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MINIMAL SCREENING ANALYSIS BASED ALGORITHM FOR DIAGNOSIS AND CLINICAL STRATIFICATION OF PATIENTS WITH ACUTE MYELOID LEUKAEMIA (AML): SINGLE CENTRE EXPERIENCE

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A b s t r a c t: In this paper we present our results from a study designed in order to establish and standardize a diagnostic algorithm for acute myeloid leukaemia (AML) in the Republic of Macedonia. A total of 146 consecutive adult patients (> 15 years) were enrolled in the study. First, we determined the correct lineage assignment of the blast cells and evaluated the incidence of the favourable PML/RARα, AML1/ETO, CBFβ/MYH11 genetic markers among the AML cases. Additionally, the obtained results were correlated with patients' age, comorbidities, and performance status, and each single AML patient was stratified to effective treatment strategy.

Our results showed that morphology and cytochemistry established a lineage in 132 (89.1%) of the patients, but not in 16 cases that presented as acute leukaemia, of which 7 were assigned as myeloid, and in two a non-haematopoietic malignancy was indicated with immunophenotyping. Mulitparameter flow cytometry immunophenotyping also changed the assigned lineage based on morphology and cytochemistry in 5 (3.3%) of the patients from lymphoid to myeloid and improved diagnosis in 21 (14.1%) cases.

By using a reverse transcriptase-polymerase chain reaction (RT-PCR) essay 28 (23.1%) patients were classified in the prognostically favourable AML genetic group; 8 patients expressed the fusion transcript PML/RARα, 5 AML1/ETO and 15 CBFβ/MYH11.

Moreover, analyses of the age, performance status and comorbidities further stratified an additional 12.5% of the patients to a different risk-adapted therapy.

The applied minimal screening-analysis-based diagnostic algorithm enabled improved and more precise diagnosis and clinical stratification in 37.2 % of AML patients from our study group.

Key words: AML, prognosis, diagnostic algorithm, genetic markers, flow citometry.

Introduction

Acute myeloid leukaemia (AML) is one of the most common types of leukaemia in adults which encompasses a family of haematologically malignancies that can be categorized according to their cytogenetic and associated genetic abnormalities (Lichtman 2010).

The latest World Health Organization (WHO) classification of acute leukaemia incorporates and interrelates morphology, cytogenetics, molecular genetics and immunologic markers and pays major attention on the importance of genetic events in the classification, prognosis 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 inv(16)/t(16,16) which generally has a favorable prognosis when treated with appropriate therapeutic agents. All other genetic events identified among AMLs had strong prognostic meaning but did not influence the therapeutic decision (Swerdlow 2008). Modern therapeutic concepts of AML are based on individual risk stratification at diagnosis and during follow-up.

The diagnosis of the diverse subtypes of AML is a major challenge for modern haematology. The routine diagnostic setting is complex and consists of classic cytogenetics, molecular cytogenetics, molecular genetics and immunophenotyping by multi-parameter flow cytometry (Döhner 2010)**.** The ultimate test of any disease diagnostic algorithm approach for AML, which guides the diagnostic procedure from basic to more specific methods, is its usefulness in guiding the selection of effective treatment strategies. (Haferlach 2007, Löwenberg 2008b). Algorithms that provide a basis for risk-adapted therapeutic choices may include immunological markers, cytogenetic factors, molecular markers as well as clinical parameters (e.g. age, attainment of an early or late complete remission) and haematological determinants (e.g. secondary AML, white blood cell count at diagnosis).

The more carefully AML is studied, the clearer it becomes that there is considerable heterogeneity among cases with respect to morphology, immunological phenotype, associated cytogenetic and molecular abnormalities and, more recently, patterns of gene expression (Lowenberg 2008a).

The diagnostic process is becoming more demanding with respect to experience, time and costs due to the expansion of diagnostic methods and ana-

lysis. There are numerous overlaps between different diagnostic methods. These can be used for an optimal pathways in the complex diagnostic proceedings and for validation of the results of single methods, which can be summarized in diagnostic algorithms.

