RESEARCH ARTICLE

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The Prevalence of *MYD88 L265P* and *TNFAIP3* Mutations and Their Correlations with Clinico-Hematological Profile in Egyptian Patients with Diffuse Large B Cell Lymphoma

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Abstract

Background: Activated B-cell–like (ABC) subtype of diffuse large B-cell lymphoma (DLBCL) is characterized by chronic active B-cell receptor signaling and a constitutive activation of the NF-KB pathway. *MYD88 L265P* mutation occurs as a driving force of NF-KB overactivity in ABC-DLBCL. Nonetheless, in cases of DLBCL, the *MYD88 L265P* mutation has not yet been investigated in association with the tumour necrosis factor alpha induced protein3 (*TNFAIP3*) mutation. **Objective:** To investigate the frequency of MYD88 and *TNFAIP3* mutations in DLBCL and their association to the clinico-hematological profile. **Material and Methods:** We used real-time polymerase chain reaction in order to search for *MYD88 L265P* and *TNFAIP3* mutations in 100 DLBCL patients. **Results:** *MYD88 L265P* In 20% of cases, the CT heterozygous genotype was discovered. CT heterozygous genotype was more common in ABC type, stage IV, greater IPI groups, extra-nodal infiltration, and BM infiltration. It was also linked to a shorter OS. *TNFAIP3* mutation GA heterozygous genotype was detected in 18% of the patients, with ABC-DLBCL subtype accounting for 77.8%. The GA heterozygous genotype was usually related with stage IV, extranodal infiltration, and a reduced life expectancy. **Conclusion:** *MYD88 L265P* and to lesser extent *TNFAIP3* mutations are major mutations in ABC- DLBCL and may be predictive factors for poor OS in ABC- DLBCL patients.

Keywords: DLBCL- MYD88- *TNFAIP3*- Molecular biology- Real time PCR

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Introduction

DLBCL is considered the most frequent subtype of adult lymphoma, representing 31% of non-hodgkin lymphoma (NHL) cases (Martelli et al., 2013). DLBCL accounts for around 49% of NHL cases at the National Cancer Institute (NCI) in Cairo, Egypt (Abdelhamid et al., 2011). DLBCL may be separated into two main subcategories depending on origin of cell utilizing gene expression profiling: activated B-cell-like (ABC) & germinal centre B-cell-like (GCB) (Martelli et al., 2013). When compared to GCB DLBCL, ABC-DLBCL has a worse prognosis (Yu et al., 2018).

MYD88, an adapter protein that promotes interleukin and toll receptor signaling and enhances nuclear factor-B (NF-B) pathways, was discovered to be the first Toll-interleukin-1 (IL-1) receptor (TIR) member of the family. Non-GCB DLBCL is distinguished by constitutive activation of NF-B pathways (Pasqualucci and klein, 2022). However, it has recently been discovered that *MYD88 L265P* alone is insufficient for carcinogenesis and that a second genetic factors, like BCL2 overexpression or deletion of *TNFAIP3* gene which encodes for A20 protein, are required (Knittel et al., 2016).

TNFAIP3 deletions or mutations on 6q23 are common in DLBCL, and when associated with a MYD88 mutation, they might result in abnormal activation of NF-B. A20 is an inducible enzyme which modifies ubiquitin and regulates the negative feedback signals of NF-κB (Wenzl et al., 2018). It has also been demonstrated that A20 expression is upregulated more rapidly in the cells to decelerates MYD88-driven proliferation and NF-B activation (Wang et al., 2014). With the introduction of various targeted therapy drugs that target NF-kB pathways, analysis of a small number of genes mutations in these pathways may aid in the selection of an effective curative plan for DLBCL (Lopez-Santillan et al., 2021).

Almost all patients with ABC-DLBCL who received the R-CHOP regimen in treatment had bad prognostic outcomes, raising alarms about the *MYD88 L265P* and

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TNFAIP3 mutations (Pasqualucci and klein, 2022).

Many studies reported that MYD88 and *TNFAIP3* are frequently mutated in DLBCL however; the prevalence of these genes in Egyptian population needs further investigations. So, the scope of our study to detect the prevalence of MYD88 and *TNFAIP3* mutations in a cohort Egyptian population and explore their prognostic value and their relationship with other clinical parameters.

