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

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Online Supplementary Design and Methods

Material

Samples obtained at diagnosis from 476 patients with acute myeloid leukemia (AML), 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 was approved by the Ethics Committee for Research with Human Subjects, (037/2008). 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.[1] Samples were taken anonymously.

Quantitative real-time reverse transcriptase polymerase chain reaction

Two micrograms of total RNA isolated from cell pellets with the RNeasy Mini Kit (Qiagen, Germany) were used for cDNA synthesis (SuperScript™II RNase HRT; Invitrogen, CA, USA). Gene expression was quantified with an ABI Prism 7,500 (Applied Biosystems, CA, USA) with 20 ng of cDNA. Quantitative real-time reverse transcriptase-polymerase chain reaction (RT-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), which includes all the *EVI1* transcripts; and *GATA2* (Hs00231119_m1). Specific assays-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 *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.[2] Over-expression of *EVI1* was defined when the level of at least one *EVI1* transcript was higher than the average and three times the standard deviation of seven bone marrow 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.[3] The panel was developed to quantify the DNA methylation status of 27,578 CpG sites located within the proximal promoter regions (1 kb upstream and 500 bp downstream of transcription start sites) of 14,475 well-annotated genes. Briefly, genomic DNA was 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 of the M beads to the total locus fluorescence intensity. The beta value is a quantitative measure of DNA methylation levels of specific CpG, and ranges from 0 (completely unmethylated) to 1 (completely methylated). The methylation status of the CpG islands of *EVI1* (island 1 and 2) and *MDS1EVI1* (island 1 and 2) were analyzed by bisulfite sequencing PCR (*Online Supplementary Table S3*). DNA modification was performed 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 with $10x10^6$ 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 dimethyl sulfoxide and glacial acetic acid.

Chromatin immunoprecipitation

HEL, TF1, OCI-AML2, NOMO-1 and MV4-11 cell lines were subjected to chromatin immunoprecipitation (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.[4] Ten million cells were cross-linked with 1% formaldehyde for 10 min, and then 0.125 M glycine

was used to stop the reaction. Subsequently, chromatin was fragmented by sonication to obtain an average fragment length of 200-900 bp (Bioruptor Diagenode, Belgium). Antibodies used were antiacetyl- 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 quantitative RT-PCR, using specific primers for *EVI1* and *MDS1EVI1* promoter regions (*Online Supplementary Table S3*). PCR results were calculated using the ΔΔCt method. They are presented as the fold enrichment of chromatin DNA precipitated by the specific antibody *versus* chromatin DNA precipitated by no antibody, as the 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 1 mM $NaVO_4$ (Sigma, MO, USA), and the concentration was determined using Bradford's method prior to each use (Bio-Rad Laboratories, Inc., CA, USA). Western blot analysis was carried out with 50 μg of total proteins electrophoresed on 10% Tris/Glycine SDS-polyacrylamide solution gel, and transferred to a nitrocellulose membrane. Anti-Evi1 antibody (Cell Signaling, MA, USA) and antilamin A/C antibody were used (Cell Signaling, 2032). Detection was performed with phosphatase alkaline-conjugated anti-rabbit Ig (Sigma, MO, USA), and enhanced chemiluminescence (Amersham Pharmacia Biotech, GE Healthcare, Sweden).

