Phenotypic quantitative features of patients with acute myeloid leukemia*

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The recent WHO classification for acute myeloid leukemias (AML) separates entities by recurrent cytogenetic abnormalities and immunophenotypic features presenting prognostic impact. We have examined the expression of several lineage and maturation linked antigens used in routine immunophenotyping of patients with de novo AML, using a 3-color two-step panel. Cases were diagnosed by peripheral blood counts, bone marrow cytology, cytochemistry, cytogenetics and immunophenotyping (CD2, CD3, CD7, CD19, CD13, CD33, myeloperoxydase – MPO, CD14, CD15, HLA-DR, CD34, CD56 and CD45). Antigen expression was measured by mean fluorescence intensity (MFI) by flow cytometry (Paint-a-gate software). Thirty five patients were analyzed. Median age: 51 years (15–79). Predominant FAB types were M2 and M4. In 6 cases more than one phenotypically distinct blast subpopulation could be detected. Although our set was small, we tried to analyze the impact of MFI of the examined antigens on the overall survival of the patients. In Cox univariate analysis, age, peripheral leukocytes (WBC) at diagnosis, MFI of CD45, and MPO were significant for worse a survival. In the multivariate analysis only MFI of CD45 and WBC remained in the model (p=0.018 and p=0.014 respectively). After bootstrap resampling, MFI of CD45 entered the model in 69%, WBC in 60%, age in 42% and MFI of MPO in 35% of the sets. Analysis of antigen expression by MFI permitted to detect cases presenting phenotypically distinct blast subpopulations. This may represent a pitfall in studies of minimal residual disease by flow cytometry, as chemotherapy may select one of these subsets.

Key words: acute myeloid leukemia, immunophenotype, flow cytometry, antigen expression, sub-populations

One of the first well established applications of flow cytometry was immunophenotyping of acute leukemias [1]. This technique helps to disclose the lineage of origin and the degree of maturation of leukemic blasts, besides detection of aberrant expressions. However, there have been many controversies in the literature concerning the optimal panel to be used for the diagnosis of entities that receive different treatment [2–14]. Usually, the panel includes lineage-associated antigens such as CD13, CD33, myeloperoxydase (MPO) and CD14, those associated with immature precursors (CD34, CD117, TdT, etc) or more mature cells (CD15, CD65, etc), as well as those observed in lymphoid cells and considered as aberrant expressions (CD2, CD19, etc).

Usually leukemic blasts have been considered positive for a given antigen if at least 20% of the cells are positive. This has produced some misleading results, especially if a two-color analysis is performed. Therefore, more recently, at least a three-color analysis has been recommended, in order to separate blasts electronically [1–3]. Few reports have analyzed AML blasts according to intensity of antigen expression by the mean fluorescence intensity (MFI) and the presence of subpopulations [9].

Immunophenotyping can be a very expensive technique if many antibodies are used. In the present study we try to apply a two-stage three-color platform using few monoclonal antibodies for the diagnosis of AML using the main antigens that can detect lineage and maturation stage, as well as the most common known aberrant expressions. The antigen expression was analyzed by the Paint-a-gate software looking for the MFI of each marker. The influence in overall survival of antigen expression was compared to that of well-established prognostic parameters in AML.

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Material and methods

Patients. From August 2002 through April 2004, all adult untreated patients with acute leukemia diagnosed at the Hematology Service of the State University of Campinas were enrolled in this study. Diagnosis was based on peripheral blood counts, bone marrow cytology (Wright-Giemsa-stained smears and cytochemical stains: myeloperoxidase, Sudan black B, and alpha-naphtyl-acetate-esterase), cytogenetics and immunophenotyping by flow cytometry using a two-step protocol (Tab. 1). Blasts were gated in the CD45/SSC gate. Classification of the cases was made by FAB criteria. Patients were treated by the TAD Protocol from the German AML Cooperative Group [10, 15, 16]. This study was approved by the Hospital's Ethics Committee.