In order to improve and simplify the diagnosis and management of AML patients who are diagnosed and treated at the University Haematology Clinic, – Skopje, we conducted a prospective study to establish and standardize a diagnostic algorithm based on minimal screening tests which will facilitate risk-adapted therapy for each single AML patient. The aims of our study were: first, to establish the correct lineage assignment of the blast cells, second, to evaluate the incidence of the favourable genetic markers PML/RARα, AML1/ETO and CBFβ/MYH11 among the AML cases, then to correlate the obtained results with the patient's age, comorbidities, and performance status as well as consecutively to select the effective treatment strategy for each single acute leukaemia patient.

Material and methods

Patients and samples

A total of 146 adult (> 15 years) patients (from 148initially tested) with acute leukaemia who were consecutively admitted to the Hematology Clinic, Skopje, from January 2009 to January 2012 were enrolled in this study. The median age of the patients (81 men, 67 women) was 49 ± 18.66 years (range 14–82), and most of the patients, 71 (48.6%), were between 51 and 76 years old. 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 (Bennet 1976) and confirmed by immunophenotyping of bone marrow aspirates and/or peripheral blood samples following the criteria of the European Group for the Immunological Classification of Leukaemia (EGIL) (Bain 2002, Bene 1995). The exact lineage assignment of the blast cells was guided to implementation of specific molecular analyses in AML cases. They were all tested for the presence of the fusion transcript of the mayor recurrent cytogenetic abnormalities in AML (PML/RARα, AML1/ETO, CBFβ/MYH11) by RT-PCR, according to standard procedures (Gabert 2003) and further stratified in the adequate genetic AML entities. The samples contained more than 20% of blast cells (most had more than 50%).

Morphology and Cytochemical analysis

The morphology and cytochemical analyses were made by microscopic examination of > 200 nonerythroid cells on air-dried bone marrow smears stained by May Gruenwald Giemza and for MPO, non-specific esterase (NSE)

and periodic acid-Schiff (PAS) according to the manufacturers' guidelines (Bennet 1976, Döhner 2010, Panovska-Stavridis 2008).

Immunophenotyping

The muliparameter flow cytometry (MPF) was performed at the University Hematology Clinic, Skopje by using a BD FAXCANTO II analyser, on lysed whole blood and/or bone marrow samples, as previously described. Briefly, cell suspensions were stained for simultaneous detection of cytoplasmic/nuclear and membrane antigens with multiple panels of three monoclonal antibodies (McAb) labelled with fluorescein (FITC), phycoerythrin (PE) and allophycocyanin (APC) as third colour (Panovska-Stavridis 2008). The slightly modified panel of monoclonal antibodies (McAb) against myeloid- and lymphoid-associated antigens as suggested by the EGIL was utilized (Bain 2002, Panovska-Stavridis 2008). The antibodies and their manufacturers are listed in Table 1.

Acquired data were analyzed with BD FACS Diva™ software version 6.0 by using CD45 gating strategy. All cases were first screened by the primary McAb panel and, if necessary, further characterized by the McAb of the secondary panel (Bain 2002).

Antigen expression was considered positive if 20% or more blast cells reacted with a particular antibody, excluding reactivity of blasts cells with MPO. It was considered positive if 10% or more of mononuclear cells were MPO positive (Bain 2002, Döhner 2010).

Table1

Panel of monoclonal antibodies for diagnosis of acute leukemias

	B-lineage	*T-lineage*	*Myeloid markers*	*Non-lineage restricted*
First line	CD19, cyt CD79b, cyt CD22	CD2, CD7, cyt CD3	CD117, CD13, CD33, CD14, CD15, anti-MPO, anti-lysosyme	TdT, CD34, HLA-DR, CD56
Second line	SmIg (kappa/lambda)**, cytIgM[2], CD138	CD1a, mCD3[1], CD4, CD5, CD8, antiTCRα/β, antiTCRγ/δ***	CD41, CD61, CD42, CD71 Anti-glycophorin A	CD38

cyt – cytoplasmic; 2.m – membrane; *MPO – myloperoxidase; ** SmIg – surface immunoglobulin; *** TCR – T cell receptor

Molecular Analysis

Mononuclear cell preparation, RNA isolation, and cDNA synthesis were performed 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. The RQ-PCR reaction was performed in a 25 μl reaction volume using 12.5 μl (1×) Master Mix (Applied Biosystems), 300 nM primers and 200nM probes on a M × 3005P$^{(TM)}$ QPCR System (Stratagene) under the following conditions: 95°C for 10 min, followed by 50 cycles of 95°C for 15 s, 50°C for 1min. 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.

