Original Article

BCL2 and Subtype as Prognostic and Predictive Markers of Diffuse Large B-cell Lymphoma

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Abstract

Introduction: Since patients with similar International Prognostic Index scores have varied outcomes, molecular signatures, including BCL2 overexpression have been studied to prognosticate diffuse large B-cell lymphoma (DLBCL), which have shown varied outcomes. **Objective:** The aim of this study is to correlate BCL2 protein expression with survival in two biologic subgroups of DLBCL. **Materials and Methods:** A total of 112 adults with DLBCL between 2008 and 2012 were identified. BCL2 overexpression was determined using immunohistochemistry. **Results:** Median survival was greater in BCL2 negative (n = 52) than positive (n = 44) (36 vs. 24.5 months; P = 0.003). In nongerminal center B-cell type (NGCB), BCL2 negativity had a survival advantage over BCL2 positive (36.5 vs. 17 months; P = 0.02), similarly in GCB (36 vs. 33 months; P = 0.032). Of 109, 66 received CHOP and 43 R-CHOP. R-CHOP arm had a significant survival advantage over CHOP arm (38 vs. 24 months; $P < 0.05$). In CHOP group, GCB had a survival advantage over NGCB (32 vs. 14 months; $P < 0.05$). In R-CHOP group, no significant difference was seen. BCL2 negativity had a survival advantage in CHOP (31 vs. 20.5 months; $P < 0.05$) as well as R-CHOP group (39 vs. 26.5 months; $P < 0.05$). Analysis was performed in each treatment arm (CHOP and RCHOP) based on BCL2 expression (positive or negative) in GCB and NGCB arms. No statistically significant difference was seen in the four arms. **Conclusions:** BCL2 although an indicator of poor outcome, its use to predict outcomes alone in the absence of study of the expression of concomitant markers, such as myc/BCL6 would cause a bias in results. Furthermore, its relevance in the rituximab era needs further validation.

Keywords: *BCL2, CHOP, diffuse large B cell lymphoma, germinal centre B-cell type, nongerminal center B-cell type, R-CHOP*

Introduction

Diffuse large B-cell lymphoma (DLBCL) is a heterogeneous disease that displays a highly variable clinical outcome. International Prognostic Index (IPI) has been used to prognosticate and risk stratify the patients with DLBCL. Furthermore, the favorable prognosis of germinal center B-cell-like (GCB) subtype has been confirmed in some, but not other studies. BCL2 is an antiapoptotic factor required for normal development and differentiation of B cells. BCL2 overexpression causes resistance to chemotherapy and provides a survival advantage for malignant B cells. The main mechanism of overexpression is t(14;18)(q32;q21) in which IgH enhances the BCL2 expression. Otherwise, it is also seen in ABC subgroup of DLBCL which lacks this translocation. The prognostic impact of BCL2 overexpression is seen in various studies. Some studies have shown no difference, whereas others have shown reduced survival.[1]

In the postrituximab era, the prognostic significance of BCL2 expression has been found to be controversial,[2] with some studies showing and others not showing any prognostic significance.

In this study, we have analyzed the correlation of BCL2 protein with survival in two biologic subgroups of DLBCL. Furthermore, we have compared the prognosis of BCL2 overexpression in the DLBCL patients treated with CHOP and R-CHOP chemotherapy.

Materials and Methods

Adult patients with DLBCL diagnosed between 2008 and 2012 were identified from the tumor registry of a tertiary level hospital in south India. Patients fulfilled the following criterion to be included in the study (1) histologically proven diagnosis

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of DLBCL, according to the WHO classification of tumors of hematopoietic and lymphoid tissues (2) availability of adequate amount of paraffin-embedded biopsy material (3) age >18 years (4) no previous treatment (5) no previous neoplasm and no second primary malignancy (6) no severe coincident diseases (7) did not have primary central nervous system lymphoma or posttransplant lymphoproliferative disorder or transformed lymphoma. All patients were staged using the Ann Arbor staging system and evaluated using the IPI. Hans algorithm was used to categorize into GCB and non-GCB (NGCB) subtype.

Formalin-fixed paraffin-embedded sections were utilized for immunohistochemistry (IHC). These tissues were stained with conventional H and E, and immunostaining to demonstrate CD10, BCL6, and MUM-1. Five-micrometer sections were cut and stained with antibodies to BCL2. Immunostains were considered positive if 30% or more of tumor cells were stained by the antibodies. Evaluation of the immunostaining was performed within 7 days to avoid antigen degradation. The kit used for BCL2 immunostaining was Leica Biosystems.

Overall survival (OS) was analyzed from the date of initial diagnosis to the date of death of any cause, or last follow-up visit. Chi-square test was used to compare the categorical data. Survival analysis was performed using the Kaplan–Meier method, and the log-rank test was used for comparing the variables. The value of $P < 0.05$ was considered statistically significant. Statistical analysis was performed using the R software.

