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FREQUENCY OF ABERRANT PROMOTER METHYLATION OF P15^{INK4B} AND O^6-METHYLGUANINE-DNA METHYLTRANSFERASE GENES IN B-CELL NON-HODGKIN LYMPHOMA: A PILOT STUDY

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Abstract – The methylation status of the target promoter sequences of *p15^{INK4B}* (*p15*) and O^6-*methylguanine-DNA methyltransferase (MGMT)* genes was studied by methylation-specific PCR in 10 adult patients with *de novo* B-cell non-Hodgkin lymphoma (B-NHL). The aberrant hypermethylation of the *p15* gene was more frequent (50%) compared to the hypermethylation of the *MGMT* gene (30%), and was detected in different types of B-NHL in both genes. Hypermethylation of the *MGMT* gene occurred exclusively in association with the hypermethylation of the *p15* gene. All lymphoma patients with hypermethylation of the *p15* and/or *MGMT* genes had a higher clinical stage of the disease (IV - V). We show the association of anemia and/or thrombocytopenia with the hypermethylation of the *p15* gene, ascribing the *p15* gene as a potential prognostic marker in B-NHL. Comethylation of *MGMT* with the *p15* gene represents a novel finding and presents both genes as candidates for future studies of the hypermethylation profiles of B-NHL.

Key words: Methylation status, *p15^{INK4B}*, O^6-methylguanine-DNA methyltransferase, B-cell non-Hodgkin lymphoma

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INTRODUCTION

The initiation and development of cancer involves several genomic alterations which are generally caused by genetic and epigenetic mechanisms (Hanahan & Weinberg, 2000; Verma & Srivastava, 2002; Baylin & Ohm, 2006). In normal mammalian cells, the methylation of cytosine (C) bases within the CpG dinucleotide islands of gene promoter regions represents the most important epigenetic mechanism for the modulation of gene activity (Jones et al., 1998; Verma & Srivastava, 2002). DNA methylation patterns, called epigenotypes (Esteller, 2003), are profoundly deranged in different types of human cancer (Esteller et al., 2001), including acute leukemias and lymphoproliferative disorders (Baylin et al., 1998; Rush & Plass, 2002; Esteller, 2003; Galm et al., 2005). Aberrant hypermethylation of CpG islands, resulting in the silencing of gene expression, has been observed for many cancer-related genes (Galm et al., 2005). Molecular pathways affected by DNA methylation changes include cell cycle control, cell invasion and adhesion, regulation of apoptosis, DNA damage repair and growth-factor response (Galm et al., 2005).

In normal cells, the protein P15, which is a product of the gene located at chromosome 9p21 of the human genome, acts as a tumor suppressor (Herman et al., 1996b) by inhibiting cyclin-dependent kinase 4 and 6 activity and inducing cell cycle arrest (Sherr & Roberts, 1999). It is also known that P15 is one of the intracellular mediators of transforming the growth factor β (TGF-β) signaling pathway, a strong negative regulator of cell proliferation and promoter of hematopoietic stem cells differentiation (Visser & Kast, 2002). Hypermethylation of normally unmethylated CpG islands of the promoter region of the *p15^{INK4B}* (*p15*) gene was detected in some tumor cell lines (Paz et al., 2003) and primary human

cancers, with highest frequencies in glioma as well as in acute leukemia (AL) and non-Hodgkin lymphoma (NHL) (Herman et al., 1996b; Drexler, 1998; Baur et al., 1999; Galm et al., 2005).

O^6-Methylguanine-DNA Methyltransferase (MGMT) is a unique DNA repair enzyme (E.C. 2.1.1.63) that removes mutagenic and cytotoxic alkyl adducts from the O^6 position of guanine (Gerson, 2002; Margison et al., 2003). A deficiency of MGMT protein induces the accumulation of premutagenic lesions, predominantly O^6-methylguanine, which leads to the fixation of transition G → A and the generation of a "mutator phenotype" (Esteller & Herman, 2004). Hypermethylation of the promoter region of the *MGMT* gene, located at chromosome 10q26 of the human genome, was identified in some tumor cell lines (Paz et al., 2003; Bogdanovic et al., 2007) and some of the primary human cancers, including NHL (Esteller et al., 1999; Margison et al., 2003). It has been shown that increased methylation of the *MGMT* gene may be a new prognostic marker of survival in patients with diffuse large B-cell lymphoma (DLBCL) treated with alkylating drugs such as cyclophosphamide (Esteller et al., 2002).

