Conventional Cytogenetics and Breakpoint Distribution by Fluorescent *In Situ* Hybridization in Patients with Malignant Hemopathies Associated with inv(3)(q21;q26) and t(3;3)(q21;q26)

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Abstract. *Inv(3)(q21q26)/t(3;3)(q21;q26) is recognized as a distinctive entity of acute myeloid leukemia (AML) with recurrent genetic abnormalities of prognostic significance. It occurs in 1-2.5% of AML and is also observed in myelodysplastic syndromes and in the blastic phase of chronic myeloid leukemia. The molecular consequence of the inv(3)/t(3;3) rearrangements is the juxtaposition of the ribophorin I (RPN1) gene (located in band 3q21) with the ecotropic viral integration site 1 (EVI1) gene (located in band 3q26.2). Following conventional cytogenetics to determine the karyotype, fluorescent in situ hybridization (FISH) with a panel of bacterial artificial chromosome clones was used to map the breakpoints involved in 15 inv(3)/t(3;3). Inv(3) or t(3;3) was the sole karyotypic anomaly in 6 patients, while additional abnormalities were identified in the remaining 9 patients, including 4 with monosomy of chromosome7 (–7) or a deletion of its long arm (7q-). Breakpoints in band 3q21 were distributed in a 235 kb region centromeric to and including the RPN1 locus, while those in band 3q26.2 were scattered in a 900 kb region located on each side of and including the EVI1 locus. In contrast to most of the inversions and translocations associated with AML that lead to fusion genes, inv(3)/t(3;3) does not generate a chimeric gene, but rather induces gene overexpression. The wide dispersion of the breakpoints in bands 3q21 and 3q26 and the heterogeneity of the genomic consequences could explain why the mechanisms leading to leukemogenesis are still poorly understood. Therefore, it is important to further characterize these chromosomal abnormalities by FISH.*

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The revised 2008 WHO classification of tumors of hematopoietic and lymphoid tissues recognized acute myeloid leukemia (AML) with inv(3)(q21q26)/ t(3;3)(q21;q26) as a distinctive entity of AML with recurrent genetic abnormalities of prognostic significance (1). Patients with inv(3)/t(3;3) frequently demonstrate normal or elevated platelet count, atypical megakaryocytes and multilineage dysplasia in bone marrow as well as minimal to no response to chemotherapy and poor clinical outcome (2-4). Inv(3) and t(3;3) occur in 1-2.5% of all FAB types of AML, except M3 (5, 6). They are also observed in myelodysplastic syndromes (MDS) (7) and in blastic phase of chronic myeloid leukemia (CML) (8).

The molecular consequence of the inv(3)/t(3;3) rearrangements is the juxtaposition of the RPN1 gene (ribophosphorin 1 gene located in band 3q21) with the *EVI1* gene (ecotropic viral integration site-1 gene located in band 3q26.2) (9). *EVI1* is a nuclear transcription factor that plays an essential role in the proliferation and maintenance of hematopoietic stem cells (10-12) and can inhibit myeloid differentiation (13). Two alternative forms exists, one generated from *EVI1*, the other *MECOM* (MDS1 and *EVI1* complex locus) through intergenic splicing with MDS1 (myelodysplasia syndrome 1), a gene located 140 kb upstream of *EVI1* (14). It has been suggested that the promoter of the house-keeping *RPN1* could be responsible for the activation of the *EVI1* gene in 3q26 (15). Indeed, the overexpression of *EVI1* can be achieved not only through

rearrangements of band 3q26, but also without the presence of 3q26 abnormalities, therefore indicating that other mechanisms can lead to *EVI1* activation (16-22). Moreover, a substantial number of patients with 3q26 rearrangements do not overexpress *EVI1* (23-25).

Furthermore, overexpression of *GATA2* (GATA binding protein 2), located in band 3q21, was found in leukemia cell lines, as well as in patient samples with inv(3)/t(3;3), compatible with the hypothesis that deregulation of its transcription could contribute to leukemogenesis (26, 27). Indeed, *GATA2*, a member of a family of zinc finger transcription factors, is required in the early proliferative phase of hematopoietic development and is essential for proliferation of definitive hematopoietic stem cells (HSC) (28, 29).

