Using quantification of the *PML-RARα* transcript to stratify the risk of relapse in patients with acute promyelocytic leukemia

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ABSTRACT

Background and Objectives

The detection of *PML-RARα* by real-time polymerase chain reaction (RQ-PCR) is becoming an important tool for monitoring minimal residual disease (MRD) in patients with acute promyelocytic leukemia (APL). However, its clinical value remains to be determined. Our aim was to analyze any associations between the risk of relapse and RQ-PCR results in different phases of treatment, comparing these data with those yielded by conventional qualitative reverse transcriptase-PCR.

Design and Methods

Follow-up samples from 145 APL patients treated with the PETHEMA protocols were evaluated by the RQ-PCR protocol (Europe Against Cancer program) and by the RT-PCR method (BIOMED-1 Concerted Action). Hematologic and molecular relapses and relapse-free survival were recorded. We then looked for associations between relapse risk and RQ-PCR results.

Results

After induction therapy, no association was found between positive RQ-PCR results and relapse. The PCR result here did not imply any change in the scheduled therapy. After the third consolidation course, two out of three cases with positive RQ-PCR relapsed in contrast to 16 out of 119 (13%) patients with negative RQ-PCR. During maintenance therapy and out-of treatment, all patients with >10 *PML-RARα* normalized copy number (NCN) (n=19) relapsed while all patients with <1 NCN at the end of the study remained in hematologic remission ($p<0.0001$). In the intermediate group (NCN 1-10) (n=18), the relapse-free survival at 5 years was 60%. Hematologic relapses were predicted if a positive RQ-PCR result had been obtained in a follow-up sample within the previous 4 months.

Interpretation and Conclusions

Based on the information provided by RQ-PCR in samples obtained after the end of consolidation and subsequently, a relapse risk stratification could be established for APL patients. This stratification divides patients into three groups: those at high risk of relapse, those with an intermediate risk and those with a low risk of relapse.

Key words: acute promyelocytic leukemia, RQ-PCR, minimal residual disease.

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Combined treatment with anthracycline-based chemotherapy and all trans retinoic acid (ATRA) is highly successful in acute promyelocytic leukemia (APL), providing long-lasting remissions and probable cures in up to 70% of newly diagnosed patients.[1-5] Nevertheless, the persistence of resistant clones causing relapse and low survival still represents a problem in 15-25% of patients.[6-9] Currently, detection of *PML/RARα* transcripts by molecular techniques constitutes an important tool for monitoring minimal residual disease (MRD) and predicting evolution in APL patients.[10] Conventional qualitative reverse transcriptase polymerase chain reaction (RT-PCR) has been widely used for genetic diagnosis and therapeutic monitoring of APL. Several reports have shown that RT-PCR positivity after consolidation treatment predicts hematologic relapse, whereas persistent RT-PCR negativity test is associated with long-term survival and a low relapse rate.[9,11-12] However, this technique has several disadvantages such as the occurrence of false positive results due to cross-contamination and false negatives due to poor RNA quality or RT-PCR failures at different stages. In addition, the sensitivity of RT-PCR for measuring MRD is relatively low[13-14] and the method is associated with significant inter-laboratory variability.[15] Finally, qualitative RT-PCR requires significant post-PCR handling which is time and labor-consuming and often leads to contamination of samples.

Recently, quantification of the *PML/RARα* copy number based on real-time PCR approaches (RQ-PCR) has become a new alternative for monitoring disease outcome. Although this approach suffers from some of the same problems as conventional RT-PCR, it has several advantages such as being highly sensitive, facilitating assessment of kinetics and being highly reproducible.[13,16] Although several protocols have been developed for quantitative monitoring in APL,[8,16-19] there is currently no consensus concerning threshold levels to discriminate between low and high relapse risk, and the optimum calendar for sampling remains to be defined.[10,20] Moreover, the clinical value of these investigations still needs to be confirmed.[10] In addition, comparisons between qualitative (RT-PCR) and quantitative (RQ-PCR) approaches as suitable techniques for predicting relapse have not been made. The present work analyzes the value of RQ-PCR for predicting relapse in APL and compares quantitative results with those of the conventional RT-PCR approach, according to treatment phase.

