

# The prognostic importance of double-expressor subgroup and AID, UNG and mismatch repair protein expressions in diffuse large B-cell lymphomas

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## ABSTRACT

**Objective:** The cases of diffuse large B-cell lymphoma, (DLBCL not otherwise specified (NOS)) which immunohistochemically exhibit MYC and BCL2 expressions are defined as double-expressor lymphomas (DELs). This study aimed to assess the prognostic impact of DEL and the expressions of other proteins that may have role in tumorigenesis.

**Materials and Methods:** In this study, 90 tumor samples from patients diagnosed with DLBCL NOS were evaluated retrospectively. Immunoections of MYC, BCL2, activation-induced cytidine deaminase (AID), uracil-DNA glycosylase (UNG) and DNA mismatch repair proteins including MLH1, MSH2, MSH6 and PMS2 were analyzed.

**Result:** Eleven cases (12.2%) which exhibited  $\geq 40\%$  MYC and  $\geq 50\%$  BCL2 immunexpressions were classified as DEL DLBCL. Patients with MYC positivity displayed lower overall survival rate than MYC negative cases. A trend of lower overall survival was observed in the double-expressor lymphoma group, however, this was not proven to be statistically significant. Significant relationship between AID, UNG and p53 immunexpressions with double-expressor lymphoma or overall survival was not detected. The correlation between immunexpressions of p53 and MYC was observed. The loss of expression of mismatch repair proteins was not observed in any cases.

**Conclusion:** In this study, a relationship between low overall survival and MYC expression is detected. However, our result does not demonstrate that double-expressor lymphoma can be associated with poor outcomes.

**Keywords:** Diffuse large B-cell lymphoma, Double-expressor lymphoma, MYC, BCL2, AID, Mismatch repair proteins

## 1. INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is the most common aggressive B-cell lymphoma and a heterogenous group of diseases in terms of morphologic appearances, immunohistochemical phenotypes and molecular aberrations.

Several studies have been made with a great effort to subtype DLBCL at all levels to predict clinical outcomes and determine suitable treatment regimes. The main treatment for DLBCL not otherwise specified (NOS) is the use of rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone (R-CHOP). The disease recurs in nearly half of patients. In order to predict the clinical course, the National Comprehensive Cancer Network-International Prognosis Index (NCCN-IPI) score system has been established with clinical and laboratory data [1-3].

It has been reported that double-expression of MYC and BCL2 in DLBCL is a prognostic factor by earlier studies of Johnson

et al. and Green et al. Numerous studies have contributed to our knowledge about these cases. DLBCL cases which show overexpression with both MYC and BCL2 antibodies with the cut-off values of 40% and 50% respectively, have been mentioned as double-expressor lymphoma (DEL), according the last World Health Organization (WHO) lymphoma classification update [3]. It is advised to identify these cases because of the fact that DEL subset in DLBCL may have the role in prognostic significance [4-8].

Translocation mutations have an important role in the lymphomagenesis process in the majority of lymphomas. It is thought that DNA fractures that occur during physiological somatic hypermutation and isotype switching in normal B-cell activation related to the formation of these mutations may play a role. Activation-induced cytidine deaminase (AID), uracil-DNA glycosylase (UNG), mismatch repair proteins (MLH1,

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MSH2, MSH6, PMS2) and p53 proteins have roles during somatic mutation and isotype switching [9-14].

The aim of this study is to assess the prognostic impact of double-expression with MYC, BCL2 antibodies and the expression of other proteins that may have role in tumorigenesis.

## 2. MATERIALS and METHODS

### Case Selection and Clinical Data

Tumor samples from patients who were diagnosed with DLBCL NOS between January 2007 and June 2015 and treated with R-CHOP regimen, were searched retrospectively. The samples were obtained from Pathology archive of Marmara University Hospital. Ninety cases among 187 cases were identified who met the inclusion criteria. The inclusion criteria were: to have formalin-fixed paraffin-embedded (FFPE) tumor block and have enough tumor volume in it (more than 100 cells), samples without artefact and have the survival data. Tumors that developed as secondary to low-grade lymphoma and were primarily located at the mediastinum, central nervous system, skin, intravascular and the body cavity were excluded.