Breakpoints in both regions are scattered over several hundred kilobases (kb). A first breakpoint cluster region (BCR) of about 30 kb located 15 kb centromeric of the *RPN1* gene was identified (called telomeric BCR or BCR-T) (15, 30) but a number of breakpoints were found to map centromeric to that BCR region in another BCR located up to 60 kb from the first described BCR (called centromeric BCR or BCR-C) (5, 31-33). Breakpoints in band 3q26 are distributed on each side of the *EVI1* gene, with t(3;3) and inv(3) breakpoints more likely to be located 5' and 3' of the *EVI1* gene, respectively (9, 15, 16).

Over the past years, several groups have used P1-derived artificial chromosome (PAC) and bacterial artificial chromosome (BAC) clones to locate the breakpoints involved in inv(3)/t(3;3) in bands 3q21 and 3q26 (5, 33-39). A sole study tried to locate the breakpoints more precisely in 7 patients (27). In the present study, we used two BAC libraries to map the 3q21 and 3q26 breakpoints in 11 patients with inv(3) and 4 patients with t(3;3). We also compared our results to those obtained with the commercially available Cytocell Aquarius *EVI1* Breakapart three-color probe (AmpliTech, Compiègne, France).

Patients and Methods

Patients. Patients diagnosed with malignant hemopathies at the Brest University Hospital (Hôpital Morvan) or at the Centre Hospitalier Le Foll in St Brieuc, a hospital located 150 km from Brest, are referred to the Cytogenetics Laboratory of the Brest University Hospital for conventional and molecular cytogenetic analyses. All the patient files from 1998 to 2010 were checked for the presence of inv(3)(q21q26)/t(3;3)(q21;q26) and included in the present study.

Conventional cytogenetics. Conventional cytogenetic analysis was performed on bone marrow cells at the time of diagnosis. Briefly, 24-hour unstimulated bone marrow culture was synchronized with fluorodesoxyuridine (10^{-7} M) for 17 h followed by thymidine (10^{-5} M) for 6 h before colcemide exposure and standard harvesting. R-Banding chromosomal analyses were performed according to standard procedures and the karyotypes described according to the International System for Cytogenetic Nomenclature (ISCN 2009) (40).

Table I. *Location and length of the BAC clones. The base pair positions (bp) are predicted on Build 39 National Center for Biotechnology Information (http://www,ncbi,nlm,nih,gov) and assembly February 2009 by The UCSC Genome Browser Database (http://genome,ucsc,edu/index,html).*

BAC name	Centromeric start (Mb from telomere)	Telomeric start (Mb from telomere)	BAC length (bp)
Band 3q21			
RP11-781E1	127,902,296	128,072,081	169786
CTC-771J4	127,972,360	128,128,021	155662
CTD-3248G10	128,037,896	128,255,193	217298
CTD-3241A20	128,072,114	128,255,217	183104
CTD-3165G11	128,203,529	128,324,553	121025
RP11-912D20	128,204,293	128,338,368	134076
RP11-456K4	128,233,583	128,437,803	204221
CTD-3022D16	128,255,226	128,359,388	104163
RP11-662G11	128,285,184	128,449,816	164633
Band 3q26			
RP11-62L21	168,454,987	168,618,867	163881
CTD-3047I22	168,565,551	168,708,453	142903
RP11-1134G12	168,577,352	168,705,013	127662
CTD-2548L11	168,627,825	168,830,068	202244
RP11-1087G18	168,633,617	168,802,313	168697
RP11-277B2	168,657,645	168,830,955	173311
CTD-2224H5	168,661,905	168,762,910	101006
RP11-33A1	168,704,881	168,869,852	164972
CTD-3219L13	168,711,974	168,925,074	213101
RP11-721P22	168,716,851	168,900,575	183725
RP11-94J18	168,775,990	168,957,538	181549
RP11-816M2	168,830,101	169,020,244	190144
RP11-57B5	168,869,869	169,026,609	156741
RP11-141C22	168,883,796	169,061,277	177482
CTD-2116C5	168,956,972	169,072,363	115392
RP11-13M6	168,987,497	169,159,253	171757
CTD-2383O23	168,995,459	169,090,874	95416
CTD-2006F22	169,016,698	169,125,184	108487
RP11-137H17	169,064,808	169,219,503	154696
RP11-80D15	169,125,660	169,288,166	162507
RP11-1141L22	169,153,042	169,306,102	153061
RP11-1151I22	169,188,143	169,337,916	149774
RP11-3K16	169,246,575	169,449,333	202759
CTD-2582E13	169,408,931	169,606,612	197682
RP11-990E14	169,410,645	169,588,155	177511
CTD-2188E5	169,417,258	169,514,389	97132
RP11-732M7	169,467,881	169,645,626	177746
RP11-778I3	169,492,904	169,717,073	224170
CTD-2532G12	169,522,012	169,743,467	221456
RP11-1145C18	169,538,472	169,701,521	163050
RP11-1123G4	169,566,610	169,701,515	134906
RP11-259C1	169,632,862	169,813,179	180318

