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ORIGINAL ARTICLE

Prognostic Significance of Beclin 1 Expression in Diffuse Large B Cell Lymphoma Patients Receiving Immuno-chemotherapy at Zagazig University and Health Insurance Hospitals

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ABSTRACT

Background: Diffuse large B-cell lymphoma (DLBCL); the commonest subtype of NHL, is genetically, biologically and clinically heterogeneous disorder which is potentially curable with combination chemo-immunotherapy, however, Prognostic assessment is important for tailoring therapy. Beclin-1, a mammalian ortholog of the yeast autophagy-related gene 6 protein, and important mediator of autophagy was found to predict clinical outcomes in many cancer patients.

Methods: This prospective cohort study was carried out at medical oncology department, Zagazig University and Health insurance hospitals and included 32 patients with CD20 positive de novo DLBCL, they were subjected to routine clinical and laboratory assessment with immunohistochemical analysis for beclin-1 status which further divided the patients into 2 groups of high and low beclin-1,Patients received first line therapy with R-CHOP regimen, then assessed for therapy response and followed up after treatment for estimating overall (OS) and disease free survival (DFS).

Results: High Beclin-1 expression was found in 12 patients (37.5%) and it wasn't associated with significant correlation with clinic-demographic patient characteristics. The Complete remission rate was 59.4% and beclin-1 expression didn't significantly affect clinical outcome, except for the significant death rate (p= 0.02). The 3-year OS and DFS were 78.1% and 45.0% respectively and high beclin-1 was significantly associated with better OS (by multivariate analysis) but not DFS.

Conclusions: Beclin-1 didn't provide a prognostic indicator for response to treatment. However, it's found to be independent predictor for OS in DLBCL patients.

Keywords: DLBCL; Prognostic; Autophagy; Beclin-1.

INTRODUCTION

Non-Hodgkin's lymphomas (NHL) are variable group of lymphoproliferative disorders arising from B-lymphocytes, T-lymphocytes or natural killer cells and are considered the 7th most common cancer among men and women, representing 4% of new cancer cases and 3% of cancer-related deaths [1]. Diffuse large B-cell lymphoma (DLBCL) accounts for about one-third of all cases of adult non- Hodgkin's lymphoma and is characterized by a wide heterogeneity in tumor type, genetic abnormality, clinical features, response to therapy, and outcome [2], DLBCL is an aggressive, but potentially curable malignancy and combination chemo-immunotherapy regimens can cause durable remissions in more than two thirds of patients [3].

Patients with relapsed or refractory disease had very bad outcome and novel biomarkers that can stratify patients to be targeted with

alternate agents are needed [4]. Autophagy is a homeostatic and catabolic process whereby damaged proteins and organelles are gathered within autophagosomes and then lysed by combining with lysosomes to maintain cellular metabolism and stabilization [5].

Stress in tumor cells and tumor microenvironment, originating from high metabolic demands, inadequate nutrient and oxygen supply, cytotoxic drugs and other similar stressful states, is a potent stimulator of autophagy [6]. Several studies have verified that autophagy actively regulates cancer formation. Nevertheless, the role of autophagy is far more complex than expected as it either positively or negatively regulates carcinogenesis [7]. Interest in the clinical importance of autophagic state in different types of malignancies is increasing, with particular focus on the prognostic value of evaluating autophagy-related markers in different malignancies [8]. Beclin-1, a mammalian ortholog of the yeast autophagy-related gene 6 (Atg6) protein, is an important mediator of autophagy [9]. Beclin-1 is deactivated by binding to B cell leukemia 2 (BCL2) and stimulated by its release from BCL2 either by phosphorylation of BECN1 or of BCL2 [10]. Free Beclin-1 is an inducer of autophagy and so widely used as a marker of monitoring the onset of autophagy [11]. It was confirmed to have prognostic impact in patients with various solid malignancies, including cancers of the gastrointestinal tract, liver, breast and ovary [12].

Previous studies demonstrated that high expression of beclin-1 was related to good outcome in colon cancer and bad outcome in nasopharyngeal carcinoma [13].

