Interstitial Insertion of Retinoic Acid Receptor-α Gene in Acute Promyelocytic Leukemia With Normal Chromosomes 15 and 17

By L.R. Hiorns, T. Min, G.J. Swansbury, A. Zelent, M.J.S. Dyer, and D. Catovsky

The translocation t(15;17)(q22;q21) is seen exclusively in patients with acute promyelocytic leukemia (APL) and in the promyelocytic blast crisis of chronic myeloid leukemia (CML). This translocation juxtaposes the promyelocytic leukemia (PML) gene on chromosome 15 and the retinoic acid receptor-α (RARA) gene on chromosome 17, resulting in the formation of a chimeric mRNA transcript. We describe a patient with the microgranular variant form of APL, with no detectable cytogenetic abnormality of either chromosomes 15 or 17, who nevertheless had juxtaposition of PML and RARA genes and expressed a chimeric transcript. Conventional cytogenetics showed the karyotype 46,XY,d-.

THE RECIPROCAL translocation t(15;17)(q22;q21) is associated exclusively with acute promyelocytic leukemia (APL) and the promyelocytic blast crisis of chronic myeloid leukemia (CML). With the correct culturing conditions, it is thought to be present in at least 95% of patients with APL of both conventional and variant morphologic forms (French-American-British classification, AML M3 and M3v). The breakpoints of this translocation have been cloned, and the genes involved or both chromosomes 15 and 17 have been identified. The promyelocytic leukemia (PML) gene on chromosome 15 is approximately 50 kb in size and consists of 9 exons. The RNA is expressed in all fetal and adult tissues examined so far. The protein has a zinc finger-like region, suggesting a role as a potential transcription factor. The gene involved on chromosome 17 is retinoic acid receptor-α (RARA), consisting of 9 exons. The protein also contains a zinc finger DNA-binding domain in addition to the RA-binding region.

The breakpoints in PML in the t(15;17) translocation are clustered in two regions, either spanning exons 3 and 4 or 6 and 7. The breakpoints in RARA occur almost exclusively in the 17-kb second intron. This results in the DNA binding and RA-binding domains of RARA being translocated to the DNA-binding domain of PML on chromosome 15. The reciprocal translocation product on chromosome 17 is transcribed but not in all patients. The 15q+ product is transcribed in all patients and, therefore, is considered to be an essential component of leukemogenesis in APL with t(15;17).

We have studied a patient with APL with no abnormalities of chromosomes 15 or 17 observed by conventional cytogenetics or by chromosome painting using fluorescent in situ hybridization (FISH). We examined the cytogenetic position of the PML and RARA genes using FISH and the rearrangement of these and other genes using Southern blotting and showed the existence of a hybrid fusion product by reverse transcriptase-polymerase chain reaction (RT-PCR).
cultures were terminated with colcemid (final concentration, 0.1 pg/mL). The cells were suspended in 0.075 mol/L KCl for 15 minutes and fixed in 3:1 methanol:glacial acetic acid. Slides were banded by incubation in 2× SSC (1× SSC: 0.15 mol/L NaCl, 0.015 mol/L trisodium citrate) at 60°C for 1 hour, followed by a brief rinse in trypsin (0.05% in pH 8.6 buffer) and stained with 6% Giemsa. Unused sample was stored at −20°C.

**In situ hybridization.** Fresh slides were prepared from the stored, fixed material and allowed to dry overnight at room temperature. Hybridization was performed under standard conditions including pretreatment with RNAse, dehydra- tion with ethanol, and denaturation in 70% formamide, 2× SSC for 3 minutes at 70°C. After dehydra- tion through a series of ethanols on ice, the slides were warmed to 37°C and hybridized under a coverslip either with labeled chromosome specific paints (Cambio, Cambridge, UK) or with labeled probe(s) in Hybrisol VI (Oncor, Gaithersburg, MD). The probe labeled probe(s) in Hybrisol VI (Oncor, Gaithersburg, MD). The probe labeled probe(s) in Hybrisol VI (Oncor, Gaithersburg, MD). The probe labeled probe(s) in Hybrisol VI (Oncor, Gaithersburg, MD). The probe labeled probe(s) in Hybrisol VI (Oncor, Gaithersburg, MD). The probe labeled probe(s) in Hybrisol VI (Oncor, Gaithersburg, MD).

