Acute Promyelocytic Leukemia: Clinical Relevance of Two Major PML-RARα Isoforms and Detection of Minimal Residual Disease by Retrotranscriptase/Polymerase Chain Reaction to Predict Relapse

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Recent data have shown that the PML-RARα fusion gene resulting from translocation t(15;17) is a highly reliable molecular marker of acute promyelocytic leukemia (APL). In this study performed on 97 Chinese patients with APL, the retrotranscriptase/polymerase chain reaction (RT/PCR) was used to evaluate the clinical relevance of the long (L) or short (S) PML-RARα fusion mRNA isoforms and to study minimal residual disease during clinical remission (CR). There were more early deaths during the all-trans retinoic acid (ATRA) induction treatment and more relapses within 2 years of CR in the S-type (6 of 19 cases) than in the L-type group (2 of 33 cases) (P < .025). Among 12 cases analyzed before and after the ATRA-induced CR, 9 cases showed positive RT/PCR, whereas only 3 cases showed a negative result, justifying the need for chemotherapy after ATRA-induced CR. Eleven of 62 APL patients in CR, after ATRA-induced CR and chemotherapy consolidation (follow-up, from 3 to 72 months), showed positive RT/PCR. Five of them relapsed within 1 to 8 months after the positive test; one converted to negative after further chemotherapy; and 5 remained in CR status without further PCR data. However, the latter 5 cases all received further intensive consolidation therapy after the PCR positivity. These results show that a positive RT/PCR of PML-RARα is a sensitive predictor of relapse in APL.

ACUTE PROMYELOCYTIC leukemia (APL) is characterized by the presence of the specific chromosomal translocation t(15;17)(q22;q11) and the response to differentiation therapy with all-trans retinoic acid (ATRA). Recently, a number of laboratories, including ours, found that the t(15;17) results in a fusion gene PML-RARα by juxtaposing the PML gene on chromosome 15 and the RARα gene on chromosome 17. Two major isoforms of PML-RARα can be produced, depending on the location of the PML breakpoint. Breakpoints located within PML introns 3 or 4 (bcr 3) are associated consistently with production of short (S) type fusion mRNA between PML exon 3 (p3) and RARα exon 3 (r3), whereas those located within or downstream of PML intron 6 (bcr1) are associated consistently with formation of long (L) type chimeras between PML exon 6 (p6) and RARα exon 3 (r3). This heterogeneity of fusion genes may have clinical relevance. We and others have shown that the retrotranscriptase/polymerase chain reaction (RT/PCR) can be used to amplify both PML-RARα mRNA isoforms across the p6-r3 or p3-r3 junctions using appropriate oligonucleotide primers. Furthermore, this RT/PCR is sensitive enough to identify minimal residual disease (MRD), and may predict early relapse during clinical remission (CR). Therefore, we used RT/PCR to study the PML-RARα fusion mRNAs from 97 Chinese patients with APL. We show that the S-type fusion gene is related to poor prognosis in 52 de novo APL cases. We also show that RT/PCR can be used to predict relapse of the disease in 62 patients observed for 3 to 72 months.

MATERIALS AND METHODS

Patients. In total, 97 APL cases were studied from January 1990 to October 1992. These include 55 men and 42 women, from 5 to 74 years of age. The diagnosis of APL (AML-M3) was according to French-American-British (FAB) nomenclature. Thirty-five cases were studied with RT/PCR only at diagnosis, and 18 cases had RT/PCR data available both at diagnosis and during CR. Cytogenetic studies were performed in all 53 of these cases. In another 44 cases, it was possible to perform RT/PCR analysis only during CR. The follow-up in 62 cases during CR was between 3 to 72 months. All cases were treated with ATRA for remission induction (45 to 60 mg/m2/d), followed by consolidation with chemotherapy (HOAP or DA). The HOAP protocol is H (daunorubicin), 0.05 to 0.07 mg/kg intravenously from day 1 to day 5 or 6; O (vincristine), 1 to 1.5 mg/m2/day; A (cytosine arabinoside), 100 mg/m2/d, day 1 to day 6; P (prednisone), 30 mg/d orally, day 1 to day 6. The DA protocol is D (daunorubicin), 20 to 30 mg/m2/d intravenously for day 1 to day 3 and A (cytosine arabinoside), 100 mg/m2/d, day 1 to day 7. After the consolidation, patients in CR were treated with combined ATRA-chemotherapy protocols recommended by the Chinese national cooperation group for treatment of APL. The first protocol consists of consolidation by chemotherapy (HOAP) in the first month, ATRA treatment in the second month (30 to 45 mg/m2/d), and maintenance chemotherapy in the third month (MTX 15 mg/wk, 6-MP 100 mg/d orally). Four courses of this 3-month protocol are used the first to third years. Three courses are applied during the fourth year, and two courses during the fifth year. Another recommended protocol consists of chemotherapy with DA in the first month and ATRA for the second month. Six courses of this 2-month protocol are used the first to third year while four courses are used during the fourth year, and two courses the fifth year. All treatment is discontinued after the fifth year. Bone marrow samples from patients during CR were obtained at intervals between two...
chemotherapy courses. Most patients (90%) were treated with the HOAP consolidation and postremission therapy protocols. Bone marrow mononuclear cells were isolated by Ficoll-Hypaque gradient separation and stored in liquid nitrogen. Approval was obtained from the Institutional Review Board for these studies. Informed consent was provided according to the Declaration of Helsinki.