The standard CHOP regimen included cyclophosphamide 750 mg/m^2, doxorubicin 50 mg/m^2, and vincristine 1.4 mg/m^2 (maximum dose 2 mg). Rituximab was administered at the standard dose of 375 mg/m^2 on day 1 with CHOP regimen on day 2. The assessment of treatment response was done in accordance with International Working Group recommendations for response criterion for non-Hodgkin's lymphoma.

Results

A total of 112 patients of DLBCL were identified and included in the study. The median age was 54 years (18–78 years), with a male/female ratio of 1.8:1. Bone marrow involvement was seen in 15 patients (13%) and B symptoms in 48 patients (43%). Disease was localized (Stage I/II) in 73 (65%) patients and advanced (Stage III/IV) in 39 (35%) patients. Mean follow-up was 60 months.

Subtype analysis into GCB and non-GCB was done in all the patients, and 64 were recognized as GCB and 48 as non-GCB subtype. No significant correlation was found among the groups with regard to age, B symptoms, bulky disease, stage, and extra nodal involvement. Median OS in GCB subtype was 34 months and in non-GCB was 22 months ($P = 0.043$) [Figure 1].

A cut-off of 30% was used to classify as BCL2 positive or negative. The relationship between baseline clinical features and BCL2 was done using Chi-square test. No significant association was detected with age, B symptoms, bulky disease, stage, extranodal involvement, and treatment received (CHOP or R-CHOP). Median survival was greater in BCL2 negative patients ($n = 52$) as compared to the positive ones ($n = 44$) (36 vs. 24.5 months; $P = 0.003$). In the non-GCB type, patients with BCL2 negativity had a survival advantage over the BCL2 positive ones (36.5 vs. 17 months; $P = 0.02$), similarly in the GCB subtype (36 vs. 33 months; $P = 0.032$). In the high IPI arm, BCL2 negativity had a survival advantage (30 vs. 18.5 months; $P = 0.002$). In the low IPI arm, similar results were obtained (44 vs. 39 months; $P = 0.037$) [Table 1].

Further subgroup analysis was performed based on treatment received. Treatment was received by

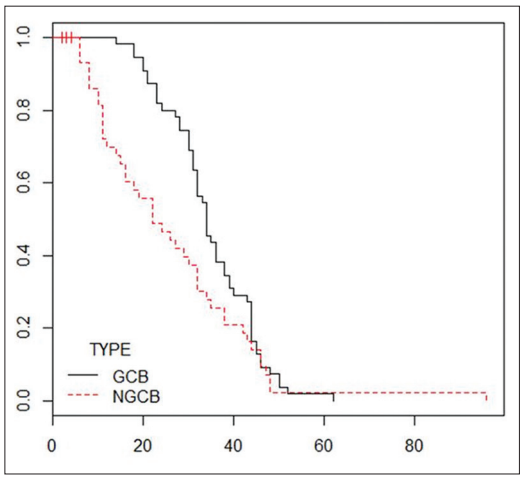

Figure 1: GCB vs non-GCB

Table 1: Summarizing the impact of immunohistochemistry on survival irrespective of treatment received

	n	Median OS	*P*
BCL2			0.0003
Positive	44	24.5	
Negative	52	36	
GCB type			0.032
BCL2 positive	18	33	
BCL2 negative	39	36	
Non-GCB type			0.02
BCL2 positive	26	17	
BCL2 negative	13	36.5	
High IPI			0.002
BCL2 positive	30	18.5	
BCL2 negative	25	30	
Low IPI			0.037
BCL2 positive	14	39	
BCL2 negative	27	44	

OS: Overall survival, GCB: Germinal centre B-cell type, IPI: International Prognostic Index

109 patients, CHOP in 66 and R-CHOP in 43 patients. R-CHOP arm had a significant survival advantage over the CHOP arm (38 vs. 24 months; $P < 0.05$) [Figure 2]. The two treatment arms were first analyzed independently based on cell type (GCB/NGCB) and BCL2 (positive/negative). In the CHOP group, GCB had a survival advantage over NGCB (32 vs. 14 months; $P < 0.05$), whereas, in the R-CHOP group, no significant difference was seen (44 m 34.5 m; $P = 0.76$). BCL2 negativity had a survival advantage in CHOP (31 vs. 20.5 months; $P < 0.05$) as well as R-CHOP group (39 vs. 26.5 months; $P < 0.05$). Later, analysis was performed in each treatment arm (CHOP and R-CHOP) based on BCL2 expression (positive or negative) in GCB and NGCB arms. No statistically significant difference was seen in any of the four arms; 29 versus 32 months in ($P = 0.173$) in BCL2 positive versus negative in CHOP-GCB arm, 11 versus 16 months ($P = 0.71$) in BCL2 positive versus negative in CHOP-NGCB arm, 34 versus 44 months ($P = 0.28$) in BCL2 positive versus negative in R-CHOP-GCB arm, and 26 versus 40 months ($P = 0.28$) in BCL2 positive versus negative in R-CHOP-NGCB arm [Table 2].

