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Downregulation of *EVI1* is associated with epigenetic alterations and good prognosis in patients with acute myeloid leukemia

Running title: *EVI1 expression and epigenetic alterations in AML*

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Key words: AML, EVI1, overexpression, 3q, epigenetics.

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Abstract

Background. The *EVI1* gene (3q26) codes for a zinc finger transcription factor with important roles in both mammalian development and leukemogenesis. Overexpression of *EVI1* through either 3q26 rearrangements, MLL fusions, or other unknown mechanisms confers poor prognosis in acute myeloid leukemia.

Design and methods. We analyzed the prevalence and prognostic impact of *EVI1* overexpression in a series of 476 acute myeloid leukemia patients, and investigated the epigenetic modifications of the *EVI1* locus that could be involved in the transcriptional regulation of this gene.

Results. Our data provide further evidence that *EVI1* overexpression is a poor prognostic marker in acute myeloid leukemia patients <65 years. Moreover, we found that patients with no basal expression of *EVI1* had better prognosis than patients with expression/overexpression (p=0.036). We also show that cell lines with overexpression of *EVI1* have no DNA methylation in the promoter region of the *EVI1* locus, and have marks of active histone modifications: H3 and H4 acetylation, and trimethylation of histone H3 lysine 4. Conversely, cell lines with no expression of *EVI1* have DNA hypermethylation and are exclusively marked by repressive trimethylation of histone H3 lysine 27 at the *EVI1* promoter.

Conclusions. Our results identify *EVI1* overexpression as a poor prognostic marker in patients <65 years in an independent large cohort, and show that the total absence of *EVI1* expression has a prognostic impact in the outcome of acute myeloid leukemia patients. Furthermore, we demonstrated for the first time that an aberrant epigenetic pattern involving DNA methylation, H3 and H4 acetylation, and trimethylation of histone H3 lysine 4 and histone H3 lysine 27 might play a role in the transcriptional regulation of *EVI1* in acute myeloid leukemia. This study opens new routes to further understand the regulation of *EVI1* expression at transcriptional level.

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Introduction

The *EVI1* gene (3q26) codes for a zinc finger transcription factor with important roles in both mammalian development and leukemogenesis. Since the identification of *EVI1* as a murine common locus of retroviral integration in myeloid tumors[1] this evolutionarily conserved gene has been implicated in human myeloid disorders, and in the development and progression of high-risk acute myeloid leukemia (AML).[2,3] Recurrent 3q26 rearrangements are the only known mechanisms that lead to *EVI1* overexpression[4,5,6,7] however, overexpression of this gene has been reported in 9-20% AML with no 3q aberrations, where it is also associated with an unfavorable outcome.[8,9,10,11,12,13] Moreover, a recent report showed that *MLL-ENL* activates the transcription of Evi1.[7] Therefore, transcriptional activation of *EVI1* through chromosome rearrangements or other yet to be identified mechanisms lead to particularly aggressive forms of human myeloid leukemia.[2,3] The *EVI1* locus gives rise to several alternatively spliced variants,[2,3,14,15] including the intergenic splicing *MDS1EVI1* which codes for a larger protein with a PR-domain.[3,16] Besides, *EVI1* is transcribed into several 5'-end mRNA transcripts that have the same translation start site (Figure S1).

To date, only three studies in large series of AML patients have analyzed the prevalence and prognostic value of *EVI1* overexpression, discriminating *EVI1* from *MDS1EVI1* (Table S1).[8,9,10,17] The first study found that *EVI1-1D* was overexpressed in 13.7% cases, and was significantly associated with a shorter overall and event-free survival.[8] Two recent studies, one by the same group, included the analyses of other *EVI1* 5'-end transcripts and confirmed the prevalence and the poor impact that *EVI1* overexpression has in AML.[9,10] Lately, this group has proposed a diagnostic assay that quantifies all *EVI1* 5'-end transcripts, including *MDS1EVI1*. In this study, high expression of *EVI1/MDS1EVI1* was found in 10.7% cases, and predicted an adverse disease-free and event-free survival.[17]

Our aim was to study the prevalence of *EVI1* overexpression and its impact on survival in a large series of AML patients, and to investigate the mechanisms of regulation of *EVI1*. We performed extensive analyses in both cell lines and patient samples to investigate the

genetic and epigenetic mechanisms that could control the expression of *EVI1* in AML. Our results open new routes to better understanding the prognostic impact of *EVI1* in AML, and the regulation of its expression at transcriptional level.

Design and Methods

Material

Samples obtained at diagnosis from 476 AML patients, other than acute promyelocytic leukemia, were provided by the Hospital La Fe (Valencia), Hospital Santa Creu i Sant Pau (Barcelona), Hospital Universitario de Salamanca (Salamanca), Hospital Dr. Negrin (Las Palmas), and Department of Genetics of the University of Navarra (Pamplona), which belong to the Myeloid Malignancies Group of the Spanish Network of Cancer Research. Of these patients, 194 were categorized as elderly (≥ 65 years old), and 249 constituted the group of younger AML individuals. The study has been approved by the Ethics Committee for Research with Human Subjects, (037/2008). Survival analysis was performed in the 213 AML patients that were eligible for treatment and were uniformly treated according to the Spanish Pethema Co-operative Group protocol LAM99.[18] Samples were taken anonymously. Normal bone marrow (BM), peripheral blood (PB), and 19 normal tissues from the human total RNA Master Panel II (Clontech, Takara-BIO, CA, USA) were used. Characteristics of the 16 myeloid cell lines used (DSMZ, Braunschweig, Germany) are summarized in Table 1. Cell lines were cultured following manufacturers advice.

Cytogenetic and mutation analysis

Cytogenetic and FISH analysis were performed as previously described[5] using 6 BAC clones: RP11-390G14 (3q21), RP11-475N22 (*GATA2*), RP11-689D3 (*RPN1*), RP11-82C9 (*EVI1*), RP11-115B16 (*MDS1*), RP11-196F13 (*TNFSF10*), and a probe for chromosome 3 centromere. The PR domain of *MDS1EVI1* was amplified by RT-PCR, followed by a semi-nested with specific primers (Table S2). Gene mutation analysis of *FLT3* and *NPM1* was performed as previously described.[19,20,21] PCR products were purified and sequenced.

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Quantitative real-time RT-PCR

Two micrograms of total RNA isolated from cell pellets using the RNeasy Mini Kit (Qiagen, Germany) were used for cDNA synthesis (SuperScript™II RNase HRT; Invitrogen, CA, USA). Gene expression quantification was carried out with an ABI Prism 7,500 (Applied Biosystems, CA, USA) with 20ng of cDNA. Quantitative real-time RT-PCR (qRT-PCR) was performed with predesigned TaqMan gene expression assays for *EVI1-1A* (Hs01118676_m1), *EVI1-1B* (Hs01118674_m1), *EVI1-1C* (Hs01118675_m1) and *EVI1 11-12* (Hs01115406_m1), that includes all the *EVI1* transcripts; and *GATA2* (Hs00231119_m1). Specific assay-by-design were designed for *EVI1-1D*, *EVI1-3L*, and *MDS1EVI1*. Triplicate cycle threshold values were averaged; concentrations of the target gene were interpolated from the standard curves and normalized to the *GAPDH* expression for each sample. Samples from the University Hospital La Fe were quantified for *EVI1-1D* expression using the P2 and P3 primers, as previously described. [8] Overexpression of *EVI1* was defined when at least the level of one *EVI1* transcript was higher than the average and 3 times the standard deviation of 7 BM samples from healthy volunteers.

Analysis of the methylation status of the *EVI1* and *MDS1EVI1* promoter regions

DNA methylation profiling of healthy donor peripheral blood (n=4), bone marrow (n=4) and CD34+ cells of bone marrow (n=4) samples was performed using the HumanMethylation27 Beadchip (Illumina, Inc., San Diego, CA, USA), according to the instructions of the manufacturer.[22] The panel is developed to quantify the DNA methylation status of 27,578 CpG sites located within the proximal promoter regions (1 kb upstream and 500bp downstream of transcription start sites) of 14,475 well-annotated genes. Briefly, genomic DNA is converted by sodium bisulfite treatment and whole-genome amplified using the manufacturer's instructions. Each CpG locus is represented by two bead types: one for the unmethylated (U) site and another for the methylated (M) site. After hybridization and single-base extension using labeled nucleotides, the intensity of the U and M beads is measured with a microarray reader. The methylation status of a CpG is

determined by the beta-value calculation, which is based on the ratio of the fluorescent signals between the M bead to the total locus fluorescence intensity. The beta value is a quantitative measure of DNA methylation levels of specific CpGs, and ranges from 0 (completely unmethylated) to 1 (completely methylated). Methylation status of the CpG islands of *EVI1* (island 1 and 2) and *MDS1EVI1* (island 1 and 2) were analyzed by bisulfite sequencing PCR (Table S2). DNA modification was performed with the CpGenome^{TM} DNA Modification Kit (CHEMICON, Millipore Corporation, MA, USA). For the treatment of the cell lines, several concentrations and time points were tested, and optimal results were obtained in $10x10^6$ cells in 10ml of media, and cultured with 4μM of 5-aza-2'-deoxycytidine (5-Aza), and 50nM of Trichostatin A (TSA) at 4 days of culture; controls were cultured with DMSO and glacial acetic acid.

Chromatin immunoprecipitation (ChIP)

HEL, TF1, OCI-AML2, NOMO-1 and MV4-11 cell lines were subjected to ChIP in order to assess the acetylation of H3 and H4, and the trimethylation of histone H3 lysine 4 and lysine 27 as previously described.[23] Ten million cells were crosslinked with 1% formaldehyde for 10 minutes, and then 0.125M glycine was used to stop the reaction. Subsequently, chromatin was fragmented by sonication to obtain an average fragment length of 200-900bp (Bioruptor Diagenode, Belgium). Antibodies used were Anti-acetyl-Histone H4 and Anti-acetyl-Histone H3 (Millipore Corporation, MA, USA) and anti-trimethyl K4 and K27 of H3 (Abcam, Cambridge, UK). The relative amount of specifically immunoprecipitated DNA was quantified by SYBR-Green fluorescent dye qRT-PCR, using specific primers for *EVI1* and *MDS1EVI1* promoter regions (Table S2). PCR results were calculated using the ΔΔCt method. They were presented as the fold enrichment of chromatin DNA precipitated by the specific antibody versus chromatin DNA precipitated by no antibody, as control, from at least two independent experiments.

Western blot analysis

Cells were lysed in lysis buffer (Cell Signaling, MA, USA) with complete protease inhibitor (Roche, IN, USA) and 1mM $NaVO_4$ (SIGMA, MO, USA), and concentration was

determined using Bradford method prior to each use (Bio-Rad Laboratories, Inc., CA, USA). Western blot analysis were carried out with 50µg of total proteins electrophoresed on 10% Tris/Glycine SDS-polyacrilamide solution gel, and transferred to a nitrocellulose membrane. Anti-Evi1 antibody was used (Cell Signaling, MA, USA) and anti-lamin A/C antibody (Cell Signaling, 2032). Detection was performed with phosphatase alkaline-conjugated anti-rabbit Ig (SIGMA, MO, USA), and detected with an enhanced chemiluminescence (Amersham Pharmacia Biotech, GE Healthcare, Sweden).

Definitions and Statistical analysis

Overall survival (OS) was defined as the time from diagnosis to death due to any cause or end of follow-up; disease-free survival (DFS) as the time from complete remission until relapse or death, whichever occurred first; and event-free survival (EFS) as the time from diagnosis until first event, in which failure to achieve complete remission, relapse or death were considered events. OS, DFS and EFS were determined according to the Kaplan-Meier method and survival comparisons were done with log-rank test. Proportional hazards models were constructed to determine whether the groups of *EVI1* expression were associated with outcome when adjusting for other prognostic variables. P values for the significance among the cytogenetic subgroups were calculated using the 2-tailed chi-square test. Sperman's Rho correlation coefficient was used to calculate the correlations between the overexpression of the *EVI1* 5´-end variants. Statistical analyses were performed using SPSS version 15.0 (SPSS Inc., IL, USA).