In this study, we conducted a prospective cohort study to investigate and determine the prognostic values of beclin-1in DLBCL patients.

METHODS

We conducted a prospective, cohort study including 32 cases between 18 and 64 years of age with Pathologically confirmed CD20 positive De novo DLBCL who had presented to medical oncology department, Zagazig University and Health insurance hospitals between July 2015 to July 2018. Patients with Age less than 18 years, Primary central nervous system lymphoma, Primary Mediastinal Large B cell lymphoma, Prior chemotherapy and/or radiotherapy, Previous malignancy or second primary tumor and Severe coincident diseases were excluded from the study. Well informed consent was obtained.

All patients were subjected to complete clinical history (with special considerations for age, sex, Co-morbidities , medications and presence of B symptoms) and physical examination (with special consideration of organomegly, lymphadenopathy and ECOG PS), routine laboratory investigations including: Complete blood counts, Serum LDH, Liver, Kidney function tests, electrolytes, virology studies (HCV Ab, HBsAg, HBcoreAb and HIV Ab), histopathological examination & immunophenotyping of paraffin embedded blocks, Bilateral iliac crest bone marrow biopsy which might not be needed if PET/CT scan was done and was clearly positive, routine radiology including: Whole-body PET/CT scan ± CT Chest/Abdomen/Pelvis with contrast and Echocardiography.

Detection of beclin-1 and BCL2 expression was done by immunohistochemistry.

They were further classified according to Beclin-1 status into 2 groups of high and low beclin-1.

Lugano Modification of Ann Arbor Staging System [14], and NCCN IPI [15] were used in this study.

All patients received six to eight cycles of R-CHOP therapy repeated at 21- day intervals [16].

Tumor response was evaluated after the fourth and the final cycle of chemotherapy according to Lugano Response criteria for NHL [14].

Patients were followed after end of the treatment with median follow up period of 23 months and the following was done: Physical examination, complete blood picture, serum LDH was performed every 3 months and computed tomography scan was performed every 6 months.

Statistical Analysis

The collected data were computerized and statistically analyzed using SPSS program version 24.

Data were tested for normal distribution using the Shapiro Walk test. Qualitative data were represented as frequencies and relative percentages. Chi square test ($\chi 2$) and Fisher exact was used to calculate difference between qualitative variables as indicated. Qualitative data compared by independent t test. All statistical comparisons were two tailed with significance Level of P-value ≤ 0.05 indicates significant. Survival analysis: Kaplan and Meier method used to estimate overall and disease free survival and log rank test compared survival curves (P value was considered significant at ≤ 0.05 levels). Cox proportional hazards regression models summarized with hazard ratios and 95% confidence intervals (CIs) was used for multivariate analysis.

RESULTS

Our study included 32 patients with CD2O positive DLBCL, 20 (62.5%) patients were male while female patients were 12 representing 37.5% & their age ranged from 18 to 64 years with mean age 44±10, as shown with other clinic-demographic data in table (1).

Beclin-1 expression was found to be high in 12 patients (37.5%) and low in 20 patients (62.5%), as shown in figures (1).

No statically significant difference was detected between both patient groups as regard Clinico-laboratory data.

As regards response to treatment, 59.4% of all patients achieved CR after 6 to 8 cycles of R-CHOP and there was no statically significant difference between both patient groups as regard response to treatment, with significantly better death rate in high beclin 1 group (p= 0.02), as in Table (2).

Beclin-1 expression wasn't correlated with BCL2 expression (P-Value = 0.098), as shown in (Table 1).

After median follow up period of 23 months (with range 4.2-32months), 7 (21.9%) out of 32 patients died with mean survival time 31.2 ± 1.5 at 95% confidence interval (28.1-34.3) while median wasn't reached. The 3-year overall survival rate was 78.1%.

By the end of follow up period 9 (37.5%) out of the 24 patients who were responder developed progressive disease with mean survival time 29.1 ± 1.1 at 95% confidence interval (26.7-31.4) while median was not reached. The 3-year disease free survival (DFS) was 45%, as in Table (3).

