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Molecular Heterogeneity in AML/MDS Patients with 3q21q26 Rearrangements

Idoya Lahortiga,[1,2] Iria Vázquez,[1,2] Xabier Agirre,[2] María J. Larrayoz,[1] José L. Vizmanos,[1] Alessandro Gozzetti,[3] María J. Calasanz,[1] and María D. Odero[1,2]*

[1]Department of Genetics, University of Navarra, Pamplona, Spain
[2]Division of Oncology, School of Medicine, University of Navarra and Foundation for Applied Medical Research, Pamplona, Spain
[3]Division of Hematology and Transplants, Department of Medicine and Immunological Sciences, University of Siena, Siena, Italy

Patients with 3q21q26 rearrangements seem to share similar clinicopathologic features and a common molecular mechanism, leading to myelodysplasia or acute myeloid leukemia (AML). The ectopic expression of *EVI1* (3q26) has been implicated in the dysplasia that characterizes this subset of myeloid neoplasias. However, lack of *EVI1* expression has been reported in several cases, and overexpression of *EVI1* was detected in 9% of AML cases without 3q26 abnormalities. We report the molecular characterization of seven patients with inv(3)(q21q26), t(3;3)(q21;q26) or related abnormalities. *EVI1* expression was detected in only one case, and thus ectopic expression of this gene failed to explain all of these cases. *GATA2* (3q21) was found to be overexpressed in 5 of the 7 patients. *GATA2* is highly expressed in stem cells, and its expression dramatically decreases when erythroid and megakaryocytic differentiation proceeds. No mutations in *GATA1* were found in any patient, excluding loss of function of *GATA1* as the cause of *GATA2* overexpression. We report finding molecular heterogeneity in patients with 3q21q26 rearrangements in both breakpoints and in the expression pattern of the genes near these breakpoints. Our data suggest that a unique mechanism is not likely to be involved in 3q21q26 rearrangements.

INTRODUCTION

Rearrangements of the long arm of chromosome 3, namely, the inv(3)(q21q26) and the t(3;3)(q21;q26), are found in approximately 2.5% of patients with acute myeloid leukemia (AML) and have also been observed in some cases with myelodysplastic syndrome (MDS) and in the megakaryoblastic crisis of chronic myeloid leukemia (CML). Patients with these karyotypes share clinical features, including multilineage involvement, in particular, erythroid and megakaryocytic dysplasia, with micromegakaryocytes that have hypolobulated nuclei, an elevated or normal (instead of low) platelet count, poor prognosis, with minimal or no response to chemotherapy, and a short survival (Bitter et al., 1985; Jenkins et al., 1989; Lee et al., 1990; Jotterand Bellomo et al., 1992; Grigg et al., 1993; Fonatsch et al., 1994; Secker-Walker et al., 1995; Shi et al., 1997; Testoni et al., 1999; Reiter et al., 2000). The chromosomal breakpoints (BPs) in 3q26 are scattered over several hundred kilobases (kb) either in the 5′ or the 3′ region of the *EVI1* gene (Morishita et al., 1992; Levy et al., 1994; Suzukawa et al., 1994). In the 3q21 region, the BPs appears to be restricted to a much smaller genomic region, and two different clusters that account for around 100 kb have been defined downstream of the *RPN1* gene (Wieser et al., 2000a). The leukemogenic mechanism in the 3q21q26 rearrangement has been suggested to be the ectopic expression of the *EVI1* gene by the housekeeping gene *RPN1* acting as an enhancer of *EVI1* expression (Suzukawa et al., 1994). This molecular mechanism, which has been described mainly in lymphoid leukemias and lymphomas, is an uncommon mechanism in myeloid leukemias, although some examples have been reported (Vinatzer et al., 2001; Cools et al., 2002).

The *EVI1* protooncogene codes for a DNA binding zinc finger protein that may act as a repressor or activator of transcription (Morishita et al., 1988; Kreider et al., 1993; Bartholomew et al., 1997; Kilbey et al., 1998). Alternative splicing of *EVI1* gives rise to the *MDS1/EVI1* transcript by fusion with *MDS1*, a four-exon gene upstream and telomeric to *EVI1* with an unknown function that is also ex-

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*Correspondence to: María D. Odero, MD, Department of Genetics, University of Navarra, C/ Irunlarrea s/n, 31008 Pamplona, Spain. Fax: +34-948-425649. E-mail: modero@unav.es

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pressed by itself. The protein encoded by *MDS1/EVI1* is identical to EVI1 except for an N-terminal extension of 188 amino acids that has 40% homology to the PR domain also present in the tumor suppressor retinoblastoma-binding protein RIZ1, another member of the PR domain family along with MDS1/EVI1 (Fears et al., 1996). The PR domain is encoded in part by *MDS1* and in part by an open reading frame in exons 2 and 3 of *EVI1* mRNA, which precedes the *EVI1* ATG start codon (Fears et al., 1996). Barjesteh van Waalwijk van Doorn-Khosrovani et al. (2003) reported an analysis that was the first to discriminate among the expression of *MDS1*, *MDS1/EVI1*, and *EVI1* by real-time quantitative polymerase chain reaction (PCR). All eight patients analyzed, who had the classical t(3;3) or inv(3), showed *EVI1* expression, but expression of *MDS1/EVI1* was also detected in seven patients. Vinatzer et al. (2003) analyzed the expression of *MDS1/EVI1* and *cEVI1*, a region common to *EVI1* and *MDS1/EVI1*. Thirteen patients analyzed with inv(3) or t(3;3) showed high expression of *cEVI1*, in 10 of whom *MDS1/EVI1* expression also was high, leading to the conclusion that *MDS1/EVI1* overexpression does not prevent the emergence of leukemia.

