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Microgranular and t(11;17)/PLZF-RARα Variants of Acute Promyelocytic Leukemia Also Present the Flow Cytometric Pattern of CD13, CD34, and CD15 Expression Characteristic of PML-RARα Gene Rearrangement

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Acute promyelocytic leukemia (APL) is a subtype acute myeloid leukemia in which leukemic promyelocytes predominate in the bone marrow (BM). Rapid diagnosis is critical for treatment decision since *all-trans*-retinoic acid must be administrated promptly. The microgranular variant may be of difficult diagnosis, as it may be confused with other diseases on morphological grounds. The purpose of this study was to determine if the microgranular variant has the same antigenic profile as the classical hypergranular type. The immunophenotype of leukemic cells from the bone marrow of 50 patients, with the PML-RARα gene rearrangement confirmed by RT-PCR, was determined by flow cytometry using a large panel of 22 monoclonal antibodies and a polyclonal anti-TdT antibody. Thirty-four cases were classified as classical APL and 16 as microgranular APL. The immunophenotypic profile of the two subtypes was indistinguishable concerning the presence or absence of these antigens, including the absence of reactivity for the HLA-DR antigen. The simultaneous immunophenotypic combination of a unique major cell population, heterogeneous intensity of expression of CD13, and the typical pattern of CD15/CD34 expression were similarly present in the hypergranular and microgranular subtypes. Homogeneous expression of CD33 was observed in 76% of the classical APL cases and in 100% of the microgranular cases. Additionally, we have studied two cases of PLZF-RARα APL that also displayed the same immunophenotype described for classical APL. Thus, the immunophenotypic profile highly characteristic of the PML-RARα gene rearrangement was also observed in microgranular and PLZF-RARα variants of APL. Am. J. Hematol. 76:44–51, 2004. © 2004 Wiley-Liss, Inc.

Key words: acute promyelocytic leukemia; flow cytometry; immunophenotype; PML-RARα gene rearrangement; PLZF-RARα gene rearrangement

INTRODUCTION

Acute promyelocytic leukemia (APL) is a distinct subtype of acute myeloid leukemia (AML) with characteristic clinical and molecular features [1,2]. APL is invariably associated with a chromosomal aberration involving the RARα locus on chromosome 17 [3]. In the vast majority of cases, the t(15;17)(q12;q22) translocation is detected, which leads to the formation of the PML-RARα fusion gene [4]. Nevertheless, four other partners of the RARα gene have been also described: the genes PLZF, NPM, NuMA, or STAT5b [2,5].

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The utilization of *all-trans*-retinoic acid (ATRA) together with conventional chemotherapy has substantially increased the number of patients that can be cured of APL [6], but this drug is ineffective when the PLZF or STAT5b genes are involved in the rearrangement [7]. The impressive improvement in treatment outcomes observed with ATRA is, at least in part, due to the rapid correction of the coagulopathy that follows APL, implying that the administration of the drug must occur in the beginning of the treatment [8,9]. Thus, rapid diagnosis is critical for treatment decision since ATRA must be given promptly.

Unequivocal diagnosis of APL can only be established by cytogenetic or molecular genetic studies. However, these tests take some days to be performed and on the time of diagnosis, only morphology, cytochemistry, and immunophenotype are available to support the decision to introduce ATRA. The morphological pattern of APL is distinctive in the majority of cases [10]. The classical hypergranular and the microgranular (hypogranular) variant are the two morphological subtypes representative of the great majority of APL cases with the t(15;17) translocation [11]. The bone marrow morphology of both subtypes, however, may cause confusion with other diseases in some instances, and problems emerge when morphology is solely used for diagnostic confirmation of APL [12]. In particular, the microgranular variant may be of difficult diagnosis, as it may be confused with other types of AML on morphological grounds [13,14].