Design and Methods

Patients and samples

From June 1996 to September 2005, 145 patients (aged 8 to 84 years) were referred to our molecular diagnostic laboratory at the University Hospital of Salamanca. The diagnosis of APL was confirmed though morphological, immunophenotypic, and cytogenetic criteria,[21] as well as by both RT-PCR and RQ-PCR analysis for *PML/RARα* rearrangements.[16,22] Post-induction and post-consolidation samples were analyzed by both methods using cDNA samples from RNA stored at –80°C. Any positive result after consolidation therapy was confirmed in a second sample or a repeated analysis with new cDNA. Patients who died of a cause related to induction therapy were not included in the present study.

Treatment protocol

Treatment was carried out according to the PETHEMA-LPA 96 protocol (before November 1999)[2] or PETHEMA-LPA 99 (subsequently).[23] Both protocols included an induction phase with ATRA plus idarubicin and three consolidation courses with idarubicin, mitoxantrone and idarubicin, followed by a maintenance phase with ATRA, methotrexate and mercaptopurine for 2 years.[2] In the APL-99 protocol the consolidation phase was modified such that ATRA plus higher doses of idarubicin were given to patients with a white blood cell (WBC) count higher than 10000/μL and /or platelets counts lower than 40000/μL, who were considered as being at high-risk.[23]

Remission and relapse definition

Hematologic remission was defined as normal bone marrow cellularity with <5% leukemic promyelocytes and normalization of peripheral blood counts. Consequently, hematologic relapse was defined as the reappearance of ≥5% leukemic promyelocytes in the bone marrow.[2,4] Molecular remission (MR) was defined as the disappearance on an agarose gel stained with ethidium bromide of the *PML/RARα*-specific band visualized at diagnosis, using a qualitative RT-PCR assay with a sensitivity level of 10^{-4} in any follow-up sample, after the end of consolidation therapy.[6,22] Regarding the RQ-PCR assay, MR was defined as present when less than 1 *PML/RARα* normalized copy number (NCN) was detected (sensitivity of 10^{-5}).[16] Molecular relapse was defined as the reappearance of a positive molecular result according to either method in two consecutive bone marrow samples at any time after consolidation therapy.[6] A result was considered to be false-positive when a positive molecular result appeared by any method but hematologic relapse was not observed within the subsequent 6 months.

RNA extraction and cDNA synthesis

Total RNA was obtained from leukocytes using the acid guanidium thiocyanate-phenol chloroform extraction method.[24] Reverse transcription was performed as previously described:[22] 1-2 μg of total RNA were added to a 20-μL volume containing random hexamers as primers and 200 U of SuperScript RNase H reverse transcriptase (Invitrogen, California, USA). The mixture was incubated at 42°C for 60 min, followed by 3 min at 99°C and 2 min at 4°C. Aliquots were stored at –80°C prior to further analysis.

RT-PCR qualitative assays

To amplify the *PML/RARα* fusion gene, a two step qualitative RT-PCR analysis was performed as previously described.[22,25] A volume of 5 μL (100 ng) of cDNA was diluted into 45 μL of a PCR mixture containing a final concentration of 400 nM primers, 2.5 mM $MgCl_2$, 200 μM dNTP, PCR buffer (20 mM Tris-HCl, 50 mM KCl, pH 8.3) and 1.0 U of TaqGold DNA polymerase. PCR cycles included an initial denaturation at 95°C for 10 minutes. Melting, annealing and extension were carried out at 95°C for 30 sec, 65°C for 1 min, and 72°C for 1 min, respectively, for a total of 35 cycles. Nested PCR was performed under the same conditions, but using 2.5 μL of PCR product from previous reaction and internal primers. Finally, 25 μL of the PCR product were analyzed in a 2.5% agarose gel stained with ethidium bromide and visualized under UV light. Two negative controls (one with non-*PML/RARα* RNA and one with distilled water) and a NB4 cell line as a positive control were included in the experiments. The assay sensitivity was 10^{-4}, using several 10-fold microdilutions.[22] The quality control of the cDNA preparation was assessed by amplification of the normal *ABL* gene.

