original paper

haematologica 2001; 86:577-585
http://www.haematologica.it/2001_06/0577.htm

The *PML-RARα* transcript in long-term follow-up of acute promyelocytic leukemia patients

PAULA GAMEIRO,*† SARA VIEIRA,* PAOLA CARRARA,† ANA LUISA SILVA,*JOANA DIAMOND,* AIDA BOTELHO DE SOUSA,# ATUL B. MEHTA,° H. GRANT PRENTICE,† JOSÉ EDUARDO GUIMARÃES,‡ A. VICTOR HOFFBRAND,† LETIZIA FORONI,† ANTONIO PARREIRA*

*Department of Hematology, Instituto Português de Oncologia, Lisboa; †Royal Free and University College School of Medicine, Royal Free Campus, London, UK; #Department of Hematology, Hospital S. A. dos Capuchos, Lisboa; ‡Instituto de Patologia e Imunologia Molecular da Universidade do Porto, Portugal

Correspondence: Letizia Foroni, M.D., Royal Free and University College of Medicine, Royal Free Campus, Pond Street, London, NW3 2QG, United Kingdom. Phone: international +44-20-73802965 – Fax: international +44-20-78302092 – E-mail: letizia@rfc.ucl.ac.uk

Background and Objectives. Detection of *PML-RARα* transcripts by RT-PCR is now established as a rapid and sensitive method for diagnosis of acute promyelocytic leukemia (APL). Although the majority of patients in long-term clinical remission are negative by consecutive reverse transcription polymerase chain reaction (RT-PCR) assays, negative tests are still observed in patients who ultimately relapse. Conversion from negative to positive PCR has been observed after consolidation and found to be a much stronger predictor of relapse. This study reports on 47 APL patients to determine the correlation between minimal residual disease (MRD) status and clinical outcome in our cohort of patients.

Design and Methods. The presence of *PML-RARα* t transcripts was investigated in 47 APL patients (37 adults and 10 children) using a semi-nested reverse transcriptase-polymerase chain reaction to evaluate the prognostic value of RT-PCR tests.

Results. All patients achieved complete clinical remission (CCR) following induction treatment with all-trans retinoic acid (ATRA) and chemotherapy (CHT) or ATRA alone. Patients were followed up between 2 and 117.6 months (median: 37 months). Relapses occurred in 11 patients (9 adults and 2 children) between 11.4 and 19 months after diagnosis (median: 15.1 months) while 36 patients (28 adults and 8 children) remained in CCR. Seventy-five percent of patients carried the *PML-RARα* long isoform (bcr 1/2) which also predominated among the relapsed cases (9 of 11) but did not associate with any adverse outcome (p= 0.37). For the purpose of this analysis, minimal residual disease tests were clustered into four time-intervals: 0-2 months, 3-5 months, 6-9 months and 10-24 months.

Interpretation and Conclusions. Children showed persisting disease for longer than adults during the first 2 months of treatment. At 2 months, 10 (50%) of 20 patients who remained in CCR and 4 (80%) of 5 patients who subsequently relapsed were positive. Patients who remained in CCR had repeatedly negative results beyond 5.5 months from diagnosis. A positive MRD test preceded relapse in 3 of 4 tested patients. The ability of a negative test to predict CCR (predictive negative value, PNV) was greater after 6 months (>83%), while the ability of a positive test to predict relapse (predictive positive value, PPV) was most valuable only beyond 10 months (100%). This study (i) highlights the prognostic value of RT-PCR monitoring after treatment of APL patients but only from the end of treatment, (ii) shows an association between conversion to a positive test and relapse and (iii) suggests that PCR assessments should be carried out at 3-month intervals to provide a more accurate prediction of hematologic relapses but only after the end of treatment.

Key words: APL, RARα, minimal residual disease

The *PML-RARα* fusion transcript is associated with the t(15;17)(q21;q22) translocation and results from the rearrangement of the PML gene on chromosome 15 and the *RARα* gene on chromosome 17.[1-4] The fusion product is transcriptionally active in all cases of acute promyelocytic leukemia (APL) (M3 subtype in the FAB classification) whilst only 70% of the cases carry the reciprocal *RARα-PML*.[5-8] A breakpoint in intron 2 is the common site on chromosome 17, while three different breakpoint regions have been described in the *PML* gene on chromosome 15. This results in the generation of three different *PML/RARα* transcripts, depending on whether the breakpoint in the *PML* gene affects intron 6 (bcr1; long type), exon 6 (bcr2; variant type) or intron 3 (bcr3; short type).[5,9]

Amplification of the *PML-RARα* transcripts by reverse transcriptase polymerase chain reaction (RT-PCR) is now established as a rapid and sensitive method for diagnosing APL. This also enables identification of patients with the three different isoforms, and finally provides a valuable method for the assessment of minimal residual disease (MRD) in these patients.[5,10-15]

Recent investigations have shown that the majority of patients in long-term clinical remission have consecutively negative RT-PCR tests. Nevertheless, clinical relapses are often preceded by negative tests.[16-18] The short half-life of the *PML-RARα* mRNA and poor sensitivity of the technique may be responsible for false negative results. Despite the increased sensitivity of recent assays,[19] negative tests are still observed in patients who ultimately relapse. More recently, conversion from negative to positive PCR has been observed after consolidation and found to be a much stronger predictor of relapse.[17] However, no consistency in the time-interval between PCR conversion and time of relapse was observed. Only larger studies or information from more APL patients will improve the future interpretation of MRD data and their significance as prognostic indicators of clinical outcome in APL.

For this reason, we report here on a two-step RT-PCR investigation of the *PML-RARα* transcript in 47 APL patients . Although the patients studied were from different institutions, they were all treated with all trans retinoic acid (ATRA) and chemotherapy (CHT). This study aimed to determine the correlation between MRD status and clinical outcome in our cohort of patients.

