Long *FLT3* internal tandem duplications and reduced *PML-RARα* expression at diagnosis characterize a high-risk subgroup of acute promyelocytic leukemia patients

María Carmen Chillón,[1,2] Carlos Santamaría,[1,3] Ramón García-Sanz,[1,2,3] Ana Balanzategui,[1] María Eugenia Sarasquete,[1,2] Miguel Alcoceba,[1] Luis Marín,[1] María Dolores Caballero,[1] María Belén Vidriales,[1] Fernando Ramos,[4] Teresa Bernal,[5] Joaquín Díaz-Mediavilla,[6] Alfonso García de Coca,[7] María Jesús Peñarrubia,[8] José Antonio Queizán,[9] Pilar Giraldo,[10] Jesús F. San Miguel[1,3] and Marcos González[1,2,3]

[1]Servicio de Hematología, Hospital Universitario de Salamanca, Salamanca; [2]Unidad de Genómica, Servicio de Investigación, Hospital Universitario de Salamanca, Salamanca; [3]Centro de Investigación del Cáncer-IBMCC (USAL-CSIC), Salamanca; [4]Complejo Hospitalario de León and Ibiomed, Universidad de León, León; [5]Hospital Central de Asturias, Oviedo; [6]Hospital Clínico San Carlos, Madrid; [7]Hospital Clínico de Valladolid, Valladolid; [8]Hospital Río Hortega, Valladolid; [9]Hospital General de Segovia, Segovia, and [10]Hospital Miguel Servet, Zaragoza, Spain

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Correspondence: María del Carmen Chillón Santos, PhD, Department of Hematology, University Hospital of Salamanca, Paseo San Vicente 58-182, Salamanca, 37007, Spain. E-mail: chillon@usal.es

ABSTRACT

Background
Internal tandem duplications of the *FLT3* gene (*FLT3*-ITDs) are frequent in patients with acute promyelocytic leukemia (APL), however its clinical impact remains controversial.

Design and Methods
We analyzed the prognostic significance of *FLT3*-ITD mutant level and size, as well as *FLT3*-D835 point mutations, *PML-RARα* expression and other predictive factors in 129 APL patients at diagnosis enrolled on the Spanish LPA96 (n=43) or LPA99 (n=86) PETHEMA trials.

Results
FLT3-ITDs and D835 mutations were detected in 21% and 9% of patients, respectively. Patients with increased ITD mutant/wild-type ratio or longer ITD size displayed shorter 5-year relapse-free survival (RFS) (P=0.048 and P<0.0001, respectively). However, patients with D835 mutations did not show differences in RFS or overall survival (OS). Moreover, patients with initial normalized copy number (NCN) of *PML-RARα* transcripts less than the 25th percentile had adverse clinical features and shorter 5-year RFS (P<0.0001) and OS (P=0.004) compared to patients with higher NCN. Patients with low NCN showed increased incidence of ITDs (P=0.001), with higher ratios (P<0.0001) and/or longer sizes (P=0.007). Multivariate analysis showed that long *FLT3*-ITD (P=0.001), low *PML-RARα* levels (P=0.004) and elevated WBC counts (>10×10^9/L) (P=0.018) were independent predictors for shorter RFS. We identified a subgroup of patients with high WBC, long *FLT3*-ITD and low NCN of transcripts that showed an extremely bad prognosis (5-year RFS 23.4%, P<0.0001).

Conclusions
In conclusion, *FLT3*-ITD size and *PML-RARα* transcript levels at diagnosis could contribute to improve the risk stratification in APL.

Key words: acute promyelocytic leukemia, *FLT3*-ITD size, *PML-RARα* level, prognosis.

