# ARRAY COMPARATIVE GENOMIC HYBRIDISATION AS A TOOL FOR A RAPID MAPPING OF BREAKPOINTS IN UNBALANCED TRANSLOCATIONS IN LEUKEMIA

# ARRAY KOMPARATIVNÍ GENOMICKÁ HYBRIDIZACE JAKO NÁSTROJ PRO RYCHLÉ MAPOVÁNÍ ZLOMOVÝCH MÍST NEBALANCOVANÝCH TRANSLOKACÍ U LEUKÉMIÍ

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**Summary**
**Background:** Chromosomal translocations involving immunoglobulin loci (14q32/*IGH*, 2p11/*IGK* and 22q11/*IGL*) play an important role in pathogenesis of B cell leukemia and lymphoma. These aberrations lead to deregulated transcription of targeted oncogenes by their juxtaposition with the *IGH* transcriptional enhancer(s). Fluorescent *in situ* hybridization (FISH) showed to be a potential tool for identification of cancer-related genes located in breakpoint regions of chromosomal translocations. However, the commonly used „probe-mapping" FISH strategy requires numerous experiments with consecutively selected probes from the narrowed down region and uses a significant amount of cytogenetic material. One of the alternative approaches, array comparative genomic hybridisation (aCGH), is a rapid technique that operates on DNA level and uses only a small amount of tumor material. In contrast to FISH, however, it analyzes only unbalanced aberrations. The aim of this study was to evaluate array comparative genomic hybridisation (aCGH) as a potential tool for a rapid mapping of breakpoint of non- reciprocal IGH-associated translocation in B cell leukemia and lymphoma. **Material and methods**. For this study, we selected one case of B cell chroniclymphocytic leukemia (CLL) with a complex karyotype including unbalanced der(14)t(1;14)(q25;q32) involving IGH. Genomic profiling of this case was performed using 1 megabase (Mb) aCGH. Validation of aCGH results was done by metaphase FISH with Bacterial Artificial Chromosome (BAC) clones and chromosome painting probes.
**Results and conclusions**. In one single aCGH experiment eight regions of genomic imbalances (4 gains and 4 losses) were identified. As expected, these imbalances included also duplication of 1q due to the der(14)t(1;14). Two consecutive BAC clones flanking the proximal breakpoint at 1q21.3 have been identified. These clones were further applied for metaphase FISH analysis that confirmed aCGH findings. Despite of 1 Mb resolution of the applied platform, these particular clones are separated by approximately 3 Mb. Given that this region is gene-rich, further BAC-mapping is required to identify the candidate gene located in the breakpoint region. Moreover, aCGH data helped us to correct original cytogenetic findings and precisely define karyotypic changes in this case. Our data provide additional evidence that aCGH is a powerful technique for molecular karyotyping of tumors and allows a rapid mapping of genomic imbalances, including breakpoints of non-reciprocal translocations. As shown in this study, the latter can be detected with high accuracy and sensitivity during a single experiment.

**Keywords**: array comparative genomic hybridisation, aCGH, unbalanced translocation, oncogene, IGH, chronic lymphocytic leukemia

**Souhrn**
**Východiska:** Chromosomální translokace zahrnující imunoglobulinové lokusy (14q32/*IGH,* 2p11/*IGK* a 22q11/*IGL*) hrají důležitou roli v patogenezi B-buněčných leukémií a lymfomů. Jejich výsledkem je deregulace transkripce onkogenů zahrnutých do těchto translokací, která je způsobená jejich juxtapozicí s *IGH* transkripčními enhancery. Pro identifikaci nádorových genů lokalizovaných v blízkosti zlomových míst chromosomových translokací lze použít fluorescenční *in situ* hybridizaci (FISH). Nicméně běžně užívaná mapovací strategie metodou FISH vyžaduje velký počet experimentů se sondami vybranými ze zkoumané oblasti a spotřebuje značné množství cytogeneticky zpracovaného nádorového materiálu. Jedním z alternativních přístupů je array komparativní genomická hybridizace (aCGH), rychlá technika na úrovni DNA, která používá jen malé množství nádorového materiálu. Narozdíl od metody FISH však dovoluje určit pouze nebalancované změny. Cílem této práce bylo ukázat, že aCGH je efektivní nástroj k rychlému mapování zlomových míst nereciprokých *IGH* translokací u B-buněčných leukémií a lymfomů.
*Materiál a metody*. Pro tuto studii jsme vybrali jednoho pacienta s B-buněčnou chronickou lymfocytární leukémií (CLL) s komplexním karyotypem a nebalancovanou translokací der(14)t(1; 14)(q25;q32) zahrnující *IGH*. Ke genomickému profilování tohoto případu jsme použili metodu aCGH s rozlišením 1 megabáze (Mb). Validace výsledků aCGH byla provedena pomocí metafázové FISH s BAC klony a celochromosomovými malovacími sondami. **Výsledky a závěry**. Během jednoho aCGH experimentu bylo identifikováno osm aberantních oblastí (4 zmnožení a 4 ztráty genetického materiálu). Podle našeho očekávání tyto abnormality