Design and Methods

Patients' data

All 47 patients were diagnosed as having APL (AML-M3) according to the FAB classification.[20] With the exception of patient #5 (Figure 1) (who suffered secondary APL following treatment for breast cancer), all cases had *de novo* APL. Thirty-seven were adults (median age 37.8 years, range: 16-69; 21 were males and 16 females) and ten were children (median age: 7.0 years, range: 1.5-14; 4 males and 6 females). Forty of the 47 patients were analyzed at time of referral using RT-PCR: 33 were analyzed at presentation, 4 at 1 month (cases #12, 21, 35, 43), 2 at 2 months (cases #31 and 46) and 1 at 10 months (case #39). In four other patients (cases #2, 4, 6 and 24) the presence of the t(15;17) was confirmed by conventional cytogenetics. In the 3 remaining cases (#1, 3 and 8) there was no material available for molecular or cytogenetic analysis at presentation and diagnosis was based on morphologic and clinical evaluation. Following diagnosis, all patients were monitored by RT-PCR for an average of 19.3 months (range: 1.1 to 97.9 months). Patients received different therapeutic protocols but all included ATRA during the induction period. The number of consolidation cycles varied between 1 and 4. Eleven patients (10 children and 1 adult) received maintenance treatment. Five patients (#7, 9, 28, 38 and 43) have been previously reported (Devaraj *et al.*, 1996).

Samples and RNA preparation

The number of tests performed varied between 1 and 9 tests per patient (average 4 tests). Bone marrow (BM) (n=184) and peripheral blood (PB) (n=5) samples were used for follow-up investigations. The mononuclear cells were isolated by density gradient centrifugation in Ficoll-Hypaque (Nycomed Pharma AS, Oslo, Norway) and washed twice in phosphate-buffered saline (PBS). Cells were lysed in 4M guanidium thiocyanate (GITC) solution and stored at -70°C. Total RNA was extracted by the method of Chomczynsky and Sacchi.[21] In 14 patients RNA was prepared using the Gentra extraction kit (Gentra, UK). Four time periods were analyzed: 0-2 months, 3-5 months, 6-9 months and 10-24 months. The result of the last sample tested was used for MRD analysis in patients with more than 1 test in each time period.

RT-PCR

The protocol and the primers used to amplify the *PML-RARα* fusion transcript were adapted from a previously described protocol.[5] Briefly, 1 μg of total RNA was reverse transcribed (RT) into cDNA in a 20 μL reaction for 90 min at 37°C using random hexamers (Promega, Madison, WI, USA), 40 U of RNAse inhibitor (Promega), 1 mM dNTP (Pharmacia Biotech, USA) and 200 U of Superscript II RNAse H Reverse Transcriptase (Gibco BRL, Bethesda Research, Gaithersburg, MD, USA) according to the manufacturers' instructions. Two and a half microliters of cDNA were first amplified in a 50 μL reaction using the oligonucleotides M4-M5 or M2-M5[5] with 2.5 U of Taq DNA polymerase (Gibco, BRL), 1.5 mM $MgCl_2$, 200 mM dNTPs and 15 pM of each primer. PCR was carried out in a DNA thermal cycler machine (Perkin Elmer-Cetus) for 35 cycles (50 sec at 94°C, 50 sec at 59°C and 90 sec at 72°C). One microliter of the first round amplification was re-amplified in a semi-nested reaction for another 35 cycles, using primers M4-R8 or M2-R8,[5] under the same conditions as for the first round amplification. The PCR products were analyzed on a 1.5% agarose gel and visualized by ethidium bromide staining. To evaluate the integrity of the RNA and assess the efficiency of the RT step, each sample was also amplified using primers for the *RARα* transcript under the same conditions as for the *PML-RARα* transcripts. A positive control (total RNA extracted from the promyelocytic cell line NB4) and a negative control (no RNA) were included in each experiment.

Sensitivity tests

To assess the sensitivity of the RT-PCR, serial dilutions of RNA from an APL patient and from the NB4 cell line were set up using RNA from the HL60 cell line. All procedures were done using RNAse free disposable material. Both the bcr1 (long form) and the bcr3 (short form) transcripts were obtained from the control amplifica-

