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Research article

Correlation of cytomorphology with flowcytometric immunophenotyping in acute myeloid leukemia

Corelația citomorfologiei cu imunofenotiparea prin citometrie în flux în leucemia acută mieloidă

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Abstract

Morphological and immuno- flow cytometry assisted analysis of peripheral blood and bone marrow are mandatory investigations in the diagnosis of acute leukemia. Cytology and immunophenotyping complement each other primarily because they have as common object malignant cell phenotype as a whole. The aim of our study was to analyze correlations between cytology and immunophenotyping on a group of patients investigated for acute myeloid leukemia. In our study the degree of correlation between blast percentage determined by cytology and immunophenotyping was low (r=0.049). The degree of correlation between myeloperoxidase positivity in cytochemistry and immunophenotyping was also low, with better results for cytochemistry. Expression of immunophenotypic markers was consistent with the composition of our group regarding French-American-British classes, except for HLA-DR (49.0%), TdT (3.77%), CD14 (5.66%), CD15 (5.66%). We also discuss the importance of interpreting with caution positivity for erythroid and megakaryocytic markers and differential diagnosis of cases simultaneously expressing CD7 and CD56. In conclusion, interpretation of immunophenotyping by flow citometry, done in close conjunction with morphology, is mandatory to facilitate the use of optimized sample processing methods and of standardized panels, for both appropriate diagnosis and follow-up.

Keywords: *malignant cells morphology, malignant cells immunophenotyping, acute myeloid leukemia*

Rezumat

Examenul morfologic şi imunofenotiparea prin citometrie in flux sunt analize indispensabile în diagnosticul leucemiilor acute. Aceste investigaţii sunt complementare, în primul rând deoarece au ca şi obiect comun fenotipul celulei maligne în ansamblul ei. Scopul studiului nostru a fost de a analiza corelaţiile intre citologie si imunofenotipare pe un lot de pacienţi investigaţi pentru leucemie acută mieloidă. În studiul nostru, gradul de

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corelare între procentul de blaşti determinat prin citologie şi imunofenotipare a fost redus (r=0.049). Gradul de corelare între expresia mieloperoxidazei determinată prin citochimie şi imunofenotipare a fost de asemenea redus, cu rezultate mai bune pentru citochimie. Expresia markerilor imunofenotipici a corespuns cu compoziția pe clase FAB a lotului nostru, mai puțin pentru HLA-DR (49.0%), TdT (3.77%), CD14 (5.66%), CD15 (5.66%). Am pus in discuție de asemenea importanța interpretării cu prudență a rezultatelor pozitive pentru markeri eritroizi şi megakariocitari şi diagnosticul diferențial al cazurilor care exprimă simultan CD7 şi CD56. În concluzie, interpretarea rezultatelor imunofenotipării prin citometrie in flux în strânsă corelație cu morfologia, optimizarea metodelor de analiză şi utilizarea de paneluri standardizate sunt indispensabile atât pentru diagnostic, dar şi pentru urmărirea în evoluție a cazurilor.

Cuvinte cheie: *morfologia celulelor maligne, imunofenotipul celulelor maligne, leucemia acută mieloidă*

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Introduction

Flow cytometric (FC) immunophenotyping and morphological analysis of peripheral blood and bone marrow are mandatory investigations in the diagnosis of acute leukemia. They allow classification of acute leukemia by establishing the proliferating cell line and the degree of maturation of the neoplastic cells.

The 2008 World Health Organization (WHO) (1) classification of hematological neoplasms gives priority to cytogenetics, molecular biology, and even patient history, in an attempt to classify patients primarily regarding prognosis. It should be noted that this hierarchy is operative for clinical practice, giving the most useful data for therapeutic decision making. In terms of the diagnostic algorithm however, the first investigations remain cytology and immunophenotyping. In the majority of cases, these two laboratory techniques establish positive diagnosis of acute leukemia and direct further investigations.

Cytology and immunophenotyping complement each other primarily because they have as common object malignant cell phenotype as a whole (morphology, i.e. surface and intracellular marker expression). On the other hand, morphology is burdened by a high degree of subjectivity and flow cytometry techniques did not reach consensus standard protocols yet. That is why correlation of information provided by the two techniques is still absolutely necessary.

