Hypermethylation of *p15* Gene in Diffuse - Large B-Cell Lymphoma: Association with Less Aggressiveness of the Disease

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Abstract

In this study, methylation-specific polymerase chain reaction was used to investigate the potential prognostic significance of the methylation status of *p15*, *p16, MGMT*, and *DAPK* genes in 51 specimens of diffuse large B-cell lymphoma (DLBCL). Hypermethylation of *p15* gene was significantly more prevalent in patients without relapse ($p = 0.001$) and there was a trend toward more frequent presence of *p15* methylation in patients without death outcome within 5-year follow-up period ($p = 0.086$) Also, there was a trend toward accumulation of *p15* methylation with favorable clinicopathological parameters including: age ≤ 60 years ($p = 0.091$), normal levels of lactate dehydrogenase ($p = 0.090$), Eastern Cooperative Oncology Group performance status < 2 ($p = 0.095$), and low/intermediate low International Prognostic Index ($p = 0.076$). In the female group and group of the patients without bulky tumor mass, treated with chemotherapeutic regimens including rituximab, methylation of *p15* was significantly related to longer overall survival ($p = 0.036$ and 0.027, respectively). Our results suggest that promoter methylation of *p15* gene could have prognostic value in DLBCL patients treated with rituximab when used in combination with gender and tumor size. Clin Trans Sci 2014; Volume 7: 384–390

Keywords: diffuse large B-cell lymphoma, methylation, *p15*, prognosis, rituximab

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the aggressive subgroup of non-Hodgkin's lymphoma (NHL) and comprises approximately 30–40% of all cases. It is clinically and biologically heterogeneous disease, characterized with highly variable response to the treatment and clinical outcome.[1] The International Prognostic Index (IPI) defines risk groups based on the clinical parameters at presentation.[2] However, patients with identical IPI still exhibit marked variability in survival, suggesting the presence of significant biological heterogeneity within the same IPI category.[3] In the recent years, much effort has been made to establish new, molecular prognostic parameters in order to further stratify patients into different risk groups and choose appropriate treatment strategy.[4–6] The addition of rituximab to the standard CHOP chemotherapy (R-CHOP) has led to a marked improvement in clinical outcome of patients with DLBCL, but in the same time, it's introduction has altered the significance of previously recognized prognostic parameters.[7] So, it is important to investigate additional molecular markers and reexamine the formerly established in aim to improve prognosis in the era of rituximab.

Aberrant DNA methylation of CpG islands in the promoter region of tumor suppressor genes represents an important mechanism of their inactivation. There are increasing evidences that hypermethylation of certain genes in cancer could serve as marker for monitoring the clinical behavior of the disease and prediction of patients outcome.[8] Recent investigations showed that multiple genes could be affected through aberrant DNA methylation in DLBCL, leading to deregulation of multiple cell pathways, including cell cycle control,[9,10] DNA damage repair, apoptosis,[11] and many others. Though there are strong evidences that such epigenetic changes could contribute to lymphomagenesis and the biological behavior of the disease,[12–14] the impact of particular genes methylation on patients' prognosis and outcome, especially in the era of new treatment strategies, remains unsolved. Hence, the aim of our study was to investigate the methylation status of four selected, cancer-related genes in the group of 51 DLBCL patients, in order to determine the possible association of their methylation with clinicopathogical features and outcome. The selected genes included: *p15* and *p16* tumor suppressor genes, encoding for cyclin-dependent kinase inhibitors important for G1 cell cycle arrest,[10] gene for O^6-methylguanine-DNA methyltransferase (*MGMT*), a DNA repair enzyme that removes mutagenic and cytotoxic adducts from the O^6 position of guanine,[15] and *DAPK* (death-associated protein kinase) gene that encodes a serine-threonine kinase involved in the extrinsic pathway of apoptosis, initiated by γ-interferon, FAS ligand, and tumor necrosis factor-α.[16] The promoter hypermethylation of these four genes has frequently been observed in DLBCL, indicating the important role of such epigenetic changes in the pathogenesis of this tumor type.[10–14] However, the data considering the relationship between methylation status of selected genes and patients' prognosis are contradictory, which needs more comprehensive investigations.

