe B | TCTTAGACGAATTTTACAATGTGAAGTTCTGCATAGATG |
| PBGD forward primer | GGCAATGCGGCTGCAG |
| PBGD reverse primer | GGGTACCCACGCGAATCAC |
| PBGD probe | CATCTTTGGGCTGTTTTCTTCCGCC |

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

| Patient | FAB | Cytogenetic abnormalities* | Relative expression of *EVI1*† | Relative expression of *MDS1-EVI1*† | Expression of *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.[22]

†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.

### Northern blotting

Northern blotting was carried out on mRNA isolated from healthy bone marrow samples as well as AML samples. A portion (20 μg) of total RNA from each sample was separated on a 1% agarose, 6% formaldehyde gel and blotted with 10 × SSC (sodium chloride/sodium citrate; Amersham) onto Hybond-$N^+$ nylon membrane (Amersham). The blot was hybridized in 1N $NaH_2PO_4$ buffer containing 7% sodium dextran sulfate and 1 mM EDTA (ethylenediaminetetraacetic acid), pH 8.0. As probe, human *EVI1* (600-bp *Hin*dIII-NcoI fragment) and murine GAPDH (777-bp *Hin*dIII-*Eco*RI fragment)[23] 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 2 × SSC/0.5% sodium dodecyl sulfate (SDS) and for 15 minutes at 65°C in 1 × 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 being in the favorable-risk category. The unfavorable-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 the International System for Human Cytogenetic Nomenclature.[22]

### 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.[24] Briefly, cDNA (derived from 50 ng total RNA) and genomic DNA (1 μg) were subjected to PCR using primers 11F 5′-CAATTTAGGTAT-3′ and 11R 5′-CAAACTCTAAATTTTCTCT-3′. The PCR cycling conditions were as follows: 3 minutes at 94°C; 30 cycles of 1 minute at 94°C, 1 minute at 54°C, 1 minute at 72°C; and a final step of 10 minutes at 72°C. PCR products were resolved on a 2.5% agarose gel.

### Statistical analysis

Statistical analysis was performed with Stata Statistical Software, Release 7.0 (Stata, College Station, TX). Spearman rank correlation, Pearson $\chi^2$ test, and Kruskal-Wallis test were used to assess the association between *EVI1* and *MDS1-EVI1* expression and the clinical and hematologic 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 hazards model was applied to determine the association of high *EVI1* expression with OS and EFS, without and with adjustment for other factors such as age, cytogenetic risk, and FLT3 internal tandem duplication (FLT3-ITD). All tests were 2-sided, and a $P$ of less than .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 analyzed by 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 and copy numbers 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 from patient 2, was approximately 1.00 for *EVI1*, 0.95 for *MDS1-EVI1*, and 0.96 for PBGD. To evaluate whether the δδCt method 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 was expressed (+) or not (−).

As calibrator, we used the average expression of *EVI1* and *MDS1-EVI1* in 9 bone marrow samples from 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 normalized 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 from 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

**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;?)(q1?5;?) [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 | 46,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.

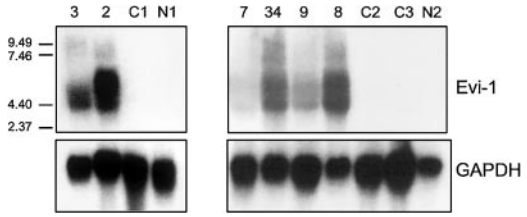


**Figure 2.** *EVI1* mRNA expression in AML samples as determined by Northern blotting. Human 600-bp *Hin*dIII-*Nco*I *EVI1* probe (see Figure 1) was used, which does not discriminate between *EVI1* and *MDS1-EVI1*. Murine GAPDH fragment was used as control. Patients 2, 3, and 7 carry a 3q26 abnormality (Table 3); patients 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.

patient *MDS1-EVI1* expression was high and *EVI1* expression was comparable to that of 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 (patients 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 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 patients newly diagnosed with AML. This cohort did not include the 7 patients from Table 3. Of these 319 patients, 44 expressed *EVI1*, *MDSI-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 patients (group IV), neither *EVI1* nor *MDS1-EVI1* was expressed. In 15 patients the *MDS1* gene expression (+) was detectable. *MDS1* expression was always associated with high levels of *EVI1* plus *MDS1-EVI1* (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 size 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*