Patients with high beclin-1 expression had significantly higher OS as compared with low beclin-1 expression patients (P-value = 0.025), as shown in Figure (2).

Patients with Positive BCL-2 expression had significantly lower OS as compared with Negative BCL-2 expression patients (P-value = 0.032).

There was no statistically significant difference between Disease free survival in both patient groups, as shown in Figure (3).

Patients with positive BCL-2 expression had significantly lower DFS as compared with negative BCL-2 patients (P-value =0.016).

According to multivariate Cox regression analysis, high beclin 1 expression, good PS and extranodal involvement were found to be independent predictors of longer OS. Whereas, Low NCCN IPI score was an independent predictor of longer DFS, as in Tables (4&5).

Table 1: Characteristics of the studied population

		Beclin 1 High N=12		Low N=20		Total N=32		P-Value
Age (Mean ± SD)		43±11		45±10		44±10		0.8
Sex	Female	6	50.0%	6	30.0%	12	37.5%	0.258
	Male	6	50.0%	14	70.0%	20	62.5%	
PS	<2	6	50.0%	14	70.0%	20	62.5%	0.258
	=>2	6	50.0%	6	30.0%	12	37.5%	
Stage	<III	3	25.0%	3	15.0%	6	18.8%	0.483

		Beclin 1 High N=12		Low N=20		Total N=32		P-Value
	Stage III/IV	9	75.0%	17	85.0%	26	81.3%	
NCCN-IPI	<2	2	16.7%	2	10.0%	4	12.5%	0.581
	=>2	10	83.3%	18	90.0%	28	87.5%	
LDH	Normal	5	41.7%	8	40.0%	13	40.6%	0.926
	High	7	58.3%	12	60.0%	19	59.4%	
Extranodal disease	Negative	6	50.0%	9	45.0%	15	46.9%	0.784
	Positive	6	50.0%	11	55.0%	17	53.1%	
Extranodal site	Bone Marrow	3	25.0%	4	20.0%	7	21.9%	0.23
	breast	0	0.0%	1	5.0%	1	3.1%	
	GIT	0	0.0%	5	25.0%	5	15.6%	
	ovarian	1	8.3%	0	0.0%	1	3.1%	
	Paraspinal mass	0	0.0%	1	5.0%	1	3.1%	
	Peritoneum	1	8.3%	0	0.0%	1	3.1%	
	shoulder	1	8.3%	0	0.0%	1	3.1%	
B-Symptoms	Negative	8	66.7%	10	50.0%	18	56.3%	0.358
	Positive	4	33.3%	10	50.0%	14	43.8%	
Bone Marrow infiltration	Negative	8	66.7%	15	75.0%	23	71.9%	0.612
	Positive	4	33.3%	5	25.0%	9	28.1%	
BCL-2	Negative	9	75.0%	9	45.0%	18	56.3%	0.098
	Positive	3	25.0%	11	55.0%	14	43.8%	
HCV	Negative	9	75.0%	17	85.0%	26	81.3%	0.483
	Positive	3	25.0%	3	15.0%	6	18.8%	
HBV	Negative	11	91.7%	20	100.0%	31	96.9%	0.19
	Positive	1	8.3%	0	0.0%	1	3.1%	

PS: performance status; **NCCN-IPI**: National Comprehensive Cancer Network-International Prognostic Index

Table 2: Response and outcome of patients according to Beclin 1 status

		Beclin 1 High N=12		Low N=20		Total N=32		P-Value
Response	CR	8	66.7%	11	55.0%	19	59.4%	0.699
	PD	0	0.0%	2	10.0%	2	6.3%	
	PR	2	16.7%	3	15.0%	5	15.6%	
	SD	2	16.7%	4	20.0%	6	18.8%	
CR	CR	8	66.7%	11	55.0%	19	59.4%	0.515
	No.CR	4	33.3%	9	45.0%	13	40.6%	
Progression	No	5	50.0%	10	71.4%	15	62.5%	0.285
	Yes	5	50.0%	4	28.6%	9	37.5%	
Death rate	Alive	12	100.0%	13	65.0%	25	78.1%	0.02
	Dead	0	0.0%	7	35.0%	7	21.9%	

