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Technical Advances

Pulsed-Field Gel Electrophoresis Analysis of Retinoic Acid Receptor-α and Promyelocytic Leukemia Rearrangements

Detection of the t(15;17) Translocation in the Diagnosis of Acute Promyelocytic Leukemia

Yong-Hong Xiao,*‡ Wilson H. Miller, Jr.,† Raymond P. Warrell, Jr.,†§ Ethan Dmitrovsky,† and Andrew D. Zelenetz*‡

From the Divisions of Hematologic Oncology and Solid Tumor Oncology,† Department of Medicine, Memorial Hospital‡ and the Programs in Molecular Biology and Experimental Therapeutics,§ Sloan-Kettering Institute, Memorial Sloan-Kettering Cancer Center, New York, New York*

Acute promyelocytic leukemia (APL) is characterized cytogenetically by a balanced reciprocal chromosomal translocation t(15;17) (q22;q21). This translocation involves the retinoic acid receptor-α (RAR-α) on chromosome 17 and the promyelocytic leukemia locus (PML) on chromosome 15 and results in the transcription of novel fusion messenger RNAs. In this study, pulsed-field gel electrophoresis (PFGE) was applied to the detection of the t(15;17) translocation in twenty-six clinical specimens cytologically diagnosed by French-American-British criteria as APL. This technique could readily be applied to both fresh and nonviably frozen tumor samples. In 24 of 26 samples, rearrangements of the PML and RAR-α, loci could be detected by Southern blotting after digestion with MluI and BssHII. Furthermore, co-migration of the rearranged fragments, detected by hybridization to probes for the PML and RAR-α genes, demonstrated that these loci were juxtaposed. The translocation was detected in specimens at the time of initial diagnosis, on differentiation therapy with retinoic acid and at the time of relapse. The diagnostic accuracy was compared to cytogenetics and the reverse transcriptase-polymerase chain reaction for the novel PML-RAR-α fusion transcript. The samples from two patients were negative by all three diagnostic methods, and both of these patients failed to respond to all-trans retinoic acid. In the other 24 APL samples, cytogenetics was positive in only 76.9% of the cases, whereas both reverse transcriptase-polymerase chain reaction and PFGE methods detected the translocation in 100% of the cases. Thus, PFGE can readily detect the t(15;17) translocation in both viable and nonviable clinical specimens and can improve the diagnostic accuracy of morphology and cytogenetics in APL. In contrast to conventional electrophoresis based on rearrangement of RAR-α, the ability to demonstrate directly co-migration of the PML and RAR-α loci enables this method to distinguish the t(15;17) translocation from variant translocations such as the t(11;15). Because PFGE can be performed on nonviable, frozen tumor samples, it could be diagnostically useful in APL when the RNA-based reverse transcriptase-polymerase chain reaction cannot be performed. (Am J Pathol 1993, 143:1301–1311)

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Address reprint requests to Dr. A.D. Zelenetz, Memorial Sloan-Kettering Cancer Center, Box 330, 1275 York Avenue, New York, NY 10021.

Acute promyelocytic leukemia (APL; French-American-British [FAB] classification M3) is characterized by a predominance of malignant promyelocytes that carry a balanced reciprocal chromosomal translocation of t(15;17) (q22;q12–21) that is uniquely found in this disease.[1–3] The translocation breakpoints occur within the retinoic acid receptor-α (RAR-α) gene on chromosome 17 and within a novel gene named PML (for promyelocytic leukemia) on chromosome 15.[4–9] This translocation results in the transcription of novel fusion messenger RNAs: PML-RAR-α from the der(15) chromosome and RAR-α-PML from the der (17) chromosome.[5,10–14] These transcripts direct the synthesis of fusion proteins that likely play a role in either leukemogenesis or retinoid response in APL. The accurate identification of the t(15;17) translocation is critically important in the management of patients with APL, because its presence predicts response to differentiation therapy with all-trans retinoic acid (ATRA).[15,16]

Previous studies in other laboratories have shown that the chromosome 17 breakpoints occur within the second 20-kb intron of the RAR-α gene. Using a variety of restriction enzymes and molecular probes, Diverio et al.[17] have demonstrated universal detection of rearrangements in the RAR-α locus in patients with APL. However, because of the variability of the breakpoints within the PML locus on chromosome 15, comigration between chromosome 15 and 17 markers cannot be readily detected. Thus, methods directed at rearrangement of RAR-α cannot distinguish the t(15;17) translocation from the variant t(11;17) translocations.[18,19] These limitations are inherent in analysis of chromosomal translocations that can occur over large distances relative to the average fragment size generated with typical restriction endonucleases having a 6-bp recognition sequence. However, some restriction enzymes generate very large average fragment lengths either because their recognition sequence is greater than 6 bp or the recognition sequence contains CpG dinucleotide pairs (which are markedly underrepresented in mammalian genomes[20]) or both. These large fragments can be resolved by pulsed-field gel electrophoresis (PFGE), which uses alternating electric fields to separate very large DNA fragments (>50 kb to ~10 Mb).[21,22] Because the average size fragment generated with these enzymes is large, the ability to detect chromosomal translocations is improved. We have recently demonstrated that the molecular diagnosis of the t(14;18) translocation seen in 90% of follicular lymphomas was most comprehensively made by Southern blotting of DNA cleaved with rare-cutting restriction enzymes and resolved by PFGE as compared to conventional Southern blotting, polymerase chain reaction (PCR) amplification of the translocation breakpoint, and cytogenetic analysis.[23] Similar observations have been made by Hooberman et al[24] in the detection of the Philadelphia chromosome seen in acute lymphoblastic leukemia. Thus, we undertook this study to determine if PFGE could also be useful in the detection of the t(15;17) translocation in clinical specimens of patients with acute leukemia.

