Acute promyelocytic leukemia: four distinct patterns by flow cytometry immunophenotyping

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A total of 97 acute promyelocytic leukemia (APL) patients with adequate flow cytometry (FC) data, bone marrow aspirates and presence of t(15;17)/*PML-RARA* by cytogenetics and/or FISH studies were analyzed for immunophenotypic pattern. Leukemic cells had the following phenotype: $CD11b^{-}$, $CD11c^{-}$, $CD13^{+}$, $CD33^{+}$, $CD45^{+}$, $CD64^{+/-}$, $CD117^{+}$, and $HLA\text{-}DR^{-}$. A subset of cases showed also an expression of CD2, CD4, CD34, and CD56. Based on the immunophenotype and side scatter properties (SSC), four FC patterns were recognized. The majority of cases represented classical (hypergranular) APL and were characterized by high SSC, positive CD117, lack of CD34, heterogeneous CD13, and bright CD33 (pattern 1). Second most common type, corresponding to the hypogranular (microgranular) variant of APL differed from classical APL by low SSC and frequent co-expression of CD2 and CD34 (pattern 2). Rare cases of APL (pattern 3) showed a mixture of neoplastic cells (low SSC/$CD2^{+}$/$CD13^{+}$/$CD33^{+}$/$CD34^{+}$/$CD117^{+}$) and prominent population of benign granulocytes/maturing myeloid precursors (high SSC/$CD10^{+/-}$/$CD16^{+/-}$/$CD117^{-}$). One case showed two APL populations, one with hypogranular and one with hypergranular characteristics (pattern 4). Apart from a well-known FC pattern of hypergranular APL, we presented less common immunophenotypic variants of APL, which helps to identify an additional group of patients who would benefit from fast confirmatory FISH and/or PCR testing for t(15;17)/*PML-RARA*.

Key words: flow cytometry, leukemia, acute promyelocytic leukemia, immunophenotyping.

Introduction

Acute myeloid leukemia (AML) represents a heterogeneous group of disorders with variable clinical presentation, cellular morphology, immunophenotype, therapeutic response and overall prognosis. Acute promyelocytic leukemia (APL, AML-M3) is a distinct subtype of AML with characteristic cytomorphology, maturation arrest at the promyelocytic stage of granulocytic differentiation and t(15;17)/*PML-RARA* that responses to maturation inducing treatment with all trans-retinoic acid (ATRA) [1-11]. Because of tendency for disseminated coagulopathy, a typical and life-threatening complication of APL, establishing correct diagnosis of APL in a timely fashion is critical in management of patients with APL. Characteristic translocation between the long arms of chromosomes 15 and 17, which fuses the promyelocytic leukemia gene (PML) on chromosome 15 to the retinoic acid receptor-α (RARA) gene on chromosome 17 resulting in the chimeric gene encoding PML/RARA fusion protein [1]. The characteristic cytomorphology and immunophenotype allow for correct identification of cases suggestive of APL, leading to mandatory chromosomal/molecular testing, either by fluorescence in situ hybridization (FISH) or by reverse transcriptase polymerase chain reaction (RT-PCR) for definite confirmation of APL diagnosis [5, 8-10, 12, 13]. Here we present four distinct subtypes of APL as defined by flow cytometric analysis.

Material and methods

A total of 97 APL patients evaluated between 2007 and 2008 with adequate flow cytometry (FC) data, bone marrow aspirates and presence of t(15;17)/*PML-RARA* by conventional cytogenetics and/or fluorescence in situ hybridization (FISH) studies were included in this study. Cases negative for t(15;17)/*PML-RARA* or cases in which the cytogenetics/FISH results were not available were excluded. Only cases with a new diagnosis of APL from untreated patients were included. At the time of original sample processing and analysis, we used heparinized bone marrow (BM) aspirate and blood, and processed the specimens within 24 hours of collection. We obtained a leukocyte cell suspension from blood and BM specimens after red blood cell (RBC) lysis with an ammonium chloride lysing solution for 5 minutes, followed by 5 minutes of centrifugation. The cell pellet was suspended with an appropriate amount of RPMI 1640 (GIBCO, New York). To minimize nonspecific binding of antibodies, we incubated the cells in RPMI media supplemented with 1% heat-inactivated fetal bovine serum (FBS) in a 37°C water bath for 30 minutes. The samples were washed with 0.1% sodium azide/1% FBS phosphate-buffered saline (PBS) buffer and assessed for viability using either trypan blue or 7-aminoactinomycin D (Sigma Chemical Co., St. Louis, Missouri) exclusion assays. Immunophenotypic analysis was performed on Becton Dickinson Immunocytometry System FACS Canto instruments (San Jose, California) using conventional methodology with 6-color directly labeled combinations of antibodies (used at a saturating concentration). Internal negative controls within each tube and controls for immunoglobulin G1 (IgG1), IgG2a and IgG2b were used as isotypic (negative) controls. The following parameters and immunophenotypes were analyzed by flow cytometry (FC): forward scatter (FSC), orthogonal side scatter (SSC), HLA-DR, CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD11b, CD11c, CD13, CD14, CD16, CD19, CD20, CD33, CD34, CD45, CD56, CD64, and CD117. Since leukemic cells in APL have a tendency for a high level of non-specific fluorescence, FC evaluation of antigen expression was carefully correlated in each case with a battery of isotypic negative controls for each fluorochrome used in the analysis, to insure identification of positive versus negative antigen expression.

