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ORIGINAL ARTICLE

The importance of Myd88 L265P mutation, clinical and immunohistochemical prognostic factors for the survival of patients with diffuse large B-cell non-Hodgkin lymphoma treated by immunochemotherapy in southeast Serbia

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Summary

Purpose: *Immunochemotherapy used in the treatment of non-Hodgkin diffuse large B-cell lymphoma (DLBCL) modifies the course of disease and has a positive effect on overall survival (OS). The purpose of this study was to verify the existence of the important Myd 88 mutation and other immunohistochemical factors on disease prognosis in patients with DLBCL in southeast Serbia.*

Methods: *Immunohistochemical expression of CD10, Bcl-2, Bcl-6, Ki-67 and MUM 1 was performed using paraffin blocks of DLBCL. Molecular-genetic study of MyD88 L265P gene polymorphism was done by isolation of genomic DNA from paraffin embedded tissue by means of polymerase chain reaction (PCR).*

Results: *Immunochemotherapy (rituximab+CHOP/R-CHOP) significantly improved the overall survival (OS) of patients with DLBCL compared with patients treated with CHOP alone (p<0.0001). OS in the R-CHOP group was longest in patients with International Prognostic Index (IPI) 2 score (p=0.012) and IPI 4 score (p=0.024). Patients with Bcl-2 +, and MUM 1+ benefited from R-CHOP and their expression had no effect on OS. Analysis of restriction fragment length on the genomic DNA showed a homozygous normal TT genotype.*

Conclusion: *Addition of rituximab to CHOP standard protocol improved the OS rate in patients with DLBCL and altered the character and significance of previously recognized prognostic factors. IPI score in the immunochemotherapy era could not reveal possible predictive factors of poor prognosis which would help identify a high-risk subgroup of newly diagnosed DLBCL. In the patient population from Southeast Serbia pathological signaling pathway achieved by Myd 88 L265 mutation was not responsible for the development of DLBCL.*

Key words: *DLBCL, immunochemotherapy, IPI, Myd 88 L265*

Introduction

DLBCL represents diffuse proliferation of large neoplastic B-lymphocytes and is the most common subgroup of non-Hodgkin lymphoma. The studies in the field of molecular genetics and immunohistochemistry identified two clinically relevant groups DLBCL: a group originating from the germinal center (GCB) and a second group originating from activated B-cells- non-GCB type [1-4]. As the gene expression profiling is not available for routine work, Hans et al. proposed an al-

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gorithm based on the expression of markers CD10, Bcl-6 and MUM-1 [5]. As possible predictive factors of poor prognosis, expression of antiapoptotic protein Bcl-2 and Ki-67 proliferative index play an important role. Myd 88 is a signaling adapter protein that activated NFkB pathway during normal immune response. The L265P mutation was by far the most common variant observed, occurring in 29% of non-GCB subtype DLBCL. This mutation was rare or absent among DLBCLs of the GCB [6]. Although there are numerous studies dealing with this topic, our aim was to verify the existence of Myd 88 mutation and other immunohistochemical factors of disease prognosis, as well as IPI, in a real situation, using authentic unselected clinical material without elimination criteria, in the era of immunochemotherapy.

Methods

A total of 57 patients with DLBCL, aged 24-82 years, treated at the Clinic of Hematology and Clinical Immunology, Clinical Center Nis, Serbia from 2009 to 2013, were included in the study. All of them were previously untreated. According to the presence of risk factors including age ≥ 60 years, disease stage III/IV, ≥1 extranodal manifestations (infiltrates in bone marrow, spleen, stomach, bladder and tonsils), elevated lactate dehydrogenase (LDH) and ECOG performance status ≥ 2, they were classified into 4 IPI groups: IPI 1 (low risk), IPI 2 (intermediate-lower risk), IPI 3 (intermediate-higher risk) and IPI 4 (high risk). A total of 57 patients were treated with standard R-CHOP for 6-8 cycles (rituximab, cyclophosphamide, doxorubicin, vincristine, prednisolone in cycles of 21 days). The control group consisted of 14 patients with DLBCL, aged 41-75 years, during the period from 1991 to 2002, treated according to standard CHOP chemotherapy. Genetic testing of MyD88 L265P polymorphism for the control group was not done. The histological sections were processed by standard techniques, and stained with hematoxylin and eosin (HE) at the Center of Pathology, Clinical Center Nis, Serbia.

