Association of 3q21q26 syndrome with different *RPN1/EVI1* fusion transcripts

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Background and Objectives. Patients with acute myeloblastic leukemia (AML) with features of myelodysplastic syndrome and abnormalities of megakaryocytopoiesis often have cytogenetic aberrations of 3q21 and 3q26 bands involving the paracentric inversion [inv(3)(q21q26)] or a reciprocal translocation [t(3;3)(q21;q26)]. These abnormalities frequently cause inappropriate expression of the *EVI1* gene located at 3q26. Other genes that have been implicated at the rearrangement breakpoint are *GR6* and *RPN1* (both on 3q21). The aim of this study was to investigate the expression of the *EVI1* fusion genes in AML patients with 3q21q26 syndrome.

Design and Methods. We used reverse transcription polymerase chain reaction to evaluate the expression of *EVI1* and *GR6*, and particularly of the fusion genes *RPN1-EVI1* and *GR6-EVI1* in 9 AML patients with either inv(3)(q21q26) (7 cases) or t(3;3)(q21;q26) (2 cases).

Results. *EVI1* and *GR6* were always expressed, as was *RPN1-EVI1*; *GR6-EVI1* was absent. In 8/9 patients, the part of *EVI1* retained in *RPN1-ΔEVI1* contained blocks B and C of the PR domain commonly found in the *MDS1-EVI1* gene. In the remaining patient [with inv(3)(q21q26)], only block C was retained: we named this variant fusion gene *RPN1-ΔEVI1*. This patient lacked the micromegakaryocytopoiesis frequently found in 3q21q26 syndrome.

Interpretation and Conclusions. These findings support the hypothesis that *EVI1* activation plays a dominant role in the pathogenesis of the 3q21q26 syndrome. *EVI1* expression might occur either as a consequence of rearrangements leading to the formation of different fusion transcripts, such as *RPN1-EVI1* and *RPN1-ΔEVI1* or following disruption of the PR activation domain of the *MDS1-EVI1* gene.

Key words: AML, *RPN1-EVI1*, PR domain, 3q21q26 syndrome.

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Specific chromosomal abnormalities involving bands 3q21 and 3q26 have been observed in all FAB subtypes of acute myeloid leukemia (AML) (but in subtype M3 only as a second event),[1,2] in myelodysplastic syndrome (MDS),[3] and in the blastic phase of chronic myeloid leukemia (CML).[4] The rearrangements of 3q encountered in AML are the paracentric inversion inv(3)(q21q26) and a reciprocal translocation t(3;3)(q21;q26).[5] A recurrent translocation or inversion between the regions of 3q21 and 3q26 gives rise to the so-called 3q21q26 syndrome, which is found in 0.5%–2% of adult patients with MDS or AML.[6-8] This syndrome is accompanied by specific, but not invariable, clinical features including: normal or elevated platelet counts at the initial diagnosis, hyperplasia with dysplasia of megakaryocytes, poor response to chemotherapy, and poor prognosis.[9,10]

The 3q26.2 chromosome band contains *EVI1*, which was originally identified as a retroviral integration site leading to myeloid tumors in susceptible strains of mice.[11,12] The *EVI1* gene, which is highly conserved through evolution, encodes a nuclear DNA-binding protein with two domains containing two sets of seven and three repeats of Cys_2Hys_2 type of zinc finger motif. During murine embryogenesis, *EVI1* is expressed in many organs[13] and is involved in organogenesis.[14] In cell lines, *EVI1* expression inhibits terminal myeloid differentiation induced by granulocyte colony-stimulating factor (G-CSF) or erythropoietin.[15-17]