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Introduction

Internal tandem duplications (ITDs) of the *FLT3* gene are present in 30-40% of acute promyelocytic leukemia (APL) patients.[1-4] *FLT3*/ITDs are associated with poor outcome in most published studies in acute myeloid leukemia (AML).[1-3,5] However, this correlation has not been found in APL where ITDs have only been associated with some adverse diagnostic clinical features (high WBC count, M3 variant morphology or bcr3 isoform), but not poor survival.[4,6-9] Previous studies analyzing the clinical utility of ITDs quantitative determination have suggested that only patients with high *FLT3* mutant levels have a real inferior prognosis, but the majority of these reports only included AML with normal karyotype,[10,11] or excluded APL patients.[5] Moreover, studies analyzing the impact of ITD length on outcome have yielded contradictory results, since the shorter survival attributed to patients with long ITDs[12] has not been confirmed by all authors.[5,13]

A number of pre-treatment characteristics have been described as prognostic factors in APL patients.[14] Among them, presenting leukocyte counts have the highest impact on outcome.[15-18] In the same line, the level of *PML-RARα* transcripts before treatment could have some predictive value. However, clinical studies with quantitative assessment of *PML-RARα* transcripts at diagnosis are scanty and have produced conflicting results.[19,20] These discrepancies may reflect technical variations or the lack of correction according to the blast cell percentage.[21] As far as other AML fusion transcripts are concerned, there are also conflicting results. For instance, some groups have observed a correlation between high levels of the *AML1-ETO* transcript at diagnosis and shorter survival,[20,22] while some others have failed to observe this.[23,24]

Our aim in the present study was to assess the prognostic relevance of *FLT3*-ITD quantification and sizing in addition to other factors such as *PML-RARα* expression and clinical characteristics in a series of uniformly treated APL patients at diagnosis.

Design and Methods

Patients

Pre-treatment bone marrow (BM, n=124) or peripheral blood (PB, n=17) samples received at our reference laboratory of the University Hospital of Salamanca (Spain) were obtained from 141 adult APL patients who were entered into either the Spanish LPA96[25] (n=46) or LPA99[26] (n=95) PETHEMA trials. 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 two years.[25] In the LPA99 protocol, the consolidation phase was modified by including ATRA plus higher doses of idarubicin for patients who were considered as being at intermediate- and high-risk of relapse.[26] In addition to using standard criteria,[26] as well as immunophenotyping,[27] all patients were confirmed by both RT-PCR and RQ-PCR analysis for *PML/RARα* rearrangements.

RNA isolation and cDNA synthesis

Total RNA was isolated from BM and PB samples using the guanidinium tyocyanate/phenol chloroform method. Reverse transcription was performed on 1μg of total RNA according to the rules and protocols approved in the "Europe Against Cancer" (EAC) Program.[21]

Determination of FLT3 mutation status

FLT3-ITD was examined by RT-PCR amplification of the juxtamembrane domain as previously described.[4] To obtain the size and the relative level of mutations, RT-PCR was performed using a fluorescently labeled primer with 6-FAM. Products were analyzed by Genescan analysis on a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The relative mutant level was calculated using the area under the peak and expressed as the ratio of mutant and wt *FLT3* alleles.[3] To detect *FLT3*-D835 point mutations, the restriction fragment length polymorphism-mediated (RFLP) PCR assay was used by amplifying the exon 20 of the *FLT3* tyrosine kinase domain.[4] In all cases, the presence of a D835 mutation was confirmed by sequencing of the amplified products with the BigDye Terminator cycle sequencing chemistry (Applied Biosystems, Foster City, CA, USA).

Quantification of PML-RARα transcripts by real-time quantitative PCR

Absolute quantification of *PML-RARα* transcripts was carried out by RQ-PCR using an ABI PRISM 7700 DNA Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to the EAC protocol.[21,28] Standard curves were produced for all three *PML-RARα* breakpoint variants using commercial plasmids (IpsoGen Laboratories, Marseille, France). To minimize variability in the results due to differences in the efficiency of cDNA synthesis and RNA integrity among the patient samples, the absolute *PML-RARα* copy number was normalized to the expression of Abelson gene (*ABL*) as previously reported.[28,29] The normalized copy number (NCN) was defined as the copy number (CN) of the fusion gene per one copy of the control gene transcript. According to EAC guidelines, the normalized values of the *PML-RARα* copies were reported as the ratio [*PML-RARα* CN] / [ABL CN] x 10000, after correction for blast cell percentage. All experiments were carried out in triplicate.