In this study, we examined 26 blood and/or bone marrow specimens from 25 patients with the cytological diagnosis of APL and five patients with other leukemias for the presence of the t(15;17) translocation by PFGE. Rearrangements of both the PML and RAR-α loci were detectable in all these cases of APL, whereas they were absent in the other forms of leukemia. Furthermore, in all cases, co-migration of the fragments detected with the chromosome 15 and 17 probes directly demonstrated that we were detecting the t(15;17) translocation. Based on these data, we conclude that PFGE analysis of RAR-α and PML rearrangements can be used as an alternative for the accurate diagnosis of APL.

Materials and Methods

Patient Samples

Bone marrow or peripheral blood samples were obtained from patients with acute and chronic leukemias after obtaining informed consent. Leukemic cells were fractionated on Ficoll-Hypaque density gradients (Pharmacia, Piscataway, NJ) as recommended by the manufacturer. Some samples were snap-frozen and stored in liquid nitrogen. Other samples were immediately processed as described below. The diagnoses were made by standard clinical and cytological criteria, according to the FAB criteria by the attending hematologist and the hematopathology staff of Memorial Hospital.[1,2] Karyotyping was performed by the G-band technique[25] in the clinical cytogenetics laboratory at Memorial Hospital.

DNA Probes

Molecular probes for hybridization were prepared by incorporation of [α-^{32}P]dCTP into gel-purified DNA fragments by the method of random hexamer priming.[26] The RAR-α full-length complementary DNA clone was kindly provided by Dr. Pierre Chambon (Strasburg, France).[27] The PML full-length cDNA was obtained as previously described.[28] Genomic RAR-α gene probes consisted of a 2.9-kb

*Bam*HI-*Not*I fragment and 4.5-kb *Bam*HI-*Not*I fragment.[4] LCN4A3/B probe was derived from the 3′ end of the second intron of RAR-α as well as the third exon. The LCN4A3/A probe was derived from the region immediately upstream of the LCN4A3/B probe (Figure 1).

Preparation of DNA for PFGE

Sample preparation was performed as previously described with minor modifications and adapted for use in nonviably frozen material.[23] Snap-frozen material was fragmented and a small portion of the specimen was quickly weighed and then resuspended in 500 µl of 1× phosphate-buffered saline (1.4 mmol/L $KH2PO_4$, 4.3 mmol/L Na_2HPO_4, pH 7.4, 137 mmol/L NaCl, 2.7 mmol/L KCl), and the number of viable (generally 0) and nonviable cells was determined by staining with 0.05% trypan blue and counting on a hemocytometer. This number was used to calculate the number of cells per milligram of frozen material. A second piece of frozen material was weighed and the total cell number determined as follows:

$$\text{total cells} = \frac{\text{cells/mg in first sample}}{\text{weight of second sample (mg)}}$$

The second frozen sample was then dissolved in $T_{10}E_{100}$ (10 mmol/L Tris, pH 8.0, 100 mmol/L ethylenediaminotetraacetic acid) at 4 × 10^7 cells/ml. Fresh cells were resuspended at a concentration of 4 × 10^7 cells/ml in $T_{10}E_{100}$. From this point on, both fresh and frozen material were processed in the same manner. Cell suspensions were mixed with an equal volume of molten agarose solution (2% InCert agarose [FMC, Rockland, ME] in $T_{10}E_{100}$ cooled to 42 C) and 250 µl aliquots distributed to wells of a sample mold (Bio-Rad, Richmond, CA). After allowing the blocks to solidify for 30 minutes at 4 C, blocks were suspended in 10 ml of $T_{10}E_{100}$ containing 1% N-lauryl sarcosine (Sigma Chemical Co., St. Louis, MO), 0.3 mol/L 2-mercaptoethanol (Sigma), and 200 µg/mL Proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, IN) and incubated at 55 C for 16 hours. The plugs were washed and stored as previously described.[23]

PFGE

Restriction endonuclease digestions were performed on agarose-embedded DNA as previously described.[23] PFGE was performed in a contour-clamped homogeneous electric field (CHEF)[29] in either a CHEF Mapper or CHEF DRII apparatus (Bio-Rad). Gels were cast as recommended by the manufacturer using 1% SeaKem GTG agarose (FMC) in 0.5× TBE. PFGE was performed in 0.5× TBE at 14 C in an electric field of 6V/cm. For a window of resolution of 25 kb to 400 kb (used for separation of the *Bss*HII digests) the switch time (T_p)