The primary aim of this pilot study was to optimize the methylation-specific PCR (MS-PCR) assay for the analysis of *p15* and *MGMT* genes, and to evaluate the frequency of aberrant *de novo* methylation of these genes in a selected group of adult patients (pts) with different types of *de novo* B-NHL. The second aim of the study was to test the potential association of epigenetic changes with the clinicopathological characteristics of studied patients.

MATERIALS AND METHODS

Patients

Our study included specimens of 10 adult patients with diagnosis (dg) of *de novo* B-NHL, collected between 2001 and 2004. The patients were diagnosed and treated at the Institute of Hematology, Clinical Center of Serbia. Patients signed informed consent prior to specimen collection according to the requirements of the Institutional ethical committee. All patients underwent standard diagnostic procedures, including cytomorphology with cytochemistry (Houwen, 2000), histopathology (Jack et al., 2005), immunophenotyping by flow cytometry (4/10 pts) (Rothe and Schmitz, 1996; Stelzer et al., 1997) or by immunohistochemistry (6/10 pts) (Jack et al., 2005). Patients were diagnosed according to the criteria given by the World Health Organization (WHO) (Harris, 2001; Jaffe et al., 2008), and treated by standard chemotherapy protocols for adults with dg B-NHL (Hiddemann et al., 2005; National Cancer Institute, 2002).

Peripheral blood and bone marrow specimens were anticoagulated by acid-citrate-dextrose (Tatsumi et al., 2002). Six out of ten DNA samples were isolated from mononuclear cells (MNC) obtained from peripheral blood (4 pts) or bone marrow (2 pts). The MNC were isolated using a Lympho Separation medium (1.077 g/ml, ICN, USA) as reported by Boyum (1968). Aliquots of 10 x 10^6 MNC were spun down at 720g/5 min, and the pellets were frozen at -20ºC. In all analyzed specimens, lymphoma cells were the predominant population (>90% of total cells). Four out of ten DNA samples were isolated from tissue sections (7-8 μm) of formalin-fixed paraffin embedded lymph node blocks, according to the protocol by Goelz et al., (1985).

Control Specimens

Three specimens of peripheral blood, taken from healthy laboratory personnel, as well as three commercial cell lines, were tested in parallel with the patients' specimens. DNA from commercial cell lines. K562 (European Collection of Cell Cultures, ECACC No 89121407), established from a patient with Chronic Myeloid Leukemia undergoing erythroblastic transformation; SuDHL-4 (kindly provided by Dr. A. L. Epstein, Stanford University, California, USA), established from a patient with Follicular cell NHL, and RAJI (European Collection of Cell Cultures, ECACC No. 85011429), established from a patient with Burkitt type B-NHL, were used for optimization of PCR and MS-PCR assays.

DNA extraction and PCR assay

Genomic DNA was extracted by standard sodium dodecyl sulfate-proteinase K treatment, phenol-chloroform-isoamylalcohol extraction and ammonium acetate-isopropanol precipitation (Sambrook et al., 1989). PCR amplification of unmodified genomic DNA (150 ng) using primer sets specific for wild-type promoter sequences (W) of *p15* and *MGMT* genes (Table 1), was performed according to the protocol by Herman et al., (1996a). Wild-type sequences of unmodified DNA for both genes corresponded to the position of target modified DNA sequences selected for methylation status analysis.

Bisulfite modification of DNA

Bisulfite modification of 2 μg of genomic DNA was performed according to the method originally described by Herman et al., (1996a), with slight modifications (Grunau et al., 2001; Krtolica et al., 2007). The bisulfite-treated DNA was purified using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany).

MS-PCR assay

An MS-PCR assay was performed to detect the methylation status of selected promoter sequences of *p15* and *MGMT* genes. Forty μl of bisulfite-modified DNA was prepared from each sample and 4 μl was amplified using 300 ng of primers specific for the methylated (M sets) and unmethylated (U sets) sequences (Table 1, Herman et al., 1996a; Esteller et al., 1999). DNA amplification was carried out using 1.25 U Taq DNA polymerase (MBI Fermentas, Lithuania) in 50 μl reaction mixture, in a Hybaid OmniGene temperature cycler (Hybaid, Teddington, UK). Reactions were hot started as an initial denaturation step, at 95°C for 5 min, followed by 33 cycles of denaturation at 95°C, annealing at 57°C and extending at 72°C for 30 s each, with a final 4 min extension step at 72°C.