Although the exact mechanism of transformation by *EVI1* is obscure, several studies have shown that ectopic expression of this gene in immature hematopoietic cells interferes with erythroid and granulocytic development (Kreider et al., 1993). It is generally accepted that *EVI1* is inappropriately expressed in leukemia cells after rearrangements of the 3q26 chromosome band; however, Barjesteh van Waalwijk van Doorn-Khosrovani et al. (2003) and Zoccola et al. (2003) found *EVI1* overexpression in 9% (28 of 315) and 20.6% (7 of 34) of AML and myeloid neoplasias without 3q26 rearrangements, respectively. Moreover, Barjesteh van Waalwijk van Doorn-Khosrovani et al. (2003) also showed that only 12.5% (4 of 32) of the patients that overexpressed *EVI1* carried a 3q26 abnormality and that *EVI1* expression is a poor prognosis marker. It was found that in several cases with 3q21q26, *EVI1* was not expressed (Fichelson et al., 1992; Morishita et al., 1992; Soderholm et al., 1997; Langabeer et al., 2001). Thus, ectopic expression of *EVI1* is a mechanism that fails to explain the characteristics of all patients with 3q21q26 rearrangements (Wieser, 2002).

Several chimeric gene fusions involving *MDS1/EVI1* and *EVI1* have been described in cases with t(3;21)(q26;q22) and t(3;12)(q26;p13) (Nucifora et al., 1994; Peeters et al., 1997). However, the BPs in the 3q21q26 rearrangements usually occurred outside the genes. Fusion transcripts involving *EVI1* with *RPN1* have been reported in 9 patients with AML and either inv(3) or t(3;3) (Martinelli et al., 2003) and in the USCD-AML1 cell line, which carries a t(3;3) (Pekarsky et al., 1997). This cell line also carried the only fusion transcript involving *EVI1* with *GR6* described to date (Pekarsky et al., 1997).

Here, we report the molecular characterization of seven patients with inv(3)(q21q26) or t(3;3)(q21;q26). A wide heterogeneity in both of the BPs in these regions and in the expression pattern of the genes near the BPs was found. This study confirms that ectopic expression of *EVI1* fails to explain all cases with 3q21q26 rearrangements. In addition, *GATA2*, a gene in the breakpoint cluster region of 3q21, was overexpressed in 83% of cases, suggesting a role for this gene in a more complex mechanism involved in the development of 3q21q26 rearrangements.

MATERIALS AND METHODS

Case Reports

Seven patients with myeloid neoplasias and inv(3)(q21q26) (5 cases) or t(3;3)(q21;q26) (2 cases) studied at the University of Navarra (Spain) and at the University of Siena (Italy) were included in the present investigation. Five had AML de novo, and two had MDS. Clinical data are shown in Table 1. All samples were obtained with informed consent.

G-Banding Karyotype

Cytogenetic studies were done on unstimulated short-term bone marrow (BM) cultures. G-banded karyotypes, described according to the ISCN (Mitelman, 1995), are shown in Table 1.

Fluorescence In Situ Hybridization

Fluorescence in situ hybridization (FISH) studies were performed by use of eight BACs and one PAC located in 3q21 and 3q26. The order of the probes according to the current mapping data is: centromere—RP11 202D20—RP11 390G14—RP11 525K18—RP3 519C2—RP11 475N22—RP11 689D3—RP11 221E20—RP11 82C9—RP-11 115B16—telomere. The clones were obtained from the Roswell Park Cancer Institute (Buffalo, NY). Information about these probes, including their relative physical positions and the genes the probes cover, is shown in Figure 1A. The probes were labeled with SpectrumGreen® and SpectrumOrange® (Vysis, Downers Grove, IL) by

TABLE 1. Cytogenetics and Clinical Characteristics of the Patients Analyzed

Case	Age/ Sex	Diagnosis	Karyotype	BM blasts (%)	Hb (g/dL)	Platelet count ($\times 10^6$/L)	Survival (months)
1	65/M	AML-M4	46,XY, inv(3)(q21q26)×2[30]	49	10.6	167,000	24
2	57/M	MDS (RAEB-2)	44,X,−Y,inv(3)(q21q26),−7[30]	12	9.6	531,000	14
3	27/F	AML-M5a	46,XX,inv(3)(q21q26)[28]/46,XX[2]	47	7.3	624,000	11
4	55/F	AML-M7	45,XX,inv(3)(q21q26),−7[30]	39	11.7	260,000	5
5	54/M	AML-M0	46,XY,t(1;7)(p32;p15),inv(3)(q21q26), del(22)(q13)[36]/46,XY[14]	37	7.7	151,000	21
6	65/F	MDS (RAEB-1)	46,XX,t(3;3)(q21;q26)[20]	6.5	7.5	195,000	Data not available
7	69/M	AML-M2	45,XY,t(2;7)(q21;q11),t(3;3)(q21;q26),del(5)(q13q33), del(12)(p?11p13), del(13)(q13q34), add(17)(p13),−18,add(20)[18]/46,XY[2]	70	8.2	187,000	5

nick translation and used pairwise. A commercial probe for *BCL6* also was used (Vysis). FISH analysis was performed on BM samples as previously described (Odero et al., 2001).

Nucleic Acid Isolation

The RNeasy Mini Kit (Qiagen, Hilden, Germany) was used to extract total RNA from frozen cell pellets from BM of the patients, and from BM and peripheral blood (PB) from healthy donors. $CD34^+$ cell isolation was performed from mononuclear cells of normal BM using the Direct CD34 Progenitor cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Genomic DNA was obtained with the QIAamp DNA Mini Kit (Qiagen) from fixed cells of the patients and from BM and PB from healthy donors. DNA from BAC and PAC clones was extracted by use of a Qiaprep® Spin Miniprep kit (Qiagen).