Results

Expression pattern of the alternative forms of *EVI1*

High expression of different splice-forms of *EVI1* has been implicated in the development of high-risk AML.[9,10] In order to fully understand the mechanisms leading to *EVI1* overexpression, we first analyzed the *EVI1* 5′-end variants, including *MDS1EVI1*, in a panel of human tissues, in AML cases, and in 16 myeloid cell lines. In each tissue, expression levels of the *EVI1* transcripts were similar, and all transcripts could be detected in normal BM, although at low levels (Figure S2). Next, we quantified the *EVI1* 5′-end

variants in a series of AML patients selected as a representation of the heterogeneity of AML cases (Table S3) and in the myeloid cell lines (Table 1 and Figure S3), and completed the analysis of the cell lines with the expression of the EVI1 protein. Expression levels of *EVI1* transcripts in both patient samples and cell lines correlate with each other in a statistically significant manner (Table S4). Among the cell lines overexpressing *EVI1* we found two groups: AML cell lines overexpressed transcripts *-1A*, *-1B*, *-1C*, and *-1D*, whereas cell lines with CML-BP had only *EVI1-1B* overexpression as a common feature. Western blot analysis detected the EVI1-FL isoform (145kDa) in cell lines with overexpression of at least one *EVI1* transcript (Table 1 and Figure S3). As an exception, MEG-01 (CML-BC) had overexpression of *EVI1-1B* and no EVI1-FL protein. Moreover, we found no association between the expression of any *EVI1* transcript and the amount of protein (Table 1 and Figure S3). Seven cell lines had no basal expression either of *EVI1* or *MDS1EVI1* (Table 1).

The fragility of the PR domain, which is a hotspot in both retroviral insertions and 3q rearrangements,[24] prompted us to perform a mutation analysis of the PR domain in the cell lines. We found no mutations in this region; however, we detected a novel *MDS1EVI1* alternative splice form in four cell lines. The analysis of the normal human tissues panel demonstrated that this novel alternative splice form is not expressed in peripheral blood, but is present in most of tested tissues (Figure S4). This form would codify for a truncated protein of 38 amino acids; however, a second ORF is possible from the *EVI1* ATG start codon in exon 3, which would codify for the Evi1-FL protein (NCBI Accession GQ352634) (Figure S4).

Prevalence of *EVI1* overexpression in AML patients

Since *EVI1* alternative transcript forms correlated significantly, we investigated the expression of *EVI1-1D, EVI1-1C*, and *MDS1EVI1* in a series of 476 AML patients (Table 2). *EVI1* (*-1C* and/or *-1D*) was overexpressed in 92 out of the 476 patients (19.3%). Table 2 shows the prevalence of *EVI1* overexpression, and its association with relevant clinical and molecular parameters. Statistical correlations for *-1C* and *-1D* were also calculated

separately and showed similar results (data not shown). The prevalence of *EVI1* overexpression was significantly different among the cytogenetic prognostic groups (p<0.001). *EVI1* overexpression was found in 72% cases with 3q rearrangements, including all 25 cases with 3q26 (p<0.001). Other cytogenetic abnormalities associated with *EVI1* overexpression were *MLL* translocations (p<0.001), and monosomy 7 (p=0.003), but not del(7q) (p=0.562). Prevalence of *EVI1* overexpression in patients with normal karyotype was 7.7%, and an inverse correlation was found between *EVI1* overexpression and both trisomy 8 and *NPM1* mutations; in fact, none of the patients with either trisomy 8 (16 cases) or *NPM1* mutations (79 cases) had *EVI1* overexpression.

Prognostic impact of *EVI1* expression in AML patients

Clinical follow-up data of patients who received induction therapy and were uniformly treated were available in 213 patients (110 males and 103 females), with a median age at diagnosis of 58 years (range: 16-83). Median follow-up was 159 weeks, with a minimum of 24 weeks. Median OS of this cohort was 45.7 weeks (95% CI 36.5-54.8). Kaplan-Meier analysis showed significant differences in well-recognized risk factors such as age and cytogenetic group (p<0.001). In a stratified analysis by age group, patients under 65 years old with *EVI1-1C* overexpression had a significantly lower OS (p=0.005) and EFS (p=0.008) (Figure 1 and Figure S5), while no significant differences were found in DFS. However, we could not confirm the independent prognosis significance of *EVI1-1C* overexpression in a multivariate model (Table S5). *EVI1-1D* overexpression had no significant impact either on OS, DFS or EFS. In the global cohort, the group of patients with *EVI1* overexpression and no *MDS1EVI1* expression had the worst outcome (p=0.017). When comparing patients with no basal expression, expression and overexpression of *EVI1* in the group of patients under 65 years old, patients with no basal expression have a better OS (p=0.020) (Figure 1). Furthermore, patients with no basal expression of *EVI1* have better OS than patients with expression/overexpression in both the global cohort (p=0.036) and in the group of patients <65 years (p=0.005) (Figure 1).

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EVI1 overexpression and 3q26 rearrangements

For a better understanding of the role of 3q rearrangements in the expression of *EVI1*, we characterized the 3q21q26 region by FISH, and quantified *EVI1* expression in 16 myeloid cell lines and in 25 cases with myeloid neoplasias. The HEL and TF-1 cell lines had overexpression of *EVI1* and several copies of probes located on 3q26; however, a similar pattern was found in NOMO-1 and OCI-AML2, with no *EVI1* expression; moreover, OCI-AML2 has an inv(3)(q21q26) (Table 1 and Figure S6). In the patient samples, FISH analyses show wide heterogeneity and complex 3q rearrangements. Cases were classified in four distinct groups: 3q21q26, 3q26, 3q21, and other 3q aberrations. Cases with either 3q21q26 (8 cases) or 3q26 (7 cases) breakpoints had *EVI1* overexpression, except case 21872s, the only one with breakpoints located between the 689D3 (3q21; 128.4Mb) and 82C9 (3q26; 168.8Mb) probes. Cases with other 3q rearrangements and breakpoints located between these probes had no *EVI1* overexpression either (Table S7). Three cases with a single breakpoint on 3q21 had *EVI1* overexpression (25704, 24316 and 14066s). The 3q26 breakpoints associated with *EVI1* overexpression were mainly located centromeric to *EVI1* in cases with inv(3), and telomeric to *MDS1EVI1* in t(3;3) and other 3q26 rearrangements. Besides, 3q21 breakpoints associated with *EVI1* overexpression were located centromeric to probe 390G14 (3/4 cases) (Table S7).

Aberrant epigenetic pattern of *EVI1* in AML

Results showing that *EVI1* overexpression sometimes occurs irrespective of 3q21q26 rearrangements, and the finding that normal basal expression of *EVI1* and *MDS1EVI1* was not detected in several patient samples and cell lines (including OCI-AML2, with 3q21q26) (Table 1 and Figure S3) prompted us to study whether *EVI1* transcription could be regulated by epigenetic mechanisms. For the analysis, we selected 5 cell lines that represented the heterogeneity detected in patient samples: HEL and TF-1 had 3q aberrations and *EVI1* overexpression; OCI-AML2 and NOMO-1 had 3q and no *EVI1* expression; and MV4-11 had neither 3q nor *EVI1* expression (Figure 2A). Treatment of *EVI1*-/*MDS1EVI1*- cell lines with TSA in combination with the demethylating agent 5-Aza

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induced *EVI1* expression (Figure 2B), confirming our hypothesis. The expression does not exceed the expression levels of PB or BM. To assess whether the aberrant methylation status of the promoter region of the *EVI1* locus was the epigenetic mechanism involved, we first analyzed the methylation status of the CpG islands predicted in the proximal promoter region of *EVI1* and *MDS1EVI1* in normal samples. High-resolution genome-wide methylation arrays from Illumina (Infinium HumanMethylation27 BeadChip, Illumina, CA, USA) showed the total absence of methylation in two probes of *EVI1* and two of *MDS1EVI1* in CD34+ progenitor cells (high *EVI1* expression) and normal BM and PB (very low *EVI1* expression) (data not shown). These results would indicate that an aberrant hypomethylation of the promoter of *EVI1* is not the mechanism of *EVI1* overexpression; nevertheless, this could be the mechanism involved in the *EVI1* gene silencing. The methylation status of *EVI1*-Island 1 and *MDS1EVI1*-Island 2 showed concordance between *EVI1* and *MDS1EVI1* expression: the *EVI1*-/*MDS1EVI1*- cell lines had these regions hypermethylated (Figure 2C). However, we observed no significant changes in the methylation status of the *EVI1*-Island 1 before and after the treatment with TSA in combination with 5-Aza (Figure 2D). This result prompted us to analyze the trimethylation status of histone H3 lysine 4 (H3K4me3) and histone H3 lysine 27 (H3K27me3), and the acetylation of histone H3 and H4. Quantification of the amount of chromatin immunoprecipitated with anti-trimethyl Lys4 and Lys27 showed that HEL and TF-1 have enrichment of the active H3K4me3 pattern, while NOMO-1, MV4-11 and OCI-AML2 had the opposite signature, a mark of repressive pattern H3K27me3 (Figure 3A). However, there was no difference in the histone methylation status of the cell lines with no expression of *EVI1* after the treatment with TSA and 5-Aza (Figure S7). We also observed an enrichment of the acetylation of histone H3 and H4, especially H3, in HEL and TF-1 (Figure 3B), and ChIP analysis of the *EVI1* promoter showed an enrichment of acetylated histone H3 and H4 in treated cell lines (Figure 3C). The enrichment of the active marks both in cell lines with *EVI1* overexpression and treated cell lines, strongly suggests that histone acetylation might play a role in *EVI1* expression regulation. Regarding to the *MDS1EVI1* locus, we observed slight changes in the methylation status of the *MDS1EVI1*

promoter in MV4-11 after treatment with TSA and 5-Aza (Figure 2D); however, *MDS1EVI1* gene expression was not induced after treatment, and we found no difference either in the histone methylation or acetylation pattern. Taken together, these results indicate that expression of *EVI1* in AML is regulated at least in part by epigenetic mechanisms.

Discussion

EVI1 has been recognized as one of the most aggressive oncogenes associated with AML.[2,3] Our results confirm that *EVI1* overexpression is an adverse prognostic factor in AML patients, not always restricted to 3q26 aberrations. Notably, we show that the total absence of *EVI1* expression might have a prognostic impact on the outcome of AML patients, and that this atypical pattern may be regulated by epigenetic mechanisms.

Our results confirm in an independent large cohort the prevalence of *EVI1* overexpression and its adverse prognostic outcome in AML.[8,10,17] For the first time, we have included the quantification and survival analysis of the *EVI1-1C* 5'-end variant, and identified the overexpression of this transcript as a poor prognostic marker in younger AML patients in both OS (p=0.005) and EFS (p=0.008) (Figure 1 and S5), suggesting that this variant could be a genetic marker in this subgroup. However, this correlation could not be confirmed in multivariate analysis. The significant impact of *EVI1* overexpression in OS in a multivariate analysis has only been confirmed in the two largest studies: Lugthart et al. for *EVI1-1A* and *EVI1-1B*; [10] and Groschel et al. for *EVI1/MDS1EVI1*, that did not discriminate *EVI1* from *MDS1EVI1* (Table S1);[17] thus, it is possible that our sample size is not large enough for being statistically significant. Of note, we found that younger AML patients with no *EVI1* expression have a significantly better outcome than patients with either *EVI1* expression or overexpression (Figure 1), although this event could not be confirmed in multivariate analysis. To our knowledge, this is the first time this finding is reported. Further studies in independent cohorts are needed to confirm the importance of this result.

