

Genetic alterations in B-cell non-Hodgkin's lymphoma

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Background. Although the patients with diagnosed B-NHL are classified into the same disease stage on the basis of clinical, histopathological, and immunological parameters, they respond significantly different to the applied treatment. This points out the possibility that within the same group of lymphoma there are different diseases at molecular level. For that reason many studies deal with the detection of gene alterations in lymphomas to provide a better framework for diagnosis and treatment of these hematological malignancies. **Aim.** To define genetic alterations in the B-NHL with highest possibilities for diagnostic purposes and molecular detection of MRD. **Methods.** Formalin fixed and paraffin embedded lymph node tissues from 45 patients were examined by different PCR techniques for the presence of IgH and TCR γ gene rearrangement; K-ras and H-ras mutations; c-myc amplification and bcl-2 translocation. There were 34 cases of B-cell non-Hodgkin's lymphoma (B-NHL), 5 cases of T-cell non-Hodgkin's lymphoma (T-NHL) and 6 cases of chronic lymphadenitis (CL). The mononuclear cell fraction of the peripheral blood of 12 patients with B-NHL was analyzed for the presence of monoclonality at the time of diagnosis and in 3 to 6 months time intervals after an autologous bone marrow transplantation (BMT). **Results.** The monoclonality of B-lymphocytes, as evidenced by DNA fragment length homogeneity, was detected in 88 % (30/34) of B-NHL, but never in CL, T-NHL, or in normal PBL. Bcl-2 translocation was detected in 7/31 (22.6%) B-NHL specimens, c-myc amplification 9/31 (29%, all were more than doubled), K-ras mutations in 1/31 (3.23%) and H-ras mutations in 2/31 (6.45%) of the examined B-NHL samples. In the case of LC and normal PBL, however, these gene alterations were not detected. All the patients (12) with B-NHL had dominant clone of B-lymphocyte in the peripheral blood at the time of diagnosis while only in 2 of 12 patients MRD was detected 3 or 6 months after BMT. **Conclusion.** Because it is quick and simple, PCR analysis of clonal IgH rearrangements is very useful when diagnostic assistance is required. This technique is also very efficient for tracking minimal residual disease in lymphomas and leukemias and for monitoring clonal evolution in acute and chronic lymphoblastic leukemias and lymphomas. The presence of other genetic alterations, which we detected, should serve as an additional prognostic or predictive factor in the patients with B-NHL.

Key words: lymphoma, non-Hodgkin; genetic phenomena; mutation; polymerase chain reaction.

Introduction

Malignant lymphomas are a heterogeneous group of B- or T-cell malignancies, which usually arise in lymph nodes, but can also arise in any other organ in the body. Their overall incidence, and the incidence of the various histologic subtypes vary in different parts of the world. En-

vironmental exposures, viruses, chromosomal aberrations, and congenital or acquired immunosuppression have been associated with the development of lymphomas (1). Despite these associations, the real cause of the majority of lymphomas remains unknown.

The diagnosis of lymphoma is usually based on morphologic evaluation supplemented by the analysis of immu-

nological markers. However, in some diagnostic categories defined in this sense, the response of patients to treatment is markedly heterogeneous, arising the suspicion that there should be several molecularly distinct diseases within the same morphologic category. For that reason many studies deal with the detection of gene alterations in lymphomas to provide a better framework for the diagnosis and the treatment of these hematologic malignancies (2). Lymphomas could be very difficult to distinguish from reactive lesions, especially at the early stages of development, even with the aid of immunophenotyping. The discovery of immunoglobulin (Ig) gene rearrangements and the application of molecular probes for these genes were confirmed as suitable to demonstrate monoclonality, assign a disorder to either B- or T-lymphocyte lineage, and enable the detection of a small number of neoplastic cells. Until recently, this was carried out by a time-consuming, complex Southern blot analysis of restriction fragments which required relatively large amount of heavy DNA extracted from fresh/fresh-frozen tissue or cell suspensions (3). These limitations were overcome by the application of Polymerase chain reaction (PCR) techniques (4, 5). The rapid clonality analysis of almost any clinical sample could be carried out by PCR amplification of randomly assembled different variable (V), sometimes diversity (D), and joining (J) gene segments of antigen receptor genes.

Most of the patients suffering from hematologic malignancies achieved a complete clinical remission (CCR) after the conventional chemo- and radiation therapy-programs. The introduction of allogeneic and autologous bone marrow transplantation (BMT) in treatment protocols further increased the remission rates in lymphomas and leukemias. However, a significant proportion of patients that achieved a CCR, relapsed and died from their primary disease (lymphoma or leukemia) or complications of a subsequent therapy. It is believed that a disease recurrence is caused by a few malignant cells not eradicated with the induction therapies. The level of disease undetectable by conventional cytomorphologic methods, is referred to as the minimal residual disease (MRD). PCR techniques can be used for the detection of tumor-specific sequences, such as the junction regions of rearranged immunoglobulin (Ig) and T-cell receptor (TCR) genes, the breakpoint fusion of the regions of chromosome aberrations and mutations in tumor suppressor genes and oncogenes (6). In that way, the sensitive PCR-based techniques for detection of clonally rearranged immune genes might become useful and broadly applicable diagnostic tools for the monitoring of the patients with B- and T-cell malignancies (7).

In this study, we considered not only the clonality analysis, but also the incidence of K- and H-ras mutation, c-myc amplification and bcl-2 translocation in B-cell non-Hodgkin's lymphoma. These genes were included in the study because their products are involved in the regulation of cell cycle, proliferation and programmed cell death.