Reverse Transcriptase-PCR

Total RNA (1 μg) was used for cDNA synthesis with SuperScript™ II RNase H^- RT (Invitrogen Life Technologies, Paisley, UK) that had random hexamers. RT-PCR amplification was performed under standard conditions with AmpliTaq Gold® DNA polymerase (Applied Biosystems, Foster City, CA), with 35 cycles at the annealing temperature (AT) shown below in parentheses for each pair of primers. Primers were designed for specific assays of amplification for *EVI1* (EVI1-F and EVI1-MDS1/EVI1-R; AT: 57°C), *MDS1/EVI1* (MDS1/EVI1-F and EVI1-MDS1/EVI1-R; AT: 57°C), *MDS1* (MDS1-F and MDS1-R; AT: 53°C), *GR6* (GR6-F and GR6-R; AT: 64°C) and *GATA2* (GATA2-F and GATA2-R; AT: 60°C). All reactions were carried out on cDNA from BM of the patients and on BM, PB, and $CD34^+$ cells from healthy donors. The primers used for analyzing the three possible intergenic transcripts (ITs) described by Pekarsky et al. (1997) between *GR6* and *EVI1* were IT1: GR6-1-F and EVI1-2A-R (AT: 59°C); IT2 and its variant, IT3: GR6-4-F and EVI1-2A-R (AT: 61°C); and the possible fusion transcript between *RPN1-EVI1* IT4: RPN1-1-F and EVI1-2B-R (AT: 62°C). All reactions were carried out on BM from the patients and from a healthy donor. *BCR* amplification was performed as a control for the quality of the cDNA used, with primers BCR-F and BCR-R under standard conditions, an AT of 55°C, and 35 cycles. The sequence of the primers is shown in Table 2.

Semiquantitative RT-PCR

Expression levels of *GATA2* were compared in BM cells of case 7 and in a healthy donor by semiquantitative RT-PCR, using GATA2-F and GATA2-R primers for *GATA2* and BCR-F and BCR-R primers for *BCR*. The *BCR* gene was used as an internal control. Serial dilutions of both samples were analyzed to assure that all reactions were kept in the linear phase of amplification. The ratio of *GATA2* to *BCR* expression was determined after densitometric analysis of the gels. *GATA2* and *BCR* amplifications were carried out with the same temperature conditions described above. Primer sequences are shown in Table 2.

Rapid Amplification of cDNA Ends Polymerase Chain Reaction

3′-Rapid amplification of cDNA ends (RACE) PCR was performed with a GeneRacer™ Kit (Invitrogen Life Technologies, Paisley, UK). Briefly, first-strand cDNA was reversed-transcribed from 1