RQ-PCR (real time) assays

The different *PML-RARα* transcripts were quantified using the ABI PRISM 7700 DNA Sequence Detection System according to the Europe against Cancer Group (EAC) protocol, in which our group has actively participated.[16] *PML-RARα* transcript copy numbers were assessed in 5 μL (100 ng) of cDNA though the ΔCt method, using commercial plasmids (IpsoGen Laboratories, Marseille, France) to construct the standard curve. Primers were designed as previously reported.[16] The Abelson housekeeping gene (*ABL*) was selected as a control gene of RNA expression, as previously reported.[26] A valid result required an *ABL* Ct within a range of 21.8 to 29.5, with at least 2000 copies of the *ABL* gene in the sample.[26] A non-amplification control (NAC), containing RNA from a healthy donor and non-template control (NTC), with distilled water instead of human cDNA were included in each assay. A positive well was defined as a sigmoid amplification (log scale) with a Ct value below the Y-intercept value of the standard curve plus one Ct, as reported previously.[16] A positive result was defined with at least two out of three wells. The sensitivity, established previously in our laboratory, was 10^{-5} using dilutions of the NB4 cell line.[16] All samples were tested in triplicate and results are reported according to EAC guidelines as the normalized copy number (NCN), derived by multiplying the *PML-RARα* copy number/*ABL* copy number ratio by 10000.[16] A result of <1 NCN was reported as RQ-PCR negative.

Statistical analysis

All tests were carried out with the SPSS 12.0 program (SPSS, Chicago, IL, USA). For univariate analyses, χ^2 and Fisher's exact test were performed to evaluate factors associated with relapse. Relapse-free survival (RFS) for analysis after consolidation therapy was defined as the time between the achievement of complete remission and relapse or last follow-up. The probabilities of RFS and overall survival (OS) were calculated using the Kaplan-Meier method and compared using the log-rank test.[27] RFS was estimated using either molecular or hematologic relapse as censored events. OS was defined as the time from achievement of complete remission to death or last follow-up. The impact of multiple predictor variables on RFS was assessed using a Cox regression model.[28]

Table 1. Clinical and biological characteristics of the APL patients at diagnosis.

Parameter	*n (%)*
Age	
Median	41
Range	8-82
Sex	
Male	83 (57.2)
Female	62 (42.8)
WBC at diagnosis (×1000/μL)	
Median	2.3
Range	0.3-187.0
BM blasts at diagnosis (%)	
Median	85
Range	10-100
PB blasts at diagnosis (%)	
Median	34
Range	0-98
Platelets at diagnosis (×1000/μL)	
Median	25.5
Range	3-183
PML/RARα isoform	
Bcr1	89 (61.4)
Bcr2	8(5.5)
Bcr3	48 (33.1)
FAB classification	
M3	107 (73.8)
M3v	38 (26.2)
Treatment protocol	
PETHEMA 96	62 (42.8)
PETHEMA 99	83 (57.2)
PML/RARα at diagnosis (NCN)	
Median	3082
Range	1224-19750
Days to molecular remission	
Median	57
Range	24-141

NCN: normalized copy number. WBC: white blood cell count; BM: bone marrow; PB: peripheral blood; FAB: French-American-Bristish.