Materials and Methods

Subject

This cohort study was applied on 100 newly diagnosed patients with DLBCL between January 2019 and September 2022 in Oncology center Mansoura university based on the current WHO classification (Sabattini et al., 2010). The following were the inclusion criteria: I) Accessible clinical data and follow-up ones; and II) tumor samples available for DNA analysis at the first diagnosis. III) Patients aged up to 80 years; iv) Both genders are eligible. Exclusion criteria: I) Any DLBCL patients with a history of other cancers or autoimmune diseases will be excluded. II) Unfit DLBCL patients because of associated co-morbidities and poor performance. Immunohistochemistry was used to classify DLBCL into GCB/ABC subgroups using Hans' algorithm (Hans et al., 2004).

DNA extraction

Fresh lymph nodes were taken from all patients and kept in a saline-filled container to be preserved at -80oC until genotyping. Following the manufacturer's instructions, extraction of the DNA from samples was done by using QIAamp DNA Mini Kit from Qiagen (Catalog No. 51304 Hilden, Germany). Nanodrop 2000 was used to test the DNA quality. The yield of DNA was 50-70 µg, and the absorbance range at wave length (280) was from 1.7 to 1.9.

TaqMan SNP Genotyping Assays (Real time PCR) for MYD88 L265P (rs387907272) and TNFAIP3 (rs143002189)

This assay was carried out by using master mix preparation (Applied biosystems, lot 00723780, Foster, USA), forward and reverse primers with specific sequences to amplify the polymorphic sequence of interest and Two TaqMan® probes for the SNPs. The probes for MYD88: One detects the Allele 1 (T) sequence and is labeled with VIC® dye & the other probe detects the Allele 2 (C) sequence and is labeled with FAM™ dye while those for *TNFAIP3*: One probe detects the Allele 1 (A) sequence and is labeled with VIC® dye & the other probe detects the Allele 2 (G) sequence and is labeled with FAM™ dye. At the endpoint, fluorescence intensity was measured to determine allelic discrimination. SDS software version 1.7 (Applied Biosystem, Foster, USA) was used to analyse the measurement findings, and genotyping was determined. Each sample is interpreted according to the 2 alleles & Genotypes (Homozygous or heterozygous).

Statistical analysis

All statistical parameters were analyzed using the statistical software package SPSS 20.0 (SPSS Inc., Chicago, Illinois, USA). Quantitative variables were reported as mean and standard deviation (for normally distributed data) or median and range (for abnormal distributed data).. The qualitative variables were shown as a count and a percentage. Direct counting was used to determine genotypic and allelic frequencies. The Mann-Whitney test was used to compare non-parametric data between two groups, whereas the Kruskal Wallis test was used to compare data from more than two groups. In terms of qualitative variants, we utilized the ChiSquare test to analyse the link between two variables and the Fisher's exact test when the predicted count was less than 5 in more than 20% of cells.

PFS was computed from the time of first diagnosis until the time of illness progression, recurrence, or death from whatever cause. Overall survival (OS) was estimated from the time of first diagnosis to the time of last follow-up or time of death from whatever reason. Median follow-up time was calculated from the date of treatment completion to the date of the last follow-up or death for any reason. The Kaplan-Meier method was applied to compute OS and PFS, and the survival curves were compared between groups using the log rank p test. P 0.05 was statistically regarded as significant.

Results

Clinicopathological characteristics: This study included 100 newly diagnosed patients with DLBCL lymphoma 42 females and 58 males, median age 57 years with range (23 to 75 years); attending to Oncology Center Mansoura University (OCMU), 57.0% of them were GC subtype, as regard disease stage; 71.0% were stage III, extra-nodal involvement detected in 50.0%, BM infiltration detected in 28.0% of cases. Detection of *MYD88 L265P* mutation rs387907272 was done by real time PCR on LN samples of DLBCL. The CT heterozygous mutant genotype was observed in 20 out of 100 patients (20%) where 16 of 20 patients (80%) were ABC-DLBCL and 4 of 20 (20%) were GC subtype.

