Original Article

The relevance of preferentially expressed antigen of melanoma (*PRAME*) as a marker of disease activity and prognosis in acute promyelocytic leukemia

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ABSTRACT

Background

The gene for preferentially expressed antigen of melanoma (*PRAME*) has been shown to be over-expressed in acute promyelocytic leukemia, but its actual incidence and clinical impact are still unknown.

Design and Methods

We studied *PRAME* expression at diagnosis using real-time quantitative polymerase chain reaction in 125 patients with acute promyelocytic leukemia enrolled in the Spanish PETHEMA-96 (n=45) and PETHEMA-99 (n=80) clinical trials. In addition, *PRAME* expression was evaluated as a marker of disease activity in 225 follow-up samples from 67 patients with acute promyelocytic leukemia.

Results

At diagnosis, *PRAME* expression in patients with acute promyelocytic leukemia was significantly higher ($p<0.001$) than in patients with non-M3 acute myeloid leukemia (n=213) and in healthy controls (n=10). Furthermore, patients with acute promyelocytic leukemia with high *PRAME* expression had a favorable outcome. Thus, the 5-year relapse-free survival was better in patients with >100-fold *PRAME* expression (86% *vs.* 74%; $p=0.03$), and this cut-off established two sub-groups with different relapse-free survival rates among patients with a white cell count $<10^9$/L (5-year relapse-free survival 94% *vs.* 80%, $p=0.01$). This effect was similar in patients with a white cell count $>10^9$/L, although differences were not statistically significant. In multivariate analysis, white cell count $>10^9$/L ($p<0.001$), bone marrow blasts >90% ($p=0.001$), and *PRAME* expression <100-fold ($p=0.009$) were associated with short relapse-free survival. Samples at remission showed *PRAME* levels similar to those in normal controls while samples at relapse over-expressed *PRAME* again. Furthermore, 12/13 samples collected within the 6-month period preceding relapse showed a >10-fold increase in *PRAME* expression levels.

Conclusions

Low *PRAME* expression defines a subgroup of patients with acute promyelocytic leukemia with a short relapse-free survival. This marker could be useful as a secondary marker for monitoring patients with acute promyelocytic leukemia.

Key words: acute promyelocytic leukemia, *PRAME*, real-time quantitative PCR.

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Introduction

Preferentially expressed antigen of melanoma (PRAME) was identified as a HLA-A24–restricted antigen peptide presented to an autologous tumor-specific cytolytic T lymphocyte clone derived from a melanoma cell line.[1] The *PRAME* gene encodes a putative protein of 509 amino acids with a function that remains unknown. Most normal tissues do not express *PRAME* but weak expression has been observed in testis, placenta, endometrium, ovary and adrenal glands.[2] By contrast, this tumor-associated antigen is frequently expressed in several solid tumors such as melanomas (88% of primary lesions), non-small cell lung carcinoma, breast carcinoma, renal cell carcinoma, head and neck cancer, Wilms' tumor and Hodgkin's lymphoma.[2,3] *PRAME* is also expressed in 17-42% of acute lymphoid leukemias (ALL) and 30-64% of acute myeloid leukemias at diagnosis,[3-8] as well as in chronic leukemias.[9]

In solid tumors, *PRAME* overexpression is associated with a more advanced tumor stage, increased probability of metastasis and a poor clinical outcome.[1,2,10,11] By contrast, preliminary data suggest that high *PRAME* RNA levels correlate with good prognosis and prolonged survival in both adult[4] and childhood AML,[5] as well as pediatric acute lymphoid leukemias.[8] Furthermore, this high expression has been associated with the presence of favorable translocations, such as t(8;21) and t(12;21).[3,4] Given this particular tumor-specific expression, several authors have suggested that *PRAME* could be useful as a target for monitoring minimal residual disease (MRD) in acute leukemias.[3-6,12-14] In the largest published series of MRD evaluation, Steinbach *et al.*[12] showed, in 26 cases of childhood non-M3 AML, that *PRAME* expression decreased to control levels in patients who achieved a continuous complete remis-sion. In addition, a rise in the expression level was observed in patients who eventually relapsed.[12]

Although some reports found higher *PRAME* expression when the t(15;17) was present, these data were based on small numbers of patients with acute promyelocytic leukemia (APL).[3,4,13] The clinical impact of *PRAME* on the outcome of patients with APL has not, however, been evaluated yet. The aim of this study was to analyze *PRAME* expression and its relationship to survival and prognosis in a large series of uniformly treated APL patients, as well as to evaluate its potential value as a surrogate marker for MRD investigations.