Fluorescent in situ hybridization (FISH) analyses with BAC clones. FISH studies were performed on the same fixed material as the conventional cytogenetic analyses. Cell preparations were stored in fixative at –20˚C until use. To delineate the location of the breakpoints, FISH analyses were carried out using BAC clones mapping to bands q21 and q26 of chromosome 3.

Table II. *Clinical and cytogenetic data of 15 patients with inv(3)/t(3;3).*

Patient	Gender	Age (years)	Diagnosis	Conventional cytogenetics at diagnosis
P1	M	19.6	Blastic phase of CML	46,XY,t(9;22)(q34;q11)[7]/46,XY,inv(3)(q21q26),t(9;22)(q34;q11)[8]/46,XY,t(9;22)(q34;q11),i(17)(q10)[3]
P2	M	70.4	MDS	46,XY,inv(3)(q21q26)[4]/46,XY[17]
P3	M	45.2	AML0	46,XY,inv(3)(q21q26)[13]/46,XY,inv(3)(q21q26),t(8;12)(q22;p12)[4]/46,XY[6]
P4	M	58.6	AML	46,XY,inv(3)(q21q26)[22]
P5	M	56.6	MDS->AML	46,XY,inv(3)(q21q26)[21]/46,XY[2]
P6	M	67.6	AML6	46,XY,inv(3)(q21q26)[22]/46,XY,inv(3)(q21q26),t(13;17)(q14;q23)[6]
P7	M	50.0	bipheno AL	45,XY,inv(3)(q21q26),-7[12]/45,XY,inv(3)(q21q26),-7,t(8;21)(p23;q21)[8]/
P8	M	83.7	AML on CMML	46,XY,inv(3)(q21q26)[18]/45,XY,inv(3)(q21q26),-7[4]
P9	F	56.4	AML5	46,XX,inv(3)(q21q26)[22]
P10	F	54.3	MDS->AML	46,XX,inv(3)(q21q26)[2]/46,XX[20]
P11	M	72.1	AML	44,XY,inv(3)(q21q26),dic(?5;17)(?q13;p?11),dic(7;17)(p?11;p?13)[14]/45,XY,inv(3)(q21q26),dic(?5;17)(?q13;p?11),dic(7;17)(p?11;p?13),+r[2]/44,XY,inv(3)(q21q26),dic(?5;17)(?q13;p?11),add(7)(p11),dic(13;18)(q10;q10),der(17)[3]/46,XY[3]
P12	F	66.0	AML7	46,XX,t(3;3)(q21;q26)[1]/46,XX,t(3;3)(q21;q26),del(7)(q31.1)[19]
P13	F	36.5	AML2	44,XX,t(3;3)(q21;q26),-4,add(7)(p10),der(10)t(10;15)(q22;q11),-11,-15,-16,-17,+mar1,+mar2,+r(?)[12]/46,XX[1]
P14	F	72.3	AML	46.XX.t(3;3)(q21;q26)[1]/45.XX.t(3;3)(q21;q26).-7[1]/46.XX[18]
P15	M	57.1	AML2	46.XY.t(3;3)(q21;q26)[20]

M: Male; F: female; CML: chronic myeloid leukemia; AML: acute myeloid leukemia; AL: acute leukemia; MDS: myelodysplastic syndrome; CMML: chronic myelomonocytic leukemia.