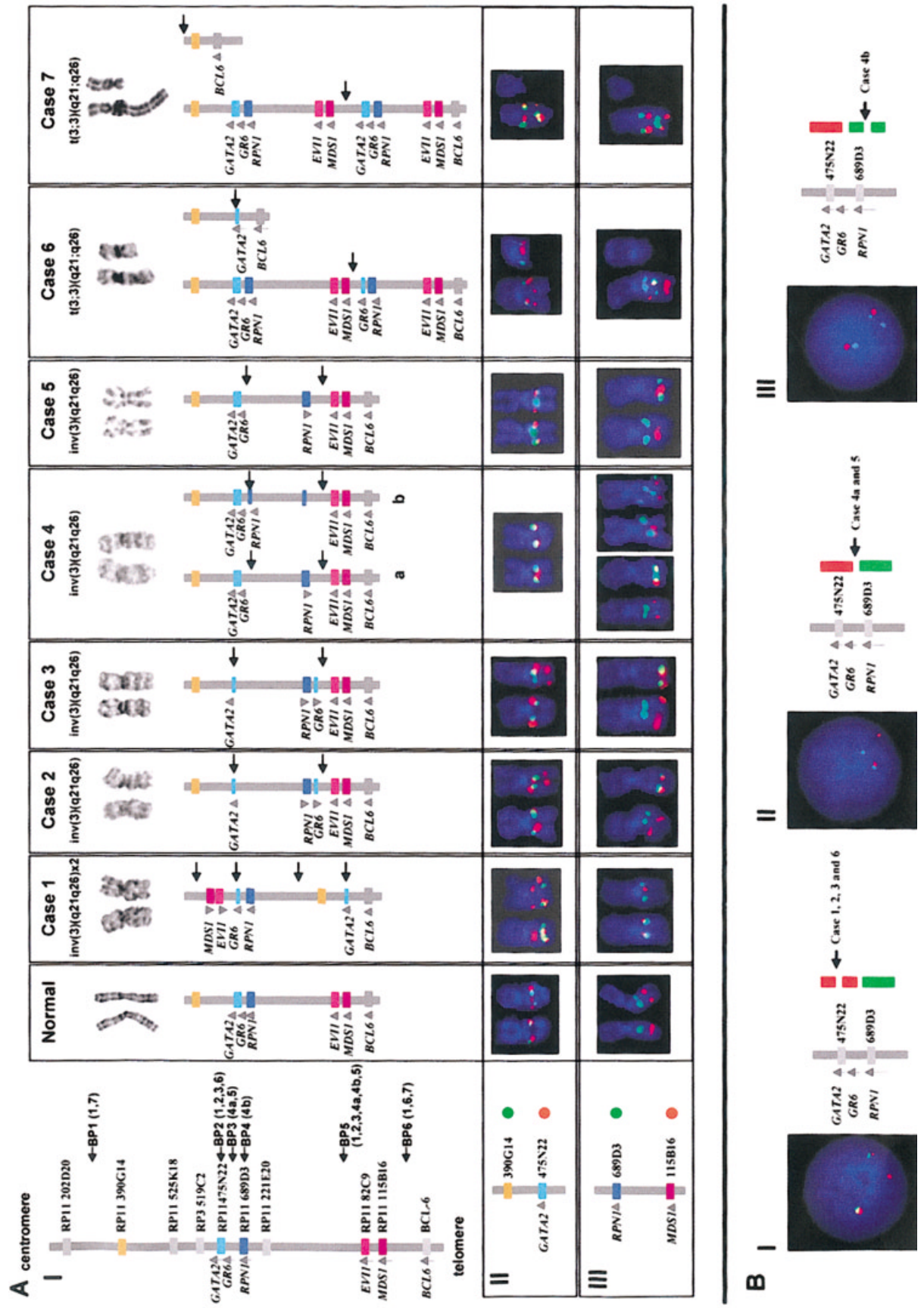

Figure 1. (A) I. A map of the region from 3q21 to 3q26 showing the relative positions of the clones used in this study and the genes involved. A G-band pair of chromosome 3 is shown for a healthy donor and for the cases analyzed. In each karyotype, normal chromosome 3 is shown on the left and derivative chromosome 3 on the right, except for cases 1, 6, and 7, where both chromosomes are involved. II. FISH analysis with probes RP11 390G14 (green signal) and RP11 475N22 (red signal). III. FISH analysis with probes RP11 689D3 (green signal) and RP11 115B16 (red signal). (B) Interphase FISH analysis with probes RP11 475N22 (red signal) and RP11 689D3 (green signal). Expected signal pattern when the BP is (I) in RP11 475N22, (II) between RP11 475N22 and RP11 689D3, and (III) in RP11 689D3.

TABLE 2. Oligonucleotide Primer Sequences*

Primer	Oligonucleotide sequence (5′–3′)	Gene	Nucleotides
EVI1-F	ACCCTTTGGCTAGATTATCTTAGACGA	*EVI1*	63–89
EVI1-MDS1/EVI1-R	CCAGCGAATCTAATGTACTTGAGC	*EVI1*	167–144
MDS1/EVI1-F	GGGCAGGACTAGGAATATGGAC	*MDS1/EVI1*	2363–2384
MDS1-F	GTGGGAGAGCAGAGGTCAAA	*MDS1*	635–654
MDS1-R	TCCCCAAATACAACCAAGAG	*MDS1*	737–718
GR6-F	CCCGGCCTGAGACAATGGAC	*GR6*	30–49
GR6-R	ACCCCCGAGTGTCTCTGGTG	*GR6*	1153–1134
GATA-2-F	AGGACGGCGTCAAGTACCAG	*GATA2*	955–974
GATA-2-R	CGCCATAAGGTGGTGGTTGT	*GATA2*	1414–1395
BCR-F	GAGAAGAGGGCGAACAAG	*BCR*	2889–2906
BCR-R	CTCTGCTTAATTCCAGTGGC	*BCR*	3265–3246
GR6-1-F	CCGGCCTGAGACAATGGA	*GR6*	31–48
GR6-4-F	GGCTGTCGGAATCAAAGAGG	*GR6*	815–834
EVI1-2A-R	TGGCATCTATGCAGAACTTCAC	*EVI1*	121–100
RPN1-1-F	CGCCGGCTTGTTTCTGCTC	*RPN1*	152–170
EVI1-2B-R	CCAGCGAATCTAATGTACTTGAGCC	*EVI1*	167–143
GATA-2-ex1-F	ATTGCCTGCCGCCACATCCATCCT	*GATA2*	60–83
GATA-2-ex2-F	CCGCGCAGCTGCTGCCTCCAGAC	*GATA2*	439–461
GATA-2-ex3-F	GGGGGACCGGCCTCCAGCTTCAC	*GATA2*	1146–1168
GATA-2-ex5-F	CCAGAAGAGCCGGCACCTGTTGT	*GATA2*	1357–1379
GATA-1-ex2-F	AAAGGAGGGAAGAGGAGCAG	*GATA1*	4460–4479
GATA-1-ex2-R	GACCTAGCCAAGGATCTCCA	*GATA1*	4850–4831
GATA-1-ex4-F	GAGGTGGGAGGGGTGGCCCAAAG	*GATA1*	5699–5721
GATA-1-ex4-R	CTGTAATCATGAGAACAGCGTTCC	*GATA1*	6022–5999

*Nucleotide coordinates refer to GenBank mRNA accession numbers X54989 (*EVI1*), SG9002 (*MDS1/EVI1*), NM_004991 (*MDS1*), NM_007354 (*GR6*), NM_032638 (*GATA2*), NM_004327 (*BCR*), and NM_002950 (*RPN1*). *GATA1* coordinates refer to GenBank genomic accession number NM_002049.

μg of total RNA using SuperScript™ II RNase H⁻ RT (Invitrogen Life Technologies) and the GeneRacer™ oligo-dT primer; 1 μl of the first-strand cDNA was then amplified by use of a *GATA2* gene-specific forward primer and the GeneRacer™ 3′ primer. A seminested or nested PCR reaction was performed using the GeneRacer™ 3′ nested primer as the reverse primer and a *GATA2* gene-specific forward primer. The gene-specific primers were designed to cover all the possible breakpoints and were used in pairs for the first and the second PCR as follows: GATA2-ex1-F and GATA2 ex1-F, GATA2-ex1-F and GATA2-ex2-F, GATA2-ex2-F and GATA2-ex3-F, GATA2-ex3-F and GATA2-ex5-F (Table 2). Amplifications were carried out under standard conditions, at an AT of 64°C, and for 35 cycles.

GATA1 Mutation Analysis

PCR was performed by use of genomic DNA with primers GATA1-ex2-F and GATA1-ex2-R for the analysis of exon 2 (Hitzler et al., 2003) and primers GATA1-ex4-F and GATA1-ex4-R for the analysis of exon 4 (Nichols et al., 2000) using a standard protocol, an AT of 60°C, for 30 cycles. Amplification products were directly sequenced after purification.

DNA Cloning and Sequencing

PCR products from the RT-PCR and the 3′-RACE experiments were cloned by use of the TOPO TA Cloning® Kit for Sequencing (Invitrogen Life Technologies). Colonies with recombinant plasmids that contained the PCR products were screened by digestion with *Eco*RI (Amersham Biosciences, Buckinghamshire, UK). Candidate plasmid clones and direct PCR products from the mutation analysis of *GATA1* were sequenced with the ABI-PRISM™ d-Rhodamine Terminator Cycle Sequencing Kit (Applied Biosystems) in an ABI PRISM™ 377 DNA Sequencer (Applied Biosystems).