Table 2

Sequences and positions of the RT-PCR primers and probes

Transcript	EAC code[a]	Primer/probe localization, 5'–'3' position (size)	Sequence
AML1/ETO	ENF701	AML1, 1005–1026 (22)	5'–CAC CTA CCA CAG AGC CAT CAA A–3'
	ENR761	ETO, 318–297 (22)	5'–ATC CAC AGG TGA GTC TGG CAT T–3'
	ENP747	AML1, 1049–295 (30)	FAM 5'–AAC CTC GAA ATC GTA CTG AGA AGC ACT CCA–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
CBFB/MYH11	ENF803	CBFB, 389–410 (22)	5'–CAT TAG CAC AAC AGG CCT TTG A–3'
	ENR862	MYH11, 1952–1936 (17)	5'–AGG GCC CGC TTG GAC TT–3'
	ENR863	MYH11, 1237–1217 (21)	5'–CCT CGT TAA GCA TCC CTG TGA–3'
	ENR865	MYH11, 1038–1016 (23)	5'–CTC TTT CTC CAG CGT CTG CTT AT–3'
	ENPr843	CBFB, 434–413 (22)	FAM 5'–TCG CGT GTC CTT CTC CGA GCC T–3' TAMRA
ABL	ENF1003	ABL, 372–402 (31)	5'–TGGAGATAACACTCTAAGCATAACTAAAGGT–3'
	ENF1063	ABL, 495–515 (21)	5'–GATGTAGTTGCTTGGGACCCA–3'
	ENF1043	ABL, 467–494 (28)	FAM 5'–CCA TTT TTG GTT TGG GCT TCA CAC CAT T–3' TAMRA

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

Statistical analysis

Statistical analyses were performed by using the statistical analyses software SPSS 18.0. The level of probability for obtaining the null hypothesis, in accordance with the international conventions for bio-medical sciences, was 0.05 or 0.01 (Armitage 2002).

Results

In a period of two years, between January 2009 and January 2012, 146 cases of acute leukaemia were diagnosed (out of 148 initially tested) at the University Hematology Clinic, Skopje. Cyto-morphological analyses showed that the average rate of the blast cells in the differential blood counts and in the bone marrow was 54.6% (3–99.0%) and 73.5% (20–98.0%) respectively. Morphology and cytochemistry established myeloid lineage in 109 (73.6%) cases and lymphoid differentiation in 23 (15.5%) cases, but did not establish lineage involvement in 16 (10.8%) cases. Basic morphological and cytochemical analyses established the lineage assignment of the blasts cells in 132 (89.1%) patients.

Statistical analyses showed that there is a statistically significant correlation between the AML and MPO positivity (X^2 with Yates's correction = 16.628 $p < 0.01$). According to the cross-reaction ratio, MPO positivity presents a statistically significant risk which improves the chance of AML diagnosis by 39 times (OR = 392.1603 (4.177 < 0R < 305.9796, CI 95%)). Also, a statistically significant correlation was noted between the PAS positivity and ALL (X^2 with Yates's correction = 5.514 $p < 0.01$).

Immunophenotyping of the cases in which lineage could not be assigned based on morphology and cytochemistry established myeloid lineage in 7 patients (AML-M0) and in two cases the immunophenotype of the malignant cells (CD45-/CD56+/CD9+) indicated a neuroectodermal origin of the malignant cells (Bain et al., 2002). Immunological analyses changed the assigned lineage based on morphology and cytochemistry in 5 (3.3%) of the patients from lymphoid to myeloid. The results of our study showed that routine immunophenoltyping improved diagnosis in 21 (14.1%) cases.

Consequently, 121 (82.8%) cases were classified as AML. The correlation between the antigen expressions and FAB morphology of AML cases is presented at Table 3.

Multivariate Cox-proportional regression analyses showed that in 89.7% of AML cases lineage assignment is defined by the following five markers: CD13, CD33, CD117, HLA-DR and anti-MPO.