Ras proteins are localised to the inner face of the plasma membrane and specifically bound to guanine nucleotides-guanosine diphosphate (GDP) and guanosine tri-

phosphate (GTP)- with high affinity and specificity. The ability of ras to alternate between inactive (GDP-bound) and active (GTP-bound) forms is a major determinant of cell growth and proliferation. The transforming activity of *ras* oncogenes in human tumors is a consequence of point mutations, most often in codons 12, 13, and 61, resulting in single amino acid substitutions. The point mutations that convert ras into an oncoprotein alter its interactions with guanine nucleotides so as to maintain the ras protein in the constitutively active GTP-bound state (8).

The members of myc gene family – c-myc, N-myc, and L-myc – have neoplastic potential, and are activated as oncogenes in a wide variety of neoplasms as a consequence of the most frequent chromosome translocation or amplification (9). The c-myc gene codes for c-myc protein which is a transcription factor that is located in the cell nucleus, where myc functions to modulate gene expression in response to the proliferative stimuli. Elevated myc expression stimulates or represses transcription of its target genes by increasing the formation of c-myc/max heterodimers, consistent with the frequent activation of myc oncogenes by genetic alterations that result in myc overexpression (10).

The bcl-2 family of related proteins is a part of an important control mechanism for the regulation of apoptosis. There are over a dozen members in the family, some of which suppress apoptosis, including bcl-2 and bcl-x_L, and others which promote it, such as bax and bak. The bcl-2 is a prototypic family member localised to the outer mitochondrial membrane, and also to endoplasmic reticular and outer nuclear membranes (11). The bcl-2 protein inhibits apoptosis induced by a variety of stimuli in many cell types, suggesting that bcl-2 is an integral, possibly central, player in cell survival programmes. The bcl-2 gene was originally found in a proportion of B-cell follicular lymphomas at the t(14; 18) chromosomal translocation breakpoint (12). In these translocations, bcl-2 gene is moved from its normal location on chromosome 18q21 into juxtaposition with the IgH locus on chromosome 14q32, which leads to increased expression of the normal bcl-2 protein. Selective overexpression of bcl-2 in the lymphoid system produces B-cell lymphoma, where progression to high-grade lymphoma often coincides with c-myc translocation (13). Overexpression of bcl-2 is found in many types of tumors, including the majority of breast carcinomas (14, 15), and has been shown to cooperate with c-myc and ras (16) family members during cellular transformation.

The aim of this study was to define the incidence of genetic alterations in non-Hodgkin's lymphoma and respectively evaluate their diagnostic potential and usefulness for the tracking of minimal residual disease (MRD). For that purpose, PCR-SSCP analysis was used to investigate the presence of point mutations in codons 12 and 13 (exon 1) of K- and H-ras oncogenes; the amplification of c-myc oncogene was analyzed using "differential" PCR; the presence of translocation t(14; 18) of bcl-2 oncogene was analyzed by the "nested" PCR; the rearrangement of gene coding of IgH was analyzed by "touch down" PCR; and the re-

arrangement of variable region of gene coding for γ chain of TCR was analyzed by "multiplex" PCR.

Methods

Patients and specimens

Formaldehyde fixed and paraffin embedded lymph node tissues from 45 patients were examined. There were 34 cases of B-cell non-Hodgkin's lymphoma (B-NHL), 5 cases of T-cell non-Hodgkin's lymphoma (T-NHL) and 6 cases of chronic lymphadenitis (CL). All patients were treated at the Clinic for hematology, Military Medical Academy Belgrade, and their diagnoses were established according to clinical parameters and routine histologic, immunohistochemical and immunophenotypic methods. The mononuclear cell fraction of the peripheral blood (PBL) of 7 healthy persons was also examined.

The PBL of 12 patients with B-NHL was also analysed for the presence of monoclonality at the time of diagnosis and at 3 to 6 months time intervals after an autologous bone marrow transplantation (BMT).

DNA extraction

Paraffin-embedded tissue was deparaffinized by xylol, followed by two ethanol washes. The mononuclear cell fraction of the peripheral blood was obtained by Lymphoprep centrifugation (Nycomed Pharma, Norway). The samples were then lysed in the digestion buffer, containing 100 mmol/l NaCl, 10 mmol/l Tris-Cl, 25 mmol/l EDTA; pH 8.0, 0.5% SDS and 0.1 mg/ml proteinase K (USB, USA). The samples were incubated for 24 hours at 37 °C. DNA was isolated by standard phenol-chloroform extraction, and precipitated with 1/10 of volume 7.5 mmol/l ammonium-acetate and 2.5 of volume cold absolute ethanol, recovered with centrifugation at $15000 \times g$ for 10 minutes at 4 °C, and resuspended in 100 μ l double distilled water (17).

PCR amplification of oncogenes

The sequences of primers for codons 12 and 13 of the K- and H-ras oncogenes were as follows: K-ras (s) ATGACTGAATATAAACTTGT and (as) CTCTATTGTTGGATCATATT; and H-ras (s): ATGACGGAATATAAGCTGGT and (as): CGCCAGGCTCACCTCTATA. The initial denaturation took 5 minutes at 95 °C, and each mixture was then subjected to 35 cycles of amplification, each of which included a denaturation step at 94 °C for 1 minute, an annealing step at 58 °C for 1 minute (H-ras), and at 50 °C for 1 minute (K-ras), and an elongation step at 72 °C for 1 minute.

To study the presence of c-myc amplification we used the differential PCR. The differential PCR assays involved simultaneous amplification of two target sequences in the same tube, with the relative amount of the two PCR products indicating the relative gene dosages. We used the primers for c-myc gene and D2R gene (dopamine D2 receptor, control gene present in one copy in human genome), which

yielded products of 150 base pairs (bp) and 110 bp, respectively. The sequences of primers for c-myc gene were: (s): GCTCCAAGACGTTGTGTGTTTCG, (as) : GGAAGGACTATCCTGCTGCCAA and D2R (s): CCACTGAATCTGTCCTGGTATG, (as): GTGTGGCATAGTAGTTGTAGTGG. The PCR protocol included starting denaturation followed by 27 cycles of 1 minute each at 94 °C, 50 °C, and 72 °C, respectively.