Orfao and others have recently reported an antigenic profile highly characteristic of the presence of the PML-RARα gene rearrangement. This profile is composed by a heterogeneous intensity of expression of CD13, a unique major blast cell population, and a typical pattern of CD15/CD34 expression [15]. However, their series had only four cases of microgranular APL and the comparison of this immunophenotypic profile between classical and microgranular APL has not been performed.

The purpose of this study was to compare the immunophenotypic features of classical and microgranular APL using a large panel of monoclonal antibodies and to test if the antigenic profile suggested by Orfao and others as highly characteristic of the PML-RARα gene rearrangement [15] was consistently present in our series, which included 16 cases displaying the microgranular morphology. Additionally, we have studied two cases of APL harboring the t(11;17)/PLZF-RARα.

PATIENTS AND METHODS

Patients

Fifty-two patients whose diagnosis of APL was made at our laboratory between March 1995 and June 2002 were included in this study. We selected 50 consecutive cases with a positive PML-RARα and 2 with PLZF-RARα gene rearrangement detected by the reverse-transcriptase polymerase chain reaction (RT-PCR). Inclusion criteria were the availability of good-quality Leishman-stained bone marrow smears and of representative flow cytometry studies from the same bone marrow sample as used for morphological review. In all cases, the bone marrow aspirates from diagnosis were used for confection of smears and for immunophenotyping and molecular biology studies.

Morphological Examination

Leishman-stained bone marrow smears from all patients were reviewed by two hematologists (EGR and RPF), and agreement concerning morphological classification of the APL cases was achieved in all cases. Cells with an abundant cytoplasm almost completely filled with azurophilic granules or Auer rods were considered to be hypergranular promyelocytes [10]. Cells without granules or containing only a few fine azurophilic granules, with a bilobed, multilobed, or reniform nucleus, were considered to be microgranular promyelocytes [11]. The count was performed on 300 nonerythroid cells, and diagnosis of the microgranular variant subtype was established when hypergranular promyelocytes represented less than 50% of the leukemic cells [11,14]. The myeloid-to-erythroid ratio and the presence or absence of Auer rods was also determined in all cases.

Immunophenotyping Studies

Bone marrow samples were studied by flow cytometry using a panel of 22 monoclonal antibodies and a polyclonal anti-TdT antibody directly conjugated with fluorochromes. Each tube contained 1×10^6 nucleated cells in suspension in 100 μL of phosphate-buffered saline after adjustment. Cells were stained with 5 μL of 1:20 dilution of various combinations of fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-labeled monoclonal antibodies against the antigens listed in Table I. For TdT staining, cells were made permeable with the Fix & Perm Permeabilization Kit (Caltag Laboratories, Burlingame, CA). Negative controls were stained with isotype-matched ($IgG1/IgG2_a$) FITC/PE-conjugated immunoglobulins of irrelevant specificity (Becton Dickinson, San Jose, CA). Samples were incubated for 20 min at 4°C in the dark. After incubation, erythrocyte lysis was performed with FACS lysing solution (Becton Dickinson).

All samples were analyzed with a FACScan flow cytometer (Becton Dickinson), equipped with an argon ion laser with a wavelength of 488 nm, by collecting 10,000 ungated list-mode events per tube.

TABLE I. Conjugated Monoclonal Antibodies Used in This Study*

Antibody anti-	Clone	Antibody anti-	Clone
CD34-PE	8G12	CD22-PE	S-HCL-1
HLA-DR-FITC	L243	CD3-FITC	SK7
CD45-FITC	2D1	CD4-PE	SK3
CD117-PE[a]	104D2	CD8-FITC	SK1
CD13-PE	L138	CD5-PE	L17F12
CD33-PE	P67.6	CD2-FITC	S5.2
CD14-PE	MØP9	CD7-FITC	4HL
CD15-FITC	MMA	CD10-FITC	W8E7
CD11b-PE	D12	CD16-FITC	NKP15
CD42a-FITC	Beb1	CD56-PE	MY31
CD19-FITC	467	TdT-FITC[a]	HT-6
CD20-FITC	L27	KLH	X40/X39

*Becton-Dickinson, San Jose, CA, was the manufacturer of all the monoclonal antibodies unless otherwise specified.
[a]Dako, Glostrup, Denmark.