All MS-PCR experiments were performed with positive and negative controls for both unmethylated and methylated alleles. Also, control experiments without DNA were performed in parallel for each set of PCR primers. The products were separated by electrophoresis, either on 2% agarose gels or nondenaturing 8% polyacrylamide gels, stained with SYBR Green I and silver nitrate, respectively.

Statistical analysis

For statistical purposes, B-NHL patients were classified into 2 different methylation groups: unmethylated (no methylated genes) and methylated (at least one methylated gene). For statistical analysis of the differences between the groups, Fisher's exact test, Chi-Square test, Student's T test and the Mann-Whitney test were used. Values were considered statistically significant if the p value was less than 0.05.

RESULTS

PCR analysis of unmodified DNA

PCR amplification of unmodified genomic DNA was performed with the aim to identify the presence of intact 5'CpG island sequences of the promoter region of *p15* and *MGMT* genes. We used wild-type DNA from the control samples with W primer sets (Table 1) The intact target sequence of the *p15* gene (137 bp, Fig. 1a) was detected in the control peripheral blood (line 1) and cell line SuDHL-4 (line 3), while it did not amplify in K562 (line 2) and RAJI (line 4) cell lines. The intact target sequence of the *MGMT* gene (93 bp Fig. 1b) was detected in all examined samples, including the control peripheral blood (line 1) and cell lines K562, SuDHL-4 and RAJI (lines 2 to 4).

Optimization of MS-PCR assay

Optimization of the MS-PCR assay for *p15* and *MGMT* genes included two steps. The first was to select the optimal annealing temperature for the assay. The range of temperatures tested was from 54°C to 60°C and from 54°C to 59°C with the U and M primer sets, respectively (results not shown). In both genes the most abundant specific amplification products were obtained at the annealing tem-

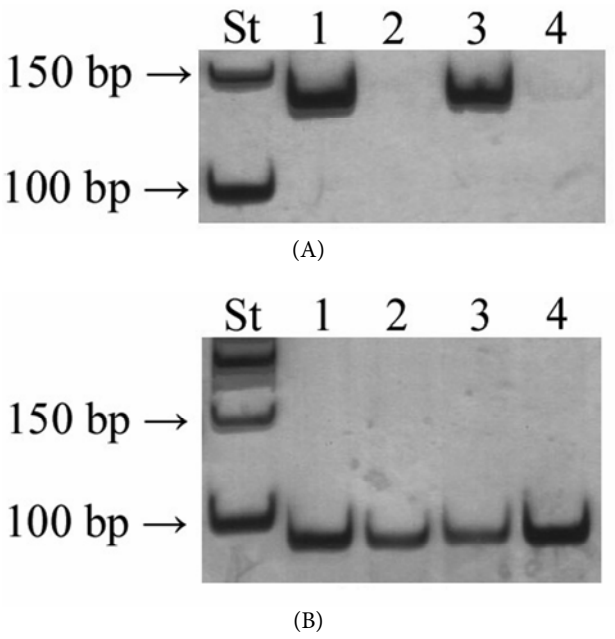

Fig. 1. Detection of the wild-type sequences of 5' region of p15 (Fig. 1a, 137 bp product) and MGMT (Fig. 1b, 93 bp product) genes in control specimens. An 8% polyacrylamide gel of PCR products with W primer set. Line 1, control peripheral blood from a healthy donor; Line 2, cell line K562; Line 3, cell line SuDHL-4; Line 4, cell line RAJI; St, Mw Standard (50 bp).

perature of 57ºC (Table 1). This annealing temperature was therefore used in further experiments with lymphoma samples.

The second step in optimization was to determine the optimal concentration of the target DNA in the MS-PCR assays. The concentration of modified DNA was tested in the range from 0.4 μl to 4 μl of the final DNA volume (40 μl). Results of MS-PCR amplification with the U and M primer sets of the *MGMT* gene as a function of DNA concentration are shown in Figure 2a (control peripheral blood) and 2b (cell line K562), respectively. The most efficient MS-PCR amplification was achieved for the highest tested DNA concentration (4 μl) for both of the examined genes.

