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RESEARCH ARTICLE

RECOGNIZING THE CONNECTION OF APOPTOTIC FACTORS IN SIGNALING PATHWAYS WITH DIFFUSE LARGE B CELL LYMPHOMA

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ABSTRACT

Background: The cell has developed mechanisms for maintaining hemostasis in defense from unfavorable external and internal influences. Apoptosis with its internal and external signaling pathways is one of those mechanisms. Failure of these mechanisms results in diseases of which the most serious are malignant diseases. Oncogenes which suppress apoptosis are bcl-2 and bclx1and c-myc, as opposed to bax, bad and bid which enhance apoptosis and which are encountered in lymphoma and which are incorporated into external and internal signaling pathway of apoptosis.
Aim: disclosure of connections between apoptotic factors in signaling pathways in diffuse large B cell lymphoma DLBCL in relation to achieving first complete remission (CR1).
Material and methods: Study was retrospective-prospective. 60 patients were analyzed with de novo DLBCL. Median was 47 months (3-91 months). Patients were divided into the following groups: germinal center B-cell like (GCB), not germinal center B-cell like (non GCB), CR1 and a group of unwanted occurrences (progressive disease, relapse and death). Groups are put in correlation to expressions CD10, CD138 i MUM1, bcl6, bcl2, bclxl, bad, bax, bid and Ki67.
Results: Positive correlation was confirmed for bad and bax in the overall sample, GCB **group** and CR1 group $p< 0.0001$. Bad that was included into external signaling had positive correlation with bcl6 $p=0.006$ and CD 10 $p=0.03$ in overall , GCB and CR 1 group. Negative correlation was confirmed for bclx1 and bid in non GCB group $p=0.022$. Positive correlation was found for MUM1 and bcl2 $p=0.004$ and negative correlation CD10 with MUM1 $p<0.0001$ and bcl2 $p=0.048$ in non GCB group.
Statistics: Spearman's analysis was used, $p<0.05$ was considered significant
Conclusion: We confirmed significant correlation for CD10, bcl6, bad and bax in GCB group. There was significantly negative correlation for bid and bclxl, together with revealing of insufficient inhibition of bid towards bclxl in both external and internal signaling pathway in non GCB group.

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INTRODUCTION

WHO Classification of Tumors of Hematopoietic and Lymphoid Tissues that was published in 2008 in Lyon (Stevan, 2008) recognized different morphological, molecular and Immunohistochemical subgroups and entities within DLBCL according to molecular, biological and clinical studies. Results of Rosenwald, Hans, Hummel et Colomo studies emphasize that cases with CD10 expression are considered to be germinal center B cell (GCB) type, as well as cases which have CD10-, bsl6+, MUM1- immunophenotype. All other cases are referred as non germinal center B cell (non-GCB) type (Rosenwald *et al*., 2002; Hans *et al*., 2004; Hummel *et al*., 2006; Colomo *et al*., 2003).

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Complex mechanisms of programmed cell death are determined by numerous oncogenes. Bcl-2 is oncogene that affects apoptosis, and when its expression is increased it blocks apoptosis. Nowadays, other proteins resembling bcl-2 are known and those are bcl-xl and c-myc, which suppress apoptosis and they are called anti-apoptotic factors. Factors that promote apoptosis, pro-apoptotic factors are: bax, bak, bad, bid and bim. Relation of activities of apoptotic and pro-apoptotic factors determines if and when the cell starts apoptosis. Relation of apoptotic and pro-apoptotic factors may also determine malignant cells' response to therapy. Programmed cell death- apoptosis is induced by signaling pathways. Two main signaling pathways, internal and external are triggers of apoptosis (Ghobrial *et al*., 2005; Jin, 2005). External pro-apoptotic signal is initiated when pro-apoptotic ligand binds to surface of pro-apototic receptor (Morange, 1994).

External signal can be triggered by toxins, hormones, growth factors and cytokines. External pathway is activated by surface tumor necrosis factor receptor (TNF) family and activates caspase-8. Bad is included in the external signaling pathway through the growth factor and Fas ligand. Internal pathway is initiated inside of the cell as a result of significant stress of the cell and DNA damage (Jin, 2005). Internal pathway includes release of intracellular pro-apoptotic proteins, which activates caspase-8, destroys essential cell proteins and stimulates fragmentation of chromosomal DNA resulting in death of the cell (Jin, 2005).