Immunohistochemical analysis

Representative sections of DLBCL were analyzed for Bcl-2, Bcl-6, CD10, CD5, MUM 1, and Ki-67 proteins by standard immunohistochemical procedure. The following primary antibodies were used: primary monoclonal mouse anti-human antibody (Dako Glostrup, Denmark) against Bcl-2 (clone 124), Bcl-6 (clone PG-B6p), CD10 (Clone C56C6), CD5 (Clone 4C7), MUM1 (Clone MUM1p), and Ki-67 (Clone MIB-1). Marked antigens were detected using DAKO EnVision kit as a universal immunoperoxidase polymer or secondary antibody. Immunohistochemical score was based on the German ImmunoReactive Score.

Molecular-genetic investigation

Molecular-genetic study of gene polymorphism MyD88 L265P was performed in the Laboratory for Functional Genomics and Proteomics, Scientific-Research Center for Biomedicine, Faculty of Medicine, Nis. The experimental work was performed using genomic DNA extracted from paraffin fixed tissues of the patients with DLBCL.

DNA isolation

DNA isolation from paraffin fixed tissues was performed using Kapa Express Extract kit (Kapa Biosystems Inc., Wilmington, MA, USA). The procedure was done in 100 µl on the Agilent Technologies Sure Cycler 8800 apparatus and included 10 µl Kapa Express Extract Buffer, 2 µl Kapa Express Extract enzyme, approximately 2 mm^3 of tissue and 86 µl of deionized water. The reaction conditions were: 10 min at 75°C ; 5 min at 95°C. The sample was then put into vortex for 2-3 sec and centrifuged for 1 min at the maximum speed of 14.5 RPM. The supernatant was extracted and transferred to a new 1.5 ml ependorfice . The DNA concentration was measured by the SPEC apparatus BIO-NANO (Shimadzu, Japan) dissolved by tris-EDTA (TE) buffer to a final concentration of 50 ng/ml. The isolated DNA was stored at -20°C until the next procedure step.

Polymerase chain reaction

PCR caused amplification of DNA segments containing single nucleotide polymorphisms (SNP) of the tested MyD88 L265P gene. For PCR KAPA2G Fast HotStart ReadyMix (Kapa Biosystems Inc., Wilmington, MA, USA) was used. Amplification of DNA segments was carried out in a reaction mixture volume of 25 µl to which 20 ng/µl DNA and 10 µM of each primer were added (Table 1). Previously known sequence primers

Table 1. Composition of the reaction mixture for the polymerase chain reaction

Composition of the reaction mixture	*Final concentration*	*Mixture composition for total volume 25 µl*
Water	/	Variable
KAPA2G Fast HotStart ReadyMix*	1X	12.5 µl
F primer (10 µM)	0.1 µM	1.25 µl
R primer (10 µM)	0.1 µM	1.25 µl
Bovine serum albumin (0.5µg/µl)	0.5 µg	1.25 µl
DNA	20 ng	Variable

* KAPA2G: Fast HotStart ReadyMix contains KAPA2G Fast HotStart DNA polymerase, KAPA2G Fast HotStart PCR buffer, dNTP (0.2 mM of each 1X dNTP-a), 1X $MgCl_2$ (1.5 mM) and stabilizators

Table 2. Sequences of primers used for determination of genetic polymorphisms of Myd88 L265P

Polymorphism	*Sequences of primers*
rs387907272	F 5'- GGG ATA TGC TGA ACT AAG TTG CCA C-3', R 5'-GAC GTG TCT GTG AAG TTG GCA TCT C- 3'

Table 3. Conditions of polymerase chain reaction

	Temperature (°C)	*Time (s)*	
Initial denaturation	95	120	
Denaturation	95	15	
Hybridization	66	30	30 cycles
Elongation	72	45	
Final extension	72	600	

were used, and the sequences of oligonucleotides were used as primers in PCR (Table 2). For PCR, PCR Thermal Cycler SureCycler 8800 (Agilent Technologies, Inc., USA) was used. PCR conditions are shown in Table 3.