Results

Ninety-seven cases of APL with t(15;17)/*PML-RARA* confirmed by conventional cytogenetics and/or FISH studies were analyzed for FC immunophenotypic features. The age of patients ranged from 17 to 81 years (average 52.5). There were 55 men and 42 women. Seventy-three cases (~74%) were characterized by high SSC (pattern 1; classic APL), 20 cases (~21%) had low SSC (pattern 2; hypogranular APL), 3 cases (~3%) showed leukemic cells and separate population of benign (residual) granulocytes/maturing myeloid precursors (pattern 3; partial involvement) and 1 case (~1%) showed two separate populations of leukemic cells, one with high SSC and one with low SSC (pattern 4; mixed classic/hypogranular APL).

Classic APL (pattern 1) was characterized by predominant population of atypical promyelocytes with markedly increased SSC (leukemic cells distributed in "granulocytic" gate on CD45 versus SSC dot plot display). Neoplastic cells were positive for CD13 (~93%), CD33 (100%), CD64 (~57%), CD117 (100%), and negative for HLA-DR, CD10, CD11b, CD11c, and CD14 (see Table I for details). A subset of cases was positive for CD2 (~19%), CD4 (~23%), CD34 (~9%), and CD56 (~16%). The expression of CD13 was dim to moderate, expression of CD33 was bright and expression of CD64, if present, was dim. Figure 1 presents a typical example of classic APL (pattern 1).

Hypogranular (microgranular) APL (pattern 2; Fig. 2) was characterized by low SSC and moderate expression of CD45 (leukemic cells distributed in the

Table I. Immunophenotypic profile of acute promyelocytic leukemia (hypergranular variant); n = 73

MARKER	FREQUENCY (%)	COMMENTS
Side scatter		markedly increased ("granulocytic" region)
$CD2^+$	3.1	
$CD4^+$	22.9	dim expression
$CD7^+$	1.4	
$CD11b^+$	0	
$CD11c^+$	0	
$CD13^+$	93.1	dim 31.5%; dim to moderate 8.2%; moderate 57.5%
$CD14^+$	0	
$CD16^+$	0	
$CD19^+$	0	
$CD33^+$	100	moderate 15.1%; bright 84.9%
$CD34^+$	4.1	4 additional cases (5.5%) showed dim expression on subset of neoplastic cells
$CD45^+$	100	moderate expression
$CD56^+$	16.4	
$CD64^+$	57.5	dim expression
$CD117^+$	100	moderate expression
HLA-DR^+	0	

