

1. Mrozek2004 & 2006 & 2007 (cited)

2. Coco2008 (cited)

6. Grimwade1998 (cited)

7. Goud2005 (not cited)

3. Slovak2000 & Byrd2002 (cited)

8. Pui2003 (not cited)

4. Sainati1997 (cited)

5. Schoch2001 (cited)

Cytogenetic analysis in acute myeloid leukemia in Tîrgu Mureş

Analiza citogenetică în leucemia acută mieloidă în Tîrgu Mureş

Claudia Bănescu^{1*}, Ionela Paşcanu¹, Katalin Csép¹, István Benedek², Erzsebét Benedek², Carmen Duicu³

1. University of Medicine and Pharmacy Tîrgu Mureş - Genetics Department

2. University of Medicine and Pharmacy Tîrgu Mureş - Hematology Clinic Tg.Mureş

3. University of Medicine and Pharmacy Tîrgu Mureş - Pediatric Clinic

Abstract

Acquired clonal chromosome aberrations can be observed after metaphase banding analyses in 50–60% of patients with acute myeloid leukemia (AML). The cytogenetic results at diagnosis provide the most important parameter for determining prognosis so far. Numerous recurrent karyotype abnormalities have been described in AML. Our aim is to evaluate the frequency of chromosomal abnormalities in patients with acute myeloid leukemia admitted in the Hematology Clinic in Tîrgu Mureş and to compare our results with those reported elsewhere.

We received for chromosomal analysis 28 samples of AML patients from the Hematology Clinic in Tîrgu Mureş between January 2006 and July 2008. We carried out bone marrow and/ or peripheral blood cell culture according to standard methods.

The leukemic karyotypes of 24 patients were successfully analyzed. Abnormal clones were detected in 66% of cases AML. In our study, the most frequent abnormality was hyperdiploidy. These findings are similar to the results obtained in other studies using a similar approach.

Key words: acute myeloid leukemia, cytogenetics, chromosomal abnormalities

Rezumat

Anomaliile cariotipice clonale sunt detectate prin analize citogenetice în 50-60% dintre pacienții cu leucemie acută mieloidă (LAM). Analizele citogenetice în momentul diagnosticului reprezintă parametrul cel mai important pentru stabilirea prognosticului. În LAM au fost descrise numeroase anomalii cromozomiale recurente. Scopul studiului nostru este să stabilim frecvența anomaliilor cromozomiale la pacienții cu LAM din Clinica de Hematologie din Tîrgu Mureş și de a compara datele obținute cu cele descrise în literatura de specialitate.

*Corresponding author: Claudia Bănescu, UMF Tg. Mureş - Genetics Department, 38, Gh. Marinescu Str, Tg.Mures 540042, Romania
Phone: +40 265 215551, E-mail: claudiamures@gmail.com

Au fost primite 28 de probe biologice de la pacienții cu LAM internați în Clinica de Hematologie din Tîrgu Mureș în perioada ianuarie 2006 - iulie 2008. S-au efectuat culturi celulare din măduva osoasă hematogenă și/ sau sânge periferic, conform metodelor standard.

Analiza citogenetică (cariotipul) a putut fi efectuată în 24 dintre cazuri. În 66% dintre acestea au fost evidențiate anomalii cariotipice clonale. Aceste date sunt similare cu rezultatele obținute în alte studii. Hiperdiploidia a fost cea mai frecventă anomalie cromozomială întâlnită în studiul nostru.

Cuvinte cheie: leucemie acută mieloidă, citogenetică, anomalii cromozomiale

Introduction

Acute myeloid leukemia (AML) is a very heterogeneous disease with regard to clinical features and acquired genetic alterations, microscopically detectable both as structural and numerical chromosome aberrations¹⁵. At present, cytogenetic aberrations detected at the time of AML diagnosis constitute the most common basis for prediction of clinical outcome^{5, 12}. Several parameters provided by cytormorphology, immunophenotyping and especially cytogenetics are needed to classify AML into biological entities in order to establish the diagnosis and to understand the pathogenesis, as well as to develop specific treatment approaches.

