***Maced J Med Sci* electronic publication ahead of print,**
published on December 5, 2008 as doi:10.3889/MJMS.1857-5773.2008.0021

Macedonian Journal of Medical Sciences. 2008;1(2):XX-XX.
doi:10.3889/MJMS.1857-5773.2008.0021
Clinical Science

OPEN ACCESS

Preliminary Results of Introducing the Method Multiparameter Flow Cytometry in Patients with Acute Leukemia in the Republic of Macedonia

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Key words:
Acute leukemia; Immunophenotyping; Molecular methods; Flow cytometry; Republic of Macedonia.

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Received: 17-Sep-2008
Revised: 15-Nov-2008
Accepted: 01-Dec-2008
Online first: 05-Dec-2008

Abstract

Background. In this paper we present the initial results of introducing the method of multiparameter flow cytometry (MPF) in patients with acute leukemia in the Republic of Macedonia.

Aim. The aim of our study is to improve the diagnosis and management of acute leukemia, to establish the correct lineage assignment of the blast cells and to select effective treatment strategy for each single acute leukemia patient.

Material and methods. A total of 44 adult (>15 years) patients (from initially 45 tested) with acute leukemia who were consecutively admitted at the Clinic of Hematology-Skopje from January through June 2008, were enrolled in this study. The MPF was introduced for the first time in the Republic of Macedonia and was performed at the Institute for Immunobiology and Human Genetics, Faculty of Medicine-Skopje.

Results. Our results showed that morphology and cytochemistry established lineage in 39 of patients, but not in 5 cases that presented as acute leukemia, of which 4 were assigned as myeloid and in one nonhematopoietic malignancy was indicated. Furthermore immunophenotyping change the lineage assigned based on morphology and cytochemistry in one case from lymphoid to myeloid. Results from our study showed that routine immunophenotyping improved the diagnosis in 6 (13.3%) cases. The exact lineage assignment of the blasts cells guides to implementation of specific molecular analyses in some subtypes of acute leukemia and their further definition, which is essential for more appropriate single patient therapeutic decisions.

Conclusion. Our data support routine implementation of MPF in the diagnostic evaluation of acute leukemia.

Introduction

The acute leukemias present heterogeneous group of malignancies of the immature haemapoietic cells. The bone marrow is infiltrated with more than 20% of blast cells and they are divided into two main groups: acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL). Although in many instances the different types of blast cells may be recognized by simple morphological and cytochemical stains, it is necessary to employ immunological reagents (monoclonal antibodies) and cytogenetic or

molecular biological techniques to identify their particular differentiation features. This is important, as the natural history and response to therapy varies according to the type of blast involved in the leukemic process (1-3).

The classification of the acute leukemias underwent many changes in recent years. The French-American-British (FAB) classification of AML and ALL was based on cytomorphological and cytohemistry details only (4,5). Since then, the diagnostic of acute leukemias had undertone a complete change and the routine diagnostic work-up incorporated immunophenotyping by multiparameter flow cytometry, classical cytogenetics, molecular cytogenetics (comprising diverse fluorescence in situ hybridization techniques and comparative genomic hybridization) and molecular genetics (mostly PCR-based techniques and sequencing) (6,7).

According to the proceedings in diagnostic methods and the improved understanding of the diversity of acute leukemia subtypes, the latest World Health Organization (WHO) classification of acute leukiemias incorporates and interrelate morphology, cytogenetics, molecular genetics and immunologic markers and pays major attention on the importance of genetic events in the classification and therapy of the AMLs. Its prognostic relevance is most clearly demonstrated in the AMLs characterized by recurrent chromosome translocation: *t(15,17),t(8,21)* and *inv16* which generally have a favorable prognosis when treated with appropriate therapeutic agents (7,8). In WHO classification of precursor B-cell and T-cell neoplasms the decisive role regarding the diagnosis, prognosis and clinical stratification of the patients has immunophenotying of the malignant cells (7).

It is anticipated that advances in molecular technology will reveal additional markers that will result in more precise classification of this heterogeneous complex of disorders. Correct diagnosis of the diverse subtypes of AML and ALL will play a central role for individual risk stratification and therapeutic decisions (8-10).