Design and Methods

Patients

We analyzed pre-treatment bone marrow samples from 125 adult APL patients enrolled in the Spanish PETHEMA-96[15] (n=45) and PETHEMA-99[16] (n=80) treatment trials. Both protocols included an induction phase with all trans retinoic acid (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.[15] The PETHEMA-96 protocol was designed with a unique consolidation arm. By contrast, in the PETHEMA-99 protocol, the consolidation phase was modified by including ATRA plus higher doses of idarubicin for intermediate or high-risk patients[16] [white blood cell (WBC) count ≥10×10^9/L and/or platelet count <40×10^9/L]. The diagnosis of APL was confirmed according to standard criteria.[16] After obtaining written consent, bone marrow samples were taken from ten healthy donors and used as controls for gene expression analysis. Additionally, 213 non-M3 bone marrow samples taken at diagnosis were analyzed as a reference group for *PRAME* expression. Informed consent to the use of biological samples and clinical data was obtained from all patients.

RNA extraction and cDNA synthesis

Total RNA was obtained from unfractionated bone marrow samples (taken at diagnosis with >70% blast cells) using the acid guanidium thiocyanate-phenol chloroform extraction method, as previously described.[17] Reverse transcription was performed using the Europe against Cancer Group (EAC) protocol.[18] Briefly, 1 μg of total RNA was added to a 20-μL volume containing random hexamers as primers and 100 U of SuperScript RNase H reverse transcriptase (Invitrogen, CA, 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.

Quantification of PRAME expression

PRAME expression was quantified using the 7900 HT Fast Real-Time PCR System and TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The cycle number at which the reaction crossed an arbitrarily placed threshold (CT) was determined and the relative expression of *PRAME* regarding a housekeeping gene (*ABL1*), used as a control of RNA quality, was calculated using the equation $2^{-\Delta\Delta Ct}$ where $\Delta CT = CT_{PRAME} - CT_{ABL1}$ and $\Delta\Delta CT = \Delta CT_{sample} - \Delta CT_{Healthy\ BM}$ (median).[19] In order to carry out the $\Delta\Delta^{CT}$ correction, we selected the median ΔCT value obtained in bone marrow samples from ten healthy donors. *PRAME* expression values were thus expressed as *relative units* (RU), where one RU is equivalent to the *PRAME* expression of the healthy donor bone marrow sample with the median Δ^{CT} value. The assay ID were: *ABL1*, Hs00245445_m1, and *PRAME*, Hs00196132_m1.

Quantification of the PML-RARA fusion gene

Absolute quantification of *PML-RARA* transcripts was carried out by real-time quantitative polymerase chain reaction (RQ-PCR) using an ABI PRISM 7700 DNA Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to the EAC protocol.[18,20] *PML-RARA* transcript copy numbers were assessed in 5 μL (100 ng) of cDNA, using commercial plasmids (IpsoGen Laboratories, Marseille,

France) to construct the standard curve. The housekeeping gene Abelson-1 (*ABL1*) was selected as a control gene for RNA expression as previously reported.[21] A non-amplification control, containing RNA from a healthy donor and a non-template control with distilled water instead of human cDNA were included in each assay. All samples were analyzed in triplicate and results are reported according to EAC guidelines as the normalized copy number, which is derived by multiplying the *PML-RARA* copy number/*ABL1* copy number ratio by 10000.

Detection of FLT3 *mutation*

FLT3-ITD was examined by amplification of the juxtamembrane region spanning exons 14 and 15 with primers 11F and 12R, using qualitative PCR[22] and Genescan analysis.[23] The up-stream primer in this latter approach was fluorescently labeled with 6-FAM to allow sizing of all products (Model 3130 Genetic Analyzer, Applied Biosystems).

Statistical analysis

All tests were carried out using the SPSS 15.0 program (SPSS, Chicago, IL, USA). For univariate analyses, the χ^2 and Student's t tests were performed to evaluate factors associated with *PRAME* expression. Relapse-free survival (RFS) was defined as the time between the achievement of complete remission and the time of the relapse or the last follow-up. Overall survival was defined as the time between the moment of diagnosis and death or the last follow-up. The probabilities of RFS and overall survival were calculated using the Kaplan-Meier method and compared using the log-rank test.[24] RFS was estimated taking hematologic relapse as a censored event. Continuous variables were dichotomized according to either the median value or relapse-risk criteria described by Sanz *et al.*[37] The impact of multiple predictor variables on RFS was assessed by multivariate analysis according to the Cox regression model.[25]