References

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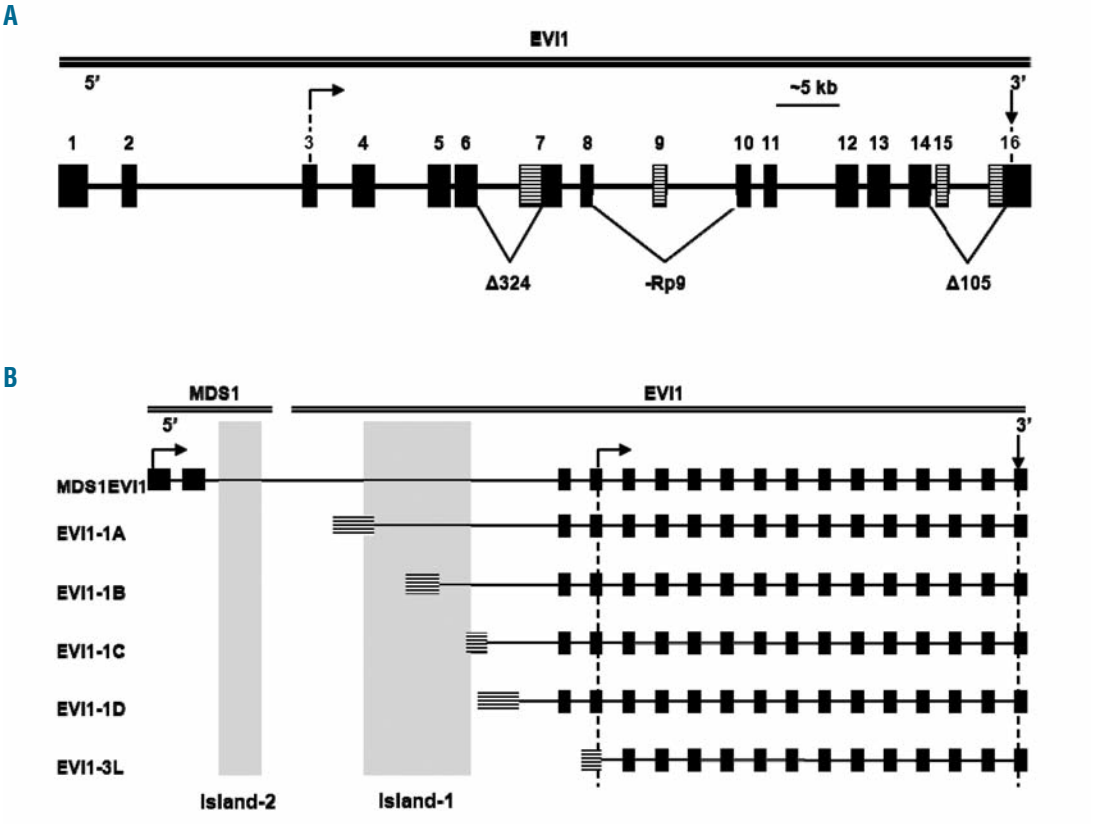
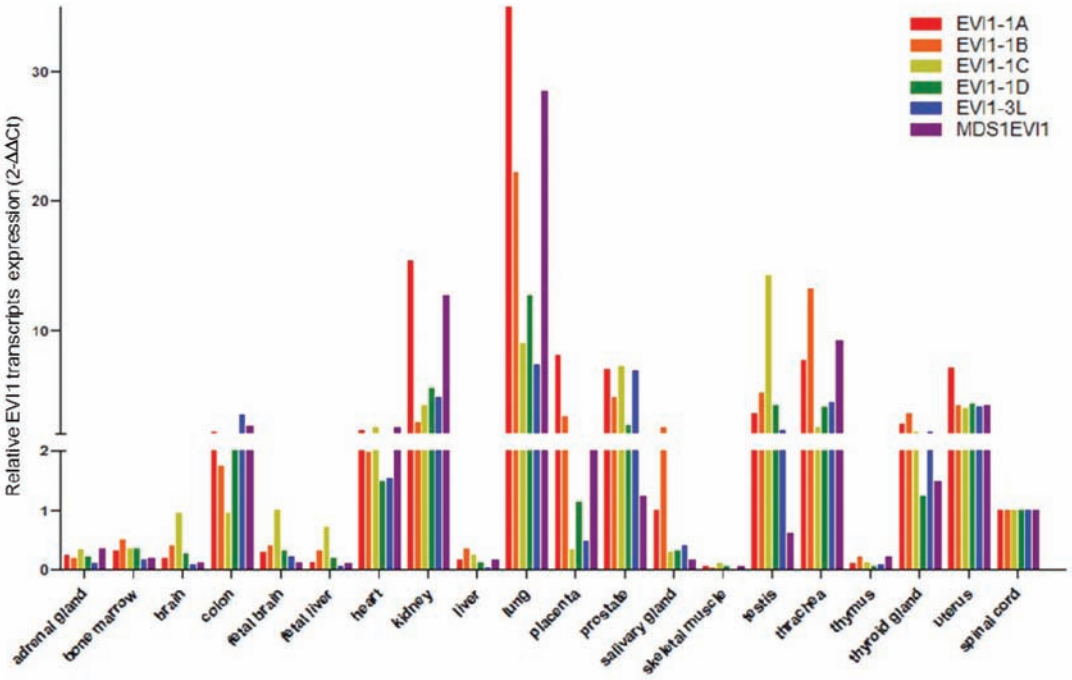
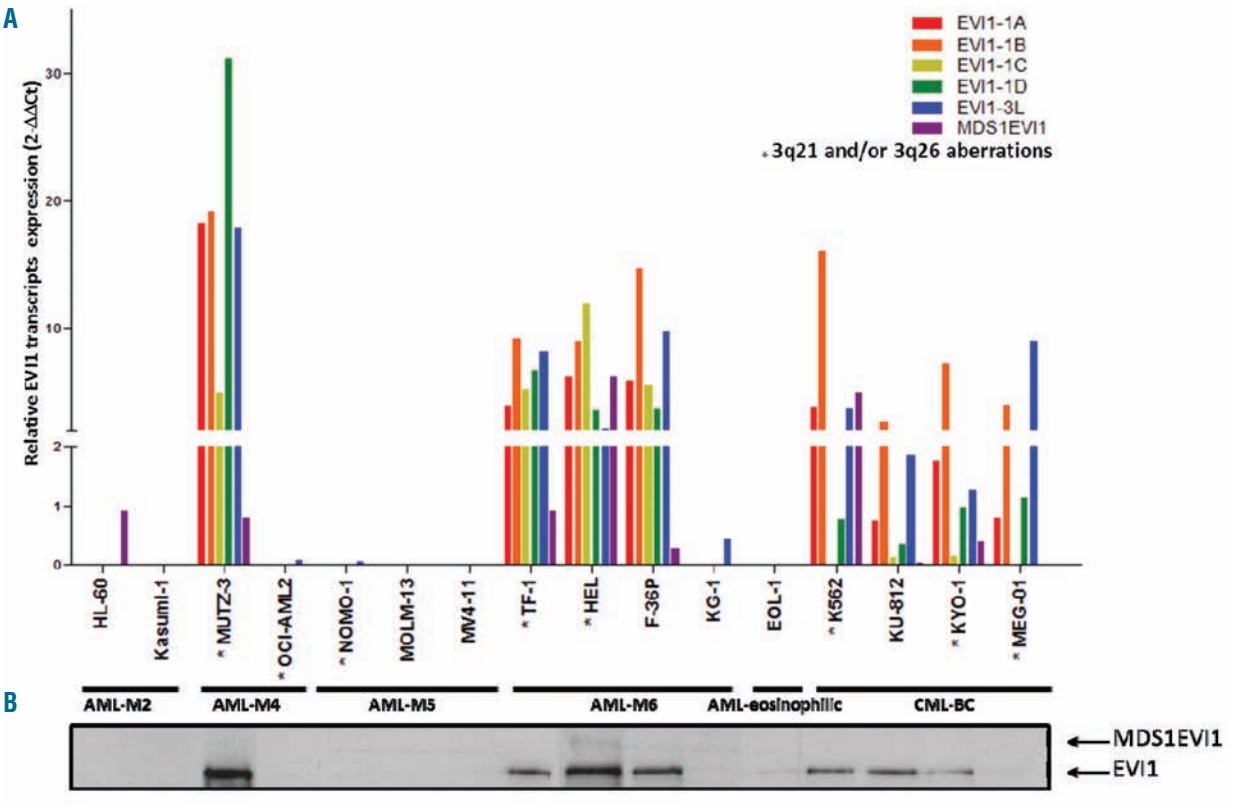
4. Paul TA, Bies J, Small D, Wolff L. Signatures of polycomb repression and reduced H3K4 trimethylation are associated with p15INK4b DNA methylation in AML. Blood. 2010;115 (15):3098-108.