RESULTS

We report here the molecular characterization of the myeloid neoplasias and 3q21q26 rearrangements of the seven patients who were studied. The G-banded karyotype showed that five patients, cases 1–5, had inv(3)(q21q26) and two patients, cases 6 and 7, had translocation t(3;3)(q21;q26) (Table 1). Six patients (cases 2–7) showed clinical characteristics consistent with a 3q21q26 rearrangement, including erythroid and megakaryocytic dysplasia, an elevated or normal platelet count, and a

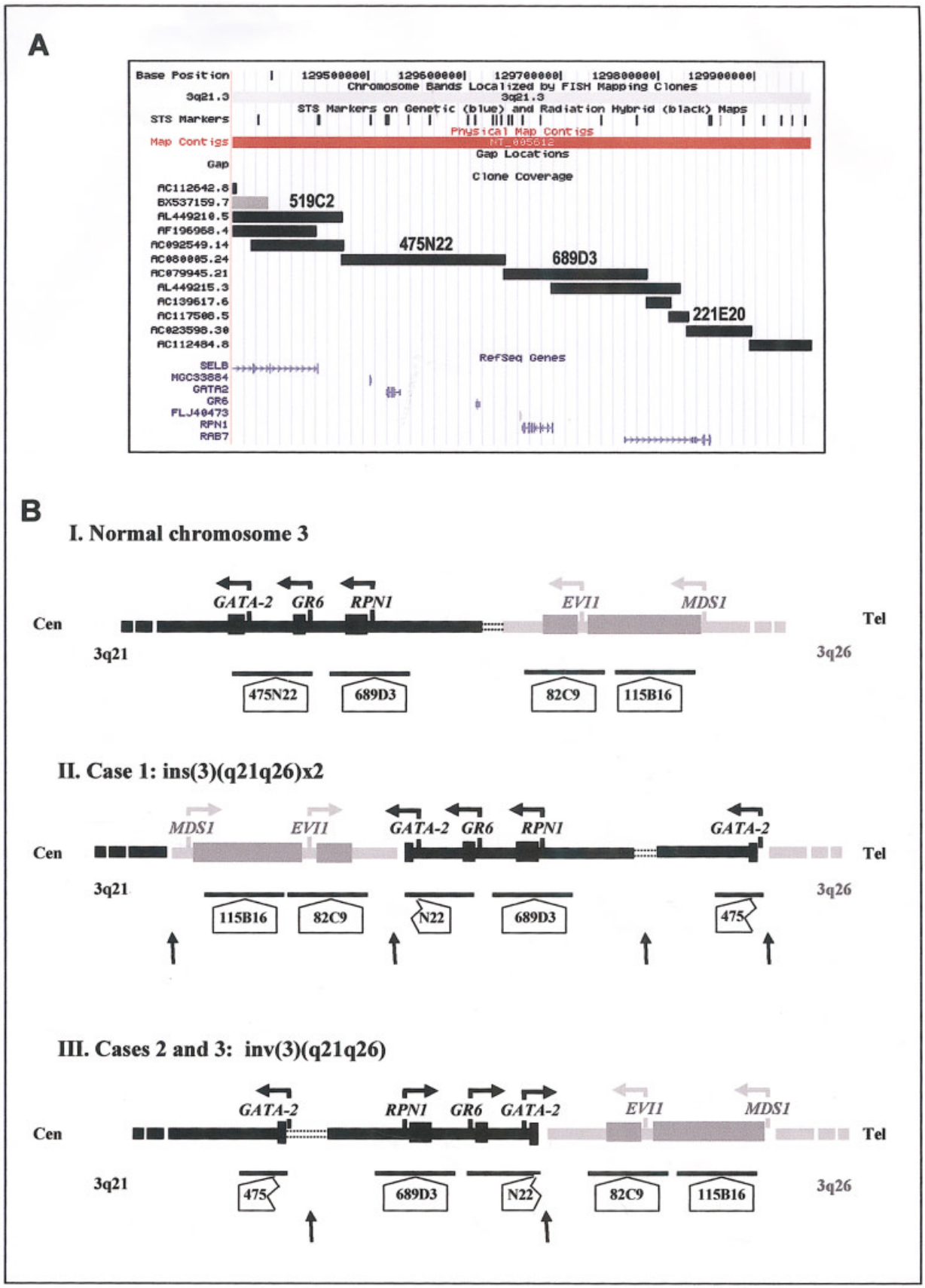

Figure 2. (A) Diagram showing the physical location of the probes that cover the *GATA2*, *GR6*, and *RPN1* genes on 3q21, from the University of Santa Cruz California Genome Bioinformatics Web site (http://genome.ucsc.edu). (B) Positional relationship of the genes and their transcriptional orientation in (I) normal chromosome 3, (II) case 1, and (III) cases 2 and 3 (III) as a consequence of the rearrangement. Vertical arrows indicate breakpoints.

poor prognosis. In case 1, neither multilineage involvement nor megakaryocytic dysplasia was found. Relevant clinical data are shown in Table 1.

FISH analysis helped to make a more precise definition of the different BPs present in the 3q21 and 3q26 regions. Four BPs in 3q21 (BP-1, BP-2, BP-3, and BP-4) and two in 3q26 (BP-5 and BP-6) were defined (Fig. 1A). Case 1 showed an inv(3)(q21q26) in both chromosomes 3 by G-banding, which was more precisely defined using FISH. Four BPs were found: BP-1 and BP-2 in 3q21 and BP-5 and BP-6 in 3q26 (Fig. 1A). BP-2 was in RP11 475N22, which split. This BAC covers the *GATA2* gene, in its centromeric region, and the *GR6* gene, in its telomeric region. Figure 2A shows the position of the clones that cover genes *GATA2*, *GR6*, and *RPN1* in 3q21. The region between BP-5 and BP-6 (3q26) contains genes *EVI1* and *MDS1* (Fig. 1A). Therefore, this patient had a complex rearrangement, with an insertion of a fragment from 3q21 into 3q26 and a second insertion of a fragment from 3q26 located between BP-5 and BP-6 into the 3q21 region.

