Molecular Monitoring of the myl/Retinoic Acid Receptor-α Fusion Gene in Acute Promyelocytic Leukemia by Polymerase Chain Reaction

By Andrea Biondi, Alessandro Rambaldi, Pier Paolo Pandolfi, Vincenzo Rossi, Giovanni Giudici, Myriam Alcalay, Francesco Lo Coco, Daniela Diverio, Enrico M. Pogliani, Eraldo M. Lanzi, Franco Mandelli, Giuseppe Masera, Tiziano Barbui, and Pier Giuseppe Pelicci

The acute promyelocytic leukemia (APL) t(15;17) translocation generates a myl/retinoic acid receptor-α (RAR-α) chimeric gene that is transcribed as a fusion myl/RAR-α messenger RNA. Using primer sets derived from RAR-cr and myl cDNAs, we were able to amplify the breakpoint sites of the fusion transcripts of all 35 APL RNA samples by reverse polymerase chain reaction (PCR) and nested primer approach of two rounds of amplification. DNA fragments of different size were obtained according to the chromosome 15 breakpoints (intron 3-bcr 3; exon 6-bcr 2; and intron 6-bcr 1). bcr 1 and bcr 3 represent the regions of the myl locus most frequently involved among APL (48.5 and 34.2 of cases, respectively); bcr 3 constitutes 62.5% of cases among M3V as compared with 25.9% of M3 cases. The feasibility of monitoring the APL clone by PCR analysis in five APL patients who received different treatment (chemotherapy, all-trans-retinoic acid or bone marrow transplantation) was evaluated. In five of nine bone marrow samples of patients in complete remission, t(15;17)-positive cells could be detected by PCR analysis. We conclude that PCR amplification of the myl/RAR-α junctions represents the easiest and rapid method for diagnosis and monitoring of the APL clone.

© 1992 by The American Society of Hematology.

From the Clinica Pediatrica Universita di Milano, Ospedale S. Gerardo, Monza; the Divisione Ematologia, Ospedali Riuniti Bergamo, Bergamo; the Istituto di Ricerche Farmacologiche “M. Negri,” Bergamo; the Istituto di Clinica Medica I, Universita di Perugia, Policlinico Monteluce, Perugia; the Dipartimento di Biopatologia Umana, Sezione di Ematologia, Universita “La Sapienza” di Roma, Roma; and the Cattedra di Patologia Medica-Sezione di Ematologia, Ospedale S. Gerardo, Monza, Italy.


Supported in part by grants from the “Associazione Italiana per la Ricerca sul Cancro” (A.I.R.C.) to P.G.P. and A.R. and by “Progetto ACRO” (A.B.), A.B., V.R., and G.G. are supported by “Fondazione Tetumani.”

Address reprint requests to Andrea Biondi, MD, Clinica Pediatrica Universita di Milano, H. “S. Gerardo,” Via Donizetti 106, 20052 Monza (Milano), Italy.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1992 by The American Society of Hematology.


492
MONITORING OF THE t(15;17) TRANSLOCATION

Table 1. Configuration of myl Locus in 35 APL Patients

<table>
<thead>
<tr>
<th></th>
<th>bcr 1</th>
<th>bcr 2</th>
<th>bcr 3</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>M3</td>
<td>15</td>
<td>5</td>
<td>7</td>
<td>27</td>
</tr>
<tr>
<td>M3v</td>
<td>2</td>
<td>1</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td>17</td>
<td>6</td>
<td>12</td>
<td>35</td>
</tr>
</tbody>
</table>

(48.5%) (17.1%) (34.2%)%

[rt(15;17) negative] recently established in our laboratory, was used (A. Rambaldi et al., manuscript submitted). Amplification of the β-actin mRNA was accomplished with 5 μL of the same cDNA preparation used to identify myl/RAR-α junctions. The following primer sequences were used: forward 5’ CTTTCTGGGATGAGTCTG-3’ and reverse 5’ GGAGGAAATGTCTTGTATCTCTTC-3’. In selected experiments, 10 μL of the PCR products fractionated by electrophoresis through a 1.5% agarose gel was transferred to nylon membranes (Genescreen Plus; New England Nuclear, Boston, MA). Prehybridization, hybridization, and washings were performed according to manufacturer’s instructions, as previously reported. The following primer was used to hybridize the PCR blots: 5’-GAGTCTGAGGAGGAAAGGA-3’, according to established procedures.