Online Supplementary Figure S1. 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 shading in gray represents the relative position of the *EVI1* CpG islands. (Adapted from Wieser 2007, and Lugthart *et al.* 2008).



Online Supplementary Figure S2. Quantification of the *EVI1* 5'-end variants in normal tissues. (Expression levels were normalized to spinal cord).



Online Supplementary Figure S3. Analysis of *EVI1* expression in 16 myeloid cell lines. (A) Quantification of the expression levels of *EVI1* 5'-end variants. Expression levels were normalized to normal bone marrow (B) Western blot analysis of *EVI1* and *MDS1EVI1*.

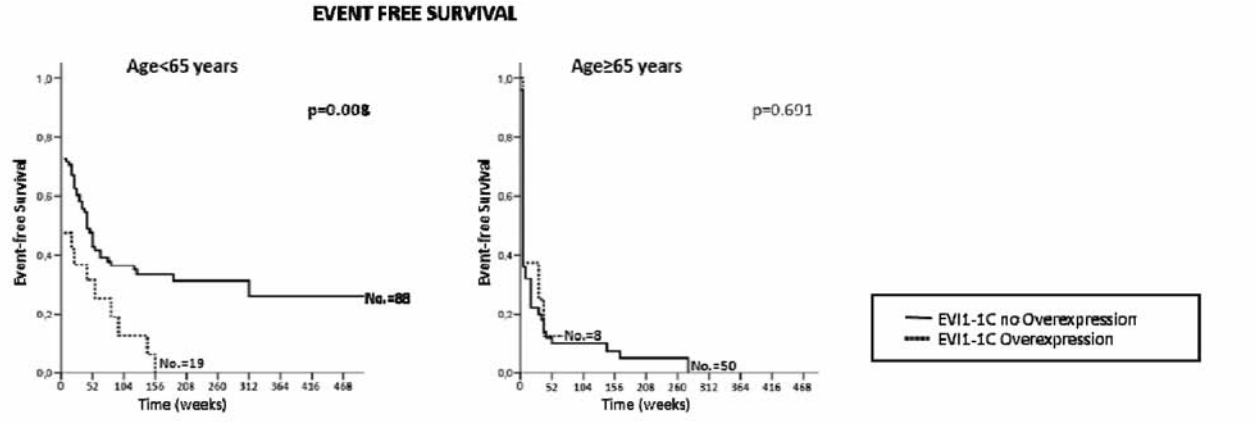
MDS1EVI1 splicing (exon 2 of MDS1 - exon 2 of EVI1)

GCGCATGTGCAAGGTGTCCAAACTGACAATGCTGGAGAGATAGCGAGTGTGGATTGAGAGAAAGGGAGAGAG
GGAGGGAGAGAGAGTGAAAGAAGAAAATACAGAGAGTGAGTGTGTGGAAGAGAGAGAGAAACAGGAGAGAAA
CAGGAGGGGGGGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGACAGGAGAGAGA
GGGAGGGAGCGAGAGGGAGAGCAAAAGAAGGAAAGGATCCAAGAAAAAAAAGCCCCAACCACACACCAGAGG
CTGCAGGACTGGGCACAGCATGAGATCCAAAGGCAGGGCAAGGAAACTGGCCACAAATAATGAGTGTGTATA
TGGCAACTACCCTGAAATACCTTTGGAAGAAATGCCAGATGCAGATGGAGTAGCCAGCACTCCCTCCCTCAA
TATTCAAGAGCCATGCTCTCCTGCCACATCCAGTGAAGCATTCACTCCAAAGGAGGGTTCTCCTTACAAAGC
CCCCATCTACATCCCTGATGATATCCCCATTCCTGCTGAGTTTGAACTTCGAGAGTCAAATATGCCTGGGGC
AGGACTAGGAATATGGACCAAAAGGAAGATCGAAGTAGGTGAAAAGTTTGGGCCTTATGTGGGAGAGCAGAG
GTCAAACCTGAAAGACCCCAGTTATGGATGGGAGATCTTAGACGAATTTTACAATGTGAAGTTCTGCATAGA
TGCCAGTCAACCAGATGTTGGAAGCTGGCTCAAGTACATTAGATTCGCTGGCTGTTATGATCAGCACAACCT
TGTTGCATGCCAGATAAATGATCAGATATTCTATAGAGTAGTTGCAGACATTGCGCCGGGAGAGGAGCTTCT
GCTGTTCATGAAGAGCGAAGACTATCCCCATGAAACTATGGCGCCGGATATCCACG