We and others have shown the association between *EVI1* overexpression and other specific cytogenetic aberrations such as *MLL* rearrangements and monosomy 7 (Table 2).[8,10,17] Interestingly, it has been recently shown that the specific *MLL-ENL* fusion

activates the transcription of Evi1 in undifferentiated hematopoietic cells.[7] In addition, in mouse models, *EVI1* overexpression induces a myelodysplastic syndrome (MDS) that does not progress to AML,[25] suggesting the necessity of cooperating mutations in the progression to AML. As demonstrated in gene therapy studies, in which enforced expression of *EVI1* in human cells leads to genomic instability, monosomy 7, and clonal progression,[24,26] our results support the putative role of monosomy 7 as a cooperating mutation in *EVI1*-positive AML. A similar cooperation has been reported in a murine model between *RUNX1* mutation D171N and *EVI1* in the AML transformation of MDS;[27] however, we found no mutations of *RUNX1* in a series of 46 cases with *EVI1* overexpression analyzed (data not shown), suggesting that this mechanism is not frequent in human AML. Finally, we also found an inverse correlation between *EVI1* overexpression and *NPM1* mutations,[8,10,12] in agreement with the better outcome of patients with *NPM1* mutations.[28]

To date, 3q rearrangements and *MLL* fusions are the only known mechanisms of *EVI1* overexpression. The characterization by qRT-PCR and FISH of 16 cell lines and a series of patient samples with myeloid malignancies confirmed that *EVI1* overexpression is associated with 3q26, although sometimes occurs irrespective of 3q rearrangements (Table S5 and Figure S6).[9,10] Moreover, the prevalence of *EVI1* overexpression among the different categories of 3q abnormalities is similar to other recent study.[29] Interestingly, we showed that FISH breakpoints in cases with 3q26 and *EVI1* overexpression were located telomeric to *MDS1EVI1* (Table S5), a hotspot locus of retroviral insertions,[26] which suggests that disruption of this region is of the foremost importance in the regulation of *EVI1* transcription. We have also demonstrated that the EVI1 protein is present even if only one *EVI1* transcript is overexpressed. As an exception, MEG-01 had overexpression of *EVI1-1B* and no EVI1-FL protein. In this cell line the protein levels might be low and therefore difficult to detect by western blot, although in the KU-812 cell line, with low expression levels of *EVI1* too, the protein could be detected. Another explanation might be that the accumulation and degradation of the protein in these cell lines would be different. Furthermore, in our study we have also identified a novel alternative spliced *MDS1EVI1*

that together with the previously described *EVI1* transcripts would codify to the same sized protein EVI1-FL. However, whether all these transcripts are used or not is difficult to know because all cell lines with EVI1-FL protein expresses more than one transcript, and we did not find any association between any specific transcript and the protein. This would point out that the mechanism of EVI1 protein regulation is complex and is still to be elucidated. Nevertheless, the fact that the EVI1 protein is present even if only one *EVI1* transcript is overexpressed supports the importance of the detection of *EVI1* expression status at diagnosis in AML patients, as indicated by the new WHO classification.[30] Moreover, AML cell lines overexpressed transcripts *-1A, -1B, -1C, and -1D*, whereas cell lines with CML-BP had only *EVI1-1B* overexpression as a common feature. This might indicate that the mechanisms of *EVI1* overexpression may depend on the action of different transcription factors in the promoter of this gene, opening directions to future studies.

In order to investigate other mechanisms of *EVI1* overexpression, we analyzed the role that epigenetic modifications could have in the regulation of the *EVI1* gene. The analysis of the promoter regions of *EVI1* and *MDS1EVI1* loci showed no methylation neither in CD34+ progenitor cells (high *EVI1* expression) nor in normal BM and PB samples (very low *EVI1* expression). These results strongly suggest that DNA methylation modifications do not have a role in the normal regulation of *EVI1* expression during the differentiation process of hematopoietic cells, and that *EVI1* promoter hypomethylation can not be the mechanism of *EVI1* overexpression. However, we had detected absence of normal basal expression of *EVI1* and *MDS1EVI1* in patient samples and cell lines, and several cell lines had 3q rearrangements and no *EVI1* overexpression; therefore, we hypothesized that epigenetic aberrations could have a role in the regulation of the expression of *EVI1* in AML. We found an aberrant hypermethylation pattern in cell lines with no *EVI1/MDS1EVI1* expression (Figure 2C), and treatment of these cell lines with TSA in combination with 5-Aza induced *EVI1* expression (Figure 2B). However, there were no significant changes in the methylation status after the treatment, suggesting that other epigenetic mechanisms could be involved (Figure 2D). Our results showed that histone modifications could be a

mechanism that contributes to silencing the normal basal expression of the *EVI1* locus in the leukemic cells (Figure 3 A-B). An important observation in this study is the active pattern of H4 and specially H3 in the HEL and TF-1 cell lines that have *EVI1* overexpression. Of note, treatment of the cell lines with no *EVI1* expression induced expression of this gene and showed an increased acetylation of both histone H3 and H4 on the *EVI1* promoter (Figure 3C). We also found that the AML cell lines with DNA methylation and no *EVI1* expression displayed reduced H3K4me3. These data supports the results of recent studies that observed that in AML there is an inverse correlation between DNA methylation and H3K4 trimethylation pattern compared with unmethylated samples. [31,32,33] The epigenetic modifications H3K4me3 and H3K27me3 are of particular interest as these modifications are catalyzed, respectively, by trithorax and polycomb-group proteins, which have key developmental functions. H3K4me3 methylation positively regulates transcription by recruiting nucleosome remodeling enzymes and histone acetylases, while H3K27me3 methylation negatively regulates transcription by promoting a compact chromatin structure. It has been described that the most highly conserved noncoding elements in mammalian genomes cluster within regions enriched for genes encoding developmentally important transcription factors, such as *EVI1*.[34] These findings suggest that these transcription factors would have key epigenetic regulatory controls involved in development. Mapping histone methylation patterns in mouse embryonic stem cells showed that *EVI1* has an open chromatin structure with a H3K4me3 pattern, as we have observed in our *EVI1* expressing cell lines, suggesting that this mechanism is involved in its regulation in early hematopoietic cells. Our results support that the same mechanism could be involved in the leukemic cells.[34] Taken together, the histone modifications could explain the atypical expression pattern of both cell lines and patient samples with no *EVI1* expression. This is of special interest since patients with no basal expression of *EVI1* tend to have a better overall survival rate in comparison with cases with either expression or overexpression (Figure 1). Nevertheless, prospective studies are needed to clarify the role of histone modifications in *EVI1* regulation.

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In summary, our results support that *EVI1* overexpression is an adverse prognostic factor in AML, and corroborate the necessity of the quantification of *EVI1* and *MDS1EVI1* expression in the diagnosis of younger AML patients, mostly in cases with 3q aberrations, monosomy 7, *MLL* rearrangements, and in the subgroup with normal karyotype and no *NPM1* mutations. Notably, we show that the total absence of *EVI1* expression may have a prognostic impact on the outcome of AML patients, and that this atypical pattern may be regulated by epigenetic mechanisms. Further studies are needed to elucidate the prevalence, prognostic impact, and the significance of no basal *EVI1* expression in the leukemic transformation of AML.

Authorship and Disclosures: Conceived and designed the experiments: IV, MM, XA, MDO. Performed the experiments: IV, MM, OMB, NM, CV, IL, CC. Analyzed the data: IV, MM, JC, XA, CV, MGB, AV. Contributed reagents/materials/analysis tools: JC, SB, EL, MTGC, JMHR, MJC, FP, MAS, JS. Wrote the paper: IV, MM, MDO. The authors reported no potential conflicts of interest.

Bibliography

1. Mucenski ML, Taylor BA, Ihle JN, Hartley JW, Morse HC, 3rd, Jenkins NA, et al. Identification of a common ecotropic viral integration site, Evi-1, in the DNA of AKXD murine myeloid tumors. Mol Cell Biol. 1988;8(1):301-8.
2. Nucifora G, Laricchia-Robbio L, Senyuk V. EVI1 and hematopoietic disorders: history and perspectives. Gene. 2006;368:1-11.
3. Wieser R. The oncogene and developmental regulator EVI1: expression, biochemical properties, and biological functions. Gene. 2007;396(2):346-57.
4. Poppe B, Dastugue N, Vandesompele J, Cauwelier B, De Smet B, Yigit N, et al. EVI1 is consistently expressed as principal transcript in common and rare recurrent 3q26 rearrangements. Genes Chromosomes Cancer. 2006;45(4):349-56.
5. Lahortiga I, Vazquez I, Agirre X, Larrayoz MJ, Vizmanos JL, Gozzetti A, et al. Molecular heterogeneity in AML/MDS patients with 3q21q26 rearrangements. Genes Chromosomes Cancer. 2004;40(3):179-89.
6. Wieser R, Volz A, Schnittger S, Jager U, Gruner H, Meran JG, et al. Mapping of leukaemia-associated breakpoints in chromosome band 3q21 using a newly established PAC contig. Br J Haematol. 2000;110(2):343-50.
7. Arai S, Yoshimi A, Shimabe M, Ichikawa M, Nakagawa M, Imai Y, et al. Evi-1 is a transcriptional target of MLL oncoproteins in hematopoietic stem cells. Blood. 2010.
8. Barjesteh van Waalwijk van Doorn-Khosrovani S, Erpelinck C, van Putten WL, Valk PJ, van der Poel-van de Luytgaarde S, Hack R, et al. High EVI1 expression predicts poor survival in acute myeloid leukemia: a study of 319 de novo AML patients. Blood. 2003;101(3):837-45.
9. Haas K, Kundi M, Sperr WR, Esterbauer H, Ludwig WD, Ratei R, et al. Expression and prognostic significance of different mRNA 5'-end variants of the oncogene EVI1 in 266 patients with de novo AML: EVI1 and MDS1/EVI1 overexpression both predict short remission duration. Genes Chromosomes Cancer. 2008;47(4):288-98.
10. Lugthart S, van Drunen E, van Norden Y, van Hoven A, Erpelinck CA, Valk PJ, et al. High EVI1 levels predict adverse outcome in acute myeloid leukemia: prevalence of EVI1 overexpression and chromosome 3q26 abnormalities underestimated. Blood. 2008;111(8):4329-37.
11. Valk PJ, Verhaak RG, Beijen MA, Erpelinck CA, Barjesteh van Waalwijk van Doorn-Khosrovani S, Boer JM, et al. Prognostically useful gene-expression profiles in acute myeloid leukemia. N Engl J Med. 2004;350(16):1617-28.
12. Santamaria CM, Chillon MC, Garcia-Sanz R, Perez C, Caballero MD, Ramos F, et al. Molecular stratification model for prognosis in cytogenetically normal acute myeloid leukemia. Blood. 2009;114(1):148-52.
13. Daghistani M, Marin D, Khorashad JS, Wang L, May PC, Paliompeis C, et al. EVI-1 oncogene expression predicts survival in chronic-phase CML patients resistant to imatinib treated with second-generation tyrosine kinase inhibitors. Blood. 2010;116(26):6014-7.
14. Aytekin M, Vinatzer U, Musteanu M, Raynaud S, Wieser R. Regulation of the expression of the oncogene EVI1 through the use of alternative mRNA 5'-ends. Gene. 2005;356:160-8.
15. Morishita K. Leukemogenesis of the EVI1/MEL1 gene family. Int J Hematol. 2007;85(4):279-86.
16. 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(4):1642-7.
17. Groschel S, Lugthart S, Schlenk RF, Valk PJ, Eiwen K, Goudswaard C, et al. High EVI1 expression predicts outcome in younger adult patients with acute myeloid leukemia and is associated with distinct cytogenetic abnormalities. J Clin Oncol. 2010;28(12):2101-7.
18. Suarez L, Vidriales MB, Moreno MJ, Lopez A, Garcia-Larana J, Perez-Lopez C, et al. Differences in anti-apoptotic and multidrug resistance phenotypes in elderly and young acute myeloid leukemia patients are related to the maturation of blast cells. Haematologica. 2005;90(1):54-9.
19. Nakao M, Yokota S, Iwai T, Kaneko H, Horiike S, Kashima K, et al. Internal tandem duplication of the flt3 gene found in acute myeloid leukemia. Leukemia. 1996;10(12):1911-8.