Methods

Patients and samples

Fifty-one patients (26 male, 25 female; median age 52.4 years, range 19–83 years) with DLBCL included in this study were diagnosed and treated in the Institute of Hematology, Clinical Center of Serbia, Belgrade, Clinic of Hematology MMA, Belgrade, Serbia and Oncology Institute of Vojvodina, Sremska Kamenica, Serbia, from 2001 to 2012. Biopsy samples of lymph node, bone marrow, or other involved organs from patients were collected at diagnosis. The data according to the all clinicopathological parameters, overall survival (OS), as well as the presence of Bcl2, Bcl6, CD10, and Ki67 expression were taken from medical documentation of listed institutions. Tumor samples were

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Primer set	Sense primer	Antisense primer	Size (bp)/AT	References
p15-M	5′-GCGTTCGTATTTTGCGGTT-3′	5′-CGTACAATAACCGAACGACCGA-3′	148 bp/57°C	20
p15-U	5′-TGTGATGTGTTTGTATTTTGTGGTT-3′	5′-CCATACAATAACCAAACAACCAA-3′	154 bp/57°C	20
p16-M	5′-TTATTAGAGGGTGGGGCGGATCGC-3′	5′-GACCCCGAACCGCGACCGTAA-3′	150 bp/65°C	20
p16-U	5′-TTATTAGAGGGTGGGGTGGATTGT-3′	5′-CAACCCCAAACCACAACCATAA-3′	151 bp/60°C	20
MGMT-M	5′-TTTCGACGTTCGTAGGTTTTCGC-3′	5′-GCACTCTTCCGAAAACGAAACG-3′	81 bp/57°C	21
MGMT-U	5′-TTTGTGTTTGATGTTTGTAGGTTTTTGT-3′	5′-AACTCCACACTCTTCCAAAAACAAAAA-3′	93 bp/57°C	21
DAPK-M	5′-GGATAGTCGGATCGAGTTAACGTC-3′	5′-CCCTCCCAAACGCCGA-3′	98 bp/60°C	22
DAPK-U	5′-GGAGGATAGTTGGATTGAGTTAATGTT-3′	5′-CAAATCCCTCCCAAACACCAA-3′	106 bp/60°C	22

M = primer set for methylated modified DNA sequence; U = primer set for unmethylated modified DNA sequence; AT = annealing temperature.

Table 1. Primer sets used for MSP.

considered positive for Bcl-2, Bcl-6, or CD10 when at least 50%, 10%, or 20% of tumor cells expressed Bcl2, Bcl6, or CD10 protein, respectively. Expression of Ki67 protein in 0–30% of tumor cells was considered as weak, in 30–60% of tumor cells as moderate, and in more than 60% of the cells as strong. All procedures were carried out with the prior informed consent of the patients and with the approval of the local Ethic Committee. Treatment consisted of CHOP/R-CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone with or without rituximab) regimen. Some patients had adjuvant radiotherapy and/or surgery, and some of them underwent autologous stem-cell transplantation. Response criteria and survival outcomes were defined according to the recommendation of Cheson et al.[17]

Analysis of methylation status of the *p15*, *p16*, *MGMT*, and *DAPK* genes

DNA methylation patterns in the promoter CpG islands of the *p15*, *p16*, *MGMT*, and *DAPK* genes were determined in all of 51 samples by methylation-specific polymerase chain reaction (MSP) following the bisulfite modification of isolated genomic DNA, as described earlier.[18] Briefly, DNA was isolated from deparaffined tumor specimens using standard proteinase K, phenol/chloroform/isoamyl alcohol extraction, and ethanol precipitation.[19] Two micrograms of isolated DNA were denatured by NaOH (final 0.3 mol/L) at 42°C for 30 minutes and modified by sodium bisulfite (5.20–5.69 mol/L, pH 5.0, Sigma, St Louis, MO, USA) for 18 hours at 50°C. After incubation, DNA was purified using the DNA extraction KIT (MBI Fermentas, Lithuania), again treated by NaOH (final 0.3 mol/L), at 37°C for 20 minutes, precipitated with ethanol/ammonium acetate and resuspended in 40 µL of 1 mmol/L Tris-HCl, pH 8.0. Aliquots of 4 µL of bisulfite-modified DNA were used for MSP reactions. The polymerase chain reaction (PCR) mixture contained 1 × PCR buffer (16 mmol/L ammonium sulfate, 67 mmol/L Tris-HCL, pH 8.8, 10 mmol/L 2-mercaptoethanol), 6.7 mmol/L $MgCl_2$, dNTP (each at 1.25 mmol/L), and primers (300 ng each per reaction) in a final volume of 50 µL. Reactions were hot-started at 95°C for 5 minutes before the addition of 1.25 units of Taq polymerase (MBI Fermentas). Amplification was carried out in an Applied Biosystems (Foster City, CA, USA) 2720 temperature cycler for 40 cycles (45 seconds at 95°C, 45 seconds at the annealing temperature being specific for each reaction, and 60 seconds at 72°, followed by final extension for 4 minutes at 72°C). Primers sequences used for each reaction are listed in *Table 1*. DNA from peripheral blood lymphocytes from a healthy donor was used as negative control for methylated alleles. The same leukocyte DNA was methylated *in vitro* with excess SssI methyltransferase (New England Biolabs, Ipswich, MA, USA) to generate completely methylated DNA at all CpG sites and used as positive control for all genes. PCR products were separated by electrophoresis on 6% acrylamide gels, stained with silver nitrate and visualized by sodium carbonate.