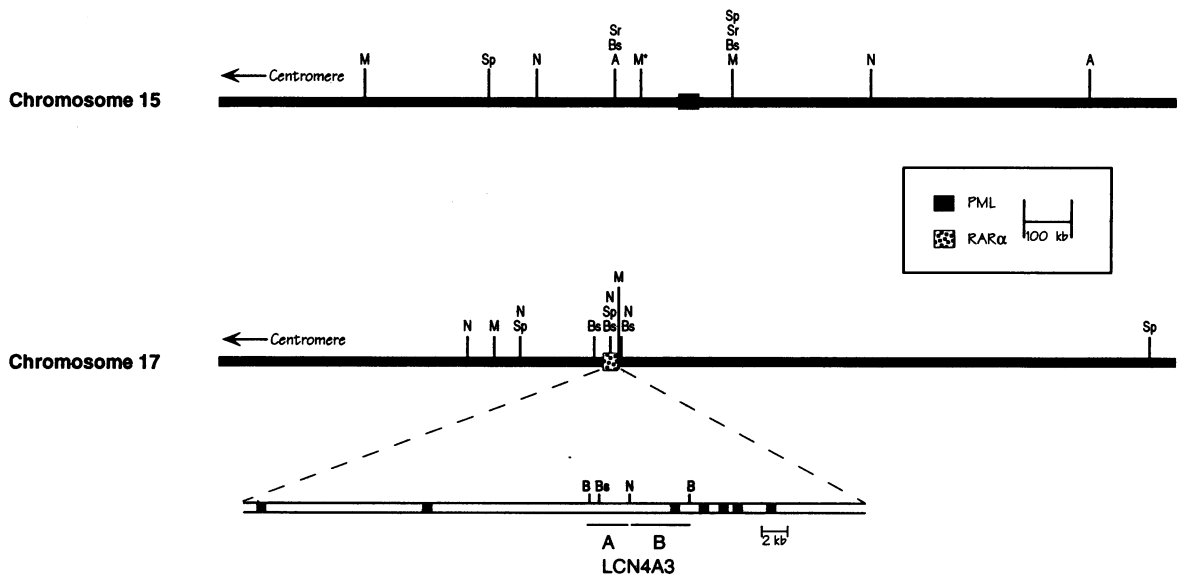

Figure 1. *Physical maps of the PML and RAR-α loci on chromosomes 15 and 17, respectively. The maps were prepared by standard techniques using PBLs, normal placenta, APL tumor specimens, and the NB4 cell line. Note the PML gene is flanked by two CpG islands. The distance between the two CpG islands varies from 220 kb to 310 kb, consistent with a megabase polymorphism. The approximate positions of the RAR-α and PML genes are shown. The exploded region illustrates the position of the LCN4A3/A and B probes relative to the genomic organization of RAR-α as previously described[13,34]; only the relevant BamHI (B) sites are shown. Other restriction enzyme sites: A, AscI; Bs, BssHII; M, MluI; N, NotI; Sp, SpoI; Sr, SrII. The asterisk (*) represents a partially methylated site.*

was linearly ramped from 1.8 seconds to 35.4 seconds for 20.2 hours; a 50-kb to 1.2-Mb window of resolution (used for separation of the *Mlu*I digests) was achieved with a linearly ramped T_p of 1.8 seconds to 114.5 seconds for 20.9 hours. Saccharomyces cerevisiae chromosomes and phage λ multimers size standards were obtained commercially (Bio-Rad).

Reverse Transcriptase - (RT) PCR

Preparation of RNA, cDNA, and amplification of the PML-RAR-α fusion transcript by PCR has been previously described in detail.[30] RNA was prepared from fresh tumor material, and then in some instances the remainder of the tumor sample was snap-frozen in liquid nitrogen.

DNA Hybridization

DNA transfer and hybridization were performed as previously described in detail[23] with two minor modifications: all the hybridizations and washes were performed in a hybridization oven (Robbins Scientific, Inc., Sunnyvale, CA) and the final wash step was performed at 42 C rather than 55 C. The alteration of washing conditions did not substantially increase hybridization background and permitted shorter exposure times.

Results

Megabase Restriction Maps of the RAR-α and the PML Loci

Megabase restriction maps were generated for the RAR-α and PML loci by standard techniques in both normal peripheral blood lymphocytes (PBLs) and in t(15;17)-positive cell line NB4[31] derived from an APL specimen using single and double digests and are shown in Figure 1. The map for RAR-α locus is consistent with that published by Borrow et al.[4] The PML locus was flanked by two CpG islands as indicated by the clustering of cleavage sites for restriction enzymes sensitive to cytosine methylation. The restriction map of the PML locus was complex; at least four distinctly sized bands of 220 kb, 240 kb, 270 kb, and 310 kb were noted in DNA prepared from PBLs of normal individuals and digested with *Bss*HII; though a given individual had only one or two of these bands. Identical results were obtained with *Mlu*I and *Asc*I double digestion of normal PBL DNA (data not shown). These data suggest that there is an unusually large polymorphism in the region of the PML locus (in preparation). The orientation of the PML locus was determined by mapping the PML locus in t(15;17)-bearing cells and inferring the orientation from the known structure of the translocation. The exact position of the PML gene within the *Bss*HII fragment has not been determined.