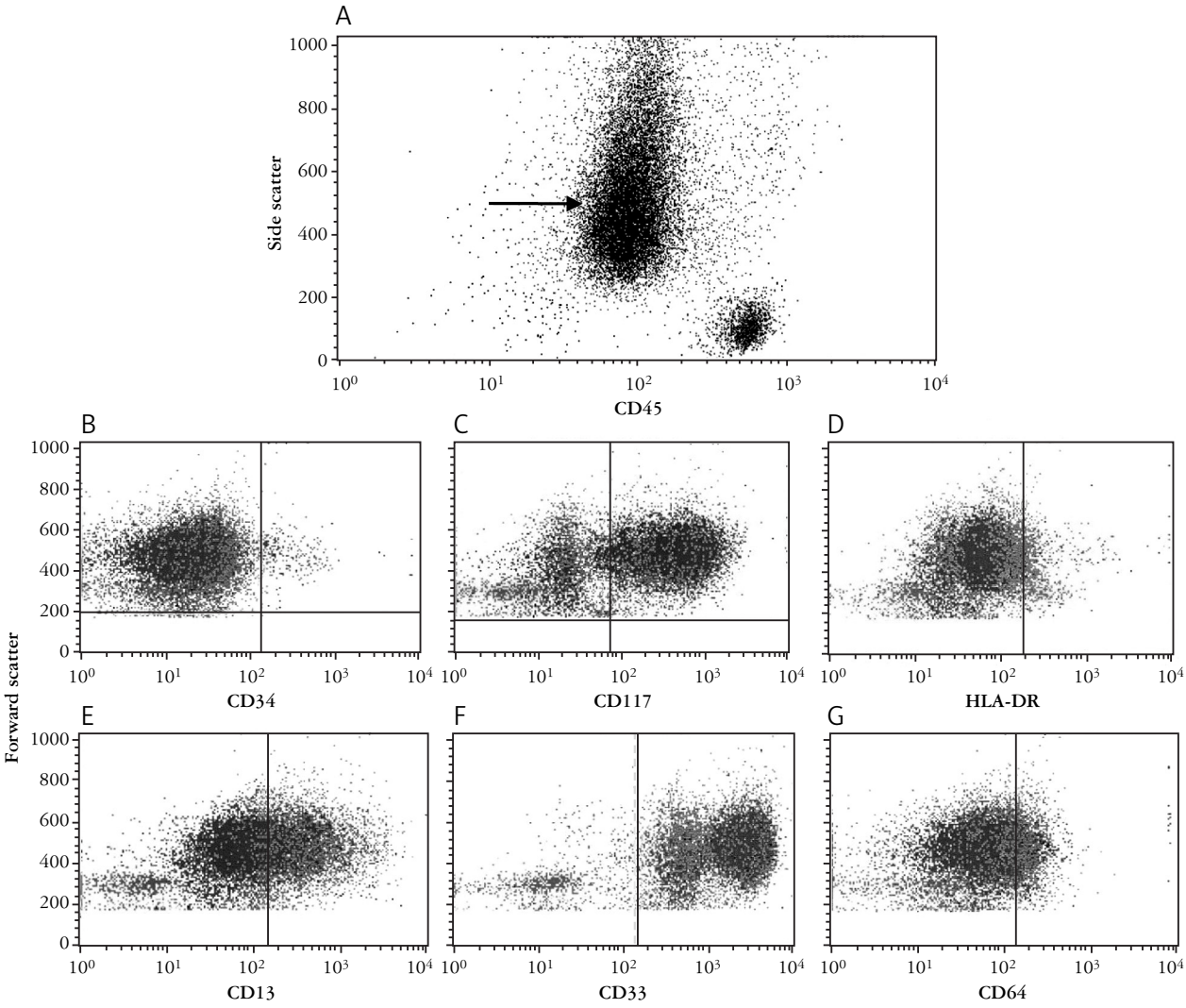

Fig. 1. Flow cytometry of classic APL (pattern 1): leukemic cells (arrow) have characteristic high SCC (A); they are negative for CD34 (B) and HLA-DR (D), and are positive for CD117 (C), CD13 (dim expression; E), and CD33 (bright expression; F). CD64 is negative to dim on subset (G)

"blast" region on CD45 versus SSC dot plot display, similar to blasts in non-APL acute myeloid leukemias). Neoplastic cells in this variant were positive for CD2 (80%), CD4 (30%), CD13 (95%), CD33 (100%), CD34 (75%), and CD117 (100%), whereas HLA-DR, CD10, CD11b, and CD11c were always negative (see Table II for details).

The third pattern by flow cytometry showed a mixture of neoplastic promyelocytes with decreased side scatter and expression of CD117 (Fig. 3; green dots) and a significant proportion of benign granulocytes with typical high SSC (Fig. 3; gray dots), negative CD117, and partially positive for CD10, CD11b, and CD16. The immunophenotype of neoplastic cells in pattern 3 was similar to that observed in hypogranular APL. The least common immunophenotypic variant of APL (pattern 4) showed two neoplastic populations, one with high SSC and the other with low SSC. Both populations were positive for CD117 and negative for HLA-DR and CD11c. The cells with high SSC expressed CD13 (dim), CD33 (moderate), and CD64 (dim), and cells with low SSC had brighter CD45 and were positive for CD34 and CD2 (Fig. 4). This pattern was seen in one case.

Discussion

Immunophenotyping by flow cytometry plays an important role in the diagnosis and subclassification of acute leukemias. Based on side scatter and phenotypic characteristics of blasts FC allows for differentiating between major types of acute leukemias (e.g. minimally differentiated AML versus ALL, acute monoblastic leukemia versus NK-cell lymphoma/leukemia or B-ALL versus T-ALL), suggests specific diagnoses such as acute promyelocytic leukemia or acute monoblastic leukemia, and helps to monitor patients after treatment [14-32]. Classic APL has a well-recognized flow cytometric pat-