Statistical analysis

The association between variables was analyzed by the X^2 and the Fisher's exact tests for categorical variables and by the Student's t-test for the mean values of continuous variables. The comparison between PB and BM was performed with the non-parametric Wilcoxon paired test. The probabilities of relapse-free survival (RFS) and overall survival (OS) were estimated according to the Kaplan-Meier method and compared using the log-rank test.[30] OS was calculated from the date of diagnosis to the date of death or last follow-up and RFS was calculated from the date of complete remission (CR) achievement to the date of relapse, death or last follow-up. A landmark analysis[31] was used to evaluate differences between groups in OS avoiding the influence of competing risks. Early death was defined as death occurring during induction therapy or during the aplasia period following chemotherapy. The Cox regression model[32] was used to assess the predictive value of multiple variables at diagnosis in relation to OS and RFS in multivariate analysis considering the parameters either as continuous or categorical variables. These analyses were performed using the SPSS 15.0 statistical software package (SPSS Inc., Chicago, IL, USA).

Results

Relationship between FLT3 status, PML-RARα expression and pre-treatment characteristics

FLT3 mutations were determined in 129 of the original cohort of 141 patients because some samples had been

used up in previous studies.[4,28,33] In total, 39 (30%) of 129 patients had mutations: 27 (21%) had ITD and 12 (9%) had D835 point mutations. Concerning *FLT3*/ITD quantification and sizing, both ITD mutant/wt ratio and ITD size varied in the overall group (median ratio 0.66, range: 0.3-1.0; median size 60 nucleotides (nt.), range: 18-105), and no correlation between mutant level and size was observed.

Among the 141 APL patients, 86 (61%) expressed the *PML-RARα* L-form (bcr1), 4 (3%) expressed the V-form (bcr2) and 51 (36%) expressed the S-form (bcr3). Because of the relative rarity of the bcr2 isoform, results for patients with bcr1 and bcr2 were joined for further analyses, as in other studies.[15-18] After correction for blast cell percentage, there were no differences in the distribution of *PML-RARα* copy numbers between BM and PB (12681 *vs.* 9176, *P*=0.317). Moreover, twelve paired BM/PB samples were analyzed for comparison of normalized *PML-RARα* expression at diagnosis, and no significant differences were found (*P*=0.374, Wilcoxon test), as previously reported by the EAC group and others.[19,21] The median NCN was 4532 with a wide range of expression (1106 - 29760), and a higher copy number was associated with fusion types bcr1 or bcr2 versus bcr3 (6982 *vs.* 4725, *P*=0.003). The PCR efficiencies were very similar for the three *PML-RARα* probe and primer sets when using plasmids to construct the standard curves (bcr1: 2.01, bcr2: 1.94 and bcr3: 1.99).

To define low and high *PML-RARα* expression at diagnosis in our series and to further analyze its prognostic value on RFS and OS, patients were separated into two groups, respectively, based on the 25th percentile (NCN=2700) of the initial NCN. This cut-off point was chosen after testing other quartiles (50 and 75) because it provided a better discrimination between prognostic groups. The main clinical and biological characteristics of the two APL subgroups are detailed in Table 1. Low levels of transcripts (NCN<2700) were significantly associated with adverse clinical features at diagnosis: higher white blood cell (WBC) count (26.8 *vs.* 8.2×10^9/L, *P*=0.002), higher blast cell percentage in BM (87.9 *vs.* 80.3, *P*=0.003) and in PB (56.1 *vs.* 37.4, *P*=0.008) and elevated LDH levels (1052 *vs.* 655, *P*=0.003). Moreover, patients with low NCN showed a higher incidence of ITDs (40% *vs.* 14%, *P*=0.001) with increased ITD levels (26% *vs.* 4%, *P*=0.001) and ITD sizes (23% *vs.* 6%, *P*=0.004) (Table1). Furthermore, the low expression group did not show D835 point mutations (*P*=0.026).