Moreover, molecular analyses of AML cases classified 28 (23.1%) of the patients in the adequate prognostic favourable AML genetic entities. Eight patients expressed the PML/RARα fusion oncogene and were diagnosed as acute promyelocitic leukaemia (APL)/AML-M3. RT-PCR analysis also detected molecular abnormalities in the Core binding factor (CBF) in 20 AML patients; the presence of the AML/ETO1 fusion gene was confirmed in 5 patients and CBFβ/MYH11 in 15 patients.

Evaluation of the distributions of the patients from our study group in the different grades of the Eastern Cooperative Oncology Group (EKOG) performance status scale (Oken 1982) is presented in Fig. 1.

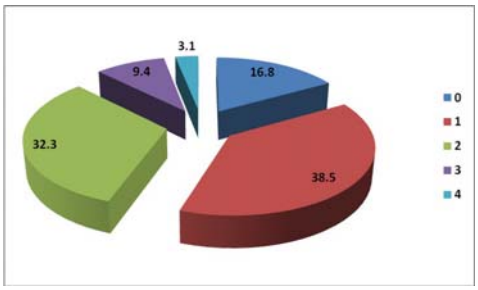

Figure 1 – Distribution of patients according to grades of EKOG performance status

Fifteen (12.5%) patients from our study group had an EKOG performance status higher than 2, but only 4 of those patients were younger than 60 years of age. Analyses of the incidence of serious comorbidities in our study group showed that 9 (8.2%) patients had serious comorbidities which limited the application of intensive chemotherapy in their treatment (Sorror 2001). Seven of them were older than 60 years.

Furthermore, all the obtained results were correlated and a consecutively effective treatment strategy for each single acute leukaemia patient was selected.

Discussion

A basis for every therapeutic decision in AML cases should be provided by a multimodal diagnostic approach. The optimal therapeutic conditions are based on exact classification and prognosis of the AML subtype at diagnosis and on delineation of sensitive markers for MRD studies during the complete haematological remission. MRD methods have many potential applications in the clinical management of patients with acute leukaemia. Today, a multimodal

diagnostic approach which combines different diagnostic techniques is needed to meet these requirements (Haferlach 2007, Lichtman 2010).

Our diagnostic algorithm for AML (shown in Fig. 2) starts with basic cytomorphology and cytochemistry analyses that established the lineage assignment of the blasts cells of 132 (89.1%) patients in our study group. These methods should always be performed in combination because they allow rapid classification of acute leukaemia and further enable the choice of the antibody panel for flow cytometric analyses. Routine immunophenotyping further improved the diagnosis in 21 (14.1%) more cases. In case cytomorphology and immunological characteristics give indices of characteristic aberrations of the FAB subtypes M3/M3v for t(15;17)/PML/RARα, RT-PCR analyses, rearrangement should be promptly applied (Grimwade 2002, Wang 2008).