Table 1. Panel of monoclonal antibodies used

Screening Panel
CD19 FITC/CD3 PE/ CD45 PerCP
CD7 FITC/ CD33 PE/ CD45 PerCP
HLA-DR FITC/ CD13 PE/ CD45 PerCP
Second Panel for AML
CD56 FITC/ CD14 PE/ CD45 PerCP
CD15 FITC/ CD34 PE/ CD45 PerCP
CD2 FITC/ CD45 PerCP
MPO FITC/ CD45 PerCP

Cytogenetic analysis. Cytogenetic procedures were carried out on pretreatment fresh bone marrow samples. The chromosome analysis was performed after 24 hours of short-term culture according to standard methods. In each case, at least 20 mitoses were analyzed and the karyotypes were reported according to the International System for Human Cytogenetic Nomenclature. The cytogenetic abnormalities detected were classified in risk groups as described in the WHO classification of acute leukemias [17].

Flow cytometric analysis. EDTA anticoagulated bone marrow aspirates were processed within 24 h after collection using a whole blood lysis technique. Peripheral blood specimens were diluted to a concentration of $5x10^6$/ml, if necessary. Bone marrow samples were washed once in RPMI 1640 culture medium (Cultilab, Campinas, SP, BR) and re-suspended to a concentration of $5x10^6$/ml and $1x10^6$/ml for cytoplasmatic staining. One hundred microliters of the cell suspensions were incubated with 5 µl of each fluorochrome-conjugated monoclonal antibodies at room temperature in the dark for 20 minutes. Specimens were lysed using 2 ml of FACS Lysing Solution (Becton Dickinson, San Jose, CA, USA) diluted 1/10 in distilled water and another incubation for 10 minutes at room temperature in the dark was performed. Then, cells were centrifuged and the cell pellet washed in 2 ml of phosphate buffered saline (PBS) per tube. Finally, the cells were ressuspended in 1 ml/tube of PBS until the analysis was performed. Cytoplasmatic MPO, anti-Kappa and anti-Lambda were assessed on paraformaldehyde (4% in PBS) – fixed and Tween 20 (0.5% vol/vol in PBS) – permeabilized cells. Antigenic expression was detected using triple combinations of MoAbs (Tab. 1) conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE) and peridin clorophyll protein (PerCP) fluorocrome: CD2 FITC, CD3 PE, CD7 FITC, CD13 PE, CD14 PE, CD15 FITC, CD19 FITC, CD20 PE and HLA-DR FITC (BD Biosciences Pharmingem, San Diego, CA, USA) and CD10 FITC, CD19 PerCP, CD34 PE, CD45 PerCP, CD56 FITC, myeloperoxydase (MPO) FITC, KAPPA FITC, LAMBDA FITC, negative controls IgG1 FITC/IgG1 PE and IgG1 FITC (BD Biosciences Immunocytometry Systems, San Jose, CA, USA).

The cases were screened by the first panel and further characterized by the second panel for AML or B-ALL or T-ALL. In all cases an isotype-matched negative control was used.

Data acquisition was performed on a FACSCallibur (Becton Dickinson, CA, USA) flow cytometer using CellQuest™ software (Becton Dickinson). At least 10,000 ungated list mode events were collected and analysis was performed by selecting an appropriate gate on the leukemic blasts on dim CD45 versus low side scatter on the screening panel and AML panel [18]. The data analysis was performed using Paint-A-Gate™ software (Becton Dickinson). The expression of each antigen was recorded as MFI in arbitrary units.

Statistical analysis. Correlations were examined by the Spearman rank test. The influence of categorical variables on overall survival was tested by the Kaplan-Meier method. Then, age, peripheral blood counts, and MFI for the several antigens were analyzed in Cox proportional hazard model ($p=0.05$ for input and $p=0.10$ for output, backward conditional step-wise selection). Finally the stability of the Cox model was tested by bootstrap resampling [19–23]. This is a useful procedure to test the internal stability of the model proposed. It consists of creating new data sets of equal size by random sampling of the original data with replacement. In an individual new bootstrap sample, a patient may be represented once, multiple times or even not at all. A new Cox regression model (with the same conditions as in the original data set) is then calculated for each of these new data sets in order to obtain the bootstrap parameter estimates. This procedure is very useful in order to point out the most important variables. SPSS 8.0 and WinStat software were used for calculations.