Polyacrylamide gel electrophoresis of the differential PCR products demonstrated two discrete bands of the predicted size (c-myc = 150 bp, D2R = 110 bp). The c-myc copy number was estimated by comparing the relative intensity of c-myc and D2R receptor bands. The band intensities were quantitated by scanning densitometry which yielded two peaks (one for c-myc, and another for D2R), together with data about peak heights and relative areas, which were obtained by integrating the peaks, and which were directly proportional to concentration of amplified DNA. To control D2R gene, which was a referent amount in each sample, the relative area of the band was 100%. Thus, there was an internal control in each sample, and relative areas obtained for c-myc band were presented with reference to that control (c-myc/D2R).

The translocation t(14; 18) of bcl-2 oncogene was analyzed using the nested PCR with two sets of primers specific for the MBR region of bcl-2 gene and J_H region of IgH (18). The product of the first PCR reaction was reamplified by the second PCR reaction using the second set of primers internal to the primers used for the initial amplification, which increased the specificity of the reaction. The product of the second reaction was 140–250 bp long.

The starting denaturation was followed by 25 cycles of the initial amplification. The reamplification of a 5- μ l aliquot of the amplified mixture was performed in 30 cycles using primers internal to the original ones, with 1 minute of denaturation at 94 °C, annealing at 58 °C, and of extension at 72 °C.

PCR amplification of 3 frameworks of CDR region IgH gene (B clonality)

The rearrangement of the genes encoding of IgH (framework regions I, II and III) was analyzed using the touch down PCR (19, 20). That PCR increased specificity of the reaction. In the first cycle of PCR, the temperature was by several degrees higher than it was optimal for annealing (60 °C), which reduced nonspecific binding of primers to DNA template at the beginning of the reaction. The annealing temperature decreased gradually to the optimal value (56 °C), and remained unchanged in the succeeding cycles. The amplified fragments were 100–150 bp long.

PCR amplification of γ chain T-cell receptor gene (T clonality)

The variable region rearrangements of the genes encoding of the γ chain of the T-cell receptor were analyzed using multiplex PCR (21). The multiplex PCR used numer-

ous primers, in this case 5 primers for V and 3 primers for J gene regions, which enabled simultaneous amplification of several sequences. The amplified fragments were about 200 bp long.

All PCR amplifications were performed in (Mastercycler gradient, Eppendorf, Germany) a 50 µl final volume using 400–600 ng of genomic DNA, 0.4 µmol/l of primers (Applied Biosystems, 380B DNA Synthesizer), 250 µmol/l each of dNTP (Applied Biosystems), 1.2 U Taq polymerase (Applied Biosystems) in 1xPCR buffer with 15 mmol/l MgCl₂ (Applied Biosystems).

For the identification of amplified products (bcl-2 and c-myc oncogenes, as well as rearranged antigen receptor genes) we used 10% vertical polyacrylamide gels in 0.5 x TBE buffer (45 mmol/l Tris-borate, 1 mmol/l EDTA) (LKB, Pharmacia). The gels were stained with silver nitrate (Serva). A DNA molecular weight marker (PCR Markers, Promega) was used to provide a size estimation in base pairs. A PCR fragment was considered to be monoclonal only if one or two discrete narrow bands were observed within the appropriate size range expected for a particular primer pair. For FRI expected size range was 300–400 bp, for FRII 240–260 bp and for FRIII 100–150 bp.

range by polymerase chain reaction in order to compare the efficiency of the chosen primers. The IgH rearrangements were considered to be of tumor origin if the PCR product was predominantly monoclonal in at least two amplifications. The primer sets showed different efficiencies in the amplification of monoclonal B-cell populations (Table 1).

The amplification of genomic DNA with V_H1/J_{Ha} primer pair produced monoclonal pattern only in 8/34 cases of B-NHL, while a smear of amplified DNA was found in all other examined samples. In the second pair from FRI (V_H3/J_{Ha}), however, the monoclonal pattern was found in 25/34 B-NHL. A clear dominant band 100–150 bp in length was seen in 30 of 34 studied B-NHL, and in none of 5 T-NHL, 6 CL and in all normal PBL's when primers for FRIII were used. In the case of negative results, i.e. when lymphocyte population was polyclonal, a diffuse smear or many discrete bands were found. Examples of the findings are shown in Figure 1. The same samples (except CL) were examined for the presence of K- and H-ras (codons 12 and 13) point mutations, amplification of c-myc and translocation t(14;18) of bcl-2 (Table 2).

Table 1

Distribution of B-cells clonality detection rates among different primer pairs

Diagnosis	Primer pairs					
	V _H 1/J _{Ha}	V _H 3/J _{Ha}	V _H 4a/J _{Ha}	FRIIa/J _{Hb}	FRIII/J _H	TcR _γ
B-NHL	8/34	25/34	15/34	12/34	30/34	0/5
T-NHL	0/5	0/5	0/5	0/5	0/5	5/5
CL	0/6	0/6	0/6	0/6	0/6	0/6
PBL	0/7	0/7	0/7	0/7	0/7	0/7

Table 2

Distribution of gene alterations among different non-Hodgkin's lymphomas

Diagnosis	Gene alterations			
	H-ras	K-ras	bcl-2	c-myc
B-NHL	1/31	2/31	7/31	9/31
T-NHL	Ø	0/5	Ø	1/5
PBL	0/7	0/7	0/7	0/7

Point mutations of K- and H-ras oncogenes, codons 12 and 13 (exon 1), were detected using SSCP (single stranded conformational polymorphism) method on silver stained 10% polyacrylamide gels.

To quantify the products of differential PCR for c-myc and control D2R gene, the scanning densitometry was used (LKB Ultrascan XL, Sweden).