Apoptotic protein family regulates apoptosis by regulating mitochondrial permeability. Antiapoptotic proteins bcl-2 and bcl-xl are found in external mitochondrial wall where they inhibit release of citochrome-c. Proapoptotc proteins bad, bid, bax and bim are found in cytoplasm from where they transfer in mitochondria following lethal signal, where they help release of cytokine-c. Control of apoptosis in normal cell hemostasis je linked to external voltage-dependent mitochondrial anion channel (VDAC-2) that interacts with apoptotic proteins and maintains latent membrane potential and apoptotic effect under control. When lethal signal is received, products of activated cascade affect VDAC-2 through activation of proapoptotic signals. Bax and bid moves to mitochondria as response to lethal stimulation and in interreaction with bcl-xl and bcl-2 (which they inhibit) affect VDAC-2. Result of this is release of cytochrom-c. After release from mitochondria, cytochrom- c binds to Apaf-1 and forms activation complex with caspases (9,3) (Dejean *et al*., 2006). Direct activator of cell's death is caspase-3 (Ghobrial *et al*., 2005).

Internal and external pathways of apoptosis converge through activated caspase 3, and this results in activation of PARP (poli-ADP-ribose polymerase) and nucleosomal DNA fragmentation eventually leading to death of the cell.Cells with bcl-2 gene alteration live longer and accumulate (Ghobrial, 2005; Jin, 2005). Therapeutic approach in DLBCL includes application of an anthracycline-containing regimen, such as R-CHOP which is the cornerstone of first-line therapy. The intergroup Collaborative Trial in Relapsed Aggressive Lymphoma (CORAL) set the limits for this standard of treatment after first comparing 2 salvage regimens: rituximab, ifosfamide, etoposide, and carboplatin (R-ICE) and rituximab, dexamethasone, aracytine, and cisplatin (R-DHAP).

Salvage chemotherapy followed by high-dose therapy and autologous stem cell transplantation (ASCT) is the standard of treatment for chemosensitive relapses in diffuse large B-cell lymphoma. Patients with an non GCB subtype or c-MYC translocation responded poorly to the treatment. More than 70% of patients will not benefit from standard salvage therapy. Biological characteristics of the tumour dominantly define its behavior, prognosis and outcome of the disease. They can identify new target locations onto which new therapeutic options could be directed and for this continued research is needed (Christian Gisselbrecht, 2012). Future progress can be achieved from critical analysis and understanding of wide characteristic range of DLBCL. Analysis of specific clinical, immunohistochemical, oncogenic (bcl6, bcl2, bclxl, bax, bad, bid, c-mic) and molecular (XIAP, pAKT) characteristics can have significant pretreatment prognostic and predictive importance.

Aim: Disclosure of inter-relations between apoptotic factors in signaling pathways in diffuse large B cell lymphoma DLBCL in relation to achieving first complete remission (CR1).

MATERIALS AND METHODS

This was retrospective-prospective study. The patients are monitored for clinical characteristics to achieve first complete remission (CR1/CRu).

Selection and Description of Participants

60 patients were analyzed as de novo DLBCL Median was 47 months (minimum 3, maximum 91 months). Patients aged >18 years, de novo DLBCL, *naive* patients that were not previously treated, that were planned to be treated with 4+4 cycles of R-CHOP or were treated according to this protocol, with adequate biopsy material for analysis.

Non inclusion criteria

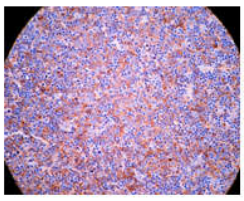
Patients with the following were not included in study: transformed low grade lymphomas into DLBCL, grey zone lymphomas, active hepatitis infection, and HIV infection. All patients provided a written informed consent before inclusion therapy. The protocol, amendments, and patient-informed consent documents were reviewed and approved by institutional review boards or ethics committees at participating study sites. The study was performed in compliance with good clinical practices and the principles of the Declaration of Helsinki.

