Abstract The PML/RARα fusion RNA can be detected in acute promyelocytic leukemia (APL), cytogenetically characterized by the translocation t(15;17). Our study included ten newly diagnosed patients with APL who were investigated during the course of their diseases using reverse transcription polymerase chain reaction (RT-PCR). At diagnosis, aberrant fragments with a size heterogeneity due to alternative spliced products were detected in all patients, we observed breakpoints within bcr3 (short type) in two patients and bcr1 and 2 breakpoints (long type) in eight patients. Treatment consisted of all-trans retinoic acid (ATRA) in all patients; six patients received simultaneous cytostatic therapy during remission induction. At the time of complete hematological remission (CR), only two patients showed a negative RT-PCR result; eight of the ten patients were still PCR positive when nested primers were used. Subsequently, eight patients received consolidation chemotherapy and became PCR negative. Seven of eight patients are in continuous complete remission (median remission duration: 21 months, range: 11+-26+ months). The remaining two patients who were treated only with ATRA relapsed, received induction chemotherapy, and are in second and third complete remission, respectively. In conclusion, PCR negativity can be achieved only by chemotherapeutic consolidation; patients treated with ATRA alone remain PCR positive. Relapse is always preceded by a positive PCR result. Surprisingly, also patients without measurable PML/RARα-mRNA in sequential analyses after cytostatic treatment became PCR positive and experienced relapse.

Key words Acute promyelocytic leukemia · Reverse transcriptase polymerase chain reaction · Minimal residual disease · Promyelocytic leukemia · Retinoic acid receptor α

Introduction

Acute promyelocytic leukemia (APL), characterized by the chromosomal translocation t(15;17), is associated with a unique transcriptional product, promyelocytic leukemia/retinoic acid receptor-α (PML/RARα) [1]. This rearrangement results in a functionally altered retinoic acid receptor, thus explaining the response of promyelocytic blasts to all-trans retinoic acid (ATRA) as a differentiating agent in vitro and in vivo [2,3]. ATRA gives a high complete remission rate in APL patients and reduces the incidence of fatal disseminated coagulopathy significantly [3–5]. Nevertheless, patients maintained with ATRA alone have a high relapse rate and therefore need chemotherapeutic consolidation [6].

Several research groups investigated the different PML/RARα fusion proteins in APL patients and established RT-PCR tests for evaluation of minimal residual disease [7,8]. Detection of PML/RARα fusion RNA in remission correlates well with impending relapse [9–11]. Since April 1992 we have investigated ten newly diagnosed patients with APL, applying a two-step RT-PCR method with "nested" primers. Our study supports previous reports concerning the need for chemo-
therapy to eradicate PML/RARα fusion RNA, the significance of PCR positivity, and the size heterogeneity of fusion transcripts. PCR negativity is the therapeutic aim in APL patients, although it is no predictor of cure.

### Materials and methods

#### Patients

Bone marrow and peripheral blood samples were obtained from ten newly diagnosed patients with APL (AML:M3) classified according to the FAB criteria, including detection of the t(15; 17) translocation. All patients received ATRA 45 mg/m²/day for induction treatment, six of them in combination with cytotoxic therapy (patients 1, 5, and 10 received a standard chemotherapy, "3 + 7", i.e., daunorubicin 60 mg/m²/day 1–3, cytosine arabinoside 100 mg/m² day 1–7; patients 4, 6, and 7 received high-dose cytosine arabinoside chemotherapy 3 g/m² every 12 h for 2 days, as previously reported for hyperleukocytosis [5]. All patients except nos. 2 and 3, who were treated with ATRA maintenance, underwent consolidation therapy with schedules containing daunorubicin/cytosine arabinoside.