Results

Patients and samples

A total of 1064 bone marrow aspirates and 145 peripheral blood samples obtained from 145 APL patients were

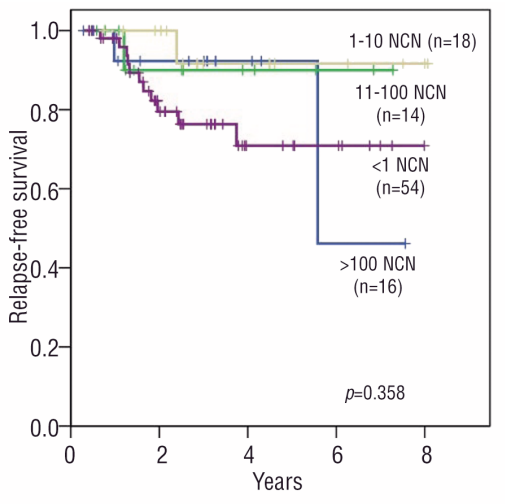

Figure 1. Kaplan-Meier analysis comparing relapse-free survival in 102 APL patients, according to *PML-RARα* normalized copy number (NCN) at the post-induction test. No NCN cut-off point was able to identify a group with a shorter RFS.

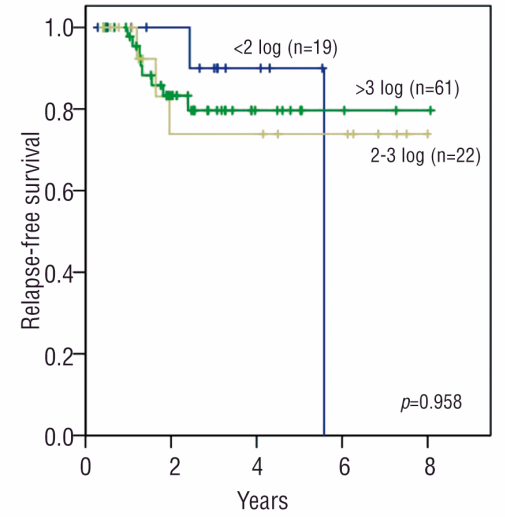

Figure 2. Kaplan-Meier analysis comparing relapse-free survival in 102 APL patients according to kinetics of *PML-RARα* normalized copy number (NCN) reduction assessed by RQ-PCR, in the post-induction phase with respect to at diagnosis. No correlation was found between any logarithmic cut-off point and shorter RFS.

included in the present study. There was a median of nine samples per patient (range, 4 to 39). The distribution of samples in different treatment phases was as follows: 102 samples were taken following induction therapy, 122 following consolidation therapy, 442 during maintenance therapy and 398 once maintenance treatment had finished. All 145 patients achieved hematologic remission with the therapeutic protocol, but 23 of them relapsed after the last consolidation course, 13 during maintenance therapy (median 18.3 months after diagnosis, range 8-23.5) and 10 out of treatment (median 40 months after diagnosis, range 28.3-70). Two additional patients had a second relapse after salvage treatment. Patients testing PCR-positive at the end of induction therapy received therapy as scheduled.

Characteristics at presentation and influence on survival

The main clinical characteristics of patients at diagnosis are summarized in Table 1. The 3-year probabilities of RFS and OS were 83.3% and 89.3%, respectively. When we evaluated the impact of the clinical features on RFS, APL FAB M3 variant subtype (p=0.016) and WBC count higher than 10000/μL (p=0.021) at diagnosis were the only parameters associated with a shorter RFS (Table 2). Multivariate analysis showed that a high WBC count was the only independent factor associated with poor RFS (p=0.026). By contrast, other parameters such as number of blast cells in bone marrow or peripheral blood, platelets counts and *PML/RARα* isoform had no prognostic impact on relapse risk (Table 2).

RQ-PCR in different phases of treatment and influence on survival

At diagnosis, all samples showed >1000 NCN (median 3082, range 1224-19750) by RQ-PCR. After induction therapy, 48 out of 102 (47%) patients in hematologic remission displayed a positive result, which was not correlated with the probability of relapse (71% and 80% RFS at 5 years in the negative and positive RQ-PCR groups, respectively; p=0.105). Several cut-off points were evaluated to determine the prognostic value of the NCN on RFS but none of them correlated with a shorter survival (Figure 1). In addition, the kinetics of tumor burden reduction (log reduction in NCN between diagnosis and post-induction) did not predict clinical outcome (Figure 2).

