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

Iria Vázquez,[1]* Miren Maicas,[1,2]* José Cervera,[3] Xabier Agirre,[1] Oskar Marin-Béjar,[1] Nerea Marcotegui,[1] Carmen Vicente,[1] Idoya Lahortiga,[4] Maria Gomez-Benito,[1] Claudia Carranza,[5] Ana Valencia,[3] Salut Brunet,[6] Eva Lumbreras,[7] Felipe Prosper,[1] María T. Gómez-Casares,[8] Jesús M. Hernández-Rivas,[7] María J. Calasanz,[2] Miguel A. Sanz,[3] Jorge Sierra,[6] and María D. Odero[1,2]

[1]Division of Oncology, CIMA, University of Navarra, Pamplona, Spain; [2]Department of Genetics, University of Navarra, Pamplona, Spain; [3]University Hospital La Fe, Valencia, Spain; [4]Human Genome Laboratory, Department of Molecular and Developmental Genetics, VIB, Leuven, Belgium; [5]Instituto de Investigación Genética, Milpas Altas, Guatemala; [6]Hospital Santa Creu i Sant Pau, Barcelona, Spain; [7]Hospital Universitario de Salamanca y CSIC, University of Salamanca, Salamanca, Spain; [8]Hospital Dr. Negrin, Las Palmas de Gran Canaria, Spain

ABSTRACT

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

Design and Methods
We analyzed the prevalence and prognostic impact of *EVI1* over-expression in a series of 476 patients with acute myeloid leukemia, and investigated the epigenetic modifications of the *EVI1* locus which could be involved in the transcriptional regulation of this gene.

Results
Our data provide further evidence that *EVI1* over-expression is a poor prognostic marker in acute myeloid leukemia patients less than 65 years old. Moreover, we found that patients with no basal expression of *EVI1* had a better prognosis than patients with expression/over-expression (P=0.036). We also showed that cell lines with over-expression of *EVI1* had no DNA methylation in the promoter region of the *EVI1* locus, and had 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 marked by repressive trimethylation of histone H3 lysine 27 at the EVI1 promoter.

Conclusions
Our results identify *EVI1* over-expression as a poor prognostic marker in a large, independent cohort of acute myeloid leukemia patients less than 65 years old, and show that the total absence of *EVI1* expression has a prognostic impact on the outcome of such 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 avenues for a better understanding of the regulation of *EVI1* expression at a transcriptional level.

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

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Correspondence:
María D. Odero, Division of Oncology, CIMA, University of Navarra, C/ Pío XII, 55, 31008-Pamplona, Spain.
Phone: international +34.948.194700.
Fax: international +34.948.194714.
E-mail: modero@unav.es

The online version of this article has a Supplementary Appendix.

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 common murine 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* over-expression;[4-7] however, over-expression of this gene has been reported in 9-20% AML with no 3q aberrations, where it is also associated with an unfavorable outcome.[8-13] Moreover, a recent study showed that *MLL-ENL* activates the transcription of Evi1.[7] Transcriptional activation of *EVI1* through chromosome rearrangements or other yet to be identified mechanisms, therefore, leads 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 (*Online Supplementary Figure S1*).

To date, only three studies in large series of AML patients have analyzed the prevalence and prognostic value of *EVI1* over-expression, discriminating *EVI1* from *MDS1EVI1* (*Online Supplementary Table S1*).[8-10,17] The first study found that *EVI1-1D* was over-expressed in 13.7% cases, and was significantly associated with 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* over-expression 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 adverse disease-free and event-free survival.[17]

Our aim was to study the prevalence of *EVI1* over-expression 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 patients' samples to investigate the genetic and epigenetic mechanisms that could control the expression of *EVI1* in AML. Our results open new avenues to a better understanding of the prognostic impact of *EVI1* in AML, and the regulation of its expression at a transcriptional level.