Results

DNA samples extracted from formalin fixed, paraffin embedded lymph nodes from 34 cases of B-NHL, as well as 5 cases of T-NHL, 6 cases of CL and the mononuclear cell fraction of the peripheral blood (PBL) of 7 healthy persons were examined for immunoglobulin heavy chain gene rear-

K-ras point mutations were found in 2 of 31 case of B-NHL (6.45%), whereas H-ras point mutations were found in only 1 of 31 case of B-NHL (3.23%) (Figure 2). No point mutations in K- and H-ras were found in all other patients and healthy persons.

Using differential PCR with two sets of primers for the c-myc and control gene, dopamine D2 receptor (D2R), according to c-myc/D2R ratio patients were divided in three groups. The first group contained the patients with c-myc/D2R ratio < 100%, the second group 100–200%, and the third group > 200%. Nine patients from the third group (c-myc/D2R ratio > 200%) had a double or higher c-myc amplification compared with the D2R gene, and were treated as positive. No amplification was detected in any other patients and healthy persons (Figure 3).

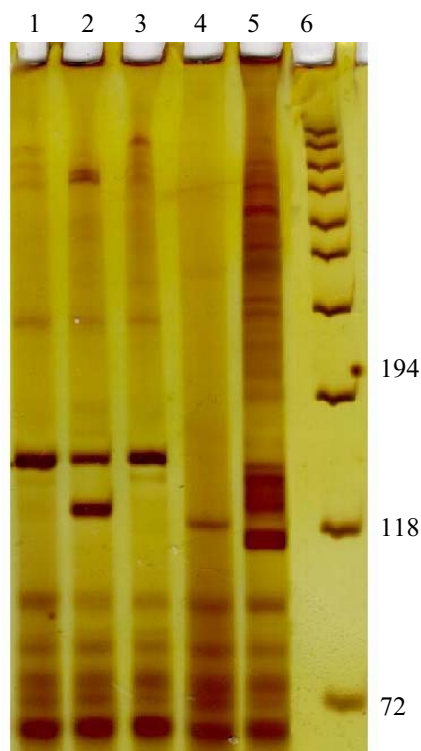


Fig. 1 – Detection of B-cell clonality in patients with B-NHL. Lanes 1, 2, 3 and 4 have dominant clone of B-lymphocytes, while in lane 5 we have detected polyclonal smear. Lane 6 is molecular weight marker (in base pairs).

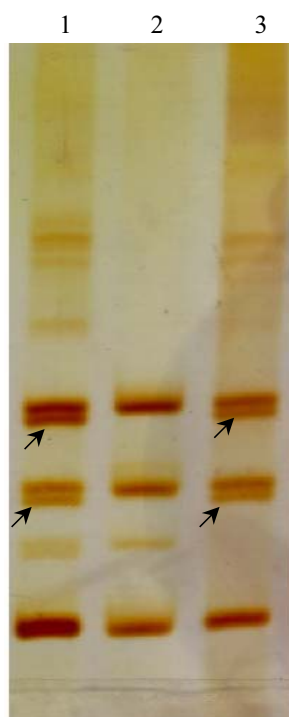


Fig. 2 – Detection of H-ras point mutations in lymph nodes of patients with B-NHL by PCR-SSCP method on 10% polyacrylamide gel (silver stained). Lane 2 – negative sample; Lanes 1 and 3 – positive samples.

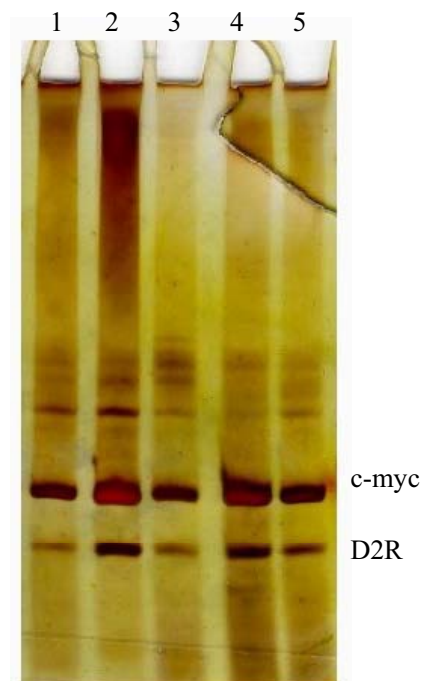
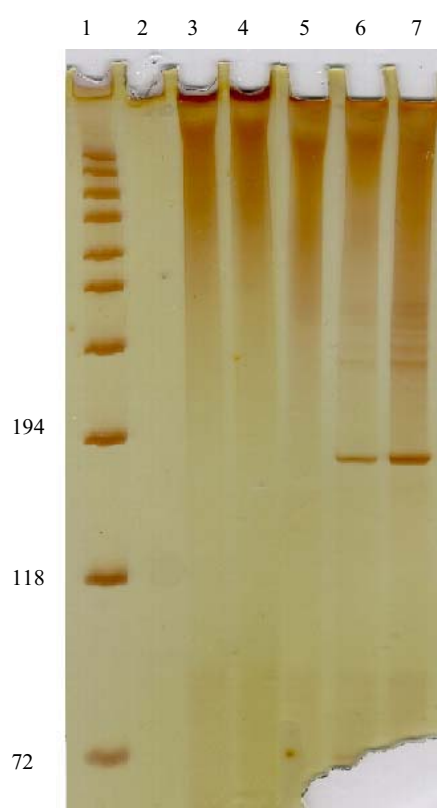


Fig. 3 – Detection of c-myc oncogene amplification in lymph nodes of patients with B-NHL by differential PCR. C-myc was amplified in all samples.