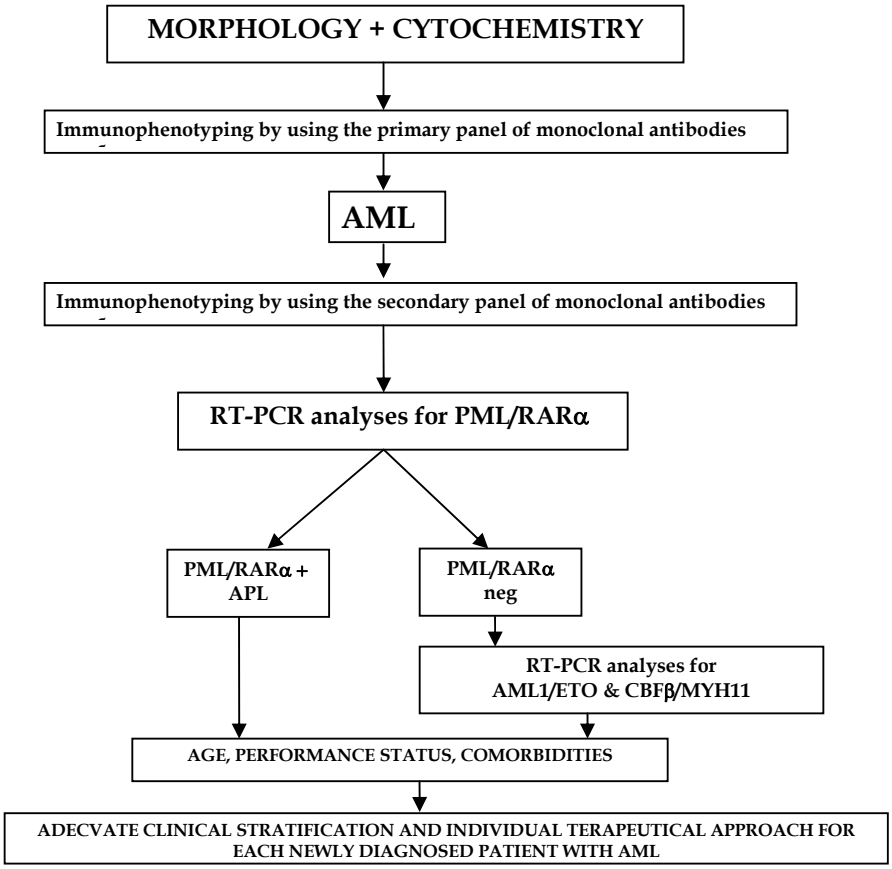

Figure 2 – Diagnostic algorithm and algorithm for risk-adapted therapy in AML patients

Literature data also suggests this analysis that should be initiated as soon as possible if even only clinical symptoms indicate a suspicion of APL (Schoch 2002). However, our experience showed that initial clinical features, morphology, cytochemistry and immunophenotype do not always correlate with AML-M3 diagnosis and therefore in our algorithm we recommended all AML cases to be tested by RT-PCR for the fusion oncogene PML/RARα. The RT-PCR method is the most sensitive and rapid technique for the detection of this oncogene and provides an optimal basis for MRD analyses (Wang 2008).

Further we suggest all AML cases which are PML/RARα negative be tested for the presence of the reciprocal fusions genes that describe CBF-AML, AML1/ETO and CBFβ/MYH11. These oncogenes present molecular markers of the two most prevalent cytogenetic subtypes of adult primary or *de novo* AML. Detection of these genetic abnormalities in adult patients with primary AML is a favourable independent prognostic indicator for the achievement of cure after intensive chemotherapy or bone marrow transplantation (BMT), and may serve as a paradigm of the risk-adapted treatment in AML (Döhner 2010, Marcucci 2000). In almost all studies of adult primary AML, the highest CR rate (approximately 90%) and the longest disease-free survival (DFS) at *5* years (approximately 50%) have been associated with CBF-AML cases. The prognostic impact of the CBF gene rearrangements appears equally significant in the setting of chemotherapy or stem cell transplant (SCT) of AML patients in first CR. However, the iatrogenic morbidity and mortality of BMT suggest that patients with CBF AML should not receive this therapeutic modality as initial treatment. The collective analysis of all data regarding the implementation of all BMT in AML treatment suggests that although allogeneic SCT and autologous SCT may have a potential role in the initial management of AML other than CBF AML, the treatment-related morbidity and mortality of SCT represent a therapeutic limitation for treatment of CBF-AML patients. Considering the high probability of cure that these patients can achieve with intensive chemotherapy, it is reasonable to spare them the toxicity of SCT as consolidation in a first complete remission (Löwenberg 2008a, Koreth 2009, Marcucci 2000, Marcucci 2001 & Perea 2006).

Although standard cytogenetic analysis is currently the most common method for identifying the cytogenetic abnormalities t(8;2l)(q22;q22) and inv (16)(pl3q22) or t(16;16)(p13;q22) that define CBF-AML patients, we decided to test all patients for the presence of the CBF fusion genes by using RT-PCR essay. Despite the recent improvements in the cytogenetic methodology and the use of complementary techniques such as fluorescence *in situ* hybridization and comparative genomic hybridization to increase the rate of successful karyotyping, the possibility that subtle structural chromosomal aberrations are missed still remains (Marcucci 2000). In t(8;21)(q22;q22) or inv(16)(p13q22), failure to

detect submicroscopic (cryptic) rearrangements of the involved genes leads to false-negative results that may ultimately impact the correct stratification of this patient population in prognostic and risk-adapted therapy groups. On the other hand, sensitive molecular methodologies such as RT-PCR have been widely successfully used to detect cryptic CBF abnormalities in diagnostic samples of AML patients with karyotypes that are otherwise negative for t(8;21)(q22;q22) or inv(16)(p13q22). The authors of these studies concluded that all primary AML should be routinely tested for the presence of the CBF fusion genes by molecular screening to improve genomic stratification of AML patients in risk-related treatment groups (Marcucci 2000).