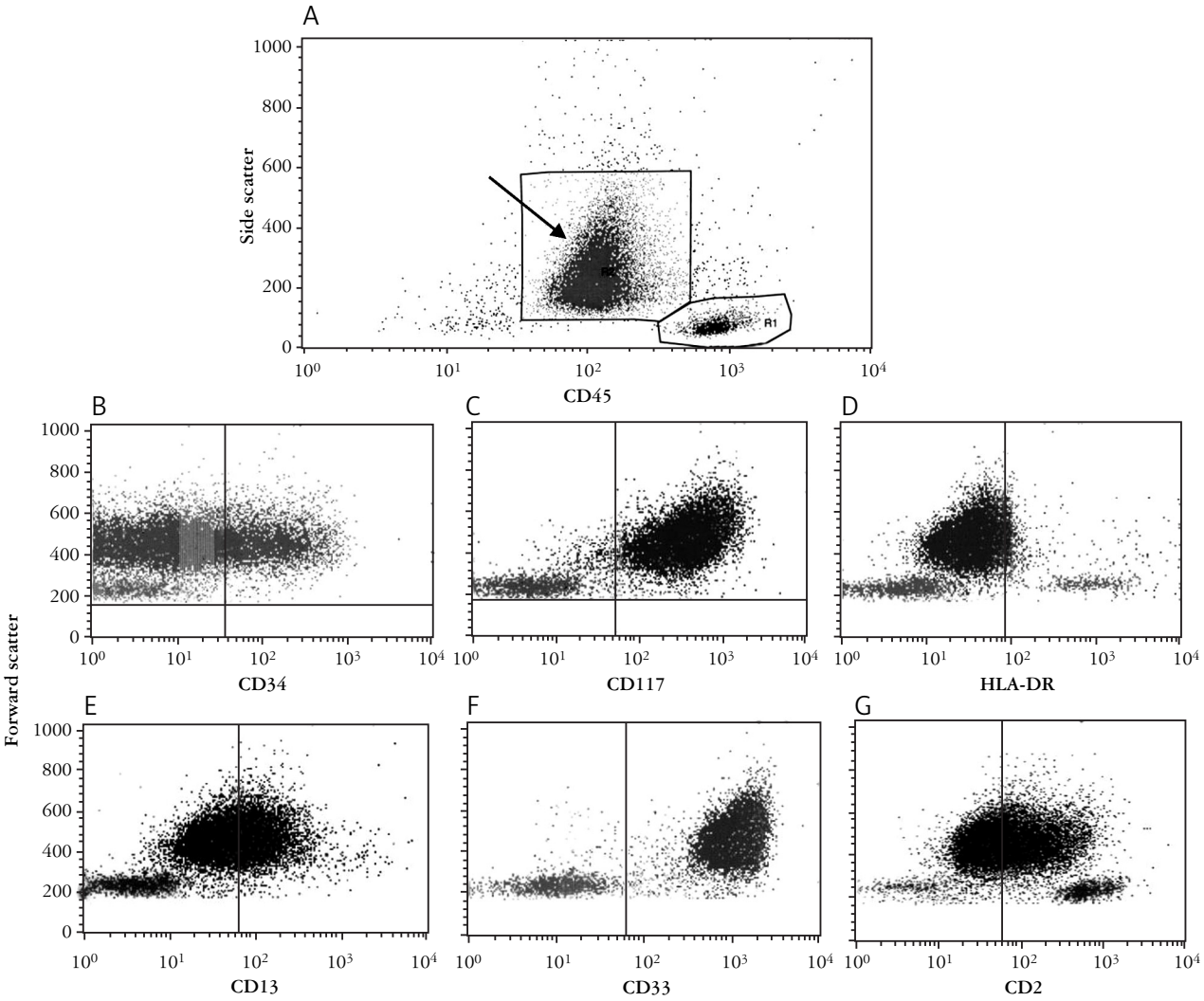

Fig. 2. Flow cytometry of hypogranular APL (pattern 2): leukemic cells (arrow) have low SCC (A); they are partially positive for CD34 (B), and strongly positive for both CD117 (C) and CD33 (F). HLA-DR is negative (D). CD13 (E) and CD2 (G) are positive but dimly expressed

tern with increased side scatter, lack of expression of HLA-DR, CD11a, CD11b, CD18, positive CD117, negative or weakly positive CD15 and CD65, negative CD34, often positive CD64, variable (heterogeneous) CD13 and bright CD33 [31, 33-37]. Kussick *et al.* described HLA-DR-/CD34- phenotype in AML with normal karyotype by conventional cytogenetics and association with the *FLT-3* gene internal tandem duplication [38]. Albano *et al.* reported the association of CD34 expression with the hypogranular APL variant and a higher proportion of $CD2^+$ and $HLA\text{-}DR^+$ cases [39]. In the same study, $CD34^+$ APL patients had a significantly higher percentage of peripheral blood leukemic promyelocytes at presentation, were more frequently female and had a higher proportion of bcr3 expression, but there were no differences between the two groups in terms of complete remission, overall survival and disease-free survival [39].

In our series of 97 cases, acute promyelocytic leukemia (APL) with t(15;17)/*PML-RARA* had the following phenotype: $CD11b^-$, $CD11c^-$, $CD13^+$, $CD33^+$, $CD45^+$, $CD64^{+/-}$, $CD117^+$, and $HLA\text{-}DR^-$. A subset of cases showed also an expression of CD2, CD4, CD34, and CD56. The majority of cases were characterized by high SSC, positive CD117, lack of CD34, heterogeneous ("smeary") CD13, and bright CD33. This immunophenotype (pattern 1) represents classical (hypergranular) APL. Second most common type, representing a hypogranular (microgranular) variant of APL differed from classical APL by low SSC and frequent co-expression of CD2 and CD34 (pattern 2). Rare cases of APL (pattern 3, partial involvement) showed a mixture of neoplastic cells (low SSC/$CD2^+$/$CD13^+$/$CD33^+$/$CD34^+$/$CD117^+$) and prominent population of residual granulocytes/maturing myeloid precursors (high SSC/$CD10^{+/-}$/$CD16^{+/-}$/$CD117^-$).

Table II. Immunophenotypic profile of acute promyelocytic leukemia (hypogranular variant); n = 20

MARKER	FREQUENCY (%)	COMMENTS
Side scatter		low ("blastic" region)
$CD2^+$	80	dim expression
$CD4^+$	30	dim expression
$CD7^+$	0	
$CD11b^+$	0	
$CD11c^+$	0	
$CD13^+$	95	dim 30%; moderate 65%
$CD14^+$	0	
$CD16^+$	0	
$CD19^+$	0	
$CD33^+$	100	moderate expression 10%; bright expression 90%
$CD34^+$	75	5 cases (25%) were completely negative; 5 cases (25%) had moderate CD34, 1 case had variable ("smeared") expression, 4 cases (20%) showed dim to moderate expression and the remaining 4 cases (20%) showed dim expression on all blasts (2 cases) or significant subset of blasts (2 cases)
$CD45^+$	100	moderate expression
$CD56^+$	20	
$CD64^+$	80	dim expression 55%, moderate expression 25%
$CD117^+$	100	moderate expression
HLA-DR^+	0	

One case showed two APL populations, one with hypogranular and one with hypergranular characteristics (pattern 4).

In flow cytometry analysis, differential diagnosis of hypergranular APL (pattern 1) includes normally maturing myeloid cells, bone marrow with myelodysplasia, chronic myeloproliferative neoplasms with myeloid leftward shift, benign marrow proliferations (e.g. recovering marrow after treatment), occasional cases of acute monoblastic leukemia and rare cases of AML with maturation, which have high SSC and lack HLA-DR expression. Differential diagnosis of hypogranular APL (pattern 2) includes acute myeloid leukemia with or without maturation, acute monoblastic leukemia and MDS with prominent dysgranulopoiesis (e.g. hypogranular cytoplasm). Blasts in non-APL acute myeloid leukemia usually have low SSC and are positive for HLA-DR, whereas atypical hypergranular promyelocytes in APL have high SSC (they are located in the same area as normal granulocytes on CD45 versus SSC dot plot display; Fig. 5) and lack the expression of HLA-DR. Rare cases of AML with or without maturation may be HLA-DR negative, but they differ from hypogranular APL, by positive CD11c and negative CD2. Rare cases of AML with maturation may be characterized by high SSC ("granulocytic" gate); those blasts may lack CD34 and CD117 expression.