Results

Efficiency of PRAME *RQ-PCR*

The efficiency of the quantification method for *PRAME* and *ABL1* was examined by constructing standard curves made using cDNA from five APL bone marrow samples that were strongly positive for both markers with a 10-fold dilution in distilled water (1 to 10^{-4}). Linear correlations between C_T values and expression levels were obtained for *PRAME* and *ABL1*, with median correlation coefficients of 0.996 (range 0.995 to 0.998) and 0.998 (range 0.997 to 0.999), respectively. The median value of amplification efficiency was 95.71% (range, 88.65 to 104.31%) for *PRAME* and 92.31% (range, 87.42 to 105.98%) for *ABL*, indicating that the $2^{-\Delta\Delta Ct}$ method used in our study for evaluating *PRAME* expression was indeed applicable.

PRAME *expression in APL at diagnosis*

PRAME expression was assessed in bone marrow samples taken at diagnosis from 125 APL patients treated within PETHEMA multicenter trial protocols. For the $2^{-\Delta\Delta Ct}$ method we used bone marrow samples from ten healthy volunteers as a calibrator. The median ΔCT (CT_{PRAME}-CT_{ABL}) value in healthy samples was 12.06 (range, 9.51 to 15.75). For APL samples, the median Δ^{Ct} value was 4.48 (range, -1.75 to 11.63). Accordingly, the median *PRAME* expression was 1.0 RU (range, 0.1-5.8) for healthy donors and 191.0 RU (range, 1.3-14301.6) for APL patients. As a reference, we also tested *PRAME* levels in 213 cases of newly diagnosed AML without t(15;17). In these latter patients, *PRAME* expression levels were significantly lower than in the APL cases (median value of 10.1 RU; range, 0.1-59531.2; $p<0.001$) (Figure 1). It is worth noting that the median *PRAME* value of non-M3 AML samples corresponds to the 15th percentile of the APL samples.

Characteristics of APL patients and PRAME *expression*

The main clinical and biological features of the 125 APL patients are summarized in Table 1. To define low and high *PRAME* expression in the series, we selected a cut-off value of 100 RU (2 logs). This cut-off was chosen because it represented the 10-fold level of the highest value observed in normal bone marrow samples and it was near to the median value in APL samples.

When we compared the clinical and biological characteristics of the two sub-groups of APL patients defined according to *PRAME* expression levels (low and high), no significant differences were observed, except for a trend towards a higher hemoglobin level ($p=0.059$) within the high-expression group than in the

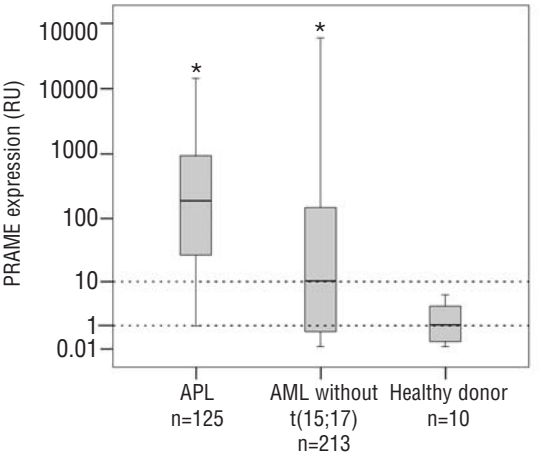

Figure 1. ***PRAME*** **expression at diagnosis in APL and in patients with other AML.** ***PRAME*** **levels were estimated with the $2^{-\Delta\Delta Ct}$ method, using** ***ABL1*** **as the control gene and bone marrow samples from ten healthy donors as calibrators. This latter group is also shown as a reference point. A significant difference was observed between all groups (*$p<0.001$).**

low-expression group. It should be noted that the WBC and platelet counts as well as the *PML-RARA* normalized copy number were similar in both groups (Table 1).

Table 1. Clinical and biological characteristics of acute promyelocytic leukemia patients at diagnosis (n=125), divided according to *PRAME* expression.