MS-PCR analysis of B-NHL samples

The methylation status of the target promoter sequences of *p15* and *MGMT* genes was studied by MS-PCR in 10 patients with *de novo* B-NHL (Fig. 3 and 4) The unmethylated allelic form of the *p15* (*u-p15*, 154 bp product) and *MGMT* (*u-MGMT*, 93 bp product) genes was detected in all examined patients (10/10, 100%). The presence of the methylated allelic form of the *p15* gene (*m-p15*, 148 bp product) was shown in half of the tested patients (5/10, 50% pts), while in the case of the *MGMT* gene, a lower frequency (3/10, 30%) of methylated allelic form (*m-MGMT*, 81 bp product) was detected.

Three of the five patients with aberrant methylation of the *p15* gene had aberrant methylation of the *MGMT* gene (*m-p15/m-MGMT* profile), whereas 2/5 patients had aberrant methylation of the *p15* gene as a sole aberration (*m-p15/u-MGMT* profile, Table 2). Half of tested patients (5/10, 50%) had only unmethylated allelic forms of both genes (*u-p15/u-MGMT* profile, Table 2).

While expression of *m-p15/m-MGMT* or *m-p15/u-MGMT* profiles did not associate itself with

Table 1. Primer sets used for MS-PCR

Primer set	Sense primer	Antisense primer	Size (bp) / AT
p15-WT	*5′-CGCACCCTGCGGCCAGA-3′*	*5′-AGTGGCCGAGCGGCCGG-3′*	137bp / 65 ºC
p15-M	*5′-GCGTTCGTATTTTGCGGTT-3′*	*5′-CGTACAATAACCGAACGACCGA-3′*	148bp / 57 ºC
p15-U	*5′-TGTGATGTGTTTGTATTTTGTGGTT-3′*	*5′-CCATACAATAACCAAACAACCAA-3′*	154bp / 57 ºC
MGMT-WT	*5′-TTTGCGTCCCGACGCCCG-3'*	*5'-AGCTCCGCACTCTTCCGG-3'*	93bp / 62 ºC
MGMT-M	*5′-TTTCGACGTTCGTAGGTTTTCGC-3'*	*5'-GCACTCTTCCGAAAACGAAACG-3'*	81bp / 57 ºC
MGMT-U	*5′-TTTGTGTTTGATGTTTGTAGGTTTTTGT-3'*	*5'-AACTCCACACTCTTCCAAAAACAAAAA-3'*	93 bp / 57 ºC

Abbreviations: WT, primer set for unmodified wild-type DNA sequence; U, primer set for unmethylated modified DNA sequence; M, primer set for methylated modified DNA sequence; AT, annealing temperature.

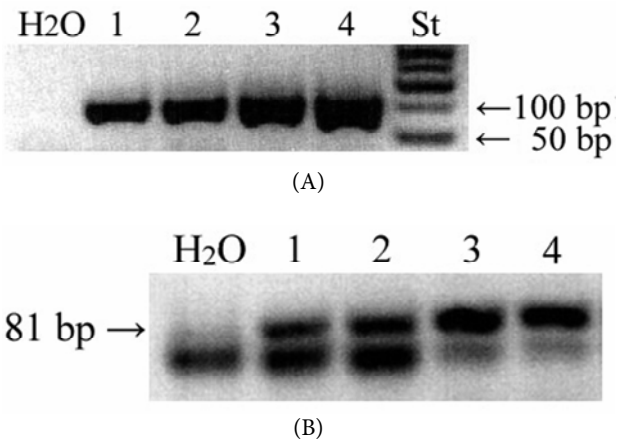

Fig. 2. Testing of the efficiency of MS-PCR amplification of the sequence of 5' region of the MGMT gene as a function of the concentration of modified DNA from: a) control peripheral blood with U primer set and MS-PCR product of 93 bp; b) cell line K562 with M primer set and MS-PCR product of 81 bp. The lines 1-4 present volume of DNA loaded on 2% agarose gel: 0.4µl; 1 µl; 2µl; 4 µl; H_2O, reaction mix without DNA.