Design and Methods

Material

Samples were obtained at diagnosis from 476 patients with AML, other than acute promyelocytic leukemia: the details are given in the *Online Supplementary Design and Methods*. Survival analysis was performed in the 213 AML patients who 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, peripheral blood, and 19 samples of normal tissues from the human total RNA Master Panel II (Clontech, Takara-BIO, CA, USA) were used. The characteristics of the 16 myeloid cell lines used (DSMZ, Braunschweig, Germany) are summarized in *Online Supplementary Table S2*. Cell lines were cultured according to the supplier's recommendations.

Cytogenetic and mutation analysis

Cytogenetic and fluorescence *in situ* hybridization (FISH) analyses were performed as previously described[5] using six 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 reverse transcriptase polymerase chain reaction (RT-PCR), followed by a semi-nested reaction with specific primers (*Online Supplementary Table S3*). Gene mutation analysis of *FLT3* and *NPM1* was performed as previously described.[19-21] PCR products were purified and sequenced.

Quantitative real-time reverse transcriptase polymerase chain reaction

The RNA isolation and EVI1 quantitative real-time RT-PCR conditions are described in the *Online Supplementary Design and Methods*.

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 manufacturer's instructions.[22] Further details are provided in the *Online Supplementary Design and Methods*.

Methylation status of the CpG islands of *EVI1* (islands 1 and 2) and *MDS1EVI1* (islands 1 and 2) was analyzed by bisulfite sequencing PCR (*Online Supplementary Table S3*). DNA was modified with the CpGenome™ 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 for 10×10^6 cells in 10 mL of medium, cultured with 4 μM of 5-aza-2'-deoxycytidine (5-Aza), and 50 nM of trichostatin A (TSA) for 4 days; controls were cultured with dimethylsulfoxide and glacial acetic acid.

Chromatin immunoprecipitation

HEL, TF1, OCI-AML2, NOMO-1 and MV4-11 cell lines were subjected to chromatin immunoprecipitation 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] Further details are provided in the *Online Supplementary Design and Methods*.

Western blot analysis

Protein isolation and western blot conditions are described in the *Online Supplementary Design and Methods*.

Definitions and statistical analysis

Overall survival was defined as the time from diagnosis to death due to any cause or end of follow-up; disease-free survival as the time from complete remission until relapse or death, whichever occurred first; and event-free survival as the time from diagnosis until first event, in which failure to achieve complete remission, relapse or death were considered events. Overall, disease-free and event-free survival rates were determined using the Kaplan-Meier method and survival comparisons were done with the 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 a two-tailed χ^2 test. Spearman's rho correlation coefficient was used to calculate the correlations between the over-expression 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 understand the mechanisms leading to *EVI1* over-expression better, 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 bone marrow, although at low levels (*Online Supplementary 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 (*Online Supplementary Table S4*) and in the myeloid cell lines (*Online Supplementary Table S2* and *Online Supplementary Figure S3*), and completed the analysis of the cell lines with investigation of the expression of EVI1 protein. Expression levels of *EVI1* transcripts in both patients' samples and cell lines correlated with each other in a statistically significant manner (*Online Supplementary Table S5*). Among the cell lines over-expressing *EVI1*, we found two groups: AML cell lines over-expressed transcripts *-1A, -1B, -1C*, and *-1D*, whereas cell lines with chronic myeloid leukemia in blast phase (CML-BP) had only *EVI1-1B* over-expression as a common feature. Western blot analysis detected the EVI1-FL isoform (145 kDa) in cell lines with over-expression of at least one *EVI1* transcript (*Online Supplementary Table S2* and *Online Supplementary Figure S3*). As an exception, MEG-01 (CML-BC) had over-expression 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 (*Online Supplementary Table S2* and *Online Supplementary Figure S3*). Seven cell lines had no basal expression of either *EVI1* or *MDS1EVI1* (*Online Supplementary Table S2*).

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; we did, however, detect a novel *MDS1EVI1* alternative splice form in four cell lines. The analysis of the panel of normal human tissues demonstrated that this novel alternative splice form was not expressed in peripheral blood, but was present in most of the tissues tested (*Online Supplementary Figure S4*). This form would codify for a truncated protein of 38 amino acids; however, a second open reading frame is possible from the *EVI1* ATG start codon in exon 3, which would codify for the Evi1-FL protein (NCBI Accession GQ352634) (*Online Supplementary Figure S4*).