We identified the BAC clones of interest through the Human Genome Browser Database of the Genome Bioinformatics Group at the University of California at Santa Cruz (http://genome.ucsc.edu/). They were then ordered from the Children's Hospital Oakland Research Institute in California (http://bacpac.chori.org/). Bacterial cultures were prepared from a single colony picked from a selective plate in the presence of chloramphenicol. Plasmids were obtained from bacterial cultures grown in the presence of chloramphenicol (10 mg/l). After lysing bacteria using 1% SDS/0.2 N NaOH, DNA was purified from RNA, proteins and other cellular contaminants. Probes were then labeled by nick translation in Spectrum Orange (Nick Translation Kit, Abbott, Rungis, France) or in fluorescein isothiocyanate (FITC; Prime-it Fluor Fluorescence Labeling Kit, Stratagene, Amsterdam, the Netherlands). All BAC clones were applied to normal lymphocyte metaphases to confirm their chromosomal location.

After hybridization, the slides were counterstained with 4-6-diamino-2-phenyl-indole-dihydrochloride. The preparations were examined using a Zeiss Axio Plan Microscope (Zeiss, Le Pecq, France). Image acquisition was performed using a CCD camera and analyzed using the In Situ Imaging System program (MetaSystems, Altlussheim, Germany).

Two libraries of 9 and 32 overlapping BACs covering bands 3q21 and 3q26 respectively were applied to the patients (Table I).

FISH analyses with commercially available probe. A FISH study using the Cytocell Aquarius *EVI1* Breakapart probe (AmpliTech, Compiègne, France) was carried out on the metaphase preparations from all patients, as recommended by the manufacturer. The *EVI1* Breakapart probe contains three probes: a probe labeled in Aqua of 562 kb in size centromeric to the *EVI1* gene, a probe labeled in Spectrum Green of 181 kb covering *EVI1* and its flanking regions and a probe labeled in Spectrum Orange of 124 kb telomeric of the EVI1 gene (telomeric of *MYNN* and covering *LRRC34*).

Results

Patients. From 1998 to 2010, 15 patients were found to have an inv(3)(q21q26) or a t(3;3)(q21;q26), (Table II). There were 10 males and 5 females. The mean age at the time of diagnosis was 57.8 years (standard deviation: 15.9 years). Nine patients had a diagnosis of AML and one of acute biphenotypic leukemia. One patient had AML evolving on chronic myelomonocytic leukemia and another was in the blastic phase of CML. Three patients had MDS, two of them developing AML within 2 years following the diagnosis of MDS.

R-Banding conventional cytogenetics showed 11 patients to have an inv(3)(q21q26) and the remaining 4 a t(3;3)(q21;q26). Inv(3) or t(3;3) was the sole karyotypic anomaly in 6 patients. Additional abnormalities were identified in 9 patients. Various anomalies led to a partial deletion of the short arm of chromosome 17 in 3 patients, while 4 other patients had monosomy 7 (–7) or a deletion of its long arm (7q-) (Table II).

Breakpoint mapping with BAC clones. Sequential FISH analyses with BAC clones were applied on metaphases of all 15 patients. Breakpoints in band 3q21 were distributed in a