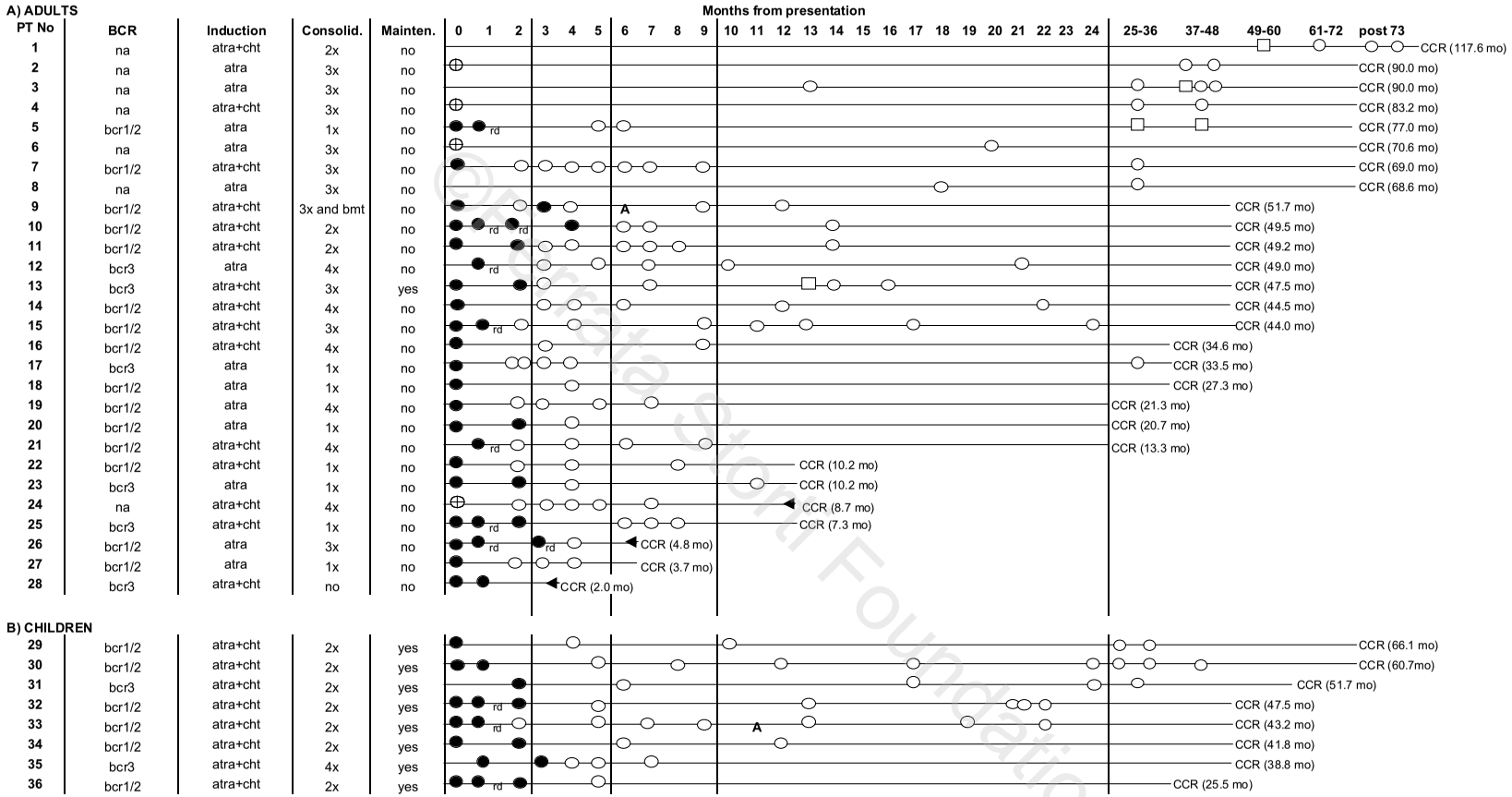

Figure 1. RT-PCR amplification of *PML-RARα* transcript during follow-up of 36 APL patients who remained in CCR: A) Adults and B) Children. Symbols are as follows: ● MRD positive test; ○ MRD negative test; □ MRD negative test (PB); ⊕Cytogenetic positive analysis. ◄ Death in CCR: Abbreviations are as follows: CCR: complete clinical remission in months from presentation; mo: months; Pt: patient number; BCR: *PML-RARα* transcript isoform; A: autologous bone marrow transplant; Consolid: consolidation treatment; Mainten: maintenance treatment; rd: resistant disease; x: number of chemotherapy cycles.

tion tests using 0.1 ng of total positive RNA (i.e. sensitivity of $1:1\times10^4$).

Statistical analysis

Standard statistical tests were carried out (Fisher's exact test, χ^2 contingency tests, and parametric and non-parametric t-tests) using the statistic programs GraphPad Prism, and SPSS. Disease-free survival (DFS) curves were generated using the Kaplan-Meier method,[22] and compared with the logrank test. The impact of multiple predictor variables on DFS was assessed and compared to MRD status using the Cox regression model.[23]

Results

Patients' clinical data

Thirty-six patients (28 adults and 8 children) remained in CCR between 2 and 117.6 months (Figure 1) from when they were first referred (median period: 43.7 months). The median period of DFS in this group was 41.7 months (range 1-114.7 months). Eleven patients (9 adults and 2 children) relapsed between 11.4 and 19 months (median 15.1) after presentation (Figure 2), following CCR for a median period of 13.3 months (range 10-16.5). The clinical and biological characteristics of patients at the time of diagnosis are presented in Table 1. Age appeared to have no impact on overall clinical outcome (p= 1.0, Fisher's exact test). All patients had achieved CCR following induction treatment with ATRA and chemotherapy or ATRA alone (median time: 54 days; range 28-102 days; 56 days in adults and 50 in children). Patients who later relapsed achieved first CCR between 35 and 90 days (median 57 days; adults 56 and children 58 days) which was comparable with the time in patients who remained in remission (range 28 and 102 days; median 53 days; adults 55 and children 48 days)(p=0.32, Mann-Whitney t-test).

The induction therapy using ATRA or ATRA plus chemotherapy did not result in better overall survival (Figure 3A) (p=0.94, logrank test) as we observed relapses in 4 (24%) of 17 patients in the ATRA group and in 7 (23%) of 30 cases in the ATRA plus chemotherapy group. Patients who received 3 or 4 cycles of chemotherapy following induction did not appear to have a better overall survival compared to the group who received 1 or 2 chemotherapy cycles as we observed relapses in 11% and 33% of patients in these two groups, respectively (p=0.07, logrank test; Figure 3B) although a trend towards a better outcome was associated with the higher number of cycles.

PML/RARα isoforms

The *PML/RARα* isoforms (bcr1/2 and bc3) were identified by RT-PCR in 40 patients. Thirty-three patients were analyzed at presentation, 4 at 1 month, 2 at 2 months and 1 patient (case #39) at 10 months, in CCR. The bcr1/2 (long form) was detected in 30 (75%) patients, 21 of them remained in CCR and 9 relapsed. The bcr3 (short form) was detected in 10 (25%) patients, 8 remained in CCR and 2 relapsed. There was no difference in the distribution of the isoforms between the two age groups (p=1.00, Fisher's exact test). Although 9 of 11 patients who relapsed carried the bcr1/2 form, there was no statistically significant difference in the incidence of the *PML/RARα* isoform types between the relapsed and CCR group (p=0.69, Fisher's exact) and neither isoform conferred better or worse disease-free survival (p=0.72, logrank test; Figure 3C).