CR: Complete respons**; PR:** Partial respons**; PD:** Progressive disease**; SD:** Stable disease

Table 3: Kaplan– Meier survival analysis illustrating OS and DFS rate differences in patients as regard Beclin 1 expression

Beclin 1	Total Number	Number of Events	Censored		Survival Rate %	Log Rank Test	Significance
			No.	%			
OS							
High	12	0	12	100.0%	100.0%	0.049	0.025
Low	20	7	13	65.0%	65.0%		
Overall	32	7	25	78.1%	78.1%		
DFS							
High	12	5	7	58.3%	50.0%	0.210	0.649
Low	20	10	10	50.0%	42.7%		
Overall	32	15	17	53.1%	45.0%		

OS: Overall survival; **DFS**: Disease free survival

Table 4: Univariate and multivariate Cox regression analyses of different prognostic factors for Overall survival

Variable	Univariate			Multivariate (stepwise)		
	HR	95.0% CI	P-value	HR	95.0% CI	P-value
Sex	1.37	0.68-2.74	0.377			
Age	1.02	0.95-1.10	0.622			
LDH	1.07	0.26-4.49	0.923			
Extranodal Disease	0.29	0.06-1.42	0.039*	0.041	0.003-0.503	0.012*
NCCN IPI	1.05	0.64-1.73	0.845			
B-symptoms	0.38	0.08-1.88	0.235			
BM involvement	0.37	0.05-2.99	0.350			
HCV	0.76	0.34-1.69	0.501			
HBV	4.63	0.00-15343.93	0.711			
Albumin	0.77	0.18-3.23	0.722			
Beclin1	0.11	0.01-1.07	0.049*	0.004	0.001-0.399	0.019*
Bcl2	5.00	0.98-25.41	0.052			
PS	1.61	0.40-6.48	0.041*	18.831	1.655-214.316	0.018*
Stage	0.55	0.07-4.52	0.582			

PS: performance status; **NCCN-IPI**: National Comprehensive Cancer Network-International Prognostic Index; **BM: bone marrow**

Table 5: Univariate and multivariate Cox regression analyses of different prognostic factors for disease-free survival

Variable	Univariate			Multivariate (stepwise)		
	HR	95.0% CI	P-value	HR	95.0% CI	P-value
Sex	1.64	0.33-8.26	0.546			
Age	1.01	0.94-1.08	0.811			
LDH	3.90	0.45-33.56	0.215			

Variable	Univariate			Multivariate (stepwise)		
	HR	95.0% CI	P-value	HR	95.0% CI	P-value
Extranodal Disease	6.20	0.69-55.23	0.102			
PS	2.73	0.48-15.55	0.257			
Stage	0.04	0.00-323.25	0.475			
NCCN IPI	4.16	1.56-11.11	0.004*	4.163	1.561-11.107	0.004*
B-symptoms	1.62	0.32-8.29	0.560			
BM involvement	0.48	0.06-4.15	0.507			
HCV	0.90	0.30-2.68	0.854			
HBV	5.10	0.01-2397.58	0.604			
Albumin	0.34	0.07-1.70	0.189			
Beclin1	0.15	0.02-1.42	0.097			
Bcl2	9.08	1.04-78.93	0.046*			

PS: performance status; **NCCN-IPI**: National Comprehensive Cancer Network-International Prognostic Index; **BM: bone marrow**