The cytometer was set up using standard operation procedures, and quality control was performed using the Calibrite Beads Kit (Becton Dickinson) for calibration and compensation. A gate was selected in the combination of forward scatter and side scatter, and analysis was performed on cells with the most appropriate promyelocyte gate (intermediate-to-high SSC and intermediate-to-high FSC) (Fig. 1A). Cell Quest software (Becton Dickinson) was used for data acquisition and analysis. Results were considered positive if 20% or more of the cells expressed a particular antigen.

Antigenic expression was considered to be homogeneous if distribution of the cells occupied up to one logarithmic decade on the scale of fluorescence intensity (Fig. 1B), otherwise it was considered heterogeneous (Fig. 1C). A blast cell subpopulation was defined as the existence of more than 10% of the gated cells with a phenotypically different cell subset. Typical expression of CD15/CD34 was considered as any combination of negativity or low-to-intermediate intensity expression for both markers, in such a way

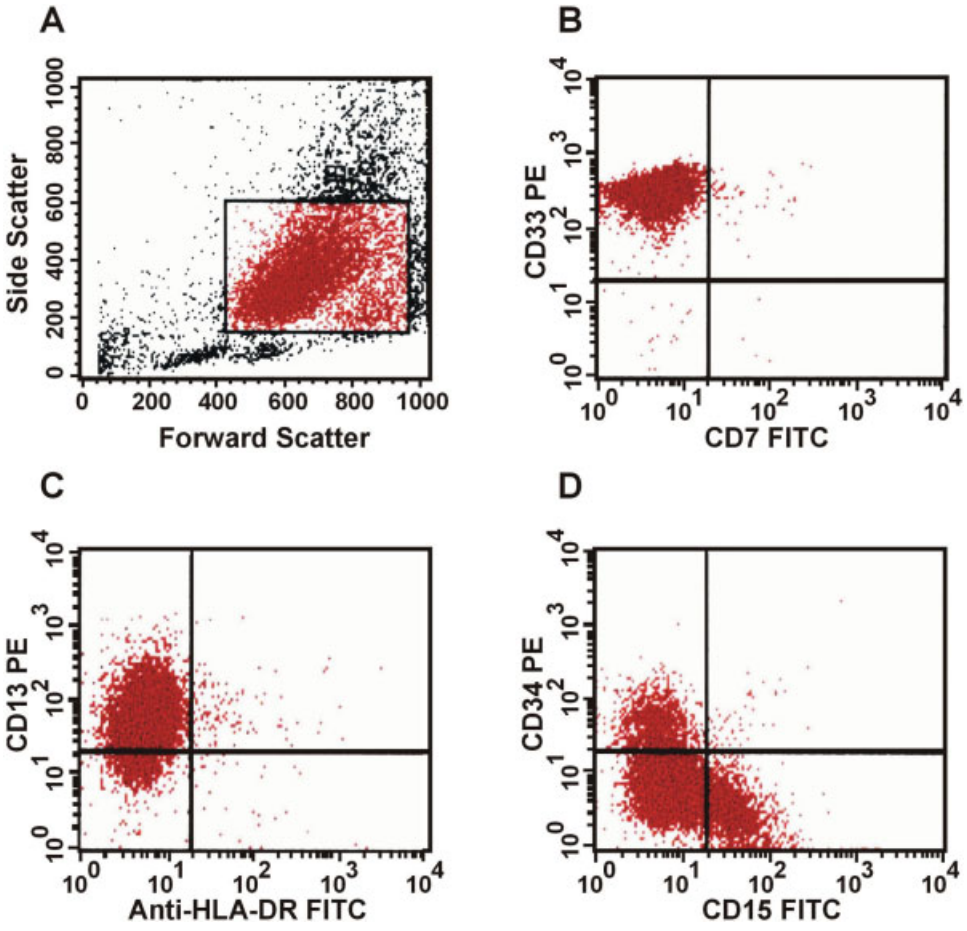

Fig. 1. Typical flow cytometry patterns in acute promyelocytic leukemia. Forward scatter (FSC) and side scatter (SSC) dot plot showing the gate strategy used in all cases (A). Typical homogeneous expression of CD33 (B) and heterogeneous expression of CD13 (C). CD15/CD34 dot plot showing low-intensity expression of both markers and absence of double positive cells (D). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

that each one of them never achieved a high expression and there never was a cell subpopulation expressing CD15 and CD34 simultaneously (Fig. 1D).

RT-PCR and Southern Blot Analyses of RARα Gene Rearrangements

RNA was extracted from bone marrow samples in all cases using the Trizol LS Reagent (Gibco BRL, Gaithersburg, MD) according to the manufacturer. The RT-PCR experiments for detection of the PML-RARα gene rearrangement were performed, with minor modification, by the methods described by Borrow et al. [16]. Briefly, reverse transcription was performed on 5 μg of total RNA after heating at 65°C for 5 min, with 10 units of M-MLV reverse transcriptase (Gibco BRL) in its furnished buffer, 0.5 μL of RNAguard RNase inhibitor (Amersham Pharmacia Biotech, Piscataway, NJ), and at 1.25 mM of each dNTP, for a final volume of 20 μL. Two samples were primed from 2.5 mM of *primer 3* for the PML gene or *primer 7* for the RARα gene in two different tubes. Samples were incubated