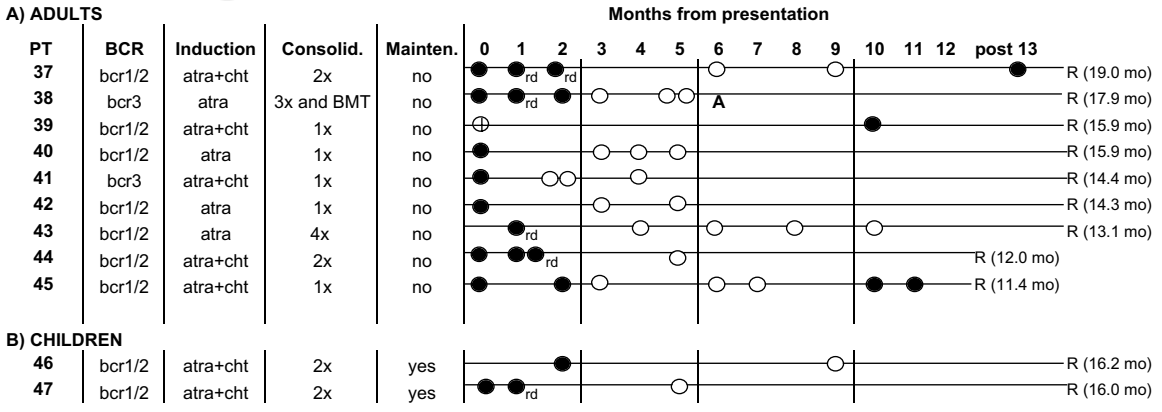

Figure 2. RT-PCR amplification of *PML-RARα* transcript during follow-up of 11 APL patients who relapsed. Symbols and abbreviations are as in Figure 1. R: relapse time (in months) from presentation.

Table 1 . Clinical characteristics of patients at time of diagnosis and type of therapy administered. Abbreviations are as followS: f; female; m, male; ATRA: all-trans retinoic acid; DA: daunarubicin + Ara-C; DAV: daunarubicin + Ara-C+etoposide; MTZ: mitoxantrone; MTZA: mitoxantrone+ Ara-C; IDACCNU: idarubicin + CCNU; IDAARA-C: Idarubicin +Ara-C; IDAARA-C6TG: idarubicin +Ara-C+6-thioguanine: MP, mercaptopurine.

N	*Age*	*Sex*	*WBC (x10⁹/L)*	*RT-PCR*	*Induction*	*1st consolidation*	*2nd consolidation*	*3rd consolidation*	*Maintenance*	*Protocol*	*Event*
1	39	m	2.0	NA	ATRA + 2x(MTZA)	MTZA	MTZA	no	no		CCR
2	47	m	0.8	NA	ATRA	DA	DA	DA	no	APL91	CCR
3	65	f	1.1	NA	ATRA	DA	MTZA	IDACCNU	no		CCR
4	34	m	1.2	NA	ATRA+DA	DA	DA	DA	no	APL91	CCR
5	53	f	27.7	bcr1	ATRA	DAV	no	no	no		CCR
6	45	m	4.6	NA	ATRA	DA	DA	DA	no	APL91	CCR
7	25	f	7.7	bcr1/2	ATRA+CHT	3xCHT			no		CCR
8	27	m	0.8	NA	ATRA	DA	DA	DA	no	APL91	CCR
9	16	f	10.4	bcr1/2	ATRA+CHT	3xCHT+autoBMT			no		CCR
10	65	f	9.0	bcr1	ATRA+DAV	DAV	IDAARA-C	no	no		CCR
11	38	f	NA	bcr1	ATRA+DAV	DAV	IDAARA-C	no	no		CCR
12	33	f	NA	bcr3	ATRA	4xCHT			no		CCR
13	29	f	1.8	bcr3	ATRA+IDA	DAV	ARA-C+Amsacrine	MTZA	ATRA	AIDA	CCR
14	34	m	NA	bcr1/2	ATRA +CHT	4xCHT			no		CCR
15	20	m	1.2	bcr1	ATRA+IDA	IDAARA-C	MTZVP16	IDAARA-C6TG	no	AIDA	CCR
16	53	m	1.8	bcr1/2	ATRA+CHT	4xCHT			no		CCR
17	43	m	9.9	bcr3	ATRA	Cytarabine+MTZ	no	no	no	Ref 35	CCR
18	38	f	1.4	bcr1	ATRA	Cytarabine+MTZ	no	no	no	Ref 35	CCR
19	52	m	8.6	bcr1/2	ATRA	4x CHT			no		CCR
20	47	f	1.4	bcr1	ATRA	Cytarabine+MTZ	no	no	no	Ref 35	CCR
21	21	m	1.9	bcr1/2	ATRA+CHT	4xCHT			no		CCR
22	27	m	106.0	bcr1	ATRA+Cytarabine	Cytarabine+MTZ	no	no	no	Ref 35	CCR
23	55	f	1.3	bcr3	ATRA	Cytarabine+MTZ	no	no	no	Ref 35	CCR
24	53	f	2.2	NA	ATRA+CHT	4xCHT			no		CCR
25	27	m	13.7	bcr3	ATRA+Cytarabine	Cytarabine+MTZ	no	no	no	Ref 35	CCR
26	52	f	53.0	bcr1/2	ATRA	3xCHT			no		CCR
27	47	f	0.6	bcr1	ATRA	Cytarabine+MTZ	no	no	no	Ref 35	CCR
28	56	m	NA	bcr3	ATRA+CHT	no			no		CCR
29	7	m	3.7	bcr1	DA	DA	DA	no	MP+MTZ	APL93	CCR
30	8	m	1.2	bcr1	DA	DA	DA	no	ATRA	APL93	CCR
31	4	f	35.8	bcr3	DA	DA	DA	no	ATRA	APL93	CCR
32	1.5	f	9.6	bcr1	DA	DA	DA	no	ATRA+MP+MTZ	APL93	CCR
33	10	m	76.9	bcr1	DA	DA	DA	no	AutoBMT	APL93	CCR
34	14	f	2.1	bcr1	DA	DA	DA	no	MTZ+MP	APL93	CCR
35	10	f	2.6	bcr3	ATRA+CHT	4xCHT			1x		CCR
36	5	f	NA	bcr2	DA	DA	DA	no	ATRA+MP+MTZ	APL93	CCR
37	16	m	2.4	bcr1	(ATRA+DAV)+(MTZA)	(ARA-C+Ansacrine)	MTZA	no	no		relapse
38	25	m	2.0	bcr3	ATRA	3x CHT +autoBMT			no	Baltimore	relapse
39	16	m	9.5	bcr1	ATRA+salvage therapy (2xDA)	DA	no	no	no	APL91	relapse
40	41	m	1.3	bcr1	ATRA	Cytarabine+MTZ	no	no	no	Ref.35	relapse
41	23	m	35.0	bcr3	ATRA+Cytarabine	Cytarabine+MTZ	no	no	no	Ref.35	relapse
42	26	f	3.1	bcr1	ATRA	Cytarabine+MTZ	no	no	no	Ref.35	relapse
43	18	m	1.0	bcr1/2	ATRA	4x CHT			no	Baltimore	relapse
44	69	m	NA	bcr1	ATRA+DAV	DAV	DAV	no	no		relapse
45	22	f	11.2	bcr1	ATRA+Cytarabine	Cytarabine+MTZ	no	no	no	Ref.35	relapse
46	6	m	3.0	bcr1	DA	DA	DA	no	1x	APL93	relapse
47	5	f	1.5	bcr1	DA	DA	DA	no	1x	APL93	relapse