Application of immunophenotyping together with the cytomorpohology and cytohemistry have a crucial role in the initial diagnosis of all cases with a suspected or proven diagnosis of acute leukemias (11,12). Immunophenotyping allows the discrimination of different cell population on the basis of their size, granularity, and antigen expression patterns. Flow cytometry is a powerful technology for characterizing and analyzing cells. It simultaneously measures and analyzes multiple physical characteristics of single particles, usually cells, as they move in a fluid stream through a beam of light through an optical and/or electronic detection apparatus. Flow cytometry uses the principles of light scattering, light excitation, and emission of fluorochrome molecules to generate specific multiparameter data from particles and cells in the size range of 0.5 μm to 40 μm diameter (13).

The applied methodology detects cell surface antigens in a suspension of viable cells and cytoplasmic and nuclear antigens in previously fixed and stabilized cell suspension with the application of monoclonal antibodies conjugated with different fluorochromes. It permits simultaneous detection (multiparameter analyzes) of more than two membrane and nuclear or cytoplasmic antigens by means of double or multiple immunostaining.

In this paper we present the initial results of introducing the method multiparameter flow cytometry (MPF) in patients with acute leukemia in the Republic of Macedonia. The aim of our study is to improve the diagnosis and management of acute leukemia, to establish the correct lineage assignment of the blast cells and to select effective treatment strategy for each single acute leukemia patient.

Material and Methods

Patients and samples

A total of 44 adult (>15 years) patients (from initially 45 tested) with acute leukemia who were consecutively admitted at the Clinic of Hematology-Skopje from January through June 2008, were enrolled in this study. The median age of the patients (28 men, 16 women) was 47.3 years (range, 15-77 years). The diagnosis was made by standard morphological examination and cytochemical analyses of bone marrow smears according to the criteria established by the FAB Cooperative Study Group (14-17) and confirmed by immunophenotyping of bone marrow aspirates and/or peripheral blood samples (14-19) following the criteria of the European Group for the Immunological Classification of leukemias (EGIL) and the British Committee for Standards in Hematology (BCSH) (20, 21). Slightly modified panel of monoclonal antibodies (McAb) against myeloid- and lymphoid-associated antigens as suggested by the EGIL was utilized (18-20). The samples contained more than 20% of blast cells (most of which had more than 50%). Patients diagnosed as FAB, AML M3 were tested for the

presence of the fusion transcripts PML-RARa by Reverse Transverese-Polymerase Chain Reaction (RT-PCR) analysis. Also, in ALL cases occurrence of the BCR-BL fusion transcript was evaluated by RT-PCR (18).

Morphology

The morphology was analyzed by microscopic examination of >500 nonerythroid cells on May Gruenwald Giemza stained air-dried bone marrow smears (18).

Cytochemical analysis

Air-dried bone marrow smears were stained for myeloperoxidase (MPO), non specific esterase (EST) and periodic acid-Schiff (PAS) according to the manufacturers guidelines. The percentage of positive cells for either stain was assessed by microscopic examination of 200 nonerythroid cells (18).

Immunophenotyping

The MPF was introduced for the first time in the Republic of Macedonia and was performed at the Institute for Immunobiology and Human Genetics, Faculty of Medicine-Skopje. Immunophenotyping was done using Cytomation (DAKO-Cytomation) flow-cytometer on whole blood and/or bone marrow specimens using lysing solutions (BD-Biosciensies, San Jose, CA, USA) (21). We prepared the samples for simultaneous detection of three cytoplasmic/nuclear and membrane antigens. The first step involved immunostaining of cell suspension with multiple panels of tree monoclonal antibodies (McAb) labeled with fluorescein (FITC), phycoerythrin (PE) and phycoerythryn-Cy5 tandem complex (Pe-Cy5) as third color. Panel of used antibodies and their manufacturers are listed at Table 1.

Table 1: Panel of monoclonal antibodies for the diagnosis of acute leukemia.