Procedures/Technical Information

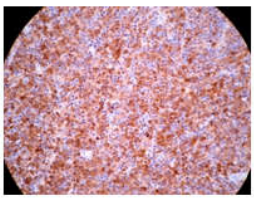
Biopsy material was analyzed to diagnose DLBCL based on imunohistologic staining using following markers: CD20, bcl-2, bcl-6, rarely CD10, Cyclin D1. Additional imunohistologic staining was performed at Institute of pathology and cytology in Clinical Center Sarajevo using the same biopsy material. Paraffin cubes were sliced and stained.
Slices were incubated with primary antibody including:

Anti-CD20 (1:150, clone L26, DakoCytomation, Glostrup, Denmark), *Anti-CD10* (1:150, clone 56C6, Novocastra Laboratories, Newcastle, Tyne, UK), *Anti-Bcl-6* (1:40, clone PG.B6p, DakoCytomation, Glostrup, Denmark), *Ki-67* (1:100, MIB1, DakoCytomation, Glostrup, Denmark), *Anti-Bcl-2* (1:20, clone bcl-2/100/D5, Novocastra Laboratories), *CD138* (dilution 1:10, clone AM 411-10 M, BioGenex,CA USA, IRF4/MUM1 (dilution 1:40, clone sc 6059, Santa Cruz Biotehnology, INC, CA, USA), Bcl-xl (dilution 1:25, clone 2H11, Zymed, Sauth San Francisco CA, USA), Bad (C-7): clone sc-8044, startin dilution 1:200 Santa Cruz CA, USA, Bax (dilution 1:40, code A 3533, Dako SA, Glostrup, Denmark) and Bid (FL-195): sc-11423, Santa Cruz startin dilution 1: 200, CA, USA. Visualization was performed using *EnVision® method* (DakoCytomation, Glostrup, Denmark) following manufacturers instruction. Adequate positive and negative controls were used. Expression of bcl2 was considered weak when it was <=30%. Expression of bcl-6 and CD10 was quantified using *histo-score* system, using method described by McCarty *et al*. Score system was adapted using 40x lens of microscope. Positive expression for MUM1 and CD138 proteins was considered when it was positive in more than 25% of malignant cells.

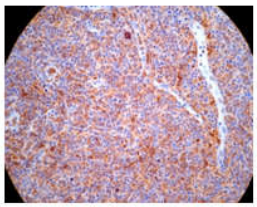
BadX40, ZEISS Scope A1



Bax X40, ZEISS Scope A1



Bid X40, ZEISS Scope A1



Bcl xl X40, ZEISS Scope A1

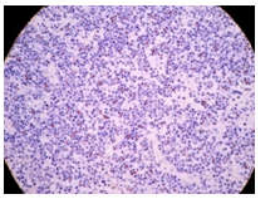


Table1.Patients' clinical characteristics in relation to CR1and overall three-year survival

Characteristics		n=60	CR1 n 47=, %	sig	OS % n=44	sig.
Age	Younger than 60 y.	50 (83,3%)	40 (80%)	NS	37 (74,0%)	NS
	Older than 60 y.	10 (16,7 %)	7 (70%)		7 (70,0%)	
B symptoms	No	14 (23,3%)	12 (85,7%)	NS	12 (85,7)	NS
	Yes	46 (76,7 5)	35 (76%)		32 (69,6)	
Performance status	ECOG 0,1,2/low	53 (88,4%)	44 (93,7%)	p=0,008	41 (93,2%)	p=0,05
	ECOG 3,4/high	7 (11,6%)	3 (6,3%)		3 (6,8%)	
Ann Anbor stage	I/II	26 (43,3%)	25 (96,15%)	0,012	25 (96,2)	p<0,0001
	III/IV	34 (56,7%)	22 (64,7%)		19 (55,9)	
LDH-P	High	22 (63,3%)	12 (25,5%)	p=0,003	33 (75%)	p=0,002
	Normal	38 (36,7%)	35 (74,5%)		11 (25%)	
International Prognostic Index IPI	Low risk + Low/intermediate	39 (65%)	36 (76,6%)	p=0,014	35 (79,5%)	p<0,0001
	High/intermediate + High risk	21 (35%)	11 (23,4%)		9 (20,5%)	