RESULTS

PCR amplification of the myl/RAR-α fusion gene in APL. We have previously reported that the RAR-α breakpoint of APL occurs consistently within an approximately 16-kb DNA fragment of the RAR-α intron 2.8 Further studies on mapping of chromosome 15 breakpoints have indicated that at least three regions of the myl locus are involved in the translocation breakpoints (intron 3-bcr 3; exon 6-bcr 2; and intron 6-bcr 1).13 According to the nucleotide sequence of a myl/RAR-α cDNA,10 a set of amplimers was selected. Figure 1 shows the positions of M2, M4, R5, and R8 relative to the sequence of myl/RAR-α derived from our previous report.10 Primer M4, derived from myl exon 3, and primer R5 were used as a first cycle of PCR; then a second round of amplification was performed by using R8 as a nested primer. Figure 2A shows the results of PCR amplification of three different APL cases, representative of bcr 1, bcr 2, and bcr 3. Nucleotide sequence analysis of the different

Fig. 1. Partial cDNA sequence of the myl/RAR-α fusion gene derived from our previous report.10 The positions of the primers M4, M2, R6, and R5 are indicated.
myl/RAR-α junctions generated by PCR amplification has shown that the additional heterogeneity observed is due to alternative splicings of the myl portion. To obtain a better resolution of the myl/RAR-α junctions corresponding to bcr 1 and bcr 2, a primer derived from a more 3' myl sequence (M2 from myl exon 5) was used for PCR amplification. As shown in Fig 2B, single fragments of 326 bp and 290 bp were observed for bcr 1 and bcr 2, respectively. We have previously reported the absence of RAR-α aberrant transcript in a variety of non-APL RNAs, including both normal and neoplastic cells (leukemias and solid tumors). Similarly, when the same RNAs were analyzed by PCR amplification for the presence of myl/RAR-α fusion gene, no PCR products were obtained (data not shown). Having defined “universal” primers able to amplify the breakpoint sites in all t(15;17), BM samples from 35 APL patients were analyzed. Table 1 summarizes the results on the distribution of chromosome 15 breakpoints among M3 and M3V APL patients. bcr 1 and bcr 3 did represent the regions of the myl locus most frequently involved among APL (48.5% and 34.2% of cases, respectively); bcr 3 constitutes 62.5% of cases among M3V as compared with 25.9% of M3 cases.

Detection of minimal residual disease (MRD) in APL. The possibility of using PCR amplification of the myl/RAR-α fusion gene to monitor patient status in APL was investigated in five APL patients during the course of treatment. Patients were selected for the availability of frozen remission samples and because they were representative of different therapeutic strategies currently used for the treatment of APL. The main presenting features of the five APL patients are summarized in Table 2. Four of five patients were studied at diagnosis and received induction therapy with anthracyclines and cytosine-arabinoside. Patient no. 5 was investigated at the time of second relapse when he received reinduction therapy with all-trans retinoic acid (ATRA), according to the protocol previously reported. The APL patients were found to have their 15 chromosome breakpoint within bcr 1 (patients no. 1, 2, and 3) or bcr 3 (patients no. 4 and 5) by Southern blot (data not shown). The products of amplification (obtained after one or two cycles of nested PCR) were blotted to nylon membranes and hybridized as described in Materials and Methods.
was not available for molecular analysis, but a positive PCR result was still apparent even after the patient received a reinuduction cycle with idarubicin. Patient no. 1 underwent allogeneic BM transplantation (BMT) and the BM aspirate performed 8 months after BMT did not show the presence of MRD. Similarly, in patient no. 4, as shown in Fig 4, no cells carrying the t(15;17) transcripts could be detected in a remission sample 21 months after autologous BMT. Despite being in clinical remission, as confirmed by the absence of any metaphases carrying the t(15;17), the fusion transcript myl/RAR-α could be detected in BM of patient no. 5 after 7 months of ATRA treatment. Similar findings on the persistence of myl/RAR-α fusion gene as assessed by PCR amplification were obtained in three additional patients during the time they received ATRA alone (data not shown). Table 3 summarizes the findings on MRD in APL patients obtained either by PCR amplification of the myl/RAR-α junctions or by standard cytogenetics. With the exception of patient no. 3 (BM sample 6 months after diagnosis), in none of the cases positive for minimal residual cells by PCR could the t(15;17) be detected. To determine the sensitivity of PCR amplification in our study, RNA from patient no. 3 was serially diluted by mixing with GF-D8 RNA, reverse transcribed and amplified as described in Materials and Methods. As shown in Fig 5, the PCR can detect the presence of myl/RAR-α fusion gene in the presence of less than 0.1 ng total RNA, which represents a final dilution between 10^-4 and 10^-5.

