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 megakaryocytopenia 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.

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 micromegakaryocytopenia 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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In general, the PR domain contains three blocks of homology, block A, B and C, which are conserved among the members of the family. 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.

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. 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.29 Expression of RPN1-EVI1, GR6-EVI1, EVI1 and GR6 was detected by PCR using previously described sets of primers and PCR conditions.19,31,32 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 bp, 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 non 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.,33 we were unable to detect any type of fusion transcript between GR6 and EVI1.33 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 micromegakaryocytopenia. 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 3.34 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

EVI1 leading to EVI1 activation and leukemia have been reported in mice infected with an ecotropic retrovirus.\textsuperscript{12}

\textbf{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-\(^\Delta\)EVI1 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-\(^\Delta\)EVI1 with the HRX, SET1 (yeast), Su(var)3-9 (Drosophila) and ASH1 (Drosophila) proteins.

\textbf{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...
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. The open reading frame of the gene starts in the third exon where the first in-frame ATG codon is located. 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.

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, 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 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.
Figure 3. PR domain sequence alignments. The PR domain region of RIZ1, BLIMP1, MDS-EVI1 and RPN1/EVI1 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 motif20,23 and it appears to function as a negative regulator of tumorigenesis.21-23 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.

References

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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.