Cases 2 and 3 were the only ones sharing the same BPs in 3q21 (BP-2) and 3q26 (BP-5). The locations of the BPs indicate that the *GR6* and *RPN1* genes, which came from 3q21, are adjacent to *EVI1* and *MDS1* in 3q26, with their transcriptional orientation changed as a consequence of the inversion (Figs. 1A and 2B). In case 1, the same BPs were also found; however, the presence of an additional BP in each region led to a different final gene orientation. In this case, it is the *EVI1* and *MDS1* genes whose transcriptional sense has changed and resulted in locations near and toward *GR6* and *RPN1*, but in the 3q21 region (Figs. 1A and 2B).

Case 4 had two clones that differed in the location of the 3q21 BP. Clone 4a had, as did case 5, a BP in 3q21 between RP11 475N22 and RP11 689D3 (BP-3). In clone 4b, the BP in 3q21 was in RP11 689D3, a BAC that covers *RPN1* in its centromeric region (BP-4; Fig. 2A). In 3q26, BP-5 was present in both clones of cases 4 and 5. In clone 4a and case 5, *GR6* remained in its original position, whereas *RPN1* changed its orientation and appeared near to and oriented toward *EVI1* and *MDS1* in 3q26 (Fig. 1A). In clone 4b, all of the genes of interest remained in their original orientation.

Cases 6 and 7 had a t(3;3)(q21;q26). The location of the BPs in 3q26 was the same in both cases (BP-6), whereas in 3q21 the BP was BP-2 in case 6, but BP-1 in case 7 (Fig. 1A). The final position of *GATA2* in cases 1–3 and 6 is unknown. This is because in these cases, BAC RP11 475N22, which covers this gene, showed a split signal, but we did not know whether the BP was upstream, downstream, or within the gene. All of these possibilities were considered when we prepared Figure 2B.

We used RT-PCR to analyze the expression of *MDS1*, *MDS1/EVI1*, *EVI1*, *GR6*, and *GATA2* in BM from the five patients with inv(3) and the one patient with t(3;3) (case 7) and in BM, PB, and $CD34^+$ from healthy donors (Fig. 3). No material from case 6 was left for molecular studies. We detected expression of *MDS1/EVI1* and *GATA2* in normal BM but only expression of *GATA2* in normal PB. In $CD34^+$ cells, expression of *MDS1*, *MDS1/EVI1*, and *EVI1* was detected, and there was high expression of *GATA2* compared with that in normal BM (Fig. 3). No expression of *GR6* was found in normal BM, PB, or $CD34^+$ cells. Substantial heterogeneity in the pattern of gene expression in the cases analyzed was found (Fig. 3). Whereas *MDS1* was expressed only in case 3, *MDS1/EVI1* expression was detected in all cases with variable intensity (Fig. 3). Expression of *EVI1* and *GR6* was detected only in case 2. Intergenic transcripts were not detected between *GR6* and *EVI1* or between *RPN1* and *EVI1* in any patients.

GATA2 expression was detected in all patient samples with inv(3) and in the patient sample with t(3;3). Compared with its expression in the *BCR* control of normal BM, *GATA2* seems to have been overexpressed in cases 1, 2, 3, 5, and 7 (Fig. 3). There was no material left from cases 1, 2, 3, and 5 for quantitative analysis. We designed a semiquantitative RT-PCR experiment to compare the expression of *GATA2* in the BM of case 7 with its expression in the BM of a healthy donor. Amplification of *BCR* was used as an internal control. The expression of *GATA2* was 10 times higher in the patient sample than in the control.

Because of the location of *GATA2* beside the breakpoint cluster region in 3q21 (BP-2), a possible deregulation of this gene caused by its breakage was investigated using 3′-RACE PCR; however, no new sequences fused to *GATA2* in 3q26 were found.

A recent study showed that GATA1 directly represses *GATA2*, displacing the GATA2 protein from its binding site and allowing cellular differentiation. To check whether the dysfunction of GATA1 could be the cause of the *GATA2* overexpression, we analyzed the mutational status of exons 2 and 4 of *GATA1* (Xp11). No mutations of *GATA1* were found in patients 2, 3, and 5. Patients

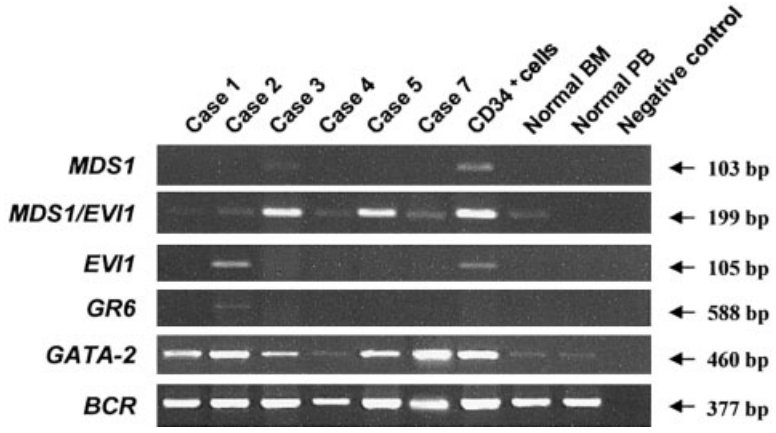

Figure 3. Analysis of the expression of *MDS1*, *MDS1/EVI1*, *EVI1*, *GR6*, *GATA2*, and *BCR* in cDNA from BM of cases 1–5 and 7 in $CD34^+$ cells, BM, and PB from healthy donors.