Translocation t(14; 18) of bcl-2 was detected in 7 of 31 cases of B-NHL (22.6%) (Figure 4). This translocation wasn't found in any other patients and healthy persons.

Fig. 4 – Detection of t(14;18) translocation of bcl-2 oncogene in lymph nodes of patients with B-NHL. Lanes 3, 4 and 5 – negative samples; lanes 6 and 7 – positive samples. Lane 1 – molecular weight marker (in base pairs) and lane 2 – negative control of PCR reaction.

The clonality of T-cells was detected by the multiplex PCR, with 5 primers for V and 3 primers for J variable re-

gion gene segments coding for γ -chain of TCR. The presence of dominant T-cell clone was detected in all the cases of T-NHL, whereas all other patients and healthy persons were TCR γ negative.

In order to evaluate the usefulness of two primer pairs ($V_H3/J_H\alpha$ and FR_{III}/J_H) for molecular detection of MRD in 12 patients with B-NHL, the presence of a dominant clone, in the peripheral blood was examined before, and after an autologous bone marrow transplantation. In all the patients included in this study the presence of dominant clone was detected in the peripheral blood at the time of diagnosis. In 3–6 months after transplantation the dominant clone was detected in 2 patients, while in other patients polyclonal pattern was found.

Discussion

Numerous IgH-CDR3 PCR-based techniques were described, but NHL specimens often contain the significant number of the non-neoplastic B cells in addition to the malignant B cell clone. Thus, a mixture of unrelated IgH-CDR3 junctions is usually amplified by PCR, together with clonal IgH-CDR3 junctions giving sometimes false negative results (22). Moreover, they occur as a result of somatic mutations or deletion of the specific region (22; 6). Although the amplification of FR_{III}/J_H was preferred as the products were small and therefore more readily amplified from degraded samples and easier resolved by electrophoresis it seemed that it was necessary to use more than one pair of primers and to establish the strict criteria for the definition of monoclonality in order to minimize the probability of false positive and negative results (23, 24). Therefore, in this study we also used primers from FR_{II} and three pairs of primers from FR_{I} because it was helpful in the cases when FR_{III} primers failed to bind, although they required the template DNA of a reasonably good quality to allow for the longer PCR products (about 250 bp for FR_{II} and 350 bp for FR_{I} as compared to 120 bp when primers for FR_{III} were used). For that purpose we chose the panel of primer sequences with high sensitivity and specificity (20, 24, 25). The best results in detection of monoclonality were obtained when we used primers for FR_{III} . The monoclonality of B-lymphocytes, as evidenced by DNA fragment length homogeneity, was detected in 88% of B-NHL, but never in T-NHL, CL or in normal PBL. Our results suggest that the clonality analysis of IgH gene rearrangement is reliable when using paraffin-embedded samples in approximately 70–80% cases (6, 26) of the B-cell lymphomas, confirming the diagnostic potential of the technique. Recent reports suggest that up to 96% of the low grade B-cell, non-follicular lymphomas and 82% of high or intermediate grade lymphomas could be amplified using multiple primers (6, 22). In our study low- and high-grade lymphomas were included, and using 5 pairs of primers we succeed in detecting monoclonality in all cases, although paraffin embedded samples were used. The primers from FR_{I} ($V_H3/J_H\alpha$),

compared to the primers from FR_{III} , were more sensitive (73%) in detection of monoclonality and when used together 91% of the cases were defined as monoclonal. We believe that additional use of $V_H3/J_H\alpha$ primers could be valuable in some cases when the discovery of monoclonality is doubtful and when fresh or fresh/frozen samples are used.

The finding of three clonal PCR products indicates that more than one B-cell clone is present (27). Such oligoclonal rearrangements are detected in the rare cases of follicular center cell-derived lymphomas and in 15 to 45% of the patients with acute lymphoblastic leukemia (ALL) (28). The oligoclonality of B-cell ALL is caused by ongoing V_H gene rearrangement reflecting recombinase activity in an immature leukemic clone (4). The presence of clonally related and yet diverse population of leukemic blasts is clinically significant as these clones may in time predominate and compromise the ability to detect minimal residual disease at the molecular level (28, 29). The detection of clonal IgH rearrangements by PCR has an advantage over Southern blot procedure since only the complete VDJ rearrangements will be amplified enzymatically, but not the incomplete rearrangements or rearrangements resulting from chromosomal translocations involving the IgH gene locus. Because of its speed and simplicity, the PCR analysis has priority when diagnostic assistance in a difficult case is required (30). But, it should be kept in mind that molecular test results have to be considered in the context of morphology and immunohistochemistry. The detection of a clonal lymphocyte population in clinical samples is usually abnormal but it should not be considered as a proof of malignancy.

This technique is very efficient in tracking minimal residual disease in lymphomas and leukemias (31, 32) and in monitoring clonal evolution in acute and chronic lymphoblastic leukemias and lymphomas (33) as well as in detecting clonal rearrangements of T-cell receptor for differential diagnosis and monitoring of T-cell lymphoma (21, 34). The use of CDR3 as clonal marker of multiple myeloma showed an excellent sensitivity as it was demonstrated that circulating malignant cell could be identified in the majority of patients with the frequency of 0.001% to 1% (24, 35). Our study included 12 patients with B-NHL who had the dominant clone of B-lymphocytes in the peripheral blood at the time of diagnosis. Those patients were treated with autologous BMT and within the first 3 to 6 months after BMT were tested for the presence of dominant clone in PBL. In 2/12 patients the dominant clone was found and one of the patients died while the other one remained in stable remission. In the group of patients (10 pts) without the presence of dominant clone in PBL, four patients developed relapse, while other six were in remission. These results were in compliance with the findings that detection of residual disease on molecular level cannot be interpreted in one way, i.e. some investigators found that detection of residual disease immediately following induction or during the first 6

months predicts likelihood of relapse, others reported that MRD can persist for 24 months and even longer. Our results and the results of other authors underline the importance/necessity of the serial assessments and quantification of MRD (36).