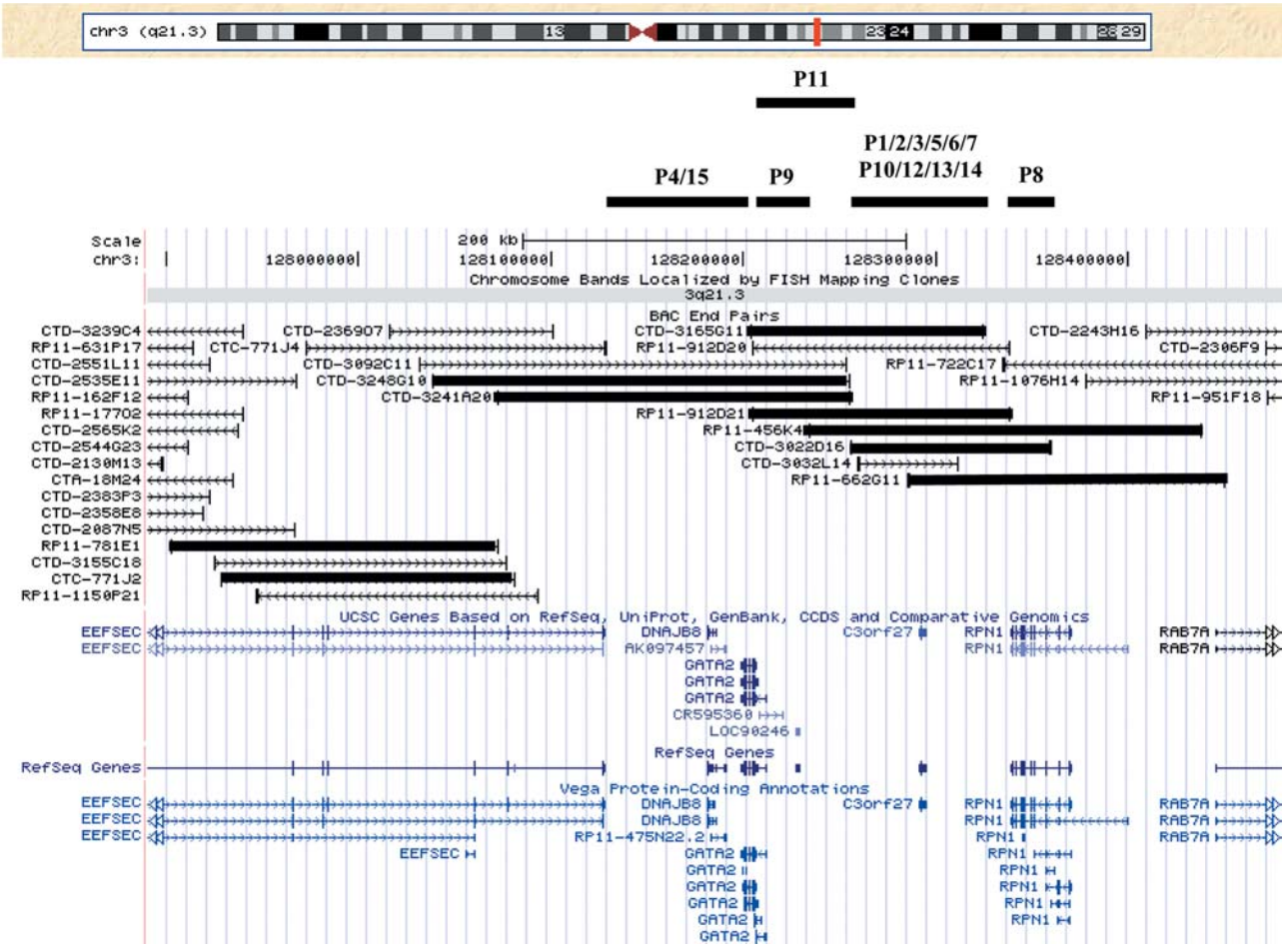

Figure 1. *3q21 breakpoint distribution of the 15 patients with inv(3)/t(3;3). Black rectangles show the BAC clones used for mapping the breakpoint.*

235 kb region centromeric to and including the *RPN1* locus (Figure 1). The breakpoint to one patient (P8) was localized in the *RPN1* gene, whereas the breakpoints for 10 other patients mapped to a 72 kb region situated about 15 kb from the *RPN1* locus.

Breakpoints in band 3q26.2 were distributed in a 900 kb region located on each side of and including the *EVI1* locus (Figure 2). The breakpoints of all 11 inv(3) were localized in or centromeric to the *EVI1* gene, in a region extending up to 180 kb from the *EVI1* locus. The 3q26 breakpoints of the four t(3;3) were scattered in a wider region, one breakpoint (P14) being some 250 kb and another (P12) some 650 kb telomeric of the *EVI1* locus.

Comparison of the breakpoint mapping using the EVI1 Breakapart probe and the BAC clones. Two sets of signal combinations were found among the 11 patients carrying an inv(3)(q21q26). Three patients (P2, P4 and P5) showed a split green signal in bands 3q21 and 3q26, with the blue signal being translocated to band 3q21 and the red signal remaining in band 3q26. This combination is compatible with a break having occurred in or close to the *EVI1* gene. The remaining inv(3) patients showed the blue signal translocated to band 3q21, whereas both green and red signals were located in band 3q26. This combination is compatible with breakpoints localized farther centromeric of the *EVI1* locus (Figure 2).