Prevalence of EVI1 over-expression in patients with acute myeloid leukemia

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 1). *EVI1* (*-1C* and/or *-1D*) was over-expressed in 92 out of the 476 patients (19.3%). Table 1 shows the prevalence of *EVI1* over-expression, and its association with relevant clinical and molecular parameters. Statistical correlations for *-1C* and *-1D* were also calculated separately and yielded similar results (*data not shown*). The prevalence of *EVI1* over-expression was significantly different among the cytogenetic prognostic groups ($P<0.001$). *EVI1* over-expression was found in 72% of cases with 3q rearrangements, including all 25 cases with 3q26 ($P<0.001$). Other cytogenetic abnormalities associated with *EVI1* over-expression were *MLL* translocations ($P<0.001$), and monosomy 7 ($P=0.003$), but not del(7q) ($P=0.562$). The prevalence of *EVI1* over-expression in patients with a normal karyotype was 7.7%, and an inverse correlation was found between *EVI1* over-expression 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* over-expression.

Prognostic impact of EVI1 expression in patients with acute myeloid leukemia

Clinical follow-up data of patients who received induction therapy and were uniformly treated were available for 213 patients (110 males and 103 females), with a median age at diagnosis of 58 years (range, 16-83 years). The median follow-up was 159 weeks, with a minimum of 24 weeks. The median overall survival 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* over-expression had significantly lower overall survival ($P=0.005$) and event-free survival ($P=0.008$) (Figure 1 and *Online Supplementary Figure S5*), while no significant differences were found in disease-free survival. However, we could not confirm the independent prognostic significance of *EVI1-1C* over-expression in a multivariate model (*Online Supplementary Table S6*). *EVI1-1D* over-expression had no significant impact on overall, disease-free or event-free survival. Among the whole cohort, the group of patients with *EVI1* over-expression and no *MDS1EVI1* expression had the worst outcome ($P=0.017$). When comparing patients with no basal expression, expression and over-expression of *EVI1* in the group of patients under 65 years old, patients with no basal expression had a better overall survival ($P=0.020$) (Figure 1). Furthermore, patients with no basal expression of *EVI1* had a better overall survival than patients with expression/over-expression in both the whole cohort ($P=0.036$) and in the group of patients less than 65 years ($P=0.005$) (Figure 1).

EVI1 over-expression 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 over-expression 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 had an inv(3)(q21q26) (*Online Supplementary Table S2* and *Online Supplementary Figure S6*). In the patients' samples, FISH analyses showed wide heterogeneity and complex 3q rearrangements. Cases were classified into four distinct

groups: 3q21q26, 3q26, 3q21, and other 3q aberrations. Cases with either 3q21q26 (8 cases) or 3q26 (7 cases) breakpoints had *EVI1* over-expression, except case 21872s, the only one with breakpoints located between the 689D3 (3q21; 128.4 Mb) and 82C9 (3q26; 168.8 Mb) probes. Cases with other 3q rearrangements and breakpoints located between these probes had no *EVI1* over-expression either (*Online Supplementary Table S8*). Three cases with a single breakpoint on 3q21 had *EVI1* over-expression (25704, 24316 and 14066s). The 3q26 break-