The members of ras proto-oncogenes were present in a variety of human malignancies, and are among the most frequent genetic aberrations detected in human tumors (acute myeloid leukemias 25%; colon carcinomas 50%; lung carcinomas 25%; and pancreatic carcinomas 90%). The mutations of N- and K-ras genes are the most frequent genetic aberrations in acute myeloid leukemia (AML) and their detection under preleukemic conditions such as the myelodysplastic syndrome (MDS) suggests their significance for the earliest stages of leukemogenesis (37). Despite these observations, our results show that ras mutations are rare event in B-NHL and also suggest that their presence could serve as an additional factor in the assessment of tumor biology in individual patients with NHL.

We found that almost one third of B-NHL (29%) has a significant amplification of the c-myc gene. Although our method for measurement of gene amplification is semiquantitative, it implies that the more precise measurement of c-myc amplification (38) can provide better understanding of lymphoma cell biology. Normally, c-myc expression is induced during cell proliferation after the action of a mitogenic stimulus or is suppressed by the action of factors which arrest the cell in the G1 stage of the cell cycle. Obviously, c-myc has a dual role: (a) to promote proliferation in the presence of a relative abundance of the appropriate growth factors; and (b) to induce apoptosis when c-myc is expressed in cells during the growth arrest (39). The expression of bcl-2 gene in pre-B cells of transgenic mice with a high expression of c-myc promotes survival of these cells, meaning that bcl-2 cooperates with c-myc to immortalize pre-B cells (13).

The translocation of bcl-2 oncogene was detected in 7/31 cases, and 6 were low grade lymphoma and 1 high grade. Bcl-2 oncogene was first identified as a gene overexpressed in human follicular B-cell lymphomas, following reciprocal chromosomal translocation t(14; 18). When overexpressed, bcl-2 inhibits apoptosis in a variety of cells. The bcl-2 and bax protein products can form homodimers or heterodimers. The bax gene has an antagonizing effect on bcl-2 and it is a key effector gene of the p53 gene in the induction of apoptosis. The bcl-2 cooperates with deregulated c-myc expression to promote hematopoietic tumor development in transgenic mice. Since bcl-2 inhibits the induction of apoptosis by c-myc, the basis of this cooperation is presumably the preservation from apoptosis by bcl-2 of c-myc expressing target cells which then can proliferate (40, 41).

The deregulation of bcl-2 expression is in a relation with the development of neoplastic transformation, and tumors with high level of bcl-2 expression show an increased resistance to different cytotoxic drugs routinely used in chemotherapy. Apart from its role in protecting against physiologic cell death, bcl-2 renders cells resistant to radiation and to cytotoxic drugs, but CTL killing is not inhibited. When bcl-2 translocation is found in PBL at the time of diagnosis it can be further used as a molecular marker of MRD, especially in the group of patients treated with monoclonal antibody that reacts with the cell-surface protein CD20 expressed by normal and malignant mature B-cells (42).

Clinical assessment and histology remain the "golden standard" for evaluating hematologic malignancies and cancers in general. However, in the postgenomic era the new goal is to assess the possibility to use molecular abnormalities for diagnostic, prognostic and predictive purposes. Some expectations go even further trying to define tumor phenotype and to use this as a new golded standard for disease staging and treatment (43, 44). Thus, in our study we tried to define genetic alterations in B-NHL with the greatest diagnostic potential for molecular detection of MRD.

R E F E R E N C E S

1. Stewart AK, Schuh AC. White cells 2: impact of understanding the molecular basis of haematological malignant disorders on clinical practice. *Lancet* 2000; 355(9213): 1447–53.
2. Staudt LM. Molecular diagnosis of the hematologic cancers. *N Engl J Med* 2003; 348(18): 1777–85.
3. Deane M, Norton JD. Immunoglobulin gene 'fingerprinting': an approach to analysis of B lymphoid clonality in lymphoproliferative disorders. *Br J Haematol* 1991; 77(3): 274–81.
4. McCarthy KP, Sloane JP, Wiedemann LM. Rapid method for distinguishing clonal from polyclonal B cell populations in surgical biopsy specimens. *J Clin Pathol* 1990; 43(5): 429–32.
5. Inghirami G, Szabolcs MJ, Yee HT, Corradini P, Cesarman E, Knowles DM. Detection of immunoglobulin gene rearrangement of B cell non-Hodgkin's lymphomas and leukemias in fresh, unfixed and formalin-fixed, paraffin-embedded tissue by polymerase chain reaction. *Lab Invest* 1993; 68(6): 746–57.
6. Diss TC, Pan L. Polymerase chain reaction in the assessment of lymphomas. *Cancer Surv* 1997; 30: 21–44.
7. Scrideli CA, Queiroz RG, Bernardes JE, Valera ET, Tone LG. PCR detection of clonal IgH and TCR gene rearrangements at the end of induction as a non-remission criterion in children with ALL: comparison with standard morphologic analysis and risk group classification. *Med Pediatr Oncol* 2003; 41(1): 10–6.