20. Yamamoto Y, Kiyoi H, Nakano Y, Suzuki R, Kodera Y, Miyawaki S, et al. Activating mutation of D835 within the activation loop of FLT3 in human hematologic malignancies. Blood. 2001;97(8):2434-9.
21. Falini B, Nicoletti I, Bolli N, Martelli MP, Liso A, Gorello P, et al. Translocations and mutations involving the nucleophosmin (NPM1) gene in lymphomas and leukemias. Haematologica. 2007;92(4):519-32.
22. Bibikova M, Fan JB. Genome-wide DNA methylation profiling. Wiley Interdiscip Rev Syst Biol Med. 2010;2(2):210-23.
23. Jose-Eneriz ES, Roman-Gomez J, Cordeu L, Ballestar E, Garate L, Andreu EJ, et al. BCR-ABL1-induced expression of HSPA8 promotes cell survival in chronic myeloid leukaemia. Br J Haematol. 2008;142(4):571-82.
24. Ott MG, Schmidt M, Schwarzwaelder K, Stein S, Siler U, Koehl U, et al. Correction of X-linked chronic granulomatous disease by gene therapy, augmented by insertional activation of MDS1-EVI1, PRDM16 or SETBP1. Nat Med. 2006;12(4):401-9.
25. Buonamici S, Li D, Chi Y, Zhao R, Wang X, Brace L, et al. EVI1 induces myelodysplastic syndrome in mice. J Clin Invest. 2004;114(5):713-9.
26. Stein S, Ott MG, Schultze-Strasser S, Jauch A, Burwinkel B, Kinner A, et al. Genomic instability and myelodysplasia with monosomy 7 consequent to EVI1 activation after gene therapy for chronic granulomatous disease. Nat Med. 2010;16(2):198-204.
27. Watanabe-Okochi N, Kitaura J, Ono R, Harada H, Harada Y, Komeno Y, et al. AML1 mutations induced MDS and MDS/AML in a mouse BMT model. Blood. 2008;111(8):4297-308.
28. Lowenberg B. Diagnosis and prognosis in acute myeloid leukemia--the art of distinction. N Engl J Med. 2008;358(18):1960-2.
29. Lugthart S, Groschel S, Beverloo HB, Kayser S, Valk PJ, van Zelderen-Bhola SL, et al. Clinical, Molecular, and Prognostic Significance of WHO Type inv(3)(q21q26.2)/t(3;3)(q21;q26.2) and Various Other 3q Abnormalities in Acute Myeloid Leukemia. J Clin Oncol. 2010.
30. Lowenberg B. Acute myeloid leukemia: the challenge of capturing disease variety. Hematology Am Soc Hematol Educ Program. 2008:1-11.
31. Mohn F, Weber M, Rebhan M, Roloff TC, Richter J, Stadler MB, et al. Lineage-specific polycomb targets and de novo DNA methylation define restriction and potential of neuronal progenitors. Mol Cell. 2008;30(6):755-66.
32. Weber M, Hellmann I, Stadler MB, Ramos L, Paabo S, Rebhan M, et al. Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. Nat Genet. 2007;39(4):457-66.
33. Paul TA, Bies J, Small D, Wolff L. Signatures of polycomb repression and reduced H3K4 trimethylation are associated with p15INK4b DNA methylation in AML. Blood. 2010;115(15):3098-108.
34. Bernstein BE, Mikkelsen TS, Xie X, Kamal M, Huebert DJ, Cuff J, et al. A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell. 2006;125(2):315-26.

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Table 1. Clinical and molecular characteristics of the 16 human myeloid cell lines, including the analysis of *EVI1* expression.

Cell line	FAB	Partial 3q Karyotype	Overexpression (qRT-PCR)							PR domain		Protein	
			EVI1-1A **0.085**	***EVI1-1B*** **0.033**	***EVI1-1C*** **0.099**	***EVI1-1D*** **0.036**	***EVI1-3L*** **0.801**	***MDS1EVI1*** **1.969**	***GATA2*** **2.820**	**Normal**	**Novel splicing variant**	**MDS1EVI1**	**EVI1-FL**
HL-60	AML-M2	no 3q aberrations	0	0	0	0	0.001	0.069	2.566	**Yes**	No	No	No
Kasumi-1	AML-M2	no 3q aberrations	0	0	0	0	0.001	0	0.691	No	No	No	No
MUTZ-3	AML-M4	t(1;3)(q43;q13)inv(3)(q21q26)	**0.389**	**0.276**	**1.971**	**0.460**	0.700	0	2.620	No	No	No	**Yes**
OCI-AML2	AML-M4	+der(1)t(1;3)(q?;q26),inv(3)(q21q26),t(1;3)(q?;q26)	0	0	0	0	0.004	0	1.192	No	No	No	No
NOMO-1	AML-M5	+der(?)t(3;?)(q21;?)	0	0	0	0	0.002	0	0.438	No	No	No	No
MOLM-13	AML-M5	no 3q aberrations	0	0	0	0	0.002	0	0.680	No	No	No	No
MV4-11	AML-M5	no 3q aberrations	0	0	0	0	0.001	0	0.021	No	No	No	No
TF-1	AML-M6	+der(?)t(?;3)(?;q21)x2	**0.187**	**0.134**	**1.835**	**0.125**	0.313	0.419	**18.427**	**Yes**	No	No	**Yes**
HEL	AML-M6	t(3;?6)(q21;q?),+3	**0.181**	**0.140**	**0.244**	**0.121**	0.102	0.313	**21.730**	**Yes**	**Yes**	**Yes**	**Yes**
F-36P	AML-M6	no 3q aberrations	**0.169**	**0.219**	**0.344**	**0.076**	0.395	0.090	**5.520**	**Yes**	**Yes**	No	**Yes**
KG-1	AML-M6	no 3q aberrations	0	0	0	0	0.017	0	**9.450**	No	No	No	No
EOL1	AML	no 3q aberrations	0	0	0	0	0.002	0	0.384	No	No	No	No
KYO-1	CML-BP	no 3q aberrations	0.074	**0.110**	**0.167**	0.025	0.053	0.163	**19.830**	**Yes**	**Yes**	No	**Yes**
K562	CML-BP	+der(?)t(3;?)(q26;?)	**0.139**	**0.216**	0	0.020	0.139	**2.321**	**4.176**	**Yes**	**Yes**	No	**Yes**
KU-812	CML-BP	no 3q aberrations	0.038	**0.045**	0	0.010	0.080	0.009	**56.600**	**Yes**	No	No	**Yes**
MEG-01	CML-BP	?inv(3)(p25q26)*	0.039	**0.065**	0	0.028	0.370	0.256	**6.640**	**Yes**	No	No	No

Overexpression is highlighted in bold; with the cut-off calculated of 7 normal BM and 3 times the standard deviation; * not confirmed by FISH analysis; cut-offs values calculated of 7 normal BM and 3 times the standard deviation are included for each EVI1 transcript

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Table 2. Clinical and molecular characteristics of a series of 476 patients with AML and the association between *EVI1* overexpression (*-1C* and/or *-1D*) and clinical and genetic parameters.

		No. Cases	No. *EVI1*-	No. *EVI1*+	*P*
EVI1 (-1C and/or -1D)		476	384 (80.7%)	92 (19.3%)	
Sex		**450**	**367**	**83**	p=0.057
Male		245	192 (78.4%)	53 (21.6%)	
Female		205	175 (85.4%)	30 (14.6%)	
Age		**445**	**366**	**79**	**p=0.002**
<65 years		249	192 (77%)	57 (23%)	
≥65 years		194	172 (88.7%)	22 (11.3%)	
CR		**220**	**174**	**46**	p=0.515
No		56	46 (82%)	10 (18%)	
Yes		164	128 (78%)	36 (22%)	
Diagnosis		**476**	**384**	**92**	
AML-M0		34	22 (64.7%)	12 (35.3%)	
AML-M1		79	72 (91%)	7 (9%)	
AML-M2		126	111 (88%)	15 (12%)	
AML-M3		16	15 (93.8%)	1 (6.3%)	
AML-M4		62	52 (84%)	10 (16%)	
AML-M5		57	47 (82.5%)	10 (17.5%)	
AML-M6		25	18 (72%)	7 (28%)	
AML-M7		7	4 (57%)	3 (43%)	
AML-NOS*		70	43 (61.4%)	27 (38.6%)	
sAML		**292**	**243**	**49**	**p=0.013**
No		243	209 (86.0%)	34 (14.0%)	
Yes		49	34 (69.4%)	15 (30.6%)	
Prognostic group		**476**	**384**	**92**	**p<0.001**
Good		55	53 (96.4%)	2 (3.6%)	
Intermediate		269	234 (87%)	35 (13%)	
Poor		152	97 (63.8%)	55 (36.2%)	
Cytogenetic group		**415**	**329**	**86**	
normal karyotype	No	272	197 (72.4%)	75 (27.6%)	**p<0.001**
	Yes	143	132 (92.3%)	11 (7.7%)	
MLL (11q23) balanced translocation	No	402	326 (81%)	76 (19%)	**p<0.001**
	Yes	13	3 (23%)	10 (77%)	
trisomy 8	No	460	368 (80%)	92 (20%)	**p=0.034**
	Yes	16	16 (100%)	0	
3q aberrations	No	372	317 (85.2%)	55 (14.8%)	**p<0.001**
	Yes	43	12 (28%)	31 (72%)	
monosomy 7	No	393	317 (80.7%)	76 (19.3%)	**p=0.003**
	Yes	22	12 (54.5%)	10 (45.5%)	
del(7q)	No	407	322 (79%)	85 (21%)	p=0.562
	Yes	8	7 (87.5%)	1 (12.5%)	
complex karyotype	No	345	272 (78.8%)	73 (21.2%)	p=0.626
	Yes	70	57 (81.4%)	13 (18.6%)	
***MDS1EVI1* overexpression**		**288**	**222**	**66**	**p<0.001**
No		259	219 (84.6%)	40 (15.4%)	
Yes		29	3 (10.3%)	26 (89.7%)	
***NPM1* mutated**		**223**	**203**	**20**	**p<0.001**
No		144	124 (86%)	20 (14%)	
Yes		79	79 (100%)	0	
***FLT3*-ITD**		**362**	**329**	**33**	p=0.166
No		295	265 (89.8%)	30 (10.2%)	
Yes		67	64 (95.5%)	3 (4.5%)	

* AML-NOS: AML not otherwise specified

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Figure Legends

Figure 1. Survival analysis of a series of patients with acute myeloid leukemia according to the *EVI1* expression status. (A) In Kaplan Meier analysis stratified by age, patients <65 years and *EVI1*-1C overexpression show an inferior overall survival in comparison to patients with no *EVI1*-1C overexpression. (B) In Kaplan Meier analysis, patients <65 years and no basal expression of *EVI1 (-1C/-1D)* show a better overall survival in comparison to patients with either expression or overexpression of *EVI1*, and a trend to better outcome in the global cohort. (C) In Kaplan Meier analysis, patients <65 years and no basal expression of *EVI1 (-1C/-1D)* show a better overall survival in comparison to patients with *EVI1* expression/overexpression. The same results are found in the global cohort.

Figure 2. Analysis of the epigenetic status of the *EVI1* locus in five myeloid cell lines. (A) Quantification of the relative expression of the *EVI1* splice variants, with BM as control sample. (B) Quantification of the relative expression of *EVI1* (*EVI1* 11-12) after treatment with 5'Aza and TSA. Statistical significance was estimated using non-parametric Wilcoxon matched pairs test; $p < 0.05$ was considered significant (*). (C) Diagram of the methylation status of the *EVI1*-Island 1 and *MDS1EVI1*-Island 2 by direct sequencing after bisulfite treatment (white: non-methylated; black: methylated).(D) Diagram of the methylation status of the *EVI1*-Island 1 and *MDS1EVI1*-Island 2 after treatment with 5'Aza and TSA (white: non-methylated; black: methylated).