The database of 90 eligible cases was collected from clinical records of the hematology department. Age, sex and tumor location of the all cases were recorded while Ann-Arbor stage, revised (R)-IPI/NCCN-IPI scores were obtained from 58 and 52 patients, respectively.

Hematoxylin and Eosin stained pathology slides were re-reviewed by one hematopathologist and one general pathologist on the basis of morphology and immunohistochemistry for the purpose of confirmation of diagnosis according to the last 2016 WHO classification [15].

This study was approved by Marmara University Clinical Research Ethics Board (Protocol number: 09.2016.578). All methods were performed in accordance with the relevant guidelines regulations.

### Immunohistochemistry

Sections with a thickness of 4 µm per tumor were obtained from formalin-fixed paraffin-embedded (FFPE) tumor blocks for immunohistochemical analysis. Immunohistochemical staining with MYC, BCL2, BCL6, CD10, MUM1, p53 and Ki67 antibodies was performed using standard methods on the Ventana platform (Roche, Basel, Switzerland). The proper external control tissues were used for each antibody as recommended. The percentage of cells that demonstrated staining for each antibody was recorded. The intensity of staining also was recorded and moderate or strong intensity of immunoreexpression were accepted as positive staining, while weak immunoreexpression was not. According the last WHO recommendation, thresholds for immunoreexpression of tumor cells with MYC and BCL2 antibodies are accepted as 40% nuclear staining and 50% cytoplasmic staining, respectively [15]. The cases that were stained with MYC and BCL2 antibodies greater than these percentages were defined as DEL. Fluorescence in situ hybridization (FISH) analyses for MYC and BCL2 were not performed.

Cell of origin (COO) was determined according to Hans algorithm with CD10, BCL6 and MUM1 antibodies and cases were grouped as germinal center B-cell like (GCB) DLBCL and non-germinal center B-cell like (non-GCB) DLBCL [16]. Ki67 proliferation index recorded in the area of tumor showed maximum percentage of staining. Cases grouped according the percentage of staining as below 50%, between 50-90 and more than 90%. Nuclear staining of tumor cells for p53 antibody was evaluated and then cases were grouped as below or above 50% staining according to p53 immunoreexpressions. Immunohistochemical staining with AID, UNG and DNA mismatch repair proteins including MLH1, MSH2, MSH6, and PMS2 antibodies were also studied. Cut-off percentages for staining were accepted as 15% and 20% for AID and UNG respectively. The loss of staining with MLH1, MSH2, MSH6, and PMS2 antibodies were recorded for each tumor.

### Statistical Analysis

Fisher's exact test and Pearson  $\chi^2$  tests were used for group comparison of categorical variables. Overall survivals were calculated from the date of diagnosis to death or last follow up and were estimated using the Kaplan-Meier curves. The log-rank test was used to compare overall survival (OS) between subgroups. Prognostic factors that would affect OS were analyzed by using univariate and multivariate Cox proportional hazard regression models adjusting for confounding variables. The statistical significance level was considered as  $p < 0.05$ . All statistical analyses were performed by using Statistical Package for Social Sciences (IBM, Chicago, USA).

## 3. RESULTS

### Patient Characteristics and Clinical Datas

Data from 90 patients were analyzed. Median age at diagnosis was 59 years. Forty-two patients (53%) were older than 60 years of age. Forty-four (49%) of all patients enrolled to this study were male 46 (51%). Female and sex ratio (M/F) was 0.96. Median follow-up time was 31 months (range, 1 to 104 months). Forty-eight tumor samples were taken from lymph nodes and forty-two from extranodal sites. Twelve of the tumors were taken from the gastrointestinal system, 11 from Waldeyer's ring, 11 from the bones, two from the lungs and the remaining tumor samples each was from other organ sites. Forty-four of 58 cases (76%) had high Ann-Arbor stage (III-IV). Twenty seven of 52 cases had poor R-IPI score and 31 of 52 cases had high or high/intermediate NCCN-IPI scores.