The expression of HLA-DR, CD11b and CD11c helps to differentiate phenotypically acute monoblastic leukemia (HLA-DR^+/$CD11b^{+/-}$/$CD11c^+$) from the microgranular variant of APL (HLA-DR^-/$CD11b^-$/$CD11c^-$). Moreover, monoblasts may show a positive, often variable ("smeary") expression of CD14, and positive CD10, CD16 and/or CD23 (those markers are negative in APL). Only rare cases of acute monoblastic leukemia are HLA-DR^-. Both APL and acute monoblastic leukemia express CD64, but the expression is usually dim in APL and bright in acute monoblastic leukemia. Acute monoblastic leukemia often is $CD56^+$, whereas CD56 is only rarely expressed in APL.

Analysis of CD11b versus HLA-DR (Fig. 6) and CD10, CD16 and CD117 (Fig. 7) distinguishes a benign process from APL. Neutrophilic maturation from blasts through promyelocytes, myelocytes, metamyelocytes, bands and neutrophils is characterized by loss of CD34 and HLA-DR expression at the promyelocytic stage and loss of CD117 expression at the myelocytic stage, and acquisition of CD11b and CD11c expression at the myelocytic stage and CD10 expression by neutrophils [40]. CD64 is expressed by promyelocytes through metamyelocytes. Granulocytes/maturing myeloid precursors with dyspoiesis (e.g. MDS) and/or leftward shift (e.g. CML) may display aberrant down-regulation of CD10, CD11b and CD16, but in contrast to neoplastic promyelocytes lack CD117 expression and are (at least partially) $CD11c^+$.

Flow cytometry plays an important role in identifying cases highly suggestive of APL, which allows for immediate reflex testing by FISH and/or PCR for t(15;17)/*PML-RARA* for final confirmation of the diagnosis. Apart from a well-known flow cytometric pattern of hypergranular APL, we presented less common immunophenotypic variants of APL. Awareness of the unusual flow cytometric pattern of APL may help to identify an additional group of patients who would benefit from fast confirmatory FISH and/or PCR testing.