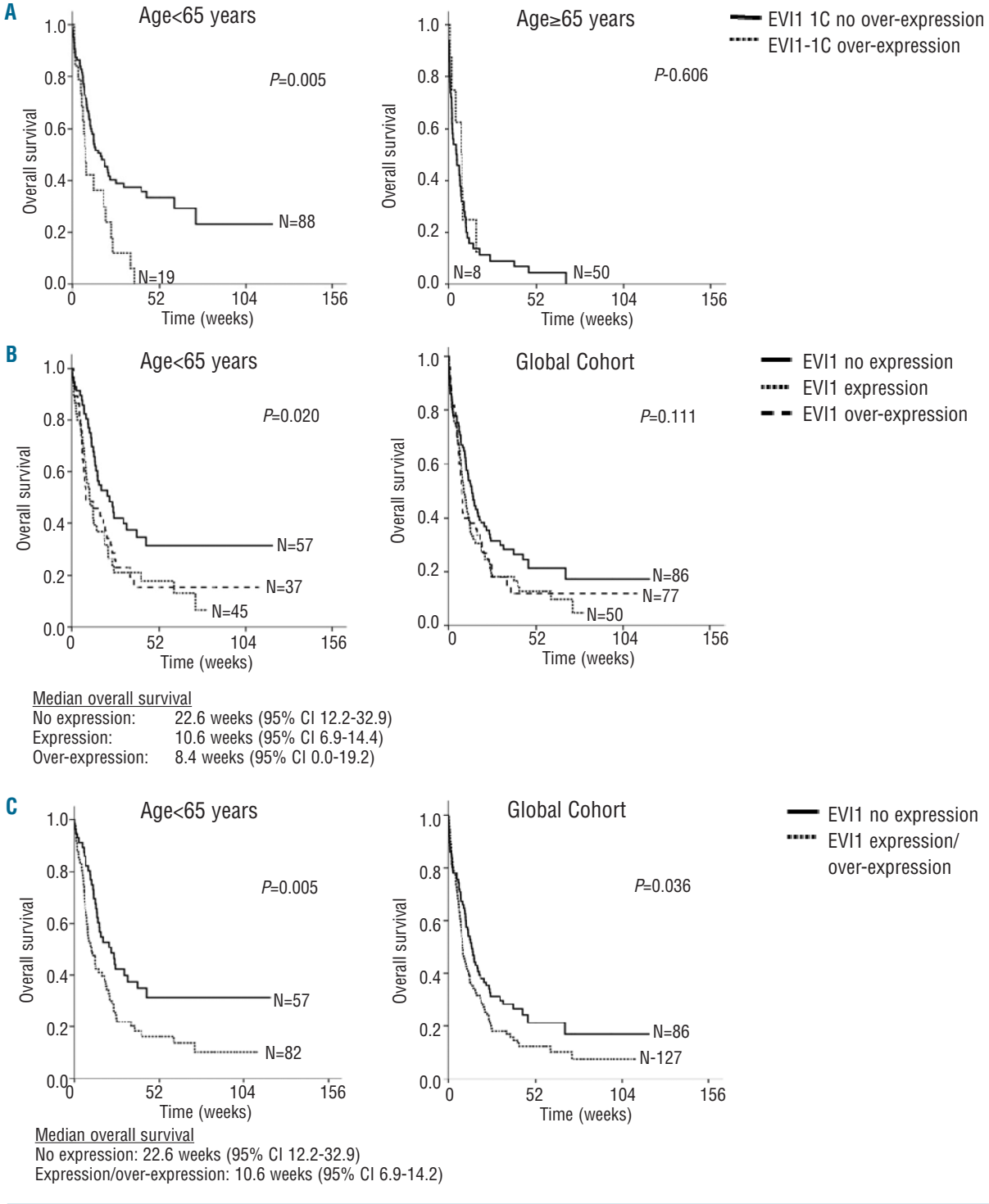

Figure 1. Survival analysis of a series of patients with acute myeloid leukemia according to *EVI1* expression status. (A) In Kaplan-Meier analysis stratified by age, patients <65 years and with *EVI1*-1C over-expression showed an inferior overall survival in comparison to patients with no *EVI1*-1C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

points associated with *EVI1* over-expression 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* over-expression were located centromeric to probe 390G14 (3/4 cases) (*Online Supplementary Table S8*).