Table 2. Correlation with Bcl-xl

BCL-XL								
		Total	A	B	CR	F	Ki67<50	Ki67≥50
Bax%	Spearman's rho	,101	,237	-,063	,109	,217	,264	-,075
	p	,443	,146	,785	,464	,476	,151	,700
	N	60	39	21	47	13	31	29
Bad%	Spearman's rho	,046	,192	-,170	,126	-,196	,075	,010
	p	,728	,241	,460	,400	,521	,687	,960
	N	60	39	21	47	13	31	29
Ki67%	Spearman's rho	,102	-,067	,205	-,110	,585(*)	,047	,358
	p	,436	,687	,372	,460	,036	,801	,057
	N	60	39	21	47	13	31	29
Bid%	Spearman's rho	-,010	,168	-,495(*)	,116	-,515	-,163	,133
	p	,942	,307	,022	,437	,072	,380	,490
	N	60	39	21	47	13	31	29
Starosna dob	Spearman's rho	-,119	-,053	-,234	,005	-,550	,050	-,277
	p	,366	,748	,307	,976	,051	,791	,145
	N	60	39	21	47	13	31	29
Trajanje CR1	Spearman's rho	-,319(*)	-,046	-,474(*)	-,098	.	-,091	-,565(**)
	p	,013	,780	,030	,510	.	,625	,001
	N	60	39	21	47	13	31	29
bcl-6%	Spearman's rho	,135	,177	,055	,260	-,002	,240	,060
	p	,303	,282	,811	,077	,996	,193	,758
	N	60	39	21	47	13	31	29
bcl-2%	Spearman's rho	-,175	-,132	-,186	-,130	.	-,222	-,147
	p	,180	,423	,420	,385	.	,231	,448
	N	60	39	21	47	13	31	29
CD 10%	Spearman's rho	-,026	,198	.	,086	-,188	,104	-,176
	p	,847	,227	.	,564	,539	,578	,360
	N	60	39	21	47	13	31	29
CD 138%	Spearman's rho	-,009	,101	-,246	,065	.	,273	-,263
	p	,945	,542	,282	,662	.	,137	,168
	N	60	39	21	47	13	31	29
Mum1%	Spearman's rho	,180	-,175	,147	-,018	,298	,151	,231
	p	,169	,287	,526	,903	,322	,417	,228
	N	60	39	21	47	13	31	29

Explanation of groups: (group A = GCB, group B = non-GCB, group CR = group with achieved complete remission, group F = group with unwanted occurrences, group Ki65<50% = group with proliferative index lesser than 50%, group Ki65%>50% = group with proliferative index greater and equal to 50%).

Table 3. Correlation with IPI

IPI								
		Total	A	B	CR	F	Ki67<50	Ki67≥50
bcl-6%	Spearman's rho	-,006	,183	-,350	,086	-,103	,109	-,114
	p	,963	,266	,120	,566	,738	,558	,554
	N	60	39	21	47	13	31	29
bcl-2%	Spearman's rho	-,082	,031	-,235	,017	.	,031	-,198
	p	,532	,850	,306	,909	.	,868	,303
	N	60	39	21	47	13	31	29
CD10%	Spearman's rho	-,115	,047	.	-,074	,298	,036	-,267
	p	,382	,778	.	,621	,323	,849	,162
	N	60	39	21	47	13	31	29
CD138%	Spearman's rho	-,158	-,170	-,266	-,090	.	-,209	-,133
	p	,228	,302	,244	,547	.	,258	,491
	N	60	39	21	47	13	31	29
MUM1%	Spearman's rho	,210	-,060	-,056	,128	-,421	,106	,278
	p	,107	,716	,810	,393	,152	,569	,144
	N	60	39	21	47	13	31	29
bcl-xl%	Spearman's rho	,261(*)	,178	,290	,063	,179	,135	,394(*)
	p	,044	,279	,201	,676	,558	,470	,035
	N	60	39	21	47	13	31	29
Bax%	Spearman's rho	-,012	,009	-,063	,043	-,090	,055	-,074
	p	,929	,956	,785	,777	,771	,770	,702
	N	60	39	21	47	13	31	29
Bad%	Spearman's rho	-,117	-,074	-,168	-,098	-,181	-,096	-,121
	p	,373	,654	,467	,514	,553	,609	,532
	N	60	39	21	47	13	31	29
Ki67%	Spearman's rho	,185	,112	,181	,109	,684(**)	,183	,109
	p	,158	,496	,431	,464	,010	,324	,573
	N	60	39	21	47	13	31	29
Bid%	Spearman's rho	-,020	,033	-,309	-,081	,030	-,112	,041
	p	,877	,844	,173	,590	,921	,549	,833
	N	60	39	21	47	13	31	29
Age	Spearman's rho	,108	,175	,047	,098	,147	,310	-,008
	p	,411	,287	,839	,511	,632	,089	,966
	N	60	39	21	47	13	31	29
Duration CR1	Spearman's rho	-,434(**)	-,319(*)	-,417	-,187	.	-,449(*)	-,470(*)
	p	,001	,048	,060	,208	.	,011	,010
	N	60	39	21	47	13	31	29