Parameter	*PRAME expression ≤100 RU (n=53)*	*PRAME expression >100 RU (n=72)*	*p*
Age, years, median (range)	45 (12-76)	35 (9-81)	0.096
Sex, Male, n (%)	30 (56.6)	43 (59.7)	0.434
WBC, $\times 10^9$/L, median (range)	2.6 (0.3-97.0)	2.6 (0.4-146.8)	0.238
Hemoglobin, g/dL, median (range)	9.0 (6.0-14.6)	10.0 (6.5-15.3)	0.059
Percentage of PB blasts, median (range)	36 (0-100)	38 (0-100)	0.945
Platelets, $\times 10^9$/L, median (range)	26 (3-183)	22 (7-158)	0.502
Percentage of BM blasts, median (range)	90 (70-100)	88 (70-100)	0.827
PML/RARA NCN1, median (range)	3737 (827-15587)	2934 (839-19750)	0.728
PML/RARA isoform, n (%):			
Bcr1, n=77	37 (69.8)	40 (55.6)	0.256
Bcr2, n=5	2 (3.8)	3 (4.2)	
Bcr3, n=43	14 (26.4)	29 (40.3)	
FAB classification, n (%):			
M3, n=94	42 (79.2)	52 (72.2)	0.493
M3v, n=31	11 (20.8)	20 (27.8)	
FLT3-ITD, n (%):			
No, n=82	38 (71.7)	44 (61.1)	0.298
Yes, n=43	15 (28.3)	28 (38.9)	
Treatment protocol			
PETHEMA 96, n=45	17 (32.1)	28 (38.9)	0.276
PETHEMA 99, n=80	36 (67.9)	44 (61.1)	
Relapse-risk group[a], n (%)			
Low risk, n=26	15 (28.3)	11 (15.3)	0.172
Intermediate risk, n=64	26 (49.1)	38 (52.8)	
High risk, n=35	12 (22.6)	23 (31.9)	
Consolidation treatment arm[b], n (%)			
Standard consolidation	25 (47.2)	36 (50.0)	0.448
Reinforced consolidation + ATRA (PETHEMA 99)	28 (52.8)	36 (50.0)	
Response to induction treatment, n (%):			
Complete remission, n=111	48 (90.6)	63 (87.5)	0.406
Death during treatment, n=14	5 (9.4)	9 (12.5)	
ATRA syndrome, n (%)			
Absent, n=91	39 (73.6)	52 (72.2)	0.516
Indeterminate/present, n=34	14 (26.4)	20 (27.8)	

NCN: normalized copy number; FAB: French-American-British; PB: peripheral blood; BM: bone marrow; [a]according to Sanz et al., 2000.[37] [b]According to Sanz et al., 2004.[15,16] This stratification was used only in the PETHEMA 99 protocol.

PRAME *expression according to response to therapy and relapse status*

Of the 125 evaluated APL patients, 111 (88.8%) achieved complete remission after induction treatment (median 34 days after diagnosis; range, 22-88 days). The remaining 14 patients died during induction treatment due to hemorrhage (n=7), therapy-related infection (n=6) or ATRA syndrome (n=1) at a median of 14 days after diagnosis (range, 1-29 days). Regarding *PRAME* expression, no statistically significant difference was observed between patients who achieved complete remission (median 184.3 RU; range, 1.3-14301.6) and patients who died during induction therapy (median 460.6 RU; range, 7.0-11764.8; p=0.502).

Among patients achieving complete remission, we investigated *PRAME* RNA levels in order to discern whether these levels were different between patients who relapsed later (n=16) and those who did not (n=95). Interestingly, patients in continuous complete remission had significantly higher *PRAME* expression (median 207.6 RU; range, 1.3-14301.6) at diagnosis than had those patients who eventually relapsed (median 39.8 RU, range 1.5-5060.1; p=0.05).

Relapse-free survival and overall survival

When we compared the RFS of patients with low and high *PRAME* expression levels, defined according to the threshold previously mentioned (100 RU), we observed that the latter group of patients had a significantly

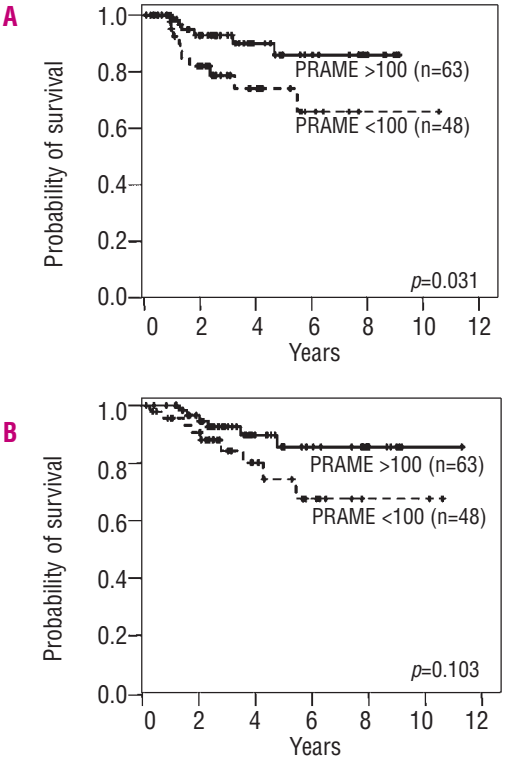

Figure 2. Relapse-free survival (A) and overall survival (B) of APL patients divided according to *PRAME* expression levels at diagnosis. Only patients who survived beyond the 34th day are included in this analysis.