Cytogenetic analysis among the 44 patients who were positive for *EVI1* and/or *MDS1-EVI1* revealed that only 4 AML patients (patients 12, 23, 35, and 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 unfavorable karyotypes

3q26 defects in AML are frequently accompanied by additional unfavorable cytogenetic abnormalities (eg, −7/7q−). In fact, in 2 cases shown in Table 3 (patients 1 and 6) and all 4 cases with a 3q26 aberration shown in Table 4 (patients 12, 23, 35, and 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% of patients (4 of 6) from group I (*EVI1* only) and 42% of patients (11 of 26) from group II (*EVI1*$^+$ and *MDS1-EVI1*$^+$), unfavorable karyotypic abnormalities (ie, −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 unfavorable karyotypes. Thus *EVI1* expression (groups I and II) correlated with unfavorable karyotypes ($P < .0001$), whereas the presence of *MDS1-EVI1* alone (group III) did not. Moreover, favorable-risk karyotypes, that is, 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 favorable-risk karyotypes (Table 5).

Among the 212 cases that belong to the leukemias with intermediate-risk karyotypes, 14 showed an 11q23 translocation (*MLL* rearrangement). Interestingly, 8 of these 14 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% to 30% of patients with AML. Moreover, this mutation appears to confer a poor prognosis in many studies carried out in the past 5 years.[25,26] In 85 (27%) of the 319 cases investigated, an FLT3-ITD was found. As shown in Table 5, high *EVI1* and *MDS1-EVI1* expression rarely

**Table 5. Distribution of different karyotypic risk categories and Flt3-ITD mutations among groups of AML patients**

| | | | Risk karyotype, no. | | |
|---|---|---|---|---|---|
| | n | Flt3-ITD, 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.

**Table 6. Therapy response and actuarial 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) |

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 unfavorable 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 cutoff 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 in group II (*EVI1*$^+$ and *MDS1-EVI1*$^+$) died within 30 months (Table 6; Figure 3A,B).

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,B). Thus patients in groups I and II had a significantly shorter OS ($P = .002$). The EFS 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 ($P = .002$) was also observed between EFS in patients with high *EVI1* expression (groups I and II) and that in AML patients without *EVI1* expression (groups III and IV). We also analyzed survival using alternative cutoff values (ie, 10, 25, and 100). Although the 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).

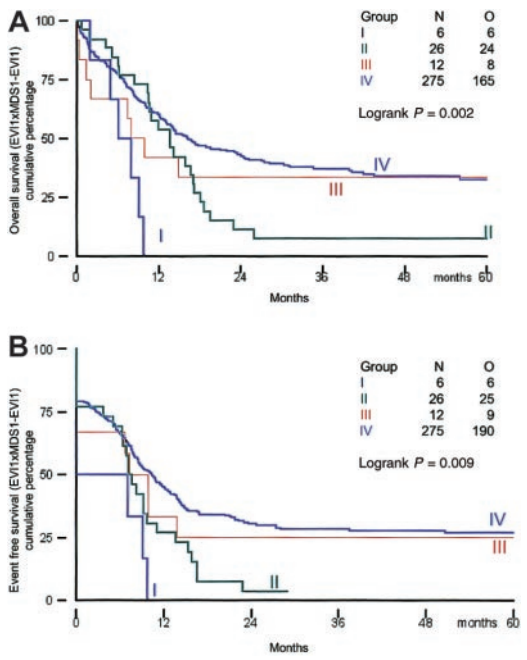


**Figure 3. Overall survival and event-free survival in AML patients based on *EVI1* and *MDS1-EVI1* expression.** (A) Overall survival; (B) event-free survival.

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 in a multivariable analysis, high *EVI1* expression was still associated with an increased hazard ratio ($P = .09$). As high *EVI1* expression is often associated with unfavorable karyotypes, its prognostic value seemed to be overshadowed. To exclude the effect of unfavorable cytogenetics, we decided to investigate the prognostic value of *EVI1* in the 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 (groups I and II) belonged to this risk group. As shown in Figure 4A-B, intermediate-risk patients with high *EVI1* expression (n = 16) had significantly shorter OS and EFS ($P = .05$ and $P = .03$) than their *EVI1*-negative counterparts (n = 196). Furthermore, the disease-free survival was significantly shorter in patients with high *EVI1* expression ($P = .007$). None of the *EVI1*-overexpressing patients carried an FLT3-ITD, whereas FLT3-ITD was observed in 36% (70 of 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).