To better establish the risk of relapse at diagnosis, patients were stratified according to the Spanish score[18] into 3 risk groups: 31 cases at low-risk (WBC count ≤10×10^9/L, platelet count > 40×10^9/L), 69 cases at intermediate-risk (WBC count ≤10×10^9/L, platelets ≤40×10^9/L) and 41 at high-risk of relapse (WBC count >10×10^9/L) (Table 1). There was a correlation between these categories and the subgroups of *PML-RARα* expression, since the major proportion of patients at high risk of relapse belonged to the low-expression group (54% *vs.* 20% *P*=0.001).

As far as the immunophenotypic characteristics of leukemic cells were concerned, we only focused on CD34 and CD15 markers according to our previous results.[27] The group with low NCN had more CD34$^+$ (30% *vs.* 11%, *P*=0.040) and CD15- cases (80% *vs.* 60%, P=0.099). These findings were translated into a higher frequency for immature phenotypes (CD34$^+$/CD15$^-$) in the group with less than 2700 NCN of the PML-RARα transcript (30% *vs.* 10%, *P*=0.025).

Response to treatment, Relapse Free Survival and Overall Survival

A total of 141 patients were treated and evaluated for response. Of these, 122 (86.5%) achieved complete remission (CR). The remaining 19 patients (13.5%) died during induction treatment due to hemorrhage (n=9), therapy-related infection (n=9) or ATRA syndrome (n=1) at a median of 14 days after diagnosis (range 1-29 days).

Survival analysis of patients with *FLT3* mutations revealed that although small differences existed between ITD-positive and negative cases (*P*=0.071) in terms of OS,

Table 1. Presenting characteristics, *FLT3* status and response to treatment of APL patients according to *PML-RARα* level at diagnosis.

Characteristic	<2700 NCN* n=37	>2700 NCN* n=104	*P*
Age (years)	39±17	41± 17	0.488
Male sex, n (%)	22 (59)	68 (65)	0.519
WBC count (x10^9/L)	27±34	8±14	0.002
BM blasts (%)	88±12	80±13	0.003
PB blasts (%)	56±37	37±34	0.008
Platelets (x10^9/L)	34±25	37±36	0.589
Hemoglobin (g/dL)	9.7±2.6	9.6±2.2	0.722
LDH (U / L)	1052±688	655±356	0.003
FAB subtype, n (%)			
Typical	25 (68)	83 (80)	0.131
Variant	12 (32)	21 (20)	
PML-RARα isoform, n (%)			
bcr1/2	22 (60)	68 (65)	0.519
bcr3	15 (40)	36 (35)	
FLT3 status, n (%)			
ITD, n=27	14 (40)	13 (14)	0.001
D835, n=12	0 (0)	12 (13)	0.026
Wild type, n=90	21 (60)	69 (73)	
FLT3 ITD, n (%)			
High mut/wt ratio (>0.66), n=13	9 (26)	4 (4)	0.001
Long size (>60 nt.), n=14	8 (23)	6 (6)	0.004
Immunophenotype (%)			
CD34$^+$	30	11	0.040
CD15$^-$	80	60	0.099
CD34$^+$ / CD15$^-$	30	10	0.025
Treatment protocol			
PETHEMA 96, n=46	13 (35)	33 (32)	0.704
PETHEMA 99, n=95	24 (65)	71 (68)	
Relapse-risk groups**, n (%)			
Low-risk, n=31	5 (14)	26 (25)	0.001
Intermediate-risk, n=69	12 (32)	57 (55)	
High-risk, n=41	20 (54)	21 (20)	
Response to induction treatment, n (%)			
Complete remission, n=122	30 (81)	92 (89)	0.259
Early deaths, n=19	7 (19)	12 (11)	