	B-lineage	T-lineage	Myeloid markers	Nonlineage restricted
First line	CD19,CD10, cytCD79a, cyt CD22,	CD2,CD7, cytCD3	CD117,CD13, CD33, CD14, CD15, antiMPO*, LYZ***	TdT, CD34, HLA-DR CD56
Second line	SmIg(kappa/lambda),** CytIgM, CD138,	CD1a, mCD3, CD4,CD5,CD8 Anti-TCR	CD 41, CD 61, CD 42, CD 71, anti-glycophorin A	CD9

All markers were manufactured by BD Biosciencies. San Jose. CA.USA, except antilysozyme (DAKO).
cyt = cytoplasmic; m = membrane.
*MPO=myeloperoxidase; LYZ=antilysozyme.
** SmIg(kappa/lambda) = surface immunoglobulin/light chain immunoglobulin.

For the detection of cytoplasmic and nuclear antigens we used commercially available permeabilization/fixation solutions (FACS Permeabilization solution BD Biosciencies, San Jose, CA, USA) (21).

We incubated 100 μl of specimens (peripheral blood or bone marrow) and appropriate McAb for 15 min at room temperature, than add the permeabilization solutions and/or lysing solution and repeat the incubation procedure. After the incubation we washed the samples for three times with PBS-A, than re-suspended the cell with isotones solution and acquired the data (21, 22). As control we used lysed, but unstained sample.

Acquired data were analyzed with software by using CD45 gating strategy. This technique involves incubation of all samples with fluorochrome-labelled CD45 McAb and with the McAb for which reactivity needs to be established with an alternative fluorochrome (22, 23). In the initial step of the analyses, gating is set up on a CD45-positive versus side-scatter dot plot. The procedure allows the discrimination between the blast cell population and normal cells and the exclusion of platelets and debris. Thereafter, if necessary, another gating is possible to be performed, to separate the cells positive with the McAb under study (23). A sample of CD45 consecutive gating is displayed at Figure 1.

Leukemias were first tested by the primary panel and, if necessary, further characterized by the McAb of the secondary panel (21).

Antigen expression was considered positive if 20% or more blast cells reacted with a particular antibody, except reactivity of blasts cells with MPO. It was considered positive if 10% or more of mononuclear cells were MPO positive (22).

Molecular Analysis

Mononuclear cell preparation, RNA isolation, and cDNA synthesis were performed at the Department of Molecular Biology, Immunology and Pharmacogenetics, Faculty of Pharmacy-Skopje, according to standard procedures. Aliquots of 5 μL of cDNA (100 ng RNA equivalent) were used for Real-time Quantitative polymerase chain reaction (RQ-PCR) with primers and dual-labeled probes as described by Gabert et al (24). Positions and nucleotide sequences of the primers and probes are shown in Table 2. The RQ-PCR reaction was performed in a 25-μl reaction volume using 12.5 μl (1x) Master Mix (Applied Biosystems),