Electrophoresis on agarose gel

The success of the PCR amplification was checked by electrophoresis on 2% agarose gel. For preparation of 2% agarose gel 1.2 g of agarose (Serva, Germany) and 60 ml of 1X TBE buffer containing 0.89 M Tris-HCl, 0.89 M boric acid, 0.5 M EDTA pH 8.0. were used. Electrophoresis was carried out in the same buffer for 30 min for MyD88 rs387907272 polymorphism at current intensity 100 mA and voltage 100 V. After completion of electrophoresis, the gel was stained in ethidium bromide solution for 30 min, and then visualization under UV light transilluminator was performed (MacroVue-up 20 transilluminator, Hoefer Inc., Holliston, MA, USA). For determination of the PCR size products the marker GeneRuler 100bp DNA Ladder (Fisher Scientific GmbH, Schwerte, Germany) was used.

Determination of restriction fragment length

After the verification of PCR products on agarose gel, restriction digestion (Fisher Scientific GmbH, Schwerte, Germany) with restriction endonucleases, the enzymes recognizing specific palindromic sequence, was performed. By hydrolysis of the phosphodiester linkages of DNA they yielded fragments of the appropriate length (Table 4). The reaction mixture for the reaction restriction digestion included 2 µl of buffer Tango, 0.5 µl BsiEI enzymes, 10 µl of PCR product and water to the total volume of 20 µl . The reaction was carried out in a water bath overnight at 37 °C.

Polyacrylamide gel electrophoresis (PAGE)

Identification of the obtained restriction fragment was performed on 8.8% polyacrylamide gel (polyacrylamide gel electrophoresis), obtained by polymerization

Table 4. Restriction enzymes used for determining tested polymorphisms of Myd88 L 265P

Polymorphism	*Restriction enzyme*	*Palindromic sequences*
rs387907272	BsiEI	5´...CGRY▾CG...3´ 3´...CG▴RYGC...5´

Table 5. Alleles and the size of the corresponding restriction fragments for tested polymorphisms of Myd88 L265P

Polymorphism	*Allele*	*Size of restriction fragments*
rs387907272	T	726 bp
	C	726bp+448bp+278bp

of acrylamide-bisacrylamide and the use of appropriate catalysts. The process of polymerization in the reaction mixture was carried out by simultaneous addition of 10% ammonium persulfate (APS) and N, N, N', N'- tetramethylethylenediamine (TEMED) to the 30% solution of acrylamide (29: 1 acrylamide: N'-methylenebisacrylamide in water).

Electrophoresis was carried out in 1X TBE buffer for 90 min at a current intensity of 20 mA and voltage of 120 V. After completion of electrophoresis, the gel was stained in a solution of ethidium bromide for 30 min, and then visualization under UV light transilluminator was performed (upward MacroVue -20 Transilluminator, Hoefer Inc., Holliston, MA, USA). For determination of the restriction fragment length the marker GeneRuler 50bp DNA Ladder (Fisher Scientific GmbH, Schwerte, Germany) was used. The sizes of restriction fragments for the tested polymorphisms are shown in Table 5.

PCR-restriction fragment length polymorphism method

The genotype of the examined polymorphisms was determined by analyzing the restriction fragment length. Homozygous, normal wild type TT genotype was represented by the presence of a 726bp fragment, whereas in the presence of two alleles CC genotype was formed observed as two 448bp and 278bp fragments on acrylamide gel. The presence of all three fragments (726bp, 448bp and 278bp) corresponded to heterozygote TC genotype.

Statistics

Comparison of the frequency distribution of attributive characteristics between groups was performed by Pearson x^2 test or Fisher exact test. Comparison of the