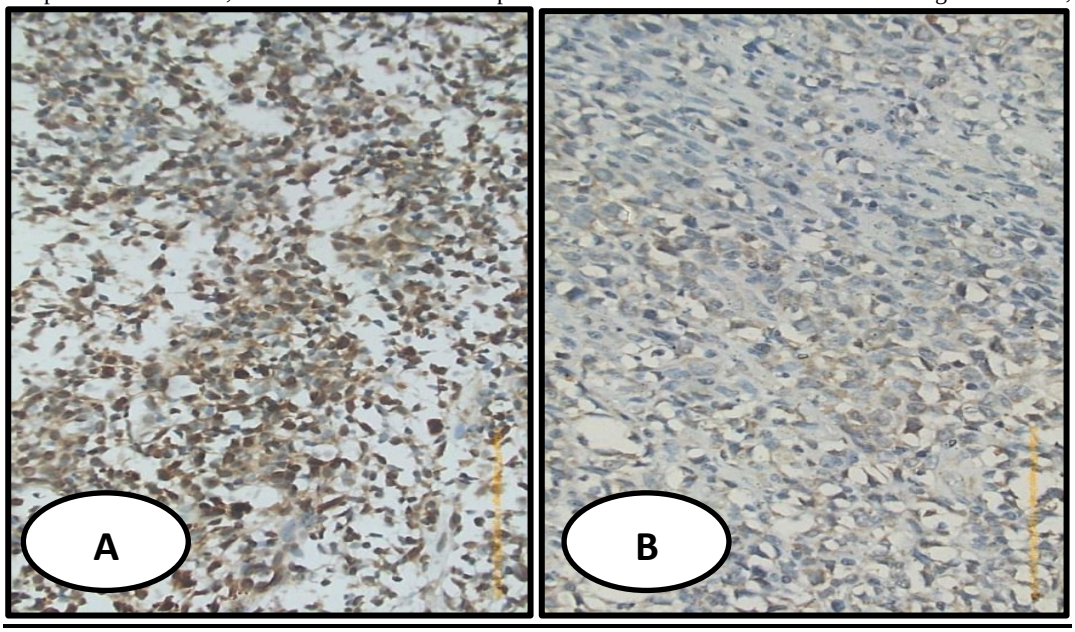

Figure (1): Diffuse large B-cell lymphoma showing high expression of Beclin1 with strong immunostaining (Slide A). While low expression of Beclin1 with weak immunostaining is shown in (Slide B) (H&E X400).

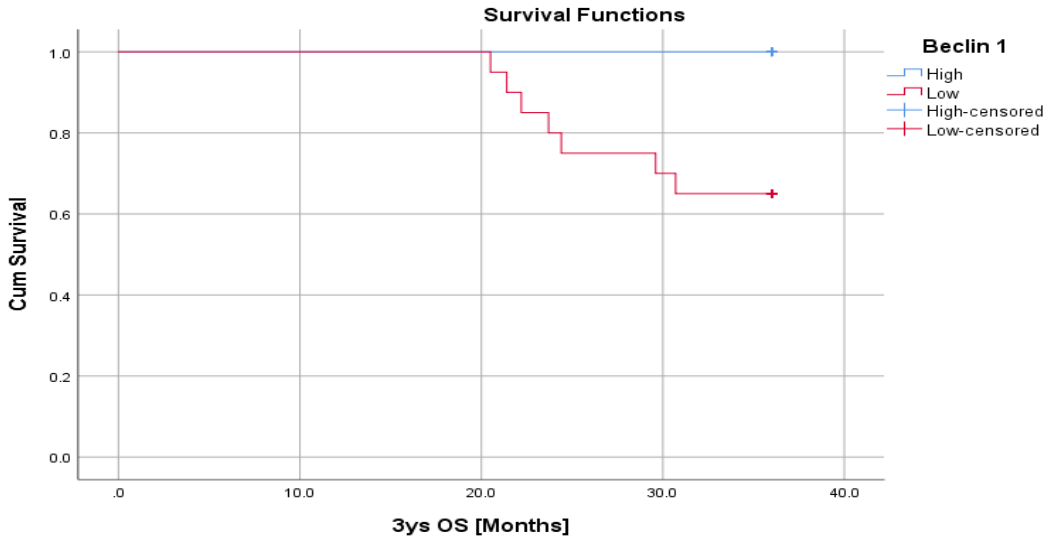

Figure (2): Comparison between Overall survival in both groups as regard Beclin-1 expression