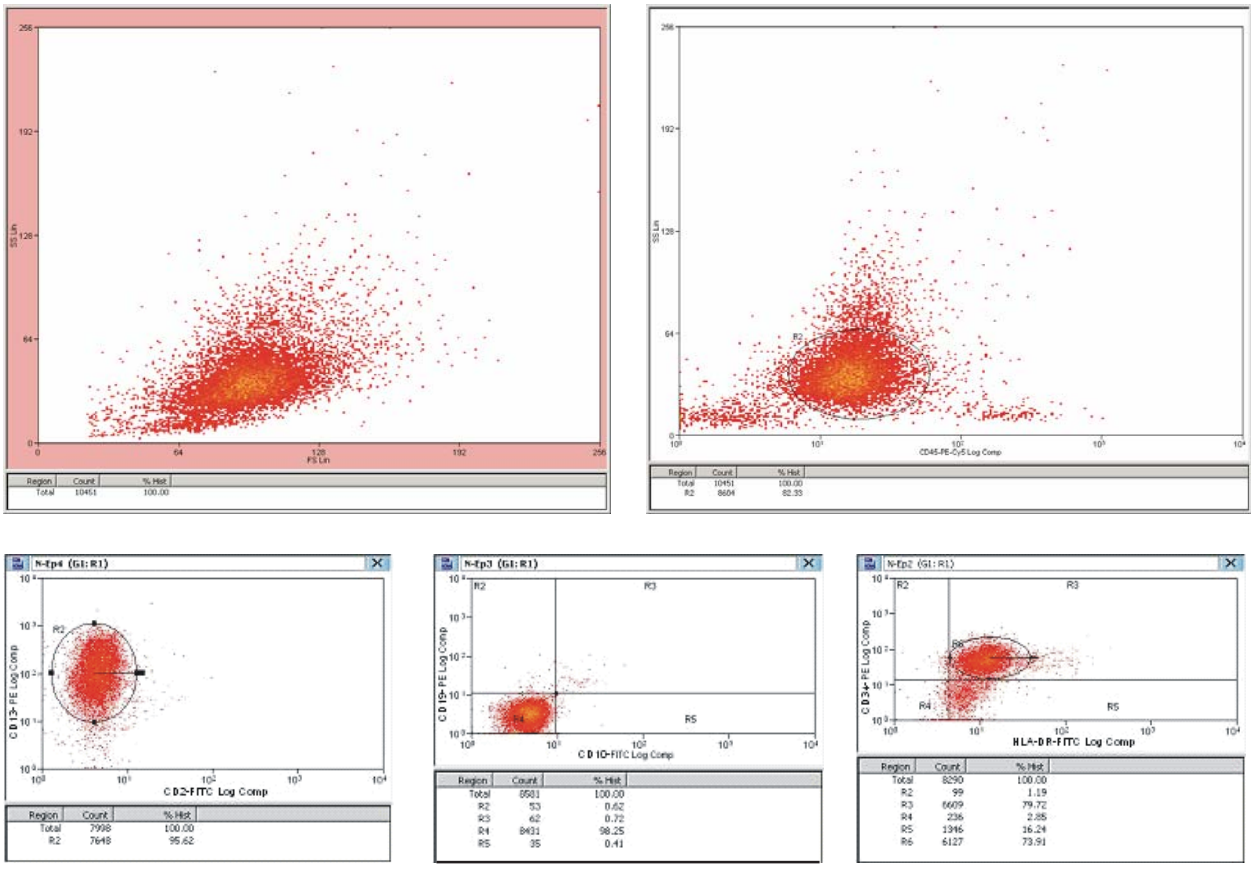

Figure 1: CD45 consecutive gating demonstrated in AML-M1 case. MPF of bone marrow sample presented in the charts demonstrated CD45/HLADR/CD34/CD13positive and CD2/CD10/CD19 negative mononuclear (blast) cells.

300 nM primers and 200 nM probes on a Mx3005P(TM) QPCR System (Stratagene) under the following conditions: 95°C for 10min, followed by 50 cycles of 95°C for 15 s, 50°C for 1 min. In order to correct variations in RNA quality and quantity and to calculate the sensitivity of each measurement, a control gene (CG) transcript was amplified in parallel to the fusion gene (FG) transcript. Since ABL (Abelson) gene transcript expression did not differ significantly between normal and leukemic samples, ABL was used as a control gene in this study (24). Positions and nucleotide sequences of the primers and probe are shown in Table 2.

Results

In the period of six months, between January and June 2008, 45 patients were submitted and tested for acute leukemia at the Clinic of Hematology-Skopje. Morphology and cytochemistry established myeloid lineage in 34 (75.5%) cases and lymphoid differentiation in 6 (13.3%) cases. Morphology and cytochemistry did not established lineage in 5 (11.1%) cases.

Further analyses of cases in which lineage could not be assigned based on morphology and cytochemistry, by MPF established myeloid lineage in 4 (AML-M0) and in one case indicated nonhematopoietic malignancy. In this case immunophenotype of the malignant cells (CD45-/CD56+/CD9+) indicated neuroectodermal origin of the malignant cells (21). Later neuroblastoma was diagnosed in this patient.

Consequently, based on FAB and immunologic criteria of EGIL and BTSH, 39 acute leukemias were

Table 2: Sequences and positions of the RQ-PCR primers and probes.