Pattern of Cytogenetic Abnormalities in AML

Clonal chromosome abnormalities, defined as an identical structural aberration or gain of the same structurally intact chromosome detected in at least two metaphase cells, or the same chromosome missing from a minimum of three cells, are consistently found in the majority of AML patients at diagnosis. However, in contrast to patients diagnosed with chronic myeloid leukemia, who are invariably positive for t(9;22) or its variants, the cytogenetic picture of AML is much more complex¹¹. To date, approximately 200 different structural and numerical aberrations such as reciprocal translocations, inversions, insertions, deletions, unbalanced translocations, isochromosomes, isodicentric chromosomes, isolated trisomies and monosomies have been found to be recurring chromosome changes in AML. Many of these

aberrations are very rare, being so far detected in few patients worldwide, whereas others occur more frequently. These more common abnormalities, together with their frequencies among adults and children with AML, are presented in Table 1.

The incidence of abnormal karyotypes in AML has been reported to be 55% to 78% in adults and 77% to 85% in children^{1, 6}. However, a substantial proportion of patients show no chromosome abnormalities.

Prognostic significance of cytogenetic abnormalities in AML

The karyotype of the leukemic blasts is the most important independent prognostic parameter in AML⁷. A favorable outcome under currently used treatment regimens was observed in several studies in patients with t(8;21) (q22;q22), inv(16)(p13q22) or t(15;17) (q22;q11-12). Chromosome aberrations with an unfavorable clinical course are inv(3)/t(3;3), -5/del(5q), -7/del(7q) and complex aberrant karyotype. All others, i.e. patients with abnormal karyotype and rare chromosome aberrations, are assigned to an intermediate prognostic group^{3, 15}.

The incidence of distinct chromosome abnormalities varies with age, but the prognosis of defined cytogenetic aberrations is age-independent¹.

The higher incidence of the chromosomal abnormalities demonstrates the importance of cytogenetic evaluation in patients with acute myeloid leukemia. Our findings suggest that cytogenetic analysis is a useful tool in the investigation of these patients for confirmation of clin-

Table 1. Frequencies of the most common cytogenetic abnormalities in patients with acute myeloid leukemia in different study groups^{3,11,17}.

| Cytogenetic Abnormality | MRC study n=2337 | CALGB study n=1311 | SWOG/ECOG study n=609 |
|------------------------------------|------------------|--------------------|-----------------------|
| -5 | 79 | 26 | - |
| -7 | 136 | 47 | - |
| -Y | - | 58 | 20 |
| del(5q) | 104 | 42 | - |
| del(7q) | 73 | 19 | - |
| del(9q) | 37 | 33 | 17 |
| del(11q) | - | 12 | - |
| +8 | 211 | 123 | 53 |
| +21 | 51 | 28 | - |
| t(15;17)(q22;q21) | 210 | 88 | 27 |
| t(8;21)(q22;q22) | 104 | 81 | 50 |
| inv(16)(p13q22); t(16;16)(p13;q22) | 53 | 96 | 53 |
| t/inv(11q23) | 45 | 54 | 42 |
| t(9;22)(q34;q11) | 16 | 10 | 8 |
| Complex karyotype | 222 | 234 | 124 |

MRC- United Kingdom Medical Research Council; CALGB- Cancer and Leukemia Group/B; SWOG/ECOG -Southwest Oncology Group/Eastern Cooperative Oncology Group;

ical diagnosis. The karyotype of the leukemic blast has been shown to be the most important independent prognostic parameter in AML. These techniques should be performed in each patient with AML at diagnosis as well as in the case of relapse.

Our aim was to evaluate the frequency of chromosomal abnormalities in patients with acute myeloid leukemia in the Hematology Clinic in Tîrgu Mureş and to compare our results with those reported elsewhere. We also included our patients in different cytogenetic risk groups. There are few reports about cytogenetic analysis in malignant hemopathies in Romania. Most of them were performed in children with acute leukemia. It is the first study frequency of chromosomal abnormality in AML in this region of the country.

false cited data

Materials and Methods

Patients

Between January 2006 and July 2008, 28 samples of bone marrow and/or peripheral blood from patients with acute myeloblastic leukemia were sent to the Genetic Laboratory of the University of Medicine and Pharmacy in Tîrgu Mureş, Romania, for cytogenetic evaluation. The study included children and adults with a diagnosis of acute myeloblastic leukemia. Their ages ranged from 2 to 62 years (median 35 years). All the cases were classified according to the French-American-British (FAB) classification². There were 13 males and 15 females from the Hematology Clinic in Tîrgu Mureş. The patients were from the central part of Romania.