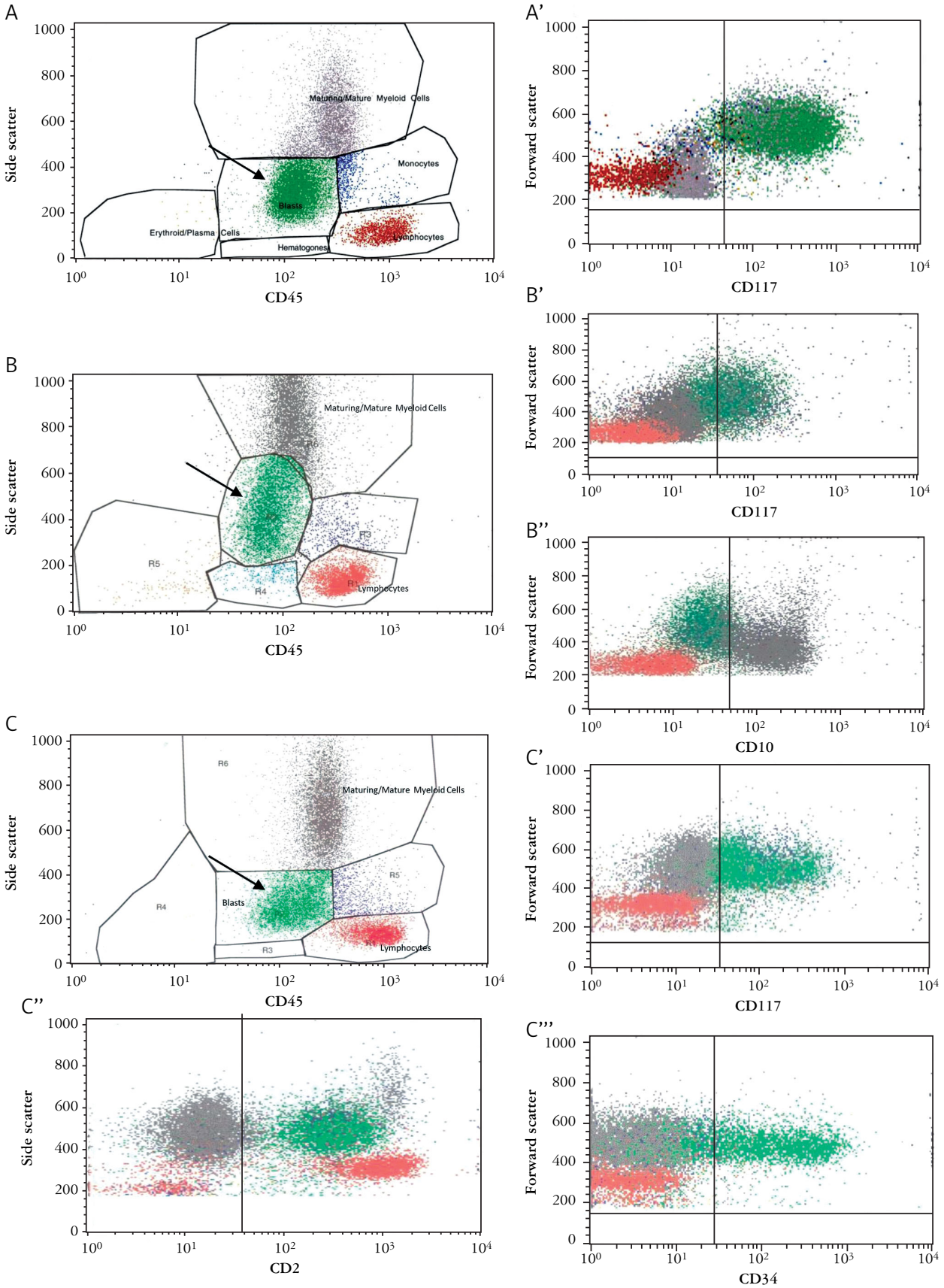

Fig. 3. Flow cytometry of APL displaying partial involvement (pattern 3), three cases (A, B, and C). Leukemic cells have low SCC (green dots, arrow; A, B, C) and are positive for CD CD117 (A', B' and C'), CD2 (C'') and CD34 (C'''), as seen in hypogranular APL (APLv). In contrast to typical APLv, there is a significant admixture of normal (benign) maturing myeloid precursors and granulocytes (gray dots; A, B, C), which are negative for CD117 (A', B', C'), but express CD10 (B'')

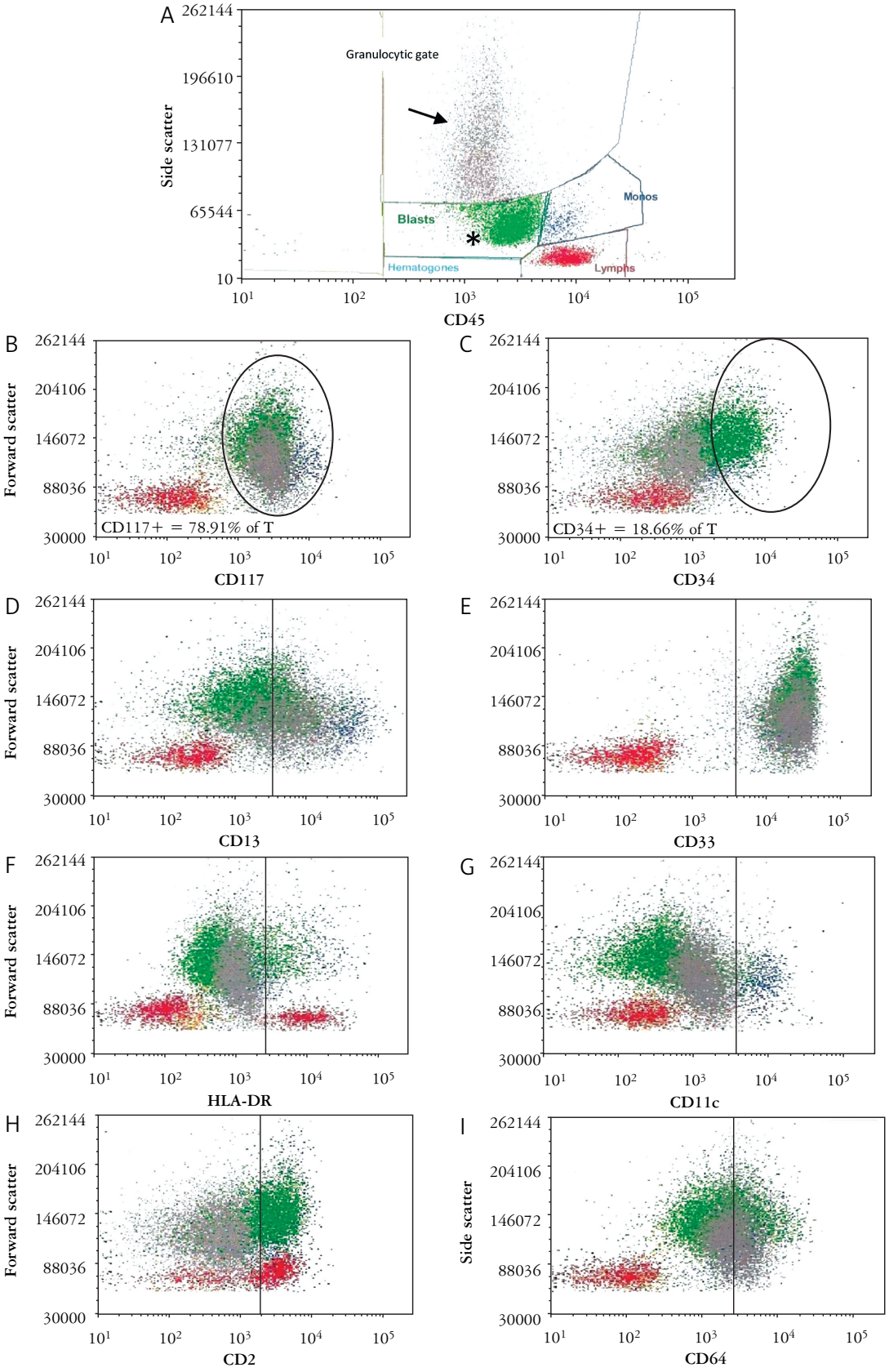

Fig. 4. Flow cytometry of APL, mixed variant (pattern 4). In this rare immunophenotypic variant of APL, two populations of leukemic cells are identified (A): one with high SSC (gray dots; arrow) and one with low SSC and brighter CD45 (green dots; *). Both populations are positive for CD117 (B), but only cells with low SSC express CD34 (C) and CD2 (H). CD13 and CD64 (I) are dimly expressed (D), and CD33 expression is bright (E). Both populations are negative for HLA-DR (F) and CD11c (G)