**Patient groups were made considering the 25th percentile of the normalized values of PML-RARα transcripts before treatment. FLT3 studies were available in 129 patients (35 and 94 in the low- and high-expression groups, respectively). In the same way, immunophenotypic results were feasible for 106 patients (25 and 81 in the low- and high-expression groups, respectively), and the percentages were adjusted accordingly. **Relapse-risk stratification according to Sanz et al.[18]*

patients with ITD mutations had a shorter RFS (68% *vs.* 90%, *P*=0.033). In the same way, increasing *FLT3*-ITD ratio (>0.66) was related with shorter 5-year RFS, with estimated probabilities of 90% for patients without ITDs and 79% and 57% for patients with low and high ratios, respectively (*P*=0.048) (Figure 1A). With respect to the length of ITDs, increasing size (>60 nt.) was associated with a short 5-year RFS, with estimated probabilities of 90% for patients without ITDs and 100% and 27% for patients with small and large ITDs, respectively (*P*<0.0001) (Figure 1B). By contrast, the CR rate, RFS and OS probabilities were similar between patients with or without D835 mutations.

Concerning the two subgroups of *PML-RARα* expression, the CR rate did not vary among them (81% *vs.* 89%, *P*=0.259) (Table 1). However, a worse outcome was observed in the low-expression group since they showed a highly significant shorter 5-year RFS (60% *vs.* 92%, *P*<0.0001) (Figure 1C). Since all deaths during the induction therapy in the present series were due to hemorrhagic complications, early infections, and ATRA syndrome, which are competing risks in evaluating leukemia-related mortality, we performed a landmark analysis beyond day 30 (median day for achieving the hematologic response). In this analysis, we could confirm that patients with a low *PML-RARα* expression really had a shorter 5-year OS compared to patients with high expression (70% *vs.* 88%, *P*=0.002) (Figure 1D).

Prognostic factors: multivariate analyses

A Cox multivariate analysis for RFS was performed including the following patient characteristics: WBC count, percentage of blast cells in BM and PB, LDH level, presence of *FLT3*-ITD mutation, ratio and size of ITDs and *PML-RARα* NCN. Among all the variables examined at diagnosis, multivariate analysis indicated that poor RFS was related to a long *FLT3*-ITD size (*P*=0.001), a low *PML-RARα* level (*P*=0.004) and elevated WBC counts (>10×10^9/L) (*P*=0.018) (Table 2). When *FLT3*-ITD ratio and size as well as *PML-RARα* levels were analyzed as continuous variables, they were also selected as prognostic factors (*P*=0.007, *P*<0.0001 and *P*=0.018, respectively).