Transcript	EAC code[a]	Primer/probe localization, 5'–'3' position (size)	Sequence
BCR-ABL	ENF402	BCR, 1727–1744 (18)	5'-CTG GCC CAA CGA TGG CGA-3'
	ENF501	BCR, 3173–3193 (21)	5'-TCC GCT GAC CAT CAA YAA GGA-3'
	ENR561	ABL, 277–257 (21)	5'-CAC TCA GAC CCT GAG GCT CAA-3'
	ENP541	ABL, 230–254 (25)	FAM 5'-CCC TTC AGC GGC CAG TAG CAT CTG A-3' TAMRA
PML-RARα	ENF905	PML, 1198–1216 (19)	5'-CCG ATG GCT TCG ACG AGT T-3'
	ENF906	PML, 1642–1660 (19)	5'-ACC TGG ATG GAC CGC CTA G-3'
	ENF903	PML, 1690–1708 (19)	5'-TCT TCC TGC CCA ACA GCA A-3'
	ENR962	RARA, 485–465 (21)	5'-GCT TGT AGA TGC GGG GTA GAG-3'
	ENR942	RARA, 439–458 (20)	FAM 5'-AGT GCC CAG CCC TCC CTC GC-3' TAMRA

[a]ENF = forward primer, ENR = reverse primer, ENP = TaqMan probe.

Table 3: Correlation of antigen expression with cytochemical analysis and FAB morphology in AML cases.

	CD45	CD117	CD13	CD14	CD15	CD33	CD9	CD10	CD34	HLA-DR	MPO	LYS	TdT	CD2	CD7	CD56
Number of tested patients	44	44	44	44	44	44	44	44	44	44	44	44	44	44	44	44
Number of positive patients	44	37	38	11	8	34	1	1	27	33	36	21	1	1	3	1
FAB morphology																
M0	4	4	4	0	0	2	0	0	4	4	4	0	0	0	1	0
M1	6	5	6	0	0	7	0	1	6	6	6	0	1	0	1	1
M2	13	13	13	1	0	11	1	0	9	13	13	6	0	0	1	0
M3	4	4	4	0	0	4	0	0	2	0	4	4	0	0	0	0
M4	8	8	8	8	4	8	0	0	5	6	8	8	0	0	0	0
M5	3	3	3	2	3	2	0	0	1	3	1	3	0	1	0	0
M6*	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0

M6 was positive for CD71& GlycophorinA+. Other markers as cytCD3, cytCD22, cytCD79a, CD19 were tested but no positivity was shown.

classified as myeloid. Correlation between the antigen expressions with FAB morphology of AML case is presented in Table 3. According to FAB criteria, the leukemias were classified as M1 (n=6), M2 (n=13), M3 (n=4), M4 (n=8), M5 (n=3). After MPF, 4 cases were classified as M0, and one as AML-M6 (20, 21).

Six (15.3 %) of AML cases showed expression of lymphoid antigens. Co-expression of two lymphoid markers was detected in two cases, TdT&CD7 and CD7&CD56, respectively. It is interesting to be mentioned that TdT&CD7 case cytochemically stained with MPO, EST and PAS and had M1 morphology. Expression of CD7, CD10, CD9 and CD2 was notified in four other case of lymphoid antigen positive AML.

Molecular evaluation of AML-M3 cases demonstrated presence PML-RARa fusion transcript in all four cases.

Among the 6 cases with ALL based on morphology and cytochemistry, MPF confirmed lymphoid lineage in 5, but demonstrate myeloid immunophenotype in 1 patient; AML-M6 diagnose was established in this patient .The leukemia associated immunophenotype of the mononuclear cells in this patient was (CD45-/HLA-DR+/CD71+/GlycophorinA+). Characteristics of ALL cases are summarized in Table 4.

One ALL case demonstrated expression of the myeloid antigen CD15 and was positive for the fusion transcript BCR-ABL. In this case blasts cells cytochemically stained for PAS and EST.

Table 4: Characteristics of ALL cases.

Case No	FAB Morphology	Cytochemical Staining	ANTIGEN EXPRESSED	SUBTYPES OF ALL	BCR-ABL EXPRESSION
1	L2	PAS	HLA-DR;CD19; cytCD79a; cytIgM; CD10;TdT	Pre-B-ALL	Negative
2	L1	PAS	HLA-DR;CD34; CD19; cytCD79a;cytCD22;TdT	Pro-B-ALL	Negative
3	L2	PAS + EST	HLA-DR; CD19; CD34; cytCD22;CD10;TdT; CD15	Common-ALL	Positive
4	L2	PAS	HLA-DR; CD19;TdT cytCD79a;cytCD22;cytIgM;	Pre-B-ALL	Negative
5	L2	PAS	HLA-DR; CD19;TdT; cytCD79a;cytCD22;CD10	Common-B-ALL	Negative

Discussion

Modern diagnostic approach for acute leukemias combines the cytomorphology, cytochemistry, multiparameter flow cytometry, chromosome banding analysis, accompanied by diverse fluorescence in situ hybridization techniques, and molecular analyses (25-27). The correct diagnosis is essential for classification of this heterogeneous complex of disorders and plays a central role for individual risk stratification and therapeutic decisions (26, 27).