No significant OS difference was observed when the cases grouped according to gender. Patients who were older than 60 years had significantly worse OS rates ( $p = 0.001$ ). No significant OS difference was detected for tumors taken from lymph nodes or extranodal sites ( $p = 0.222$ ), although, slightly better clinical course was observed in extranodal tumors. OS was better for low Ann-Arbor stages, R-IPI and NCCN-IPI scores that was statistically significant ( $p = 0.026$ ,  $p < 0.001$ ,  $p < 0.001$ , respectively).

### Immunohistochemical Analysis Results

Of the 90 cases enrolled in the study, 19 cases showed MYC positivity and 53 cases showed BCL2 positivity. Among them 11 cases (12.2%) were double-expressor lymphoma that stained both antibodies. It was also observed that there was no overexpression with either MYC or BCL2 antibodies in 20 patients that were defined as double-negative cases (Figures 1 and 2). The demographic,

clinicopathologic and immunohistochemical data of DEL subgroup are summarized in Table I. Patients with MYC positivity displayed poorer OS than MYC negative cases ( $p=0.018$ ), whereas no significant difference was determined for BCL2 antibody ( $p=0.073$ ). Double-expressor lymphoma cases statistically did not show any OS difference when compared with other cases ( $p=0.169$ ), albeit, these cases tended to show poor OS (Figure 3).

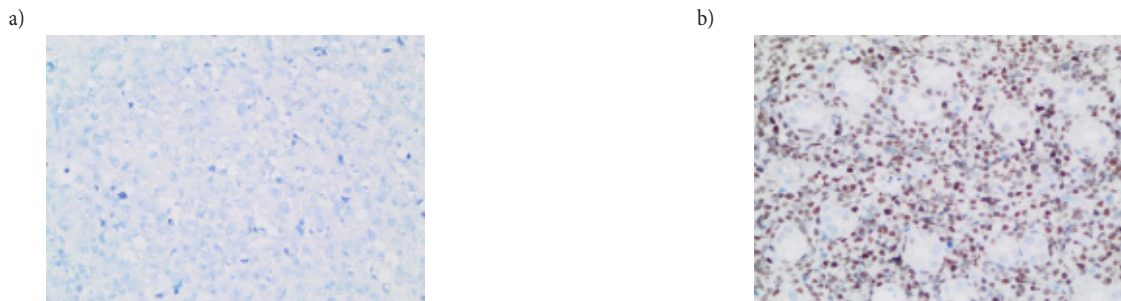


Figure 1. MYC <40% (a), and  $\geq 40\%$  (b) immunopositivity(200X).

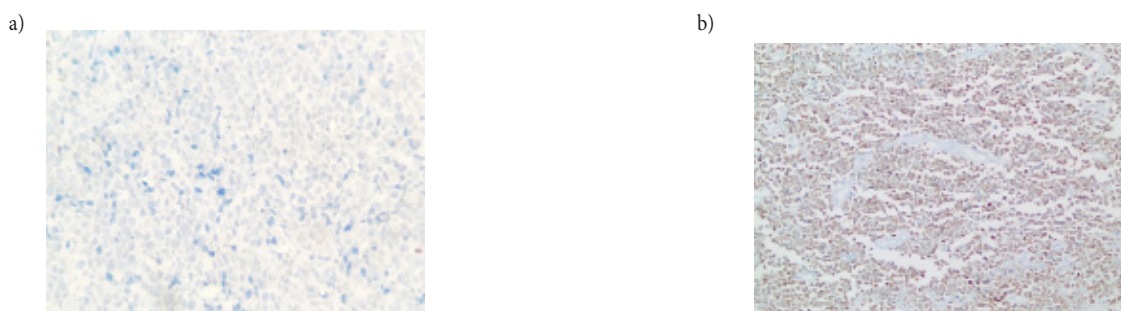


Figure 2. BCL2, <50% (a), and  $\geq 50\%$  (b) immunopositivity (200X).