Aberrant epigenetic pattern of EVI1 in acute myeloid leukemia

Results showing that *EVI1* over-expression sometimes occurs irrespectively of 3q21q26 rearrangements, and the finding that normal basal expression of *EVI1* and *MDS1EVI1* was not detected in several patients' samples and cell lines (including OCI-AML2, with 3q21q26) (*Online Supplementary Table S2* and *Online Supplementary Figure S3*) prompted us to study whether *EVI1* transcription could be regulated by epigenetic mechanisms. For the analysis, we selected five cell lines that represented the heterogeneity detected in patients' samples: HEL and TF-1 had 3q aberrations and *EVI1* over-expression; OCIAML2 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 induced *EVI1* expression (Figure 2B), confirming our hypothesis. The expression did not exceed the levels in peripheral blood or bone marrow. 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 bone marrow and peripheral blood (very low *EVI1* expression) (*data not shown*). These results indicate that aberrant hypomethylation of the promoter of *EVI1* is not the mechanism of *EVI1* over-expression; 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 regions were hypermethylated in *EVI1*$^-$/*MDS1EVI1*$^-$ cell lines (Figure 2C). However, we observed no significant changes in the methylation status of the *EVI1*-island 1 before or after 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 had enrichment of the active H3K4me3 pattern, while NOMO-1, MV4-11 and OCI-AML2 had the opposite signature, a mark of the 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 treatment with TSA and 5-Aza (*Online Supplementary Figure S7*). We also observed an enrichment of the acetylation of histones H3 and H4, especially H3, in HEL and TF-1 (Figure 3B), and chromatin immunoprecipitation analysis of the *EVI1* promoter showed enrichment of acetylated histones H3 and H4 in treated cell lines (Figure 3C). The enrichment of the active marks both in cell lines with *EVI1* over-expression and treated cell lines, strongly suggests that histone acetylation might play a role in *EVI1* expression regulation. Regarding

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

		N. Cases	N. *EVI1*-	N. *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%)	
Complete remission		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%)	
Secondary AML		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 over-expression		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-internal tandem duplication		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*

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 in either 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* over-expression is an adverse prognostic factor in AML patients, not always restricted to 3q26 aberrations. Notably, we found 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 the prevalence of *EVI1* over-expression and its adverse prognostic outcome in AML in an independent large cohort.[8,10,17] For the first time, we included quantification and survival analysis of the *EVI1-1C* 5'-end variant, and found that in younger AML patients over-expression of this transcript was a poor prognostic marker with regards to both overall survival (P=0.005) and event-free survival (P=0.008) (Figure 1 and *Online Supplementary Figure S5*), suggesting that this variant could be a genetic marker in this subgroup. However, the correlation could not be confirmed in multivariate analysis. The significant impact of *EVI1* over-expression on overall survival in a multivariate analysis was shown only in the two largest studies – by Lugthart *et al.* for *EVI1-1A* and *EVI1-1B*[10] and by Groschel *et al.* for *EVI1/MDS1EVI1* – which did not discriminate *EVI1* from *MDS1EVI1* (*Online Supplementary Table S1*);[17] it is, therefore, possible that our sample size was not large enough to give statistically significant results. Of note, we found that younger AML patients with no *EVI1* expression had a significantly better outcome than patients with either *EVI1* expression or over-expression (Figure 1), although this could not be confirmed in multivariate analysis. To our knowledge, this is the first time this finding has been reported. Further studies in independent cohorts are needed to confirm the importance of this result.

We and others have shown the association between *EVI1* over-expression and other specific cytogenetic aber-