Therefore we chose to utilize in our algorithm the molecular analyses that additionally improved the exact classification of 28 (23.1%) of our cases in the specific AML genetic entities and made a specific therapeutic approach available for those the AML patients.

The applied multimodal diagnostic approach consisting of a minimal number of cytomorphological, cytochemistry, immunological and molecular analyses enabled an improved and more precise diagnosis and clinical stratification in 37.2% acute laeukemia patients in our study.

Moreover, when we correlate those results with the results obtained from the analyses of the EKOG performance status and the incidence of serious comborbidities in our study group, an additional 12.5% of the patients were stratified to a different risk-adapted therapy.

Our initial results are consistent with literature data and indicate that our applied multimodal diagnostic approach improved the diagnosis of the specific genetic entities of AML in which a specific treatment approach is indicated and allows individual clinical stratification in treatment protocols of the patients.

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Р е з и м е

ПРИКАЗ НА ПРИМЕНАТА НА АЛГОРИТАМ БАЗИРАН НА МИНИМАЛЕН БРОЈ ДИЈАГНОСТИЧКИ АНАЛИЗИ ВО ДИЈАГНОСТИКА И КЛИНИЧКА СТРАТИФИКАЦИЈА НА АКУТНИТЕ МИЕЛОИДНИ ЛЕУКЕМИИ (АМЛ): ИСКУСТВА ОД ЕДЕН ЦЕНТАР

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Во овој труд се презентирани резултатите од студијата спроведена со цел да се воспостави и стандардизира дијагностички алгоритам за акутната миелоидна леукемија (АМЛ) во Република Македонија. Во неа беа вклучени вкупно 146 адултни (> 15 години) последователни пациенти со АМЛ.

Најпрво, беше одредена линиската припадност на бласните клетки и евалуирана инциденцата на поволните генетски маркери PML/RARα, AML1/ETO, CBFβ/MYH11 помеѓу АМЛ случаите. Потоа, добиените резултати дополнително беа корелирани со возраста, коморбидните состојби и општата состојба и за секој поединечен пациент со АМЛ беше стратифицирана ефикасна тераписка стратегија.

Нашите резултати покажаа дека со морфологијата и цитохемијата се одреди линиската припадност кај 132 (89,1%) од пациентите, но не и кај 16 од нив. Имунофенотипизацијата кај 7 од овие пациенти покажа миелоидна припадност, а кај два беше инидицирано постоење на нехематолошки малигнитет. Мултипараметарската имунофенотипизација со проточен цитометар ја промени назначената линиска припадност врз основа на морфолошките и цитохемиските испитувања кај 5 (3,3%) од пациентите од лимфоидна во миелоидна и ја подобри дијагнозата во 21 (14,1%) од случаите.

Со примена на есејот реверзна-транскрипција-полимераза верижна реакција (РТ-ПВР) 28 (23,1%) пациенти беа класифицирани во прогостички поволната генетска група на АМЛ; 8 пациенти го експримираа фузиониот транскрипт PML/RARα, 5 AML1/ETO и 15 CBFβ/MYH11.

Анализата на возраста, општиот статус и коморбидните состојби дополнително стратифицираа уште 12,5% од пациентите во различни адаптирани на ризик тераписки групи.

Применетиот дијганостички алгоритам базиран на минимален број дијагностички анализи овозможи подобра и попрецизна дијагностика и клиничка стратификација на 37,2% пациенти со АМЛ од нашата студиска група.

Клучни зборови: акутна миелоидна леукемија (АМЛ); прогноза; дијагностички алгоритам; генетски маркери; проточна цитометрија.

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