at 42°C for 1 hr and at 52°C for 30 min; next, 5 μL of the reverse transcription product was diluted with 120 μL of 1× TE buffer, and aliquots of 10 μL of the dilution were used for PCR amplification. Polymerase chain reactions were performed with a PerkinElmer 2400 GeneAmp PCR System thermal cycler, using nested primers [16]. Each sample was submitted to three PCR assays: two for control amplification of normal PML and RARα cDNA and one for diagnostic amplification of the PML-RARα cDNA. This latter reaction was performed using the cDNA primed with the RARα *primer 7* in the reverse transcription. Each tube had a final volume of 25 μL, containing 10 μL of the cDNA pool, 2.5 μL of 10× Promega buffer, 2 μL of dNTP at the concentration of 5 mM, 1.5 μL of each external primer, 1 unit of Promega *Taq* polymerase (GIBCO, BRL), and dH_2O. Forty cycles of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C were run, followed by a final extension of 7 min at 72°C. A 2-μL aliquot of the product was used as the starting template for a second round of 40 cycles using the same conditions, but with the internal primers. Samples were separated by electrophoresis on 1.5% agarose gels with a 100-bp size ladder.

In order to determine the frequency of the two major PML-RARα isoforms, referred to as long (L) and short (S) transcripts, we used a second PCR strategy as described by van Dongen et al. [17]. The long transcript is generated when PML gene breakpoint (bcr) is located in intron 6 (bcr1) or exon 6 (bcr2), whereas the S transcript is produced when PML intron 3 (bcr3) is affected.

In those cases of APL in which the PML-RARα transcript was not detected by RT-PCR, we tested for rearrangement of the RARα gene by Southern blot analysis. DNA was extracted from frozen bone marrow cells using the Puregene Genomic DNA Purification Kit (Gentra Systems, Minneapolis, MN), digested with *Eco*RI and *Hin*dIII restriction enzymes, size-fractionated by 0.8% agarose gel electrophoresis, and transferred to nylon membranes. RARα genomic fragments (H18, X5′, and K3 were used as probes as described by Longo et al. [18]) were ^{32}P-labeled with the Rediprime II kit (Amersham Pharmacia, Little Chalfont, England) and allowed to hybridize according to established procedures [18]. The RNA from cases with rearranged RARα gene was submitted to RT-PCR for PLZF-RARα gene rearrangement using the primers and protocols described by Licht et al. [19]. Positive results were confirmed to be PLZF-RARα transcript by sequencing of the PCR product.