**Table 7. Univariable and multivariable analysis of high *EVI1* expression as prognostic factor 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.

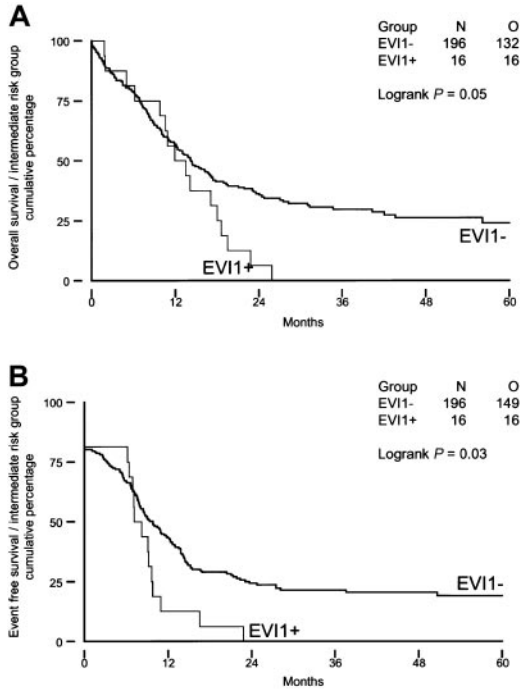


**Figure 4. Overall survival and event-free survival in AML patients with immediate-risk karyotype based on *EVI1* expression.** (A) Overall survival; (B) event-free survival.

## 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.[3,27-29] 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 unfavorable cytogenetic abnormalities. Favorable-risk karyotypes were not present among the *EVI1*-expressing groups.

*Evi1* was first discovered as a proto-oncogene in retrovirally induced myeloid leukemias in the mouse. Retroviral insertions in the *Evi1* locus in those tumors mostly occurred in close vicinity of the first 2 or 3 exons of *Evi1*, causing *Evi1* overexpression.[30] 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 cohort of de novo AML patients investigated in the present study, *EVI1* and not *MDS1-EVI1* expression correlated with unfavorable-risk leukemias.

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 patients. Langabeer et al[31] 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 favorable-risk karyotype, indicating that these patients are most likely expressing *MDS1-EVI1* rather than *EVI1*. Furthermore, previous studies were carried out using classical reverse transcriptase–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 favorable-risk and unfavorable-risk groups of 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 favorable-risk from unfavorable-risk patients within this heterogeneous group of patients by means of molecular biologic approaches. Among the patients with intermediate-risk karyotype studied, 33% (70 of 212) harbored an FLT3-ITD mutation that predicts poor prognosis. Sixteen (8%) of the 212 patients had high *EVI1* expression and showed very poor survival. Interestingly, none of the *EVI1*-positive patients harbored an FLT3-ITD, indicating that the *EVI1*-expressing group represents a distinct subclass of poor-response leukemias. Within the same group of patients with intermediate-risk karyotype, a subpopulation of poor responders has been defined with very low mRNA levels of the CEBPα gene.[32] Again, no overlap was found with the other 2 molecularly defined classes. Moreover, mutation analysis revealed another subset of intermediate-risk AML patients that harbored 3′ mutations within the CEBPα gene. These cases could be categorized as leukemias with a good prognosis.[32] 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* and 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 of 29 *EVI1*-positive patients studied 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.[33] In de novo leukemia, 75% of the breakpoints in *MLL* are mapped to the centromeric half of the breakpoint cluster region (BCR),[34] which is located in the repression domain. Disruption of the repression domain in these particular 11q23 translocations might lead to an alteration of the

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

| | 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.

repressor function of MLL, leading to an up-regulation 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 hypothesize that MLL normally represses *EVI1* and *MDS1-EVI1* expression. This repression might then be disrupted as a result of a chimeric protein generated by 11q23 translocation, as 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 (S.B.v.W.v.D.-K. et al, unpublished data, January 2001). Previous studies[10,35] 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 patients 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 that *EVI1* overexpression in AML patients correlates with poor treatment outcome. We propose that *EVI1* gene expression measurements with real-time PCR should be incorporated into the diagnostic procedures for de 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.