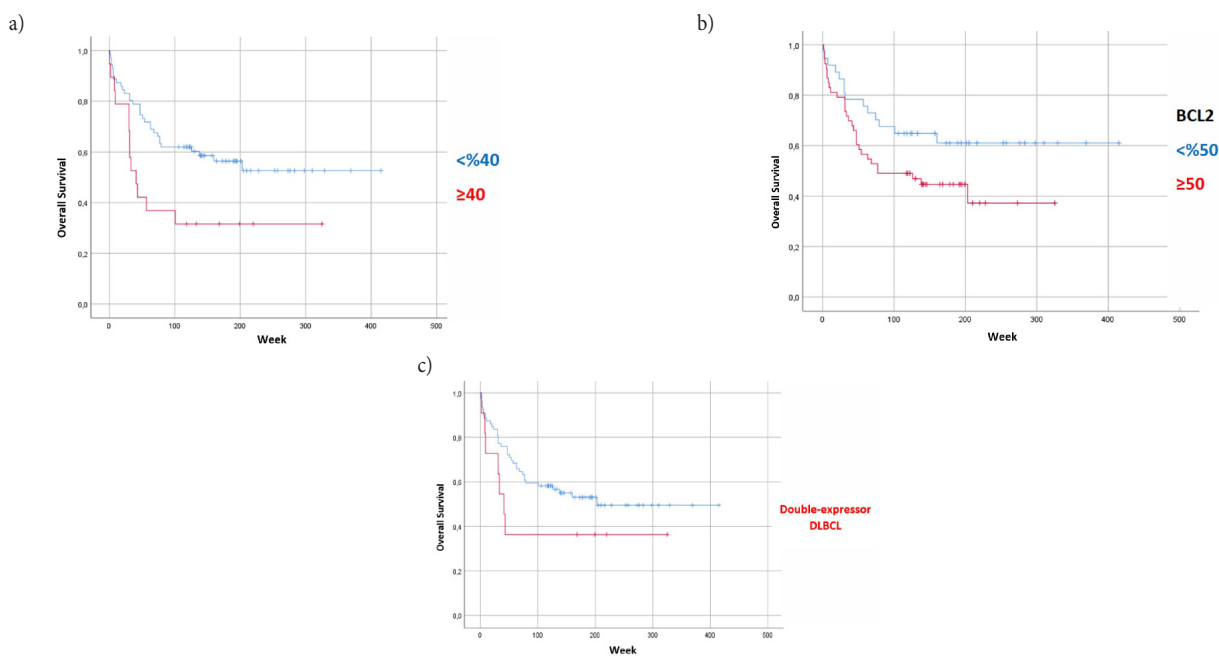


Figure 3. MYC expression-Overall survival (a), BCL2 expression-Overall survival (b), Double-expressor lymphoma-Overall survival (c).

When cases grouped as only MYC positive, only BCL2 positive, double-expressor lymphoma and double negative lymphoma; double negative cases showed markedly better OS than other three groups ( $p=0.015$ ). There was no significant association between double-expressor lymphoma and the factors including sex, age and tumor localization, Ann-Arbor stage and R-IPI/NCCN-IPI scores were distributed independently (Table I).