Statistical analysis

Contingency tables were analyzed using Pearson' s χ^2-test or Fisher' s exact two-tailed test, when expected frequencies were lower than five. Continuous variables were compared with the use of Student's *t*-test. OS distributions were estimated by the Kaplan-Meier method and differences were evaluated by the Log-rank test. In all tests, a *p*-value less than 0.05 were considered as statistically significant. All statistical analyses were performed using the Sigma Plot 10.0 licensed statistical analysis software package.

Results

Correlation between promoter methylation status and clinicopathological features

Hypermethylation of *p15*, *p16*, *MGMT*, and *DAPK* genes was detected in 23% (12/51), 37%, (19/51), 39% (20/51), and 55% (28/51) of samples, respectively. Representative examples of the methylation analysis are shown in *Figure 1*. Overall, 74% (38/51) of cases showed at least one hypermethylated gene, and 25% (13/51) of cases show no methylation of any genes examined. We observed the significant correlations between methylation of *p15* and *p16* genes ($p = 0.037$), *p16* and *DAPK* genes ($p = 0.047$), and *MGMT* and *DAPK* genes ($p = 0.025$), and observed comethylations were present in 16%, 27%, and 29% of cases, respectively.

Study of correlations between promoter methylation status of each gene and clinicopathological features are summarized in *Table 2*. Methylation of *p16* gene was significantly more prevalent in patients with normal level of lactate dehydrogenase (LDH) ($p = 0.035$) and low/intermediate low IPI score ($p = 0.034$). We observed a trend toward more prevalent methylation of *p15* gene in patients younger than 60 years ($p = 0.091$), normal level of LDH ($p = 0.090$), Eastern Cooperative Oncology Group (ECOG) performance status <2 ($p = 0.095$), and with low/intermediate low IPI score ($p = 0.076$). In the same time, we observed significant correlation between *p15* methylation status and relapse of