The breakpoints of t(3;3)(q21;q26) have been mapped approximately 10-330 kb upstream of the *EVI1* gene, while those of inv(3)(q21q26) are mainly downstream of the *EVI1* gene coding region,[18] suggesting that one or more genes involved in inappropriate *EVI1* activation are probably located at 3q21. The molecular cloning and analysis of the breakpoint junctions has implicated the *RPN1*[15,16,19] and *GR6*[19] genes as fusion partners of *EVI1*. In normal tissues, *EVI1* is also found in a longer isoform, named *MDS1-EVI1*, which encodes a protein with an additional proximal extension of 188 amino acids. This extension results from the splicing of most of the *MDS1* gene to the second exon of *EVI1*, which is not translated in *EVI1*. The protein extension present in MDS1-EVI1 has homology to the PR domain of several zinc finger proteins that appear to function as negative regulators of tumorigenesis.[20] In addition to MDS1-EVI1, other members of this protein family include PRDI-BF1/BLIMPI (a transcription repressor of c-MYC)[21] and RIZ (which binds to RB, the retinoblastoma tumor-

Table 1. Morphologic and clinical features of patients at diagnosis.

Pts.	*Sex/age*	*Hb g/dL*	*WBC ×10⁹/L*	*Plts ×10⁹/L*	*% Peripheral Blast Cells*	*Previous MDS*	*FAB*	*Micro MKC*	*Dysmyelo-poiesis*	*Dyserythro-poiesis*
1.	F/45	9.8	59.0	304	50	NO	M1	++	–	–
2.	M/42	11.1	20.4	64	60	RAEB	M1	++	++	+
3.	M/42	8.6	13.3	225	19	NO	M1	+++	++	+
4.	M/52	6.4	2.1	142	28	RAEB	M1	+++	++	++
5.	M/36	4.7	1.3	92	19	NO	M1	+	+	+
6.	M/53	6.5	23.4	56	90	NO	M1	–	–	–
7.	F/26	8.3	7.7	230	37	RAEB	M1	+++	++	–
8.	F/43	8.3	6.2	249	26	NO	M1	+++	–	–
9.	F/41	5.3	18.3	26	76	NO	M1	+++	–	–

WBC: white blood cell count; Olts: platelets; MDS: myelodysplastic syndrome; RAEB: refractory anemia with excess of blasts; Micro MKC: micromegakaryocytes; abnormal and elevated number of mostly mononuclear megakaryocyte-megakaryoblasts, all of which show evidence of deficit of cytoplasmic maturation and granulation ("blue-gray granulated cytoplasm").

Table 2. Karyotype and molecular analysis of patients at diagnosis.

Patient	*Karyotype*
1.	46,XX,inv(3)(q21q26) [15/15]
2.	45,XY,inv(3)(q21q26), -5,del(7)(q22q34)[22/23]
3.	45,XY,inv(3)(q21q26),-7 [17/17]
4.	45,XY,t(3;3)(q21;q26),-7 [12/14]
5.	45,XY,t(3;3)(q21;q26),-7 [21/23]
6.	45,XY,inv(3)(q21q26),-7 [52/52]
7.	45,XY,inv(3)(q21q26),-7, del(4)(p15) [30/30]
8.	46,XX,inv(3)(q21q26) [31/32]
9.	46,XX,inv(3)(q21q26) [30/30]

suppressor protein).[22-25] In general, the PR domain contains three blocks of homology, block A, B and C, which are conserved among the members of the family.[21] The PR domain of *MDS1-EVI1* has a complex genomic structure. The first block of homology, block A, is derived from the small *MDS1* gene located about 300 kb upstream of *EVI1*. Blocks B and C are encoded by exon 2 and part of exon 3 of *EVI1* upstream of the translation start site of *EVI1* in exon 3. The chromosomal breakpoints at 3q26 usually disrupt the region encoding the PR domain of MDS1-EVI1 and therefore result in a transcript which still maintains the region encoding block B and C but which has lost the upstream translation start site.[19,20,26]

To investigate the expression of the *EVI1* fusion genes in 3q21q26 syndrome, we used reverse transcription polymerase chain reaction (RT-PCR) in nine AML patients with either inv(3)(q21q26) (7 cases) or t(3;3)(q21;q26) (2 cases). In all but one patient *EVI1* was transcribed from the RPN1 promoter as an *RPN1-EVI1* fusion gene containing the first exon of *RPN1* spliced in frame to *EVI1* (*RPN1* was spliced to exon 2 of *EVI1*). We discuss the role of *RPN1-EVI1*, including this novel variant form, in a cohort of cytogenetically defined patients with abnormalities of 3q26. Despite our series of AML patients having 1) only FAB M1 subtype, and some of them having 2) low platelets counts and 3) not pre-existing MDS aspects, we will refer to these patients as affected by *3q21q26 syndrome*.