PFGE Detection of the t(15;17) Translocation in a Cell Line as well as in Fresh and Frozen Leukemic Samples

In constructing the megabase restriction enzyme maps, nongermline bands were observed in DNA extracted from the APL cell line, NB4, which were absent in the myeloid cell line, HL-60, derived from an acute myelogenous leukemia (AML) (FAB-M2).[32] In NB4 DNA digested with *Bss*HII, two nongermline bands of 150 and 200 kb were detected with a PML cDNA probe that co-migrated with two nongermline bands detected with a RAR-α cDNA probe (Figure 2, left panel). Two rearranged bands were expected corresponding to the PML-RAR-α rearrangement on the der(15) chromosome and the RAR-α-PML rearrangement of the der(17) chromosome. The blots were rehybridized with an RAR-α genomic probe, LCN4A3/B, which included the 3′ end of the second intron and extended to the proximal portion of the third intron including the third exon (Figure 1); this probe detected both of the rearranged bands, suggesting that the translocation breakpoint in the NB4 cell line mapped to the region spanned by the probe (Figure 2, left panel). To test this hypothesis, another probe, LCN4A3/A, which lies immediately upstream of the LCN4A3/B probe (Figure 1), was used to hybridize these blots. This probe detected only the 200-kb nongermline band. Because the LCN4A3/A probe is proximal to the translocation breakpoint, the 200-kb band must correspond to the der(17) chromosome. When DNA from the NB4 cell line was digested with the restriction endonucleases *Mlu*I and hybridized with the PML cDNA probe, two germline bands of approximately 200 kb and 800 kb and nongermline bands of 450 kb and 760 kb were observed (Figure 2, right panel). The 200-kb germline band is readily observed in the cell lines HL-60 and NB4 (Figure 2, right panel) but weak or absent in clinical specimens (Figure 3, bottom, left). This 200-kb fragment was generated from

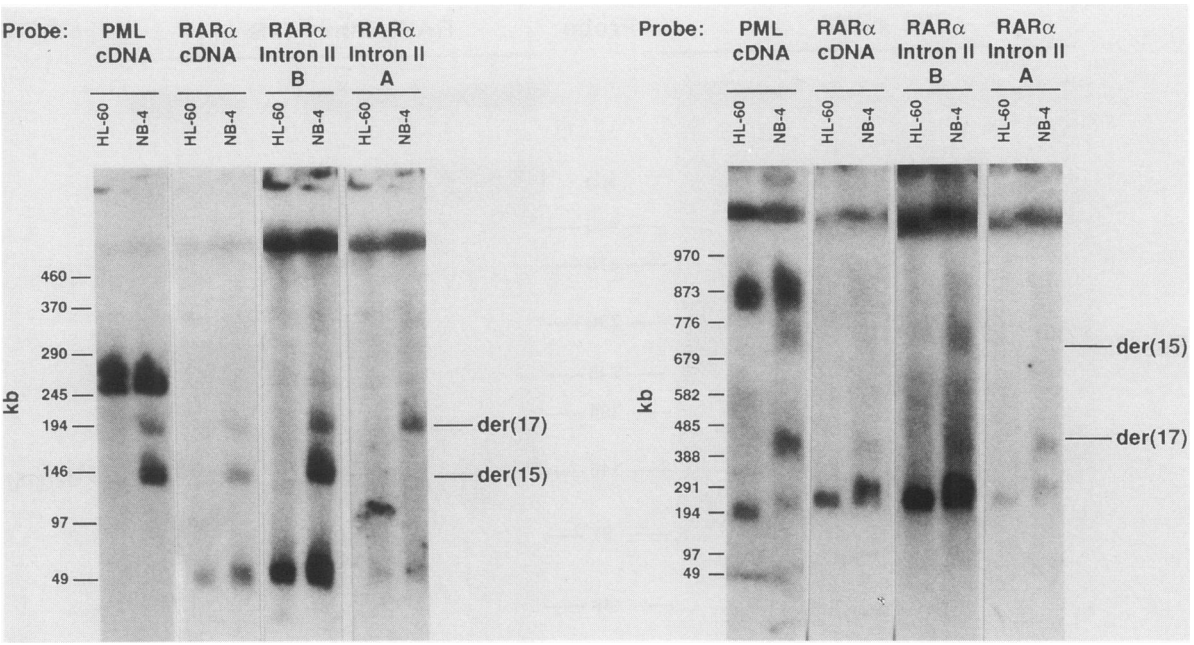

Figure 2. *Analysis of t (15:17) by PFGE in the NB4 cell line. Megabase DNA was prepared from the NB4 and HL-60 cell lines and digested with either BssHII* (**left panel**) *or MluI* (**right panel**), *resolved by PFGE, and blotted to a positively charged nylon membrane as described in Materials and Methods. The filters were sequentially probed with a PML cDNA probe, RAR-α cDNA probe, and RAR-α genomic probes LCN4A3/A and LCN4A3/B. Phage λ concatemers and S. cerevisiae chromosomes were used as size standards. The position of the two rearrangements that were detected are indicated as corresponding to the der(**15**) and der(**17**) chromosomes* (***see text***).

cleavage at a *Mlu*I site (see map in Figure 1) that is partially methylated in the cell lines and more completely methylated in the tumor and PBL samples (data not shown). Both nongermline bands were also detected with the LCN4A3/B RAR-α intron probe. The 450-kb band was detected with the LCN4A3/A probe, demonstrating that this band corresponds to the der(17) chromosome. Thus, the t(15;17) chromosomal translocation was directly demonstrated in the NB4 cell by co-migration of nongermline bands detected with the PML and RAR-α probes after either *Bss*HII or *Mlu*I digestion.