longer RFS (RFS rates of 86% versus 74% at 5 years, p=0.031; Figure 2A). We evaluated the impact of the main biological and clinical features of patients on RFS using the Kaplan-Meier model as a univariate approach (Table 2). Multivariate analysis for RFS was carried out including the parameters with significant differences in the univariate analysis: WBC counts, *PRAME* expression level and bone marrow blasts. Only two of these variables were selected as having an independent prognostic value for a shorter RFS: WBC >10×10^9/L (p=0.003) and *PRAME* expression <100 RU (p=0.011).

In APL, the number of WBC has a marked prognostic impact and may determine treatment choices.[26-29] Following a recent analysis by Adès *et al*. comparing the French-Belgian-Swiss and PETHEMA results,[29] we wanted to investigate the prognostic influence of the main clinical and biological features described in Table 2 in patients with WBC<10×10^9/L or WBC ≥10×10^9/L. In patients with low WBC, only *PRAME* expression (100 RU cut-off) could define two subgroups with significantly different RFS at 5 years (80% *vs*. 92% in low and high *PRAME* expression group, respectively; p=0.032; Figure 3A). The Kaplan-Meier curves were not statistically significantly different between subgroups by any of the other clinical and biological parameters. There were few patients (n=22) in the group with a high WBC count, so it is not surprising that there were no parameters associated with statistically significant differences in risk of relapse. Regarding the 100 RU cut-off, the two subgroups showed different RFS curves, but the differences were not statistically significant (p=0.231) (Figure 3B).

Because all deaths during induction therapy in the

Table 2. Influence of the clinical-biological characteristics of acute promyelocytic leukemia patients at diagnosis on their relapse-free survival (RFS).

	n	*5-year RFS*	*Univariate*	*Multivariate*
WBC at diagnosis (×10^9/L)[a]				
≤10	89	87%	0.002	0.003
>10	22	58%		
PRAME expression (RU)				
≤100	48	74%	0.031	0.011
>100	63	86%		
Bone marrow blasts at diagnosis (%)[b]				
≤86	56	92%	0.008	0.076
>86	55	69%		
FAB classification				
M3	88	84%	0.096	–
M3v	23	71%		
Peripheral blood blasts at diagnosis (%)[b]				
≤37	55	86%	NS	–
>37	56	75%		
Platelet count at diagnosis (x10^9/L)[a]				
≤40	83	83%	NS	–
>40	28	76%		
Sex				
Male	64	79%	NS	–
Female	47	81%		
Age (years)[b]				
≤39	56	81%	NS	–
>39	55	83%		
PML/RARA (Normalized copy number)[b]				
≤3093	56	77%	NS	–
>3093	55	85%		
PML/RARA isoform				
Bcr1	70	76%	NS	–
Bcr2	5	80%		
Bcr3	36	89%		
Hemoglobin (g/dL)[b]				
≤9.5	55	83%	NS	–
>9.5	56	79%		
FLT3-ITD, n (%):				
No	80	84%	NS	–
Yes	31	73%		
Treatment protocol				
PETHEMA 96	40	81%	NS	–
PETHEMA 99	71	82%		
Consolidation treatment arm[c], n (%)				
Standard consolidation	56	82%	NS	–
Reinforced consolidation + ATRA (PETHEMA 99)	55	81%		
ATRA syndrome				
Absent	84	80%	NS	–
Indeterminate/present	27	83%		

NS: not statistically significant, p>0.1. [a]Dichotomization based on criteria for high-risk patients from Sanz et al., 2000.[37] [b]Dichotomization based on median value. [c]According to Sanz et al., 2004.[15,16] This stratification was used only in the PETHEMA 99 protocol.