Design and Methods

Patients and cytogenetic studies

Among the 416 cases of AML that have been cytogenetically studied at our institution, 14 (2%) displayed inv(3)(q21q26) or t(3;3)(q21;q26) at diagnosis. Morphologic assessment of AML was made according to the French-American-British (FAB) classification.[27] Here, we consider the 9 patients with inv(3)(q21q26) (7 cases) or t(3;3)(q21;q26) (2 cases) for whom cryopreserved bone marrow samples from the time of diagnosis were available for molecular studies (Table 1 and Table 2). Karyotyping and G-

banding with Wright's stain were performed after a short-term culture (24-48 h) without stimulation, as described elsewhere.[28] Karyotypic descriptions follow the recommendations of the International System for Human Cytogenetic Nomenclature.[29]

Molecular studies

Samples and RNA isolation

Mononuclear cells from samples were obtained by Ficoll-Hypaque density gradient centrifugation and were stored at -80°C in guanidinium thiocyanate. Extraction of total RNA and its qualitative and quantitative controls were performed as previously described.[30]

RT-PCR analysis

The random primer-based RT assay was performed as previously reported.[31] Expression of *RPN1-EVI1*, *GR6-EVI1*, *EVI1* and *GR6* was detected by PCR using previously described sets of primers and PCR conditions.[19,32,33] The HL60 cell line was used as a negative control for the *RPN1-EVI1* fusion transcript. As a positive control, we used the cDNA of patient #1, in which the *RPN1-EVI1* fusion transcript was confirmed by sequencing.

Nucleotide sequencing

The PCR products were gel purified and cloned into the pCR2.1 vector using a TA cloning kit (Invitrogen, Milan, Italy) according to the manufacturer's directions. Sequencing was performed using an Applied Biosystems (Monza, Italy) model 373A automated DNA sequencer using dye terminator reactions.

Computer analysis

Translation computer analysis was performed using Translate Tool (*www.expasy.ch/tools/dna.html*). The protein identity search was performed using PSI-BLAST at NCBI (*www.ncbi.nlm.nih.gov*).

Results

Clinical and cytogenetic characteristics of the patients

The clinical and cytogenetic characteristics of the 9 patients are summarized in Tables 1 and 2, respectively. Leukemic cells from all patients had a myeloid phenotype with a similar stage of maturation and were classified as M1 according to the FAB classification. Three patients (#2, 4 and 7) who were initially diagnosed as having MDS developed AML within 6 months. All but one (#9 in Table 1) patient showed normal or only mildly (#2 and 6) reduced platelet counts. All patients had inv(3) or t(3;3). In addition, 5 patients had monosomy 7, one had del(7) and monosomy 5, and another had del(4) (Table 2). All patients responded poorly to chemotherapy. All but one failed to achieve remission after induction chemotherapy. Patient #5 achieved complete hematologic and cytogenetic remission after allogeneic bone marrow transplantation from an HLA-matched unrelated donor. The median disease-free and overall survival was 0.5 months (range 0-18) and 16.5 months (range 6-28), respectively.