Cytogenetic analysis

Heparinized bone marrow and/or peripheral blood samples were collected in syringes or test tubes and sent to the laboratory at room temperature. Three different cultures (for direct harvest, 24-hour culture, and 72-hour culture with hematopoietic growth factors) were prepared as described elsewhere⁸, starting from these samples. Culture media contained RPMI 1640 medium, 20% FCS (fetal calf serum), L-glutamine and penicillin/streptomycin (50 IU/ml and 50 µg/ml, respectively). Any samples without anticoagulant or in which EDTA was used as an anticoagulant, were classified as unsuitable and excluded from the analysis. Metaphases were harvested by adding colcemid (10µg/ml) solution followed by hypotonic KCl (0.075 M) treatment and fixation using standard 3:1 methanol: glacial acetic acid fixator. We used the conventional Giemsa banding (GTG banding) technique. Five to ten slides were screened in each case and 10 - 20 metaphases were analyzed for each sample. At least three cells were karyotyped according to the International System for Human Cytogenetic Nomen-

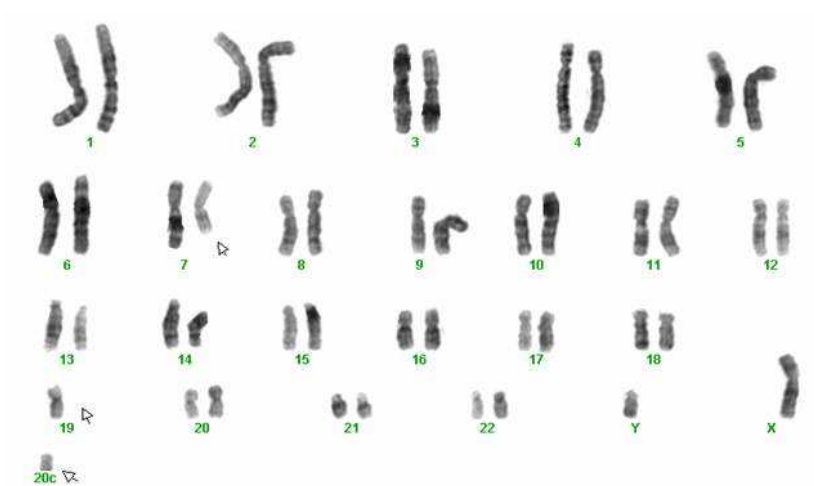


Figure 1. Karyotype 46,XY,del(7q),-19,+mar

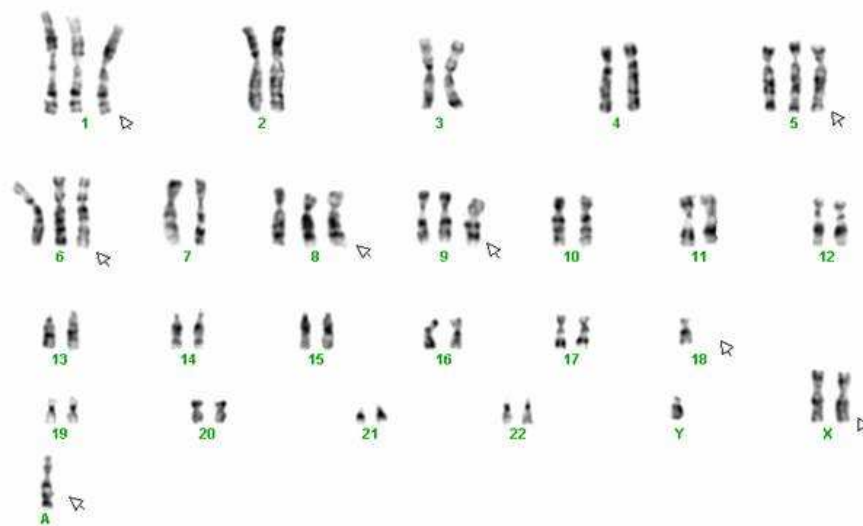


Figure 2. Hyperdiploidy. Karyotype: 52,XXY, +1,+5,+6,+8,+9,-18,+mar

clature (ISCN 1995) ¹¹. Analysis was carried out using a BX51 Olympus microscope and images captured with an automated image analysis system (Cytovision, Applied Imaging). Cell culture failure was defined as cases with poor-quality metaphases or with less than 10 analyzable metaphases.

Results

The 28 analyzed samples from patients with acute leukemia included: 26 cases at the

moment of diagnosis and 2 after relapse. Among the 28 samples received, 2 were unsuitable for cytogenetic analysis due to clotting or the use of an unsuitable anticoagulant; these samples were consequently excluded from the study. A karyotype was not definite in 7.6 % of the cases because of a very low mitotic index or poor-quality metaphases obtained from the cell culture.

We successfully analyzed the leukemic karyotype of 24 patients, and identified 16 cases with chromosomal abnormalities. The results

Table 2. Distribution of FAB groups by ploidy in AML at diagnosis in our patients