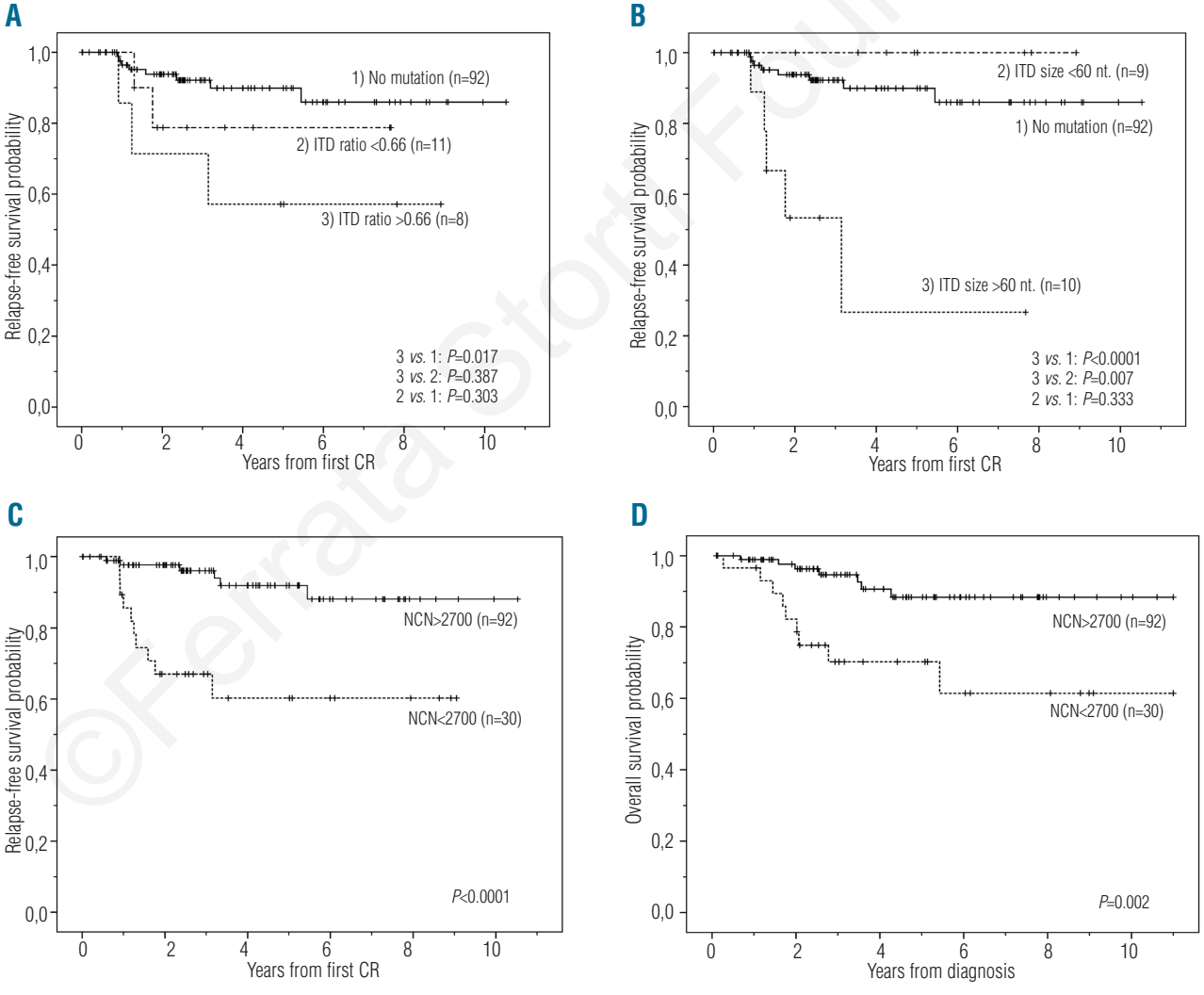

Figure 1. Kaplan-Meier analysis for RFS of APL patients regarding *FLT3*-ITD mutant/wild-type ratio (A) and ITD size (B), the median was selected for both cut-off values. Kaplan-Meier analysis comparing RFS (C) and OS (D) of APL patients according to *PML-RARα* expression levels at diagnosis. Only patients who survived beyond the 30th day are included in this analysis. To define low and high expression groups in the series, the 25th percentile of the NCN was selected as cut-off value.

However, at the final step of the analysis, due to extreme values, only categorical variables were selected (Table 2). The independent value of these molecular parameters was not demonstrated for OS, because of the high statistical power of the WBC count in this analysis ($P<0.0001$).