Table 4.Correlation with Bax

BAX								
		Total	A	B	CR	F	Ki67<50	Ki67≥50
Bad%	Spearman's rho	,507(**)	,576(**)	,368	,511(**)	,496	,631(**)	,360
	p	,000	,000	,101	,000	,085	,000	,055
	N	60	39	21	47	13	31	29
Ki67%	Spearman's rho	-,072	-,041	-,169	-,025	-,205	,114	-,272
	p	,587	,806	,464	,867	,501	,543	,153
	N	60	39	21	47	13	31	29
Bid%	Spearman's rho	,069	,121	-,064	,082	,027	,154	-,009
	p	,600	,462	,784	,583	,930	,409	,963
	N	60	39	21	47	13	31	29
Age	Spearman's rho	,019	,073	-,114	-,004	,116	-,064	,066
	p	,888	,660	,624	,980	,705	,731	,733
	N	60	39	21	47	13	31	29
Duration CR1	Spearman's rho	,074	,145	-,053	,064	.	,034	,121
	p	,575	,377	,818	,669	.	,854	,531
	N	60	39	21	47	13	31	29
bcl-6%	Spearman's rho	,134	,202	-,011	,142	,089	,087	,182
	p	,309	,218	,962	,342	,773	,643	,345
	N	60	39	21	47	13	31	29
bcl-2%	Spearman's rho	,021	,087	-,170	,014	.	-,252	,271
	p	,871	,600	,462	,924	.	,172	,154
	N	60	39	21	47	13	31	29
CD10%	Spearman's rho	,080	,190	.	,160	-,356	,357(*)	-,260
	p	,543	,246	.	,283	,233	,048	,173
	N	60	39	21	47	13	31	29
CD138%	Spearman's rho	,176	,430(**)	-,138	,192	.	,203	,170
	p	,179	,006	,551	,196	.	,274	,378
	N	60	39	21	47	13	31	29
MUM1%	Spearman's rho	,024	,030	-,042	-,046	,583(*)	-,168	,184
	p	,856	,856	,858	,757	,036	,366	,340
	N	60	39	21	47	13	31	29
bcl-xl%	Spearman's rho	,101	,237	-,063	,109	,217	,264	-,075
	p	,443	,146	,785	,464	,476	,151	,700
	N	60	39	21	47	13	31	29

Positive expression for bcl-xl, bax, bad and bid proteins was considered when there were more than 9% of positive malignant cells. ZEISS Scope A1 microscope was used.

Randomisation

The patients were divided into: A-GCB and B-non GCB group, group with first complete remission (CR1), F-group with unwanted occurrences **(**partial remission, progressive disease, early relaps and death); then the groups were placed in correlation according to Prognostic Index Score (0,1,2 vs >2) and expression CD10, CD138 i MUM1and oncogene expression bcl2, bclxl, bcl6, bax,bad, and bid, whose expressions were defined on bioptic material taken during the process of diagnosing the disease.