Such results contrast with those obtained after the third consolidation course, when only three out of 122 patients were RQ-PCR positive and two of them, with NCN of 10 and 133, relapsed 3 and 4.5 months later, respectively. The third patient (NCN of 4) received salvage therapy and remains in continuous molecular remission after maintenance therapy (follow-up of 19 months). With regards to the group with NCN<1 according to RQ-PCR, 16 out of 119 patients had a hematologic relapse (13.4%) at a median of 19.2 months after hematologic remission (range 8-70). According to these results, the RFS curves were markedly different with a probability of remaining in continuous complete remission at 5 years of 33% in the NCN≥1 group versus 84% in the NCN<1 group at the end of the consolidation therapy (p<0.0001).

During the maintenance phase, 442 samples from 96 patients were analyzed. In 75 patients (78.1%), all samples were constantly negative, while in 21 patients at least one positive sample was detected. Within this latter group, 12 had >10 NCN and the remaining nine had between 1 and 10 NCN. The RFS at 3 years was 94%, 67% and 0% for the NCN<1, NCN1-10 and NCN>10 groups, respectively

Table 2. Influence of clinical and biological characteristics at diagnosis on relapse-free survival (RFS) in patients with APL.

	n	*RFS* Univariate	Multivariate
Age (years)[1]			
≤ 60	117	NS	NS
> 60	28		
Sex			
Male	83	NS	NS
Female	62		
WBC at diagnosis (x1000/μL)[1]			
≤ 10.0	111	0.021	0.026
> 10.0	34		
BM blasts at diagnosis (%)[2]			
≤ 85	73	NS	NS
> 85	72		
PB blasts at diagnosis (%)[2]			
≤ 34	74		
> 34	71	NS	NS
Platelets at diagnosis (x1000/μL)[1]			
≤ 40	104	NS	NS
> 40	41		
PML/RARα isoform			
Bcr1	89		
Bcr2	8	NS	NS
Bcr3	48		
FAB classification			
M3	107	0.016	0.119
M3v	38		
Treatment protocol			
PETHEMA 96	62	NS	NS
PETHEMA 99	83		
Days to molecular remission[2]			
≤ 57	74	NS	NS
> 57	71		

NS: not statistically significant NCN: normalized copy number; [1]based on criteria of high-risk patients from Sanz et al., 2004; [2]based on median value. WBC: white blood cell count; BM: bone marrow; PB: peripheral blood; FAB: French-American-British.

(Figure 3a). Relapses occurred in all patients with NCN>10 within the 4 months following a positive molecular result (median 41 days, range 0-153 days).

Table 3. RQ-PCR results of patients during maintenance treatment and out of treatment.

	Maintenance		*Out of treatment*	
	n	*Relapse n (%)*	*n*	*Relapse n (%)*
All samples negative	75	8[a] (10.7)	62	0 (0)
At least one positive sample	21	15 (71.4)	16	10 (62.5)
At least one sample 1–10 NCN	9	3 (33.3)	9	3 (33.3)
At least one sample 11–50 NCN	5	5 (100)	0	–
At least one sample 51–100 NCN	3	3 (100)	1	1 (100)
At least one sample more than 100 NCN	4	4 (100)	6	6 (100)
TOTAL	**96**	**23 (23.9)**	**78**	**10 (12.8)**

[a]*All eight patients relapsed during the period out of treatment.*

Seventy-eight patients were monitored after the end of the treatment, through the investigation of a total of 398 samples. Sixty-two patients had continuously negative RQ-PCR tests while 16 had at least one positive result. All patients from the first group remained in complete remission until the end of this study (Table 3) while ten from the second group had already relapsed, which provides 5-year RFS probabilities of 100% and 38% for patients with negative vs. positive results, respectively (p<0.0001). Analogous to the findings during maintenance therapy, all patients with NCN >10 (n=7) relapsed at a median of 45 days (range, 0-107) after the positive test, while most of patients who had NCN between 1 and 10 (six out of nine) remained in complete remission (Table 3). Accordingly, three groups with different probabilities of RFS could be established based on the *PML/RARα* NCN: <1 NCN, 1-10