Figure 3. Analysis of the epigenetic status of the histones of the *EVI1* locus in five myeloid cell lines. (A) qRT-PCR performed on fragmented chromatin, showing the enrichment of trimethylation of histone H3 lysine 4 (H3K4me3) and histone H3 lysine 27 (H3K27me3) on the *EVI1* promoter. (B) qRT-PCR performed on fragmented chromatin, showing the enrichment of acetylated histones H3 and H4 on the *EVI1* promoter. The results were calculated using the ΔΔCt method. They were presented as the fold enrichment of chromatin DNA precipitated by the specific antibody versus chromatin DNA precipitated by no antibody, as control. (C) qRT-PCR performed on fragmented chromatin, showing the enrichment of acetylated histones H3 and H4 on *EVI1* promoter regions after treatment with 5'Aza and TSA. The results were calculated and presented as described above, and comparing with or without the treatment. Statistical significance was estimated using non-parametric Wilcoxon matched pairs test; $p < 0.05$ was considered significant (*), and $p < 0.01$ very significant (**).

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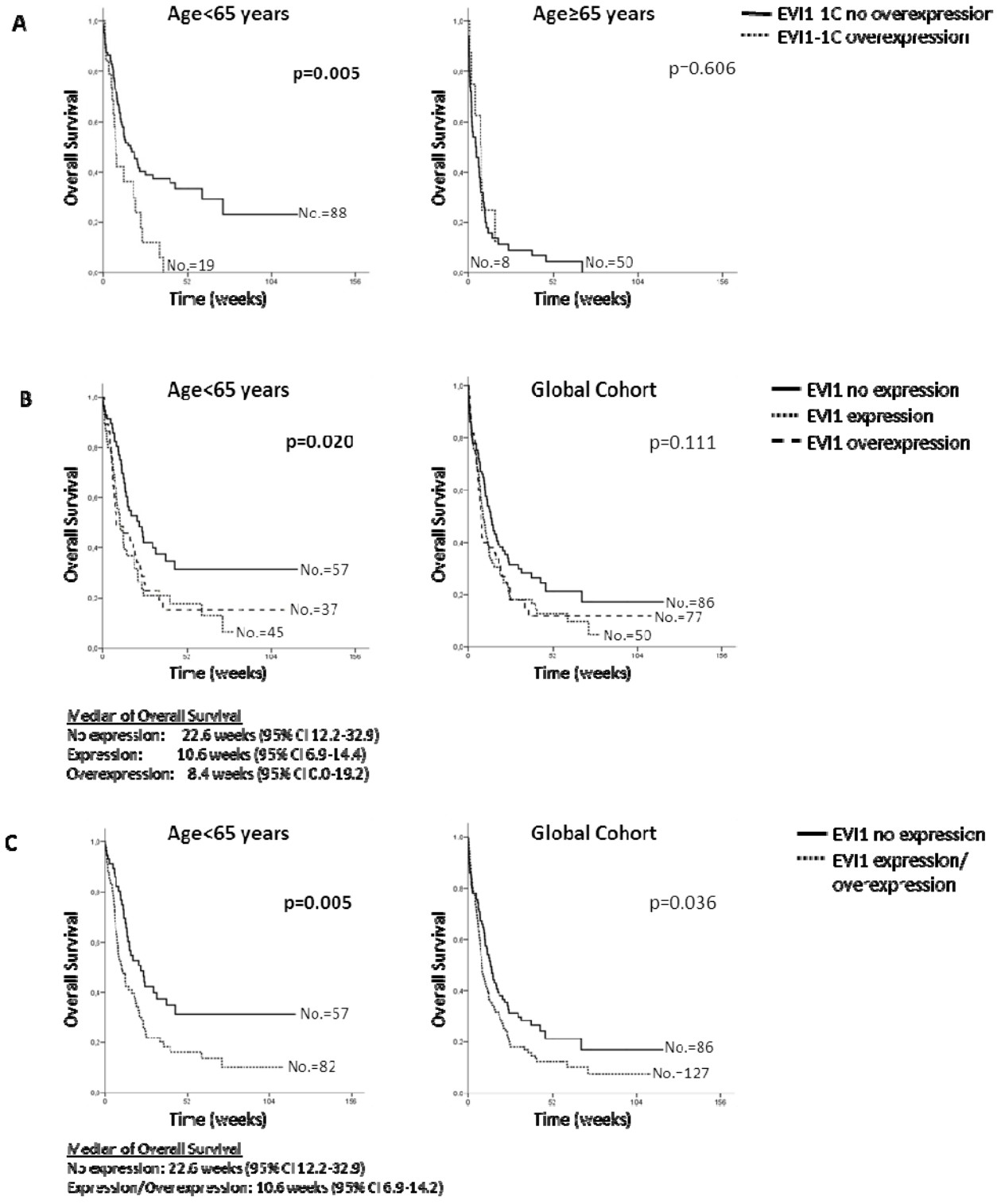

Figure 1

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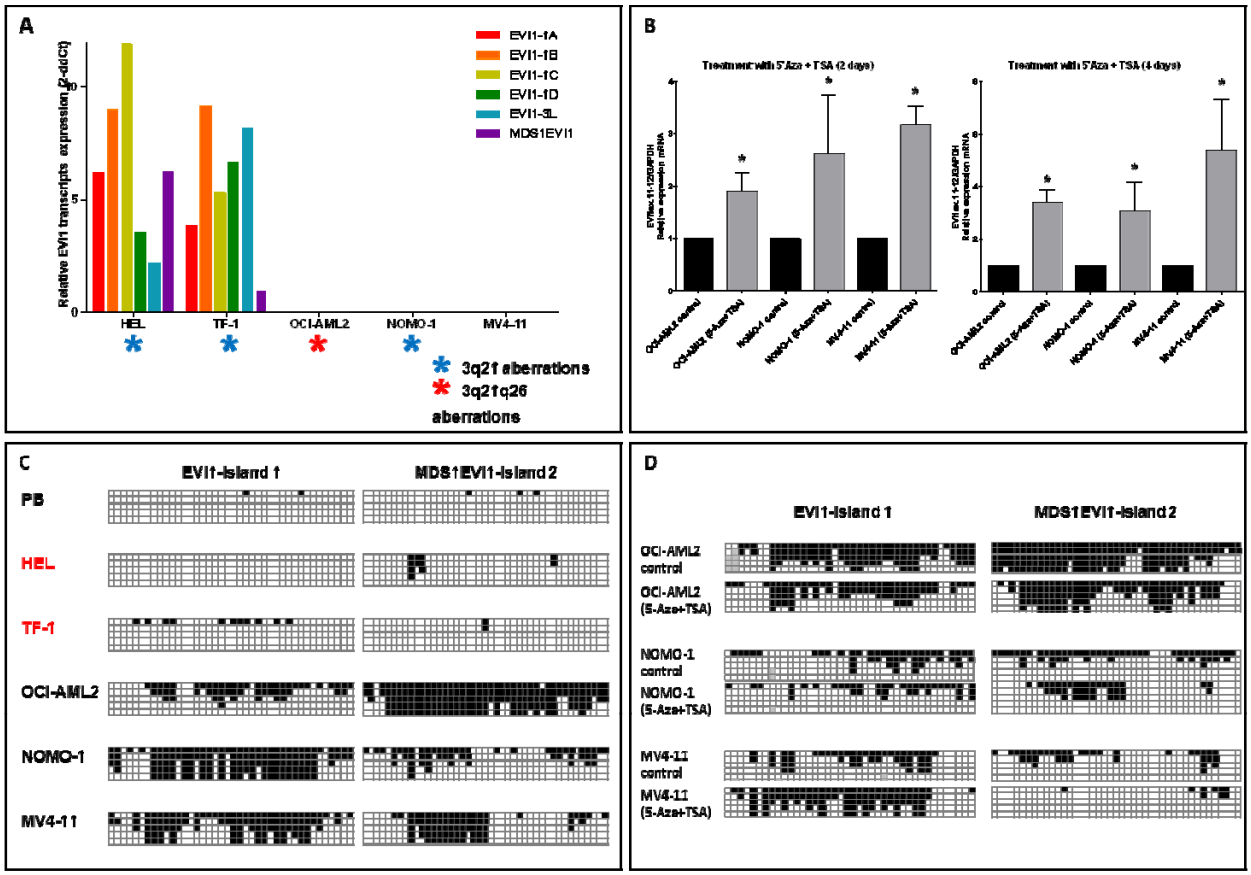

Figure 2

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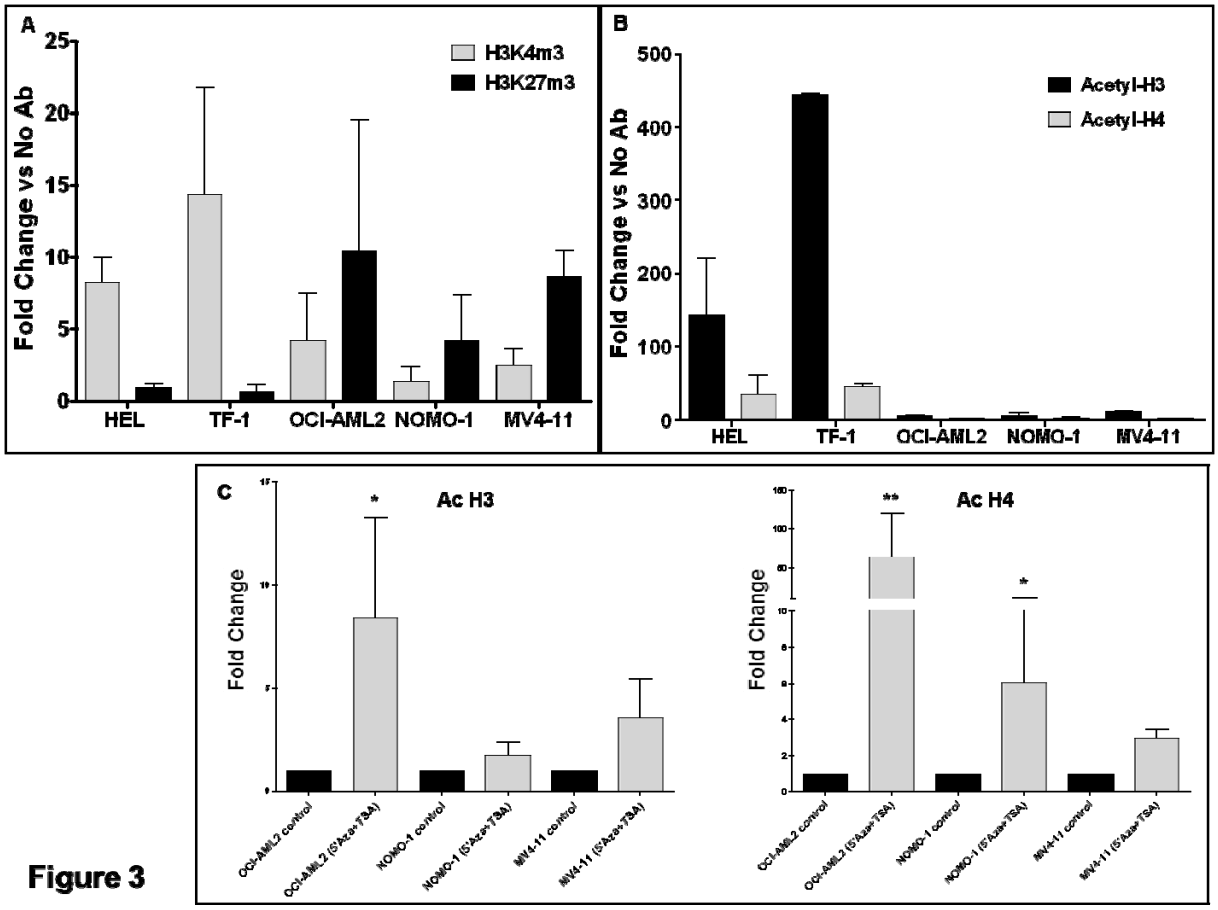

Figure 3

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Legends to Supplementary Figures

Supplementary Figure 1. Genomic locus of the human *EVI1* gene and *EVI1* splice variants. A) Genomic structure of the human *EVI1* gene with the three alternative splice variants: EVI1-Δ324, EVI1-Rp9 and the EVI1-Δ105. (B) Alternative mRNA 5′-end variants of the human *EVI1* gene. The shadow in grey represents the relative position of the *EVI1* CpG islands. (Adapted from Wieser 2007, and Lugthart et al. 2008).