Detection of EVI1, GR6 and RPN1-EVI1 transcripts in all 9 patients

RT-PCR analysis of all 9 cases showed two amplified fragments of 254 and 281 bases, corresponding to the two previously reported splice forms of *EVI1*.[33] In addition, in all cases we observed a third band which appeared as about 120 bp (shown in Figure 1a, lanes 6 and 7). Sequence analysis of this third band revealed it to be an artefact, probably due to not specific annealing of primers. *GR6* expression (280 bp band in Figure 1b) was detected in all samples. However, in contrast to the findings of Pekarsky *et al*.,[19] we were unable to detect any type of fusion transcript between *GR6* and *EVI1*.[19] On the other hand, *RPN1-EVI1* fusion transcripts were found in all 9 cases. Eight cases showed a 324 bp amplified fragment containing the junction between exon 1 of *RPN1* and exon 2 of *EVI1*. This result was confirmed both by digestion with the restriction endonuclease Hae III, which yielded the expected three fragments of 192 bp, 84 bp, and 48 bp (Figure 1C), and also by sequence analysis (*data not shown*).

Recognition of a novel RPN1-EVI1 fusion transcript in one case

In one patient (#6), a fragment of 189 bp instead of 324 bp was detected after amplification of the *RPN1-EVI1* fusion junction (Figure 1D). Sequence analysis showed that this smaller cDNA fragment contained the junction between nucleotide 276 of *RPN1* and nucleotide 213 of *EVI1* corresponding to the beginning of the third exon. The fusion retained the correct reading frame. We called this fusion transcript *RPN1-ΔEVI1*. Interestingly, this patient, unlike the other eight patients, did not show micromegakaryocytopoiesis. Computer analysis using Translate Tool predicted a protein product of 1160 amino acids, as compared to 1205 amino acids for *RPN1-EVI1*. The so-called *non-coding* region of *EVI1*, which in the other *RPN1-EVI1* junctions included the second exon (45 in-frame codons) and part of the third exon (18 in-frame codons), was reduced to the 18 codons upstream of the translational start site of *EVI1* in exon 3.[16] The analysis of the *RPN1-ΔEVI1* junction sequence suggests that the breakpoint in *EVI1* occurs within intron 2. This *EVI1* breakpoint has not been previously described in human leukemia with EVI1 rearrangements. However, retroviral integration sites in intron 1 and 2 of