The patients with chemosensitive relapse were given High dosis therapy – HDT, followed with autologous stem cell transplantation (ASCT).

Statistical Analysis

The results are shown in tables and charts. Nonparametric analysis of correlation coefficient according to *Spearman and Pearson* was used. P values < 0.05 were accepted as significant.

RESULTS

The study included 60 patients with de novo diffuse large B cell lymphoma (DLBCL).

Table 5. Response to the therapy in first line: groups GBC vs non GBC

Response to the therapy	Immunohistochemical groups		Sig.
	A-GCB (n=39)	B-non-GCB (n=21)	
CR1	36 (92,3%)	11 (52,4%)	P = 0,001
PR	3 (7,7%)	5 (23,8%)	N.S.
ORR	39 (100%)	16 (76,2%)	
PB	0 (0%)	5 (23,8%)	p = 0,01
Death during monitoring	3 (7,7%)	13 (61,9%)	p < 0,0001

Table 5.Correlation with Bad

BAD								
		Total	A	B	CR	F	Ki67<50	Ki67≥50
Ki67%	Spearman's rho	-,118	-,048	-,174	-,025	-,432	,082	-,180
	p	,371	,771	,450	,870	,140	,663	,349
	N	60	39	21	47	13	31	29
Bid%	Spearman's rho	,239	,313	,128	,220	,331	,194	,310
	p	,066	,052	,580	,137	,269	,295	,102
	N	60	39	21	47	13	31	29
Age	Spearman's rho	,086	,198	-,160	,098	,079	-,153	,281
	p	,514	,227	,488	,514	,797	,412	,140
	N	60	39	21	47	13	31	29
Duration of CR1	Spearman's rho	-,066	-,099	-,027	-,144	.	-,163	,073
	p	,614	,550	,907	,335	.	,382	,706
	N	60	39	21	47	13	31	29
bcl-6%	Spearman's rho	,348(**)	,304	,429	,347(*)	,443	,248	,467(*)
	p	,006	,060	,052	,017	,129	,178	,011
	N	60	39	21	47	13	31	29
bcl-2%	Spearman's rho	-,087	-,116	-,039	-,108	.	-,230	,070
	p	,510	,483	,865	,471	.	,214	,718
	N	60	39	21	47	13	31	29
CD10%	Spearman's rho	,279(*)	,395(*)	.	,322(*)	,130	,441(*)	,064
	p	,031	,013	.	,027	,671	,013	,742
	N	60	39	21	47	13	31	29
CD138%	Spearman's rho	,166	,355(*)	-,021	,183	.	,096	,269
	p	,204	,026	,927	,217	.	,609	,159
	N	60	39	21	47	13	31	29
MUM1%	Spearman's rho	-,110	-,197	,014	-,115	-,001	-,287	,087
	p	,405	,229	,953	,440	,996	,117	,655
	N	60	39	21	47	13	31	29
bcl-xl%	Spearman's rho	,046	,192	-,170	,126	-,196	,075	,010
	p	,728	,241	,460	,400	,521	,687	,960
	N	60	39	21	47	13	31	29
Bax%	Spearman's rho	,507(**)	,576(**)	,368	,511(**)	,496	,631(**)	,360
	p	,000	,000	,101	,000	,085	,000	,055
	N	60	39	21	47	13	31	29

This was homogeneous group of patients, regarding therapy**.** First line therapy was R-CHOP protocol, (rituximab day 1. 375mg/m^2 iv + CHOP/ day 1. cyclophosphamid 750 mg/m^2 iv, doxorubicin 50 mg/m^2 iv, vincristin max. 2 mg/ iv, day 1-5. Prednizone 100 mg p.o.). **(1)** Radiotherapy was applied when there was bulky disease, extranodal presentation and on residual disease. Response to therapy was determined according to conventional criteria (normalization of metabolic results and absence of tumor mass). Second line therapy was conventional chemotherapy DHAP (dexamethasone, cytarabine, and cisplatin).