Patients with CT heterozygous genotype were significantly older patients. They had significant increases in WBC and LDH compared to patients with TT wild genotype. Also, ABC type, stage IV, higher IPI groups, extra-nodal infiltration and BM infiltration were significantly more frequent in patients with CT heterozygous genotype versus patients with TT wild genotype, table (1). Regarding *TNFAIP3* rs14002189, 18 out of 100 (18%) of the patients were GA heterozygous mutant genotype. Fourteen out of those eighteen patients (77.8%) were ABC-DLBCL and only four (22.2%) were GC. GA heterozygous genotype patients had significant lower Hb level than those with GG wild genotype also, stage IV, and extra-nodal infiltration were significantly frequent in patients with GA heterozygous genotype compared to GG wild genotype patients, table (2). *TNFAIP3* GA genotype is present in 60.0% of MYD88 CT genotype patients while *TNFAIP3* GG genotype is present

Table 1. Comparison of Clinicopathological Characteristics and Laboratory Parameters as Regards rs387907272 (MYD88) Genotypes in Patients with DLBCL

Parameters		TT (n=80)	CT (n=20)	P-value
Age	Median (Min-Max)	56.0 (23-75)	64.0 (37-75)	0.007
Gender	Male, Count (%)	47 (58.8%)	11 (55.0%)	0.761
	Female, Count (%)	33 (41.2%)	9 (45.0%)	
WBCS $\times 10^9$ /L*	Median (Min-Max)	6.0 (2.2-71.0)	11.8 (3.8-102.0)	0.006
Hemoglobin g/dl*	Median (Min-Max)	12.3 (6.6-13.9)	10.8 (6.5-13.6)	0.374
PLT $\times 10^9$ /L*	Median (Min-Max)	169.0 (55.0-412.0)	151.0 (95.0-240.0)	0.608
LDH*	Median (Min-Max)	263.0 (160-797)	323.0 (197-1548)	0.021
Type	GC, Count (%)	39 (48.8%)	4 (20.0%)	0.024
	ABC, Count (%)	41 (51.2%)	16 (80.0%)	
Disease Stage	Stage II, Count (%)	0 (0.0%)	2 (10.0%)	<0.001
	Stage III, Count (%)	63 (78.8%)	8 (40.0%)	
	Stage IV, Count (%)	17 (21.2%)	10 (50.0%)	
ECOG score	<2, Count (%)	45 (56.2%)	9 (45.0%)	0.367
	≥2, Count (%)	35 (43.8%)	11 (55.0%)	
IPI	Low risk, Count (%)	2 (2.5%)	0 (0.0%)	0.004
	Low intermediate risk, Count (%)	66 (82.5%)	11 (55.0%)	
	High intermediate risk, Count (%)	12 (15.0%)	7 (35.0%)	
	High risk, Count (%)	0 (0.0%)	2 (10.0%)	
Extra-nodal involvement	Count (%)	35 (43.8%)	15 (75.0%)	0.012
BM infiltration	Count (%)	17 (21.2%)	11 (55.0%)	0.003
B symptoms	Count (%)	36 (45.0%)	9 (45.0%)	1.00

Mann-Whitney test*; chi square test (fisher's exact test), P between both groups.

Table 2. Comparison of Clinicopathological Characteristics and Laboratory Parameters as Regard rs143002189 (TNFAIP3) Genotypes

Parameters		GG (n=82)	GA (n=18)	P-value
Age	Median (Min-Max)	57.0 (30-75)	56.0 (23-75)	0.726
Gender	Male, Count (%)	46 (56.1%)	12 (66.7%)	0.411
	Female, Count (%)	36 (43.9%)	6 (33.3%)	
WBCS $\times 10^9$ /L*	Median (Min-Max)	6.2 (2.2-102.0)	6.6 (2.6-102.0)	0.683
Hemoglobin g/dl*	Median (Min-Max)	12.9 (6.5-13.9)	10.6 (7.0-13.5)	0.010
PLT $\times 10^9$ /L*	Median (Min-Max)	169.0 (55-412)	146.0 (85-233)	0.805
LDH*	Median (Min-Max)	263.0 (160-1548)	297.5 (197-797)	0.229
Type	GC, Count (%)	39 (47.6%)	4 (22.2%)	0.066
	ABC, Count (%)	43 (52.4%)	14 (77.8%)	
Disease Stage	Stage II, Count (%)	0 (0.0%)	2 (11.1%)	0.003
	Stage III, Count (%)	62 (75.6%)	9 (50.0%)	
	Stage IV, Count (%)	20 (24.4%)	7 (38.9%)	
ECOG score	<2, Count (%)	45 (54.9%)	9 (50.0%)	0.707
	≥2, Count (%)	37 (45.1%)	9 (50.0%)	
IPI	Low risk, Count (%)	2 (2.4%)	0 (0.0%)	0.010
	Low intermediate risk, Count (%)	68 (82.9%)	9 (50.0%)	
	High intermediate risk, Count (%)	11 (13.4%)	8 (44.4%)	
	High risk, Count (%)	1 (1.3%)	1 (5.6%)	
Extra-nodal involvement	Count (%)	37 (45.1%)	13 (72.2%)	0.037
BM infiltration	Count (%)	22 (26.8%)	6 (33.3%)	0.578

Mann-Whitney test*; chi square test (fisher's exact test), P between both groups.