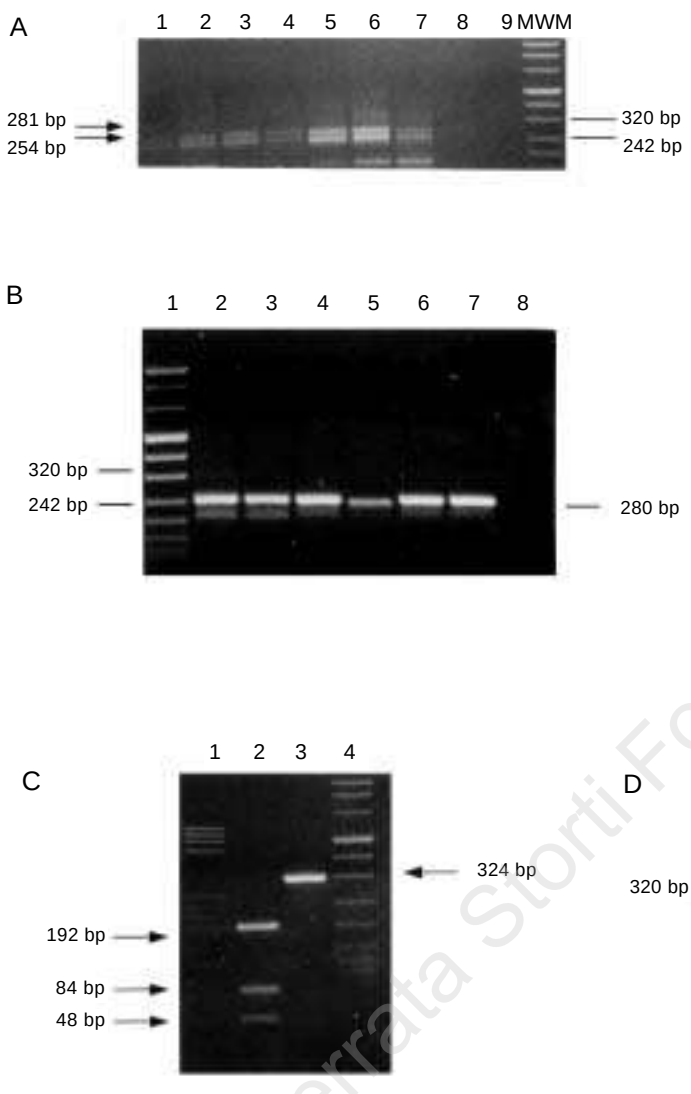

Figure 1. (A). Amplification of *EVI1* transcript. Electrophoretic separation on 2% agarose gel of the amplification products of *EVI1* transcript. Lanes 1 to 6 represent PCR products from patients' samples, lane 7 represents the positive control (patient #1); lanes 8 and 9 represent negative controls. MWM: molecular weight marker VIII from Boehringer Mannheim. Expected *EVI1* PCR products of 254 bp and 281 bp are indicated on the left side by arrows. Two MWM of 320 bp and 242 bp are indicated. (B). Amplification of *GR6* transcript. Electrophoretic separation on 2% agarose gel of the amplification of *GR6* transcript. Lane 1 represents MWM VIII as in Figure 1A, lanes 2 to 7 represent PCR products from patients' samples, lane 8 represents a negative control. At present, we have no explanation for the faint band of amplification obtained in line 5. (C). Amplification of *RPN1-EVI1* transcript. Lane 3 shows the electophoretic separation on 2% agarose gel of a 324 bp PCR product (arrow on the right side) corresponding to the *RPN1* (exon 1) – *EVI1* (exon 2) junction. The same product was HaeIII restriction digested (lane 2) and the expected three fragments of 192 bp, 84 bp and 48 bp were electrophoretically separated (arrows on the left side). Lanes 1 and 4 represent MWM V and VIII of Boehringer Mannheim. (D) Amplification of *RPN1-ΔEVI1* transcript. Electrophoretic separation, as in Figure 1C, of *RPN1-EVI1* transcript in patient #1 (lane 2) and *RPN1-ΔEVI1* transcript in patient #6 (lane 3). The expected PCR product of 324 bp was reduced (189 bp) in *RPN1-ΔEVI1* transcript, as can be seen from the arrowed portion on the right. The MWM, as in Figure 1C, is partially shown.

EVI1 leading to *EVI1* activation and leukemia have been reported in mice infected with an ecotropic retrovirus.[12]

PSI-BLAST searches

Using the PR domain peptide sequence of EVI1 as a query, we performed PSI-BLAST searches of the non-redundant protein database of the NCBI. All known PR proteins were identified. The PSI-BLAST search showed that all the RPN1-EVI1 fusion proteins maintain homology with blocks B and C of the PR domain. Significant matches were also found for the *RPN1-ΔEVI1* protein: in this case, however, the homology was limited to block C. Figure 3 shows the alignment of the PR domain region of the RIZ1, BLIMP1, MDS-EVI1, RPN1-EVI1 and RPN1-ΔEVI1 with the HRX, SET1 (yeast), Su(var)3-9 (*Drosophila*) and ASH1 (*Drosophila*) proteins.

Discussion

All nine cases of AML with abnormalities of 3q26, which we arbitrarily refer to as patients affected by *3q21q26 syndrome*, had *RPN1-EVI1* fusion transcripts. In addition, one of the cases also showed a previously non-described variant form of the fusion junction. These observations are in line with the hypothesis that leukemic transformation may