Table 6. Characteristics of experimental and control group

Variable	*Control group CHOP*		*Experimental group R-CHOP*		*Test*
	N	*%*	*N*	*%*	
Sex					
male	7	50.00	17	29.82	x^2=2.04, n.s.
female	7	50.00	40	70.18	OR =2.35
Total	14	100.00	57	100.00	CI (0.62 – 9.07)
Age, years					
<60	6	42.86	26	45.61	x^2=0.03, n.s.
>60	8	57.14	31	54.39	OR =0.89
Total	14	100.00	57	100.00	CI (0. 24 – 3.35)
B-symptoms					
+	11	78.57	49	85.96	Fisher exact=0.44; n.s.
-	3	21.43	8	14.04	OR =0.60
Total	14	100.00	57	100.00	CI (0.11 – 3.41)
ECOG PS					
0	1	7.14	10	17.54	x^2=3.28, n.s.
1	6	42.86	25	43.86	
2	4	28.57	18	31.58	
3	2	14.29	3	5.26	
4	1	7.14	1	1.75	
Total	14	100.00	57	100.00	
Extranodal manifestations					
Yes	4	28.57	31	54.39	x^2=3.00, n.s.
No	10	71.43	26	45.61	
Total	14	100.00	57	100.00	
Clin. stage					
1	1	7.14	4	7.02	x^2=5.12, n.s.
2	3	21.43	25	43.86	
3	8	57.14	15	26.32	
4	2	14.29	13	22.81	
Total	14	100.00	57	100.00	
IPI					
1	1	7.14	5	8.77	x^2=8.41 p=0.038
2	4	28.57	26	45.61	
3	3	21.43	20	35.09	
4	6	42.86	6	10.53	
Total	14	100.00	57	100.00	

IPI: International prognostic index, n.s.: non significant, OR: odds ratio, SI: 95% confidence interval

Table 7. Overall survival (mean±SD) in relation to IPI

	Group	*IPI 1*	*IPI 2*	*IPI 3*	*IPI 4*
Mean±SD (Months)	CHOP	12.00	8.75±2.75	8.00±6.93	10.17±8.73
	RCHOP	20.00±13.04	25.54±13.82	21.53±13.09	28.83±13.36
Range (Months)	CHOP	12	6-12	4-16	2-24
	RCHOP	6-40	4-60	2-48	12-48
Mann-Whitney U		1,500	10,000	10,000	4,000
Z		-,594	-2,506	-1,775	-2,250
P		0.552	0.012	0.076	0.024

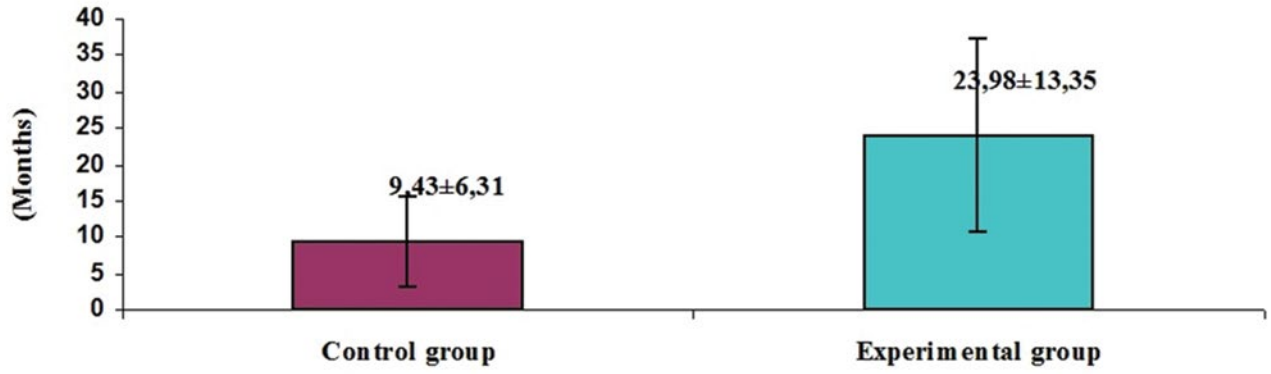

Figure 1. Overall survival (mean±SD, months) in the experimental and control group (p<0.0001).