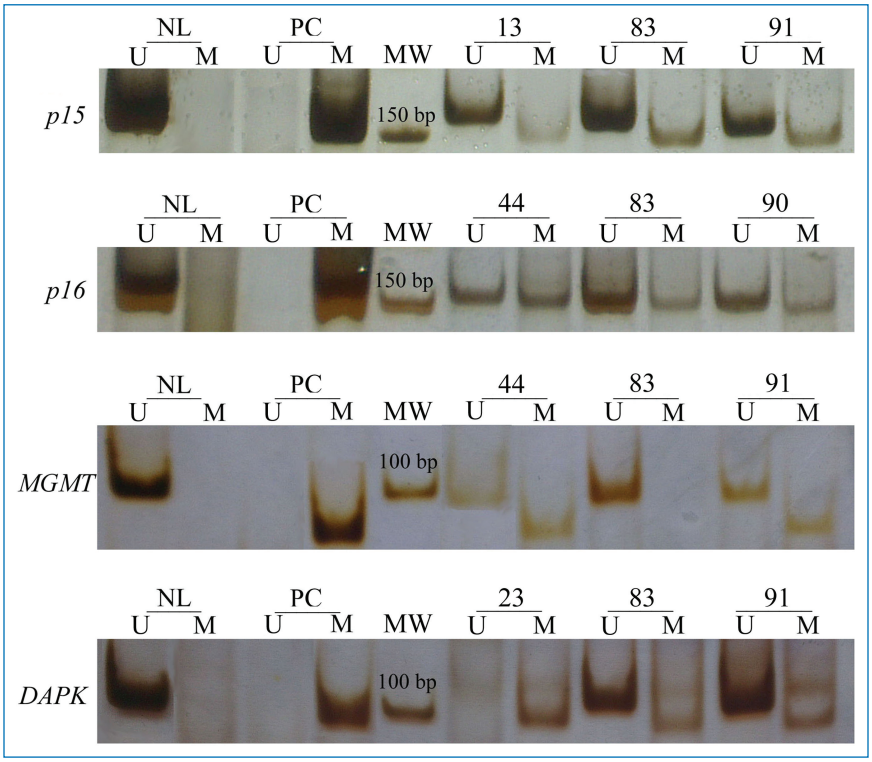

Figure 1. Analysis of *p15*, *p16*, *MGMT*, and *DAPK* genes methylation by MSP in representative cases of DLBCL. The presence of a visible PCR product in lanes U indicates the presence of unmethylated allels (154, 151, 93, and 106 bp, respectively), while the presence of product in lanes M indicates the presence of methylated allels (148, 150, 81, and 98 bp, respectively). NL = normal lymphocytes as a positive control for unmethylated allels; PC = *in vitro* methylated DNA from normal lymphocytes as a positive control for methylated allels; MW = molecular weight marker (50 bp).

the disease; no one patient with relapsed DLBCL showed *p15* methylation ($p = 0.001$, *Table 2*). Also, there was a trend toward more frequent presence of *p15* methylation in patients without death outcome within 5-year follow-up period ($p = 0.086$). There was no correlation between methylation of *MGMT* or *DAP*K genes with any clinicopathological characteristic, Bcl2, Bcl6, CD10, and Ki67 expression, response to the therapy or patients' outcome.

Clinicopathological features of the group with concomitant *p15* and *p16* methylation *(p15m/p16m)*

We found that methylation of *p15* gene occurs significantly more frequent with simultaneous methylation of *p16* gene than as a single event (67% vs. 33%, $p = 0.037$). We have also observed a tendency toward accumulation of *p15m/p16m* with clinicopathological characteristics related to less aggressiveness of the disease, including: female gender ($p = 0.140$), normal LDH levels ($p = 0.100$), age less than 60 years ($p = 0.119$), ECOG performance status < 2 ($p = 0.127$), low/intermediate low IPI score ($p = 0.099$). In addition, none of the patients with *p15m/p16m* had relapsed disease during follow-up period ($p = 0.031$).

Survival analysis in the whole group of DLBCL patients

Follow-up data were available for 46/51 patients and the median follow-up period was 30.5 months (range 1–111 months). Five-year survival for the entire group was 61% (95% CI: 45.25–74.42). OS was significantly worse for patients who were in any of the following categories: intermediate high/high IPI risk group ($p = 0.033$), ECOG performance status ≥ 2 ($p < 0.001$) B symptoms present ($p = 0.027$), no Bcl6 expression ($p = 0.050$), no response to the initial therapy ($p < 0.001$), and not treated with rituximab ($p = 0.031$). Among all patients with DLBCL, there was no significant difference in the OS between those with hypermethylated and unmethylated of any examined genes ($p > 0.05$ in all cases, *Figure 2*). However, patients with methylated *p15* tended to have longer OS in contrast to those with unmethylated *p15* gene, though this difference was not statistically significant ($p = 0.155$, *Figure 2*A). There was no significant difference in OS between patients with hypermethylated and unmethylated of any examined genes in the different groups according to the IPI or any other group of the patients classified according to the parameters given in the *Table 2*.

Survival analysis in the group of the patients treated with multidrug regimens including rituximab

Considering our finding that DLBCL patients treated with rituximab in addition to the standard chemotherapy had significantly longer OS ($p = 0.031$, *Figure 3*A), we further evaluated the impact of the methylation status of four examined genes on survival in this group of the patients. Five-year survival for the 38 patients who received rituximab was 65% (95% CI: 47.93–79.32). OS was significantly worse for patients with ECOG performance status ≥ 2 ($p < 0.001$), no Bcl6 expression ($p = 0.043$), no response to the initial therapy ($p < 0.001$), and with relapsed disease ($p = 0.009$). As in the whole group of the patients, we observed a trend toward longer OS for the patients with methylated *p15* gene ($p = 0.093$, *Figure 3*B). We found no significant difference in OS between patients with hypermethylated and unmethylated *p15* gene according to the IPI (results not shown). However, *p15* methylation was significantly related to longer OS in the female group of the patients ($p = 0.036$, *Figure 3*C) and in those with no bulky tumor mass ($p = 0.027$, *Figure 3*D). Moreover, in both cases, all patients with *p15* methylation were alive during follow-up period. No significant impact on OS was seen for the other three genes in the any group of the patients treated with R-CHOP (results not shown).