2 and 5 were males. No material from cases 1 and 7 was available for these experiments.

We also designed a simple and efficient FISH assay for the detection of all possible BPs in 3q21 in patients with 3q21q26 rearrangements and for the assignment of any BP found to either the breakpoint cluster region telomeric (BCR-T) or breakpoint cluster region centromeric (BCR-C), as previously reported (Wieser et al., 2000a). Our assay consists of a unique experiment using only two probes (RP11-475N22 and RP11 689D3) that cover a region of 320 kb including both BCRs (Figs. 1B and 2A). FISH analysis of eight cases with translocations involving 3q21 other than inv(3) or t(3;3) showed that in those cases, the BPs were outside this region (data not shown).

DISCUSSION

The molecular characterization by FISH and RT-PCR of five patients with inv(3)(q21q26) and two patients with t(3;3)(q21;q26) showed wide heterogeneity in both the BPs and the expression pattern of the genes near the BPs. FISH analysis enabled a more precise definition of the BP in these cases, providing data about the new positional relationship of the genes and the possible implication for transcription derived from the rearrangements (Figs. 1A and 2B). Consideration of the FISH and RT-PCR results together showed that all of the cases were different. The only two cases with the same BPs according to the FISH analysis (cases 2 and 3) showed different expression patterns. FISH analysis also allowed clarification of the karyotype in case 1, resulting in a complex ins(3)(q21q26) in both chromosomes 3. This suggests that during the neoplastic process there was duplication of the abnormal chromosome 3, with loss of the normal chromosome 3.

In our study, we discriminated among the expression of the *EVI1*, *MDS1*, and *MDS1/EVI1* genes. Surprisingly, expression of *EVI1* was detected only in one case (case 2). Even case 1, which had two abnormal chromosomes 3, and case 3, which shared with case 2 the same BPs in both 3q21 and 3q26, showed no expression of *EVI1*. The lack of expression in these two cases suggests that *EVI1* overexpression is independent of 3q21q26 aberrations. Although *EVI1* overexpression is a common finding in 3q21q26 rearrangements (Barjesteh van Waalwijk van Doorn-Khosrovani et al., 2003; Martinelli et al., 2003; Vinatzer et al., 2003), several studies have yielded other results. Langabeer et al. (2001) detected no *EVI1* expression in 3 of 19 patients with 3q26 rearrangements, one of whom had an ins(3)(q26;q21q26) that could be similar to our case 1. Morishita el al. (1992) also reported no expression of *EVI1* in seven patients with inv(3), and additional cases with the same characteristics have been described (Fichelson et al., 1992; Soderholm et al., 1997). Although some of these studies analyzed the transcript of *cEVI1*, a region common to *EVI1* and *MDS1/EVI1*, the negative result for both transcripts confirms the lack of *EVI1* expression. In addition, overexpression of *EVI1* was detected in 9% (28 of 315) of patients who had AML but no 3q26 rearrangements (Barjesteh van Waalwijk van Doorn-Khosrovani et al., 2003), confirming that expression of this gene cannot be considered a specific leukemogenic mechanism for the 3q21q26 rearrangement. Another study on myeloid neoplasias showed the same results, with a higher percentage of cases without 3q21q26 rearrangements expressing *EVI1* (7 of 34, 21%; Zoccola et al., 2003). Barjesteh van Waalwijk van Doorn-Khosrovani et al. (2003) showed that overexpression of *EVI1*, not of *MDS1*/

EVI1, was a poor prognosis factor in patients with AML, independently of 3q26 rearrangements. In our study, *MDS1/EVI1* expression was detected in normal BM, in $CD34^+$ cells, and in all patients analyzed. Zoccola et al. (2003) reported the same results. Putting these data together, it is possible to affirm that both overexpression of *EVI1* and 3q21q26 rearrangements are poor prognostic factors in patients with myeloid neoplasias, although the poor prognosis associated with 3q21q26 rearrangements could be independent of *EVI1* expression.

EVI1 is rarely involved in fusion transcripts in 3q21q26 rearrangements. *RPN1–EVI1* has been recently reported in 9 patients with AML with either inv(3)(q21q26) or t(3;3)(q21;q26) (Martinelli et al., 2003). Besides, the UCSD-AML1 cell line, with a t(3;3), has both *RPN1–EVI1* and *GR6–EVI1* fusion transcripts (Pekarsky et al., 1997). We found no ITs in our cases. The position and the transcription orientation of the genes involved as a consequence of the rearrangements (Figs. 1A and 2B) make it impossible for these ITs to occur in cases with inv(3) (cases 2–5). In cases 6 and 7, which had a t(3;3), ITs could arise. Only material from case 7 was available for analysis, and in the analysis, no fusion transcripts were detected. Our results are consistent with previous studies showing that overexpression of *EVI1* is unlikely to be a consequence of the formation of fusion transcripts, which are not a common finding in patients with 3q21q26 rearrangements (Pekarsky et al., 1997; Wieser, 2002).

Additional potential oncogenes in 3q21 and 3q26 are still under consideration (Russell et al., 1994; Rynditch et al., 1997; Wieser, 2002). The suggested role of *RPN1* as an enhancer of *EVI1* expression seems to be improbable because enhancer elements in 3q21 have not yet been identified (Rynditch et al., 1997; Wieser, 2002). Moreover, the heterogeneity found by FISH in our series in BP location and, in consequence, in the relative position of the genes around them, makes a unique mechanism improbable and adds support for the hypothesis that there is a complex mechanism involving several genes (Figs. 1A and 2B). The data reported by Pekarsky and Rynditch suggest that the 3q21 region is gene-rich and that additional genes could be involved in these rearrangements (Pekarsky et al., 1997; Rynditch et al., 1997). We detected *GR6* expression in a patient with inv(3)(q21q26) (case 2), analyzing the most frequent transcript, the splicing form from exon 1 to exon 3 of *GR6*. The *GR6* gene is downstream and centromeric to *RPN1*, within the 3q21 BCR-T, and is normally expressed in early fetal development but not in adult tissues. *GR6* was first found to be activated in the UCSD-AML1 cell line and in a leukemic sample, both carrying a t(3;3)(q21;q26) (Pekarsky et al., 1997). Recently, *GR6* expression also was reported in nine patients with AML and 3q21q26 rearrangements (Martinelli et al., 2003). However, the low incidence of *GR6* ectopic expression suggests that this cannot be considered a general mechanism for rearrangements in 3q21, although a role in a more complex mechanism cannot be refuted completely.