## Acknowledgments

A. Hagemeijer of the Center for Human Genetics, Catholic University of Leuven, Belgium, is acknowledged for the cytogenetic analysis performed on patient samples obtained from that institute. We thank our colleagues from the bone marrow transplantation group and the molecular diagnostics laboratory, Institute of Hematology, Erasmus Medical Centre, for preparation and storage of AML samples. We wish to thank Karola van Rooijen for preparation of the figures.

## References

1. Jolkowska J, Witt M. The EVI-1 gene—its role in pathogenesis of human leukemias. Leuk Res. 2000;24:553-558.
2. Morishita K, Parganas E, William CL, et al. Activation of EVI1 gene expression in human acute myelogenous leukemias by translocations spanning 300-400 kilobases on chromosome band 3q26. Proc Natl Acad Sci U S A. 1992;89:3937-3941.
3. Reiter E, Greinix H, Rabitsch W, et al. Low curative potential of bone marrow transplantation for highly aggressive acute myelogenous leukemia with inversion inv (3)(q21q26) or homologous translocation t(3;3) (q21;q26). Ann Hematol. 2000;79:374-377.
4. Nucifora G. The EVI1 gene in myeloid leukemia. Leukemia. 1997;11:2022-2031.
5. Kawagoe H, Kawagoe R, Sano K. Targeted down-regulation of MLL-AF9 with antisense oligodeoxyribonucleotide reduces the expression of the HOXA7 and -A10 genes and induces apoptosis in a human leukemia cell line, THP-1. Leukemia. 2001;15:1743-1749.
6. Russell M, Thompson F, Spier C, Taetle R. Expression of the EVI1 gene in chronic myelogenous leukemia in blast crisis. Leukemia. 1993;7:1654-1657.
7. Xi ZF, Russell M, Woodward S, Thompson F, Wagner L, Taetle R. Expression of the Zn finger gene, EVI-1, in acute promyelocytic leukemia. Leukemia. 1997;11:212-220.
8. Russell M, List A, Greenberg P, et al. Expression of EVI1 in myelodysplastic syndromes and other hematologic malignancies without 3q26 translocations. Blood. 1994;84:1243-1248.
9. Dreyfus F, Bouscary D, Melle J, Ribrag V, Guesnu M, Varet B. Expression of the Evi-1 gene in myelodysplastic syndromes. Leukemia. 1995;9:203-205.
10. Privitera E, Longoni D, Brambillasca F, Biondi A. EVI-1 gene expression in myeloid clonogenic cells from juvenile myelomonocytic leukemia (JMML). Leukemia. 1997;11:2045-2048.
11. Morishita K, Parganas E, Parham DM, Matsugi T, Ihle JN. The Evi-1 zinc finger myeloid transforming gene is normally expressed in the kidney and in developing oocytes. Oncogene. 1990;5:1419-1423.
12. Kreider BL, Orkin SH, Ihle JN. Loss of erythropoietin responsiveness in erythroid progenitors due to expression of the Evi-1 myeloid-transforming gene. Proc Natl Acad Sci U S A. 1993;90:6454-6458.
13. Fears S, Mathieu C, Zeleznik-Le N, Huang S, Rowley JD, Nucifora G. Intergenic splicing of MDS1 and EVI1 occurs in normal tissues as well as in myeloid leukemia and produces a new member of the PR domain family. Proc Natl Acad Sci U S A. 1996;93:1642-1647.
14. Sitailo S, Sood R, Barton K, Nucifora G. Forced expression of the leukemia-associated gene EVI1 in ES cells: a model for myeloid leukemia with 3q26 rearrangements. Leukemia. 1999;13:1639-1645.
15. Soderholm J, Kobayashi H, Mathieu C, Rowley JD, Nucifora G. The leukemia-associated gene MDS1/EVI1 is a new type of GATA-binding transactivator. Leukemia. 1997;11:352-358.
16. Ogawa S, Mitani K, Kurokawa M, et al. Abnormal expression of Evi-1 gene in human leukemias. Hum Cell. 1996;9:323-332.
17. Ohyashiki JH, Ohyashiki K, Shimamoto T, et al. Ecotropic virus integration site-1 gene preferentially expressed in post-myelodysplasia acute myeloid leukemia: possible association with GATA-1, GATA-2, and stem cell leukemia gene expression. Blood. 1995;85:3713-3718.
18. Boyum A. Separation of leukocytes from blood and bone marrow. Introduction. Scand J Clin Lab Invest Suppl. 1968;97:7.
19. Delwel R, Touw I, Bot F, Lowenberg B. Fucose binding lectin for characterizing acute myeloid leukemia progenitor cells. Blood. 1986;68:41-45.
20. Rombouts WJ, Lowenberg B, van Putten WL, Ploemacher RE. Improved prognostic significance of cytokine-induced proliferation in vitro in patients with de novo acute myeloid leukemia of intermediate risk: impact of internal tandem duplications in the Flt3 gene. Leukemia. 2001;15:1046-1053.
21. Bennett JM, Catovsky D, Daniel MT, et al. Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French-American-British Cooperative Group. Ann Intern Med. 1985;103:620-625.
22. Mitelman F, ed. ISCN 1995: International System for Human Cytogenetic Nomenclature (1995). Basel, Switzerland: Karger; 1995.