Results

During the period of the study, 35 patients were included. Clinical and hematological data are listed on Table 2. Median age was 51 years. FAB types are listed on Table 3. FAB-M2 and FAB-M4 were the most common types. Among the 25

cases in which karyotype was conclusive 7 were low risk, 13 were intermediate and 5 were high risk.

In all cases, the panel proposed was sufficient for the diagnosis of AML. In 29 patients, only one blast population could be individualized in the Paint-a-gate software. In 5 cases however, 2 phenotypically distinct subpopulations could be individualized. Most of these cases were FAB-M4 (Tab. 3). The case of erythroid leukemia presented 3 distinct subpopulations: one with a "myeloblast" phenotype, another with an "erythroblast" phenotype and a third one with a hybrid phenotype (Fig. 1).

CD45 was expressed in all cases. However, its MFI showed a large variation and presented a different expression in each sub-set in the cases with 2 blast populations (Tab. 4 and 5). Absence of MPO expression was observed in 12 cases. CD14 was expressed in 8 cases with one blast population and in 3 cases with 2 subpopulations. In 3 cases of FAB M4 or M5, CD14 was not expressed. The expression of CD45 showed a positive correlation with MFI of CD33 (r=0.53; p=0.0001), MFI for CD14 (r=0.45; p=0.003) and that of CD15 (r=0.31; p=0.03).

Survival analysis. Cases presenting any expression of CD14 (but not positivity for alpha-naphtyl-esterase) had a shorter overall survival (Fig. 2). FAB types as well as the presence of expression of CD56, CD19 and MPO had no influence on overall survival.

In Cox model, age (p=0.11), peripheral leukocytes at diagnosis (WBC) (p=0.02), MFI of CD45 (p=0.06) that of MPO (p=0.08) and karyotype (p=0.09) were significant in the univariate analysis. In the multivariate analysis, only WBC and MFI of CD45 remained as independent significant factors (p=0.014 and p=0.018, respectively). Using all the variables from the univariate analysis in 100 bootstrap resampling sets, MFI of CD45 entered in 69%, WBC in 60%, age in 42% and MFI of MPO in 35%.

Table 2. Clinical and hematological data of the patients

	Median	Range
Age (years)	51	15–79
Male/Female	22/13	
Hemoglobin g/dl	8.0	4.0–13.0
Leukocytes $x10^9$/l	16.7	0.8–150
Platelets $x10^9$/l	61	11–271

Table 3. Distribution of the patients by FAB types and presence of phenotypically different blast populations

		Populations			
		1	2	3	Total
FAB	M0	3			3
	M1	4	1		5
	M2	7			7
	M3	4			4
	M4	6	3		9
	M5	2	1		3
	M6			1	1
Total		26	5	1	32*

*In 3 cases, smears were inadequate to perform FAB classification. All of them presented only one blast population.

Table 4. MFI values of the several antigens in the 29 patients presenting only one phenotypically well defined blast population

Antigens	N	MFI: median and range
CD45	29	188 (5–995)
CD13	28	122 (19–1116)
CD15	8	135 (32–264)
CD33	27	196 (27–4381)
MPO	18	62 (6–232)
CD14	8	228 (36–347)
CD56	10	29 (8–156)
CD7	4	83 (51–771)
CD34	19	262 (27–737)
HLA-DR	24	252 (65–2280)
CD19	1	91
CD2	1	131

N – number of cases in which any antigen type expression was found

Discussion

In the present study we performed a two-step panel for the diagnosis of AML using a relatively small number of monoclonal antibodies. The screening panel included two myeloid antigens, two T and one B antigen. The second panel for AML was designed to search for maturation asynchrony and the presence of a monocytic component. This kind of ap-

Table 5. Expression of the several antigens in the 5 cases presenting two phenotypically different blast sub-sets

	population 1		population 2	
Antigens	N	MFI median and range	N	MFI median and range
CD45	5	172 (72–776)	5	353 (157–637)
CD13	3	61 (41–205)	5	154 (60–241)
CD15	3	46 (33–171)	3	150 (98–186)
CD33	5	66 (32–427)	4	116 (35–161)
MPO	4	44 (32–115)	1	25
CD14	0	0	3	201 (195–239)
CD56	2	110 (77–143)	2	50 (32–69)
CD7	1	35	1	385
CD34	2	287 (171–403)	2	105 (11–199)
HLA-DR	5	637 (216–1235)	2	357 (209–506)
CD19	2	17 (14–20)	0	0
CD2	0	0	1	19