| | No. of cases | FAB groups | | | | | | | |
|-----------------------|--------------|------------|----------|----------|----------|----------|----------|----------|----------|
| | | M0 | M1 | M2 | M3 | M4 | M5 | M6 | M7 |
| Hyperdiploidy (>50) | 4 | 1 | - | - | - | - | 2 | - | 1 |
| Hyperdiploidy (47-50) | 4 | 1 | 1 | 2 | - | - | - | - | - |
| Diploidy (normal) | 8 | 1 | 1 | 2 | 2 | 1 | - | 1 | - |
| Pseudodiploidy | 4 | - | 1 | 1 | 1 | 1 | - | - | - |
| Hypodiploidy | 4 | 1 | - | 1 | 1 | - | - | - | 1 |
| Total | 24 | 4 | 3 | 6 | 4 | 2 | 2 | 1 | 2 |

Table 3. Cytogenetic Risk Groups

| Risk Group | Cytogenetic abnormality | No. of cases |
|-----------------------|--|--------------|
| Favorable | t(15;17) | 1 |
| Intermediate | del(7q) | 1 |
| | +21 | 2 |
| | +19 | 1 |
| | t(14;21) | 1 |
| | Normal karyotype | 8 |
| | All other structural/numerical abnormalities (hyperdiploidy, hypodiploidy) | 8 |
| Unfavorable (adverse) | Complex karyotype | 2 |

were obtained from overnight cultures and from cultures with additional growth factors, as described in the methods.

wrong term

Cytogenetic Findings

Clonal abnormalities were detected at diagnosis in 16 patients (66%), of which 2 cases (12.5%) had complex karyotype and 14 patients (87.5%) had one or two clonal chromosome changes. An abnormal clone carrying del(7q) was detected in 1 patient with AML (Figure 1). In this patient, del(7q) was part of a complex karyotype and was associated with monosomy 19 and an extra chromosome marker (mar). A complex karyotype is defined by the presence of at least 5 clonal aberrations (12,16) or at least 3 abnormalities in the absence of t(8;21), inv(16)/t(16;16) and t(15;17).^{3, 17, 18}

Isolated trisomy 19 was present in 1 case. Isolated trisomy 21 was documented in 2

cases. Hyperdiploidy with more than 50 chromosomes (Figure 2) was detected in 4 cases.

Table 2 shows the distribution of structural abnormalities and ploidy according to FAB subgroups in AML at diagnosis. The structural rearrangements were the Robertsonian translocations t(14;21) in the M4 (1 case), reciprocal translocation t(15;17) in the M3 (1 case) and deletion del(7q) in the M1 (1 case) FAB sub - groups.