Two sets of signal combinations were found among the four patients carrying a t(3;3)(q21;q26). Two patients (P12 and P14) had a small derivative chromosome 3 (der(3) with a red signal and a large der(3) with a blue-green-blue-green-red signal combination compatible with a break having occurred telomeric to the *EVI1* gene. The two remaining patients (P13 and P15) had a small der(3) with green and red signals and a large der(3) with a blue-green-blue-green-red signal combination, compatible with a break having occurred in or close to the *EVI1* gene (Figure 2).

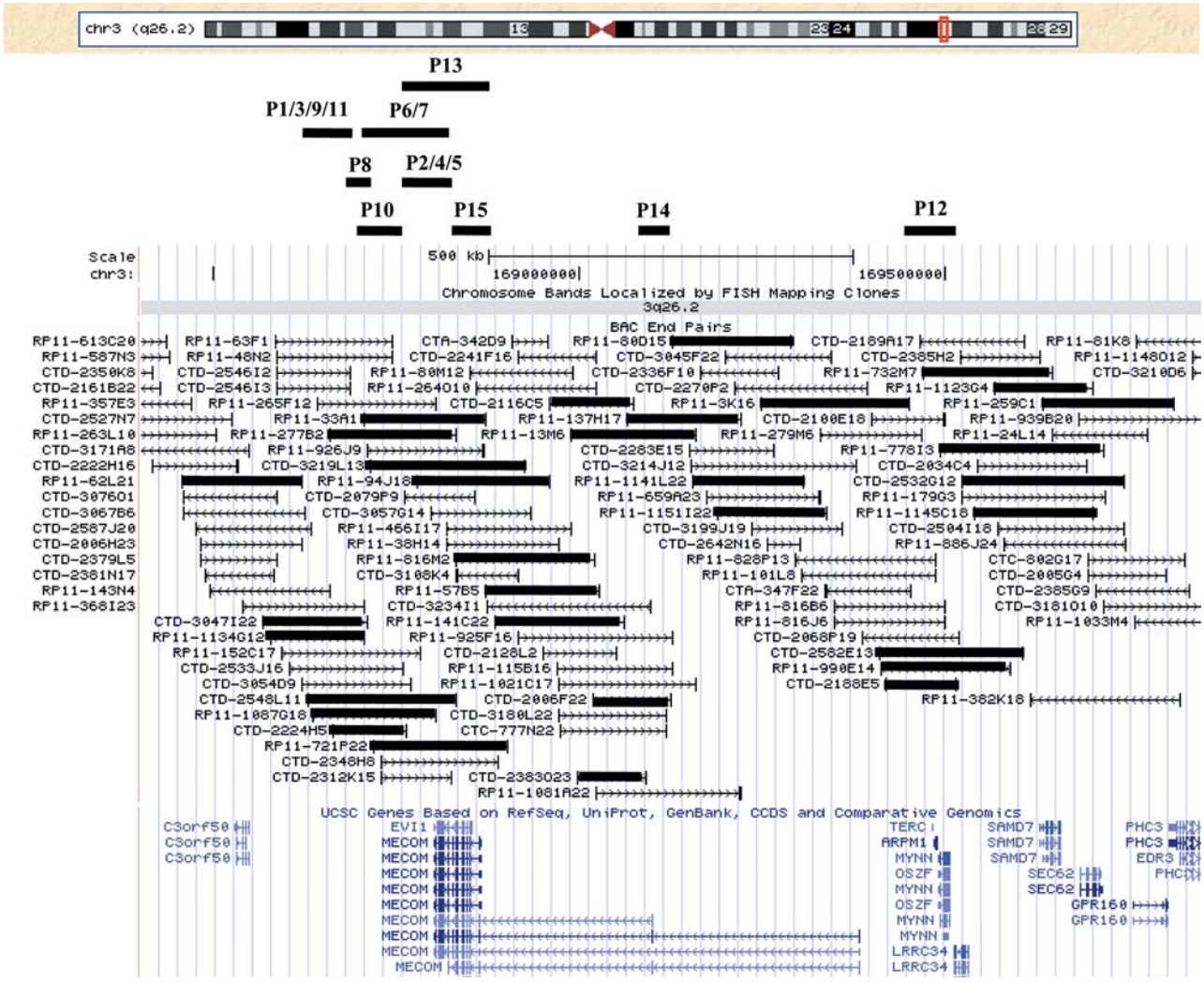

Figure 2. *3q26 breakpoint distribution of the 15 patients with inv(3)/t(3;3). Black rectangles show the BAC clones used for mapping the breakpoint.*

Discussion

Patients with inv(3)(q21q26) or t(3;3)(q21;q26) are now classified in a distinctive entity of AML with recurrent genetic abnormalities of prognostic significance (1). Although the inv(3)/t(3;3) is not restricted to AML, patients with MDS or CML in the blastic phase associated with these abnormalities have usually a progressive disease and a poor prognosis (7, 8).