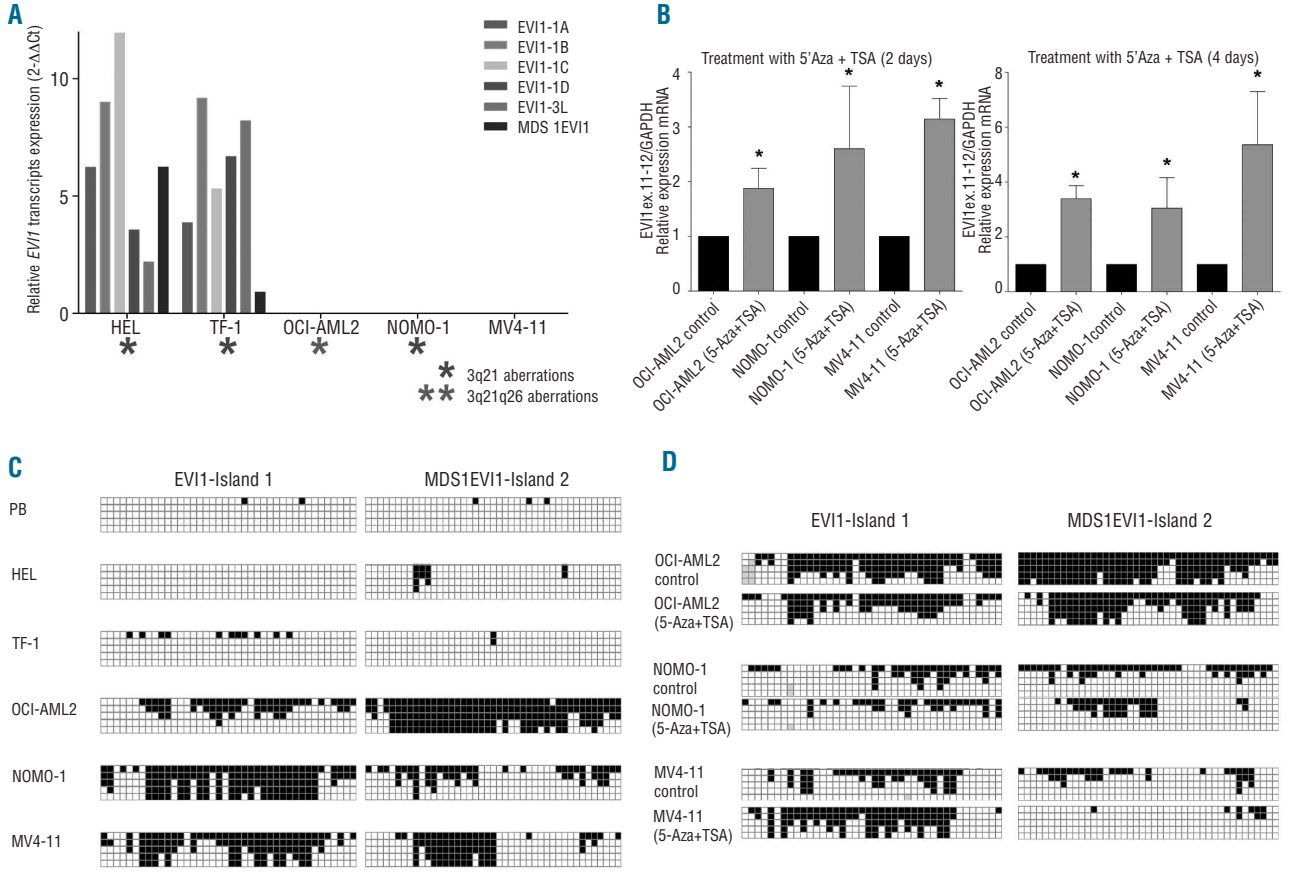

Figure 2. Analysis of the epigenetic status of the *EVI1* locus in five myeloid cell lines. (A) Quantification of the relative expression of the *EVI1* splice variants, with bone marrow as the control sample. (B) Quantification of the relative expression of *EVI1* (*EVI1* 11-12) after treatment with 5'Aza and TSA. Statistical significance was estimated using the non-parametric Wilcoxon's 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).