any particular type of B-NHL, all patients in this group presented a higher clinical stadium (CS) of the disease: CS IV or V (Table 2). Only 1/5 patients with dg B-NHL-Follicular showed hypermethylation of the studied genes, having an *m-p15/m-MGMT* profile (Table 2, pt NHL-8).

p15 and MGMT gene methylation status and clinicopathological features

We examined the relationship between the *p15* or *MGMT* gene methylation status and the clinicopathological features of the patients (Table 3). In the group with the *m-p15* allelic form of the gene, we found that there is a statistically significant lower median hemoglobin value compared to the group with *u-p15* allelic form (p=0.004, t-test). The same trend of decreased hemoglobin values, although statistically insignificant, was shown for the group of patients with an *m-MGMT* allelic form of the *MGMT* gene. In the group of patients with an *m-p15* allelic form of the gene we also found a lower, although statistically insignificant, median platelet level. Further analysis revealed a significant association between the presence of an *m-p15* allelic form of the gene and decreased platelet levels (<150.000 x 10^9/l, p=0.038, Mann-Whitney test). The methylation status of the *MGMT* gene was independent of platelet values.

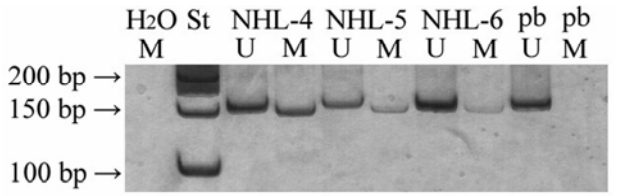

Fig. 3. Methylation status of p15 gene in patients with dg B-NHL: 8% polyacrylamide gel of MS-PCR products. U, 154 bp product with U primer set ; M, 148 bp product with M primer set; pb, control peripheral blood from a healthy donor; St, Mw Standard (50 bp); H_2O, reaction mix without DNA with M primer set.

Patients with an *m-MGMT* allelic form of the *MGMT* gene showed a trend towards a lower median leucocyte value, as well as lower proportions of lymphoma cells in the peripheral blood and bone marrow, compared to the group of patients with a *u-MGMT* allelic form of the gene (Table 3). The methylation status of the *p15* gene was independent of the leucocyte values.

There was no correlation between the methylation status of the *p15* and *MGMT* genes and the gender and age of the patients. Analysis of the response to the therapy did not reveal clear differences between the groups with different gene methylation profiles (data not shown).

DISCUSSION

The profile of CpG island hypermethylation in hematological malignancies is an epigenetic signature unique for each subtype of acute leukemia or lymphoma (Esteller, 2003). NHL exhibits non-random methylation patterns in which germinal center tumors seem to be prone to *de novo* methylation

Table 2. Basic clinical and biological characteristics of patients with dg B-NHL

Patient	Sex / Age	Sample type	Immuno-hisopathologic type / CS	p15 / MGMT gene methylation status
NHL-1	F / 34	PB	B-NHL-Follicular / VB	u / u
NHL-2	M / 65	PB	B-HLL/SLL / II	u / u
NHL-3	F / 41	PB	B-NHL-Marginal zone / IV	m / u
NHL-4	M / 66	BM	B-NHL, NOS, low grade / IVB	m / m
NHL-5	M / 38	BM	B-NHL-Lymphoplasmocytic, IgGkappa / IVB	m / m
NHL-6	M / 52	PB	B-NHL-Mantle zone / V	m / u
NHL-7	*M / 50*	*LN*	*B-NHL-Follicular / IIEA*	*u / u*
NHL-8	*F / 67*	*LN*	*B-NHL-Follicular / IVA*	*m / m*
NHL-9	*M / 81*	*LN*	*B-NHL-Follicular / IIIB*	***u / u***
NHL-10	*M / 44*	*LN*	*B-NHL-Follicular / IVB*	***u / u***

Abbreviations: M, male; F, female; PB, peripheral blood; BM, bone marrow; LN, lymph node; CS, clinical stage; NOS, not otherwise specified; u, unmethylated; m, methylated.