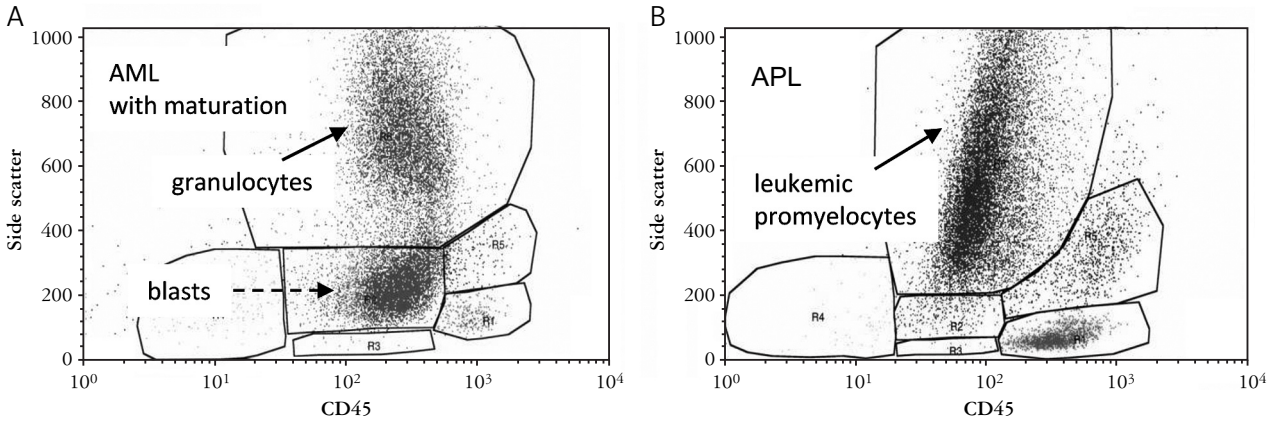

Fig. 5. Side scatter (SSC; orthogonal, right angle scatter) on Y axis corresponds to granularity of the cytoplasm (X axis presents CD45 expression). Neutrophils (A; arrow) and atypical promyelocytes in APL (B, arrow) have high SSC, whereas blasts in AML with maturation have low SSC (A, dotted arrow)