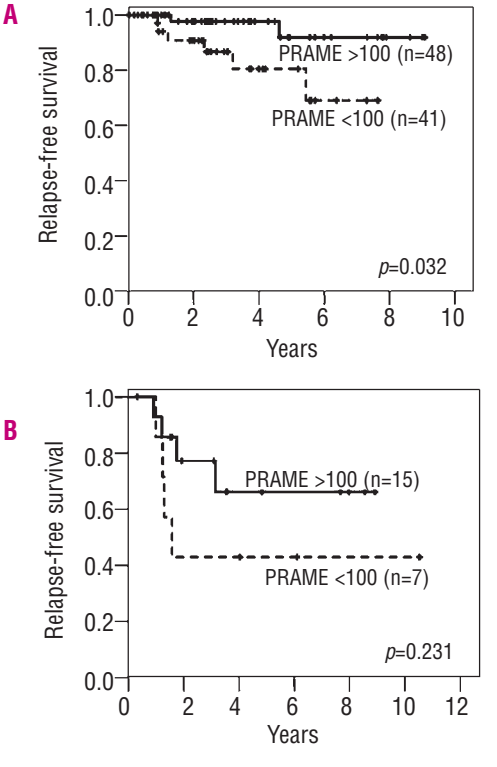

Figure 3. Relapse-free survival of APL patients based on PRAME expression among patients with a WBC count at diagnosis <10×10^9/L (A) or ≥10×10^9/L (B) following the criteria used by Ades *et al*., 2008.[26]

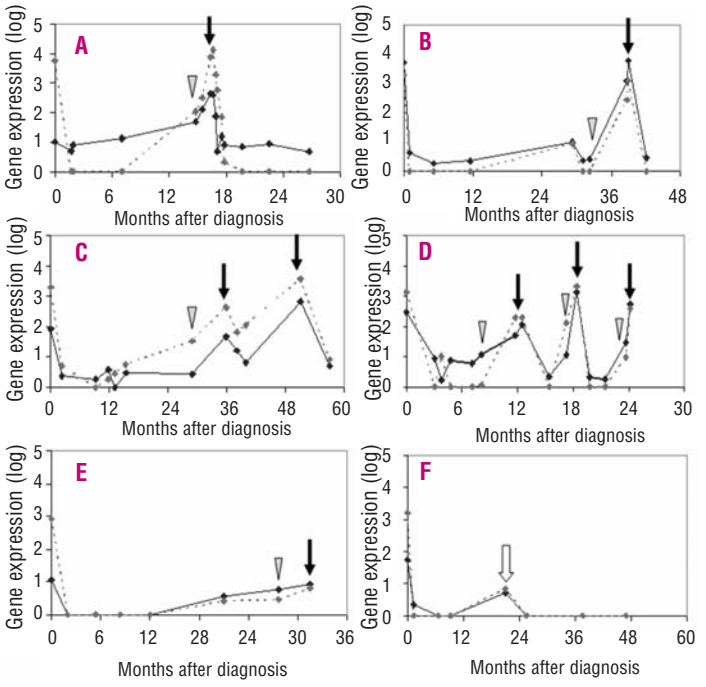

Figure 4. Comparison of minimal residual disease evaluated by *PML-RARA* and *PRAME* expression. Expression of *PML-RARA* (dotted lines) and *PRAME* (continuous lines) in follow-up samples from five patients (A-E) who relapsed and one patient (F) in continuous complete remission with one false-positive sample (open arrow). Relapses are indicated as closed arrows. Samples analyzed within 6 months before relapse are indicated as triangles. *PML-RARA* is expressed as normalized copy number on a log-scale and *PRAME* is expressed as a result of the $2^{-\Delta Ct}$ equation (log-scale).

present series were due to early infectious or hemorrhagic complications and ATRA syndrome rather than to progressive disease, which is a competing risk in evaluating leukemia-related mortality, we carried out a landmark analysis beyond day 34 (the median day for achieving a response evaluation). In the analysis of the remaining patients we observed that those with high *PRAME* expression had a higher 5-year overall survival rate compared to patients with low *PRAME* expression (85.6% versus 74.4%, Figure 2B), although the difference did not reach statistical significance (p=0.103).