**Fluorescent detection.** Slides were rinsed in a solution of 4× SSC, 0.05% Tween 20 (SSCT) at 37°C. They were then incubated in a series of solutions at 37°C for 30 minutes in the following order, with washes between in SSCT: (1) 5% Marvel in SSCT; (2) avidin-fluorescin isothiocyanate (5 μg/mL in SSCT with 2% Marvel; Vector Laboratories, Peterborough, UK) for the paints or the same solution with the addition of anti-Digoxigenin mouse monoclonal antibody (0.2 μg/mL; Boehringer) for the single copy probes; (3) biotinylated antidna (5 μg/mL in SSCT with 2% Marvel; Vector) for the paints and the same solution with the addition of antimmunoglobulin-Texas Red (7.5 μg/mL; Vector) for the single copy probes; and (4) avidin-fluorescin isothiocyanate, as before. After dehydration through an ethanol series, the samples were counterstained with propidium iode (1 μg/mL) or DAPI (0.5 μg/mL; both from Sigma, UK) as appropriate in Citifluor antifade mountant (Citifluor, Canterbury, UK). Slides were examined using a Zeiss Axiosplan microscope (Carl Zeiss [Oberkochen] Ltd, Welwyn Garden City, UK), equipped with appropriate filters for fluorescence, and photographed directly, or images were collected through a Photometrics CCD camera (Photometrics, Tuscon, AZ) to a computer using Digital Scientific software (Digital Scientific, Cambridge, UK).

**Southern bloting.** High molecular weight DNA was extracted from a mononuclear fraction of peripheral blood at diagnosis by standard techniques and digested with the restriction enzymes, BamHI, EcoRI, HindIII, Sac I and Bgl II. Southern blotting and probe labeling were performed as previously described. Probes used in this study included, for the PML gene, a mixture of M4 and M12, which are PCR-amplified cDNAs containing the sequences between bases 49 through 117 (coding for exon 1 and 5' UTR) and 931 through 1739 (coding for exons 4 through 6) from the cDNA sequence of NB4; for RARA, a 1.3-kb XbaI genomic fragment, containing sequences from the human homolog of mouse exon 3 that encodes the unique A domain of the RARA isoform; for RAR-β (RAR), a mixture of RAR genomic sequences containing the human homolog of mouse exon 1 that encodes the unique A domain of RARβ, and the human homolog of mouse exon 3 that encodes the unique A domain of RARB; and for c-mos a 2.5-kb human placental fragment containing the c-mos gene. Reverse transcription was performed using 2.5 μg total RNA, 100 ng oligonucleotide primer 3 from Table 1 and 200 U of Mo-MLV reverse transcriptase (BRL), under conditions recommended by the supplier. Subsequently, one round of 25 cycles of PCR was performed using primers 1 and 2 from Table 1. Cycling times and conditions were as previously

---

**Table 1. Sequences of Oligonucleotide Primers and Probes**

<table>
<thead>
<tr>
<th>No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>A</td>
</tr>
<tr>
<td>B</td>
</tr>
<tr>
<td>C</td>
</tr>
<tr>
<td>D</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>3' to 5' Sequence</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGATGCTCCCATGGTTC</td>
<td>PML</td>
</tr>
<tr>
<td>TTAAGACCCGTCCAGGA</td>
<td>PML exon 3</td>
</tr>
<tr>
<td>GCCATGCTGCTGACAG</td>
<td>PML exon 5</td>
</tr>
<tr>
<td>ACCATGGAGACTGTCCTGAG</td>
<td>PML exon 6</td>
</tr>
<tr>
<td>AGCCCTGAGGGACTTCTGGA</td>
<td>RARA exon IV</td>
</tr>
</tbody>
</table>

---

**Fig 1. Karyotype of patient 46,XY,der(3)t(3;8)(p25;q12) is shown. The arrow indicates the chromosome 8-derived portion of the der(3) chromosome.**
Fig 2. FISH analysis of metaphase chromosomes from this patient is shown. (A) A paint specific for chromosome 8 confirms trisomy for 8q on the der(3) chromosome. (B) A paint specific for chromosome 15 shows two apparently normal homologs. (C) A paint specific for chromosome 17 shows two apparently normal homologs. (D) Probes specific for the PML gene (red), the RARA gene (green), and the centromere of chromosome 15 (green) are shown; small arrows show RARA in its normal position on chromosome 17; large arrow shows the juxtaposition of PML and RARA genes on one homolog of chromosome 15.