zahrnovaly také duplikaci 1q zahrnuté do tranlokace der(14)t(1;14). Byly identifikovány dva po sobě následující BAC klony ohraničující zlomové místo v oblasti 1q21.3. Tyto klony byly posléze použity pro metafázovou FISH, která potvrdila aCGH nález. Navzdory 1 Mb rozlišení použitého chipu, byly od sebe tyto dva konkrétní klony odděleny oblastí přibližně 3 Mb velkou. Vzhledem k tomu, že v této oblasti se vyskytuje velké množství genů, je k identifikaci kandidátního genu ležícího v oblasti zlomu nezbytné další mapování za pomocí BAC klonů. aCGH výsledky nám navíc pomohly opravit původní cytogenetický nález a přesně určit změny karyotypu u tohoto pacienta. Naše data poskytují další důkaz toho, že aCGH je efektivní technika pro molekulární karyotypování nádorů a umožňuje rychlé mapování genomických změn, včetně zlomových míst nereciprokých translokací. Ty mohou být detekovány s vysokou přesností a citlivostí během jediného experimentu, jak ukazuje naše práce.

**Klíčová slova**: array komparativní genomická hybridizace, aCGH, nebalancované translokace, onkogen, *IGH*, chronická lymfocytární leukémie

## INTRODUCTION

Molecular cytogenetic techniques including FISH and aCGH are potential tools used to unravel tumor-associated chromosomal aberrations. They offer precise molecular karyotyping with a much higher resolution that conventional banding analysis. Among others, FISH has been succesfully applied for mapping of translocation breakpoints and identification of targeted genes. This strategy, however, requires selection of numerous DNA probes from the presumably involved region and several rounds of experiments before the breakpoint region will be narrowed down to <1 Mb. Usually this procedure is labourious and time- and material-consuming. Array comparative genomic hybridisation (aCGH) enables rapid and efficient mapping of genomic imbalances (including unbalanced translocations) in one reaction at a resolution given only by the size and density of clones on the array (1). By principle, this technique does not operate in cases with balanced rearrangements.

The aim of this study was to evaluate aCGH as a tool to identify putative oncogenes located in the breakpoint regions of non-reciprocal *IGH*/14q32-associated translocations in B cell leukemia and lymphoma. It is well known that these, usually reciprocal, translocations result in deregulated transcription of affected oncogenes by bringing them in the vicinity of regulatory sequences of *IGH*. Thus, hypothetically, each gene affected by 14q32/*IGH* translocation can be consider as a putative oncogene.

To evaluate potential of aCGH in a rapid mapping of breakpoints of *IGH*-associated translocations, we selected one case of B cell chronic lymphocytic leukemia (B-CLL) with unbalanced der(14)t(1;14)(q25;q32) and other complex chromosomal changes. Results of our studies are shown and discussed below.

## MATERIAL AND METHODS

*Patient*

Patient, 66-year-old male with clinically and immunophenotypically unambiguous B-CLL, was recently diagnosed in our center. Peripheral blood was taken at the time of diagnosis after informed consent.

*Cytogenetic analysis*

Peripheral blood cells were cultured 72 hour in presence of tetradecanoyl phorbol acetate (TPA). Chromosome preparations, R- banding and karyotyping were performed using conventional methods. Chromosomal aberrations were described according to ISCN (2005)(2).