Median was 18-72 with median age 45 years. Median of monitoring was 47 months. We analyzed 31 (51.7%) males, 29 (48.3%) were women. During the period of examination, in 60 patients who were treated by immunochemotherapy and who had DLBCL, complete remission 47(78,3%), PR-partial remission 8(13,3%), the overall response (CR+PR) 55 (91,5%) was achieved. Influence of International Prognostic Index (IPI) in relation to achieving first complete remission (CR1) was examined and considerable influence at the level of significance p<0,0001was confirmed, with confirmation of significant influence of individual risk factors:

ECOG>2 p=0,008, clinical stage (I vs II vs III vs IV) p=0,012 as (I/II va III/IV) p< 0,0001 and level LDH p=0,014 X^2. Significant influence IPI>2 in relation to three-year survival was confirmed p<0.0001, ECOG p=0,05, clinical stage I/II vs III/IV) p<0,0001 and level LDH p=0,002 X^2. Better response was confirmed in relation to achieving CR1 in immunohistochemical group GBC at the level of significance X^2 p=0,001. Expression bcl-xl which acts in anti-apoptotic manner is in positive correlation with expression Ki67 in failure group p=0,036. Expression bcl-xl is in negative correlation in relation to CR1 duration and in overall group p=0,013, group B (non-GBC) p=0,03 and group with expression Ki67 >50% p=0,001. Significant positive correlation in the overall group (IPI >2 vs Ki67≥50% p=0,035) was found. Significant positive correlation in the overall group (IPI>2 vs expression bcl-xl p=0,044) was found. Significantly negative correlation was found in relation to duration of CR1 (IPI>2 vs CR1 p=0,001) in the overall group, in group GCB p=0,048 and group non GCB insignificant p=0,06. IPI>2 in insignificantly positive correlation in group GCB and insignificant in negative correlation in non GCB group in relation to bcl-6, bcl-2, bax, bad i bid. These results suggest that high IPI >2 influence on poor sensibility to existing treatments of immunochemotherapy in first line of DLBCL. Duration of CR1 is significantly shorter if IPI>2 in groups (in overall group p=0,001,in group GCB p=0,48, group Ki67<50% p=0,011 i Ki67≥50% p=0,010). IPI >2 is in significant positive correlation regardless of expression Ki67 in failure group p=0,01.

Expression bax has positive correlation with expression bad in the overall group and GCB group at the significance level p<0,0001, in relation to group CR1 p<0,0001 and group with Ki67 less than 50% p<0,0001. Expression bax has positive correlation with expression CD10 in group Ki67 less than 50% p=0,048. Expression bax has positive correlation in relation to expression MUM1 in group with unwanted occurrence at the significance level p=0,036 Expression bad has positive correlation with bcl-6 p=0,006, CD10 p=0,031 and bax p<0,0001 in the overall group, group A (GCB) according to bax p<0,0001 i CD10 p=0,013. Expression bad has positive correlation in the group in achieving CR1 according to bcl-6 p=0,017 according to CD10 p=0,027 and according to bax p<0,0001. Expression bad has positive correlation in group Ki67 less than 50% according to bax p<0,0001 i CD10 p=0,013 and in the group Ki67 greater than 50% in relation to bcl-6 p=0,011. Pro-apoptotic synchronized influence of expressions: bcl-6 , CD10, bad and bax was found.

DISCUSSION

The primary aim of this study was to analyze of apoptotic factors in 60 patients with diffuse large B cell lymphoma (DLBCL) treated with immunochemotherapy as first-line therapy aimed at disclosing their connection in signaling pathways and significance on influence in achieving first Complete Remission (CR1). The results of this study have confirmed a better response in relation to achieving CR1 in immunohistochemical group germinal center B cell (GBC) at the significance level X^2 p=0,001. At the high significant level p<0,0001 it was proved that bad and bax are in a positive correlation in the group GCB, the group in which greater CR1 is achieved and in which CR1 lasts longer. In the same group, in the significant positive correlation are also bax with bcl-6 p=0,01 and bax with CD10 p=0,027.