Discussion

Prognostic utility of epigenetic changes in malignant lymphoma, especially in the era of new therapy regimens, is still not clearly determined. In the present study, we investigated the biological significance and the prognostic relevance of the methylation status of four cancer-related genes in a series of 51 DLBCL, most of them treated with rituximab in addition to standard chemotherapy. Aberrant methylation of *p15*, *p16*, *MGMT*, and *DAPK* genes was detected in 23%, 37%, 39%, and 55% of patients, respectively. Thirty-eight of 51 patients (75%) had methylation of more than one examined genes. Our findings indicate that epigenetic alterations are common phenomenon in DLBCL and may be important for pathogenesis of this group of NHL.

Variable	p15m	p16m	MGMTm	DAPKm	*p*
Gender					
Male	4/26 (15%)	9/26 (35%)	11/26 (42%)	14/26 (54%)	
Female	8/25 (32%)	10/25 (40%)	9/25 (36%)	14/25 (56%)	NS
Age (yr)					
≤60	10/30 (33%)	14/30 (47%)	14/30 (47%)	19/30 (63%)	
>60	2/21 (9%)*	5/21 (24%)	6/21 (29%)	9/21 (43%)	0.091*
Stage					
I, II	2/8 (25%)	4/8 (50%)	2/8 (25%)	5/8 (62%)	
III, IV	10/43 (23%)	15/43 (35%)	18/43 (42%)	23/43 (53%)	NS
LDH					
Normal	7/20 (35%)**	11/20 (55%)*	7/20 (35%)	12/20 (60%)	0.035*
Elevated (>450 u/L)	4/29 (14%)	6/29 (21%)	12/29 (41%)	14/29 (48%)	0.090**
Extranodal sites					
0, 1	10/39 (26%)	14/39 (36%)	14/39 (36%)	20/39 (51%)	
>1	2/12 (17%)	5/12 (42%)	6/12 (50%)	8/12 (67%)	NS
ECOG performance status					
<2	10/35 (29%)*	14/35 (40%)	13/35 (37%)	21/35 (60%)	
≥2	2/16 (12%)	5/16 (31%)	7/16 (44%)	7/16 (45%)	0.095*
IPI score					
Low/intermediate low	7/17 (41%)**	10/17(59%)*	7/17 (41%)	12/17 (71%)	0.034*
Intermediate high/high	5/34 (15%)	9/34 (26%)	13/34 (38%)	16/34 (47%)	0.076**
B symptoms					
Absent	4/16 (25%)	7/16 (44%)	4/16 (25%)	8/16 (50%)	
Present	7/33 (21%)	12/33 (36%)	15/33 (45%)	18/33 (54%)	NS
BM involvement					
Absent	9/32 (28%)	13/32 (41%)	13/32 (41%)	20/32 (62%)	
Present	3/19 (16%)	6/19 (32%)	7/19 (37%)	8/19 (42%)	NS
Bulky tumor (≥10 cm)					
Absent	9/38 (24%)	15/38 (39%)	15/38 (39%)	21/38 (55%)	
Present	3/11 (27%)	4/11 (36%)	4/11 (36%)	5/11 (45%)	NS
Bcl-2 expression					
Absent	6/28 (21%)	11/28 (39%)	9/28 (32%)	14/28 (50%)	
Present	4/18 (22%)	4/18 (22%)	8/18 (44%)	9/18 (50%)	NS
Bcl-6 expression					
Absent	1/12 (8%)	5/12 (42%)	5/12 (42%)	7/12 (58%)	
Present	5/23 (22%)	8/23 (35%)	6/23 (26%)	11/23 (48%)	NS
CD10 expression					
Absent	1/12 (8%)	4/12 (33%)	5/12 (42%)	6/12 (50%)	
Present	3/11 (27%)	3/11 (27%)	5/11 (45%)	7/11 (64%)	NS
Ki67 expression					
Weak/moderate	3/15 (20%)	5/15 (33%)	5/15 (33%)	7/15 (47%)	
High	6/21 (29%)	7/21 (33%)	8/21 (38%)	12/21 (57%)	NS
Response to treatment					
CR/PR	10/41 (24%)	14/41 (34%)	16/41 (39%)	21/41 (51%)	

Table 2. Continued.