PRAME *vs.* PML-RARA *fusion transcript expression*

We analyzed 225 follow-up bone marrow samples from 67 patients who achieved complete remission: 53 patients who maintained a continuous complete remission and 14 patients who eventually relapsed. The follow-up for patients remaining in complete remission (median 3.13 years, range 0.5 to 10.6) was virtually the same as the follow-up for patients who eventually relapsed (median 3.17 years, range 1.3 to 11.3, p=0.551). When we compared the reduction of *PRAME* expression with that of *PML-RARA* expression from diagnostic to post-induction samples, a strong correlation was observed between the levels of expressions (Pearson's correlation coefficient of 0.689, p<0.001). We also analyzed both MRD markers in samples from patients who relapsed, finding a similar expression patterns between *PRAME* and *PML-RARA* in all of them. The evolution of these two markers is illustrated in Figure 4 (A-E) which shows five representative patients who eventually relapsed, together with one case who remained in continuous complete remission (F). In four relapsed patients, all the samples taken within 6 months before relapse showed an increase in at least one of the two markers to above 10 normalized copy numbers or 10 RU. The fifth patient (Figure 4E) was the only case who relapsed with no *PRAME* expression or *PML-RARA* increase (false-negative case). None of the three patients in continuous complete remission with a sample false-positive for MRD during maintenance and beyond (i.e. Figure 4F) showed either *PRAME* expression >10 RU or *PML-RARA* >10 normalized copy number in subsequent analyses.

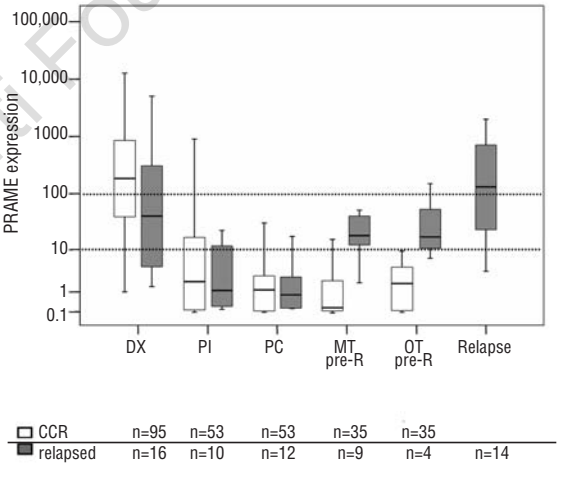

Figure 5. *PRAME*-based evaluation of minial residual disease at different stages of disease. Patients in continuous complete remission (CCR) and patients who eventually relapsed were evaluated at diagnosis (DX), post-induction (PI), post-consolidation (PC), during maintenance therapy (MT) and out of therapy (OT). As regards patients who relapsed, we analyzed 13 pre-relapse samples (pre-R) during MT (n=9) and OT (n=4), which were collected within the 6-month period before relapse, as well as 14 samples taken during the relapses. Empty boxes correspond to patients in CCR, who had not relapsed at the time of evaluation (always >1 year of follow-up). Solid boxes correspond to patients who eventually relapsed.

PRAME *expression and tumor burden evolution*

Upon analyzing *PRAME* expression during different phases of therapy, a rapid decrease was observed from diagnosis to post-induction and post-consolidation treatment. However, there were no statistically significant differences in these reductions between patients in continuous complete remission and patients who eventually relapsed (Figure 5), indicating that the kinetics of this parameter has no predictive value for relapse detection at these two time-points.

Once the consolidation treatment was concluded, virtually all samples from patients in continuous complete remission continued to show very low *PRAME* expression levels during the maintenance phase (median 0.25 RU; range, 0-18) and out of treatment (median 2.7 RU; range, 0-11). Only three out of 70 samples (4.3%) from patients in continuous complete remission showed *PRAME* expression >10 RU during maintenance therapy and beyond. These were considered as false positive results, since all subsequent samples (at least two) had *PRAME* expression <10 RU (n=9, median value 0.4; range, 0-6) and none of patients relapsed (follow-up after the positive sample of 10, 22 and 27 months). By contrast, 11 out of 13 samples taken within 6 months preceding relapse (median 72; range 22, to 173 days) from patients who eventually relapsed had *PRAME* expression >10 RU (median 18.2 RU; range, 1.9 to 150.0).

Discussion

In this study we evaluated the biological and prognostic significance of *PRAME* expression in 125 patients with APL and demonstrated that overexpression of this tumor-related antigen is associated with a better outcome and longer RFS. In addition, the RNA levels of *PRAME* can be a useful method for monitoring MRD, since levels of expression are reduced during complete remission and increased several months preceding relapse.