Impact of FLT3-ITD size and PML-RARa levels on the risk stratification of APL

Since *FLT3*-ITD size and *PML-RARα* levels were independent prognostic markers in addition to WBC count, we investigated the prognostic influence of these molecular parameters in patients with WBC ≤10×10⁹/L (n=87) and with WBC >10×10⁹/L (n=24). Patients with low counts showed a decreased incidence of ITD mutations (5%) with small sizes and elevated *PML-RARα* fusion gene levels (median NCN: 4970). By contrast, patients with a high WBC count presented more ITDs (63%) with longer sizes ($P<0.0001$) and reduced transcript levels (median NCN: 2650, $P<0.0001$). Accordingly, among these later patients the existence of an ITD longer than 60 nt. was able to identify a subgroup of patients with a very high risk of relapse: 23% *vs.* 79% probabilities of 5-year RFS ($P<0.0001$, Figure 2).

Table 2. Influence of clinical and biological variables with prognostic value at diagnosis in APL patients regarding their relapse-free survival (RFS).

Characteristic	Cases	RFS at 5 years (%)	P (Univariate)†	P (Multivariate)‡
WBC (×10⁹/L)				
≤10	85	93.3		
>10	35	62.9	0.009*	0.018
Blast cells in BM (%)				
<85	62	93.1		
≥85	58	74.9	0.009*	0.124
Blast cells in PB (%)				
<30	54	92.0		
≥30	53	75.7	0.053	–
LDH (U/L)				
<700	57	92.8		
≥700	45	73.4	0.030*	0.301
PML-RARα NCN**				
<2700	30	60.3		
≥2700	92	91.9	<0.0001*	0.004
FLT3 ITD mutation				
Negative	92	89.9		
Positive	19	67.6	0.033*	0.090
FLT3 ITD mutant/wt ratio				
wt and <0.66	103	88.6		
≥0.66	8	57.1	0.022*	0.248
FLT3 ITD size (nt)				
wt and <60	101	90.9		
≥60	10	26.7	<0.0001*	0.001
Platelets (×10⁹/L)				
<40	84	86.8		
≥40	34	82.2	0.764	–
Hemoglobin (g/dL)				
<10	71	83.6		
≥10	48	85.3	0.298	–
Age (years)				
<50	84	81.3		
≥50	37	90.0	0.176	–
FAB subtype				
Typical	97	86.2		
Variant	25	75.2	0.240	–
PML-RARα isoform				
bcr1/2	80	82.9		
bcr3	42	85.5	0.643	–
Protocol				
LPA96	37	76.8		
LPA99	85	88.2	0.107	–

*NCN: normalized copy number. wt: wild type; **NCN was dichotomized based on the 25th percentile of the initial value; *Significant variables used for multivariate analysis; †indicates that the Log-rank test was used; ‡indicates that a Cox regression model was used.*

Discussion

In this series of acute promyelocytic leukemia (APL) patients we have evaluated the clinical impact of *FLT3*-ITD mutant level and size in combination with *PML-RARα* expression before treatment. Our study demonstrated that both longer ITDs and lower *PML-RARα* level at diagnosis characterize a small subgroup of patients with a much worse prognosis.

FLT3-ITDs have been associated with a worse prognosis in non-APL AML in most published studies.[1-3,5] However, the impact of these mutations on clinical outcome of APL patients seems to be marginal, since several reports did not find any relationship between ITDs and relapse risk or survival.[6,7,34,35] However, these studies only analyzed the presence or absence of *FLT3*-ITD mutations and they did not examine either the mutant/wild-type ratio or mutant size, which have been recently recognized as the real prognostic factors in non-APL AML.[3,5,10,12] In a previous study from our group,[4] no significant differences in OS and disease-free survival were observed between *FLT3* mutated and non-mutated APL cases, despite ITDs correlated with adverse clinical features. In the present work, using a quantitative method in a larger number of patients, we have found a significant association of high *FLT3*-ITD ratios and sizes with a worse prognosis. It has been suggested that an increasing length of the inserted sequence induces constitutive activation of the kinase