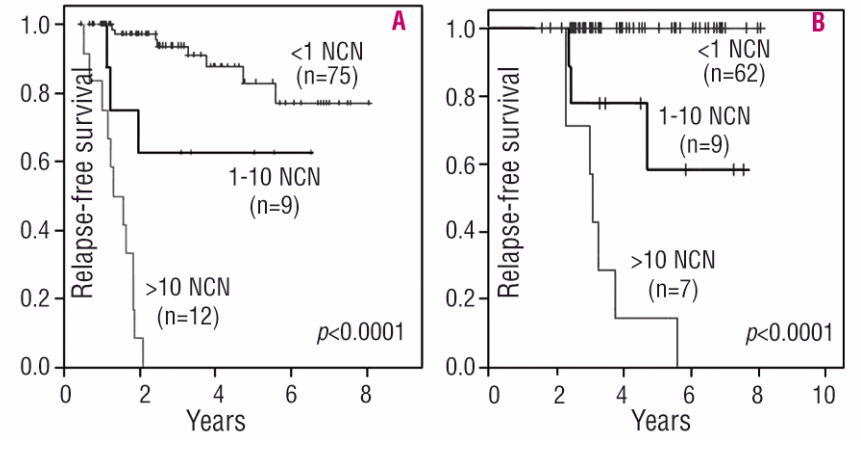

Figure 3. Kaplan-Meier analysis of RFS in APL patients according to relapse-risk stratification, based on *PML-RARα* normalized copy number (NCN) during maintenance therapy (A) and out of treatment (B), and reported according to the Europe against Cancer (EAC) protocol. A well-defined stratification of relapse-risk was obtained during maintenance therapy and after treatment: patients with a RQ-PCR result higher than 10 NCN formed a high-risk group, those with RQ-PCR results between 1 and 10 NCN formed an intermediate-risk group and those with RQ-PCR results lower than 1 NCN constituted the low-risk group.

Table 4. Comparison between RQ-PCR and RT-PCR results in samples according to therapeutic phase.

	Qualitative RT-PCR								
	Post-Induction n=102		*Post-consolidation n=122*		*Maintenance n=156*		*Out of treatment n=127*		*Discrepancies (%)*
	POS	*NEG*	*POS*	*NEG*	*POS*	*NEG*	*POS*	*NEG*	*TOTAL*
< 1 NCN	0	60	1	118	2	106	2	87	5/376 (1.3)
1-10 NCN	6	5	2	0	9	8	11	10	23/51 (45)
> 10 NCN	31	0	1	0	31	0	17	0	0/80 (0)
TOTAL	**37**	**65**	**4**	**118**	**42**	**114**	**30**	**97**	**28/507 (5.5)**

Table 5. Comparison between RQ-PCR and RT-PCR results in patients under maintenance therapy or out of treatment.

RQ-PCR	*RT-PCR*	*Patients (Relapses/Total)*		
		Maintenance	*Out of treatment*	*TOTAL*
< 1 NCN	Negative	0/51	0/48	0/99
< 1 NCN	Positive	0/1	0/1	0/2
1-10 NCN	Negative	1/5	0/5	1/10
1-10 NCN	Positive	2/4	3/4	5/8
> 10 NCN	Positive	12/12	7/7	19/19

NCN and >10 NCN: these groups had 5-year RFS probabilities of 100%, 67% and 14%, respectively (p<0.0001, Figure 3B). All patients with positive results by RQ-PCR in two consecutive samples during maintenance therapy or out of treatment, finally relapsed (n=23). By contrast, all patients with a result that was initially positive but negative in the confirmatory sample, remained in hematologic remission until the end of the study (n=12, median follow-up of 37.9 months; range, 8.3-57.0)