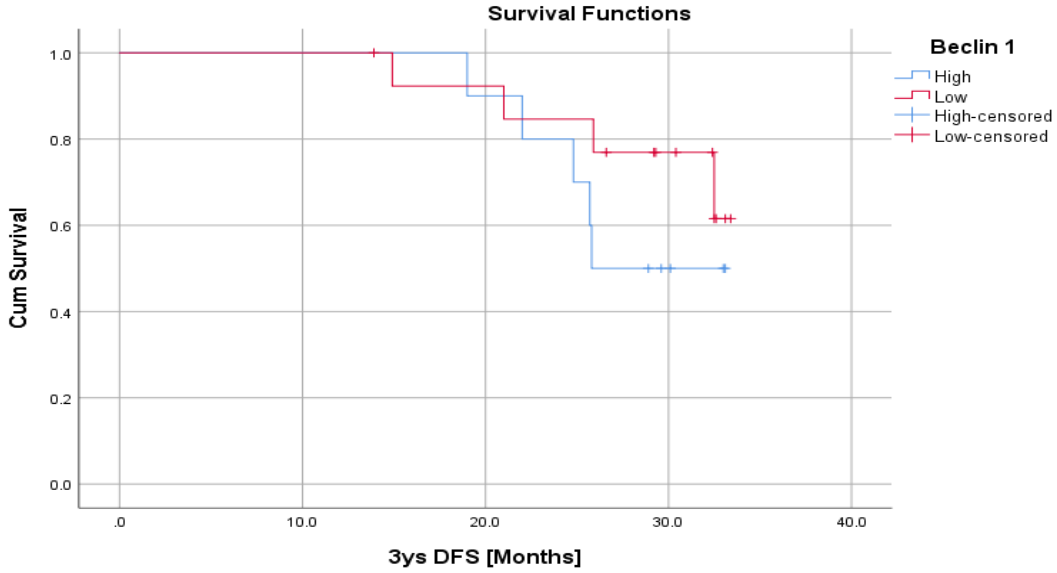

Figure (3): Comparison between Disease free survival in both groups as regard Beclin 1 expression

DISCUSSION

Diffuse large B-cell lymphoma, is a clinically, biologically, and genetically heterogeneous disease [17]. Prognostic assessment is important for designing of individualized therapy in DLBCL on the basis of accurate estimation of outcome [18].

DLBCL is more prevalent in elderly patients with a median age in the 7th decade [19], but in our study, the Median age was 48 years and this is consistent with Egyptian NCI registry [20] with median age 47.8 year and this may be due to difference in disease biology where some aggressive diseases are observed in younger patients like AML, multiple myeloma, and CML or difference in the structure of population pyramid in our country where majority of the population is below 60 years.

In our study, the ECOG PS of patients < 2 represented 62.5% and this is similar to **Nimmagadda et al**[21] where it represented 69.9%. However, in **Huang et al**[22]**,** it represented 98% and that may be attributed to different sample size and the time taken for patient diagnosis.

Elevated LDH was found in 59.4% of our patients and this is similar to **Huang et al**[22] & **Kim et al**[23] with high LDH in 53.3% & 59.5% respectively. However, in **Hammouda et al**[24] & **Samaka et al**[25] it was observed in 82.2% & 87% respectively, and this may be attributed to difference in study design and lab to lab variations.

In our study, Bone marrow is involved in 28.1% of cases and this is close **Kim et al[23]** & **Park et al[26]** which was 15.2% and 17.1% respectively.

However, in the study by, **Ma et al[27]** higher proportion of Bone marrow involvement is found and represented 29.1% of cases, and this might be due to difference in sensitivity of laboratory or imaging method used to detect bone marrow involvement.

The frequency of HCV infection in our patients was 18.8% of cases and this is near to **Haggag et al[28]**, which was 26.5% .However, In the study done by **Dlouhy et al[29]** carried out in spain, only 9.7% of DLBCL patients were found to have HCV infection and this might be attributed to difference in sample size and the very high prevalence of HCV infection among Egyptians.