Variable	p15m	p16m	MGMTm	DAPKm	*p*
NR	1/8 (12%)	4/8 (50%)	3/8 (37%)	6/8 (75%)	NS
Relapse					
Absent	11/31 (35%)*	13/31 (42%)	10/31 (32%)	17/31 (55%)	
Present	0/18 (0%)	5/18 (28%)	8/18 (44%)	8/18 (44%)	0.001*
Death outcome within 5 years					
Absent	9/27 (33%)*	11/27 (41%)	10/27 (37%)	16/27 (59%)	
Present	2/20 (10%)	6/20 (30%)	8/20 (40%)	9/20 (45%)	0.086*

LDH = lactate dehydrogenase; ECOG = Eastern Cooperative Oncology Group; BM = bone marrow; CR = complete remission; PR = partial remission; NR = no response; NS = not significant. * and ** relates significant p values with appropriate gene and examined clinicopathological characteristics. For the cases where methylation status of two genes is related to appropriate clinicopathological characteristic (LDH and IPI score), * relates appropriate p value with p16 gene, and ** with p15 gene.

Table 2. Correlation of *p15, p16, MGMT,* and *DAPK* methylation status with clinicopathological features in DLBCL.

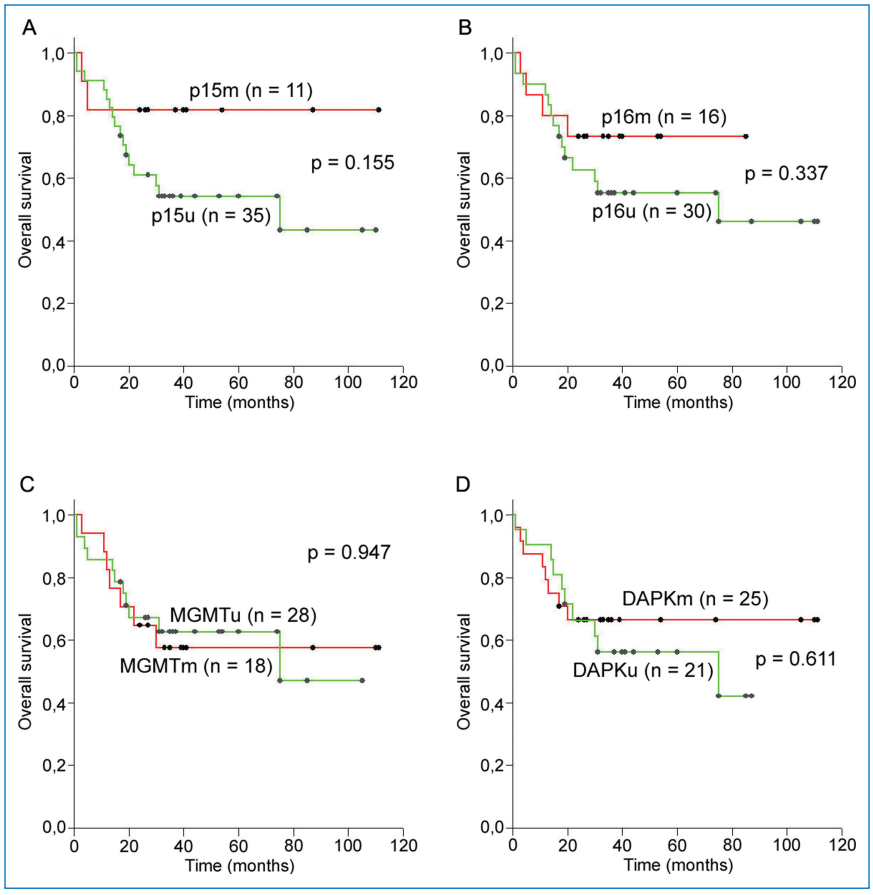

Figure 2. Overall survival (OS) in the entire group of DLBCL patients according to the methylation status of (A) *p15*, (B) *p16*, (C) *MGMT*, and (D) *DAPK* gene. No significant differences were observed in OS between patients with methylated and unmethylated any of examined genes. The greatest difference in OS is observed in the case of *p15* gene, where patients with methylated *p15* tended to have longer OS than the patients with unmethylated *p15* gene.

Tumor suppressor genes *p15* and *p16* are the members of cyclin-dependent kinase inhibitors and are frequently inactivated by aberrant hypermethylation of promoter CpG islands in various hematological tumors.[23,24] In DLBCL, methylation of *p15* and *p16* genes has been found in 32–77% and 27–54% of cases, respectively[10,13,14,24–26] and the results obtained in our study are similar to those in previous reports. However, prognostic significance of *p15* and *p16* methylation in DLBCL is unclear. While some studies have found *p16* methylation as a marker of worse prognosis[13,14,27] some others have not.[26,28] In our study, methylation of *p16* gene was not related with patients outcome. However, we demonstrated a significant correlation between *p16* hypermethylation and normal level of LDH and low/intermediate low IPI score. These two parameters have been related to better prognosis in many studies,[2,13,14,29] which is confirmed for IPI in ours. To our knowledge, we are the first to detect such associations, so the importance of our results should be confirmed in the further studies on the larger number of samples.