8. Gougopoulou DM, Kiaris H, Ergazaki M, Anagnostopoulos NI, Grigoraki V, Spandidos DA. Mutations and expression of the ras family genes in leukemias. *Stem Cells* 1996; 14(6): 725–9.
9. Facchini LM, Penn LZ. The molecular role of Myc in growth and transformation: recent discoveries lead to new insights. *FASEB J* 1998; 12(9): 633–51.
10. Sakamuro D, Prendergast GC. New Myc-interacting proteins: a second Myc network emerges. *Oncogene* 1999; 18(19): 2942–54.
11. Korsmeyer SJ. BCL-2 gene family and the regulation of programmed cell death. *Cancer Res* 1999; 59(7 Suppl): 1693s–700s.
12. Hockenbery D, Nunez G, Millman C, Schreiber RD, Korsmeyer SJ. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* 1990; 348(6299): 334–6.
13. Kramer MH, Hermans J, Wijburg E, Philippo K, Geelen E, van Krieken JH, et al. Clinical relevance of BCL2, BCL6, and MYC rearrangements in diffuse large B-cell lymphoma. *Blood* 1998; 92(9): 3152–62.
14. Magić Z. Apoptosis and cancer. In: *Antypas G*, editor. *Proceedings of 2nd Balkan Congress of Oncology*; 1998 Sep 10-14; Izmir, Turkey. Bologna: Monduzzi Editore; 1999. p. 343–8.
15. Cory S, Vaux DL, Strasser A, Harris AW, Adams JM. Insights from Bcl-2 and Myc: malignancy involves abrogation of apoptosis as well as sustained proliferation. *Cancer Res* 1999; 59(7 Suppl): 1685s–92s.
16. Reed JC, Haldar S, Croce CM, Cuddy MP. Complementation by BCL2 and C-HA-RAS oncogenes in malignant transformation of rat embryo fibroblasts. *Mol Cell Biol* 1990; 10(8): 4370–4.
17. Sambrook J, Fritsch EF, Maniatis T. *Molecular cloning: A laboratory manual*. 2nd ed. New York: Cold Spring Harbor Laboratory Press; 1989.
18. Gribben JG, Freedman A, Woo SD, Blake K, Shu RS, Freeman G, et al. All advanced stage non-Hodgkin's lymphomas with a polymerase chain reaction amplifiable breakpoint of bcl-2 have residual cells containing the bcl-2 rearrangement at evaluation and after treatment. *Blood* 1991; 78(12): 3275–80.
19. Segal GH, Jorgensen T, Masih AS, Braylan RC. Optimal primer selection for clonality assessment by polymerase chain reaction analysis: I. Low grade B-cell lymphoproliferative disorders of nonfollicular center cell type. *Hum Pathol* 1994; 25(12): 1269–75.
20. Lombardo JF, Hwang TS, Maiese RL, Millson A, Segal GH. Optimal primer selection for clonality assessment by polymerase chain reaction analysis. III. Intermediate and high-grade B-cell neoplasms. *Hum Pathol* 1996; 27(4): 373–80.
21. Whittaker S. T-cell receptor gene analysis in cutaneous T-cell lymphomas. *Clin Exp Dermatol* 1996; 21(2): 81–7.
22. Medeiros LJ, Carr J. Overview of the role of molecular methods in the diagnosis of malignant lymphomas. *Arch Pathol Lab Med* 1999; 123(12): 1189–207.
23. Diss TC, Peng H, Wotherspoon AC, Isaacson PG, Pan L. Detection of monoclonality in low-grade B-cell lymphomas using the polymerase chain reaction is dependent on primer selection and lymphoma type. *J Pathol* 1993; 169(3): 291–5.
24. van Belzen N, Hupkes PE, Doekharan D, Hoogeveen-Westerveld M, Dorssers LC, et al. Detection of minimal disease using rearranged immunoglobulin heavy chain genes from intermediate- and high-grade malignant B cell non-Hodgkins lymphoma. *Leukemia* 1997; 11(10): 1742–52.
25. Choi Y, Greenberg SJ, Du TL, Ward PM, Overturf PM, Brecher ML, et al. Clonal evolution in B-lineage acute lymphoblastic leukemia by contemporaneous VH-VH gene replacements and VH-DJH gene rearrangements. *Blood* 1996; 87(6): 2506–12.
26. Chen YT, Whitney KD, Chen Y. Clonality analysis of B-cell lymphoma in fresh-frozen and paraffin-embedded tissues: the effects of variable polymerase chain reaction parameters. *Mod Pathol* 1994; 7(4): 429–34.
27. Stewart AK, Schwartz RS. Immunoglobulin V regions and the B cell. *Blood* 1994; 83(7): 1717–30.
28. Ebeling SB, Schutte ME, Logtenberg T. Molecular analysis of VH and VL regions expressed in IgG-bearing chronic lymphocytic leukemia (CLL): further evidence that CLL is a heterogeneous group of tumors. *Blood* 1993; 82(5): 1626–31.
29. Foroni L, Coyle LA, Papaioannou M, Yaxley JC, Sinclair MF, Chim JS, et al. Molecular detection of minimal residual disease in adult and childhood acute lymphoblastic leukaemia reveals differences in treatment response. *Leukemia* 1997; 11(10): 1732–41.
30. Diaz-Cano S. PCR-based alternative for diagnosis of immunoglobulin heavy chain gene rearrangement: principles, practice, and polemics. *Diagn Mol Pathol* 1996; 5(1): 3–9.
31. Brisco J, Hughes E, Neoh SH, Sykes PJ, Bradstock K, Enno A, et al. Relationship between minimal residual disease and outcome in adult acute lymphoblastic leukemia. *Blood* 1996; 87(12): 5251–6.
32. Szczepanski T, Orfao A, van der Velden VH, San Miguel JF, van Dongen JJ. Minimal residual disease in leukaemia patients. *Lancet Oncol* 2001; 2(7): 409–17.
33. Szczepanski T, Willemse MJ, Kamps WA, van Wering ER, Langerak AW, van Dongen JJ. Molecular discrimination between relapsed and secondary acute lymphoblastic leukemia: proposal for an easy strategy. *Med Pediatr Oncol* 2001; 36(3): 352–8.
34. Szczepanski T, Langerak AW, Willemse MJ, Wolvers-Tettero IL, van Wering ER, van Dongen JJ. T cell re-