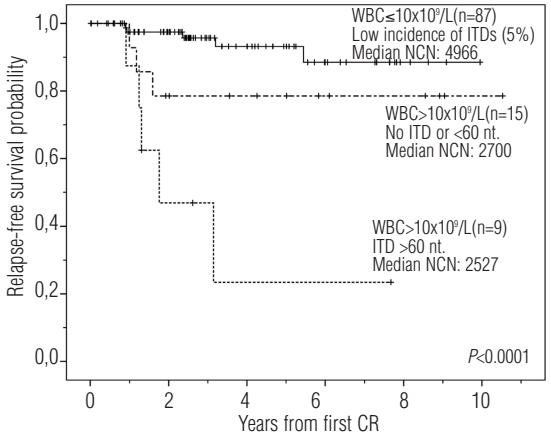

Figure 2. RFS of APL patients according to *FLT3*-ITD size in addition to WBC counts. The median size was selected as cut-off value.

domain of the FLT3 receptor leading to increased proliferation and survival of myeloid precursor cells.[36] However, studies analyzing the impact on disease outcome of ITD size have produced conflicting results, partially explained by different screening techniques.[5,12,13] Our study is in accordance with the analysis of Stirewalt *et al.*[12] in which increasing ITD size is associated with short OS and RFS in non-APL AML. Indeed, multivariate analysis for RFS confirmed that a long *FLT3*-ITD size is the most significant predictor of poor outcome, followed by low *PML-RARα* levels and high WBC count. Therefore, we investigated if both molecular parameters combined with WBC count could improve other risk classifications.[17,18] Interestingly, within the high-risk group (WBC>10×10^9/L) we could identify a small subgroup of patients at a very high risk of relapse (23% at five years) who were characterized by long ITDs and low expression of *PML-RARα*.

The prognostic impact of *PML-RARα* levels at the time of diagnosis of APL patients has been analyzed by few groups showing conflicting data.[19,20] Gallagher *et al.*[19] did not find any association between pre-treatment transcript levels and clinical outcome. By contrast, Schnittger *et al.*[20] observed a favorable prognosis for patients with low fusion gene expression. Our results do not favor any of these two studies, since low *PML-RARα* levels were associated with poor prognosis in terms of short OS and RFS. The poor outcome of low *PML-RARα* levels was in line with the presence of adverse clinical features, such as a high WBC count, high blast cell percentages in BM and PB, and elevated LDH serum levels. A clear explanation for these results was not found. However, experimental data suggest that t(15;17) cells with low levels of transcripts are more efficient to develop APL in knock-in mice than those with high levels.[37] This could indicate that the acquisition of progression mutations leading to the development of APL would depend on an "optimal pathogenic *PML-RARα* dose".[37] Moreover, in our series, those patients with a more immature and aggressive immunophenotype (CD34$^+$/CD15$^-$),[27,38] showed low *PML-RARα* levels, suggesting that immature blast cells probably have a lower ability to produce transcripts and a higher capacity to develop resistance to the therapy. However, the significance of this finding is at present unclear and needs further investigation. One potential experiment might be analyzing *PML-RARα* levels before treatment in separated subpopulations, in order to compare the fusion gene expression of mature and immature phenotypes.

In summary, the present study shows that the length of ITD mutations and *PML-RARα* transcript levels could be used as genetic markers for prognosis in APL allowing a more accurate patient risk classification. Nevertheless, these new prognostic parameters need to be validated in an independent series of patients before they can be implemented into the clinical practice.

Authorship and Disclosures

MCC and CS carried out all molecular studies and prepared the database for the final analysis. MCC performed the statistical analysis and prepared the initial version of the paper. RG-S helped in the design of the work, reviewed the database and contributed towards the statistical analysis. He provided the pre-approval of the final version. AB, MES, MA and LM participated in the generation of the molecular results. MDC, MBV, FR, TB, JD-M, AGC, MJP, JAQ 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 and were the persons responsible for the most important revision of the draft. MG approveded the final version to be sent to the editor. The authors report no potential conflicts of interest.

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