N – number of cases in which any antigen type expression was found

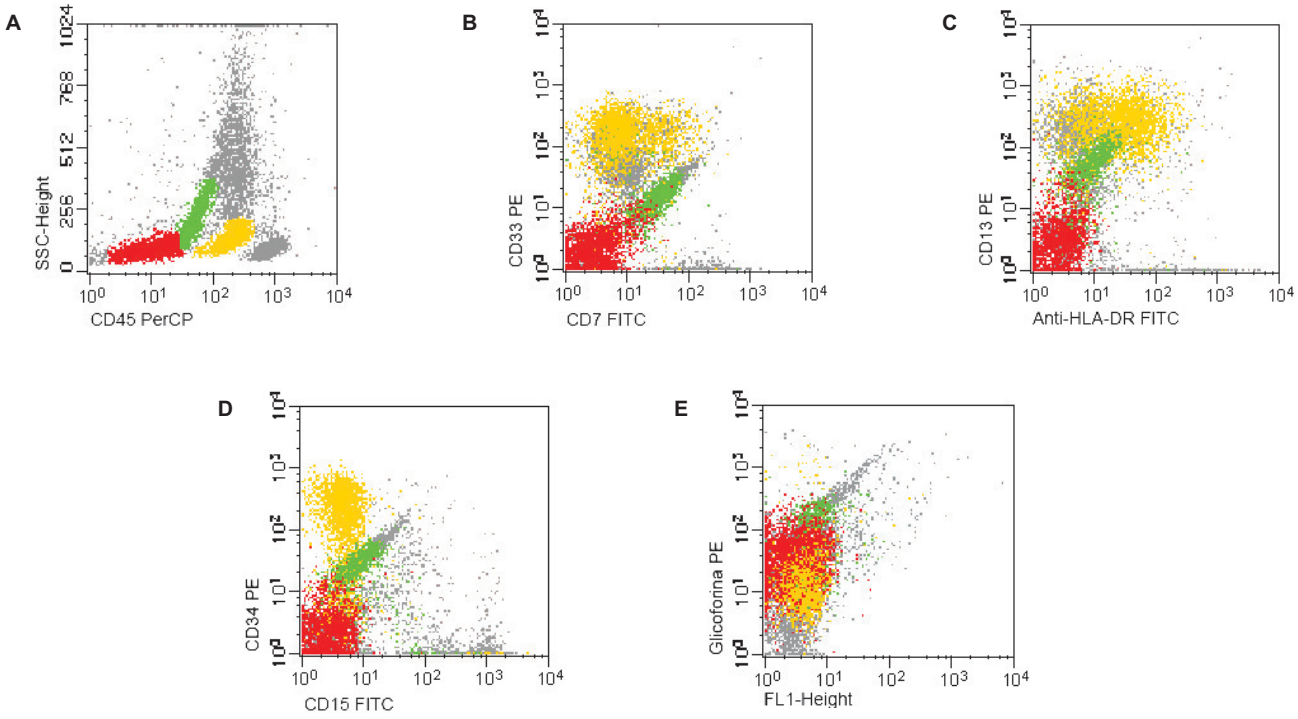

Figure 1. flow cytometric analysis of the case of erythroid leukemia: A: in the CD45/SSC plot, cells in the blast gate in yellow, erythroblasts in red and a third population (green) in the localization of pro-erythroblasts. B–E: Blasts were CD33^{high}, CD13^{high}, CD34^{high}, and Glycophorin A^{dim}. "Pro-erythroblasts" were Glycophorin A^{high}, but presented also a moderate expression of CD13 and CD34. The cells in the erythroblast gate had only expression of Glycophorin A.