*Array CGH*

Arrays were constructed using a 1Mb Clone Set (Welcome Trust Sanger Institute, UK) containing a total of 3527 BAC/PAC clones (3), in MicroArray Facility (Flanders Interuniversity Institute for Biotechnology, VIB, Leuven, Belgium). Genomic DNA was extracted according to standard procedures. Test and reference gDNA were labeled by a random prime labeling system (BioPrimeR Array CGH Genomic Labeling Module, Invitrogen, Carlsbad, CA) with Cy3-/Cy5-labeled dCTPs (Amersham Biosciences, Piscataway, NJ). Probe preparation, preblocking of the slide, hybridization and posthybridisation washes were performed with small modifications as described previously (3, 4). Slides were scanned using GenePix 4000B scanner (Axon Instruments, Foster City, CA), image and data analysis was done using GenePix Pro 6.0 (Axon Instruments) and Excel (Microsoft Inc., Diegem, Belgium). Data were normalized by dividing the fluorescent intensity ratio of each spot by the mean of the ratios of the autosomes. The normalized ratio values of the duplicates were averaged and a $\log_2$ value was calculated. For detection of copy number alterations we determined our thresholds as 0.3 for gains and -0.3 for losses.

*Interphase/metaphase FISH*

BAC/PAC clones, RP4-790G17 (148,42-148,56 Mb) and RP11-216N14 (151,95-152,11 Mb), were labeled in Spectrum Green and Spectrum Orange, respectively and used for FISH. We selected them from the 1Mb Clone Set (Welcome Trust Sanger Institute, UK) used for arrays. Other applied probes included LSI IGH, WCP 2 (Vysis Inc, IL, USA), WCP7 and WCP 8 (Cambio Ltd, Cambridge, UK) and break- apart IG kappa assay (5). BAC DNA was labeled by a random prime reaction (RadPrime DNA labeling system, Invitrogen) with Spectrum Orange/Green d-UTPs (Vysis Inc.) according to manufacturers protocols.

FISH experiments were evaluated using the Axioplan 2 fluorescence microscope equipped with the charge-coupled device Axiophot 2 camera (Carl Zeiss Microscopy, Jena, Germany) and the MetaSystems Isis imaging system (MetaSystems, Altlussheim, Germany). Three to 6 abnormal metaphases were evaluated in each FISH experiment.

## RESULTS AND DISCUSSION

Cytogenetic analysis of peripheral blood cells from the reported patient revealed presence of two related abnormal clones presented in Table 1. The second subclone showed structural aberrations of both 14q32 described as der(14)t(1;14)(q25;q32) and add(14)(q32).

The applied aCGH analysis identified 8 regions of genomic imbalances. These imbalances include loss of 10q26.3qter, 11q22.3q23.2, 13q14.2q14.3 and 14q32.33qter and duplication of 1q21.3qter, 2p14pter, 7q11.2qter and 8q21.3qter (Fig. 1A). The size of unbalanced regions varied from 2 to 97 Mb. The identified duplicated 1q region covered 94 Mb; RP4-790G17 mapped at 148,42-148,56 Mb is the first proximal

**Table 1.:** Summary of cytogenetic and aCGH/FISH results
*aCGH results described according to ISCN (2005)

| Karyotype | aCGH results* | Karyotype corrected after aCGH and FISH |
|---|---|---|
| 1.<br>46,XY,add(5)(q35),add(10)(q26),del(11)(q21q23),del(13)(q13q21) [3]/<br><br>2.<br>46,XY,del(2)(p12),t(3;13)(q27;q31),add(7)(q35),add(8)(p12),add(10)(q26),del(11)(q21q23),del(13)(q13q21),der(14)t(1;14)(q25;q32),add(14)(q32),add(15)(q26) [5] | arr cgh<br>1q21.3qter(RP4790G17→CTB-160H23)x3,<br>2p14pter(GS1-68F18→RP11-568N6)x3,<br>7q11.2qter(RP5-905H7→RP4-764O12)x3,<br>8q21.3qter(RP11-3J21→CTC-489D14)x3,<br>10q26.3qter(RP11-168C9→CTB-137E24)x1,<br>11q22.3q23.2(RP11-563P16→RP11-212D19)x1,<br>13q14.2q14.3(RP11-305D15→RP11-431O22)x1,<br>14q32.33qter(RP11-417P24→CTC-820M16)x1 | 1.<br>46,XY,add(5)(q35), der(10)t(8;10)(q21.3;q26.3),del(11)(q22.3q23.2),del(13)(q14.2q14.3) [3]/<br><br>2.<br>46,XY,t(2;14)(p12;q32),t(3;13)(q27;q31),dup(7)(q11.21qter),der(10)t(8;10)(q21.3;q26.3),del(11)(q22.3q23.2),del(13)(q14.2;q14.3),der(14)t(1;14)(q21.3;q32.33),der(15)t(2;15)(p14;q26.3) [5] |