Furthermore, we analyzed 130 paired bone marrow and peripheral blood samples (66 from 21 patients in continuous complete remission and 64 from 7 patients who finally relapsed). Regarding the second group, no significant differences were observed in NCN by RQ-PCR between bone marrow samples (median, 45 NCN: range, 6-697) and peripheral blood samples (median, 21 NCN: range, 4-343) (p=0.365). However, in three out of seven patients the molecular relapse was detected in bone marrow 24, 28 and 35 days earlier than in peripheral blood. These data suggest that bone marrow samples could be more suitable than peripheral blood for RQ-PCR follow-up. Alternatively, if peripheral blood samples are used, the monitoring should be performed more frequently. However, the reduced number of samples mean that these results should be considered preliminary.

Comparison between RQ-PCR vs. RT-PCR assays

Overall, 507 samples taken during different phases of treatment were analyzed in parallel by both methods (Table 4). Results were concordant in 479 samples (94%) (both positive in 108 samples and both negative in 371 samples). In 28 samples, however, discrepant results were obtained: 23 samples were positive by RQ-PCR but negative by RT-PCR, while in five samples the opposite was observed. As shown in Table 4, most of these discrepancies occurred in the group of samples with 1-10 NCN. It should be noted that five out of eight patients with RQ-PCR results between 1-10 NCN and positive RT-PCR relapsed, whereas only one out of ten patients with 1-10 NCN by RQ-PCR and negative RT-PCR relapsed (Table 5). This difference was statistically significant (p=0.04). Regarding discrepant cases we observed that while RQ-PCR yielded false positive results (12 cases), particularly when the NCN was lower than 10, RT-PCR was associated with both false positive (five cases) and false negative (one case) results.

Finally, it should be noted that in 11 patients the hematologic relapse was not predicted by molecular techniques (neither RQ-PCR nor RT-PCR). In all these cases, the final molecular analysis was consistently performed >5 months before relapse occurred (median 260 days, range 153-368). This indicates that the design of an optimal calendar for investigating of MRD in APL should be based on sampling intervals between 4-5 months.

Discussion

In the present study, we analyzed the prognostic value of a well-standarized RQ-PCR protocol (Europe Against Cancer program)[16] in APL patients during different phases of treatment. Our results indicate that this approach is a robust alternative for assessing MRD and a relapse-risk stratification can be established based on the *PML-RARα* normalized copy number.

As previously reported for both RQ-PCR and RT-PCR, no correlation was found between a positive test immediately after induction therapy and outcome.[2,4,8,19] Actually, no significant differences in *PML-RARα* NCN values post-induction were observed between relapsed patients and those who remained in continuous complete remission, as also reported by other groups.[19,29-30] Similarly, the kinetics

of tumor burden reduction (log-reduction in NCN between diagnosis and post-induction) did not correlate with disease outcome. These results contrast with the picture in other leukemic disorders such as t(9;22) acute lymphoblastic leukemia[31] or t(8;21) acute myeloid leukemia, in which successful induction chemotherapy produces a reduction of 2 to 3 log in the level of *AML1-MTG8*, followed by a further 2 to 3 log after consolidation/intensification chemotherapy.[32] Such differences could be explained in part by the type of therapy, since ATRA, unlike other cytotoxic treatments, promotes the differentiation of APL cells to a maturative stage instead of quickly eliminating leukemic cells.[33] Furthermore, in contrast to other acute myeloid leukemias, induction treatment of APL can be associated with delayed leukemic clearance.[10]

Regarding post-consolidation analysis, it is generally accepted that there is a correlation between positive RQ-PCR assays and a high risk of relapse,[3,13,19,30] especially when the third course of chemotherapy has been completed. However; in our series, as well as in other studies[7,23] the low number of positive cases detected at the end of consolidation limits the utility of this parameter. Interestingly, in a recent study that evaluated samples by RQ-PCR at the end of each consolidation course, there was a significant correlation between an MRD level $>10^{-3}$ after first consolidation and poor clinical outcome.[30] This value is equivalent to the 10 NCN threshold in the present study.