RNA extraction. Treatment of guanidinium-thiocyanate-phenol-chloroform method. Caution was taken during the extraction to minimize cross-contamination between samples.

"Nested" RT/PCR analysis. The PML-RARα fusion gene transcripts were analyzed using the previously described RT/PCR method with some modifications. Eight oligonucleotides (oligo) were used for retrotranscription, amplification, and detection of the long- and short-type isoforms. An additional 4 oligos were used for amplification of normal RARα transcripts as internal control (see Table I and Fig 1 for sequences and positions of these oligos). The retrotranscription (RT) is performed at 37°C for 45 minutes in a volume of 40 μL, including 1 μg of total RNA, 100 ng of oligo A, 500 mmol/L of each dNTP, and 200 U of MMLV reverse transcriptase (Bethesda Research Laboratory, Bethesda, MD). PCR conditions as follows. Reaction volume is 100 μL, including 10 μL of RT product, 150 pmol of each dNTP, 1.5 U of Taq polymerase (Promega, Madison, WI), in a buffer of Tris-HCl 10 mmol/L, pH 8.4, MgCl₂ 1.5 mmol/L, bovine serum albumin (BSA) 20 μg/mL, KCl 50 mmol/L. A programmable thermal reaction (Hybaid, UK) was used for the PCR. Each round of the "nested" PCR contains 30 cycles, each cycle being 1 minute at 94°C (denaturation), 1 minute and 30 seconds at 55°C (annealing), and 2 minutes at 72°C (elongation), with the last elongation at 72°C for 10 minutes. The first round was performed with primers B (5' primer) and D (3' primer) for L-type isoform or primers C (5' primer) and D for S-type isoform. For the second-round PCR, the system is the same as for the first round except that 10 μL of the first-round PCR product was used instead of RT product and the primers E (5' primer) and G (3' primer) for L-type isoform or primers F (5' primer) and G for S-type isoform. Two negative controls (one with non-APL RNA and one without RNA) and one positive control APL sample were included in each experiment. To avoid contamination, each RNA sample was aliquoted and analyzed for two different RT/PCR. Of 97 samples examined in this study, only 4 did not show the same result in duplicate tests. A third test was performed and the data of the two tests giving concordant results were taken (3 positive and 1 negative).

Table 1. Sequences of the Oligonucleotides Used in This Study

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>PML-RARα</td>
<td>5' TGG ATG CTG CGG CGG AAG AAG CCC TTG CAG 3'</td>
</tr>
<tr>
<td>B</td>
<td>5' GTC ATA GGA AGT GAG GTC TTC 3'</td>
</tr>
<tr>
<td>C</td>
<td>5' CGG ATG GCT TCG ACG AGT TC 3'</td>
</tr>
<tr>
<td>D</td>
<td>5' CTC ACA GGC GCT GCC CCC AT 3'</td>
</tr>
<tr>
<td>E</td>
<td>5' AAC AGC AAC CAC GTG GCC AG 3'</td>
</tr>
<tr>
<td>F</td>
<td>5' TTC AAG GTG CGC CTG CGA 3'</td>
</tr>
<tr>
<td>G</td>
<td>5' AGC CTG AGG ACT TGT CCT GA 3'</td>
</tr>
<tr>
<td>H</td>
<td>5' GCT GCT CTG GGT CTC AAT 3'</td>
</tr>
<tr>
<td>Normal RARα (internal control)</td>
<td>5' GCC TCC TTA CGC CTT CT C 3'</td>
</tr>
<tr>
<td>J</td>
<td>5' AAA GCA AGG CTT GTA GAT GC 3'</td>
</tr>
<tr>
<td>K</td>
<td>5' GAC CAC TCT CCA GCA CCA 3'</td>
</tr>
<tr>
<td>L</td>
<td>5' GCT GGG CAC TAT CTC TTC AGA ACT 3'</td>
</tr>
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</table>