The aim of our study was to analyze the correlation between cytology and immunophenotyping on a group of patients investigated for acute myeloid leukemia (AML).

Materials and methods

We have studied 88 patients admitted to the Department of Hematology of the Oncology Institute "Prof. Dr. Ion Chiricuta" in Cluj between 2006-2009, with a diagnosis of acute myeloid leukemia. All patients were investigated at diagnosis by complete blood count, blood smear, biochemistry and coagulation studies. For 84 patients results for bone marrow smear at diagnosis were available. Other investigations performed at diagnosis were immunophenotyping by flow cytometry, karyotyping and molecular biology (RUNX1/RUNX1T1, MLL-PTD, CBFB/MYH11, FLT3-TKD, PML/RARA, FLT3-ITD, AF9/MLL, NPM1). Immunophenotyping results were available for 53 patients, karyotyping for 79 patients, PCR for 73 patients. In thirty seven patients all the above mentioned techniques were employed. For comparison of immunophenotyping and morphology, depending on the available data, we analysed a homogenous group of 53 patients.

General characteristics of the group in our study (n = 88) are presented in *Table 1*.

Twenty-one patients were over 65 years old, and 8 patients were in the range of 60-65 years. Twenty one patients had at presentation

Table 1. General data of the patient group

		Median	Range
Age at diagnosis	(years)	54.5	21-79
WBC	(x 1000/µl)	20	1-200
Hemoglobin	(g/dl)	7	4-14
Platelets	(x 1000/µl)	21	10 -220
Blasts in bone marrow (n=76)	%	57	20-100
Sex ratio	-	M/F=1.32	

leukopenia (<4000/µl), and 44 patients had leukocytosis (>10 000/µl). Of these, 9 patients had over 100 000 leukocytes /µl and 8 patients had WBC in the range of 50 000 to 100 000 /µl. Most patients (n=77) had anemia at presentation. Hemoglobin level was above 8 g /dl in 31 patients and between 6 and 8 g /dL in 44 patients. Fifthy-seven patients had values below 50 000 platelets /µl, of which 16 under 20 000 /µl and 5 below 10 000 /µl.

Morphological characterization

Morphological description of the blasts was made according to French-American-British Group (FAB) (2) and WHO (3) criteria, reviewed and refined by the the International Working Group on Morphology of MDS (IWGM-MDS) (4, 5), taking into account: cell size, chromatin appearance, cytoplasm appearance, granularity, the presence of Auer rods, positivity and appearance of myeloperoxidase in cytochemistry. We used the May-Grunwald Giemsa stain and myeloperoxidase stain (the benzidine and hydrogen peroxide method, internal protocol of the Medical Analysis Laboratory Hematology Department).

Immunophenotyping

Immunophenotyping was performed in the Laboratory of Immunology, Medical Clinic III, Ulm, Germany on bone marrow aspirate samples, except for two cases in which venous blood was used. Samples were collected on anticoagulant (heparin or EDTA). In order to standardize the method, counting of nucleated elements was performed on the primary sample on an automatic hematology analyzer (Pentra 60C +).

The samples were pretreated with ammonium chloride for erythrocyte lysis and an additional stage of fixation and permeabilization (Fix & Perm, An der Grub) for the stainig of intracellular markers. For some of the samples the mononuclear cells were separated by a gradient concentration method (Ficoll-Hypaque). The nucleated cell pellet was then resuspended in PBS-BSA solution (phosphate buffer saline - bovine serum albumin) 0.1% to a final concentration of 1-1.5 x107 cells / ml, determined on an automated hematology unit (Pentra 60C +) .

Staining was done with combinations of four monoclonal antibodies (Beckman-Coulter, Dako, Becton Dickinson) in amounts of 5µl or 10µl, previously determined by titration.