On the whole, our results showed that the most frequent clonal karyotype alteration in AML was hyperdiploidy, detected in 50% of cases, while metaphases with structural anomalies were found in 4 cases (25%); another 4 (25%) presented a hypodiploid karyotype.

Cytogenetic Risk Groups

We included our patients in different cytogenetic risk groups, according to the hierar-

chical system of karyotype classification of Medical Research Council (MRC) AML 10 Trial (Table 3).

Discussion

Cytogenetic analysis of acute leukemia yields important information which has been demonstrated to be correlated with patient survival. Cytogenetic analysis is consequently standard practice in the diagnostic approach to leukemia, but very accurate culturing methods and considerable technical expertise are needed for proper karyotype analysis of acute leukemia.

In our study, acquired clonal chromosome abnormalities, structural aberrations or trisomy observed in at least 2 and monosomy found in at least 3 metaphase cells, were detected in 66% of patients with *de novo* AML. In 12.5% of patients, the abnormal karyotype was complex, i.e. contained at least 3 chromosome aberrations, whereas in almost 40% of patients no cytogenetic abnormality could be found using standard banding methods. The chromosome abnormalities frequency (66%) found in our study are within the limits reported by other authors^{4, 5, 15}.

We successfully analyzed the leukemic karyotype of 24 patients (85%) with AML. These results are similar to the findings of a BFM (Berlin-Frankfurt-Munster Group) multicenter study conducted on patients with acute leukemia. Lampert reports a cytogenetic success in 70% of AML, with a proportion of abnormal karyotypes of 68% in AML⁹. Sainati et al. have observed abnormal clones in 66% of AML cases¹⁶.

Our results indicate that in AML, good metaphases are obtained from the three short-term cultures, and the addition of growth factors is useful. Sainati et al. also demonstrated the relationship between cytogenetic success in AML and the method with three different cultures¹⁶.

In our study, the most frequent clonal karyotype alteration in AML was hyperdiploidy, detected in 50% of cases, while metaphases with structural abnormalities were found in 4 cases (25%); another 4 (25%) presented a hypodiploid karyotype. In other studies like the Medical Research Council (MRC) AML 10 Trial, AIEOP protocols (Associazione Italiana Emato-Oncologia Pediatrica), GALGB 8461 (Cancer and Leukemia Group B) and SWOG/ECOG (Southwest Oncology Group/Eastern Cooperative Oncology Group), the most frequent karyotype alterations were represented by chromosomal structural abnormalities^{3, 5}. We think that the different results concerning the most frequent clonal karyotype in AML obtained in our study are due to the low number of patients.

In our study the most frequent trisomy isolated or associated with other chromosome abnormalities was trisomy 21. According to Mrozek et al.², the most common trisomies in *de novo* AML are, in decreasing order of frequency, +8, +22, +13, +21 and +11. Trisomy 22 is a non-random secondary aberration accompanying *inv(16)/t(16;16)* and is rarely seen as an isolate chromosome abnormality. Although each of the remaining trisomies can be found as a secondary aberration, +8, +13, +11 and +21 are also detected recurrently as isolate karyotypic changes at diagnosis, with a frequency among adults with *de novo* AML of 4% for trisomy 8; 1% each for trisomy 13 and trisomy 11, and 0.4% for trisomy 21.^{4, 12}

It is also very important to continue looking for other abnormalities that might be relevant to the diagnosis, treatment and follow-up of leukemia confirming the relevance of cytogenetic analysis in acute leukemia.

Cytogenetic analyses are used not only in diagnosis. According to Slovak et al., karyotypic analysis predicts outcome of pre-remission and post-remission therapy in adult acute myeloid leukemia¹⁸.

We included our patients in three dif-

ferent cytogenetic risk groups (favorable, intermediate and unfavorable) according to the hierarchical system of karyotype classification of Medical Research Council (MRC) AML 10 Trial. According to this classification, most of our patients had an intermediate risk.

