Molecular Heterogeneity in AML/MDS Patients with 3q21q26 Rearrangements

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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)(q21q26) 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 multiligneage 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; Fontans 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-
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) (Nuñifora 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—RP11 475N15—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
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 RNasey 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 RNase H⁻ alerts (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 EVII (EVII-F and EVII-MDS1/EVII-R; AT: 57°C), MDS1/EVII (MDS1/ EVII-F and EVII-MDS1/EVII-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 EVII were IT1: GR6-1-F and EVII-2A-R (AT: 59°C); IT2 and its variant, IT3: GR6-4-F and EVII-2A-R (AT: 61°C); and the possible fusion transcript between RPN1-EVII IT4: RPN1-1-F and EVII-2B-R (AT: 62°C). All reactions were carried out on BM from the patients and on 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

<table>
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<th>Case</th>
<th>Age/Sex</th>
<th>Diagnosis</th>
<th>Karyotype</th>
<th>BM blasts (%)</th>
<th>Hb (g/dL)</th>
<th>Platelet count (x 10⁶/L)</th>
<th>Survival (months)</th>
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<td>70</td>
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**Table 1. Cytogenetics and Clinical Characteristics of the Patients Analyzed**
Figure 1. (A) 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. (B) Interphase FISH analysis with probes RP11 475N22 and RP11 689D3. Expected signal pattern when the breakpoint is (I) in RP11 475N22, (II) between RP11 475N22 and RP11 689D3, and (III) in RP11 689D3. (C) In RP11 475N22, (D) in RP11 115B16, and (E) in RP11 115B16.
μg of total RNA using SuperScript™ II RNase H−
RT (Invitrogen Life Technologies) and the Gene-
Racer™ 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 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 af-

**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 (Invitro-
gen Life Technologies). Colonies with recombi-
nant plasmids that contained the PCR products
were screened by digestion with EcoRI (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 Biosys-
tems).

**RESULTS**

We report here the molecular characterization of
the myeloid neoplasias and 3q21q26 rearrange-
ments 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 rearrange-
ment, including erythroid and megakaryocytic dys-
plasia, an elevated or normal platelet count, and a

<table>
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<th>Primer</th>
<th>Oligonucleotide sequence (5’–3’)</th>
<th>Gene</th>
<th>Nucleotides</th>
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Table 2. Oligonucleotide Primer Sequences*
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 locations between BP-3 and BP-6 (3q26) contains genes EVII 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 EVII 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 EVII 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 EVII 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/EVII, EVII, 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/EVII and GATA2 in normal BM but only expression of GATA2 in normal PB. In CD34+ cells, expression of MDS1, MDS1/EVII, and EVII 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/EVII expression was detected in all cases with variable intensity (Fig. 3). Expression of EVII and GR6 was detected only in case 2. Intergenic transcripts were not detected between GR6 and EVII or between RPN1 and EVII 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
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 EVII, MDS1, and MDS1/EVII genes. Surprisingly, expression of EVII 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 EVII. The lack of expression in these two cases suggests that EVII overexpression is independent of 3q21q26 aberrations. Although EVII 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 EVII 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. Motishita et al. (1992) also reported no expression of EVII 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 EVII and MDS1/EVII, the negative result for both transcripts confirms the lack of EVII expression. In addition, overexpression of EVII 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 EVII (7 of 34, 21%; Zoccola et al., 2003). Barjesteh van Waalwijk van Doorn-Khosrovani et al. (2003) showed that overexpression of EVII, not of MDS1/
EVII, was a poor prognosis factor in patients with AML, independently of 3q26 rearrangements. In our study, MDS1/EVII 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 EVII and 3q21q26 rearrangements are poor prognostic factors in patients with myeloid neoplasias, although the poor prognosis associated with 3q21q26 rearrangements could be independent of EVII expression.

EVII is rarely involved in fusion transcripts in 3q21q26 rearrangements. RPNI–EVII 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 RPNI–EVII and GR6–EVII 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 EVII 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 RPNI as an enhancer of EVII 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 RPNI, 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 GATA2 overexpression.

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. EVII 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 EVII 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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