Having demonstrated that the t(15;17) translocation could be detected in the NB4 cell line after DNA digestion with both *Mlu*I and *Bss*HII, we sought to determine if similar results could be obtained from clinical specimens. Figure 3 illustrates typical results in nine clinical specimens, six were APL by morphological criteria (APL53 and 73 represent the same patient at different points in time), and one case each of acute lymphoblastic leukemia, AML (FAB M4), and chronic myelogenous leukemia. Megabase DNA was prepared from clinical specimens, digested with either *Bss*HII and *Mlu*I, and transferred to positively charged nylon membranes, as described in Materials and Methods. The filters were hybridized to the LCN4A3/B probe, demonstrating a single nongermline band in five of the APL samples and two rearrangements in sample APL121. Like the NB4 cell line, the breakpoint in APL121 occurred within the region spanned by the probe; this was confirmed by hybridization with the LCN4A3/A probe (data not shown). When the filters were stripped and rehybridized with the PML cDNA probe, two nongermline bands were detected in all six APL samples in either *Bss*HII or *Mlu*I digested DNA. Thus, with two different enzymes it was possible to demonstrate the t(15;17) chromosomal translocation. Three samples from non-APL patients lacked rearrangements of either RAR-α or PML. In the six APL samples, in both *Bss*HII- and *Mlu*I-digested DNA, there was co-migration of nongermline bands detected with the PML and RAR-α probes.

Specimens APL53 and APL73 represent samples obtained on a single patient at baseline and following 28 days of therapy with ATRA. In the APL73 sample, the cells were morphologically differentiated into band forms (data not shown). However, both the baseline and treatment samples had identical rearrangements demonstrating persistence of the translocation despite ATRA therapy. Thus, analysis of clinical specimens for the t(15;17) translocation can distinguish cells of the tumor clone that undergo morphological maturation in response to ATRA therapy but retain the molecular lesion from normal neutrophils lacking the translocation.