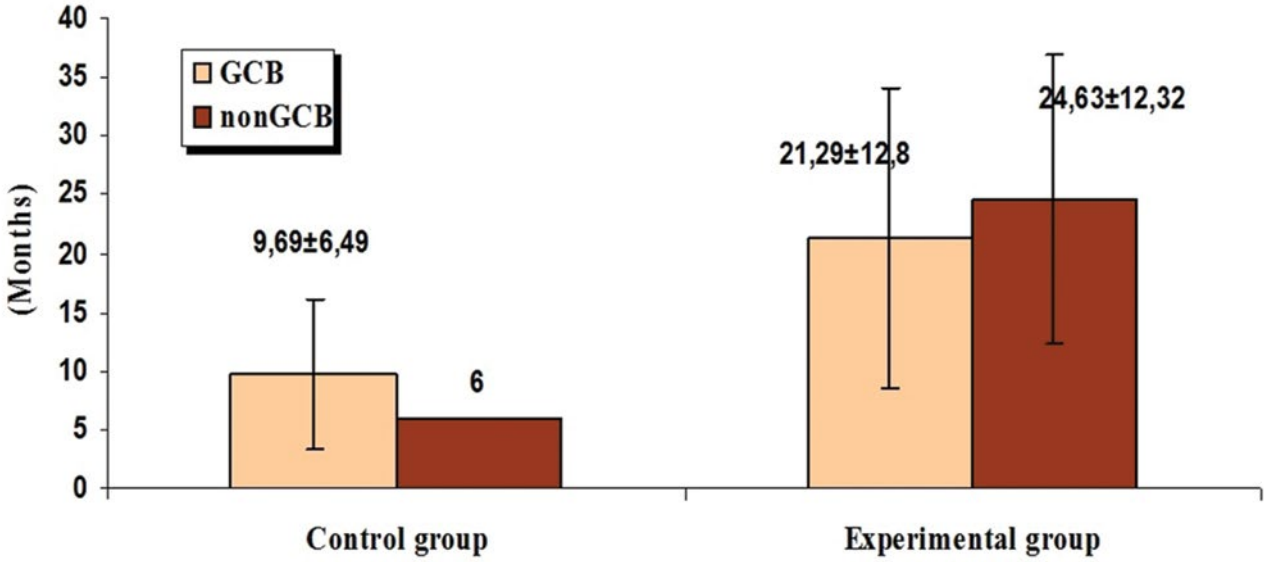

Figure 2. Overall survival (mean±SD, months) in the experimental and control group in relation to DLBCL subtype (p=0.040).

mean values of numerical characteristics between the two groups was performed using Mann-Whitney U test.

The relationship of certain characteristics was measured by correlation analysis. Predictor impact of certain markers on survival was determined by multivariate Cox regression analysis. The results were analyzed using the R computer program. P values <0.05 were considered statistically significant.

Results

No significant difference was found in relation to sex, age, ECOG performance status, clinical stage of disease, presence of "B" symptoms and extranodal manifestations between the two groups. According to IPI, significant difference between the groups was found. IPI 2 and IPI 3 were more frequently found in the R-CHOP group, IPI 4 was more common in the control group (x^2=8.41, p=0.038) (Table 6).

OS relating to the IPI 1-4 in groups was significantly longer in patients with IPI 2 (25.54 months, p=0.012) and IPI 4 (28.83 months, p=0.012) treated with R-CHOP (Table 7).

Distribution of modality evidence +/- of immunohistochemical markers showed significant difference in Bcl-2 protein expression (x^2, p=0.0079, OR =5.82, 95% CI 1.43– 24.87) and significant correlation (p=0.003) with R-CHOP. In this group Bcl-2 expression was dominantly positive in 73 (68%) patients and in the control group in 35.71%. MUM 1 expression (x^2, p=0.084, OR=6.50, 95% CI 0.75–145.57) was at borderline of significant correlation in the experimental group (p=0.056). Ki-67 expression >50% (x^2, p=0.08, OR=0.25, 95% CI 0.05–1.16) showed significant correlation with the control group (p=0.043). No significant correlation of the expression between groups was found in CD5 (p=0.564) and CD10 (p=0.097). No significant

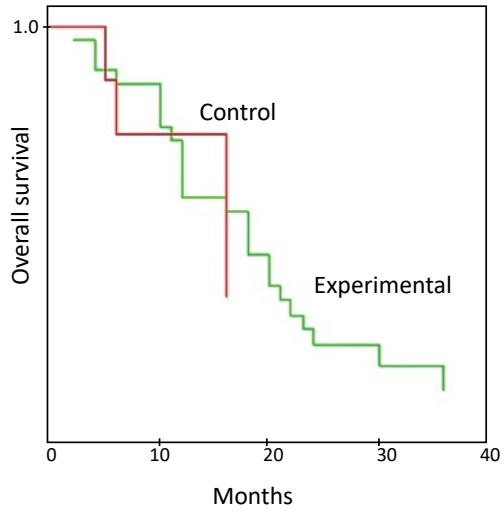

Figure 3. Predictive significance of Bcl-2 and MUM 1 expression on overall survival in the control and study group (p= 0.976; p= 0.961).