The surface marker antibody panel consisted of:
MsIgG-FITC/-PE/CD45-ECD/-PC5,
CD14-FITC/CD64-PE/CD45-ECD/CD3-PC5,
CD19-FITC/CD33-PE/CD45-ECD/CD34-PC5,
CD7-FITC/HLA-DR-PE/CD45-ECD/CD33-PC5,
CD15-FITC/CD56-PE/CD45-ECD/CD117-PC5,
CD11b-FITC/CD11c-PE/CD45-ECD/CD34-PC5,
CD61-FITC/CD235-PE/CD45-ECD/CD13-PC5,
CD38-FITC/CD133-PE/CD45-ECD/CD34-PC5,
CD41-FITC/CD42b-PE/CD45-ECD/CD34-PC5,
CD34-FITC/7.1-PE/CD45-ECD/CD184-PC5,
CD47-FITC/CD243-PE/CD45-ECD/CD34-PC5.

The intracytoplasmatic antibody panel comprised:
MsIgG-FITC/-PE/CD45-ECD/-PC5,
TdT-FITC/CD79a-PE/CD45-ECD/CD3-PC5,

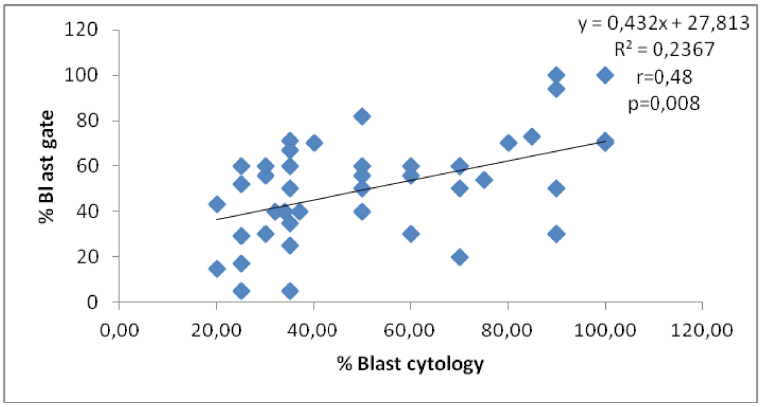

Figure 1. Comparison of blast percentage immunophenotype vs. cytology

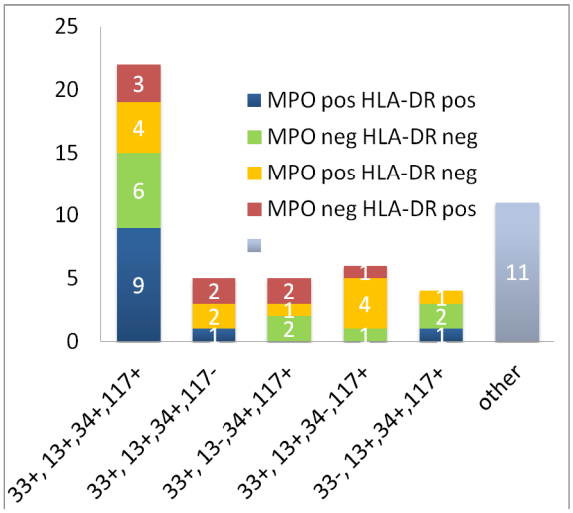

Figure 2. Concomitant expressed markers

MPO-FITC/CD22-PE/CD45-ECD/CD34-PC5.

The analysis was performed on an Epics-XL-MCL flow cytometer (Beckman-Coulter) using a 4 color protocol. Gating strategy was side scatter / forward scatter (SSC / FSC) and / or CD45 / SSC and the results were expressed as the percentage of positive cells in the gate. A population was defined as positive for a marker if it was present in 20% of the cells for surface antigens and 10% for cytoplasmic antigens.

The statistical analysis was done using the GraphPadPrism program for linear regression and Fisher's exact test. Concordance was determined using k statistics. A p-value of <0.05 was considered statistically significant.

Results

Comparison of the percentage of blasts determined by counting on the bone marrow smear and by flowcytometry could be done for 44 cases of the 53 evaluable cases. Assessment of blast percentage was not possible in some cases because of the quality of the bone marrow samples (cellularity similar to peripheral blood, hypoplastic or fibrotic bone marrow, necrosis), but even in these cases cytology and flow cytometry added valuable data regarding morphology and immunophenotype of malignant cells.