RESULTS

Correlation between different types of PML-RARα fusion gene mRNA isoforms and the prognosis of APL. Cytogenetic studies performed in 53 APL cases at diagnosis showed a t(15;17) in 52 cases and a variant chromosomal translocation in 1 case. Molecular characterization of this case allowed a new fusion gene PLZF-RARα to be found, which is described elsewhere. RT/PCR analysis of the 52 cases with
t(15;17) showed specific amplification of the p6-r3 junction in 33 cases, including one case with a slightly shorter band corresponding to the variant L-type isoform previously reported.13 The remaining cases had p3-r3 junctions. The L-type fusion gene appeared to predict a better response to treatment because 32 of 33 cases with L-type fusion gene achieved CR, whereas 16 of 19 cases with S-type achieved CR (Table 2). In the 48 cases achieving CR (CR duration, 3 to 32 months), only 1 of 32 L-type cases relapsed (at 24 months), whereas 3 of 16 S-type cases relapsed (at 9, 14, and 20 months, respectively). Note that both groups received the same treatment protocols, and the four relapsed cases, like most of the other cases, were treated with HOAP consolidation and postremission therapy. We have also examined the hematologic data of the 8 cases without CR or with early relapse. All these had typical AML-M3 morphology. At diagnosis, the white blood cell (WBC) count was extremely high in only 1 S-type case (174 × 10^9/L) whereas the median was 2.1 × 10^9/L. The range of platelet counts were 24 to 110 × 10^9/L (median, 47 × 10^9/L). This was not different from that of the rest of the patients (16 to 164 × 10^9/L; median, 54 × 10^9/L). The difference of clinical outcome between L- and S-type groups, if calculated separately in terms of the early death rate or early relapse rate (within 24 months of CR), have not reached statistical significance. Nevertheless, if the overall prognosis (combining the early deaths and relapsed cases) of both isoform types are compared, the S type is related to a poor prognosis when compared with the L-type (χ^2 = 6.03, .01 < P < .025). In agreement with our previous report,11 no statistically significant difference of sex, age distribution, or hematologic data (Table 2) exist between L and S types in this larger series, although the median WBC count seems to be higher in the S-type group than in the L-type group. The isoform types detected at relapse in the 4 cases were identical to those detected at diagnosis.

Detection of MRD during clinical remission to predict relapse. Eighteen cases were studied by RT/PCR for PML-RARα fusion gene transcripts before and after ATRA remission induction and the clinical course has been observed after chemotherapy (Fig 2, upper part). RT/PCR assays for both L- and S-type isoforms were performed in each case. In this series, 35 cases were negative and 9 were positive for PML-RARα fusion mRNA. All RT/PCR-negative cases are currently in CR. Among the 9 positive cases, hematologic relapse occurred in 4 cases (no. 29, 52, 58, and 60, who were in 24, 36, 48, and 60 months of CR, respectively) 1 (no. 52, 58, and 60) to 6 months after the RT/PCR positivity. Only 1 of these 4 cases (no. 58) had clinical findings suggesting an impending relapse (hypogranulocytosis and anemia), whereas the three others came for routine reexamination. The other 5 cases (no. 28 and 38 through 41) tested positive at 12, 24, and 28 months of CR all received consolidation chemotherapy after the positive RT/PCR assay and remain in CR. The RT/PCR will be reexamined in these cases.

### Table 2. Correlation Between PML-RARα Fusion Gene Isoform Types and the Clinical Prognosis in 52 De Novo APL Cases

<table>
<thead>
<tr>
<th>Isoform</th>
<th>No. of Cases</th>
<th>Sex (M/F)</th>
<th>Ranges of Age (yr) (median)</th>
<th>WBC Count (×10^9/L) (median)</th>
<th>Percentage of Blasts in BM (median)</th>
<th>CR (%)</th>
<th>ED* (%)</th>
<th>Early Relapse* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L</td>
<td>33</td>
<td>18/15</td>
<td>5-64 (29)</td>
<td>1.0-30.3 (2.05)</td>
<td>62.94 (88.5)</td>
<td>32</td>
<td>1</td>
<td>1†</td>
</tr>
<tr>
<td>S</td>
<td>19</td>
<td>12/7</td>
<td>6-74 (31)</td>
<td>1.72-174.0 (6.9)</td>
<td>42.90 (80)</td>
<td>16</td>
<td>3</td>
<td>3†</td>
</tr>
</tbody>
</table>

The duration of observation after consolidation therapy is from 3 to 32 months. Abbreviation: BM, bone marrow.

* Early death and relapse in S- versus L-type group: χ^2 = 6.03, .01 < P < .025.
† Three S-type cases died relatively shortly (2 weeks to 4 months) after relapse, whereas a second CR was obtained in the only L-type case.