Supplementary Figure 2. Quantification of the *EVI1* 5'-end variants in normal tissues. (Expression levels were normalized with spinal cord).

Supplementary Figure 3. Analysis of *EVI1* expression in 16 myeloid cell lines. (A) Quantification of the expression levels of *EVI1* 5'-end variants. Expression levels were normalized with normal bone marrow (B) Western blot analysis of EVI1 and MDS1EVI1.

Supplementary Figure 4. Analysis of the MDS1EVI1 PR domain. Sequences of alternative splicings of *MDS1EVI1*. The previously described intergenic splicing between *MDS1* (exon 2) and *EVI1* (exon 2), and the novel alternative splicing between *MDS1* (exon 1) and *EVI1* (exon 2). Different exons are colored with different colors; the sequence of *MDS1* is underlined.

Supplementary Figure 5. *EVI1-1C* overexpression is associated with poor survival outcome in AML patients <65 years. (A) In Kaplan Meier analysis stratified by age, patients <65 years and *EVI1-1C* overexpression shows an inferior event-free survival in comparison to patients with no *EVI1-1C* overexpression.

Supplementary Figure 6. Representation of the 3q aberrations detected by FISH in 6 myeloid cell lines, indicating the position of the probes used and the orientation of the genes located within these probes.

Supplementary Figure 7. Analysis of the histone methylation of the *EVI1* locus in three myeloid cell lines after treatment with 5-Aza and TSA. (A) qRT-PCR performed on fragmented chromatin, showing the levels of trimethylation of histone H3 lysine 4 (H3K4me3) and histone H3 lysine 27 (H3K27me3) on *EVI1* promoter before and after the treatment. The results were calculated using the ΔΔCt method. They were presented as

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the fold enrichment of chromatin DNA precipitated by the specific antibody versus chromatin DNA precipitated by no antibody, as control, and comparing with or without the treatment.

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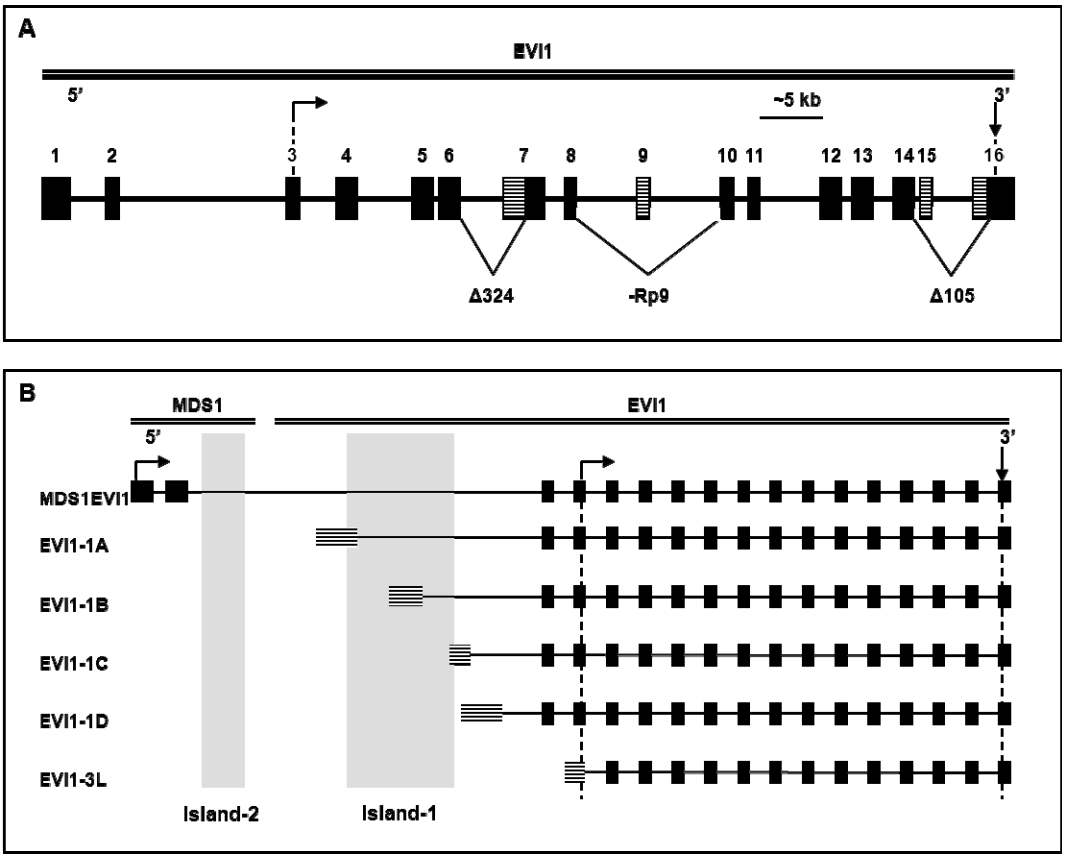

Supplementary Figure 1

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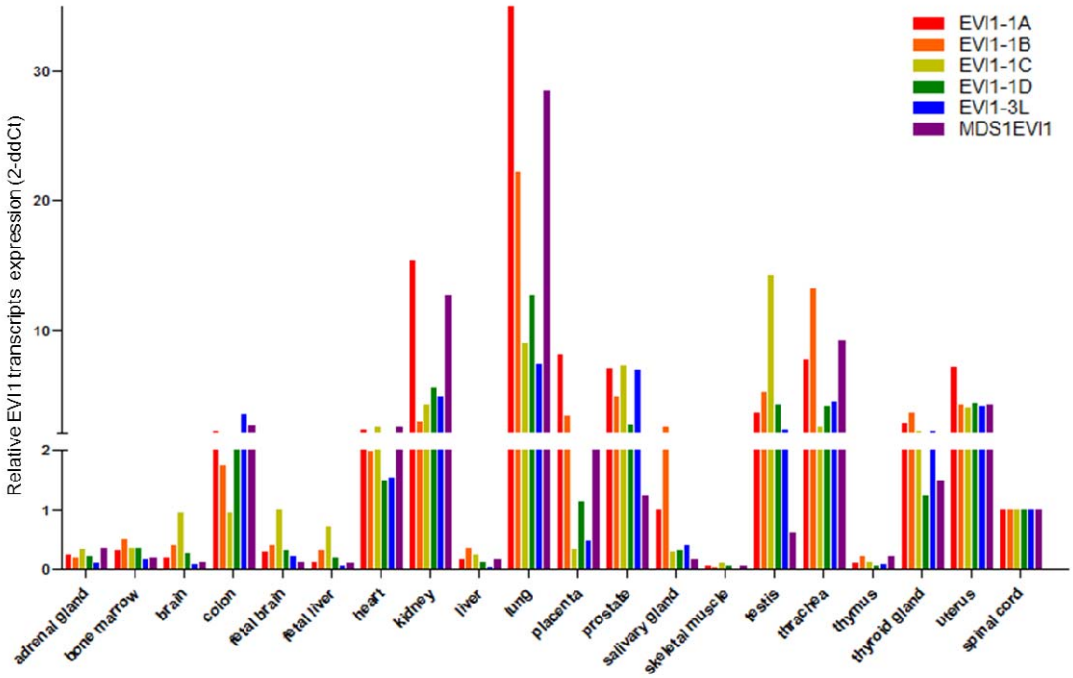

Supplementary Figure 2

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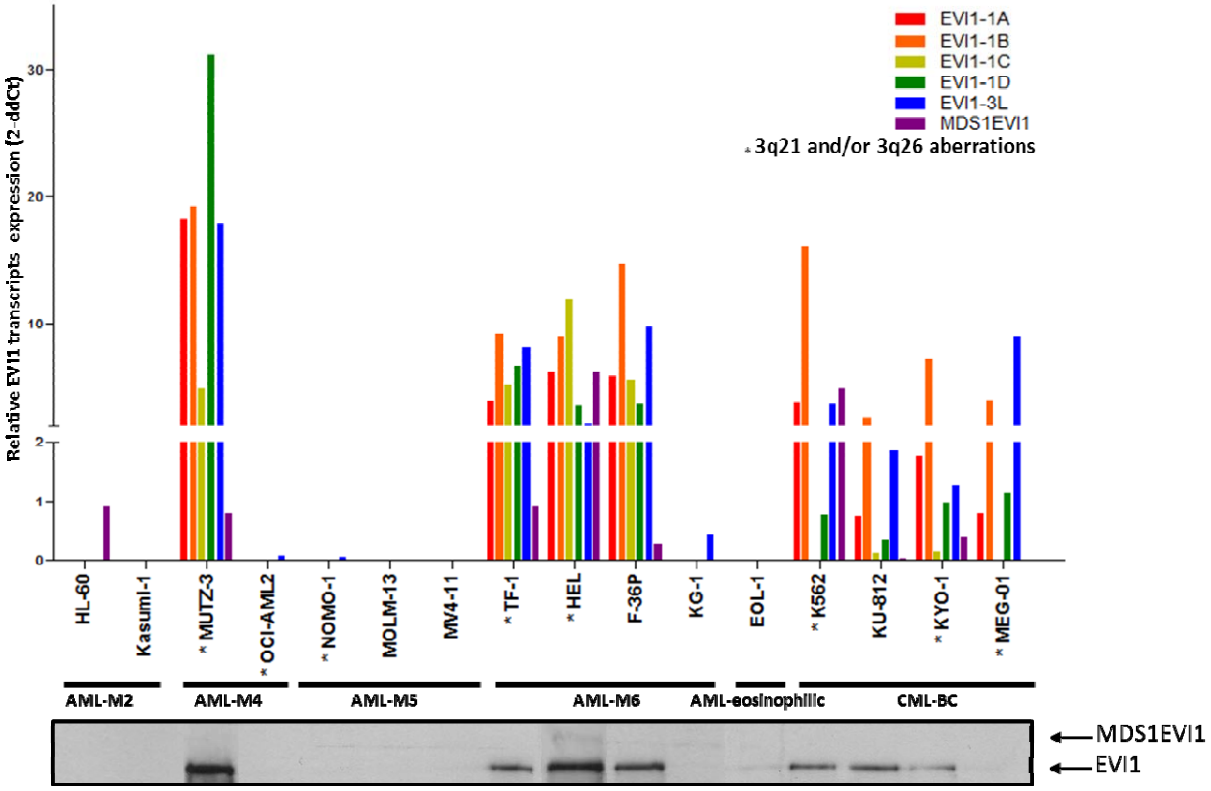

Supplementary Figure 3

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MDS1EVI1 splicing (exon 2 of MDS1 - exon 2 of EVI1)