High *PRAME* levels were initially correlated with an advanced tumor stage and poor clinical outcome for several solid tumors such as non-small cell lung, breast and renal cell carcinoma, Hodgkin's lymphoma, medulloblastoma and melanomas.[1,2,10,11] By contrast, in hematologic neoplasias such as AML and acute lymphoid leukemia preliminary reports suggest that high *PRAME* expression is associated with a favorable prognosis.[4,5,8] Only two previous studies have indicated that *PRAME* levels may be higher in AML patients with t(15;17) than in the rest of the AML subtypes; however, both studies only evaluated a limited number of t(15;17) AML cases using either conventional semi-qualitative RT-PCR (n=11)[3] or gene expression arrays (n=12).[30] When these latter patients were reanalyzed using the SYBR Green RQ-PCR approach, a correlation between high expression of *PRAME* (defined as the median expression across all AML samples) and t(15;17) AML was observed.[4]

However, the authors argued that this correlation might have been secondary to its correlation with favorable cytogenetics and they merely observed a trend towards longer overall survival in cases with higher *PRAME* expression.[4] Based on a large cohort of APL patients, our data demonstrate that PRAME expression is an independent prognostic factor in APL since its over-expression is associated with prolonged RFS. Moreover, PRAME level contributes to defining two prognostic sub-groups within low-risk APL patients according to whether their WBC count is above or below 10×10^9/L.[26-29]

The biological explanation of why high *PRAME* expression is associated with a better prognosis is unclear. *PRAME* has been described to be a repressor of retinoic acid signaling, capable of inhibiting retinoic acid-induced differentiation, growth arrest and caspase-dependent apoptosis in F9 mouse embryonic carcinoma cells.[31] Knock-down of *PRAME* by RNA interference in the retinoic acid-resistant A375 human melanoma cell line restores both retinoic acid receptor signaling and sensitivity to the antiproliferative effects of retinoic acid.[31] However, some authors have shown that this effect could be tissue-specific, since *PRAME* expression is not associated with down-regulation of retinoic acid signaling in cells from primary AML.[32] Furthermore, Tajeddine *et al.*[33] demonstrated that *PRAME* overexpression can induce caspase-independent cell death in CHO-K1 and HeLa cell lines. In addition, the repression of *PRAME* expression by a short interfering RNA increases the tumorogenicity of the K562 leukemic cell in BALB/c nude mice.[33] These latter findings would be fully concordant with a presumed beneficial effect of *PRAME* expression in the prognosis of APL.

Traditionally, MRD in APL patients is evaluated using levels of *PML-RARA*.[18,20,34,35] In the post-induction phase, about one half of the patients in complete remission after ATRA plus chemotherapy, remain positive for *PML-RARA* in the bone marrow and this evaluation is clinically not informative. By contrast, studies carried out after completion of consolidation treatment are extremely relevant since a positive *PML-RARA* test is strongly predictive of relapse.[20,28,34,36] In our series, RNA levels of *PRAME* at diagnosis were relatively lower than those of *PML-RARA*, but the kinetics of both transcripts after therapy seemed to be similar. *PRAME* levels in both the post-induction and post-consolidation phase did not have any prognostic impact. By contrast, in follow-up samples during maintenance treatment and out of therapy, we observed that an increasing level of *PRAME* was associated with impending relapse.

Accordingly, our results show that APL patients with a *PRAME* level 10-fold higher than normal values during maintenance therapy and beyond are at high-risk of relapse. *PRAME* and *PML-RARA* expression are strongly correlated and results after therapy were concordant, since a result of >10 normalized copy number during this period was almost equivalent to an immediate relapse.[20] However, it is unclear whether *PRAME* RNA levels give information additional to that provided by *PML-RARA* expression, suggesting that *PRAME* could be used only as a secondary marker during the

follow-up of APL.

In conclusion, our data demonstrate that *PRAME* is a suitable indicator of prognosis in APL, since overexpression at diagnosis is associated with a better outcome and the levels were able to identify two subgroups with significantly different RFS within low-risk APL patients (i.e. those with a WBC<10×10^9/L).

Authorship and Disclosures

CS and MCC participated equally in designing the study, carrying out all molecular studies and preparing the database for the final analysis; CS prepared the initial version of the paper; RG-S conceived the study, helped in the design of the work, reviewed the database and carried out the statistical analysis. He rewrote the paper and provided pre-approval of the final version; AB, MES and MA participated in the generation of the molecular results; FR, TB, JAQ, MJP and PG were the clinicians responsible for the patients and took care of administering the treatment protocols, taking samples and collecting clinical data; JFS-M and MG promoted the study and obtained financial support. Both were responsible for the group of researchers and were responsible for the most important revision of the draft article; MG gave final approval of the version to be submitted. The authors reported no potential conflicts of interest.

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