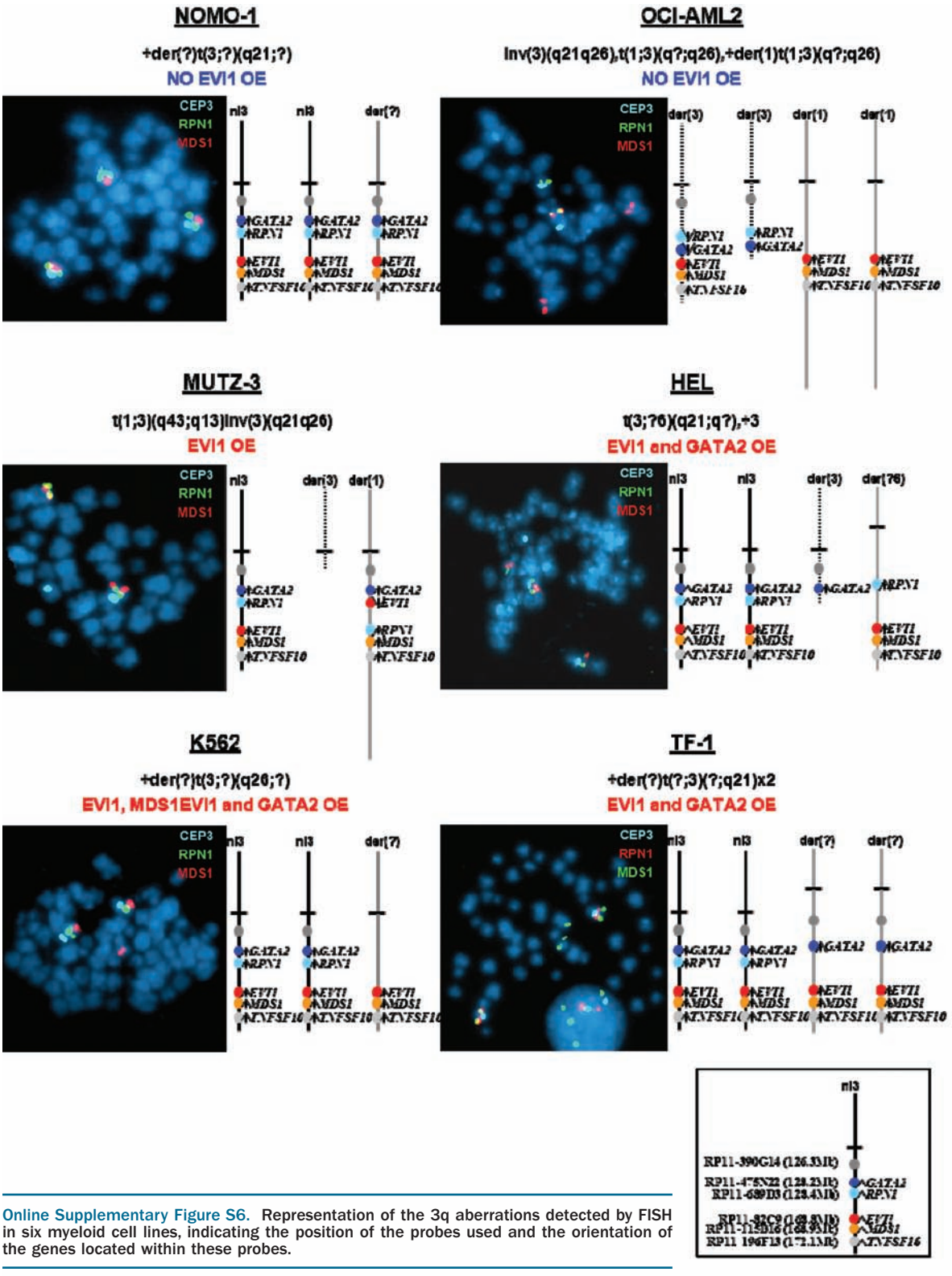
MDS1EVI1 alternative splicing (exon 1 of MDS1 - exon 2 of EVI1)

GCGCATGTGCAAGGTGTCCAAACTGACAATGCTGGAGAGATAGCGAGTGTGGATTGAGAGAAAGGGAGAGAG
GGAGGGAGAGAGAGTGAAAGAAGAAAATACAGAGAGTGAGTGTGTGGAAGAGAGAGAGAAACAGGAGAGAAA
CAGGAGGGGGGGGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGACAGGAGAGAGA
GGGAGGGAGCGAGAGGGAGAGCAAAAGAAGGAAAGGATCCAAGAAAAAAAAGCCCCAACCACACACCAGAGG
CTGCAGGACTGGGCACAGCATGAGATCCAAAGGCAGGGCAAGGAAACTGGCCACAAATCTTAGACGAATTTT
ACAATGTGAAGTTCTGCATAGATGCCAGTCAACCAGATGTTGGAAGCTGGCTCAAGTACATTAGATTCGCTG
GCTGTTATGATCAGCACAACCTTGTTGCATGCCAGATAAATGATCAGATATTCTATAGAGTAGTTGCAGACA
TTGCGCCGGGAGAGGAGCTTCTGCTGTTCATGAAGAGCGAAGACTATCCCCATGAAACTATGGCGCCGGATA
TCCACG