MRD tests

The results of MRD analysis of 189 samples are illustrated in Figure 1 for patients remaining in CCR (A: adults; B: children) and in Figure 2 for relapsed patients (A: adults; B: children). At 1 month all patients tested were RT-PCR positive (including 14/17 patients who had resistant disease, RD), irrespective of age and clinical outcome. At 2 months, 50% (10 of 20) of the patients tested in the CCR group were negative, while only 1 of the 5 patients in the relapsed group was RT-PCR negative. Tests carried out between 3-5 and 6-9 months were almost all negative (with the exception of 4 patients with a positive result who remained in CCR), including in 10 of the 11 patients who later relapsed.

We observed a rapid decrease in the level of disease during the first 2 months of monitoring, with the adults showing a more rapid molecular clearance of disease than the children: 83% (5 of 6) of the children were still positive against 41.2% (7 of 17) of the adults. However, the slower reduction in MRD cases at 2 months did not result in a higher incidence of relapses in the younger age group: 2 (20%) of 10 children *versus* 9 (24.3%) of 37 adults (p=1.0, Fisher's exact test). CCR patients had serially negative RT-PCR results beyond 5.5 months. However, 4 patients (3 adults and 1 child) who later relapsed also had negative results at 6-9 months. Moreover, only negative tests were recorded 4, 7, 8, 10, 11, 11, 12 and 13 months prior to relapse in 8 patients (Figure 2A-B). Residual disease beyond 10 months was observed in 3 of 4 adults who relapsed 2, 5 and 7 months after the positive RT-PCR (#45, 37 and 39, respectively).

Statistical analysis of MRD tests

The ability of a positive test to predict relapse (predictive positive value, PPV) was better (100%) between 10-24 months than in any previous time-interval. The ability of a negative test to predict CCR (predictive negative value, PNV) was also highest at 10-24 months with 17 (94%) of 18 MRD negative tests among patients who remained in CCR. During treatment a negative test was invariably better at predicting CCR, (PNV: 91%, 76% and 83% at 0-2 months, 3-5 months and 6-9 months, respectively), than a positive test at predicting relapse (PPV: 20%, 0% and 0% at 0-2 months, 3-5 months and 6-9 months, respectively).