Although the dispersion of the results is high, there was a positive correlation (r = 0.48, p= 0.008, *Figure 1*).

Next we compared the positivity of myeloperoxidase (MPO) in cytochemical staining and flow cytometry. We noted a large number of false negative results for flow cytometry. To show the degree of concordance between the two techniques we used the Kappa test. According to the Landis and Koch scale the degree of concordance is low (K = 0.090) and correlation of the two techniques is not significant (p =0.22) (*Table 2).*

Of the 53 cases which were immunophenotyped, the FAB class distribution was as follows: 5 cases M1, 1 case M1/M2, 18 cases M2, 2 cases M3, 16 cases M4, 4 cases M6, 7 cases were unclassifiable by FAB criteria. Immunophenotypic markers expressed in these cases are presented in *Table 3*.

Progenitor cell markers CD34, TDT and HLA-DR were expressed in 75%, 3.77%

Table 2. Myeloperoxidase in cytochemistry vs. immunophenotyping

	Immunophenotyping MPO+	Immunophenotyping MPO-	Total
Cytochemistry MPO +	24	20	44
Cytochemistry MPO -	0	2	2
Total	24	22	46

Table 3. Immunophenotypic markers / FAB categories

n=53			**M1(5)**	**M1/2(1)**	**M2 (18)**	**M3 (2)**	**M4 (16)**	**M6 (4)**	**Mx (7)**
CD13	43	81,13%	5	1	14	2	13	2	6
CD33	43	81,13%	5		15	1	12	4	5
CD34	42	79,25%	3	1	19	1	9	3	6
CD117	40	75,47%	5		17	2	8	3	5
MPO	29	54,72%	3	1	9	1	9	2	3
HLA-DR	26	49,06%	1		12	0	11	1	
CD56	10	18,87%	1		5				3
CD7	8	15,09%	2	1	4		1		
CD65	8	15,09%			3				
CD61	6	11,32%			1	1			
CD41	4	7,55%			1		2		1
CD133	4	7,55%			2			1	
CD14	3	5,66%					3		
CD15	3	5,66%	1				1		1
CD38	3	5,66%					2	1	
CD11c	2	3,77%			1		1		
CD184	2	3,77%	1						
TDT	2	3,77%			1				
CD42	1	1,89%							1
CD47	1	1,89%							1
CD64	1	1,89%							
CD235	1	1,89%						1	
7.1	1	1,89%							1

and 49% of all cases. Myeloid specific markers CD33, CD 117, CD13 and CD15 were present in 81%, 75%, 83% and 6% of cases. The most frequently expressed markers were: CD13 (83.02%), CD33 (81.13%), CD34 (79.25%), C117 (75.47%), MPO (54.72%), HLA -DR (49.06%). Some of the markers were present in a total of 6 to 10 cases (between 11 and 19% of all cases): CD 56, CD7, CD65, CD61. A small number of cases expressed CD41, CD133 (7.55% each), CD14, CD15, CD38 (5.66% each). Only two cases expressed CD11c, CD71, TDT, and one case CD42, CD47, CD64, CD235 and anti-7.1. The CD3, CD19 and CD11b markers were not expressed in any of the cases studied. The most frequently expressed markers were: CD33, CD34, CD13, CD117, HLADR and MPO. They were found in combinations, as shown in *Figure 2* and *Table 4*.

Table 4. Concomitant expressed markers within the FAB categories

	CD33+, CD13+, CD34+,CD117 +	Other combinations of positive markers *	Total
M2	12	6	18
M4	3	13	16
other FAB	7	12	19
Total	22	31	53

* CD33, CD13, CD34, CD117 - 3 or less then 3 positive markers

Table 5. Expression of immunophenotypic markers in the FAB M2 and M4 categories

FAB	CD13	CD33	CD34	CD117	MPO	HLA-DR	CD56	CD7
M2	14	15	19	17	9	12	5	4
M4	13	12	9	8	9	11	0	1
p	0.0856	1	0.0017	0.0218	0.738	1	0.0493	0.3468