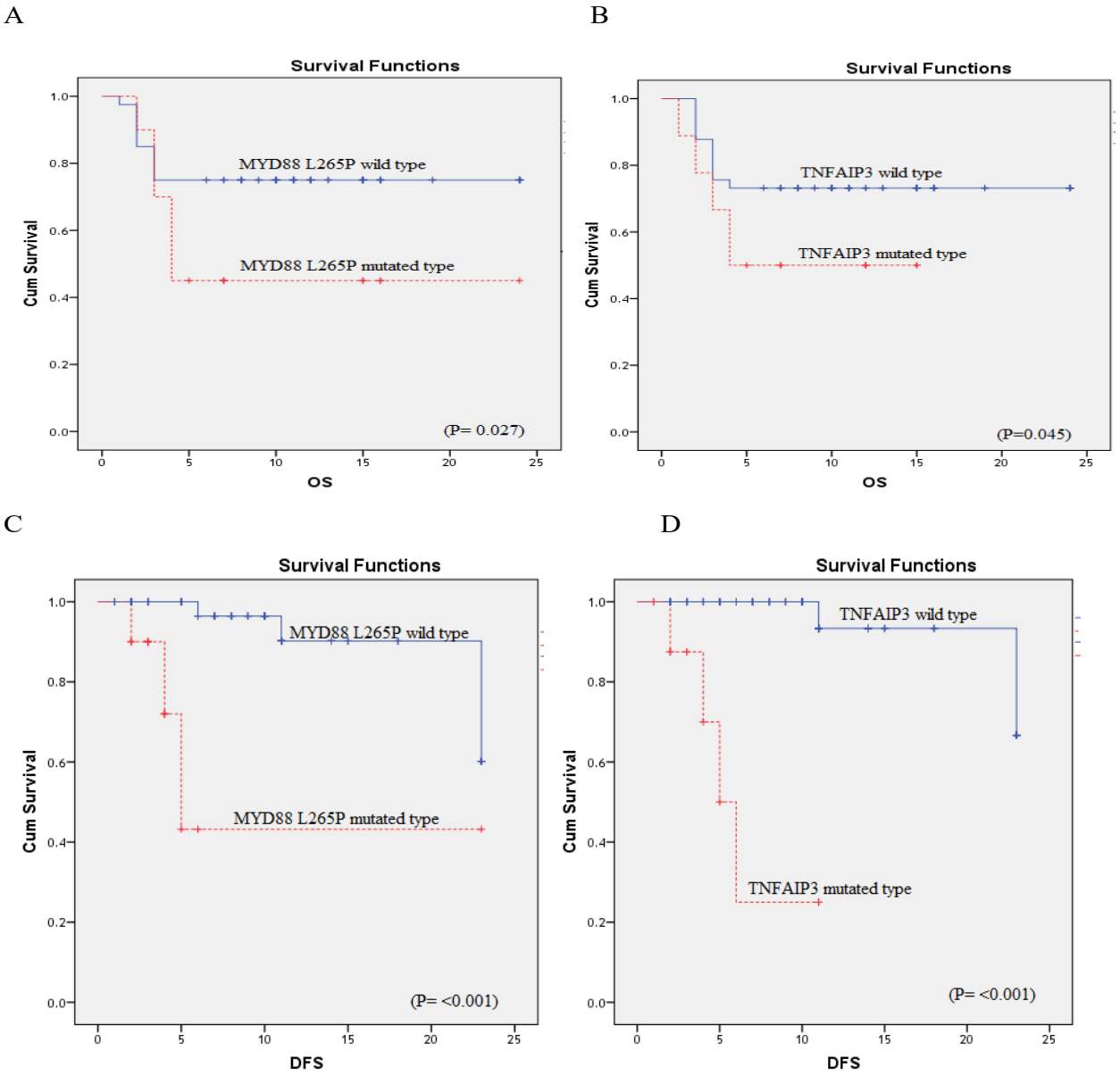

Figure 1. Kaplan-Meier Survival Curves based on MYD88 L265P (rs387907272) and TNFAIP3 (rs143002189) in DLBCL. (A,B) Overall survival of MYD88 L265P and TNFAIP3 in 100 patients with DLBCL. (C,D) Disease-free survival of MYD88 and TNFAIP3 in 100 DLBCL patients, OS and DFS time is represented in months, myeloid differentiation factor88; TNFAIP3, tumor necrosis factor alpha induced protein3; DLBCL, diffuse large B cell lymphoma.

in 40% of MYD88 CT genotype patients.