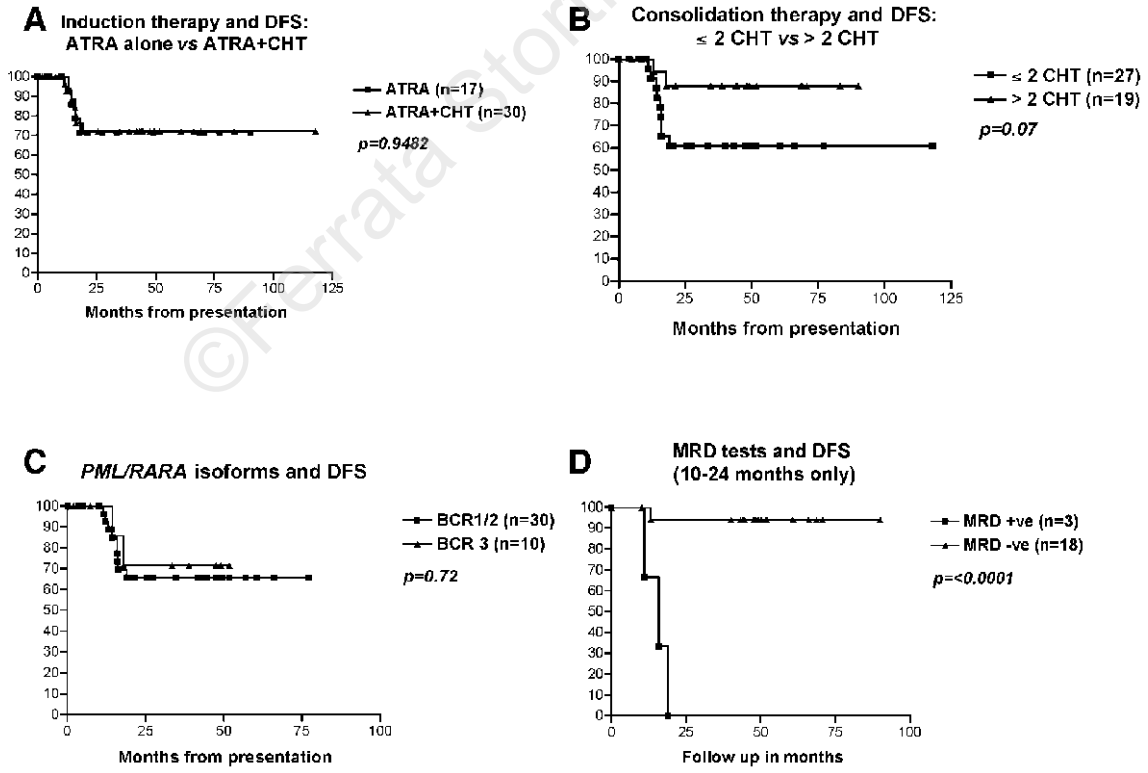

Figure 3. Survival curves (Kaplan Meier method). A) DFS and induction therapy: ATRA alone *versus* ATRA plus chemotherapy (CHT), B) DFS and consolidation therapy: 1 or 2 cycles of chemotherapy *versus* 3 or 4 cycles, C) Disease-free-survival (DFS) and *PML/RARα* isoforms: bcr1/2 *versus* bcr3 forms (n= number of patients), D) DFS and MRD tests obtained 10-24 months after presentation: RT-PCR positive *versus* RT-PCR negative results.

The correlation between MRD tests and disease-free survival (DFS) was investigated in each of the four time-intervals: 0-2 months, 3-5 months, 6-9 months,10-24 months with only tests at 10-24 months reaching statistical significance (p<0.0001, logrank test) (Figure 3D).

A Cox-regression multi-variant model was used to determine the variable that was most significantly prognostic of the DFS rate. MRD-status at each time period was compared with sex, age and days to CR (Table 2). The only co-variable to have a significant independent impact on the DFS was the MRD status at the 10-24 month mesasurement period: p=0.016 (the Wald statistics was also its highest and the Exp(B) deviated most from the numerical one).

Table 2. Cox-regression model analysis of effects on disease-free-survival of four co-variants: MRD-status, age, sex and days to CCR.

Co-variables	*Cases*	*Wald Statistic*	*Exp(B)*	*p-value*
10-24 months	(21)			
MRD status		5.848	181.229	0.016
Days to 1st CR		1.929	0.925	0.165
Sex		0.306	3.504	0.580
Age		0.031	0.989	0.859

Discussion

Treatment with ATRA and chemotherapy has dramatically improved survival in APL patients[18,19,24,25] and RT-PCR studies (using the *PML-RARα* fusion transcripts) have been extensively used to monitor the efficacy of these therapeutic regimens. The patients in our study came from different institutions which used different therapeutic strategies but these all included ATRA and one or more cycles of chemotherapy, with a trend towards a better overall outcome for patients who received more than two cycles of chemotherapy, following induction therapy, although this did not reach statistical significance (p=0.07).

The bcr3 transcript (short isoform) has been associated in different studies with adverse prognosis but no statistically significant correlation has been established with clinical outcome.[19,25] In our study relapse was observed mainly in patients carrying the bcr1/2 (long isoform) while the majority of the patients carrying the bcr3 transcript remained in CCR. We observed no difference in overall incidence and outcome between patients carrying either isoform in the two age groups analyzed.

In other studies, long-term survivors have been associated with the finding of persistently negative MRD results[11,13,15,25-27] suggesting eradication of the malignant clone, at least below the level of sensitivity of the RT-PCR test. In our series, all patients who have remained in CCR were serially RT-PCR negative from 5.5 months onwards irrespective of age (Figure 1A-B) with the PNV greatly improving beyond 5 months. However, 10 patients who later relapsed also converted to negative MRD after achieving clinical remission and tested negative at the end of therapy (time-period 6-9 months) (Figure 2A-B). This is suggestive of a poor ability of the MRD tests to detect future relapse or a rapid rise of residual disease pre-relapse (see below).

Our data indicate that during the first 9 months, the ability of a negative test to predict CCR is stronger than the ability of a positive test to predict relapse. However, with only negative tests detected among patients who later relapsed, our MRD study shows that there is little advantage in assessing patients during treatment and greater effort should be made to monitor disease beyond the end of treatment, as this is the best time to pick up patients destined to relapse (p<0.0001).

Overall there was little difference in MRD results between the two age groups. The persistence of residual disease during the first 2 months of treatment was longer in children than in adults but did not translate into a worse outcome in the younger age group. This was rather unexpected as children with acute leukemia of B-cell origin have a more rapid clearance of disease than adults, as previously suggested.[28] We did not observe a higher incidence of the bcr3 isoform in younger patients or a different clinical outcome for the patients with this isoform.

Our MRD investigation results expand data from our own previous study[16] and confirm data from other investigators[18] both in highlighting the poor ability of early tests to predict outcome and in describing the limited ability of MRD tests during treatment to predict overall outcome. Either inefficiency of the RT steps or low levels of *PML-RARα* expression[29] might explain the high rate of negative tests. However, the high level of positive tests during the 0-2 months period is a strong argument, in our view, against technical reasons being the cause. Also, more sensitive (1:10^6) competitive RT-PCR tests can detect MRD in patients in CCR who tested negative using a less sensitive conventional RT-PCR (1:1×10^4).[30] However, a more important biological feature could also explain this apparent poor performance of the tests. The rate at which cells are cleared following therapy is slower than the rate at which leukemic cells divide and re-appear preceding relapse. This has been demonstrated in studies of patients with Philadelphia chromosome positive acute lymphoblastic leukemia following bone marrow transplantation.[31] Very low or even undetectable levels of MRD preceded relapse in this group of patients. This is not the case, for instance, for patients with chronic myeloid leukemia who show

persistence of MRD for very long periods. In these patients the rate at which the *BCR-ABL* transcripts disappear is relevant in predicting outcome. Those who are slow to achieve a remission are more likely to relapse than those who reach this state quickly.[32] The kinetics of cells becoming undetectable is influenced more by the disease-type than by the technique applied.