Table 3. Clinical and hematological characteristics of B-NHL patients in correlation to the methylation status of *p15* and *MGMT* genes

Parameter	*m-p15 gene*	*u-p15 gene*	*p value*	*m-MGMT gene*	*u-MGMT gene*	*p value*
Sex (%) *male* *female*	*3/5 (60)* *2/5 (40)*	*4/5 (80)* *1/5 (20)*	*0.490*	*2/3 (67)* *1/3 (33)*	*5/7 (71)* *2/7 (29)*	*0.880*
Age (year) *(x±SD)*	*53 ± 13*	*55 ± 18*	*0.850*	*57 ± 17*	*52 ± 16*	*0.691*
Le (x10⁹/l) *(x±SD)*	*29 ± 44*	*23 ± 25*	*0.810*	*5 ± 3*	*35 ± 37*	*0.219*
Plt (x10⁹/l) *(x±SD)*	*135 ± 84*	*214 ± 71*	*0.147*	*183 ± 69*	*171 ± 95*	*0.849*
Plt (%) *(>150 x10⁹/l)* *(<150 x10⁹/l)*	*2/5 (40)* *3/5 (60)*	*5/5 (100)* *0/5 (0)*	*0.038**	*2/3 (67)* *1/3 (33)*	*5/7 (71)* *2/7 (29)*	*0.880*
Hb (g/dl) *(x±SD)*	*92 ± 21*	*134 ± 9*	*0.004**	*98 ± 21*	*119 ± 28*	*0.278*
PB LyC (%) *(x±SD)*	*59 ± 29*	*51 ± 31*	*0.676*	*41 ± 19*	*61 ± 31*	*0.337*
BM LyC (%) *(x±SD)*	*57 ± 31*	*67 ± 19*	*0.709*	*50 ± 39*	*65 ± 24*	*0.598*

Abbreviations: u, unnmethylated; m, methylated; PB LyC, peripheral blood lymphoma cells; BM LyC, bone marrow lymphoma cells; Le, leukocytes; Plt, platelet; Hb, hemoglobin; *, statistically significant ($p<0.05$).

(Shi et al., 2007). Although the list of hypermethylation-repressed genes in these neoplasms is expanding very rapidly, particular methylotypes for specific NHL subtypes have been examined in only a few studies (Rossi et al., 2004; Shi et al., 2007).

We examined the frequency of aberrant *de novo* hypermethylation of 5'CpG islands located in the promoter region of *p15* and *MGMT* genes by performing MS-PCR analysis of the bisulfite-modified genomic DNA in selected group of adult patients with different types of primary B-NHL. According to literature data, both genes *p15* and *MGMT* are unmethylated in normal "healthy" hematopoietic cells (Herman et al., 1997; Cameron et al., 1999; Margison et al., 2003) and are indicated as potential methylation markers for B-NHL (Esteller, 2003).

In this study we applied the MS-PCR assay, the most widely-used technique for qualitative methylation analysis of CpG islands of different genes involved in human cancer (Esteller et al., 2002; Galm et al., 2006). This approach applies to genes with known gene sequences. The high sensitivity of this technique, with a detection limit of approximately 0.1% of methylated DNA present in an otherwise unmethylated sample, allows qualitative DNA methylation analysis not only of fresh or frozen tissues, peripheral blood, bone marrow and body fluids, but also of paraffin-embedded samples (Herman et al., 1996a; Galm et al., 2006; Krtolica et al., 2007).

Although, the methodology of bisulfite modification of DNA as well as the MS-PCR assay have been well standardized (Herman et al., 1996a; Grunau et al., 2001), prior to initiation of the analysis we optimized two parameters - the annealing temperature for U and M primer sets and the concentration of modified target DNA. In the case of both genes *p15* and *MGMT*, we found that the most efficient specific amplification products for both primer sets U and M, were obtained using an annealing temperature of 57°C. This temperature was lower than that originally proposed for identical primer sets, 60°C for *p15* gene (Herman et al., 1996a) and 59°C for *MGMT* gene (Esteller et al., 1999). In accordance with published data, the optimal chosen concentration of modified DNA (4μl) gave the most efficient amplification product for both studied genes (Herman et al., 1996a; Grunau et al., 2001).