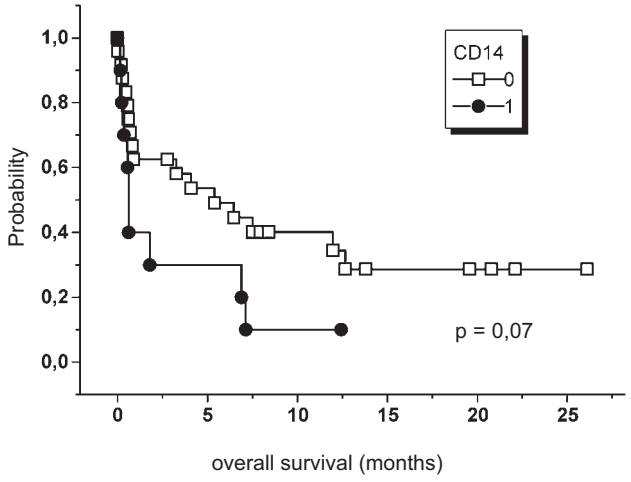

Figure 2. Kaplan-Meier estimates for overall survival in the CD14 positive cases. A different survival was observed for cases in which blasts expressed this antigen.

proach has also been recommended by other groups [7, 24–26]. In all of our cases, it was possible to conclude diagnosis for inclusion of the patients in a treatment protocol.

Comparing the diagnosis by FAB criteria based on morphology and cytochemistry with the immunophenotypic data, the most important discrepancy was seen in the cases with a monocytic component. This has also been found by other authors who observed a discrepant expression of alpha-naphtyl-acetate esterase (ANAE) and CD14 in up to 26% of the cases [13, 27–30]. However, expression of CD14 in AML, which can also be seen in FAB types M0, M1 and M2, was more consistently associated with a poor survival than that of ANAE. Expression of CD14 has also been associated with resistance to Ara-C and daunorubicin [29]. Among our patients, in 12 cases diagnosed as FAB-M4/FAB-M5 only 9 expressed CD14. FAB-M4/5 types had no impact on survival of our patients, but expression of CD14 was a negative prognostic factor, independent of FAB type. All these facts may be responsible for the conflicting results about survival in AML with a monocytic component.

The use of the analysis in the Paint-a-gate software permitted us to detect phenotypically different subpopulations in one third of the cases. Although they could represent a different maturation stage of the leukemic blasts, they frequently presented a partial or total deficiency of one or more antigens examined. This phenomenon showed no impact on survival of the patients. However, these subsets could have a different sensitivity to chemotherapeutic agents used in the treatment. It also represents a pitfall for the use of flow cytometry in the

study of minimal residual disease. This may explain the different phenotype found at recurrence of the disease in many cases [1, 3, 30].

The quantitative analysis of the expression of the several antigens studied could reveal also some interesting facts of prognostic impact in AML. Although our group of patients was relatively small, we could detect a negative impact of the expression of CD14 on overall survival and show that this feature is more important than the expression of ANAE. However, MFI of CD14 did not reach significance in the Cox model, probably due to few cases that expressed this antigen. Similar findings have been reported also by other authors trying to categorize the number of positive cells [13, 27, 28, 30]. In a recent study, DUNPHY et al [27] analyzed extensively the expression of several antigens that have been associated with the monocytic lineage such as CD11b, CD14, CD15 and CD64 in ANAE positive cells. They could demonstrate that, besides variation in expression of monocytic antigens according to cell maturation, a partial or complete deficiency of one or more antigens is frequently found in AML with a monocytic component as well as in chronic myelomonocytic leukemia. A similar phenomenon occurs with MPO in the granulocytic lineage. In our patients, as well as in several other reports [1–3, 7], up to 34% of the cases lack expression of this enzyme. CD13 and CD33 are more constantly expressed. Presence or absence of antigen expression may be associated with resistance to chemotherapy, as has already been described for CD14 [29] and CD33 [31].

Intensity of expression of CD45 is a known prognostic factor in acute lymphoid leukemia (ALL) [23, 32, 33]. However, little is known about its impact on survival in AML. Among some of our patients, we could detect that MFI of CD45 was an important independent factor of a shorter survival. This parameter showed a positive correlation with expression of CD33, CD15 and CD14, indicating a higher degree of maturity of the blasts.

Therefore, we can conclude that a quantitative analysis of the commonly expressed antigens in AML can make a better discrimination of subpopulations of blasts, helping to perform studies of minimal residual disease. It can also give prognostic information. It would be interesting to correlate this expression with cell proliferation and molecular prognostic markers in AML.