Bad had positive correlation with bax p<0,0001, bcl-6 p=0,006 i CD10 p=0,03 in the overall sample and group GCB, as well as in the group with CR1. These results confirm connection between bad and bax (anti-apoptotic proteins) in external and internal pathways, and which are more intensively epressed in the GBC group, for which we confirmed better sensitivity to existing application of immunochemotherapy. Significant positive correlation was found: CD10 and bcl-6 with pro-apoptotic oncogenes bad and bax as hidden predictors of good sensibility in immunochemotherapy use in first-line treatment of DLBCL. High expression of MUM1 is main characteristic of non germinal center B cell (non-GCB) of immunophenotypic profile in which is proved a weak sensibility to existing use of immunochemotherapy in first-line treatment of DLBCL. In this study, a positive correlation of MUM1 with anti-apoptotic oncogene bcl 2 p=0,004 and negative correlation CD10 with MUM1 p<0,0001 i bcl2 p=0,048 were confirmed. Negative expressions of MUM1 with expressions of pro-apoptotic oncogenes (bax, bad and bid) were found, but not at a significant level, as well as positive correlation of MUM1 expression with bcl-xl (joint influence of anti-apoptotic oncogenes), also at insignificant level, which does not completely correlate with the results which Bai M[11] published in his study. In the study it was mentioned that expression MUM1 shows significantly negative correlation with pro-apoptotic oncogenes bax, bad and bid expression. If we refer to the results of the study Bai M., then the results of this study are significant.

Expression Ki67 has positive correlation with bcl-xl in the failure group with unwanted occurrences at the level of significance p=0,036. Duration of CR1 is in significantly negative correlation with Ki67≥50% at the level of significance p=0,017, which is confirmed by strong joint influence of expression bcl-xl i Ki67 greater than 50%, as a predictor of failure in the existing treatment approach in first-line therapy of DLBCL. It was confirmed, at high significance level p<0,0001, that the level of expression Ki67<50% and bad, which are included in external signaling pathways, are in positive correlation with bax which is included in internal signaling pathway in the GCB group, the group that achieves higher CR1 and longer duration of CR1. Within the same group, in significantly positive correlation are also bax with bcl-6 p=0,01 and bax with CD10 p=0,027. Therefore, in the GCB group with a better response to immunotherapy was recognized greater influence of pro-apoptotic oncogenes, whose influence is in external and internal signaling pathway.

The relation of external signaling pathway through bad as well as its relation with internal signaling pathway through bax was observed. Bad is activated in external signaling pathway in which growth factor is included. Human epidermal growth factor binds with the surface of cell receptor and significantly regulates normal cell's proliferation and survival (Bai *et al*., 2004). Dysregulation includes increased expression of growth factor receptors. Human epidermal growth factor receptor (HER) is one of the fundamental elements that contributes to the growth and progression of many solid tumors (Hanahan, 2000; Spivak-Kroizman *et al*., 1992; Hynes, 1994; Linggi, 2006). In the non-GCB group a significantly negative correlation bid with anti-apoptotic oncogene bcl-xl p=0,02 was found. In external and internal signaling pathway of apoptosis, when transcriptional bid is located beside mitochondria, it blocks bcl-xl. In our study, patient group with immunophenotype non-GCB had significantly poorer

response. It can be assumed that in our patient group non-GCB group the external and internal pathway through bid is insufficiently activated (or it is related to resistance to apoptosis), which as a consequence has decreased sensibility to the therapy. The referred results mark places for targeted therapeutic possibilities, potentially new therapeutic options, in acting upon mediators of signaling pathways. Advancement of therapeutic options through existing and new antibodies can improve sensibility to the therapy and initiate apoptosis through external signaling pathway.

Conclusion

- In this study, we found significantly positive correlation CD10 and bcl-6 with pro-apoptotic oncogenes bad and bax as hidden predictors of good sensibility in the use of immunochemotherapy in first-line treatment of DLBCL.
- A positive correlation of MUM1 and bcl 2 was confirmed as well as connection of MUM1 with anti-apoptotic oncogenes in the connection that decreases sensibility of immunochemotherapy.
- Significantly negative correlation of expression of bcl-xl and expression bid was found in the non-GCB group which has a poor response to immunochemotherapy, which is in support of the finding that in external and internal signaling pathway bid inhibits bcl-xl, but insufficiently in the non GCB group.

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