RESULTS

Cytogenetics. The karyotype of this patient (Fig 1) was defined as 46,XY,der(3)(3:8)(p25;q12), arising from a translocation between 3p25 and 8q12 in addition to two normal copies of chromosome 8. This results in a net gain of material from chromosome 8 (trisomy, 8q12-24) and loss of material from the tip of 3p (monosomy, 3p26). Thirty-two metaphases were examined, and no evidence for any abnormality of either chromosome 15 or 17 was observed in any cells.

Molecular cytogenetics. To confirm the cytogenetic findings, FISH was performed on slides from the same culture as the cytogenetics, with paints specific for chromosomes 8, 15, and 17 (Fig 2A, B, and C, respectively). The paint for chromosome 8 confirmed that the material on the terminal portion of 3p was derived from chromosome 8. Analysis with chromosome 15-specific paint showed two
INTERSTITIAL INSERTION OF RARA IN APL

Fig 3. (A) Representative Southern blot shows results of probing for RARA and PML on DNA from N, normal control; 1, APL patient with t(15;17) showing rearrangements in both RARA and PML; and 2, patient in this study. (B) Schematic representation of normal PML and RARA genes showing restriction maps for B, BamHI; E, EcoRI; X, Xba I; H, HindIII; and K, Kpn I. Hatched boxes indicate exons; solid bars indicate probes used in Southern analysis.

intact copies of this chromosome. The slide painted with chromosome 17-specific probes also showed two apparently normal homologs. The standard t(15;17) is easily detected using these paints and conditions (unpublished observations, December 1992). Because of its distinctive morphology the der(3) chromosome was easily identifiable in the metaphase spreads hybridized with other paints, which served as a marker for the clone.

An example of the result of applying the PML and RARA probes to metaphase spreads from this patient is shown in Fig 2D. The probes for both PML and RARA had been applied to normal male metaphase spreads to confirm that they mapped to the expected region (results not shown). Signal from the RARA probe was seen on both chromosomes 17 and on one copy of chromosome 15, with the signal coming to lie on top of one signal for PML that remains on chromosome 15 in both homologs. The probe for RARA encompasses the second intron, which is the site of translocation in most
reported cases of APL, therefore, it is not unusual, depending on the precise breakpoint, for this probe to show signal on both the translocated chromosomes 15 and 17.

**Southern blotting.** The gels probed with the RARA probe showed only normal bands on BamHI, EcoRI, HindIII, SacI, and BglI digests (examples are shown in Fig 3A); this is probably because the probe covers only a small portion of the second intron (shown in Fig 3B). These results show that any break in RARA must lie 5' of the HindIII site in the second intron. Gels probed with PML showed rearranged bands with all enzyme digests (examples are shown in Fig 3A). Because RARB has been mapped to 3p24 and c-mos has been mapped to 8q11 close to the translocation breakpoints detected cytogenetically in this patient, we sought rearrangements of these genes by DNA blots, but none were detected (data not shown).

**RT-PCR.** The PCR for PML-RARA transcripts yielded three bands from this patient of 276 bp, 455 bp, and 623 bp. These were confirmed to be from PML and RARA by probing with oligonucleotide probes (Fig 4a) whose sequences are listed in Table 1 and whose position with respect to the exons of PML and RARA are shown in Fig 4b. All three bands hybridized to probes A and D from exon 3 of PML and exon IV of RARA, respectively; two of the bands hybridized to probes B and C from exons 5 and 6 of PML, respectively, which is consistent with alternative splicing of exon 5. However, the two longer transcripts were shorter than those in the control cell line, NB4, where the breakpoint in PML is known to be in intron 6, indicating that the breakpoint in this patient lies in exon 6. The patient in lane 1 has only a short form of the transcript consistent with a break in intron 3 of PML.

**DISCUSSION**

The translocation t(15;17)(q22;q21), or more specifically the fusion transcript PML-RARA, has not been reported in subtypes of acute myeloid leukemia other than APL and, therefore, may be used as a marker for this subtype of leukemia. In the present case, there was no cytogenetic evidence for involvement of either chromosomes 15 or 17, but there was an unusual translocation between chromosomes 3 and 8, all of which were confirmed by FISH with chromosome specific paints. FISH using cosmids probes for PML and RARA genes showed a break in the second intron of the RARA gene and an interstitial insertion of a portion of this gene juxtaposing it to the PML gene on one homolog of chromosome 15. This observation was confirmed both by Southern blotting, which showed rearrangement of the PML gene, and by RT-PCR, which showed the presence of a hybrid PML-RARA transcript.