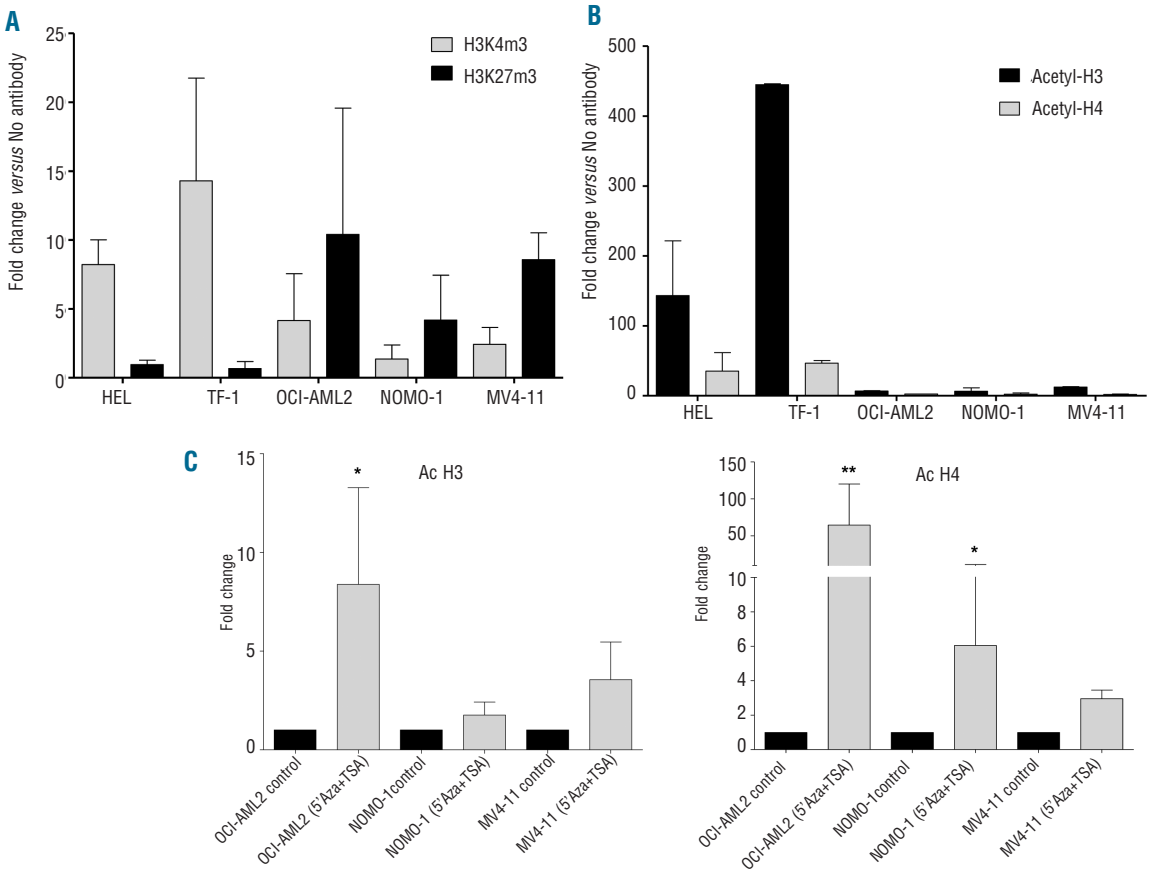

Figure 3. Analysis of the epigenetic status of the histones of the *EVI1* locus in five myeloid cell lines. (**A**) Quantitative real-time RT-PCR performed on fragmented chromatin, showing the enrichment of trimethylation of histone H3 lysine 4 (H3K4me3) and histone H3 lysine 27 (H3K27me3) on the *EVI1* promoter. (**B**) Quantitative real-time RT-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**) Quantitative real-time RT-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 and without the treatment. Statistical significance was estimated using the non-parametric Wilcoxon's matched pairs test; $P<0.05$ was considered significant (*), and $P<0.01$ strongly significant (**).