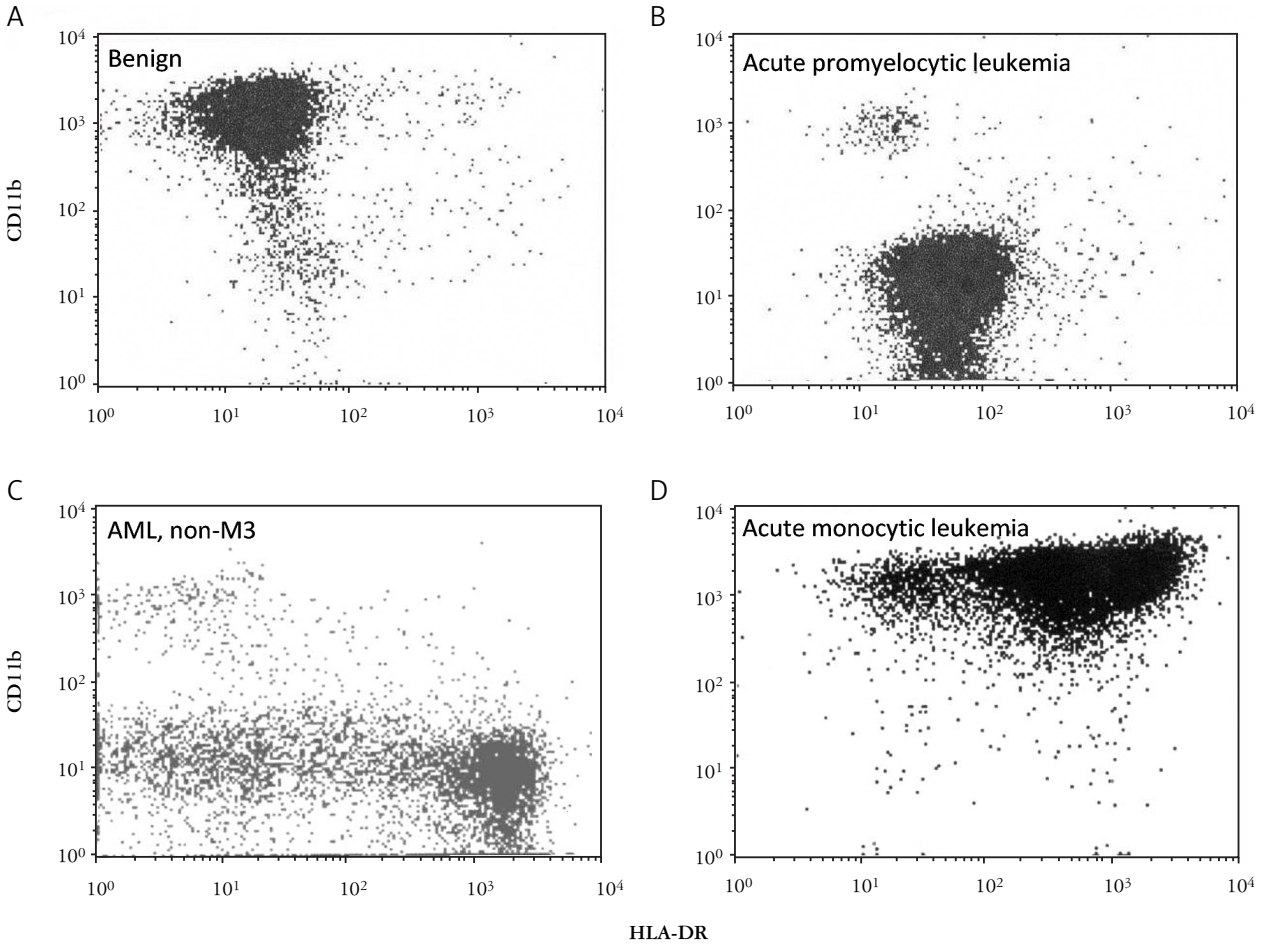

Fig. 6. APL – differential diagnosis: comparison of the expression of CD11b and HLA-DR in APL, non-M3 AML and acute monocytic leukemia. (A) Normal (control) sample. Granulocytes are brightly positive for CD11b and negative for HLA-DR. (B) APL: Promyelocytes lack the expression of CD11b and HLA-DR. (C) AML-non M3; Blasts are strongly positive for HLA-DR and negative for CD11b. (D) Monoblasts are positive for both CD11b and HLA-DR

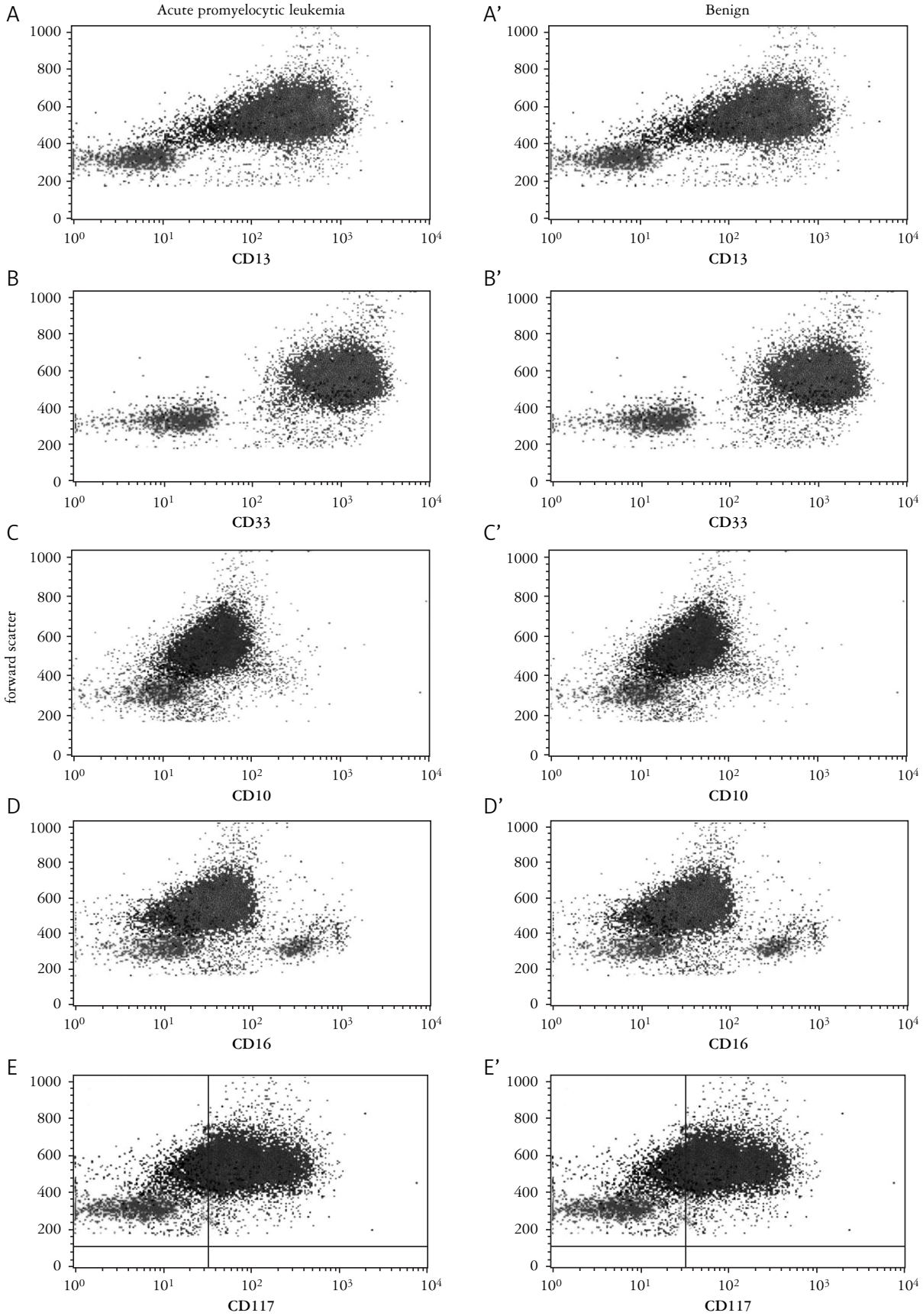

Fig. 7. APL – differential diagnosis: flow cytometric differences between promyelocytes (left column) and granulocytes (right column). Promyelocytes and granulocytes are positive for CD13 and CD33 (A, B). In contrast to granulocytes, promyelocytes are negative for CD10 (C, compare with C') and CD16 (D, compare with D'), and are positive for CD117 (E, compare with E')

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