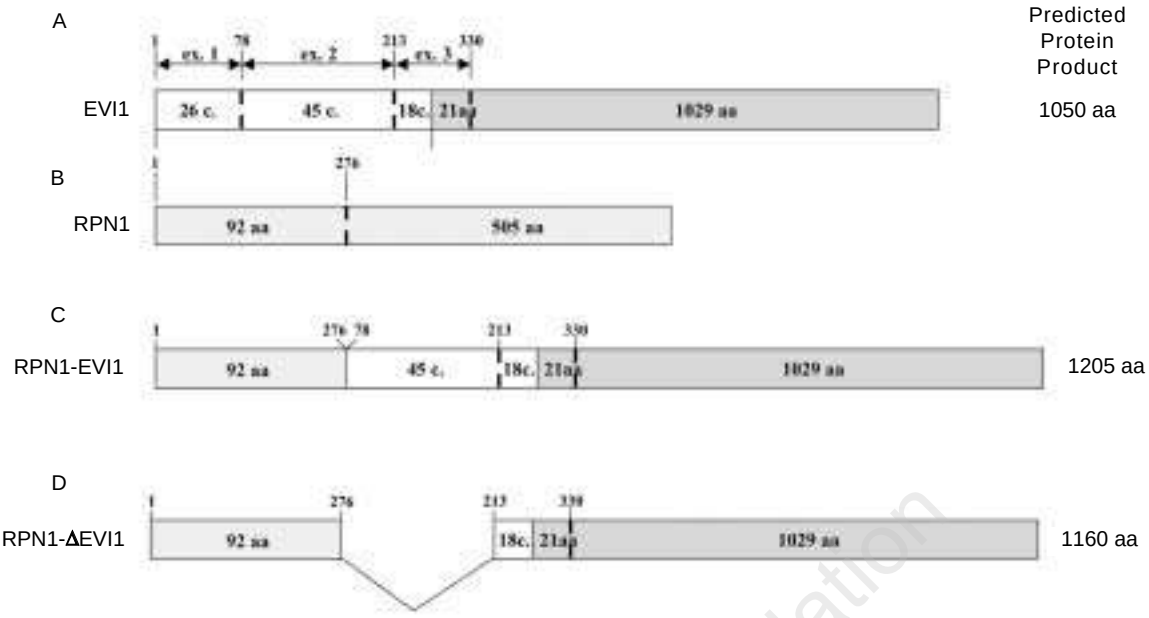

Figure 2. Schematic representation of normal *EVI1* (a) and RPN1 (b) transcripts and the 3q21-3q26-associate: *RPN1-EVI1* (c) and *RPN1-ΔEVI1* (d) fusion transcripts. Striped gray boxes correspond to non-coding parts of the *EVI1* transcript (exons 1 and 2 and part of exon 3). The dotted gray box represents the *RPN1* portion. The predicted lengths of protein (aa, amino acid) and codon (c.) are indicated inside the boxes. The numbers on the top of the bars indicate the nucleotide.

sometimes be a consequence of: 1) inappropriate expression of the normal *EVI1* gene product; and/or 2) inappropriate expression of an *EVI1* fusion gene, such as *RPN1-EVI1*; and/or 3) disruption of a normal fusion transcript containing a PR domain, such as *MDS1-EVI1*.

Regarding the first possibility, *EVI1* is not normally detected in human hematopoietic cells.[16] The open reading frame of the gene starts in the third exon where the first in-frame ATG codon is located.[16] The second exon can be activated by chromosomal rearrangements in which *EVI1* is juxtaposed to *RPN1*. Transcription of the *RPN1-EVI1* fusion gene starts from the *RPN1* promoter. It is not currently clear whether *EVI1* is also inappropriately expressed by its own promoter. Our findings strongly support the concept that inappropriate *EVI1* expression plays a primary role in the 3q21q26 syndrome. Indeed, all the blasts of our 9 patients showed aberrant expression of the *EVI1* transcript. Due to the small size of the available bone marrow samples, we were unable to perform Northern or Western blot analysis to confirm the RT-PCR results.