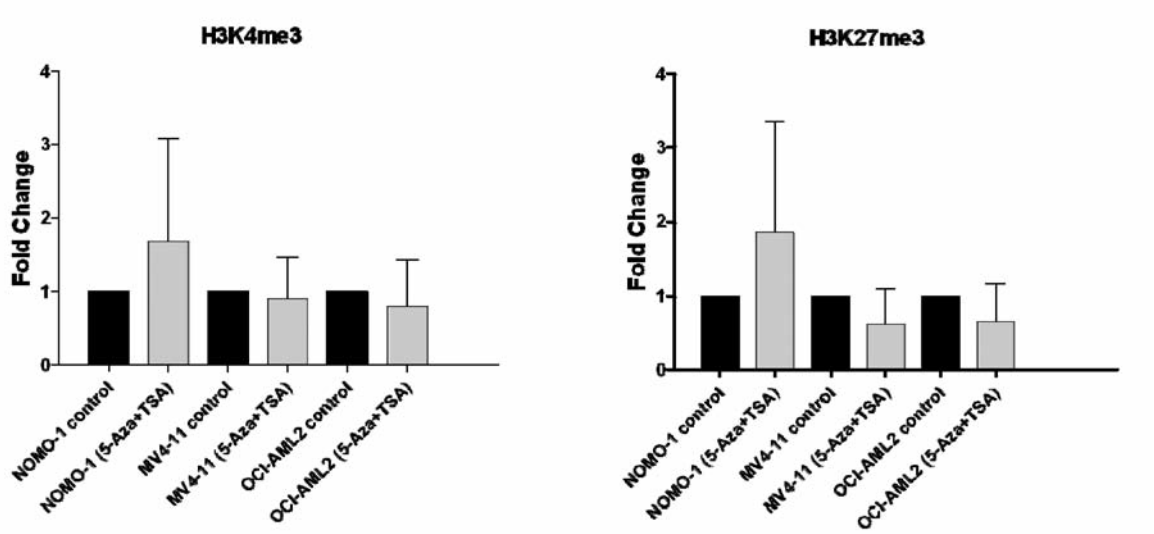
Online Supplementary Figure S4. Analysis of the *MDS1EVI1* PR domain. Sequences of alternative splicings of *MDS1EVI1*. The previously described intergenic splicing between *MDS1* (exon 2) and *EVI1* (exon 2), and the novel alternative splicing between *MDS1* (exon 1) and *EVI1* (exon 2). Different exons are show by different colors; the sequence of *MDS1* is underlined.



Online Supplementary Figure S5. *EVI1*-1C over-expression is associated with poor survival in AML patients <65 years. In Kaplan-Meier analysis stratified by age, patients <65 years and with *EVI1*-1C over-expression have an inferior event-free survival in comparison to that of patients with no *EVI1*-1C over-expression.



Online Supplementary Figure S6. Representation of the 3q aberrations detected by FISH in six myeloid cell lines, indicating the position of the probes used and the orientation of the genes located within these probes.



Online Supplementary Figure S7. Analysis of the histone methylation of the *EVI1* locus in three myeloid cell lines after treatment with 5-Aza and TSA. (A) Quantitative real-time RT-PCR performed on fragmented chromatin, showing the levels of trimethylation of histone H3 lysine 4 (H3K4me3) and histone H3 lysine 27 (H3K27me3) on the *EVI1* promoter before and after the treatment. The results were calculated using the ΔΔCt method. They are presented as the fold enrichment of chromatin DNA precipitated by the specific antibody *versus* chromatin DNA precipitated by no antibody, as the control, and comparing with or without the treatment.

Online Supplementary Table S1. Review of the literature of large series of AML adult patients for whom the prevalence and prognostic value of *EVI1* overexpression was reported.

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)

Online Supplementary Table S2. 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-offs calculated from seven normal bone marrow samples and three times the standard deviation; * not confirmed by FISH analysis.*

Online Supplementary Table S3. 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	TTTGTTTAAGTTTTTTTAATTTTTTT	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.

Online Supplementary Table S4. 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-NOS: acute myeloid leukemia, not otherwise specified; Over-expression is highlighted in bold; with the cut-offs calculated from seven normal bone marrow and three times the standard deviation.

Online Supplementary Table S5. 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 samples from AML patients and 16 myeloid cell lines in which the EVI1 transcripts had been measured. $P<0.05$ (*); $P<0.001$ (**)

Online Supplementary Table S6. Multivariate analysis of *EVI1*-1C over-expression 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

[1]*Other 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. HR: hazard ratio; CI: confidence interval.*

Online Supplementary Table S7. Multivariate analysis of *EVI1* expression groups (patients with no basal expression *versus* patients with expression/over-expression) 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. HR: hazard ratio; CI: confidence interval.*

Online Supplementary Table S8. 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.*