### DISCUSSION

The detection of MRD in patients undergoing treatment for hematologic malignancies has become an important goal, not only to monitor the effectiveness of therapy but also to detect an impending relapse. The application of techniques based on the enzymatic amplification of DNA target sequences by PCR has attracted much attention due to the extreme sensitivity and specificity of the technique. If the two primers flank a translocation junction, exponential amplification is dependent on the presence of tumor cells carrying the translocation, thus providing a very sensitive method for tumor detection.

### Table 3: MRD in APL Patients

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Cell Source</th>
<th>Months After Diagnosis</th>
<th>MRD by PCR</th>
<th>MRD by Cytogenetics</th>
<th>Clinical Follow-up (mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PB</td>
<td>+3 (pre-BMT)</td>
<td>–</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>BM</td>
<td>+3 (pre-BMT)</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>BM</td>
<td>+11 (post-BMT)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>BM</td>
<td>+2</td>
<td>–</td>
<td>–</td>
<td>CCR 15+</td>
</tr>
<tr>
<td></td>
<td>BM</td>
<td>+3</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>BM</td>
<td>+6 (stop)</td>
<td>+</td>
<td>+*</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>BM</td>
<td>+8</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>BM</td>
<td>+9 (relapse)</td>
<td>ND</td>
<td>ND</td>
<td>CCR 15+</td>
</tr>
<tr>
<td>4</td>
<td>BM</td>
<td>+10</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>BM</td>
<td>+7</td>
<td>+</td>
<td>–</td>
<td>CCR 10</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not done; CCR, continuous complete remission.

*One of 36 metaphases analyzed showed the t(15;17) translocation.
†The patient was studied at the time of second relapse.
The presence of the chimeric myl/RAR-α fusion gene, as a consequence of the reciprocal translocation between the myl and RAR-α loci, has suggested the possibility of extending to APL, a similar approach that has been extensively used for chronic myeloid leukaemia.20 The different regions of the myl locus involved in the 15 translocation breakpoints and the presence of alternative splicing of the myl portion are responsible for the great heterogeneity of myl/RAR-α junctions observed among APL patients.15 Moreover, the alternative use of two RAR-α polyadenylation sites generates myl/RAR-α transcripts of different sizes.11,12 The set of amplifiers applied in the present study allowed for the amplification of myl/RAR-α junctions in 35 APL patients, the largest caselist of APL patients studied so far by PCR amplification. The patients included were representative of different age, of M3 and M3v FAB subtype, and of different chromosome 15 breakpoints. The resolution of the fragments obtained by the two rounds of amplification and the possibility of visualizing them by standard ethidium staining (as shown in Figs 2 and 4) made the approach outlined herein the easiest and most rapid method for identifying the t(15;17), even in the cases in which conventional cytogenetics failed. As an alternative approach for the molecular identification of the t(15;17) breakpoint site of APL, we considered the amplification of the RAR-α/myl fusion transcript. The observation that the RAR-α/myl transcript was present in most, but not all, APL cases seemed to favor the use of myl/RAR-α as the target gene for PCR amplification of the APL clone.21

The detection of MRD in APL will be important in evaluating an impending relapse and the effects of different therapeutic approaches. As shown by the presented data, PCR analysis was shown to be superior in detecting residual APL cells as compared with morphology and standard cytogenetics. In fact, the relapse of patient no. 3 was heralded by PCR 3 months before the clinical appearance of leukemia. By the same approach, we could show that high doses of chemotherapy and total body irradiation associated with either autologous or allogeneic BMT represent an effective and potentially eradicating therapeutic strategy of APL. On the contrary, treatment with ATRA alone, despite resulting in complete hematologic remission as assessed by morphology, cytogenetics, and molecular analysis by Southern blot technique,16 did not result in complete eradication of the APL clone. The recent findings that the remission induced by ATRA does not appear to be long term seem to support our molecular results.22

REFERENCES


Molecular monitoring of the myl/retinoic acid receptor-alpha fusion gene in acute promyelocytic leukemia by polymerase chain reaction

A Biondi, A Rambaldi, PP Pandolfi, V Rossi, G Giudici, M Alcalay, F Lo Coco, D Diverio, EM Pogliani and EM Lanzi