Leukemic transformation in AML or MDS as well as in CML in blastic phase has also been associated with the inappropriate expression of *EVI1* in other fusion genes, such as *TEL-MDS1-EVI1* or *AML1-MDS1-EVI1* resulting respectively from a t(3;12)(q26;p13) or a t(3;21)(q26;q22).[3,4,16] It is not yet known whether inappropriate *EVI1* expression is sufficient for leukemic transformation or whether the other gene partners involved in the translocation also play a role. It has been suggested that gene rearrangements associated with the 3q21q26 syndrome must carry an *element* at 3q21 into the region of the *EVI1* gene for *EVI1* activation.[12,32-34] *RPN1* is a main candidate, and *GR6* may be another one.[19] Even though *GR6* fusion genes have been reported to be involved in *EVI1* expression,[19] no *GR6-EVI1* fusion gene expression was found among our AML samples. We have no explanation for this discrepancy apart from chance, and studies on a larger number of patients, representing all subtypes and characteristics of the syndrome, are needed to shed light on *GR6* involvement.

A third oncogenetic possibility is that the rearrangements at the 3q26 region may disrupt the normal PR-protein MDS-EVI1, which normally functions as a growth suppressor. This situation would be similar to that of the PR-protein RIZ,[22,24] and it was suggested that deletion of the PR domain of RIZ leads to a shorter protein, RIZ2, that has acquired transforming properties. A similar mechanism has been proposed for a novel EVI1