- ceptor gamma (TCRG) gene rearrangements in T cell acute lymphoblastic leukemia reflect 'end-stage' re-combinations: implications for minimal residual disease monitoring. *Leukemia* 2000; 14(7): 1208–14.
35. *Slack DN, McCarthy KP, Wiedemann LM, Sloane JP.* Evaluation of sensitivity, specificity, and reproducibility of an optimized method for detecting clonal rearrangements of immunoglobulin and T-cell receptor genes in formalin-fixed, paraffin-embedded sections. *Diagn Mol Pathol* 1993; 2(4): 223–32.
36. *van der Velden VH, Hochhaus A, Cazzaniga G, Szczepanski T, Gabert J, van Dongen JJ.* Detection of minimal residual disease in hematologic malignancies by real-time quantitative PCR: principles, approaches, and laboratory aspects. *Leukemia* 2003; 17(6): 1013–34.
37. *Neubauer A, Dodge RK, George SL, Davey FR, Silver RT, Schiffer CA, et al.* Prognostic importance of mutations in the ras proto-oncogenes in de novo acute myeloid leukemia. *Blood* 1994; 83(6): 1603–11.
38. *Saez AI, Artiga MJ, Romero C, Rodriguez S, Cigudosa JC, Perez-Rosado A, et al.* Development of a real-time reverse transcription polymerase chain reaction assay for c-myc expression that allows the identification of a subset of c-myc+ diffuse large B-cell lymphoma. *Lab Invest.* 2003; 83(2): 143–52.
39. *Amati B, Alevizopoulos K, Vlach J.* Myc and the cell cycle. *Front Biosci* 1998; 3: d250–68.
40. *Kusenda J.* Bcl-2 family proteins and leukemia. Mini-review. *Neoplasma* 1998; 45(3): 117–22.
41. *Gross A, McDonnell JM, Korsmeyer SJ.* BCL-2 family members and the mitochondria in apoptosis. *Genes Dev* 1999; 13(15): 1899–911.
42. *Howard OM, Gribben JG, Neuberg DS, Grossbard M, Poor C, Janicek MJ, et al.* Rituximab and CHOP induction therapy for newly diagnosed mantle-cell lymphoma: molecular complete responses are not predictive of progression-free survival. *J Clin Oncol* 2002; 20(5): 1288–94.
43. *Alizadeh AA, Eisen MB, Davis RE, Ma C, Lossos IS, Rosenwald A, et al.* Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature* 2000; 403(6769): 503–11.
44. *de Vos S, Hofmann WK, Grogan TM, Krug U, Schrage M, Miller TP, et al.* Gene expression profile of serial samples of transformed B-cell lymphomas. *Lab Invest* 2003; 83(2): 271–85.

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Apstrakt

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GENETSKE ALTERACIJE KOD NON-HODGKINOVIH LIMFOMA B-ĆELIJA

Uvod. Iako se, u zavisnosti od kliničkih, patohistoloških i imunoloških parametara, bolesnici sa dijagnozom B-NHL svrstavaju u isti stadijum bolesti, oni značajno različito reaguju na primenjenu terapiju. Ovo ukazuje na mogućnost da u okviru iste grupe limfoma postoje različite bolesti na molekulskom nivou. Zbog toga mnogi u svetu izučavaju genske alteracije u limfomima sa ciljem da se definišu bolji dijagnostički i prognostički parametri. **Cilj** ovog rada je bio da se definišu genske alteracije u NH-limfomima koje se mogu koristiti u dijagnostičke svrhe i za otkrivanje minimalne rezidualne bolesti (MRB). **Metode.** Limfni čvorovi 45 bolesnika, ukalupljeni u parafin, iskorišćeni su za otkrivanje: rearanžmana gena za IgH (teški lanac imunoglobulina), TCR-γ (γ-lanac T-ćelijskog receptora); mutaciju u K- i H-ras onkogenima; amplifikaciju c-myc i bcl-2 translokaciju uz pomoć različitih tehnika lančane reakcije polimeraze (PCR). Analizovano je 34 B-ne Hodgkinovih limfoma (B-NHL), 5 T-NHL i 6 slučajeva hroničnog limfadenitisa (CL). U perifernoj krvi 12 bolesnika sa B-NHL je u vreme dijagnoze, i 3 do 6 meseci nakon autologe transplantacije matičnih ćelija hematopoeze, pronađen je dominantni klon B-limfocita. **Rezultati.** Monoklonost B-limfocita je otkrivena u 88% (30/34) B-NHL. Translokacija bcl-2 je otkrivena u 22,6% uzoraka (7/31), c-myc amplifikacija u 29% (9/31, svi su amplifikovani više od 2 puta), K-ras mutacija u 3,23% (1/31), H-ras mutacija u 6,45% (2/31), kada se radilo B-NHL, dok u slučaju T-NHL, CL i periferne krvi zdravih kontrola ove alteracije nisu otkrivene. U perifernoj krvi svih bolesnika sa B-NHL je dokazano prisustvo dominantnog klona B-limfocita u momentu postavljanja dijagnoze dok je nakon autologe transplantacije matičnih ćelija hematopoeze kod samo dva bolesnika otkriven domi-

nantni klon B-limfocita (u periodu od 3 do 6 meseci nakon transplantacije). **Zaključak.** U slučajevima kada postoje sumnje u vezi dijagnoze B-NHL otkrivanje rearanžmana gena za IgH uz pomoć PCR može biti izuzetno korisno zbog brzine i jednostavnosti reakcije. Ova tehnika je korisna i za otkrivanje MRB kod limfoma i leukemija, a može se koristiti za praćenje klonske evolucije kod istih bolesti. Prisustvo ostalih genskih alteracije koje smo ispitivali može biti upotrebjeno kao dodatni prognostički ili prediktivni parametar.

Ključne reči: limfom, nehodžkinov; genetski fenomeni; mutacija; polimeraza, reakcija stvaranja lanaca.