BAC clone found to be duplicated and CTB-160H23 at 247,03-247,17 Mb is the most terminal duplicated clone. These results indicated gain of the 1q21.3qter region (B). According to cytogenetics, this region was translocated to the der(14). To validate this aCGH finding, we performed metaphase FISH analysis with SpectrumGreen-labeled RP4-790G17 (148,42-148,56 Mb) and SpectrumOrange-labeled RP11-216N14 (151,95-152,11 Mb); the latter clone represents the adjacent proximal region flanking the 1q21.3 breakpoint. Indeed, the der(14)t(1;14) was marked by a single green signal while both normal chromosomes 1 carried co-localized green/red signals (Figure 2.A). The aberrant 2F1R signal pattern was found in 36 % of interphase cells. Further FISH with LSI IGH applied on the previously analyzed metaphases showed two red signals (3'end of IGH) on der(14) and add(14) and one green signal (*IGHV*) on chromosome resembled del(2)(p12) (Fig. 2.B). Loss of the second green IGH signal was in line with the 14q32.33-qter loss found by aCGH illustrating the non- reciprocal t(1;14). The postulated reciprocal t(2;14)(p14; q32.33) was demonstrated by chromosome painting with WCP2 that hybridized to the add(14)(q32), del(2)(p12), normal chromosome 2 and unexpectedly, to add(15)(q26). The 2p12 breakpoint of t(2;14) was further mapped distally to *IGK* that retained on the der(2).

The remaining 7q11.2qter and 8q21.3qter gains were also validated by metaphase FISH using respective chromosome paintings. WCP7 hybridized with a normal chromosome 7 and add(7)(q35) indicating dup(7)( q11qter). WCP8 marked two normal chromosomes 8 and add(10)(q26) that showed to be der(10)t(8;10) (q21.3;q26.3). The latter non-reciprocal translocation was confirmed by loss the 10q26.3-qterm region found by aCGH. Losses of 11q22.3-q23.2 and 13q14.2-q14.3 remained in line with the respective del(11q) and del(13q) observed by cytogenetics. Results of cytogenetic, aCGH and FISH analysis are summarized in Table 1.

Altogether, aCGH complemented by FISH studies allowed us to correct karyotype of the reported case as follows: 46,XY,t(2;14) (p12;q32), t(3;13) (q27;q31), dup(7) (q11.21qter), der(10) t(8;10) (q21.3;q26.3), del (11) ( q22.3q23.2), del (13) (q14.2q14.3), der(14) t(1;14) (q21.3; q32.33), der(15)t (2;15) (p14;q26.3)

Particularly important, we were able to rapidly map the breakpoint of non-reciprocal IGH-mediated t(1;14)(q21;q32) expecting to affect gene involved in pathogenesis of CLL. The breakpoint was narrowed down to the 1q21.3 region flanked by two consecutive BAC clones spaced by approximately 3 Mb. Unfortunately, this chromosome region is not covered with a resolution of 1 Mb, as could be expected. We searched for potential candidate genes with the Ensembl Cytoview genome browser (www.ensembl.com). This region, however, contains dozens of genes, mostly with unknown functions. It is worth to note that none of the 4 previously described genes associated with lymphomas: *BCL9* (6), *FCGR2B* (7), *MUC1* (8) and *IRTA2* (9), is located in the breakpoint region. This suggests that t(1;14) ( q21.3;q32.33) involves a new oncogene that warrants identification and characterization. Further FISH studies with BAC and fosmid clones selected from the narrowed down 3 Mb breakpoint region will follow.