Interestingly, *GATA2* expression was detected in all patients. Although a quantitative analysis was not possible in all patients because of limited material, a comparison with the *BCR* control suggests that *GATA2* was overexpressed in patients 1, 2, 3, 5, and 7, when compared with normal BM (Fig. 3). This was confirmed by semiquantitative RT-PCR in case 7, which showed an expression of *GATA2* 10 times higher in the patient with t(3;3) than in the controls (data not shown). Wieser et al. (2000b) reported that *GATA2* was overexpressed in 7 of 9 patients with myeloid neoplasias and 3q21 rearrangements, suggesting that, in these cases, the leukemogenic mechanism could be *GATA2* deregulation. *GATA2* is one of the six members of the *GATA* family of zinc finger transcription factors, which are characterized by the ability to bind the consensus DNA sequence WGATAR (Orkin, 1992). Among these genes, *GATA1*, *GATA2*, and *GATA3* play crucial roles in hematopoiesis. *GATA1* is highly expressed in erythroid cells and megakaryocytes (Tsai et al., 1989) and is required for terminal differentiation of these lineages (Simon et al., 1992; Tsang et al., 1998). *GATA2* is highly expressed in hematopoietic stem and progenitor cells, and its expression dramatically decreases when erythroid and megakaryocytic differentiation proceeds (Tsai et al., 1994, 1997; Cantor et al., 2002). A recent study showed that *GATA1* directly represses *GATA2* by a bimodal mechanism. First, the binding of GATA1 to a region -2.8 kb upstream of *GATA2* displaces the GATA2 protein from this location, repressing *GATA2* transcription. Second, GATA1 also displaces the histone acetyltransferase CREB-binding protein (CBP), leading to the establishment of a domain-wide repressive chromatin structure. Such mechanisms seem to be critical for the control of hematopoietic differentiation (Grass et al., 2003) and could have particular relevance in 3q21q26 rearrangement, which is characterized by dysplasia of the erythroid and megakaryocytic lineages. Therefore, *GATA2* could be a candidate gene in 3q21. Moreover, all of the

BPs in 3q21 reported so far were upstream of the coding region of *GATA2*. It has been reported that in mice, properly regulated hematopoietic expression of *Gata2* depended on the presence of at least 150 kb of upstream sequences; if this observation could be extrapolated to the human gene, *GATA2* would be disrupted in all the 3q21 BPs described to date (Wieser, 2002). According to the current mapping data, *GATA2* is in RP11 475N22, the BAC that was split in three of our cases (cases 1, 2, and 3), with overexpression of *GATA2*.

To determine whether the breakpoint was within *GATA2* and whether this was the cause of the deregulation of this gene, we performed a 3′RACE PCR assay. However, no new sequences in 3q26 fused to *GATA2* were found. Similarly, Wieser et al. (2000b) found no structural aberrations of *GATA2* in the cell lines analyzed by Southern blotting. To study whether the loss of function of *GATA1* could be the cause of *GATA2* overexpression, we analyzed exons 2 and 4 of *GATA1*, looking for possible mutations. Wechsler et al. (2002) reported mutations in exon 2 of *GATA1* that led to a shorter GATA1 protein with reduced transactivation activity, potentially affecting normal megakaryocytic differentiation in children with Down syndrome and a megakaryoblastic AML (M6). On the other hand, hemizygotic mutations in exon 4 of *GATA1* (Xp11) have also been reported to cause congenital defects in males, including dyserythropoietic anemia, because of the substitution of a highly conserved valine, which is necessary for the interaction of *GATA1* with its essential cofactor FOG-1, underscoring the importance of *GATA1* in both megakaryocyte and erythroid development (Nichols et al., 2000). We detected no mutations of *GATA1*, either in exon 2 or in exon 4, in patients 2, 3, and 5, thus excluding this mechanism as well. Patients 2 and 5 were males; thus, they had only one *GATA1* allele.

The poor prognosis that characterizes patients with 3q21q26 rearrangements makes it important to discriminate among the mechanisms involved in order to design useful stratified treatment approaches.

Finally, this study has allowed us to design a simple and efficient FISH assay for the detection of all possible BPs in 3q21 in patients with 3q21q26 rearrangements and to assign these BPs to the BCR-T or BCR-C defined by Wieser et al. (2000a). Our assay consists of a unique experiment using only two probes that cover a 320-kb region including both BCRs. This assay simplified the design reported by Wieser et al. (2001), which used six BACs for the BCR-C and five BACs for the BCR-T in two separate experiments.

In conclusion, this is the first report of molecular heterogeneity in cases with 3q21q26 rearrangements both in the BPs in these regions and in the expression pattern of the genes near the BPs. The breakpoints in 3q21 and 3q26 were defined by FISH, providing data about the new positional relationship of the genes. The different expression pattern of the genes around the breakpoints confirmed this heterogeneity. *EVI1* expression was found in only one patient, suggesting that overexpression of this gene is a mechanism that fails to explain the characteristics of all these cases. These results argue for the hypothesis that the ectopic expression of *EVI1* directed by *RPN1* acting as an enhancer is unlikely to be the sole molecular mechanism in patients with 3q21q26 rearrangements and that there is a complex mechanism involving several genes. *GATA2*, which was overexpressed in 83% of our cases, might have a role in this putative complex mechanism that should be elucidated.

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