23. Jenkins JR, Ayton P, Jones T, et al. Isolation of cDNA clones encoding the beta isozyme of human DNA topoisomerase II and localisation of the gene to chromosome 3p24. Nucleic Acids Res. 1992;20:5587-5592.
24. Nakao M, Yokota S, Iwai T, et al. Internal tandem duplication of the flt3 gene found in acute myeloid leukemia. Leukemia. 1996;10:1911-1918.
25. Kondo M, Horibe K, Takahashi Y, et al. Prognostic value of internal tandem duplication of the FLT3 gene in childhood acute myelogenous leukemia. Med Pediatr Oncol. 1999;33:525-529.
26. Meshinchi S, Woods WG, Stirewalt DL, et al. Prevalence and prognostic significance of Flt3 internal tandem duplication in pediatric acute myeloid leukemia. Blood. 2001;97:89-94.
27. Grigg AP, Gascoyne RD, Phillips GL, Horsman DE. Clinical, haematological and cytogenetic features in 24 patients with structural rearrangements of the Q arm of chromosome 3. Br J Haematol. 1993;83:158-165.
28. Bitter MA, Neilly ME, Le Beau MM, Pearson MG, Rowley JD. Rearrangements of chromosome 3 involving bands 3q21 and 3q26 are associated with normal or elevated platelet counts in acute nonlymphocytic leukemia. Blood. 1985;66:1362-1370.
29. Pintado T, Ferro MT, San Roman C, Mayayo M, Larana JG. Clinical correlations of the 3q21; q26 cytogenetic anomaly. A leukemic or myelodysplastic syndrome with preserved or increased platelet production and lack of response to cytotoxic drug therapy. Cancer. 1985;55:535-541.
30. Morishita K, Parker DS, Mucenski ML, Jenkins NA, Copeland NG, Ihle JN. Retroviral activation of a novel gene encoding a zinc finger protein in IL-3-dependent myeloid leukemia cell lines. Cell. 1988;54:831-840.
31. Langabeer SE, Rogers JR, Harrison G, et al. EVI1 expression in acute myeloid leukaemia. Br J Haematol. 2001;112:208-211.
32. Barjesteh van Waalwijk van Doorn-Khosravani S, Meijer J, Erpelinck C, et al. Mutations in the basic-fork-leucine zipper and low C/EBP$\alpha$ expression levels are prognostic markers in intermediate-risk AML. Hematol J. In press.
33. Zeleznik-Le NJ, Harden AM, Rowley JD. 11q23 translocations split the "AT-hook" cruciform DNA-binding region and the transcriptional repression domain from the activation domain of the mixed-lineage leukemia (MLL) gene. Proc Natl Acad Sci U S A. 1994;91:10610-10614.
34. Broeker PL, Super HG, Thirman MJ, et al. Distribution of 11q23 breakpoints within the MLL breakpoint cluster region in de novo acute leukemia and in treatment-related acute myeloid leukemia: correlation with scaffold attachment regions and topoisomerase II consensus binding sites. Blood. 1996;87:1912-1922.
35. Gerhardt TM, Schmahl GE, Flotho C, Rath AV, Niemeyer CM. Expression of the Evi-1 gene in haemopoietic cells of children with juvenile myelomonocytic leukaemia and normal donors. Br J Haematol. 1997;99:882-887.