The positive results observed during the monitoring of three of the relapsed patients are also in accord with those of previous studies which described the reappearance of the leukemic clone prior to relapse.[11,15,17,18,27] However, in 8 of our patients, no prior PCR conversion was observed, although, no bone marrow sample was tested within the 6 to 9 months preceding relapse in 7 of these 8 patients. This strongly argues for a policy of monitoring MRD at 3-monthly intervals in the bone marrow of APL patients in any future study in order to avoid false negative tests after the end of treatment.

In conclusion, our study highlights the poor predictive value of MRD during treatment in APL patients and the requirement for regular (3-monthly) sampling for MRD investigation, after the end of treatment. In a small cohort of patients we have confirmed the value of the RT-PCR positive conversion in predicting relapse. There is great expectation that the advent of *real-time* RT-PCR technology will in the future allow for more accurate and rapid monitoring of residual disease in APL patients. Preliminary results have shown that this technology is suitable for assessing the rate of clearance of *PML-RARα* transcripts and that its sensitivity can be superior to that of the conventional RT-PCR.[33,34] It is likely that the application of this technique to large prospective trials will improve the identification of APL patients at risk of relapse.

Contributions and Acknowledgments

PG, SV, ALS and JD carried out the tests on over 30 of the patients presented here. PC carried out molecular analyses from the patients collected at the Royal Free Hospital and presented here. AM, HGP and AVH provided the material and clinical data on the patients entered into the study from the Royal Free Hospital in the UK while JEG and AP funded and provided material from the largest porportion of patients from Portugal entered into the study. LF was involved in the generation, collection and analysis of data for the whole cohort of patients, preparation of the manuscript and elaboration of it from beginning to end as well as the practical measurement of some of the tests in a restricted group of patients.

We would like to thank all clinicians who participated in this study and provided material for MRD investigation both in the UK and Portugal. We would also like to thank Dr. F J Mortuza for assistance in the statistical analysis.

Disclosures

Conflict of interest: none.

Redundant publications: yes, < 50%.

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Francesco Lo Coco, who acted as an Associate Editor. The final decision to accept this paper was taken jointly by Prof. Lo Coco and the Editors. Manuscript received February 5, 2001; accepted May 6, 2001.

Potential implications for clinical practice

Our results have a primary implication for the management and stratification of APL patients[36-39] according to MRD which is already being applied to a large cohort of patients in Europe and the USA.

References

1. Alcalay M, Zangrilli D, Pandolfi PP, et al. Translocation breakpoint of acute promyelocytic leukemia lies within the retinoic acid receptor α locus. Proc Natl Acad Sci USA 1991; 88:1977-81.
2. Borrow J, Goddard AD, Sheer D, Solomon E. Molecular analysis of acute promyelocytic leukemia breakpoint cluster region on chromosome 17. Science 1990; 249:1577-80.
3. de Thé H, Chomienne C, Lanotte M, Degos L, Dejean A. The t(15;17) translocation of acute promyelocytic leukaemia fuses the retinoid acid receptor α gene to a novel transcribed locus. Nature 1990; 347:558-61.
4. Kakikuza A, Miller WH Jr, Umesono K, et al. Chromosomal translocation t(15;17) in human acute promyelocytic leukemia fuses RARα with a novel putative transcription factor, PML. Cell 1991; 66:663-74.
5. Biondi A, Rambaldi A, Pandolfi PP, et al. Molecular monitoring of the myl/retinoic acid receptor-α fusion gene in acute promyelocytic leukemia by polymerase chain reaction. Blood 1992; 80:492-7.
6. Castaigne S, Balitrand N, de Thé H, Dejean A, Degos L, Chomienne C. A PML/retinoic acid receptor α fusion transcript is constantly detected by RNA-based polymerase chain reaction in acute promyelocytic leukemia. Blood 1992; 79:3110-5.
7. de Thé H, Lavau C, Marchio A, Chomienne C, Degos L, Dejean A. The PML-RARα fusion mRNA generated by the t(15;17) translocation in acute promyelocytic leukemia encodes a functionally altered RAR. Cell 1991; 66:675-84.
8. Longo L, Pandolfi PP, Biondi A, et al. Rearrangements and aberrant expression of the retinoic acid receptor α in acute promyelocytic leukemias. J Exp Med 1990; 172: 1571-5.
9. Pandolfi PP, Alcalay M, Fagioli M, et al. Genomic variability and alternative splicing generate multiple PML/RARα transcripts that encode aberrant PML/RARα isoforms in acute promyelocytic leukaemia. EMBO J 1992; 11:1397-407.
10. Chang KS, Lu JF, Wang G, et al. The t(15;17) breakpoint in acute promyelocytic leukemia cluster within two differ-