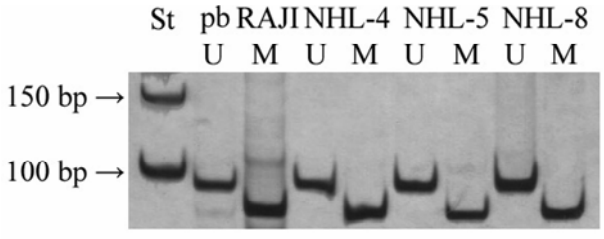

Fig. 4. Methylation status of the MGMT gene in patients with dg B-NHL: 8% polyacrylamide gel of MS-PCR products. U, 93 bp product with U primer set; M, 81 bp product with M primer set; pb, control peripheral blood from a healthy donor; St, Mw Standard (50 bp); RAJI, positive control for methylated allelic form of MGMT gene.

As a control experiment for MS-PCR, we performed PCR amplification of unmodified DNA (Herman et al., 1996a), with the aim to validate the presence of wild-type promoter sequences of *p15* and *MGMT* genes in native unmodified DNA samples. The aim of this test was to ensure the DNA integrity of potential cases in which an MS-PCR assay would fail to give any product of amplification. In all of the tested patients' samples we found intact promoter sequences of both genes. The intact target sequence of the *p15* gene was not validated for the cell lines K562 and RAJI. Absence of *p15* promoter amplification was anticipated for K562, since it contains 9p21 deletion that comprises a locus of the *p15* gene (Hatta et al., 1995; Drexler, 1998). However, our finding that RAJI also lacks this region was unexpected since the published data (Herman et al., 1996a; Drexler, 1998) indicated that the wild-type sequence of the *p15* gene was intact. A possible explanation for this is that the continuous passaging of the RAJI cell line might have induced an unknown mutation within the 5'CpG islands of *p15* gene promoter.

Since the MS-PCR assay was performed on unsorted samples of lymphoma cells which are heterogeneous and invariably contaminated with

normal cells, we could not identify the type of aberrant methylation, biallelic vs. monoallelic, of the studied genes (Esteller et al., 1999). On the other hand, we revealed the biallelic type of aberrant methylation of the *MGMT* gene in all of the tested cell lines, K562, SuDHL-4 and RAJI. This finding is in agreement with the published profile of *MGMT* gene methylation in other human cancer cell lines (Esteller et al., 1999; Paz et al., 2003).

Inactivation of the *p15* gene by promoter hypermethylation is one of the most studied epigenetic changes in hematological malignancies, especially in acute leukemias and myelodysplastic syndromes (Rush & Plass, 2002; Esteller, 2003; Galm et al., 2006). According to the published data, *p15* hypermethylation is extremely common in leukemias and relatively rare in lymphomas (Esteller, 2003; Galm et al., 2006). Moreover, inactivation of the *p15* gene by genetic mechanisms, either by point mutations (Baur et al., 1999) or by homozygous deletion of chromosome 9p21 (Herman et al., 1997), is very rare. Our preliminary data showed that the frequency of hypermethylation of the *p15* gene was 50% (5/10 pts) in the whole B-NHL group. Our data are in agreement with the results of study by Baur et al., (1999), who reported a frequency of 55% for aberrant *p15* methylation in an unselected group of low risk B-NHL.

With respect to the type of examined B-NHL, we detected *p15* hypermethylation in all the studied B-NHL types, except B-HLL/SLL. Despite the small number of cases in our study, our results were in accordance with the data reported by other groups (Baur et al., 1999; Martinez-Delgado et al., 2000).

Published data indicate that hypermethylation of the *p15* gene plays an important role in the different steps of lymphomagenesis, including the phase of disseminated disease, which is in positive correlation with a higher CS of the disease (Baur et al., 1999; Martinez-Delgado et al., 2000). All lymphoma patients with an *m-p15* allelic form had higher CS of the disease (IV - V). Hypermethylation of the *p15* gene could be generated as a primary *de novo* (Martinez-Delgado et al., 2000) or as a secondary *de novo* aberration caused by chemotherapeutic treatment (Au et al., 2003). The silencing of the *p15* gene by hypermethylation can lead to the loss of sensitivity of the lymphoma cells to growth inhibition by TGF-β and interferon-α, the potent regulators of differentiation and proliferation of lymphohematopoietic cells (Herman et al., 1997; Sherr & Roberts, 1999; Sangfelt et al., 1999; Visser & Kast, 2002).