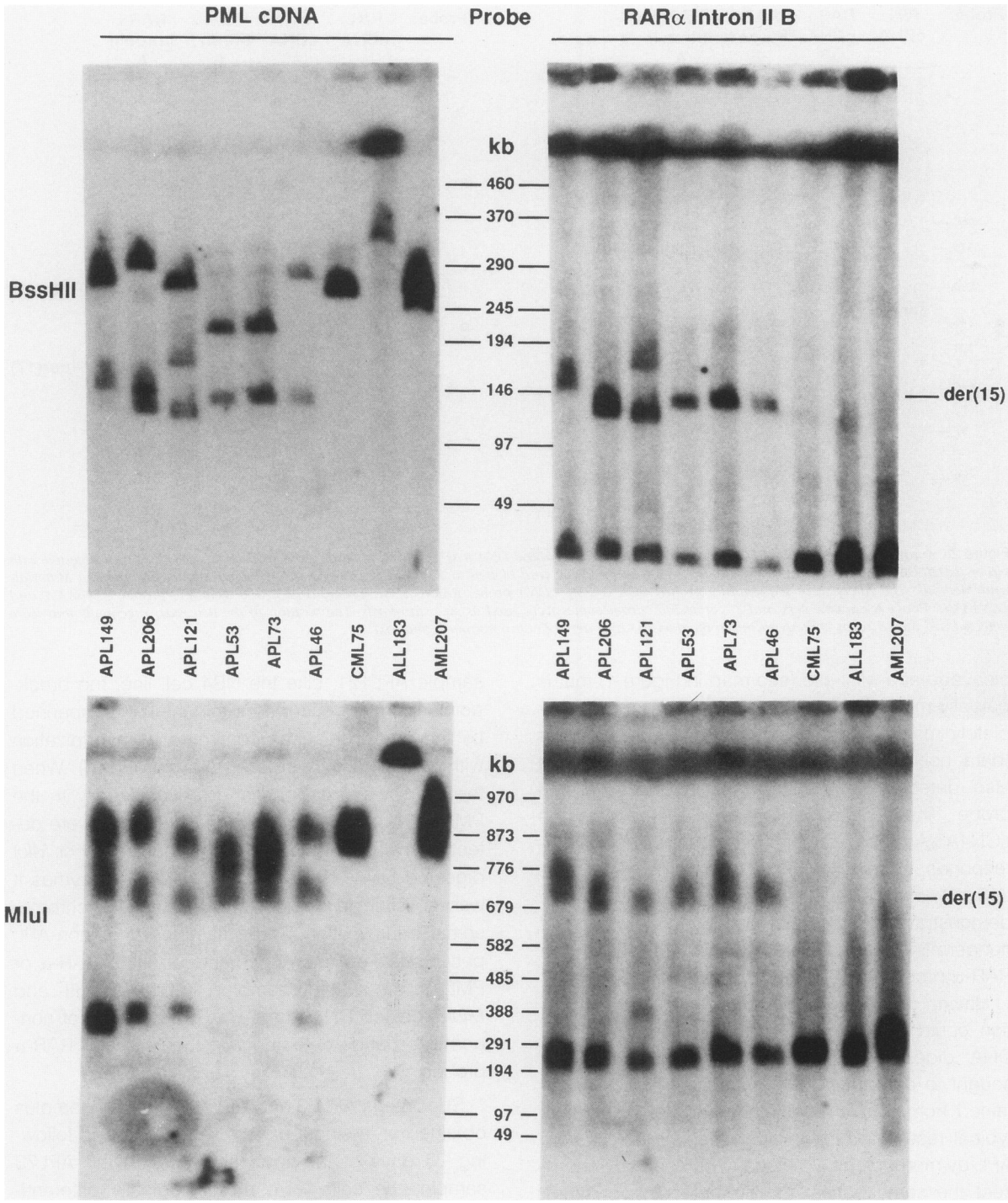

Figure 3. *Analysis of t(15:17) in clinical specimens by PFGE. Megabase DNA was prepared from nine clinical specimens and digested with either BssHII* (top) *or MluI* (bottom), *resolved by PFGE, and blotted as described in Materials and Methods. The filters were sequentially probed to a PML cDNA probe and the RAR-α genomic probe, LCN4A3/B. The first six lanes represent samples cytologically identified as APL. The last three lanes were samples from cases of chronic myelogenous leukemia, acute lymphoblastic leukemia, and AML* (FAB-M4), *respectively. The der*(15) *band is indicated and corresponds to the band that co-migrates with the two probes.*

The t(15;17) Translocation Detected in a Series of Fresh and Frozen Tumor Material

Because APL cells are rich in RNAse, RNA-based methodology for detection of the fusion transcript (the product of the t(15;17) translocation) required careful handling of the tumor specimens.[10,30] We examined a series of samples frozen early in the investigation of the t(15;17) translocation before the initiation of our PFGE studies. Residual Ficoll-Hypaque density gradient-purified cells were stored as cell pellets without regard to sample viability by snap-freezing in liquid nitrogen. To determine if these samples could be used for the diagnosis of the t(15;17) translocation by PFGE, megabase DNA was prepared as described in Materials and Methods. Of note, the cells were generally 100% nonviable as determined by trypan blue exclusion. When megabase DNA prepared from the frozen material was analyzed by PFGE without prior restriction enzyme digestion, these samples had a greater degree of degradation than fresh samples (data not shown); nonetheless, the samples were adequate for evaluation of the t(15;17) translocation (Figure 4). As with the fresh samples, rearrangement of the PML and RAR-α loci could be detected in both *Bss*HII- and *Mlu*I-digested DNA. Six frozen samples were analyzed, and the t(15;17) translocation could be detected in all those tested. Table 1 summarizes our results with PFGE in the detection of the t(15;17) translocation by PFGE in both fresh and frozen material cytologically diagnosed as APL. Overall, in 24 of 26 cases the t(15;17) translocation could be identified (see below).

Comparison of PFGE to Cytogenetics and RT-PCR in the Detection of the t(15;17) Translocation

The presence of the t(15;17) translocation is an important predictor in the clinical response of patients with APL to ATRA.[14,16,30] Previously, it has been shown that the detection of the PML-RAR-α fusion transcript by Northern analysis or RT-PCR was superior to conventional cytogenetics alone in the determination of the t(15;17) translocation.[30] Thus, we compared our results obtained with PFGE, RT-PCR, and cytogenetics. Our results in 26 cases cytologically diagnosed as APL are presented in Table 2. PFGE and RT-PCR were able to detect the t(15;17) translocations in 24 of 26 samples tested at baseline, relapse, as well as patients on therapy with