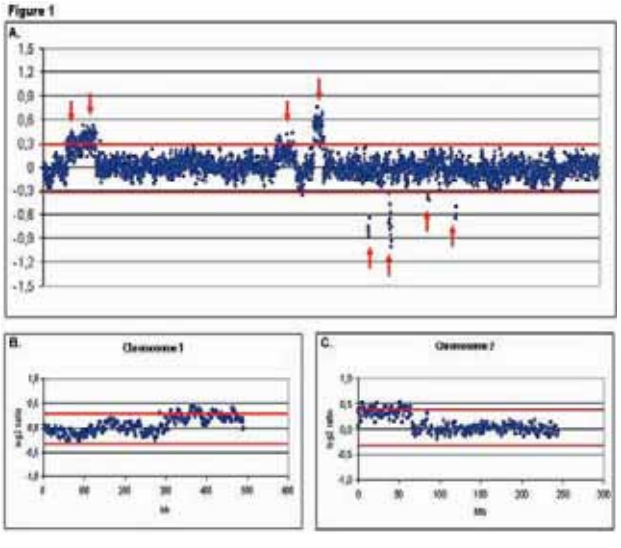


**Figure 1.:**
A. aCGH genomic profile The x-axis represents the clones ordered from the chromosome 1 to 22, X and Y. The Y-axis shows the log2 ratios of Cy5/Cy3 fluorescent intensity. The bold line indicates the thresholds for gains (0.3) and losses (-0.3). Arrows mark duplications of 1q21.3qter, 2p14pter, 7q11.2qter, 8q21.3qter and losses of 10q26.3qter, 11q22.3q23.2, 13q14.2q14.3 and 14q32.33qter, from the left side to the right.
B. Partial genomic profile of chromosome 1 showing the 1q21.3qter duplication The x-axis represents the clones ordered from 1p telomere to the 1q telomere. The Y-axis shows the log2 ratios of Cy5/Cy3 fluorescent intensity. The bold line indicates the thresholds for gains (0.3) and losses (-0.3). Lower log2 ratios of duplicated region reflect subclonal appearance of this aberration found by interphase FISH.
C. Partial genomic profile of chromosome 2 showing the 2p14pter duplication. The x-axis represents the clones ordered from 1p telomere to the 1q telomere. The Y-axis shows the log2 ratios of Cy5/Cy3 fluorescent intensity. The bold line indicates the thresholds for gains (0.3) and losses (-0.3).

In addition to der(14)t(1;14), we were able to map breakpoints of two other non-reciprocal translocations, t(2;15) and t(8;10) and to determine duplicated region of 7q. Deletions of 11q and 13q were mapped with a resolution of approximately 1 Mb. As expected, the 11q and 13q lost regions harbor respectively, *ATM* and *miR*15/*miR*16, the candidated tumor suppressor genes involved in pathogenesis of CLL (10, 11).

Finally, using FISH, we identified t(2;14)(p12;q32), the second

*IGH*-associated translocation present in this case of CLL. Given that this translocation is reciprocal, the 2p12 breakpoint could not be rapidly mapped by aCGH; FISH identification of the involved partner gene requires more labourious BAC-mapping strategy. The known 2p genes involved in *IGH*-associated translocations in B-NHL include *REL* (2p16) and *BCL11A* (2p16) (12).

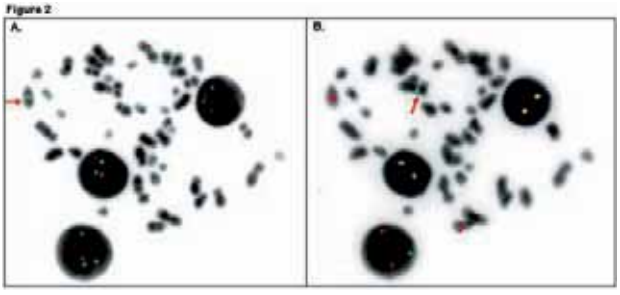


**Figure 2.**
A. FISH with RP4-790G17 (SpectrumGreen) (148,42-148,56 Mb) and RP11-216N14 (SpectrumOrange) (151,95-152,11 Mb) confirming the aCGH results. The arrow shows the der(14)t(1;14)(q21.3;q32.33) with the only RP4-790G17 signal. Other two red/green signals are localized on both chromosomes 1.
B. FISH with LSI IGH (Vysis Inc.) probe on the same rehybridized metaphase. The red signals confirm the presence of 3' end of *IGH* on der(14) and add(14). The arrow is showing one green signal (*IGHV*) localized at der(2)t(2;14)(p12;q32.33). Loss of second green signal remains in line with the 14q32.33qter loss found by aCGH. Note the aberrant signal pattern also in interphase nuclei.