GCGCATGTGCAAGGTGTCCAAACTGACAATGCTGGAGAGATAGCGAGTGTGGATTGAGAGAAAGGGAGAGAG
GGAGGGAGAGAGAGTGAAAGAAGAAAATACAGAGAGTGAGTGTGTGGAAGAGAGAGAGAAACAGGAGAGAAA
CAGGAGGGGGGGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGACAGGAGAGAGA
GGCAGGGAGCGAGAGGGAGAGCAAAAGAAGGAAAGGATCCAAGAAAAAAAAGCCCCAACCACACACCAGAGG
CTGCAGGACTGGGCACAGCATGAGATCCAAAGGCAGGGCAAGGAAACTGGCCACAAATAATGAGTGTGTATA
TGGCAACTACCCTGAAATACCTTTGGAAGAAATGCCAGATGCAGATGGAGTAGCCAGCACTCCCTCCCTCAA
TATTCAAGAGCCATGCTCTCCTGCCACATCCAGTGAAGCATTCACTCCAAAGGAGGGTTCTCCTTACAAAGC
CCCCATCTACATCCCTGATGATATCCCCATTCCTGCTGAGTTTGAACTTCGAGAGTCAAATATGCCTGGGGC
AGCACTAGGAATATGGACCAAAAGGAAGATCGAAGTAGGTGAAAAGTTTGGGCCTTATGTGGGAGAGCAGAG
GTCAAACCTGAAAGACCCCAGTTATGGATGGGAGATCTTAGACGAATTTTACAATGTGAAGTTCTGCATAGA
TGCCAGTCAACCAGATGTTGGAAGCTGGCTCAAGTACATTAGATTCGCTGGCTGTTATGATCAGCACAACCT
TGTTGCATGCCAGATAAATGATCAGATATTCTATAGAGTAGTTGCAGACATTGCGCCGGGAGAGGAGCTTCT
GCTGTTCATGAAGAGCGAAGACTATCCCCATGAAACTATGGCGCCGGATATCCACG

MDS1EVI1 alternative splicing (exon 1 of MDS1 - exon 2 of EVI1)

GCGCATGTGCAAGGTGTCCAAACTGACAATGCTGGAGAGATAGCGAGTGTGGATTGAGAGAAAGGGAGAGAG
GGAGGGAGAGAGAGTGAAAGAAGAAAATACAGAGAGTGAGTGTGTGGAAGAGAGAGAGAAACAGGAGAGAAA
CAGGAGGGGGGGCAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGACAGGAGAGAGA
GGCAGGGAGCGAGAGGGAGAGCAAAAGAAGGAAAGGATCCAAGAAAAAAAAAGCCCCAACCACACACCAGAGG
CTGCAGGACTGGGCACAGCATGAGATCCAAAGGCAGGGCAAGGAAACTGGCCACAAATCTTAGACGAATTTT
ACAATGTGAAGTTCTGCATAGATGCCAGTCAACCAGATGTTGGAAGCTGGCTCAAGTACATTAGATTCGCTG
GCTGTTATGATCAGCACAACCTTGTTGCATGCCAGATAAATGATCAGATATTCTATAGAGTAGTTGCAGACA
TTGCGCCGGGAGAGGAGCTTCTGCTGTTCATGAAGAGCGAAGACTATCCCCATGAAACTATGGCGCCGGATA
TCCACG

Supplementary Figure 4

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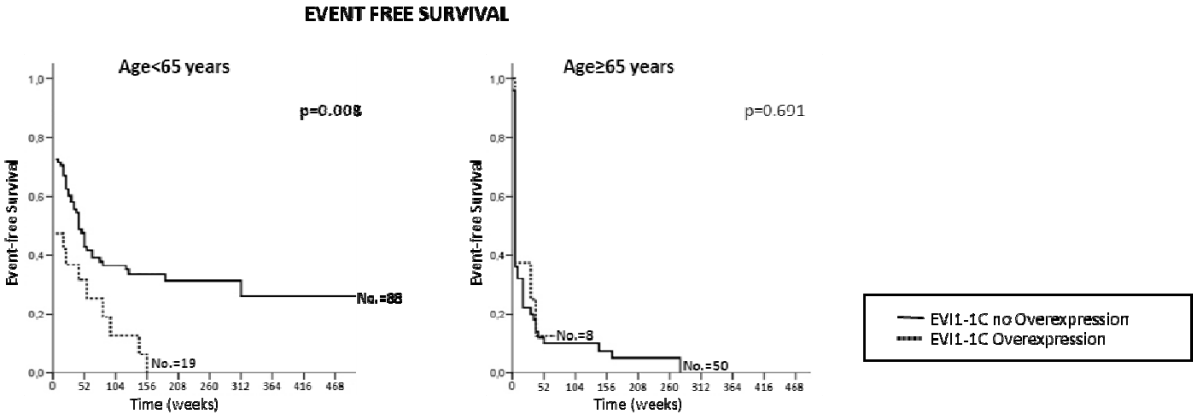

Supplementary Figure 5

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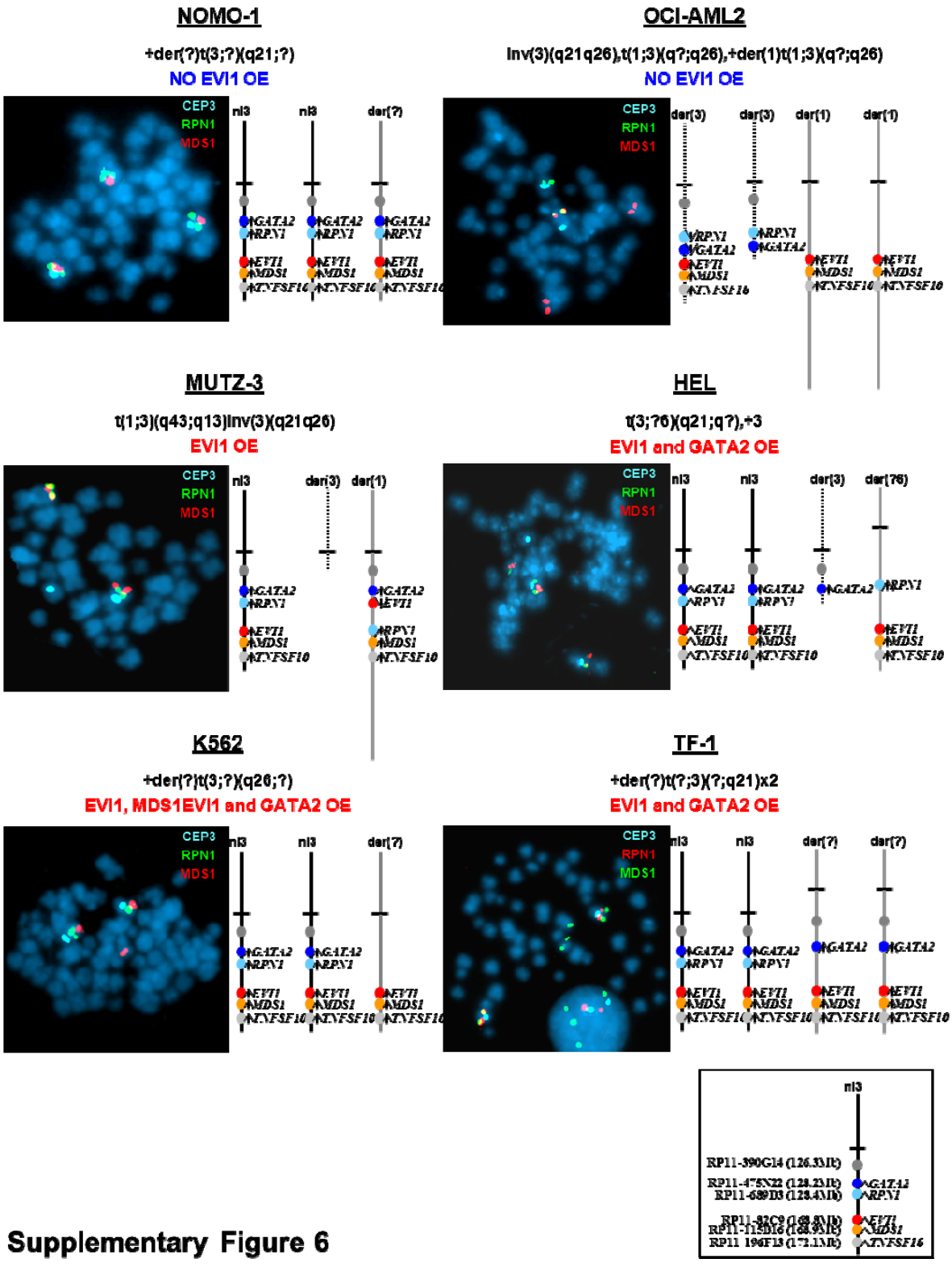

Supplementary Figure 6

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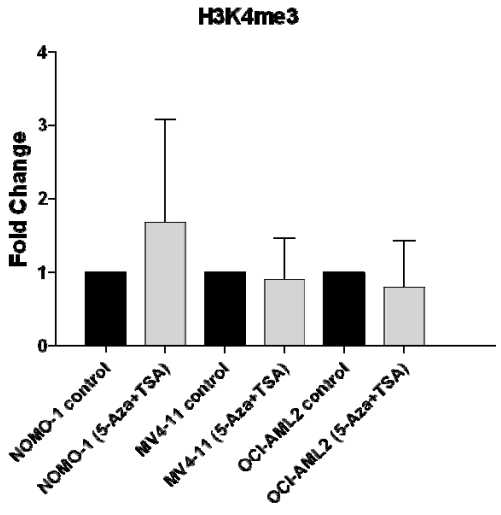

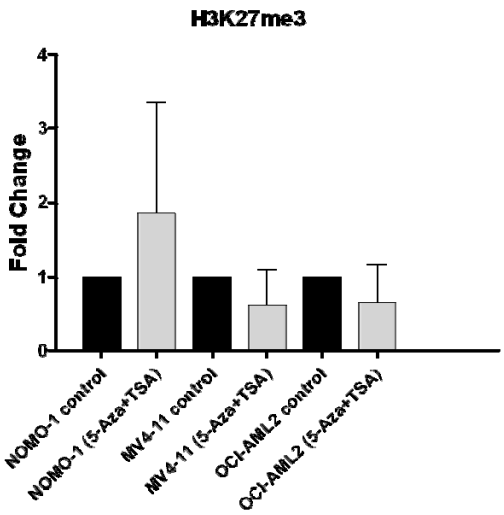

Supplementary Figure 7

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Supplementary Table 1. Review of the literature of large series of AML adult patients that reported the prevalence and prognostic value of *EVI1* overexpression.

Reference	No. cases analyzed	No. cases with EVI1 overexpression	Prevalence of EVI1 overexpression among the cytogenetic risk groups	Univariate			Multivariate	
				Global cohort	Intermediate-risk karyotype (*)	Normal cytogenetics (*)	Global cohort (*)	Intermediate-risk karyotype (*)
Barjesteh van Waalwijk van Doorn-Khosrovani et al., 2003								
	319	32 (10%)	good: 0% (0/57) intermediate: 7.5% (16/212) poor: 32% (16/50)	OS and EFS (*-1D*)	OS and EFS (*-1D*)			OS and EFS (*-1D*)
Haas et al., 2008								
	266	41 (15.4%)	no data	EFS (*-3L*) DFS (*-1A, -1B, -1D, -3L, MDS1EVI1, cEVI1*)	EFS (*-1B*) DFS (*-1A, -1B, -1D, -3L, MDS1EVI1, cEVI1*)	DFS (*-1A, -1B, -1D, -3L, MDS1EVI1, cEVI1*)		
Lugthart et al., 2008								
	534	41 (7.8%)	good: 1.1% (1/90) intermediate: 4.7% (17/364) poor: 28.8% (23/80)	OS (*-1A, -1B, -1D, -3L*) EFS (*-1A, -1B, -1D, -3L*) DFS (*-1A, -1B, -1D, -3L*)			OS (*-1A, -1B*) EFS (*-1A, -1B, -3L*) DFS (*-1A, -1B*)	
Groschel et al., 2010								
	1328	148 (10.7%)	good: 0.4% (1/263) intermediate: 7.4% (62/836) poor: 38% (73/198)	OS, EFS and RFS (*EVI1/MDS1EVI1*) <60 years			OS, EFS and RFS (*EVI1/MDS1EVI1*) <60 years	
Vazquez et al., 2011								
	476	92 (19.3%)	good: 3.6% (2/55) intermediate: 13% (35/269) poor: 36.2% (55/152)	OS and EFS (*-1C*) <65 years				

(*) Significant data; overall survival (OS); event-free survival (EFS); disease-free survival (DFS); relapse-free survival (RFS); sum of all EVI1 transcripts (cEVI1)

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Supplementary Table 2. Sequence of the primers used to analyze the PR domain of *MDS1EVI1*, the methylation status of CpG islands of *EVI1* (Island 1 and Island 2) and *MDS1EVI1* (Island 1 and Island 2), and primers used for qRT-PCR on ChIP.