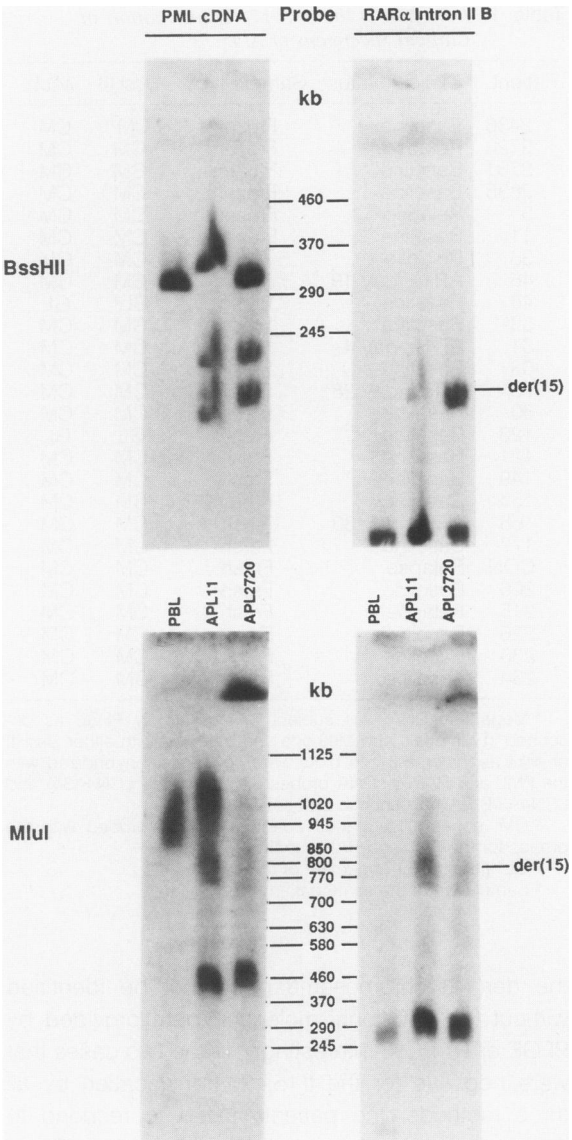

Figure 4. *Analysis of t (15:17) in frozen specimens by PFGE. Megabase DNA was prepared from snap-frozen leukemic cells as described in Materials and Methods. Agarose-embedded DNA was digested with either BssHII* (top) *or MluI* (bottom), *resolved by PFGE, and blotted. The filters were sequentially probed to a PML cDNA probe and the RAR-α genomic probe, LCN4A3/B. The first lane was DNA derived from fresh PBLs from a healthy volunteer (A.D.Z.). Samples APL11 and APL 2720 were frozen samples that had previously been cytologically identified as APL. The der(15) band is indicated and corresponds to the band that co-migrates with the two probes.*

ATRA. In one patient, after 30 days of therapy with ATRA, the karyotype was normal despite the persistence of tumor cells bearing the t(15;17) detected by both PFGE and RT-PCR. Overall, cytogenetic analysis was diagnostic of the translocation in only 18 of 26 cases; this included three normal analyses, one cytogenetic failure, one equivocal case, and two with clonal abnormalities lacking the t(15;17). In the equivocal case, a 17q breakpoint consistent with the t(15;17) translocation was observed, but

Table 1. *Detection of the t(15;17) Translocation in Clinical Specimens of APL**

Patient	Clinical status	Sample type	*Bss*HII	*Mlu*I
2436	Baseline	Frozen	CM†	CM
2720	Relapse	Frozen	CM	CM
2781	Baseline	Frozen	CM	CM
2835	Baseline	Frozen	CM	CM
5	Relapse	Frozen	CM	CM
11	Baseline	Frozen	CM	CM
38	Relapse	Fresh	CM	CM
46	ATRA, day 19	Fresh	CM	CM
48	Baseline	Fresh	GL‡	GL
53§	Baseline	Fresh	CM	CM
61	ATRA, day 4	Fresh	CM	CM
63	Baseline	Fresh	CM	CM
73§	ATRA, day 28	Fresh	CM	CM
80	Baseline	Fresh	CM	CM
120	Baseline	Fresh	GL	GL
121	Baseline	Fresh	CM	CM
149	Baseline	Fresh	CM	CM
154	Baseline	Fresh	CM	CM
168	ATRA, day 30	Fresh	CM	CM
177	Baseline	Fresh	CM	CM
CON	Relapse	Fresh	CM	CM
206	Baseline	Fresh	CM	CM
215	Relapse	Fresh	CM	CM
226	Baseline	Fresh	CM	CM
230	Baseline	Fresh	CM	CM
234	Baseline	Fresh	CM	CM

* Megabase DNA was subject to analysis by PFGE as described in Materials and Methods and digested with either *Bss*HII or *Mlu*I as indicated. The blots were sequentially hybridized with the PML and RAR-α cDNA probes as well as the LCN4A3/A and LCN4A3/B RAR-α genomic probes.

† CM: nongermline bands co-migrate when probed with the probes for the PML and RAR-α loci.

‡ GL: germline arrangement of bands.

§ Samples from the same patient.

the der(15) chromosome could not be identified without the additional molecular data provided by PFGE or RT-PCR. Interestingly, in the two cases that were negative for the t(15;17) translocation by all three methods, the patients failed to respond to therapy with ATRA, as will be discussed. Thus, PFGE and RT-PCR in this study are equivalent in the diagnosis of the t(15;17) translocation and superior to conventional cytogenetics.

Discussion

The chromosomal translocation t(15;17) (q22;q11-q21) is considered to be a hallmark of APL and is found in most cases of APL, but not in other types of leukemias.[3] The recent elucidation of the structure of this translocation involving the RAR-α and PML loci has led to the development of several molecular approaches to the diagnosis of APL.[4,5,10,14,15,17,30,33–35] In this study, we demonstrated that the t(15;17) translocation can be simply and universally diagnosed by Southern analysis of megabase DNA resolved by PFGE in both fresh and frozen clinical specimens obtained from patients with APL. Furthermore, we have compared the diagnostic sensitivity to those obtained with cytogenetics and the RT-PCR. Both PFGE and the RT-PCR methods were diagnostic in 24 cases of APL responsive to ATRA. In contrast, six of the 24 cases examined by cytogenetics were nondiagnostic because the karyotypes were normal (four cases), technical failure (one case), or equivocal (one case). Two cases were negative for the t(15;17) translocation by all three methods and failed to respond to ATRA. These two cases demonstrate that some AMLs with morphology consistent with APL do not have the characteristic molecular rearrangement and to not respond to ATRA.