B-CLL is one of the most common leukemias in the Western world showing variable clinical course. The most frequent genomic aberrations identified in CLL include del(13q), del(11q), trisomy 12, del(17p) and del(6q) found in up to 80 % of cases analyzed by FISH. Importantly, del(11)(q22q23) and del(17)(p) likely targeting the *ATM* and *p53* genes, respectively, hallmark rapid disease progression and poor survival, while del(13) (q14.3) as a single aberration is associated with a good prognosis (13, 14). Chromosomal translocations involving 14q32/ *IGH* are relatively rare in CLL; they occur in about 4 % of cases studied by FISH and usually affect the *BCL2*/18q21 and *BCL3*/19q13 genes (13, 14, 15). Particularly interesting is finding of two 14q32/*IGH* translocations in the present case. Given that der(14)t(1;14) and t(2;14) were found in a subclone with a more complex karyotype, we believe that both these translocations represent secondary chromosomal aberrations acquired during evolution of the del(11q)/del(13q)-positive karyotype.
In conclusion, using isolated CLL case, we demonstrated potential of aCGH as a tool for a rapid molecular mapping of non-reciprocal translocation. Resolution of this analysis reflects resolution of the applied aCGH platform. In the present case, the 1q21 breakpoint possibly harboring a novel CLL-associated oncogene, was narrowed down to the approximately 3 Mb region during one aCGH experiment. This approach is significantly less time- and material-consuming when compare to a standard probe-walking strategy. In most cases, the definitive mapping of breakpoint may require complementary FISH analysis. Although application of aCGH is limited to unbalanced translocations, it can be succesfully used in rare non- reciprocal translocations involving *IGH*/14q32 likely targeting lymphoma-associated oncogenes.


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**REFERENCES**
1. Fiegler H., Gribble SM., Burford DC. et al.: Array painting: a method for the rapid analysis of aberrant chromosomes using DNA microarrays. J Med Genet 40, 2003, 664-670.
2. ISCN (2005): An International System for Human Cytogenetic Nomenclature, Shaffer LG., Tommerup N. (eds); Karger S., Basel 2005
3. Fiegler H., Carr P., Douglas EJ. et al.: DNA microarrays for comparative genomic hybridization based on DOP-PCR amplification of BAC and PAC clones. Genes Chromosomes Cancer 36, 2003, 361-374.
4. Vermeesch JR., Melotte C., Froyen G. et al.: Molecular Karyotyping: Array CGH Quality Criteria for Constitutional Genetic Diagnosis. J Histochem Cytochem 53(3), 2005, 413-422.
5. Martín-Subero JI., Harder L., Gesk S. et al.: Interphase FISH assays for the detection of translocations with breakpoints in immunoglobulin light chain loci. Int J Cancer 98(3), 2002, 470-474.
6. Willis TG., Zalcberg IR., Coignet LJ. et al.: Molecular cloning of translocation t(1;14)(q21;q32) defines a novel gene (BCL9) at chromosome 1q21. Blood 91(6), 1998, 1873-81.
7. Callanan MB., Le Baccon P., Mossuz P. et al.: The IgG Fc receptor, Fc RIIB, is a target for deregulation by chromosomal translocation in malignant lymphoma. PNAS 97(1), 2000, 309-314.
8. Teruya-Feldstein J., Donnelly GB., Goy A. et al.: MUC-1 Mucin Protein Expression in B-cell Lymphomas. Appl Immunohistochem Mol Morphol 11(1), 2003, 28-32. antibodies. Clin Cancer Res 11(1), 2005, 87-96.
9. Ise T., Maeda H., Santora K. et al.: Immunoglobulin superfamily receptor translocation associated 2 protein on lymphoma cell lines and hairy cell leukemia cells detected by novel monoclonal, 3413-20.
10. Schaffner C., Stilgenbauer S., Rappold GA. et al.: Somatic ATM mutations indicate a pathogenic role of ATM in B-cell chronic lymphocytic leukemia. Blood 94(2), 1999, 748-53.
11. Calin GA, Dumitru CD, Shimizu M. et al.: Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukaemia. PNAS 99(24), 2002, 15524-15529.
12. Satterwhite E., Sonoki T., Willis TG. et al.: The BCL11 gene family: involvement of BCL11A in lymphoid malignancies. Blood 98(12), 20018.
13. Mossafa H., Huret JL: Chronic lymphocytic leukaemia (CLL). Atlas Genet Cytogenet Oncol Haematol., August 1997.
14. Reddy KS: Chronic lymphocytic leukaemia (CLL). Atlas Genet Cytogenet Oncol Haematol, May 2005.
15. Dyer MJ.: The pathogenetic role of oncogenes deregulated by chromosomal translocation in B-cell malignancies. Int J Hematol 77(4), 2003, 315-20.