During maintenance therapy and beyond the end of treatment, a positive RQ-PCR test was associated with a higher relapse risk and shorter survival. Moreover, three well-defined risk groups could be established according to the *PML-RARα* NCN assessed with the Europe Against Cancer protocol.[16] Patients with <1 NCN had a very favorable RFS, especially when the test was performed during follow-up, post-maintenance therapy. By contrast, patients with >10 NCN had a very poor prognosis since all these patients finally relapsed. These results are similar to preliminary data communicated by Cassinat *et al.,* showing that no relapse occurred in patients with <10 copies, whereas the relapse rate observed in patients with more than 100 copies was 100%.[34] The discrepancies in the thresholds could be related to methodological differences such as the control gene used (*PBGD vs ABL*) for the normalization of the *PML-RARα* copy number.

An interesting group with intermediate-risk was detected in our series. This group included patients with at least one positive result between 1 and 10 NCN during maintenance and out of treatment. There was a very high probability of relapse within this group if either a second confirmatory positive sample or RT-PCR positive assay was found. If not, this low positivity can be considered a false positive result, since all 11 patients with this pattern and negative RT-PCR remained in continuous complete remission. On the other hand, no false negative results were observed by RQ-PCR of samples taken in the post-maintenance phase. In contrast, RT-PCR can produce both false positive and false negative results in a few patients (three and two, respectively). These data suggest that RT-PCR could be used as a complementary assay for the RQ-PCR approach, especially within the subgroup with 1-10 NCN. A good correlation between RT-PCR and RQ-PCR results has recently been found in 31 newly APL diagnosed patients.[35] Furthermore, it is important to note that the relatively high specifity of RT-PCR assay is not reason enough to substitute a highly sensitive, standarized and high through-put technology such as RQ-PCR.

Interestingly, all patients who had a positive molecular result, by both techniques, had a hematologic relapses within 4 months. This emphasizes the need for frequent sampling (at least every 4 months) in order to predict impending relapses. Since our data include patients monitored not only during maintenance therapy but also out of treatment, and we have observed a similar pattern of rapid relapse in these latter cases, we can conclude that the recent recommendation to monitor MRD every 3 months during maintenance therapy[9-11,19] could be prolonged to the 2 years following treatment, although during this period sampling could be slightly less frequent (every 4-5 months). In addition, patients with adverse features such as WBC counts >10,000/μL at diagnosis should be monitored more closely, for example every 2 or 3 months.[10] Its important to note that our recommendations about levels and frequencies of sample collection should be considered within the framework of treatment schedules similar to the PETHEMA protocol used here.

In conclusion, we propose a relapse-risk stratification based on quantification of *PML-RARα* NCN, to evaluate APL patients during their maintenance therapy and beyond the end of treatment. Nevertheless, RT-PCR remains a complementary and valuable technique, particularly for patients with only one low positive RQ-PCR result. Finally, our data show that a positive molecular result with >10 NCN or reconfirmed positivity by RT-PCR is predictive of rapid clinical relapse within the subsequent 4 months.

Authors' Contributions

MG and MCC were the iniatial designers of the study; CS, MCC and CF carried out all molecular studies and prepared the database for the final analysis; CS deveoped the statistical analysis and prepared the initial version of the paper; PM-J and AB helped in the molecular analysis and data collction; RG-S reviewed the conception and design of most of the work, made the database and supervised the statistical analysis, re-wrote the paper and provided the pre-approval of the final version; FR, MCR and MJP were clinicians responsible for the patients who took care of the protocol accomplishment, sampling and collection of the clinical data; JFSM and MG were responsible for the group and the final revision of the draft. They gave final approval of the version to be published.

Conflict of Interest

The authors reported no potential conflicts of interest.

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