Using the NCCN-IPI for risk stratification, the proportion of High-intermediate to high risk score in our patients was 45% and this is consistent with **Park et al[26]** which was 44%. However, A higher proportion was reported by **Go et al[30]** which was 53.8% and that difference might be due to the clinical & biological heterogeneity of the disease.

In our study, serum Albumin level of patients ranged from 2.7-4.4 gm/dl with mean value 3.51 ± 0.4, however in the study done by **(Bairey et al[31]**, Serum albumin level ranged from 1.9–4.9 gm/dl with mean value 3.9 and in the study by **Nakayama et al[32]** serum albumin level ranged from 1.7–5.0 gm/dl with mean value 3.8 ± 0.1and that might be explained by difference in sample size and presence of other factors which may affect albumin level such as nutritional status, hepatic insufficiency or proteinuria.

We found that frequency of BCL-2 overexpression was 43.8% and this near to **Huang et al [22]** which was 61% and both findings are consistent with the range of BCL-2 overexpressed DLBCL cases reported by **Vaidya and Witzig[33]** which was 47–58%. However, in **Horn et al[34]** & **Tsuyama et al[35]**, the percent of BCL-2 overexpressed DLBCL cases was 21% & 9% respectively and that might be due to difference in cut-off levels used for the IHC analysis and biological heterogeneity among DLBCL patients.

In our study, we found high beclin-1 expression in 37.5% of DLBCL patients and this near to what's found by **Nicotra et al[36]** with high beclin-1 expression in 46.8% of DLBCL patients, However, in study by **Huang et al [22]**, Beclin-1 overexpression was found in 67.8% of DLBCL patients and that difference may be explained by their usage of relatively larger sample size (118 patients).

High beclin-1 was associated with negative Bcl-2 expression (although it didn't reach significant difference) in our study and this is consistent with **Huang et al [22]** & **Won et al[37]**. However, **McCarthy et al[38]** reported increased autophagy activity in BCL2+ DLBCL cells and this might possibly explained that the inhibition of apoptosis caused by BCL2 overexpression may switch on autophagy to eliminate damaged proteins and aged organelles.

We found that there was no statistically significant difference between high and low beclin-1 patients as regard response to treatment and progression rate after getting initial response, indicating that beclin-1 has no prognostic impact on the outcome of therapy (except for death rate). However, in the study by **Huang et al [22]**, higher CR rate was observed in high beclin-1 patients and this might be explained by their larger sample size.

In our study, Patients with high beclin-1 expression had significantly higher OS as compared with low beclin-1 expression patients and this is consistent with **Huang et al [22]** verifying the good prognostic impact of high beclin-1 expression on overall survival. However, there was no statistically significant difference between high and low beclin-1 patients as regard DFS, as the Number of progressive cases wasn't significantly different between both patient groups and this might be due to the relatively small sample size of our study.

We found that positive BCL2 expression was associated with significantly lower OS & DFS in contrast to **Mounier et al[39] & Wilson et al[40]** and that might be explained by difference in cut-off levels used for the IHC analysis between studies and the heterogeneity of the DLBCL patients with variable proportions of

GCB and ABC DLBCL (not explored in our study).

Using multivariate Cox regression analysis, we identified high beclin 1 expression, good PS and few extranodal involvement as independent predictors of longer OS & Low NCCN IPI score as independent predictors of longer DFS and this is consistent with **Nicotra et al**[36] & **Huang et al** [22], which confirmed Beclin-1 as a predictor of OS and OS & PFS respectively.

CONCLUSIONS

Beclin-1 was over expressed in 37.5% of patients with De novo DLBCL and although it didn't provide prognostic indicator for response to treatment, it's found to be independent predictor for OS in DLBCL patients.

Testing for beclin-1 expression could be done to DLBCL patients at the initial workup stage and further studies should be done on larger scale in order to verify its prognostic significance.

Conflicts of interest: None.

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