FAB	CD65	CD61	CD41	CD133	CD14	CD15	CD38	CD11c	TDT
M2	3	1	1	2	0	0	0	1	1
M4	0	0	2	0	3	1	2	1	0
p	0.2336	1	0.5820	0.4891	0.0856	0.4571	0.0217	1	1

Of the 22 cases with concomitant expression of CD33, CD13, CD34 and CD117, 9 cases were also HLADR+, MPO+, which was the most frequently encountered phenotype. They belonged morphologically to M2 (6 cases), M4Eo (2 cases), and M1 (1 case). Also in the same group of 22 cases we observed a significant association with FAB2 morphology ($p < 0.05$, *Table 5*)

The FAB subtypes which were best represented in our cohort were M2 (18 cases) and M4 (16 cases). The expression of CD34, CD117 and CD56 correlated with FAB type M2 ($p = 0.0017$, $p = 0.0218$ and $p=0.0493$, respectively), while CD14 correlates with FAB M4 ($p = 0.048$, *Table 4*). For other markers no difference in expression between the two FAB categories was obvious. Regarding the monocytic marker CD14, we noticed that of 16 cases classified cytologically as M4, only 3 cases were positive for this marker.

Aberrant expression of CD7 was present in 8 cases out of 53. Of these, 3 cases were M2, two cases M1, 1 case M1/M2, 1 case M4, 1 case unclassified. In 3 cases CD7 was associated with CD56.

Discussion

Comparison of the blast percentage determined by counting on the bone marrow smear and by flow cytometry, showed a high dispersion of results, with both positive difference for cytomphology and for immunophenotyping (18 cases with higher percentage of blasts by immunophenotyping, compared to 26 cases with higher percentage of blasts by cytomorphology). Correlation coefficient between the two measurements is positive but the two parameters are not strongly correlated ($r = 0.49$, $p = 0.008$).

The negative bias that was observed in immunophenotyping versus bone marrow smear can be attributed to many causes. In bone marrow samples received for FC evaluation there is

a degree of blood contamination (sample-dilution), while a smear requires only several highly concentrated bone marrow spicules, which are first collected during puncture. If data derived from the analysis of only peripheral blood samples would be compared, there should be a higher degree of correlation. The separation of mononuclear cells by gradient concentration techniques leads to a loss of cells. The erythrocyte lysis procedure preserves cells better, but in both cases however the primary sample requires passage through sieves in order to obtain a cell suspensions and this process can also lead to cell loss. Although erythrocyte lysis is preferred and highly recommended, there are situations which require gradient concentration separation. In our case the laboratory workflow included also sample preparation for cytogenetics, molecular biology and cryopreservation and that is why in certain circumstances immunophenotyping was performed on a mononuclear cell suspension. Samples sent for FC were usually stored up to 24 hours until analysis, therefore a difference due to cell viability, with a selective loss of the more fragile malignant clonecells, could be expected.

In cases where the bias is positive for immunophenotyping, the main cause is the gating strategy (FSC / SSC). It is known that this gating strategy has reduced capacity of discriminating blasts from normal residual cells (lymphocytes, monocytes), in contrast with the CD45/SSC gating technique (6).

The lack of correlation between blast percentage determined by the two methods can be explained as a consequence of the widely accepted fact that some statistically derived differences would be expected, as by morphology the operator can count a total of up to several hundred cells, while by FC one may acquire up to several hundred thousand events.

The degree of correlation between the two types of measurements (blast count on smear and flow cytometry) lies in the literature around the value of 0.7 when using the FSC / SSC, and 0.9 for CD45/SSC (7).

The degree of correlation between myeloperoxidase expression in flow cytometry and cytochemistry was reduced in our study ($K = 0.09$, $p < 0.05$) (8). Our results allow us to state the superiority of cytochemistry in the detection of intracellular myeloperoxidase, for the techniques we used. Because falsely positive results for myeloperoxidase have not been reported, we could consider it, at least for our study, as a "reference" method, and in this case the sensitivity for the flow cytometry procedure is quite reduced (68.75%). The main reasons for these results may be related to antibody specificity and the efficiency of permeabilisation techniques for intracellular marker (9, 10). Our results do not agree with some literature data stating superiority of immunohistochemistry and flow cytometry compared to cytochemistry. Saravanan et al. (11) found in a series of 110 cases of AML, 11 cases cytochemical negative but positive in immunohistochemistry and / or flow cytometry.