While there are reports about impact of *p15* promoter methylation on patient's prognosis in various cancer types,[30–32] such influence in DLBCL, to our knowledge, was not found till now.[13,14,26] In the present study, we observed a significant correlation between *p15* hypermethylation and longer OS in the female patients and those with no bulky tumor mass, treated with rituximab in addition to standard chemotherapy. In the same time, we observed a trend toward longer OS in the whole group of the patients under given treatment. The precise mechanism underlying such favorable impact is unknown. Of notice, the prevalence of *p15* methylation was twice as higher in female than in male patients from our study, though this difference was not statistically significant (32% vs. 15%, *Table 2*). There are some reports about gender-associated differences in DNA methylation at specific loci, but the results are controversial.[33] In addition, male gender was recently reported as an adverse prognostic factor in DLBCL patients treated with R-CHOP. The possible explanation for observed differences among genders in our study could be the difference in blood clearance of rituximab between males and females, which cause the better response of women to rituximab.[34] Though not significant, a tendency toward accumulation of *p15* and simultaneous *p15/p16* methylation with clinicopathological characteristics related to better prognosis is observed in the whole

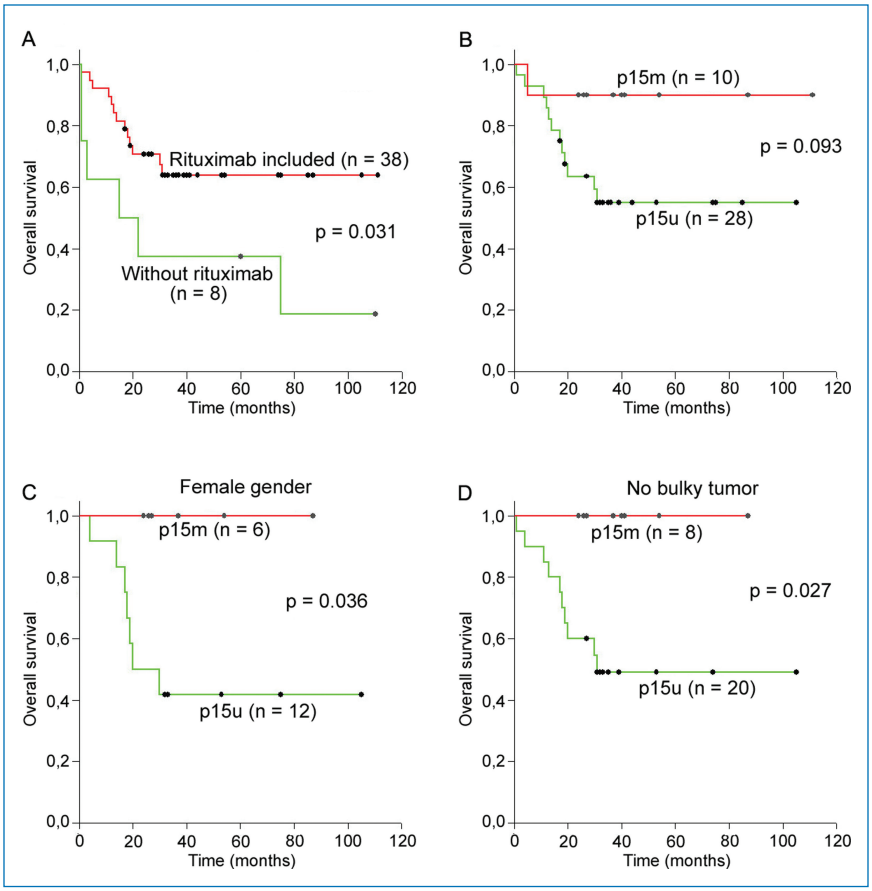

Figure 3. Overall survival (OS) in the DLBCL patients treated with rituximab. (A) OS is significantly longer in patients treated with rituximab in addition to the standard chemotherapy; (B) patients with methylated *p15* tended to have longer OS than patients with unmethylated *p15* gene; (C) female patients with methylated *p15* had significantly longer OS than those with unmethylated *p15* gene; (D) patients with no bulky tumor mass with methylated *p15* had significantly longer OS than those with unmethylated *p15* gene.