Using age, gender, laboratory data, cell of origin "activated B-cell-like (ABC) or germinal centre B-cell-like (GCB)", IHC, staging, ECOG, IPI, extranodal, B symptoms, rs387907272 and rs143002189 as covariates, cox regression analysis was conducted for prediction of shorter disease-free survival DFS and OS are summarized in Tables (3), (4). In multivariate analysis Only the CT was a significant risk factor for shorter PFS and OS while GA genotype was a significant risk factor for shorter PFS only.

MYD88 (rs387907272) and *TNFAIP3* (rs143002189) mutations and overall survival figure (1): The follow-up time throughout this cohort study was ranged from 1-24 months, with 2 year OS and PFS rates of 69% and 59.6%,

Table 3. Cox Regression Analysis for Prediction of Shorter DFS

	Univariate analysis				Multivariate analysis			
	P	HR	95% CI		p	HR	95% CI	
IPI (4-8 vs 0-3)	0.029	3.551	1.135	11.114	0.781	2.833	0.230	3.02
rs387907272 (CT vs TT)	<0.001	10.546	3.122	35.622	0.049	5.237	1.979	28
rs143002189 (GA vs GG)	<0.001	36.998	7.684	78.135	<0.001	29.23	5.081	68.173

HR, hazard ratio; CI, confidence interval; COX regression was used.

Table 4. Cox Regression Analysis for Prediction of Shorter OS

	Univariate analysis				Multivariate analysis			
	P	HR	95% CI		p	HR	95% CI	
IPI	0.690	1.384	0.942	1.964				
rs387907272 (CT vs TT)	0.041	2.154	1.032	4.496	0.043	2.032	1.027	4.287
rs143002189 (GA vs GG)	0.063	2.086	0.960	4.530				

HR, hazard ratio; CI, confidence interval; COX regression was used.

respectively. A statistical difference was found in the median OS (p= 0.027) and PFS (p <0.001) for patients with MYD88 CT genotype versus TT genotype group. Regarding *TNFAIP3* mutation, there is a significant correlation in both OS (p =0.045) and DFS (p <0.001) with patients of GA mutant genotype.

Discussion

DLBCL is considered a heterogeneous illness clinically and physiologically. Although immunochemotherapy has treated most of the patients, a significant minority remain resistant to treatment or eventually relapse. Several B-cell neoplasms have been reported to possess the *MYD88 L265P* mutation, which is important for treatment, prognosis, and diagnosis (Shekhar et al., 2021). Determining the molecular rationale which affects the prognosis of MYD88-mutant DLBCL patients may give justification for new therapy targets in the relevant pathways (Visco et al., 2020). As a result, MYD88 could be an important biomarker in ABC DLBCL.

In our research, the frequency of *MYD88 L265P* mutation CT genotype was observed in 20% of samples. Patients with CT genotype tend to be older, had mostly ABC subtype, extranodal involvement, and a poorer OS. Moreover, frequency for *TNFAIP3* mutation GA genotype was found in 18% of patients. Stage IV, higher IPI groups, and extra-nodal infiltration were significantly more frequent in GA genotype, also we found that this mutation was a significant risk factor for shorter OS in multivariate analysis.

It was reported that excessive activation of NF-KB pathways frequently exists in ABC type DLBCL which explain MYD88 mutation presence in this type (Yu et al., 2018). Some studies support our results regarding *MYD88 L265P* as Vermaat et al., (2020) study on 250 lymphoma patients found *MYD88 L265P* mutation in 17% of nodal DLBCL cases and had a statistically significant association with shorter 5-year OS. Relapse and progression occur more frequently in patients with MYD88 mutations . In addition, Niu et al., (2020) studied on 100 patients and revealed that the *MYD88 L265P* mutation was substantially corresponded to extra nodal site, Ann-Arbor stage (stages III-IV), and ECOG score in 29% of patients. Also, age (60 years), low lymphocyte number, high LDH level, rituximab therapy, Bcl-2 over expression (70%), and MYD88 mutation L265P were significant variables for shorter OS in their univariate analysis. However, in contrast to our investigation, in their multivariate analysis, age ≥60 years and a low lymphocyte count were independent predictive factors affecting patient survival. *MYD88 L265P* mutation, on the other hand, was not an independent predictive predictor of OS .