Recently, Chen et al[34] and Diverio et al[17] have demonstrated that rearrangements of the RAR-α locus can be detected in most or all patients with APL using conventional Southern blotting. However, universal detection with conventional techniques required digestion of DNA with at least two different restriction enzymes and hybridization with four different probes spanning the region from the distal portion of intron 1 to the proximal portion of intron 3. This complexity results from the fact that the t(15;17) translocation breakpoints occur throughout the approximately 20-kb second intron of RAR-α.[17,34] The need for multiple enzymes and probes limits the practical applicability of conventional Southern analysis in the routine molecular diagnosis of APL. Furthermore, in conventional Southern blot hybridization, co-migration of nongermline fragments detected by the RAR-α and PML probes is uncommon even with multiple enzymes and probes.[17,34] In contrast, Southern analysis of megabase DNA resolved by PFGE digested by a single enzyme (either *Bss*HII or *Mlu*I) and hybridized to a single probe, LCN4A3/B, resulted in universal detection of rearrangements of the RAR-α locus. When the PML cDNA probe was subsequently used to hybridized the same blot, co-migration of the PML and RAR-α loci directly demonstrated the t(15;17) translocation. Use of a second restriction enzyme primarily served to confirm the findings obtained with a single enzyme. Recently, a variant t(11;17) translocation has been characterized on the molecular level; this translocation juxtaposes the RAR-α locus with a gene called PLZF.[18,19,36] The variant t(11;15) translocation poses a problem for the molecular diagnosis of APL: the PML/RAR-α RT-PCR assay would not detect a translocation; and conventional Southern blotting with an RAR-α probe would not distinguish

Table 2. *Comparison of PFGE, RT-PCR, and Cytogenetics for the Detection of t(15;17) in APL**

	Baseline	On retinoic acid	Relapse	Total
No. of patients	17	4	5	26
PFGE +	15	4	5	24/26 (92.3%)
PCR +	15	4	5	24/26 (92.3%)
Cytogenetics +	11	3	4	18/26 (69.2%)
Normal	3	1	0	
Equivocal	0	0	1	
Failures	1	0	0	
Clonal, not t(15;17)	2			

* Twenty-six samples were obtained from 25 patients and analyzed by PFGE, RT-PCR, and cytogenetics. The samples were divided into categories with respect to the clinical status of the patient at the time the sample was obtained: baseline was at time of initial diagnosis, on therapy with ATRA, or at relapse following therapy. Cytogenetic data was divided into positive for the t(15;17) translocation, normal, equivocal, or having a clonal abnormality not including the t(15;17) translocation.

the variant from the t(15;17). Thus, the direct demonstration of co-migration of PML and RAR-α possible with the PFGE technique has diagnostic and clinical importance.

In this study, both *Bss*HII and *Mlu*I are informative for the detection of the t(15;17) translocation. We were surprised that digestion with *Bss*HII was informative in all cases because there is a *Bss*HII site in the second intron of RAR-α in the previously published megabase map of the region.[4] Our data agree that the normal RAR-α locus is cleaved by *Bss*HII within the second intron (Figure 1). However, in DNA prepared from tumor specimens and digested with *Bss*HII, the translocated fragment derived from the der(15) chromosome is 150 kb (Figure 3), which is a size consistent with cleavage at the *Bss*HII site 3′ of the RAR-α locus. Thus, it is likely that the *Bss*HII site in the second intron is methylated as a consequence of the translocation. Because this enzyme reliably detects the t(15;17) translocation and the informative bands are moderate in size (<300 kb), *Bss*HII digestion is used in our laboratory as the standard for diagnosis of the translocation.

In addition, in constructing the megabase map of the PML locus, we have detected a restriction fragment length polymorphism on the megabase scale. The PML locus is contained in a 220-kb to 310-kb region flanked by two CpG islands (Figure 1). The restriction fragment length polymorphism is demonstrated by digestion with *Bss*HII or combinations of *Asc*I and *Mlu*I, suggesting that it did not result simply from loss of a restriction enzyme site but rather represents the gain or loss of DNA within the 250-kb region that lies between the CpG islands. The DNA sequences responsible for the variation in size of the PML locus are currently under investigation. In addition, we are undertaking family studies to determine if this variation in the megabase map segregates as would be expected for a polymorphism. We are also examining the importance of this restriction fragment length polymorphism, if any, to the pathogenesis of APL.

This study reveals that PFGE is a DNA-based alternative to the RT-PCR, which is RNA-based, and can contribute to the clinical diagnosis of APL and to monitoring treatment response in APL. In some circumstances, the PFGE method may be superior to the RT-PCR. In settings where it is impossible for a clinical laboratory to perform the molecular analysis, our data demonstrate that the PFGE method can be performed on frozen material, which facilitates transfer to an outside molecular diagnosis laboratory. Furthermore, the PFGE method is not subject to minor contamination that can frustrate PCR-based assays. Lastly, in the variant t(11;17) translocation, the RT-PCR is negative, whereas the PFGE method demonstrates a rearrangement of RAR-α and a germline PML gene (Goy, Miller, and Zelenetz, unpublished data). Though morphological diagnosis of APL is usually straightforward, occasionally the microgranular variant[2] can be difficult to differentiate from other forms of AML. Recent studies have indicated that detection of the t(15;17) translocation correlates with clinical response to ATRA therapy.[15,16] Thus, accurate detection of the translocation has not only diagnostic but also therapeutic applications. For initial diagnosis, this study reveals the PFGE and RT-PCR methods are equivalent and because PFGE analysis provides direct demonstration of the co-migration of PML and RAR-α it is more informative than conventional electrophoresis. PFGE has proven to be clinically useful for the diagnosis of the t(14;18)[23] and t(9;22)[24] chromosomal translocations and may be especially useful in APL because tumor cells can be frozen and sent to a reference laboratory for analysis and it does not depend on the isolation of RNA.

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