Primer Name	**Oligonucleotide sequence (5´-3´)**	**AT(°C)**
PR domain F	CACAGCATGAGATCCAAAGG	59
PR domain R	AAGAGCGAAGACTATCCCCA	
PR domain R2	CCAGCGAATCTAATGTACTTGAGC	59.5
EVI1-Island1 F	TGTTGAGTTGAGGTTATAGAAATTTAAAG	59
EVI1-Island1 R	CCCACAATCTAACCAAAAAATC	
EVI1-Island2 F	GTAGGTTTGGTTAAATTAGGATTT	55
EVI1-Island2 R	CCTAAACTACAATATACCTTCCTCTC	
MDS1EVI1-Island1 F	TTTGTTTAAGTTTTTTTAATTTTTTTT	55
MDS1EVI1-Island1 R	CTCTCCAACATTATCAATTTAAACAC	
MDS1EVI1-Island2 1F	GGAAGGGATTTTAAGAGGTTTAAATT	56
MDS1EVI1-Island2 1R	ACCCATAAAATTAAAAAACCATTTC	
MDS1EVI1-Island2 2F	TTTTATATATATATTAGAAGTTGGATGGGA	59
MDS1EVI1-Island2 2R	TATAAACACACATCCAAACAACAAC	
ChIP -724(EVI1) F	CATTGGAACTGGGAAGGAGA	60
ChIP -724(EVI1) R	CGCGTTTCGGATTTATTGTC	
ChIP -5000(MDS1EVI1) F	GGGGAGGGAGTAGGATTGTA	60
ChIP -5000(MDS1EVI1) R	CTTGCCGTTTTGTAAATTGC	

AT, annealing temperature. The relative position of the amplicon in the ChIP primers is specified before the name of the gene.

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Supplementary Table 3. Quantification of the *EVI1* 5'-end variants in 18 patients with AML.

ID	Sex	FAB	sAML	Karyotype	*EVI1-1A* **0.085**	*EVI1-1B* **0.033**	*EVI1-1C* **0.099**	*EVI1-1D* **0.036**	*EVI1-3L* **0.801**	*MDS1EVI1* **1.969**
43	F	AML-M4	sAML	45,XX,t(3;10)(q24;q24),del(5)(q31),-7,del(13)(q21)	**2.330**	**34.960**	**12.150**	**2.369**	0.299	**18.381**
269	M	AML-NOS	sAML	46,XY	0.070	0.004	**0.161**	**0.058**	0.298	1.222
270	M	AML-M5	sAML	46,XY,add(11)(q24)	**0.574**	**20.690**	**0.465**	**0.296**	**4.606**	**6.752**
271	F	AML-M4	de novo	46,XX	0.038	0.001	0	0.017	0.127	1.047
273	M	AML-M4	de novo	46,XY	0.012	0.001	0	0.004	0.038	0.313
276	F	AML-M5	de novo	46,XY	0.003	0	0	0.002	0.035	0.022
277	F	AML-M5	sAML	48,+3,+8,t(9;11)(p21;q23)/46,XX	**0.903**	0.003	**0.440**	**0.136**	0.690	**13.767**
278	F	AML-M5	de novo	43,X,-X,der(3)t(3;10)(p23;q11),-10,del(12)(p11),-20	0.002	0	0	0.002	0.370	0.042
280	M	AML-M5	sAML	46,XY,del(3)(p21),del(5q21q34),-13,-18,add(19)(q13),+2mar/ 45,XY,-3,del(5)(q21q34),der(6),-17,-17,-18,-19,-20,+5mar	0.044	0.001	**0.108**	0.023	0.148	0.469
281	M	AML-NOS	sAML	46,XY	0.072	0.001	0.053	0.012	0.201	**6.984**
282	F	AML-M2	de novo	45,XX,t(4;11)(p12;q23),-7	**0.993**	0.002	**2.170**	**0.299**	0.201	0.330
284	M	AML-M2	de novo	47,XY,+4,t(8;21)(q22;q22)	0.014	0.000	0	0.004	0.511	0.124
286	F	AML-M0	de novo	46,XX,t(5;16)(q14;q24)	**8.767**	0.025	**7.960**	**2.086**	**4.523**	0.157
289	M	AML-M2	de novo	46,XY,t(8;21)(q22;q22)	0.019	0.003	**0.150**	0.012	0.060	0.367
290	M	AML-M4	de novo	46,XY,t(10;11)(p14;q21)	0.066	0.002	0	0.030	0.263	**3.107**
291	F	AML-M6	de novo	45,XX,-7	**2.188**	**19.570**	**10.770**	**1.568**	**1.025**	0.049
292	M	AML-M1	de novo	46,XY	0.007	0	**0.169**	0.004	0.012	0.108
471	M	AML-M5	de novo	46,XY,add(11)(q23)	0.024	0.001	0	0.009	0.131	0.112

AML not otherwise specified (AML-NOS); Overexpression is highlighted in red; with the cut-off calculated of 7 normal BM and 3 times the standard deviation.

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Supplementary Table 4. Correlations between the expression levels of the *EVI1* transcripts in AML patients and cell lines.

Correlations between the expression levels of *EVI1* transcripts in 18 AML cases:

	EVI1-1B	**EVI1-1C**	**EVI1-1D**	**EVI1-3L**	**MDS1EVI1**
EVI1-1A	0.860(**)	0.792 (**)	0.965 (**)	0.631 (**)	0.550 (*)
EVI1-1B		0.759 (**)	0.902 (**)	0.557 (*)	0.593 (**)
EVI1-1C			0.824 (**)	0.473 (*)	0.294
EVI1-1D				0.597 (**)	0.542 (*)
EVI1-3L					0.267

Correlations between the expression levels of *EVI1* transcripts in 16 AML cell lines:

	EVI1-1B	**EVI1-1C**	**EVI1-1D**	**EVI1-3L**	**MDS1EVI1**
EVI1-1A	0.970(**)	0.852(**)	0.987(**)	0.877(**)	0.677(**)
EVI1-1B		0.783(**)	0.943(**)	0.889(**)	0.660(**)
EVI1-1C			0.852(**)	0.672(**)	0.374
EVI1-1D				0.889(**)	0.657(**)
EVI1-3L					0.567(*)

Spearman's Rho correlation coefficients were calculated for the 18 AML patient samples and 16 myeloid cell lines in which the *EVI1* transcripts had been measured.

$p<0.05$ (*)

$p<0.001$ (**)

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Supplementary Table 5. Multivariate analysis of *EVI1-1C* overexpression as a prognostic marker for survival in the cohort of AML patients under 65 years old.

Prognostic Marker	*P* (univariate)	*P* (multivariate)	HR	95% CI
Overall survival[1]				
Cytogenetic risk group	<0.001	<0.001	2.026	1.410-2.371
EVI1-1C overexpression	0.006	0.211	1.436	0.814-2.942
Event-free survival[1]				
Cytogenetic risk group	<0.001	0.002	1.756	1.226-2.517
EVI1-1C overexpression	0.018	0.235	1.403	0.802-2.456

1Other parameters such as sex, type of AML, *FLT3*-ITD mutations and *NPM1* mutations were not significantly associated with survival in the univariate analysis and were not included in the multivariate model.

Abbreviations: HR, hazard ratio; CI confidence interval.

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Supplementary Table 6. Multivariate analysis of *EVI1* expression groups (patients with no basal expression versus patients with expression/overexpression) as a prognostic marker for survival in the global cohort of AML patients, and in AML patients under 65 years old.

Prognostic Marker (global cohort)	***P* (univariate)**	***P* (multivariate)**	**HR**	**95% CI**
Overall survival[1]				
Age	**<0.001**	**<0.001**	2.038	1.489- 2.790
Cytogenetic risk group	**<0.001**	**<0.001**	1.828	1.410-2.371
EVI1 with no basal expression	**0.037**	0.642	1.080	0.781-1.493
Prognostic Marker (under 65)	***P* (univariate)**	***P* (multivariate)**	**HR**	**95% CI**
Overall survival[1]				
Cytogenetic risk group	**<0.001**	**<0.001**	1.941	1.395-2.702
EVI1 expression groups	**0.006**	0.100	1.428	0.934-2.183

[1]Other parameters such as sex, age (only for the global cohort), type of AML, *FLT3*-ITD mutations and *NPM1* mutations were not significantly associated with survival in the univariate analysis and were not included in the multivariate model.

Abbreviations: HR, hazard ratio; CI confidence interval.

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Supplementary Table 7. Schematic representation of the FISH breakpoints in 25 cases with 3q aberrations.

					centr.		3q21								3q26		tel.
	Case	Partial karyotype after FISH	Gene overexpression	FAB		390G14		475N22 (GATA2)		689D3 (RPN1)			82C9 (EVI1)		115B16 (MDS1)		196F13 (TRAIL)
3q231 & 3q26	18352	der(3)t(?;3)(?;q21)inv(3)(q21q26)	**E+ME**	AML-M0					X			X					
	15525	der(3)inv(3)(q21q26)t(3;17)(q27?;q12)	**E+ME**	AML-M7					X			X					
	8689s	der(3)inv(3)(q21q26)del(3)(q21)	**E**	AML-M0			X	del				X					
	18707s	inv(3)(q21q26)	**E+ME**	AML-NOS							X					X	
	15845s	t(3;3)(q21;q26)	**E**	AML-NOS				X								X	
	10357s	t(3;3)(q21;q26)	**E**	MDS					X							X	
	30840	ins(3;3)(q21q26)	**E+ME**	RAEB-1			X				X					X	
	21872s	t(3;3)(q21;q26)	NO	AML-NOS							X	X					
3q26	21029s	t(3;?6)(q26;?q25)	**E**	AML-M0												X	der(6)
	3666v	t(2;3)(p23;q26)	**E+ME**	AML-M0												X	der(2)
	28783	t(3;3)(q26;p?)	**E** (ME n.d.)	AML-M7												X	der(3p)
	562v	t(3;12)(q26;p13)	**E+ME**	CML-BP												X	der(12)
	19491s	t(3;21)(q26;q21)	**E+ME**	MDS												X	der(21)
	1389v	t(2;3)(p15;q26)	**E**	RAEB-2												X	der(2)
	19130	der(3)t(3;?)(q26;?)	**E**	AML-M5										X	der(?)		der(?)
3q21	14066s	del(3)(q21q26)	**E**	AML-M2	X	del		del		del			del		del		del
	24188	der(3)t(3;5)(q26q21;q31)	NO	AML-M2	X	der(5)		der(5)		der(5)			n.d.		der(5)		
	24316	der(3)del(3)(q21)t(3;?9)(q26;?)	**E**	AML-M4	X	del		del		del			n.d.		der(9p)		
	25704	inv(3;3)(p?;q21)	**E**	RAEB-2	X												
	12201	del(3)(q21)	NO	RAEB-2			X	del		del							
	30157	der(3)t(3;?)(q26q21;?)	NO	AML-M6					X	der(5)			der(5)		der(5)		der(5)
	12826	-3,der(3)t(3;12)(q13;p13)	NO	MDS					X	der(12?)			der(12?)		der(12?)		der(12?)
3q	15285	t(3;12)(q?21;q?)	NO	AML-M1							X		der(12)		der(12)		der(12)
	26164	der(3)ins(3;3)(q26;p?) (probes 3q26 in both 3q and 3p)	NO	AML-M5							X		dup(3p)		dup(3p)		dup(3p)
	8706s	der(3)t(3;?)(q26;?),der(?)t(3;?)(q26;?)x2	NO	MDS							X		der(?)x2		der(?)x2		der(?)x2

EVI1 (E); *MDS1EVI1* (ME); No gene overexpression (NO); no data (n.d.); centromere (centr.); telomere (tel.); the crosses (X) show the positions of the breakpoints.