According to published criteria adopted by SWOG (Southwest Oncology Group), four cytogenetic categories were defined¹⁷. The favorable risk category included patients with abnormalities *inv(16)*/*t(16;16)*/*del(16q)*, or *t(15;17)* with any additional abnormalities, or *t(8;21)* either without *del(9q)* or being part of a complex karyotype. The intermediate risk category included patients characterized by *+8*, *-Y*, *+6*, *del(12p)*, or normal karyotype. The unfavorable risk category was defined by the presence of one or more of *inv(3q)*/*t(3;3)*, *-5/del(5q)*, *-7/del(7q)*, *del(9q)*, *t(6;9)*, *t(9;22)* and complex aberrant karyotype defined as 3 or more abnormalities. The unknown risk category included all other abnormalities. All SWOG karyotypes of unknown prognostic significance are designated as intermediate risk by MRC. Other major differences between these 2 systems are: the classification of 11q abnormality as intermediate risk by MRC but unfavorable risk group by SWOG and the classification by MRC of all *t(8;21)* studies as favorable, despite the presence of *del(9q)* or complex karyotypes. According to SWOG risk category definitions, most of our patients had an unknown risk.

During the last 30 years, cytogenetic analyses of patients with AML have discovered a great number of recurrent chromosome abnormalities. Several of the more common abnormalities have been associated with specific laboratory and clinical characteristics, and are used as diagnostic and prognostic markers. However, the prognostic importance of less frequent recurrent aberrations, both primary and secondary, is still unknown.

Because cytogenetic findings are among the most important prognostic factors, cytogenetic analysis of bone marrow is now

mandatory in the diagnostic workup of newly diagnosed patients with AML.

The genetic alterations in AML include chromosome abnormalities detectable by cytogenetic analyses i.e. translocations and numerical abnormalities, as well as subtle gene alterations that are identified by molecular techniques such as small duplications/insertions and point mutations. Recent molecular analyses of leukemic blasts from patients with AML and a normal karyotype, have revealed a striking heterogeneity with regard to the presence of acquired gene mutations and changes in gene expression. Multiple submicroscopic genetic alterations have been discovered, including internal tandem duplication of the *FLT3* (Fms-related tyrosine kinase3) gene, mutations in the *NPM1* (Nucleophosmin - nucleolar phosphoprotein B23, numatrin) gene, partial tandem duplication of the *MLL* (Myeloid/Lymphoid or mixed-lineage leukemia) gene, high expression of the *BAALC* gene, and mutations in the *CEBPA* (*CCAAT*/ enhancer binding protein, alpha) gene¹⁴.

According to the latest studies, *FLT3* and *NPM1* aberrations show apparently opposite prognostic significance, *FLT3* mutations are correlated with poor outcome and *NPM1* mutations are associated with a more favorable response to therapy.

Other chromosomal aberrations, which have not yet been well defined at the gene level (e.g. numerical abnormalities such as *-7*, *-5*, *+8*, and others) are detectable by cytogenetic or fluorescence in situ hybridization (FISH) analysis only, and are equally important in the clinic because of their association with specific entities (e.g. therapy-related AML) and with unfavorable outcomes.

Based on the above considerations, modern genetic characterization of AML combines conventional karyotyping and molecular methods - FISH, reverse transcriptase polymerase chain reaction (RT-PCR), sequencing - with the aim of analyzing all major types of

clinically relevant alterations¹⁰

Application of gene-expression profiling has also identified a gene-expression signature that appears to separate cytogenetically normal AML patients into prognostic subgroups, although gene expression signature based classifiers predicting outcome for individual patients with greater accuracy are needed. These and similar future findings are likely to have a major impact on the clinical management of cytogenetically normal AML not only in terms of prognosis but also in the selection of appropriate treatment, since many of the identified genetic alterations already constitute or will probably become targets for specific therapeutic intervention¹⁴. Because of the different results concerning the most frequent clonal karyotype in AML (hyperdiploidy) obtained in our study we will continue this study and we will look for other abnormalities.

Conclusion

The cytogenetic investigation in AML is important because it enabled us to include our patients in different cytogenetic risk groups.