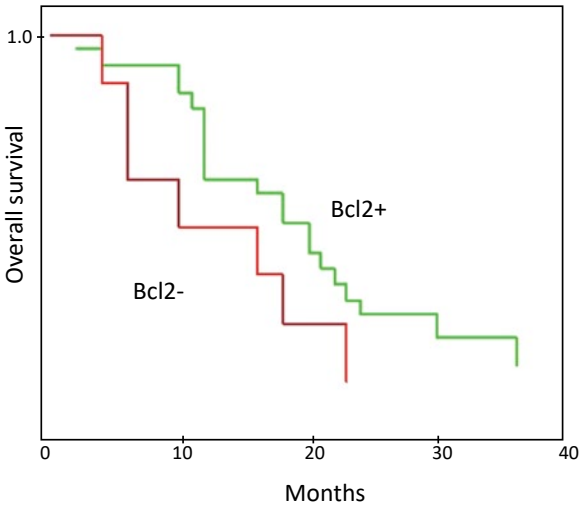

Figure 4. Overall survival in relation to Bcl-2 expression in the R-CHOP group (p=0.321).

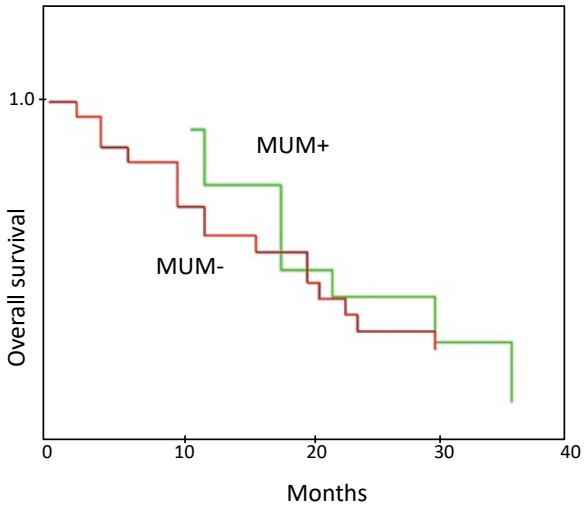

Figure 5. Overall survival in relation to MUM 1 expression in the R-CHOP group (p=0.664).

difference was found in the expression of Bcl-6 between the 2 groups (p=0.381). OS in the control group for Bcl-6+ patients was 12.6±8.71 months, and for Bcl-6 negative 7.66±4.12 months; in the R-CHOP OS for Bcl-6+ was 21.52±11.66 months, and for Bcl-6 negative 26.5±14.87 (p=0.373).

OS in the R-CHOP group was 23.98±13.35 months and in the control group 9.43±6.31 months (p=0.000, z=-3.933, Mann-Whitney U test). The difference in survival was highly significant (p<0.0001) (Figure 1).

According to the expression of CD10, Bcl-6 and MUM 1, patients were classified into GCB and non-GCB type of DLBCL. In the control group the OS of the GCB subtype was 9.69 months, while it was 6 months in the non-GCB subtype (p=0.571). In the R-CHOP group OS of GCB subtype was 21.29 months (p=0.571) and 24.69 months in the non-GCB subtype (p=0.708). OS was significantly different between the groups in relation to DLB subtype (z=2.056, p=0.040; Figure 2). Multivariate Cox regression analysis showed that Bcl-2 (Wald=0.3001, p=0.976) and MUM 1 (Wald=0.002, p=0.961) were nonsignificant predictors of OS in the groups (Figure 3). Ki-67 expression was also nonsignificant predictor of OS in both groups (Wald=0.198, p=0.656).

In the R-CHOP group no significant difference relating to survival was found between Bcl-2 + and Bcl-2 – (Z=0.992 p=0.321) (Figure 4), and between MUM 1 + and MUM 1 – (Z=0.435 p= 0.664) (Figure 5).

In multivariate Cox regression analysis Bcl-2 +/- and MUM1 +/- expressions were nonsignificant predictors of OS.