Therefore, this patient defines a new cytogenetic subgroup of APL. This subgroup is composed of patients who are t(15;17) negative but PML-RARA-positive and is analogous to the Philadelphia chromosome-negative CML patients who, nevertheless, are BCR-ABL positive. Although the molecular consequences of this cytogenetically undetectable transposition appear to be identical to those observed in regular cases with t(15;17)(q22;q21), the molecular mechanisms underlying these events must differ; a further chromosome break distal to RARA must have occurred to allow for the transposition. The amount of chromosome-17 material inserted into chromosome 15 in this case is presently being studied. In cases of Philadelphia chromosome-negative/BCR-ABL-positive CML, the amount of DNA transferred can be substantial.

It is clear that, if the correct culturing conditions are used, cytogenetically detectable t(15;17) is present in the great majority of cases of both APL and the variant form. The incidence of this new subgroup of APL is not yet known. We are presently examining other cases of APL in which no t(15;17) was detected cytogenetically. A therapeutic consequence of the definition of this subgroup of APL might
be that all-trans RA therapy should not be withheld on the basis of cytogenetics alone unless supplemented by FISH
and/or molecular investigations, because such cases may, nevertheless, have PML-RARA fusion. All-trans RA was
not administered in the present case because of rapidly progressive disease. Whether all t(15;17)-negative/PML-
RARA-negative patients will follow such an unusual clinical course is not yet known.

ACKNOWLEDGMENT

We are grateful to Dr Ellen Solomon, Imperial Cancer Research Fund (London, UK) for providing cosmid probes to PML and RARA
and to Ms Anne O'Byrne for excellent technical assistance with the
DNA blots. We thank Dr Ray Powles and his team from the Leukae-
mia Unit, Royal Marsden Hospital for allowing us to study this patient, and Dr John Luckit for hematological diagnosis.

REFERENCES

1. Castaigne S, Berger R, Jolly V, Daniel MT, Bernheim A, Marty M, Degos L, Flandrin G: Promyelocytic blast crises of chronic my-
elocytic leukemia with both t(9;22) and t(15;17) in M3 cells. Cancer
54:2409, 1984
2. Sheer D, Lister TA, Amess J, Solomon E: Incidence of the 15q+;17q− chromosome translocation in acute promyelocytic leu-
kaemia (APL). Br J Cancer 52:55, 1985
3. Swansbury GJ, Feary SW, Clink HM, Lawler SD: Cytogenetics of acute promyelocytic leukaemia: Incidence of t(15;17) at the Royal
4. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR, Sultan C: Proposals for the classification of the acute
5. Borrow J, Goddard AD, Sheer D, Solomon E: Molecular analy-

sis of acute promyelocytic leukemia breakpoint cluster region on
6. De The H, Chomienne C, Lanotte M, Degos L, Dejean A: The t(15;17) translocation of acute promyelocytic leukemia encodes a functionally
7. Leroy P, Krust A, Zelent A, Mendelsohn C, Garnier JM, Kastner P, Dienach A, Chambon P: Multiple isoforms of the mouse retinoic acid receptor α are generated by alternative splicing and
9. Watson R, Oskarsson M, Van de Woode GF: Human DNA sequence homologous to the transforming gene (mos) of Moloney
murine sarcoma virus. Proc Natl Acad Sci USA 79:4078, 1982
receptor-α (RAR-α) locus in all patients with acute promyelocytic leukemia (APL) and mapping of APL breakpoints within the RAR-
α second intron. Blood 79:3331, 1992
forms in acute promyelocytic leukemia. EMBO J 11:1397, 1992
232:31, 1971
14. Dyer MJS, Heward JM, Zani VJ, Buccheri V, Catovsky D: Unusual deletions within the immunoglobulin heavy-chain locus in acute
16. Chen Z, Chen SJ, Zhu YJ, Tong JH, Wang ZY, Waxman S, Zelent A: Fusion between a novel kruppel-like zinc finger gene and the retinoic acid receptor-α locus in all patients with acute promyelocytic leukemia (APL) and mapping of APL breakpoints within the RAR-
α second intron. Blood 79:3331, 1992
forms in acute promyelocytic leukemia. EMBO J 11:1397, 1992
Interstitial insertion of retinoic acid receptor-alpha gene in acute promyelocytic leukemia with normal chromosomes 15 and 17

LR Hiorns, T Min, GJ Swansbury, A Zelent, MJ Dyer and D Catovsky