Another cause of falsely negative results in flow cytometry is using intracellular markers positivity threshold of 10%. Of the 20 cases reported as MPO-negative in immunophenotyping, 7 cases had less than 10% MPO positive myeloblasts in cytochemistry, which justifies rethinking the positivity threshold in immunophenotyping as suggested by Peffault Latour et al (12).

Regarding the frequency of expression in myeloid markers in the whole group compared to data from literature (13), we recorded similar percentages for CD13 (81.13 vs 60-90%), CD33 (81, 13% vs 70-90%), CD 117 (75.47% vs 60-70%). A higher frequency was observed for CD34 (79.25% vs 30-40%) and a lower frequency for TDT (3.77% vs 10-20%), HLA-DR (49.06% vs 70%), CD15 (5.66% vs 40-70%), CD14 (5.66% vs 15-40%). The differences can be explained in part by the large number of M2 and M4 cases in our group. These FAB classes usually do not express TdT, which is consistent with the small percentage of TdT positive cases in our group, but it does not explain the lower expression of HLA-DR, which is usually found in a higher percentage

of M2 and M4 cases and the higher expression of CD34 which is usually present in a small number of M2 cases and variable present in M4. In our group we also would have expected a greater number of cases positive for CD 15 (monocyte marker) and CD 14 (marker myeloid / monocyte).

Also surprising is the large number of cases simultaneously expressing CD34, CD33, CD13, CD117, because this immunophenotype is assigned to M1 rather than M2 and M4 classes. Consequently these differences can be explained only on account of the small size of our group.

Regarding the aberrant expression of CD7, which currently occurs in approximately 30% of AML cases (14), in our series it was present only in 15% of cases.

A special attention deserves the cases of CD7 and CD56 positive cells, as the combination of these two markers in the presence of myeloperoxidase requires differential diagnosis between an AML with aberrant expression of lymphoid markers and mixed phenotype acute leukemia with myeloid / NK cells.

Another situation which could pose differential diagnosis questions, is the case in which CD117 and CD56 were positive, while all other myeloid markers (including myeloperoxidase) were negative. This case presented positivity for 7.1 (anti NG2), which is a marker associated with abnormalities of chromosome 11q. But in this case these abnormalities could not be demonstrated (normal karyotype, MLL-PTD negative). NG2 antigen is also positive in 60% cases of blastic plasmocytoid dendritic cell neoplasms. (15) For this reason we believe that additional testing with CD4 and CD123 in such a case, especially in circumstances of particular morphology, could aid the diagnosis.

Megakaryocytic marker positivity (5 cases) should be interpreted with caution, given that platelets can adhere to the blast cells.

The two cases with erythroid marker positivity also deserve special attention. In interpreting these cases we have to take into account the possibility that myeloid precursors express CD235 at low levels or the possible presence of residual erythroblasts in the analyzed population.

Conclusions

Immunophenotyping is an essential method in the diagnosis and classification of acute myeloid leukemia. Interpretation of immunophenotyping results must however be done in close correlation with morphological appearance. Morphology and cytochemistry could be used as a validation method of flow cytometric immunophenotyping for some markers (in our case MPO), especially in situations where inter-laboratory comparison is not possible. The use of large panels for immunophenotyping of acute leukemia is encouraged for a more accurate and complete description of surface and cytoplasmic markers, allowing both diagnosis and appropriate follow-up. Sometimes only correlation between morphology and immunophenotyping suggests the positive diagnosis of acute myeloid leukemia, draws attention on particular and rare cases and suggests further investigation.

Abbreviations

AML- acute myeloid leukemia
FAB- French American British Group
FC- flow cytometry
FSC/ SSC – forward scatter / side scatter
IWGM-MDS- International Working Group on Morphology of myelodysplastic syndrome
MPO- myeloperoxidase
PBS- BSA - phosphate buffer saline – bovine serum albumin
WHO- World Health Organization.

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