The investigation of gene polymorphism for MyD88 L265 P in the R-CHOP group included the analysis of restriction fragment length by means of which the genotype of the examined polymorphism was determined. The homozygous, normal wild-type genotype TT, represented by the presence of a 726bp fragment, was noted in all genomic DNA isolated from paraffin fixed tissue (Figure 6). There were no CC (448bp, 278bp) and TC (726bp,448bp 278bp) genotypes (Figure 7).

Discussion

The authors compared the prognostic factors between the R-CHOP and control group of patients from the same geographic area. They were homogeneous in terms of all clinical prognostic values except for the IPI value. There was a significant difference relating to the length of survival in patients with IPI 2 and IPI 4 in the R-CHOP group. The standard IPI in patients treated according to CHOP continued to have predictive value,

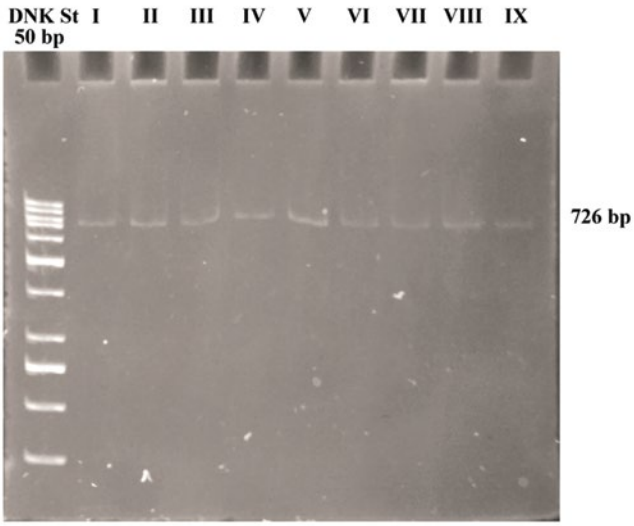

Figure 6. Polyacrylamide gel electrophoresis for MYD88 L265P polymorphism. Lane DNA St. 50 bp Ladder. Lane I represents unrestricted fragment, 1 band = 726 bp. Lane II shows homozygous (TT) GENOtyPE, II – IX Band= 726 bp. After restrictive digestion with BsiEI we didn't have any heterozygotes (TC) or polymorphic homozygotes (CC) among patients, but only wild type 726 bp size according to DNA Ladder.

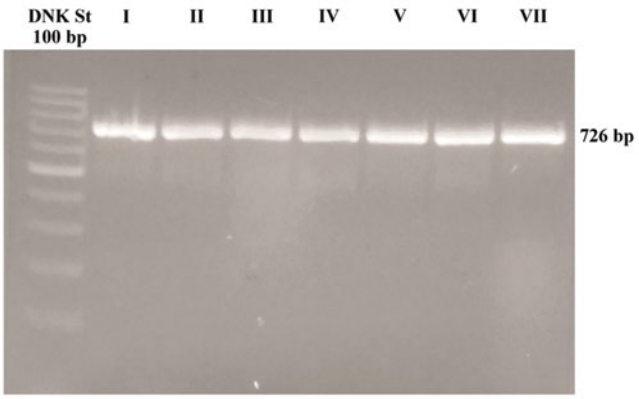

Figure 7. Agarose electrophoresis for testing PCR reaction for MYD88 L265P. Lane DNA St: 100bp Ladder. Lane I-VII PCR fragment 726 bp. Agarose gel electrophoresis of MYD88L265P fragments stained with ethidium bromide. After PCR, amplification was noted of 726 bp fragment with respect to DNA Ladder.

[9,10]. The role of Bcl-2 as prognostic factor was considered to be controversial by Igbal et al. [11]. In our study, too, the expression of Bcl-2 protein had no effect on the length of survival, although in the R-CHOP group this protein was detected significantly more often compared to the control group. In a study with a large number of patients by Sehn et al. the expression of Bcl-2 protein in patients treated with R-CHOP therapy showed no difference in survival [8]. Similar results were reported in other studies as well [12-15].

Bcl-6 protein was found to be a favorable prognostic factor in patients with DLBCL [8,13,16-21]. The results of our study showed no difference in OS between groups, compared to the expression of Bcl-6 +/-. Seki et al. [15] found that Bcl-6 protein was a significant predictor of prognosis, independent of IPI in the multivariate analysis in the R-CHOP group.