group of the patients. So, we suggest that *p16* and especially *p15* methylation could be related to better prognosis in DLBCL, though the precise mechanism is unknown. Considering that methylation of both, *p15* and *p16*, genes is more prevalent in lower IPI patients from our study, it is possible that their inactivation in higher IPI DLBCLs is achieved by some other mechanisms, including deletions. As described earlier, homozygous deletions of *p15* and *p16* are present in approximately one-third of the DLBCL, predominantly in activated B-cell subtype of DLBCL (ABC), where they are related to a poor prognosis.[27,35–37] On the contrary, methylation of these two genes is predominant mechanism of their inactivation in germinal center B-cell subtype of DLBCL (GCB),[37] which has better prognosis and distinct epigenetic and genetic signature than ABC-DLBCL.[4,5] Moreover, Guney et al.[37] have found that inactivation of *p15* and *p16* genes in GBC subtype is monoallelic, while in ABC subtype is biallelic. In addition, they demonstrated higher methylation levels of *p15* and *p16* promoters in the DLBCL cases with simultaneous heterozygous deletions of the second allel, compared to the methylation level of undeleted cases. Considering these results, we suppose that the expression of *p15* and/or *p16* in such types of DLBCL is not completely suppressed. It could be supported by finding of Cameron et al.,[38] who showed that the extent of methylation is a critical determinant of the degree to which *p15* is silenced in primary acute leukemia. It is possible that DLBCL patients with *p15* and/or *p16* methylation from our study belong to the GCB subtype, so it could be, at least, partially explanation for our results. Also, it is possible that in more aggressive DLBCL some other genetic and/or epigenetic alterations exist that are related to more aggressive behavior of the disease. Previous studies have shown that *p16* methylation is related to more aggressive phenotype only when simultaneous inactivation of *p53*, *p14*, and *p27* genes is present.[25,39] However, as we did not have enough data to distinguish our DLBCL samples on GBC and ABC subtypes, further investigations are needed to confirm our hypothesis and completely elucidate the role of *p15* and *p16* hypermethylation in DLBCL.

MGMT is a well-known DNA repair gene, important for protecting cells from mutagenic and cytotoxic adducts originated from the environmental and therapeutic alkylating agents.[15] Inactivation of *MGMT* due to promoter hypermethylation occurs at varying frequencies throughout the entire spectrum of B-cell neoplasms.[11,40] We detected *MGMT* methylation in 39% of our cases, which is in line with observed frequencies in other studies on DLBCL.[11,21,41] Previous studies on DLBCL demonstrated that *MGMT* methylation could be a useful marker for predicting survival in patients treated with multidrug regimens, including the alkylation agent cyclophosphamide.[12] However, these results were obtained before the introduction of rituximab to the chemotherapy. Recent studies on DLBCL patients who received R-CHOP showed that inactivation of *MGMT* gene does not play a role as a predictive marker of response to this treatment and in predicting survival. It is postulated that rituximab may play an important role in overcoming chemoresistance to cyclophosphamide, especially in the *MGMT* unmethylated group.[42] Our results are in concordance with this finding, as we observed no difference in the response to the therapy or OS according to the methylation status of *MGMT* gene.

Inactivation of *DAPK* gene by aberrant promoter hypermethylation is common event in B-cell malignancies, including DLBCL, where it is occurring in approximately 60% of cases.[11,14,22,41,43] Our results are similar with those in previous reports. *DAPK* is a pro-apoptotic serine/threonine kinase, involved in multiple apoptosis pathways, so its inactivation could be a key factor modulating the response to chemotherapy in human cancer.[16,44] While Amara et al.[14] have found that *DAPK* hypermethylation is an independent prognostic factor in predicting shortened OS of DLBCL patients, Nakamichi et al.[45] have found no impact of *DAPK* methylation on patients' outcome. The latter finding is in concordance with our results, so further analyses are necessary to elucidate the prognostic utility of *DAPK* in DLBCL.

Conclusion

Although obtained results need to be confirmed in larger series, our study suggests that methylation of *p15* gene could have prognostic value in DLBCL patients treated with rituximab when

used in combination with gender and tumor size. It is possible that *p15* hypermethylation is not a prognostic marker by itself, but identifies a specific pathogenetic subset of lymphomas with a more favorable outcome. However, further investigations are needed to confirm our hypothesis and clarify the role of *p15* gene methylation in the pathogenesis of DLBCL.

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