A meta-analysis of 40 studies conducted by Lee et al., (2017) revealed that 16.5% of patients had *MYD88 L265P* mutation, which was substantially found in the ABC subtype, high IPI prognostic score, advanced age, and poor survival rate.Rovira et al., (2016) observed *MYD88 L265P* mutation in 22% of 230 cases. These MYD88 mutant patients were older, with increased LDH levels, and had frequent extranodal affection, especially in the breast and testis. Apart from that, they discovered a strong correlation between the MYD88 mutation and shorter 5-year overall survival; nonetheless, IPI and COO were the most significant predictors of PFS .

Another study had the same finding regarding OS and PFS but the *MYD88 L265P* mutation was reported in 10% of 175 patients and occurred more frequently in older ages, males, cases without B symptoms and those with primary extranodal disease (Fernández-Rodríguez et al., 2014)

However, a contrary study by Dubois et al., (2017) performed on the 361 patients where 26% had MYD88 mutations, 89% of mutant patients had the ABC subtype. This mutation was linked with age but not associated with Ann Arbor stage, IPI, LDH levels, or performance status. This contrast may be resulted from the larger number of patients in this study.Regarding *TNFAIP3* mutation, Rosenquist et al., (2017) performed a genetic study by sequencing on 224 DLBCL tissue samples and found *TNFAIP3* mutation in 6% of patients. Also, Cao et al., (2016) found that this mutation was 7.14% of 196 cases.

Wenzl et al., (2016) discovered a considerably greater incidence of G>A mutation in DLBCL (2.97%), implying that this mutation plays a role in the pathogenesis of DLBCL. Another study by Dubois et al., (2016) found that *TNFAIP3* mutation presented in 17% of cases where 15% of them are ABC origin and 11% are GC-DLBC and significant association with lower OS. In contrast to our findings, Cen et al., (2015) reported that the A20 mutation is common in ABC-DLBCLs (29%) of 67 DLBCLs. However, there was no statistically significant difference in PFS or OS between patients with and without A20 mutations.

Bu et al., (2012) examined the *TNFAIP3* gene mutation status in 150 DLBCL samples and found the mutation in 4.6% of the samples. The *TNFAIP3* mutation, on the other hand, was not linked to a difference in overall survival. This disparity could be attributed to the varying amount of cases and methodologies. Another study discovered that high IPI and MUM1 expression were independent predictive indicators, although *TNFAIP3* gene status was not (Paik et al., 2013). Wenzl et al., (2018) examined 145

DLBCL patients for the relationship between MYD88 and *TNFAIP3* in DLBCL and discovered that 20 of 145 patients (13%) had the MYD88 mutation. The mutant cases were 70% ABC-DLBCL. Eleven (55%) of the MYD88 mutant patients showed *TNFAIP3* deletion . On the other hand, Dubois et al. (2017) discovered *TNFAIP3* mutation in 8% of ABC *MYD88 L265P* patients, which results in significantly lower expression levels, implying reduced NF-KB activity (Dubois et al., 2017)

In conclusion, as a result, we can conclude that *MYD88 L265P* and to lesser extent *TNFAIP3* mutations are significant mutations in the ABC-DLBCL patients which can be a powerful driver of high NF-κB activity. *MYD88 L265P* and *TNFAIP3* mutations can be associative factors for shorter OS in patients with ABC-DLBCL.

Author Contribution Statement

Osama Elbaz: Conception, preparation of the manuscript, revision, supervision. Rana M. Shaat: Conception, Interpretation and analysis of data, preparation of the manuscript, revision. Hasan A. Abd El Ghaffar: Conception, revision and preparation of the manuscript. Sameh Shamaa: Conception, Interpretation and analysis of data, preparation of the manuscript, revision Hanaa M. Abdel-masseih: Conception, interpretation and analysis of data, preparation of the manuscript, revision. Nahla Anber: Interpretation and analysis of data, preparation of the manuscript, revision. Metwaly Ibrahim Mortada: Conception, Interpretation and analysis of data, preparation of the manuscript, revision and supervision.

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Disclosure

The authors have no conflicts of interest to declare.

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