Statistical Analysis

Statistical analysis was performed using the GraphPad InStat software. The Mann–Whitney test was applied to raw data for comparison of medians. Clinical and laboratory data were plotted on a 2 × 2 contingency table, and the two-sided P value was calculated using Fisher's exact test. A P value of 0.05 or less was considered significant.

RESULTS

Some clinical and morphological findings for the 50 studied cases with the PML-RARα gene rearrangement are summarized in Table II. Thirty-four cases (68%) were classified as classical APL and 16 as the microgranular subtype (32%). There was no difference in age or in male-to-female ratio between both groups. The median percentage of hypergranular promyelocytes observed in the hypergranular APL was 92% (range 57–99%), and that observed in microgranular APL was 18% (range 10–43%). The myeloid-to-erythroid ratio and the percentage of Auer rods were similar for both subtypes.

The immunophenotypic profile of both PML-RARα APL subtypes was statistically indistinguishable concerning all antigens (Table III). In addition, the simultaneous immunophenotypic combination of a unique major cell population, heterogeneous intensity of expression of CD13, and the typical pattern of CD15/CD34 expression were similarly present in the hypergranular and microgranular APL (Table IV). Interestingly, homogeneous expression of CD33 was detected in 76% of hypergranular cases and in 100% of microgranular cases ($P = 0.04$).

TABLE II. Summary of Clinical and Morphological Data

	Classical APL	Microgranular APL	*P* value
No. of individuals	34	16	N/A[a]
Sex: M/F (ratio)	18:16 (1.1)	4:12 (0.3)	0.08
Age in years (range)	32 (7–72)	33 (6–60)	0.31
Children (age $\leq$ 15 years old)	7 (21%)	6 (37%)	0.30
Hypergranular promyelocytes (%)	92 (57–99)	18 (10–43)	<0.0001
Myeloid-to-erythroid ratio	10 (1–29)	15 (5–50)	0.06
Presence of Auer rods (%)	33 (97)	15 (94)	0.54

[a]N/A, not applicable.

TABLE III. Immunophenotypic Profile of Classical and Microgranular Variant Subtypes of Acute Promyelocytic Leukemia*

Antigen	Classical APL	Microgranular APL	*P*	Antigen	Classical APL	Microgranular APL	*P*
CD34	3/33 (9)	3/16 (19)	0.38	CD22	0/32 (0)	0/16 (0)	N/A[a]
HLA-DR	1/34 (3)	1/16 (6)	0.73	CD3	0/34 (0)	0/16 (0)	N/A
CD45	32/33 (97)	15/16 (94)	1.0	CD4	14/31 (45)	4/15 (26)	0.34
CD117	27/31 (87)	12/15 (80)	0.67	CD8	1/18 (6)	1/8 (13)	0.53
CD13	34/34 (100)	16/16 (100)	N/A	CD5	0/34 (0)	0/16 (0)	N/A
CD33	34/34 (100)	16/16 (100)	N/A	CD2	5/26 (19)	5/15 (33)	0.45
CD14	2/34 (6)	0/16 (0)	1.0	CD7	0/33 (0)	0/16 (0)	N/A
CD15	14/34 (41)	2/16 (13)	0.06	CD10	1/33 (3)	0/15 (0)	1.0
CD11b	2/33 (6)	2/16 (13)	0.59	CD16	1/21 (5)	1/12 (8)	1.0
CD42a	0/34 (0)	0/16 (0)	N/A	CD56	6/30 (20)	2/14 (14)	1.0
CD19	5/34 (15)	3/16 (20)	0.70	TdT	2/30 (7)	3/14 (21)	0.31
CD20	0/34 (0)	0/16 (0)	N/A				

*Values in parentheses represent percentages.
[a]N/A, not applicable.