**Table I.** The demographic, clinicopathologic and immunohistochemical data of DEL subgroup

Characteristics		DEL (n=11)	Non-DEL (n=79)	All	p value
Gender	Male	4	40	44	0.375
	Female	7	39	46	
Age	≤60	4	38	42	0.229
	>60	7	41	48	
Tumor location	Nodal	4	44	48	0.229
	Extranodal	7	35	42	
AID	>%20	1	15	16	0.421
	≤%20	10	64	74	
UNG	>%50	3	14	17	0.449
	≤%50	8	65	73	
p53	>%50	8	40	48	0.169
	≤%50	3	39	42	
Cell origin	GCB	7	34	41	0.199
	Non-GCB	4	45	49	

DEL: Double-expressor lymphoma, AID: Activation-induced cytidine deaminase, UNG: uracil-DNA glycosylase, GCB: Germinal center B-cell

Cell of origin subtype was determined for all patients according to Hans algorithm. Forty-one cases (46%) were GCB and 49 cases (54%) were non-GCB subtypes. There was no significant prognostic difference among patients with GCB or non-GCB subtypes ( $p=0.707$ ).

Cases were classified into two groups according to the cut-off value for AID antibody that was accepted as 20% staining of tumor cells. Sixteen cases showed immunoreactivity for AID antibody in more than 20% of tumor cells, whereas 74 cases did not show any immunoreactivity for AID antibody. Cases were divided into two groups as: immunexpression for UNG antibody more or less than 50% of cells. Immunostaining in more than half of the neoplastic cells was observed in 17 cases (18.9%). No significant difference was observed between groups for both antibodies in terms of overall survival ( $p=0.330$  and  $p=0.559$ ). In 42 cases (46.7%), more than 50% of the neoplastic cells had p53 immunexpression and in 48 cases (53.3%), less than 50% neoplastic cells had p53 immunexpression. There was no difference between these groups in terms of overall survival ( $p=0.740$ ). The loss of expression with MLH1, MSH2, MSH6 and PMS2 was not observed in any tumor sample.

Multivariate analysis demonstrated that age, MYC expression and Ki67 expression were independent prognostic factors.

#### 4. DISCUSSION

It was first reported that double-expressions of MYC and BCL2 in DLBCL were prognostic factors by earlier studies of Johnson et al. and Green et al. [5-6]. Numerous studies have contributed to our knowledge about these cases. DLBCL cases that show overexpression with both MYC and BCL2 antibodies with the cut-off values of 40% and 50% respectively, are defined as double-expressor lymphoma, according the last WHO lymphoma classification update [3,15]. It is advised to identify these cases because of the fact that DEL subset in DLBCL may be related with poor prognosis. Our study focused on immunohistochemical overexpression of MYC and BCL2 in DLBCL. Even though, there were different threshold values at earlier papers, 40% and 50% cut-off values are accepted widely nowadays. Thereby, we determined DEL cases as parallel to the WHO suggestion. Incidence of the DEL subset in DLBCL was reported as between 20%-35% in previous studies with different cut-off values but the incidence of DEL cases in our study was 12.2% [15].

It has been stated that DEL subset is associated with a poor prognosis in DLBCL in many studies. Firstly, Johnson et al. and Green et al., reported that double-expressions of MYC and BCL2 were prognostic factors independent of genetic rearrangement of MYC and BCL2 genes [5,6]. However, our study showed that the association regarding the role of DEL may not be reliable. Our results suggested that DEL cases showed poor clinical courses, but these were not independent predictors of poor survival. Although, inadequate sample size and limited number of DEL cases may have limited our ability to detect significant prognostic differences, there are some other publications that support our data. Therefore, the role of DEL as prognostic subgroup is still controversial. In this study, we found that MYC expression in more than 40% of tumor cells detected by immunohistochemistry were associated with shorter OS in DLBCL irrespectively of the NCCN-IPI scores and Ann-Arbor stage. In multivariate analysis, we confirmed that MYC overexpression was an independent prognostic factor in DLBCL. On the other hand, BCL2 expression more than 50% of tumor cells showed no significant association with poor OS. Although, many studies support poor prognostic role of DEL subset, it will be better to confirm the role of DEL with further studies with large data-sets or meta-analyses. The other limitation of our analysis was the lack of FISH analysis, thus double-hit lymphoma subgroup which was associated with poor prognosis could not be determined. This made study population heterogeneous in terms of cell origin.