#### Cell preparation and cytogenetics

For molecular and cytogenetic evaluation, mononuclear cells of bone marrow (BM) and/or peripheral blood (PB) at diagnosis and BM cells only at remission were isolated by density gradient centrifugation using Ficoll-Hypaque (density 1.077) or Percoll (1.088), respectively. Chromosome preparations were obtained from 24- to 72-h BM cell cultures stimulated by either 10% BM-conditioned or GM-CSF (100 units/ml). Twenty simultaneously R- and C-banded metaphases were analyzed.

#### PCR amplification of PML/RARα junctions

Isolation of mRNA and in vitro reverse transcription from 1 µg mRNA in a 15-µl assay) were performed using commercial kits (Pharmacia). PCR assays were set up in 25-µl aliquots containing 2 µl cDNA, 1.5 mmol/l MgCl₂, 50 mmol/l KCl, 10 mmol/l Tris-HCl, pH 8.3, 200 µmol/l dNTPs, 0.2 units of a recombinant Taq polymerase (AGS Company), and 15 pmoles of primers. The primers used were M2: 5'-AGTGTACGCCTTCTCCATCA-3', M4: 5'-AGCTGCTGGAGGCTGTGGACGCGCGGTACC-3', and M5: 5'-GACTTCTGGTGCTTTGAGTG-3' as 5'-primers and RARA 5: 5'-CCACTAGTGTAGCCTGAGGACT-3' and RARA 8: 5'-CAGAACTAGTGCTGCTTGGTTCAAT-3' as 3'-primers. R2: 5'-GCTCTGACCACTCTCCAGCA-3', a primer for the normal RARα gene, kindly donated by Dr. Andrea Biondi, was used as an internal control [1, 7]. Probes used for Dot-blot and Southern blot hybridization of the PCR products were as follows:

- PML ex.2: 5'-GACCAACAACATCTTCTTGCT-3',
- PML ex.3: 5'-ACGGCAGCTTGAAGGCTCTG-3',
- PML ex.5: 5'-AAGGCCCCTTCCTATGGAGAG-3'.

Denaturation, annealing, and extension were performed on an automated heat block (PHC-3 Dri-Block Cycler; Techne). After an initial denaturation step at 95°C for 5 min, 30 cycles, each consisting of 1 min denaturation at 95°C, 1 min annealing at 50°C, and 1 minute extension at 72°C, were performed (Detection limit 1/10⁶ cells). For quality control, and for minimal residual disease monitoring (detection limit: 1/10⁷ cells), 5 µl of the first PCR product was used for a second-round amplification for a further 30 cycles using a nested primer (R8 or RAR8). Finally, 10 µl of PCR product was run on a 2% nusieve agarose gel stained with ethidium bromide, visualized under a UV lamp, and Southern blotted. Another aliquot (2 µl) was dot blotted onto nylon membranes and hybridized with oligonucleotide probes containing sequences from PML exons II, III or V. There were no differences in the sensitivity levels between the nonradioactive ethidium-bromide detection and the dot-blot analysis when nested primers were used. Prehybridization, hybridization, and washings were done according to standard techniques. As a negative control, RNA isolated from normal buffy coat cells was used. Amplification of the normal RARα cDNA was accomplished with commercial primer combinations (Clontech) with 2 µl of the same cDNA preparation used to identify PML/RARα junctions. The integrity of the RNA preparation was further confirmed by the presence of the normal RARα PCR product in all samples, showing negative PML/RARα fusion mRNA [8].

### Results

Clinical and biological patient characteristics at diagnosis are shown in Table 1. All patients tested had the translocation (t(15; 17)) and a detectable chimeric PML/RARα mRNA. Further minimal residual disease monitoring of bone-marrow samples was carried out at least every 3 months during and after consolidation therapy and in remission. Figure 1 shows the PCR analysis of patient 7 at the time of diagnosis; aberrant fragments were detected with four different primer pairs.

At diagnosis we observed 5' breakpoints (breakpoint cluster region 3 = bcr 3, S-type) in two patients and 3' breakpoints within bcr 1 and 2 (L-type) in eight patients in the second intron of the RARα gene. We