MUM 1 expression between the groups showed a borderline significant difference (p=0.056). Positive MUM 1 expression was marked as a predictor of poor outcome by several authors [22-24]. In our study, no significant difference was found between expression MUM 1 and OS between CHOP and R-CHOP groups. The same findings were confirmed by other authors as well [15,25].

Ki-67 is a nuclear protein and evaluation marker of proliferative activity of lymphoma. In the control group, expression of Ki-67 (cut off 50%) was significantly higher than in the R-CHOP group. In our study, Cox regression analysis showed that the presence of Ki-67 was not able to predict poor outcome. In a meta-analysis by He et al. [26] including 3902 patients, Ki-67 was characterized as a powerful predictor of adverse outcome in patients with DLBCL, but its importance was dependent on the type of DLBCLC and didn't correlate with other clinical-pathological prognostic factors or with IPI score. In the studies by Koh et al. [27] and Yoon et al. [28], high expression of Ki-67 (>85%) in patients treated with R-CHOP correlated with shorter OS. This result could be explained by the Ki-67 cut off value of 85%, which was significantly higher from the Ki-67 cut off value of 50% in our study, as well as by the small number of patients, especially in the control group.

The results of the present study showed longer OS in the GCB type of the control group and the non-GCB type of the R-CHOP group. In the literature non-GCB DLBCL was identified as subtype with adverse outcome [5,22,29,30]. The use of rituximab is changing the existing knowledge, as well as the prognosis of affected people [31], a fact that was confirmed in our study.

Myd 88-adaptor protein transmits signals from toll-like receptors and receptors for IL-1 and IL-18. Myd 88 L 265P mutation is the result of replacement of leucine with proline at the 265 position. Overexpression of Myd 88 and the presence of mutations in Myd 88 L 265P leads to chronic activation of the B cell receptor signaling system and the activation of NF-kB pathway [6,32,33].

In our experimental study on 57 genomic DNAs, no Myd 88 L 265P gene polymorphism was detected. In the study by Kraan et al. [34] 177 patients with DLBCL were analyzed and Myd 88 L 265P mutations were verified in 24.3% of them, mostly of non-GCB DLBCL. The highest incidence of mutations was found in the CNS material and testicles. The lowest incidence was found in the lymph nodes. Myd 88 L 265 mutation without Myd 88 expression was analyzed by Kraan et al. [34]. On a sample of 328 biopsies Ngo et al. [6] detected Myd 88 L 265P mutation in 29% of the cases. The difference in the results of our study could be partly explained by the larger number of patients included in their study. Choi et al. [35] in their research on 124 patients, detected high Myd 88 expression in 38.7% of the cases, mostly of non-GCB DLBCL, which led to reduction of disease-free survival (DFS). Myd 88 L 265 mutation rate was 6.5% (8/124). There were no significant differences in DFS in patients with 88 L Myd 265P mutation in comparison with the patients without this mutation. These results indicated that the expression of Myd 88 represented a predictive factor of poor outcome in patients with DLBCL, but it did not correlate with Myd 88L 265P mutation. Also, Myd 88 expression might have a great significance in the prediction of DLBCL progression, independent of Myd 88 L 265P mutation [35].

The significance of this study is that we standardized PCR-RFLP method for Myd 88 L265P for DNA samples isolated from paraffin blocks. No data are available so far related to studies on Myd 88 L 265P polymorphism in patients with DLBCL in Serbia.

In conclusion, addition of rituximab to CHOP standard protocol led to improvement of OS in patients with DLBCL and altered the character of previously recognized prognostic factors. We concluded that IPI score in the era of immunochemotherapy was unable to precisely identify newly diagnosed DLBCL patients with poor prognosis. In patients from Southeast Serbia the pathological signaling pathway achieved by Myd 88 L265 mutation was not responsible for the development of DLBCL. A better understanding of other proteins involved in Myd 88 signaling pathway in studies with larger numbers of patients would contribute to a more complete understanding of DLBCL pathogenesis and development of new prognostic indices, as well as to more effective therapeutic approach.

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Conflict of interests

The authors declare no confict of interests.

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