The affect of primary localization of tumor to OS is controversial, because of several reasons including classification problems and difficulties of determination of tumor location [17]. It is still a controversial issue whether Waldeyer's ring tumors are nodal or not. Some tumors may be located at or invade both nodal and extranodal sites and it is not easy to detect actual origin of the



tumor. In this study, we detected the localization according to biopsy site and extranodal tumors, especially Waldeyer's ring and the bones tended to show better clinical course but that was not statistically significant. In the literature, studies do not have consensus about prognostic importance of primary tumor location. However, better outcomes in the head and neck DLBCLs are seen in some studies [18-21]. Patient age, Ann Arbor stage and NCCN-IPI scores were important predictors of OS in our study as parallel to studies in literature [22-28]. Meanwhile, in our study, not reaching all IPI scores and stages of patients were also considered as a limitation.

The cases in our study were examined in two groups as GCB and non-GCB according to gene expression profiles. They were determined by immunohistochemical Hans algorithm methods [29]. There was no significant difference in overall survival between GCB and non-GCB groups in our study. Although, the Hans algorithm has an acceptable application validity, it is reported that its efficacy is limited in terms of identifying 10-15% of the cases, which cannot be classified by gene expression profiles, repeatability problems, application errors, and weak prognostic value [30-35].

AID and UNG are DNA base excision repair proteins that have important role during B-cell maturation. They create DNA breaks and mutations in physiological processes as somatic hypermutations and class switch recombination [36]. Since, these proteins take role in the formation and repair of DNA breaks, which cause translocations and may have a role in lymphoma etiopathogenesis. Immunostaining of the AID protein has been reported in several lymphomas, especially in DLBCL and Hodgkin's lymphoma. There are studies showing that AID immunostaining may be associated with poor prognosis in patients with chronic lymphocytic leukemia / small lymphocytic lymphoma (CLL / SLL) and follicular lymphoma. We have limited knowledge about the expression of UNG in DLBCL [9, 37-42]. The loss of UNG and mismatch repair proteins in murine patients have been shown to increase the rate of mutation and cause DLBCL-like disease, while only loss of UNG is shown to be protective [42]. In our study, there was no relation between the expression of AID/UNG proteins and overall survival or DEL status. Few publications indicate that the loss in the immunexpressions of mismatch repair proteins in DLBCL cases is not expected [43,44]. Consistently, we did not see any loss of these proteins in our study.

It is reported that overexpression of p53 protein may be associated with mutations in the p53 gene and lymphomagenesis [45]. There are reports that show relationship between p53 expression and MYC protein expression and / or MYC gene translocation in DLBCL [10, 46-48]. In some studies, increased p53 immunoeexpression has been reported to be a poor prognostic factor in DLBCL [46,47,49,50]. In our study, there was no correlation between p53 expression and overall survival or DEL subgroup. The correlation between immunexpressions of p53 and MYC were solely observed.

In conclusion, our result did not demonstrate that double-expressor lymphoma was associated with poor outcomes, although, we showed a relationship between low overall survival

and MYC expression. We did not detect any significant relation between AID, UNG and p53 immunexpressions with double-expressor lymphoma and overall survival. It had been concluded that mismatch repair proteins did not play a significant role in the pathogenesis of DLBCL due to the lack of loss of immunoeexpression.

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### Compliance with Ethical Standards

**Ethical Approval:** This study was approved by Marmara University Clinical Research Ethics Committee (Protocol number: 09.2016.578). All methods were performed in accordance with the relevant guidelines regulations.

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**Conflict of Interest:** The authors declare that they have no conflict of interest.

**Authors' Contributions:** Design: MHT, Supervision: TT, SB, Resources: MHT, SB, TE, TT, Materials: SB, TE, TT, Data collection and/or processing: MHT, SB, TE, TT, Analysis and/or interpretation: MHT, SB, Writing manuscript: MHT, Critical review: SB.

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