References

1. Appelbaum FR, Gundacker H, David R, et al. Age and acute myeloid leukemia. *Blood* 2006 107: 3481-3485
2. Bennet JM, Catovsky D, Daniel MT, et al. Proposed revised criteria for the classification of acute myeloid leukemia. *Ann Intern Med* 1985; 103: 626-9.
3. Byrd JC, Mrozek K, Dodge RK, et al. Pretreatment cytogenetic abnormalities are predictive of induction success, cumulative incidence of relapse, and overall survival in adult patients with de novo acute myeloid leukemia: results from Cancer and Leukemia Group B (CALGB 8461). *Blood* 2002; 100: 4325-36.
4. Farag SS, Archer KJ, Mrozek K, et al. Isolated trisomy of chromosomes 8, 11, 13 and 21 is an adverse prognostic factor in adults with de novo acute myeloid leukemia: results from Cancer and Leukemia Group B 8461. *Int J Oncol* 2002; 21: 1041-51.
5. Grimwade D, Walker H, Oliver F, et al. The Importance of Diagnostic Cytogenetics on Outcome in AML: Analysis of 1,612 Patients Entered Into the MRC AML 10 Trial. *Blood* 1998 92: 2322-2333
6. Haferlach T, Schnittger S, Kern W, et al. Genetic classification of acute myeloid leukemia (AML). *Ann Hematol.* 2004; 83 Suppl 1: S97-100.
7. Haferlach T, Schoch C, Löffler H, et al. Morphologic dysplasia in de novo acute myeloid leukemia (AML) is related to unfavorable cytogenetics but has no independent prognostic relevance under the conditions of intensive induction therapy: results of a multiparameter analysis from the German AML Cooperative Group studies. *J.Clin.Oncol.* 2003, 21: 256-265
8. Haines JL, Korf BR, Morton C, et al. Current Protocols in Human Genetics. Chapter 10 Cancer Genetics Unit 10.2 Metaphase Harvest and Cytogenetic Analysis of Malignant Hematological Specimens. John Wiley & Sons, 2006
9. Lampert F, Harbott J, Ritterbach J. Chromosomen aberrationen bei akuten Leukämien in Kindesalter :Analyse von 1009 Patienten. *Klin Padiatr* 1991; 203: 311-8
10. Lo-Coco F, Cuneo A, Pane F, et al. Prognostic impact of genetic characterization in the GIMEMA LAM99P multicenter study for newly diagnosed acute myeloid leukemia. *Haematologica* 2008; 93(7).
11. Mítelman F, ed. ISCN (1995): An International System for Human Cytogenetic Nomenclature. Basel: S. Karger; 1995.
12. Mrózek K, Heerema NA, Bloomfield CD. Cytogenetics in acute leukemia. *Blood Rev.* 2004 Jun;18 (2): 115-36
13. Mrozek K, Heinonen K, de la Chapelle A, Bloomfield CD. Clinical significance of cytogenetics in acute myeloid leukemia. *Semin Oncol.* 1997; 24: 17-31.
14. Mrózek K, Marcucci G, Paschka P, et al. Clinical relevance of mutations and gene-expression changes in adult acute myeloid leukemia with normal cytogenetics: are we ready for a prognostically prioritized molecular classification? *Blood.* 2007, 109: 431-448
15. Mrózek K, Bloomfield CD. Chromosome Aberrations, Gene Mutations and Expression

Changes, and Prognosis in Adult Acute Myeloid Leukemia. *Hematology* 2006

16. Sainati L, Leszl A, Putti M, et al. Centralized Cytogenetic Analysis Of Pediatric Acute Leukemia: Results Of An Italian Collaborative Experience. *Haematologica* 1997; 82: 654-659

17. Schoch C, Haferlach T, Haase D, et al. Patients with de novo acute myeloid leukaemia and complex karyotype aberrations show a poor prognosis despite

intensive treatment: a study of 90 patients. *Br J Haematol.* 2001; 112: 118-126.

18. Slovak ML, Kopecky KJ, Cassileth PA, et al. Karyotypic analysis predicts outcome of preremission and postremission therapy in adult acute myeloid leukemia: a Southwest Oncology Group/Eastern Cooperative Oncology Group study. *Blood.* 2000; 96: 4075-4083.