TABLE IV. Comparison of Some Immunophenotypic Characteristics Highly Predictive of the PML-RARα Gene Rearrangement*

	Classical APL	Microgranular APL	*P* value
CD13 Heterogeneous	34/34 (100)	16/16 (100)	N/A[a]
One major blast cell population	32/34 (94)	16/16 (100)	1.0
Typical CD15/CD34 pattern	30/33 (91)	15/16 (94)	1.0
Negativity for HLA-DR	33/34 (97)	15/16 (94)	1.0
CD33 homogeneous	26/34 (76)	16/16 (100)	0.04

*Values in parentheses represent percentages.
[a]N/A, not applicable.

We have determined the frequency of PML bcr isoforms in 17 classical and 8 microgranular APL cases. Among the 17 classical APL cases, 4 presented PML bcr1, 2 bcr2, and 11 bcr3 isoforms, therefore 11 S- and 6 L-transcripts. On the other hand, in the eight microgranular APL cases, 1 presented the PML bcr1 and 7 the bcr3 isoform. The frequency of cases displaying the bcr3 isoform was not statistically significant between both subtypes ($P = 0.36$).

The BM morphological characteristics of the two PLZF-RARα cases were in conformity with the morphologic classification system described for APL underlying this gene rearrangement [20]. Both patients were male, at ages 14 and 52 years old. The percentages of hypergranular promyelocytes in BM were 9% and 5%, and the myeloid-to-erythroid ratios were 3:1 and 20:1, respectively. Blasts with round, regular nuclei were predominant. Pelger-like cells were observed in both cases, whereas Auer rods were observed only in one case. The immunophenotypic profile of the first case was $CD13^+CD33^+CD45^+$ and $HLA^-DR^-CD15^-CD34^-CD14^-CD2^-CD4^-CD8^-CD7^-TdT^-$. The second case was $CD13^+CD33^+CD11b^+CD56^+$ and $HLA^-DR^-CD15^-CD34^-CD117^-CD42a^-CD2^-CD7^-$. Both cases also displayed the simultaneous immunophenotypic combination described above for classical PML-RARα APL, including the homogeneous expression of CD33.

DISCUSSION

Morphology, cytochemistry, immunophenotyping, cytogenetics, and molecular genetics are the methods used for the diagnosis and classification of acute leukemias [21]. For treatment purposes, once the dichotomy between myeloid and lymphoid leukemias has been solved, the most important definition is if a given case of acute myeloid leukemia (AML) could be sub-classified as APL, since this subtype benefits from treatment with ATRA [6–9].

Although the use of morphology for initial diagnostic confirmation sounds appealing, there are some problems when only this method is used. In the recovery phase from acute agranulocytosis, for example, the bone marrow may be replenished with promyelocytes, displaying a picture closely similar to classical APL [12]. Microgranular APL may also be confused with other types of AML by morphology alone, mainly with acute myelomonocytic leukemia (FAB M4) or acute monocytic leukemia (M5) [13,14]. Furthermore, some groups have describe other morphological subtypes of APL with the PML-RARα gene rearrangement in addition to the classical and microgranular forms [22,23]. These rare subtypes (hyperbasophilic, M2-like, and M1-like) display a morphological picture quite different from classical APL and are difficult to distinguish from M1 or M2 AML [23,24].

Careful analysis of the bone marrow smear is the only method available for subclassification as classical or microgranular APL. Previous studies have reported the proportion of cases with the microgranular morphology to account for 15–20% of all APL [1], but when the analysis is restricted to children, this proportion may increase to 38% [25]. The high proportion of the microgranular subtype found in our study (32%) may be due to the presence of a significant number of children (13 cases were $\leq$15 years old). No differences were detected concerning age, male-to-female, or myeloid-to-erythroid (M/E) ratios, and the presence of Auer rods between classical and microgranular APL.