Assignment of lineage is critical in the diagnostic evaluation of acute leukemia, as treatment for AML and ALL markedly differs. Myeloid and lymphoid lineage may be distinguished based on cellular morphology, cytochemical staining, and expression of lineage-specific antigens (26, 29-30). Analyses of diagnostic evaluation of acute leukemia in our study showed that immunophenotyping was necessary for lineage assignment in 4 (8.9%) cases that were morphologically and cytochemically undifferentiated, and also corrected the lineage that was assigned based on morphology and cytochemistry in one (2.2%) additional case. In this case blasts cells had lymphoid morphology, stained with PAS and did not stained with other cytochemical stains, but nevertheless had myeloid immunophenotype, AML-M6. In one case (2.2%) with suspected acute leukemia, MPF indicate diagnosis of neuroblastoma (19).

Immunophenotyping is essential in the diagnosis of ALL and AML, demonstrates a particular lineage involvement and has a prognostic significance in ALL cases. We used the panel based on the recommendation by the EGIL group and BTSH. Our analyses

comprised a two step process with the first panel of markers being applied to all cases of acute leukemia and the second only in patients with AML which did not demonstrated a clear myeloid commitment. We also evaluate further lymphoid antigen positive AML cases by using the second panel of McAb.

Second panel is aimed for identification of uncommon types of AML, such as those with megakaryocytic or erythroid differentiation and the exclusion or conformation of diagnosis of nonhematological malignancy. In our study we apply the second AML panel in 8 cases; 2 which did not demonstrated clear myeloid commitment and 6 cases with lymphoid antigen positive AML cases. With our primary McAb we were able to differentiate AML form ALL in 97.7% of cases. Only in one patient (AML-M6-2.5%) lineage differentiation was assigned after staining with the secondary McAb panel and one case of nonhematological malignancy was confirmed.

PMF is crucial in all cases of poorly differentiated myeloid leukemia (AML-M0), megakaryoblatstic leukemia (AML-M7) and in some case of monoblastic leukemia (AML-M5) and those with primitive erythroid cells as predominant leukemic cells (AML-M6). It is important, also for recognizing AML co-expressing lymphoid-associated antigens. In addition immunophenotyping enables recognition of unusual form of acute leukemia designated acute biphenotypic or acute mixed lineage leukemia. The leukemia associated immunophenotype (LAIP) of the blast cells is a useful tool for detection of minimal residual disease in AML and ALL cases (7, 26-29).

Immunophenotying takes central role in the diagnosis of ALL and contributes to more precise and biologically oriented classification of the disease. Moreover, membrane antigen expression predicts treatment response and has prognostic significance (26).

B-lineage ALL is defined by the expression of at least two B-cell antigens, CD79a, CD19, and/or CD22; T-lineage ALL is defined by the expression of nuclear TdT and CD3. B-and T-lineage ALL can be further subclassified on the basis of cell differentiation of maturation (8).

ALL patients from our study had B-ALL immmunophenotype, two cases were diagnosed as common-B-ALL, two as pre-B-AL and one as pro-B-ALL (18-20,30,31).

In order to improve further their diagnosis, we tested them for the presence of the fusion transcript BCR-ABL. Only one patient was positive and underwent target therapy with Imatinib in combination with chemotherapy (32).

Results for our study showed that routine immunophenotyping improved the diagnosis in 5 (11.1%) cases with acute leukemia which was essential for more appropriate individual clinical stratification of the patient with acute leukemia. Results from immunophenotying guided us to perform exact molecular analyses in some of the subtypes of acute leukemia. Those further molecular testing of the patients with AMl-M3 and B-ALL for the presence of PML-RARa and BCR-ABL fusion transcripts, respectively, additionally improved the exact classification of the acute leukemia subtypes and made target therapy available for those patients (32, 33).

Our data support and validate routine clinical implementation of MPF in the diagnostic evaluation of acute leukemia.

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