References

[1] ORFAO A, ORTUNO FJ, SANTIAGO M, LOPEZ A, SAN MIGUEL J. Immunophenotyping of acute leukemias and myelodysplastic syndromes. Cytometry 2004; 58A: 62–71.

[2] BASSO G, BULDINI B, DE ZEN L, ORFAO A. New methodologic approaches for immunophenotyping acute leukemias. Haematologica 2001; 86: 675–692.

[3] BENÉ MC. Immunophenotyping of acute leukaemias. Immunology Lett 2005; 15: 9–21. Review.

[4] CASASNOVAS RO, SLIMANE FK, GARAND R, FAURE GC, CAMPOS L et al. Immunological classification of acute myeloblastic leukemias: relevance to patient outcome. Leukemia 2003; 17: 515–527.

[5] CHANG H, SALMA F, YI Q, PATTERSON B, BRIEN B, MINDEN MD. Prognostic relevance of immunophenotyping in 379 patients with acute myeloid leukemia. Leuk Res 2004; 28: 43–48.

[6] DANESHBOD Y, AMIRGHOFRAN Z, TABEI SZ. Bcl-2 expression in acute myelogenous leukemia: the relation to myeloid antigen expression and response to therapy in iranian patients. Neoplasma 2005; 52: 109–114.

[7] KLOBUSICKA M, KUSENDA J, BABUSIKOVA O. Myeloid enzymes profile related to the immunophenotypic characteristics of blast cells from patients with acute myeloid leukemia at diagnosis. Neoplasma 2005; 52: 211–218.

[8] PIEDRAS J, BARRALES-BENITEZ O, LOPEZ-KARPOVITCH X. Classification of acute leukemias according to the first latin-american consensus conference for the immunophenotyping of leukemias. La Revista de Investigación Clinica 2000; 52: 524–528.

[9] RASPADORI D, LAURIA F, VENTURA MA, RONDELLI D, VISANI G et al. Incidence and prognostic relevance of CD34 expression in acute myeloblastic leukemia: analysis of 141 cases. Leuk Res 1997; 21: 603–607.

[10] REGO MF, PINHEIRO GS, METZE K, LORAND-METZE I. Acute leukemias in Piaui: comparison with features observed in other regions of Brazil. Br J Med Biol Res 2003; 36: 331–337.

[11] REPP R, SCHAEKEL U, HELM G, THIEDE C, SOUCEK S et al. Immunophenotyping is an independent factor for risk stratification in AML. Cytometry 2003; 53B: 11–19.

[12] SCHABATH R, RATEI R, LUDWIG WD. The prognostic significance of antigen expression in leukaemia. Best Pract Res Clin Haematol 2003; 16: 613–628.

[13] TALLMAN MS, KIM HT, PAIETTA E, BENNETT JM, DEWALD G et al. Acute monocytic leukemia (French-American-British classification M5) does not have a worse prognosis than other subtypes of acute myeloid leukemia: a report from the Eastern Cooperative Oncology Group. J Clin Oncol 2004; 22: 1276–1286.

[14] VALET G, REPP R, LINK H, EHNINGER A, GRAMATZKI MM. Pretherapeutic identification of high-risk acute myeloid leukemia (AML) patients from immunophenotypic, cytogenetic, and clinical parameters. Cytometry 2003; 53B: 4–10.

[15] BUCHNER T, URBANITZ D, EMMERICH B, FISCHER JT, FULLE HH et al. Multicentre study on intensified remission induction therapy for acute myeloid leukemia. Leuk Res 1982; 6: 827–831.

[16] PAGNANO KB, TRAINA F, TAKAHASHI T, OLIVEIRA GB, ROSSINI MS et al. Conventional chemotherapy for acute myeloid leukemia: a Brazilian experience. Sao Paulo Med J 2000; 118: 173–178.

[17] VARDIMAN JW, HARRIS NL, BRUNNING RD. The World Health Organization classification of the myeloid neoplasms. Blood 2002; 100: 2292–2302.

[18] LEGRAND O, PERROT JY, BAUDARD M, CORDIER A, LAUTIER R et al. The immunophenotype of 177 adults with acute myeloid leukemia: proposal of a prognostic score. Blood 2000; 96: 870–877.