The microgranular variant has been previously associated with PML bcr3 isoform (PML-RARα S-type transcript) [25,26]. In the present study, although the majority of variant cases harbored the PML bcr3, the difference was not significant. This may be due to the small number of samples analyzed. It is interesting to point out that the frequency of PML bcr3 was higher than the one described in the literature [25,26].

Flow cytometry immunophenotyping with a large panel of monoclonal antibodies was performed in all cases. No statistically significant difference in the expression of any marker was detected between classical and microgranular APL. Only CD15 tended to be less expressed in microgranular APL ($P = 0.06$). Among ten studies selected to compare our data [14,25,27–34], no one had used so many different markers as we did. Five studies showed no difference in the antigenic expression of the tested markers between classical and microgranular APL [14,27–30]. A positive correlation of the expression of CD34 and/or of CD2 with the microgranular variant was described in five studies. Both CD34 and CD2 were shown to be positively associated with microgranular APL in two studies [25,31], only CD34 [32] in one study, and only CD2 in two other studies [33,34]. Nevertheless, CD34 and CD2 were similarly expressed in classical and microgranular APL in our study. We have no clear explanation for this discrepancy, which is not due to the cutoff criteria used to consider a given marker as positive, since it was defined as 20% of the leukemic cells in all studies. Neither does the size of our series explain the discrepancy, as 50 cases constitute a representative number of individuals concerning the study of APL. The Italian Cooperative Study [25] also analyzed, as we did, children and adults and included an expressive casuistic which showed a strong correlation of the expression of both CD34 and CD2 with microgranular APL. These findings raises the issue of a geographic difference in the biology of APL, in addition to the higher frequency of APL reported among Latinos with acute myeloid leukemia [35,36].

Orfao et al. have recently suggested a flow cytometric pattern of CD34, CD15, and CD13 expression as highly characteristic of the PML-RARα gene rearrangement [15]. However, their study included only four patients with microgranular APL. In the present investigation, we observed a heterogeneous intensity of expression of CD13, a unique major blast cell population, a typical CD15/CD34 pattern, and negativity for HLA-DR in a very high proportion of both classical and microgranular APL. In addition, homogeneous expression of CD33 was present in just 76% of the classical APL cases but in 100% of the microgranular APL cases ($P = 0.04$). However, the marginal statistical difference achieved would not obviate the necessity of a larger series to definitely conclude something about this trend.

We also studied two cases of t(11;17)/PLZF-RARα APL. Both displayed the suggestive morphologic features as describe by Sainty et al. [20], namely, predominance of blasts with regular nuclei and increased number of Pelger-like cells. Since the CD13, CD33, and CD15/34 immunophenotypic profile was similar in PLZF-RARα and PML-RARα cases, the precocious identification of the PLZF-RARα cases must be based on morphological grounds. Considering that the RARα moiety is the same in PML-RARα and in PLZF-RARα chimeric proteins, the detection of identical immunophenotypes in both variants suggest that

the leukemic phenotype depends on the retinoid pathway. In fact, Minnucci et al. demonstrated that the oligomerization of RARα transcription factor is the critical step for oncogenesis and that it occurs irrespectively of the RARα fusion partner [37]. Moreover, PML inactivation was demonstrated to increase the frequency and accelerate leukemia onset on the APL transgenic model but did not affect the leukemic phenotype [38].

In conclusion, the immunophenotypic features of classical and microgranular APL with the PML-RARα gene rearrangement are very similar, even when tested for a large panel of monoclonal antibodies. Furthermore, the antigenic combination highly characteristic of classical APL (a heterogeneous intensity of expression of CD13, a unique major blast cell population, a typical CD15/CD34 pattern of antigenic expression and negativity for HLA-DR) is also observed in microgranular and PLZF-RARα APL. These data may be useful in combination with the analysis of BM morphology as a rapid confirmatory diagnostic tool for classical APL and its variants.

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