A statistically significant positive association of different clinicopathological features (especially anemia and thrombocytopenia) with the presence of hypermethylation of the *p15* gene, represents a novel finding. Anemia and thrombocytopenia are associated with disseminated disease and bone marrow infiltration that reflect a higher CS of the disease (Jaffe et al., 2008).

MGMT is one of the genes that is frequently hypermethylated in the major types of B-NHL (Esteller et al., 1999; Esteller et al., 2002; Rush & Plass, 2002). Our preliminary data showed that the frequency of overall hypermethylation of *MGMT* gene was 30% (3/10 pts) in our B-NHL group at diagnosis. The frequency of hypermethylation of the *MGMT* gene is reported in the literature to be between 25% for an unselected group of B-NHL (Esteller et al., 1999; Rossi et al., 2004) and 36% for a selected DLBCL type (Esteller et al., 2002), and these results that are in agreement with our data.

All three patients with a hypermethylated allelic form of the *MGMT* gene had a high CS of the disease (IV) and were comethylated for the *p15* gene. There are few literature reports that explain the potential role of hypermethylation of the *MGMT* gene in lymphomagenesis. They suggest that loss of the MGMT protein from the cells in the early phase of the disease can cause the generation of a new mutator phenotype characterized by G → A transitions and the generation and accumulation of point mutations in various genes involved in cancer etiology, such as the tumor suppressor p53 and the oncogene K-ras (Esteller et al., 1999; Esteller & Herman, 2004). From this point, it is important to note that *MGMT* hypermethylation

alone is a poor prognostic factor in many human cancers (Esteller & Herman, 2004). On the other hand, loss of the MGMT protein from tumor cells due to aberrant hypermethylation can predict a better chemoresponse of human tumors to alkylating agents such as cyclophosphamide and others (Esteller & Herman, 2004).

Clinicopathological parameters showed a positive but insignificant association with the presence of hypermethylation of the *MGMT* gene, presenting a lower median value of hemoglobin and leukocytes, as well as a lower median proportion of peripheral blood and bone marrow lymphoma cells.

The comethylation of the *MGMT* with the *p15* gene, as we report in our study, represents a novel finding and selects both genes as candidates in future studies of the hypermethylation profiles of B-NHL. The biological and clinical significance of this association remains undetermined due to the small size of the patient group involved in this study. Similar comethylation of the *p16* with the *p15* gene has been previously reported (Baur et al., 1999; Martinez-Delgado et al., 2000).

In summary, aberrant DNA hypermethylation of the *p15* and *MGMT* genes in B-NHL could play a role as an epigenetic biomarker with possible applications in diagnostics and the monitoring of treatment efficacy (Rush & Plass, 2002; Esteller, 2003; Galm et al., 2006; Shi et al., 2007).

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УЧЕСТАЛОСТ АБЕРАНТНЕ МЕТИЛАЦИЈЕ ПРОМОТОРА ГЕНА *P15^{INK4B}* И *O^6-МЕТИЛГУАНИН-ДНК МЕТИЛТРАНСФЕРАЗЕ* КОД Б-ЋЕЛИЈСКОГ НЕХОЏКИНОВОГ ЛИМФОМА: ПИЛОТ СТУДИЈА

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Метилациони статус таргет промоторских секвенци гена *p15 и O^6-метилгуанин-ДНК метилтрансферазе (MGMT)* је у нашој пилот студији истраживан применом методе метилација зависним PCR-ом (MS-PCR) код 10 пацијената са дијагнозом *de novo* Б-нехочкинског лимфома (B-NHL). Аберантна хиперметилација *p15* гена је учесталија (50%) у односу на аберантну хиперметилацију *MGM*Т гена (30%), и утврђена је код различитих типова B-NHL у случају оба испитивана гена. Хиперметилација *MGMT* гена је откривена искључиво у асоцијацији са хиперметилацијом *p15* гена. Сви пацијенти са хиперметилацијом *p15* и/или *MGMT* гена имали су виши клинички стадијум болести (IV – V). Значајна асоцијација анемије и/или тромбоцитопеније са хиперметилацијом *p15* гена, указује на његов потенцијални прогностички значај у B-NHL. Кометилација *MGMT и p15* гена представља нов податак, и издваја ове гене као кандидате у будућим истраживањима хиперметилационих профила у B-NHL.