rations such as *MLL* rearrangements and monosomy 7 (Table 1).[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* over-expression induces a myelodysplastic syndrome 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 myelodysplastic syndrome;[27] however, we found no mutations of *RUNX1* in a series of 46 cases with *EVI1* over-expression (*data not shown*), suggesting that this mechanism is not frequent in human AML. Finally, we found an inverse correlation between *EVI1* over-expression 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* over expression. Quantitative real-time RT-PCR and FISH of 16 cell lines and a series of samples from patients with myeloid malignancies confirmed that *EVI1* over-expression is associated with 3q26, although sometimes occurs irrespective of 3q rearrangements (*Online Supplementary Table S8* and *Online Supplementary Figure S6*).[9,10] Moreover, the prevalence of *EVI1* over-expression among the patients with different categories of 3q abnormalities is similar to that found in another recent study.[29] Interestingly, we showed that FISH breakpoints in cases with 3q26 and *EVI1* over-expression were located telomeric to *MDS1EVI1* (*Online Supplementary Table S8*), a hotspot locus for retroviral insertions,[26] which suggests that disruption of this region is of the foremost importance in the regulation of *EVI1* transcription. We also demonstrated that the EVI1 protein is present even if only one *EVI1* transcript is over-expressed. As an exception, MEG-01 had over-expression 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, also with low levels of *EVI1* expression, the protein could be detected. Another explanation might be that the accumu-

lation and degradation of the protein in these cell lines is different. Furthermore, in our study we identified a novel alternatively spliced *MDS1EVI1* that, together with the previously described *EVI1* transcripts, might codify to the same sized protein EVI1-FL. However, it is difficult to know whether all these transcripts are used or not because all cell lines with EVI1-FL protein express more than one transcript, and we did not find any association between any specific transcript and the protein. This highlights that the mechanism of EVI1 protein regulation is complex and still to be elucidated. Nevertheless, the fact that the EVI1 protein is present even if only one *EVI1* transcript is over-expressed supports the importance of the detection of *EVI1* expression status at diagnosis in AML patients, as indicated by the new World Health Organization classification.[30] Moreover, AML cell lines over-expressed transcripts *-1A*, *-1B*, *-1C*, and *-1D*, whereas cell lines with CML-BP had only *EVI1-1B* over-expression as a common feature. This might indicate that the mechanisms of *EVI1* over-expression may depend on the action of different transcription factors in the promoter of this gene, opening directions for future studies.

In order to investigate other mechanisms of *EVI1* over-expression, we analyzed the role that epigenetic modifications could have in the regulation of the *EVI1* gene. The loci showed no methylation in either $CD34^+$ progenitor cells (high *EVI1* expression) or normal bone marrow and peripheral blood 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 process of differentiation of hematopoietic cells, and that *EVI1* promoter hypomethylation cannot be the mechanism of *EVI1* over-expression. However, we detected the absence of normal basal expression of *EVI1* and *MDS1EVI1* in patients' samples and cell lines, and several cell lines had 3q rearrangements and no *EVI1* over-expression; we, therefore, hypothesized that epigenetic aberrations could play 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 especially of H3 in the HEL and TF-1 cell lines which over-express *EVI1*. Of note, treatment of cell lines with no *EVI1* expression induced expression of this gene and increased acetylation of both histones 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 support the results of recent studies in which it was observed that in AML there is an inverse correlation between DNA methylation and the H3K4 trimethylation pattern compared with unmethylated samples.[31-33] The epigenetic modifications H3K4me3 and H3K27me3 are of particular interest as these modifications are catalyzed by trithorax and polycomb-group proteins, respectively, 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 non-coding elements in mammalian genomes cluster within regions enriched for genes encoding developmentally important transcription factors, such as *EVI1*,[34] suggesting that these transcription factors could have key epigenetic regulatory roles 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 observed in our *EVI1*-expressing cell lines, suggesting that this mechanism is involved in its regulation in early hematopoietic cells. Our results support the concept 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 patients' 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 over-expression (Figure 1). Nevertheless, prospective studies are needed to clarify the role of histone modifications in *EVI1* regulation.

In summary, our results confirm that *EVI1* over-expression is an adverse prognostic factor in AML, and corroborate the necessity of quantifying *EVI1* and *MDS1EVI1* expression during the diagnosis of AML in younger patients, mostly in cases with 3q aberrations, monosomy 7, *MLL* rearrangements, and in the subgroup with a normal karyotype and no *NPM1* mutations. Notably, we found that the total absence of *EVI1* expression may be associated with a better outcome in 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

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