ent sites of the myl gene: targets for the detection of minimal residual disease by the polymerase chain reaction. Blood 1992; 79:554-8.
11. Huang W, Sun GL, Li XS, et al. 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. Blood 1993; 82:1264-9.
12. Fukutani H, Naoe T, Ohno R, et al. Prognostic significance of the RT-PCR assay of PML-RARα transcripts in acute promyelocytic leukemia. The Leukemia Study Group of the Ministry of Health and Welfare (Kouseisho). Leukemia 1995; 9:588-93.
13. Lo Coco F, Diverio D, Pandolfi PP, et al. Molecular evaluation of residual disease as a predictor of relapse in acute promyelocytic leukaemia. Lancet 1992; 340:1437-8.
14. Miller WH Jr, Kakizuka A, Frankel SR, et al. Reverse transcription polymerase chain reaction for the rearranged retinoic acid receptor α clarifies diagnosis and detects minimal residual disease in acute promyelocytic leukemia. Proc Natl Acad Sci USA 1992; 89:2694-8.
15. Miller WH Jr, Levine K, DeBlasio A, Frankel SR, Dmitrovsky E, Warrel RP Jr. Detection of minimal residual disease in acute promyelocytic leukemia by a reverse transcription polymerase chain reaction assay for the PML/RAR-α fusion mRNA. Blood 1993; 82:1689-94.
16. Devaraj PE, Foroni L, Prentice GH, Hoffbrand AV, Secker-Walker LM. Relapse of acute promyelocytic leukemia follows serial negative RT-PCR assays: a cautionary tale. Leuk Res 1996; 20:733-7.
17. Diverio D, Rossi V, Avvisati G, et al. Early detection of relapse by prospective reverse transcriptase-polymerase chain reaction analysis of the PML/RARα fusion gene in patients with acute promyelocytic leukemia enrolled in the GIMEMA-AIEOP multicenter “AIDA” trial. GIMEMA-AIEOP Multicenter "AIDA" Trial. Blood 1998; 92:784-9.
18. Grimwade D, Howe K, Langabeer S, Burnett A, Goldstone A, Solomon E. Minimal residual disease detection in acute promyelocytic leukemia by reverse-transcriptase PCR: evaluation of PML-RARα and RARα-PML assessment in patients who ultimately relapse. Leukemia 1996; 10:61-6.
19. Burnett AK, Grimwade D, Solomon E, Wheatley K, Goldstone AH. Presenting white blood cell count and kinetics of molecular remission predict prognosis in acute promyelocytic leukemia treated with all-trans retinoic acid: result of the Randomized MRC Trial. Blood 1999; 93:4131-43.
20. Bennett JM, Catovsky D, Daniel MT, et al. Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. Br J Haematol 1976; 33:451-8.
21. Chomczynski P, Sacchi N. Single step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; 162:156-9.
22. Peto R, Pike MC, Armitage P, et al. Design and analysis of randomized clinical trials requiring prolonged observation of each patient. II. analysis and examples. Br J Cancer 1977; 35:1-39.
23. Cox DR. Regression models and life-tables. J R S Stat [B] 1972; 34:187-220.
24. Lo Coco F, Diverio D, Falini B, Biondi A, Nervi C, Pelicci PG. Genetic diagnosis and molecular monitoring in the management of acute promyelocytic leukemia. Blood 1999; 94:12-22.
25. Mandelli F, Diverio D, Avvisati G, et al. Molecular remission in PML/RARα-positive acute promyelocytic leukemia by combined all-trans retinoic acid and idarubicin (AIDA) therapy. Gruppo Italiano-Malattie Ematologiche Maligne dell'Adulto and Associazione Italiana di Ematologia ed Oncologia Pediatrica Cooperative Groups. Blood 1997; 90:1014-21.
26. Diverio D, Pandolfi PP, Biondi A, et al. Absence of reverse transcription–polymerase chain reaction detectable residual disease in patients with acute promyelocytic leukemia in long-term remission. Blood 1993; 82:3556-9.
27. Martinelli G, Remiddi C, Visani G, et al. Molecular analysis of PML/RARα fusion mRNA detected by reverse transcription-polymerase chain reaction assay in long-term disease-free acute promyelocytic leukaemia patients. Br J Haematol 1995; 90:966-8.
28. Foroni L, Coyle LA, Papaioannou M, et al. Molecular detection of minimal residual disease in adult and childhood acute lymphoblastic leukaemia reveals differences in treatment response. Leukemia 1997; 11:1732-41.
29. Seale JR, Varma S, Swirsky DM, Pandolfi PP, Goldman JM, Cross NC. Quantification of PML-RARα transcripts in acute promyelocytic leukaemia: explanation for lack of sensitivity of RT-PCR for the detection of minimal residual disease and induction of the leukaemia-specific mRNA by α interferon. Br J Haematol 1996; 95:95-101.
30. Moore H, Tobal K, Macheta M, Liu Yin JA. Predicting relapse in APL by quantitating PML-RARα transcripts with a highly sensitive competitive RT-PCR. Br J Haematol 2000; 108 (Suppl 1):50.
31. van Rhee F, Hochhaus A, Lin F, Cross NCP, Goldman JM. High BCR-ABL transcript levels precede haematological relapse in Philadelphia positive acute leukemia. Exp Hematol 1995; 2:922.
32. Radich J, Gooley T, Bryant E, Flowers M, Clift R. The significance of BCR-ABL “molecular relapse” in CML patients 18 months or more post-transplant. Blood 1999; 94 (Suppl 1):625a.
33. Grimwade D, Diverio D, Harrison G, et al. Detection of minimal residual disease (MRD) in APL by “real-time” RT-PCR : analysis of cases entered into the UK MRC ATRA trial. Blood 1999; 94 (Suppl 1):625a.
34. Cassinat B, Zassadowski F, Balitrand N, et al. Quantification of minimal residual disease in acute promyelocytic patients with t(15;17) translocation using real-time RT-PCR. Leukemia 2000; 14:324-8.
35. Sousa AB, Fernandes JP, Ferreira G, et al. Short-term intensive consolidation therapy after all-trans retinoic acid in acute promyelocytic leukemia. Am J Clin Oncol 1999; 22:294-7.
36. Minucci S, Cioce M, Maccarana M, Pelicci PG. The APL-associated fusion proteins. Haematologica 1999; 84(EHA-4 Educational Book):70-1.
37. Lo Coco F, Diverio D, Avvisati G, Mandelli F. Diagnosis, front line treatment and molecular monitoring of acute promyelocytic leukaemia. Haematologica 1999; 84(EHA-4 Educational Book):72-4.
38. Lo Coco F. Development and overcoming of ATRA resistance in acute promyelocytic leukemia. Haematologica 1999; 84(EHA-4 Educational Book):961-2.
39. Warrell RP Jr. Arsenicals and inhibitors of histone deacetylase as anticancer therapy. Haematologica 1999; 84(EHA-4 Educational Book):75-7.