[19] ALTMAN DG, ANDERSEN PK. Bootstrap investigation of the stability of a Cox regression model. Statistics in Medicine 1989; 8: 771–783.

[20] LORAND-METZE I, PINHEIRO MP, RIBEIRO E, DE PAULA EV, METZE K. Factors influencing survival in myelodysplastic syndromes in a brazilian population: comparison of FAB and WHO classifications. Leuk Res 2004; 28: 587–594.

[21] METZE K, OLIVEIRA GB, PEREIRA FG, ADAM RL, LORAND-METZE I. Spontaneous apoptosis in chronic lymphoid leukemia is not an independent prognostic factor for stability of disease when compared with AgNOR and TTM. Cell Oncol 2005; 27: 199–201.

[22] METZE K, LOBO AM, LORAND-METZE I. Nucleolus organizer regions (AgNORs) and total tumor mass are independent prognostic parameters for treatment-free period in chronic lymphocytic leukemia. Int J Cancer 2000; 89: 440–443.

[23] METZE K, SILVA RC, ADAM RA, LEITE NJ, PEREIRA FG, LORAND-METZE I. Relation between chromatin texture and phenotype in acute leukemias. Cell Oncol 2005; 27: 112.

[24] CAMPANA D, BEHM FG. Immunophenotyping of leukemia. J Immunol Meth 2000; 243: 59–75.

[25] THALHAMMER-SCHERRER R, MITTERBAUER G, SIMONITSCH I, JAEGER U, LECHNER K et al. The immunophenotype of 325 adult acute leukemias: relationship to morphologic and molecular classification and proposal for a minimal screening program highly predictive for lineage discrimination. Am J Clin Pathol 2002; 117: 380–389.

[26] VOSKOVA D, VALEKOVA L, FEDOROVA J, HUDECEK J, KUBISZ P. Leukemic cells and aberrant phenotypes in acute leukemia patients: a flow cytometric analysis. Neoplasma 2003; 50: 422–427.

[27] DUNPHY CH, ORTON SO, MANTELL J. Relative contributions of enzyme cytochemistry and flow cytometric immunophenotyping to the evaluation of acute myeloid leukemias with a monocytic component and of flow cytometric immunophenotyping to the evaluation of absolute monocytoses. Am J Clin Pathol 2004; 122: 865–874.

[28] FERGEDAL M, ASTROM M, TIDEFELT U, KARLSSON MG. Differences in CD14 and alpha-naphthyl acetate esterase positivity and relation to prognosis in AML. Leuk Res 1998; 22: 25–30.

[29] NORGAARD JM, OLESEN LH, OLESEN G, MEYER K, KRISTENSEN JS et al. FAB M4 and high CD14 surface expression is associated with high cellular resistance to Ara-C and daunorubicin: implications for clinical outcome in acute myeloid leukaemia. Eur J Haematol 2001; 67: 221–229.

[30] PEFFAULT DE LATOUR R, LEGRAND O, MOREAU D, PERROT JY, BLANC CM et al. Comparison of flow cytometry and enzyme cytochemistry for the detection of myeloperoxydase in acute myeloid leukaemia: interests of a new positivity threshold. Br J Haematol 2003; 122: 211–216.

[31] VAN DER VELDEN VHJ, BOECKX N, JEDEMA I, TE MARVELDE JG, HOOGEVEEN PG et al. High CD33-antigen loads in peripheral blood limit the efficacy of gemtuzumab ozogamicin (Mylotarg) treatment in acute myeloid leukemia patients. Leukemia 2004; 18: 983–988.

[32] BOROWITZ MJ, SHUSTER J, CARROLL AJ, NASH M, LOOK AT et al. Prognostic significance of fluorescence intensity of surface marker expression in childhood B-precursor acute lymphoblastic leukemia. A Pediatric Oncology Group Study. Blood 1997; 89: 3960–3966.

[33] NAKAMURA A, TSURUSAWA M, KATO A, TAGA T, HATAE Y et al. Prognostic impact of CD45 antigen expression in high-risk, childhood B-cell precursor acute lymphoblastic leukemia. Leuk Lymphoma 2001; 42: 393–398.