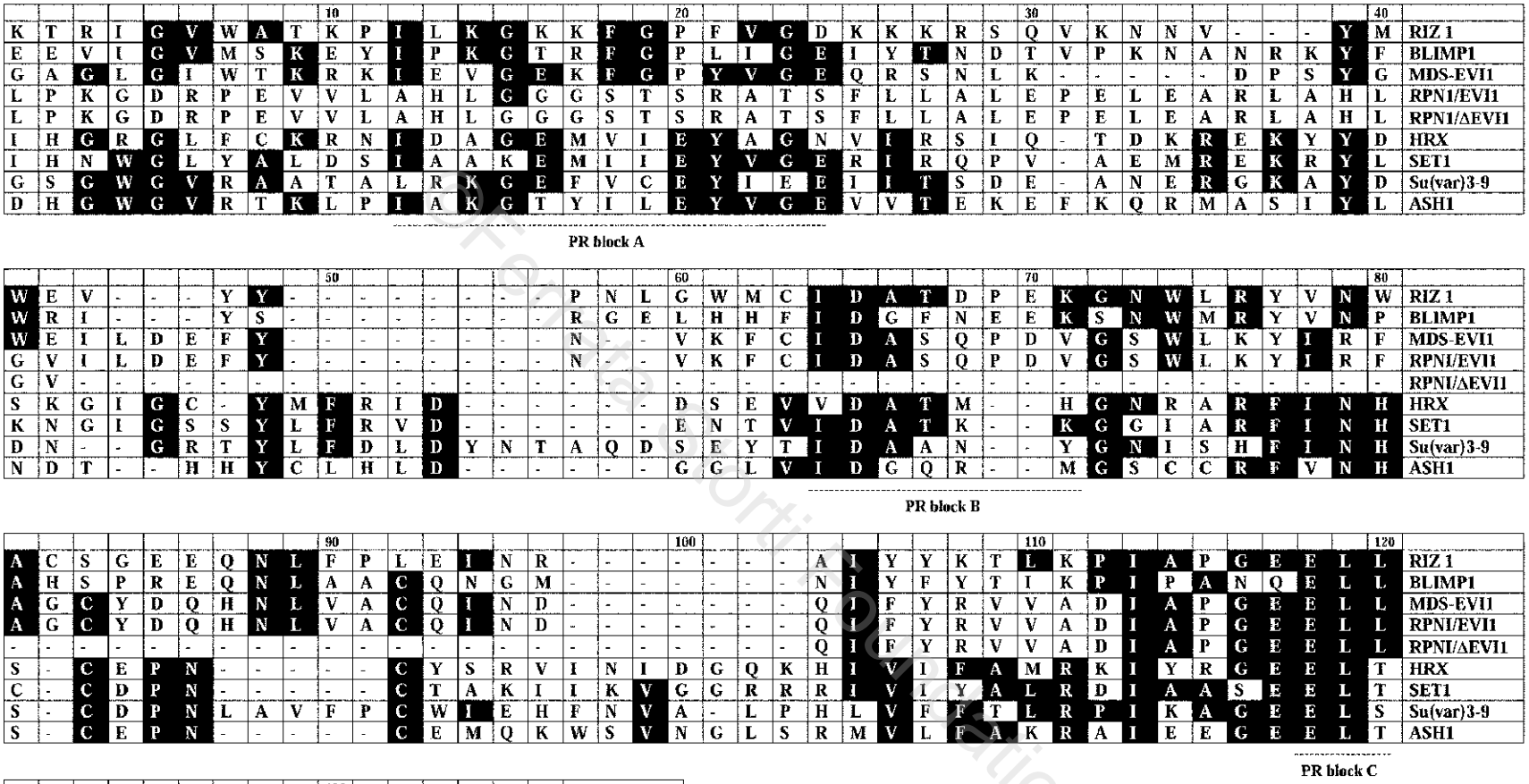

Figure 3. PR domain sequence alignments. The PR domain region of RIZ1, BLIMP1, MDS-EVI1 and RPN1-EVI1, RPN1-ΔEVI1 proteins is aligned with the HRX, SET1 (yeast), Su(var)3-9 (*Drosophila*) and ASH1 (*Drosophila*) proteins. Residues that are identical for at least three proteins are shaded.

gene family, MEL1 lacking a PR domain (MEL1S) which is mainly expressed in t(1;3)(p36;q21)-positive AML and blocks G-CSF-induced myeloid differentiation.[24] It was suggested that the PR domain may act as a protein binding motif[20,23] and it appears to function as a negative regulator of tumorigenesis.[21-23,26] In this regard, *RPN1-EVI1* may not only cause inappropriate EVI1 expression, but also produce EVI1 proteins lacking at least part of the PR domain. We found that all but one of the *RPN1-EVI1* transcripts that we have detected maintained the region of *EVI1* encoding blocks B and C of the PR domain. The remaining case (n. 6) expressed the novel transcript junction *RPN1-ΔEVI1* encoding only *block C* of the PR domain. Our sequence homology search indicated that the PR domain is maintained through evolution from yeast to mammalian cells, and that it has remarkable homology to proteins that are associated with chromatin remodeling. Thus, lack or inactivation of PR domains in a gene may lead to specific inactivation of its chromatin-associated functions, without affecting its other functions such as DNA binding and chromatin-independent transcriptional activation or repression.[35,36] The possible effects on chromatin structure regulation could cause major functional differences between products retaining larger or smaller portions of the PR domain. Our finding of the *RPN1-ΔEVI1* transcript suggests that this concept may apply for the *MDS1-EVI1* gene.

In conclusion, our findings support the hypothesis that activation of *EVI1* expression plays a role in the pathogenesis of the 3q21q26 syndrome. *EVI1* expression might occur either as a consequence of rearrangements leading to the formation of a particular fusion transcript, or following disruption of the PR activator domain of *MDS1-EVI1*: either of these two possible mechanisms could change the transcription regulatory property of EVI1 from an activator of promoters containing the AGATA DNA sequence to a repressor.

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Contributions

GM designed the study and interpreted the data; GV was responsible for the clinical data reported in the paper. EO was responsible for the Methods' section. AI and MM revised the manuscript before editing. Primary responsibility for the paper and the figures: GM.

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In the following paragraphs, Professor Cazzola summarizes the peer-review process and its outcomes.

What is already known on this topic

A small subset of patients with acute myeloid leukemia show the so-called 3q21q26 syndrome, which is typically associated with normal to elevated platelet counts, dysplastic megakaryocytes, poor response to chemotherapy and poor prognosis. Several oncogenes might be involved in its molecular pathogenesis.

What this study adds

The findings of this study support the hypothesis that EVI1 activation plays a dominant role in the pathogenesis of the 3q21q26 syndrome.