Additional chromosomal abnormalities besides inv(3)/t(3;3) are frequently found, more particularly –7/7q-. A non-exhaustive search in the literature identified 278 patients, including ours, with inv(3) (191 patients, 68.7%) or t(3;3) (87 patients, 31.3%) (2, 3, 6, 37, 39, 41-44). Among these 278 patients, 190 (68.3%) had at least one additional abnormality, including 145 (76.3%) with –7/7q-. Retroviral integration experiments have shown that overexpression of the *EVI1* gene was not sufficient to cause leukemia, indicating that development of malignancy likely depended on the occurrence of additional mutations (45). However, neither the exact link between inv(3)/t(3;3) and –7/7q-, nor the sequence of events, is fully understood (46). Recently, using array comparative genomic hybridization (array CGH), De Weer *et al*. identified two deleted regions in 7q35-36 in patients with inv(3)/t(3;3) overexpressing the *EVI1* gene (47).

In contrast to most of the inversions and translocations associated with AML that lead to fusion genes, the inv(3)/t(3;3) does not generate a chimeric gene, but rather induces gene overexpression (10, 15, 26). Indeed, *EVI1* overexpression is unlikely to be a consequence of the

formation of fusion transcripts because of the position and transcription orientation of the genes involved in the rearrangement (27). The wide dispersion of the breakpoints in bands 3q21 and 3q26 and the heterogeneity of the genomic consequences necessitate further characterization of these chromosomal abnormalities by FISH.

Wieser *et al*. developed an interphase dual-color FISH assay using PAC clones to detect rearrangements of band 3q21 (33). They assembled PAC contigs covering both BCRs (centromeric and telomeric), which allowed them to map the majority of 3q21 breakpoints involved in inv(3)/t(3;3) (5). Wieser *et al*. also developed a similar assay using several overlapping PAC clones localized on the centromeric and telomeric sides of the *EVI1* locus to detect rearrangements in band 3q26 (34). Other workers constructed dual-color probes located on either sides of the *EVI1* locus using PAC or BAC clones (35-38). These constructions differed in size, ranging from a few hundreds kilobases to more than 1 megabase.

Shearer *et al*. developed a dual-color, double fusion FISH assay to detect *RPN1–EVI1* gene fusion associated with inv(3)/t(3;3) using BAC clones. They labeled 3 BAC clones located in band 3q21 centromeric of *GATA2*, between *GATA2* and *RPN1*, and telomeric of *RPN1* in Spectrum Green and 11 BAC clones in band 3q26 covering the *EVI1*/*MDS1* (*MECOM*) gene and beyond (39). They identified the fusion in 94% of the 47 samples analyzed.

We used a different approach because our goal was to map more precisely the breakpoints involved in inv(3)/t(3;3), and not only to verify the *RPN1–EVI1* fusion by molecular cytogenetics. Our results showed a wide heterogeneity of breakpoints, as previously observed by Lahortiga *et al*. in 7 patients (27).

Breakpoints in band 3q21 were distributed in a 235 kb region, with breakpoints of 10 patients clustering in the so-called BCR-T and 2 in the BCR-C, with no preferential localization of the breakpoints involved in inv(3) or t(3;3). These results are compatible with those previously reported (15, 30-32). The distribution of the 3q26 breakpoints is much wider (about 900 kb), with a propensity for breakpoints involved in t(3;3) to be located telomeric of the *EVI1* gene, as already shown (9, 15, 16).

No discrepancy between our results obtained with BAC clones and those using the *EVI1* Breakapart probe was found. Therefore, this commercial probe could be used to screen not only patients with inv(3)/t(3;3), but presumably also patients with other chromosomal abnormalities involving band 3q26.

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