DISCUSSION

The finding that t(15;17) is present in most cases as the only cytogenetic abnormality and that the PML-RARα fusion gene exists in almost every APL case that responds to ATRA supports the idea that this translocation-based fusion gene plays an essential role in the leukemogenesis. The t(15;17) also results in the expression of the reciprocal fusion gene, RARα-PML. However, this reciprocal fusion gene is not expressed in about 30% of APL cases13 and thus should be less important in the pathogenesis of the disease. One of the features in the molecular pathology of APL is the heterogeneity of the fusion genes. Although the chromo-
Fig 2. RT/PCR analysis of the APL minimal residual disease in 62 cases after CR. The numbers on the vertical axis correspond to the patient numbers. The horizontal axis shows the follow-up in months (m) after complete remission. Each box or circle represents a PCR study from the specified patient(s). Open and filled boxes indicate negative and positive results for L-type fusion mRNAs, respectively. Open and filled circles indicate negative and positive results for S-type isoform, respectively. Cases 1 through 18 were studied according to the L- or S-type fusion mRNAs. The difference between these two isoforms is that a stretch of 158 amino acids coded by PML exons 4-6, existing in the L-type isoform, is lacking in the S-type isoform. This portion of protein is structurally an α-helical region, with a serine-rich portion for potential phosphorylation. The functional meaning of the presence or absence of this portion remains to be determined. As for the clinical relevance of the two major PML-RARα isoforms in APL, we previously observed in a smaller number of APL patients that S- or L-type fusion genes do not differ from each other by age, sex, or response to ATRA. However, clinical significance is suggested because cells carrying different PML-RARα fusion genes display distinct CD2 antigen expression. In the present larger study, there is a significant difference in the prognosis between patients carrying L- versus S-type fusion genes; the latter show more mortality before ATRA treatment and relapsed more frequently. More cases are needed to confirm this observation.

It is now well accepted that ATRA is effective in inducing CR in APL. According to our experience and that of others, more than 95% of patients with t(15;17) or positive PML-RARα RT/PCR will respond to ATRA administered for a sufficiently long time. However, if the ATRA is used alone as postremission therapy, most patients will relapse within 6 months. The 75% finding of RT/PCR positivity in APL patients after ATRA-induced clinical CR in this series and even more in other studies suggests that, although ATRA is effective in inducing the clinical CR, it is unable, in most APL patients, to eliminate the leukemic clone completely. The “resistance” to ATRA could be related to the transient changes in retinoid metabolism or a secondary genetic event within the leukemic cell. It has been shown that combination cytotoxic chemotherapy can enhance differentiation therapy in leukemic cell lines and there is no cross-resistance. Incorporation of intensive chemotherapy in postremission treatment is necessary to eliminate MRD and prevent relapse.

The best example of MRD detection in current hematology practice is the RT/PCR for the BCR-ABL fusion mRNA in CML after bone marrow transplantation (BMT). It is worth noting that the RT/PCR assay for APL is somewhat different. Methodologically, although CML has two fusion RNA isoforms, one oligonucleotide set is sufficient for each round in the “nested” PCR. However, according to our experience, the use of different primer sets to analyze the L- and S-type isoforms in APL can increase the sensitivity and specificity of the test. Second, using “nested” RT/PCR, we showed that the fusion gene transcripts can be detected in as little as 50 pg of total RNA extracted from de novo APL patient bone marrow samples, whereas the single-step PCR requires 1 to 10 ng of total RNA for detection. However, in our hands, this sensitivity seems to be lower than that of BCR-ABL RT/PCR, in which fusion transcripts are detectable in 1 to 10 pg of total RNA. Third, and more important, a number of CML patients may have a PCR positivity during the first 6 to 12 months after BMT, probably due to residual, long-lived leukemia stem cell-derived B and T lymphocytes. Most of these patients will become PCR negative later without further therapeutic intervention. In APL, the situation seems to be quite different. In our series, a total of 62 APL patients have been investigated after ATRA-induced CR and chemotherapy consolidation (including 18 cases with pretreatment data and 44 cases tested only during CR). Among 11 patients having positive PCR, 5 relapsed from 1 to 6 months after the test, 1 converted to negative after new courses of chemotherapy, and 5 remained in CR status 3 months later without further PCR data. However, the latter 5 cases all received intensive consolidation therapy after the PCR positivity. These results reinforce the idea that RT/PCR can predict relapse in APL, as suggested by LoCoco et al.
a shorter follow-up than in our series. Therefore, it may be concluded that, in APL, a positive RT/PCR during CR, after consolidation, should be considered as a serious sign of relapse and no spontaneous conversion from RT/PCR positive to negative can be expected. Accordingly, we suggest systematic use of RT/PCR to monitor MRD in APL and to initiate treatment (consolidation with already used protocols or eventually using other non-cross-resistant drugs) when a positive result is obtained during CR.

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