To summarize, GCB and BCL2 negativity had a survival advantage independently. On subgroup analysis, BCL2 negativity had a survival advantage irrespective of GCB and IPI status. In two treatment arms, GCB had a survival advantage in CHOP arm, and BCL2 negativity had a survival advantage in both CHOP and R-CHOP arm. However, on further subgroup analysis based on BCL2 (positive/negative) in GCB/NGCB arms in CHOP/R-CHOP groups, no significant differences were noticed. Therefore, the independent prognostic significance of BCL2 is nullified when grouping is done on the basis of cell type (GCB/NGCB). Furthermore, the addition of rituximab nullifies the poor prognostic effect of NGCB subtype.

Discussion

DLBCL is an aggressive lymphoma and IPI is used as a prognostic score in the clinical setting to determine outcomes. Since patients with similar IPI scores have varied outcomes, molecular signatures have been studied to prognosticate patients with DLBCL. Studies have revealed that expression of certain genes is associated with poor outcomes such as the study by Lossos *et al*. which showed that expression of 6 genes, including BCL2 of the many studied, were prognostic in DLBCL.[3] BCL2 and myc overexpression have been to be associated with poor outcomes and revised IPI scores such as the B-IPI have been proposed to predict high-risk patients.[4] Our study was undertaken to determine the relation between BCL2 overexpression and outcomes in these patients.

According to available studies, BCL2 rearrangements are detectable in 12% to 30% of DLBCL[5] and the t(14;18)(q32;q21) has been identified in 18%–20% of patients with *de novo* DLBCL.[6]

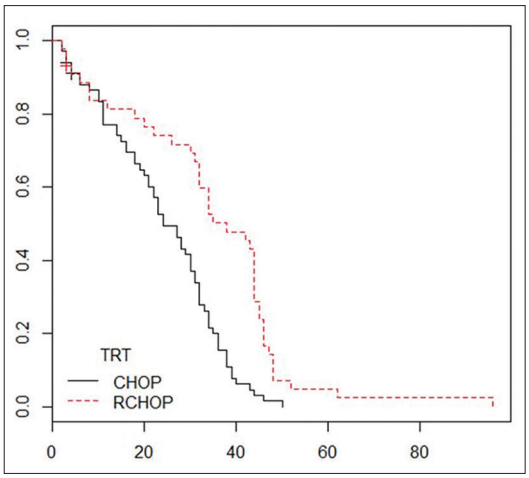

Figure 2: CHOP vs RCHOP

Table 2: Summarizing the impact of subtype and BCL2 in CHOP and RCHOP group

	n	Median OS	*P*
CHOP	66	24	<0.05
RCHOP	43	38	
CHOP group			
GCB	40	32	<0.05
NGCB	25	14	
BCL2 positive	30	20.5	0.005
BCL2 negative	28	31	
GCB			
BCL2 positive	12	29	0.173
BCL2 negative	25	32	
NGCB			
BCL2 positive	17	11	0.71
BCL2 negative	3	16	
RCHOP group			
GCB	20	44	0.76
NGCB	22	34.5	
BCL2 positive	16	26.5	0.003
BCL2 negative	23	39	
GCB			
BCL2 positive	5	34	0.28
BCL2 negative	13	44	
NGCB			
BCL2 positive	9	26	0.28
BCL2 negative	10	40	

OS: Overall survival, NGCB: Nongerminal center B-cell like

BCL2 overexpression has been identified with different methods, such as IHC, Western blot, chimeric genomic hybridization, fluorescence *in situ* hybridization, and cDNA microarray (gold standard) across studies. Studies using different techniques have shown varied results. In studies using Southern blot analysis, the presence of BCL2 gene rearrangement did not appear to be predictive of survival, with only one study reporting worse survival. In fact, some studies suggest that patients with BCL2 gene rearrangements have better survival. Multiple studies have

looked at the expression of BCL2 using immune stains, and most have found no difference in OS. Some studies have found that BCL2 expression is associated with a significantly worse OS.[7]

BCL2 positivity was identified using IHC in our patients. Neither the t(14;18) nor the NF-kB pathway overexpression which are thought to be the primary pathways for BCL2 overexpression in the GCB and ABC subtypes,[7] respectively, were identified in our patients.

The outcomes of patients with DLBCL with BCL2 overexpression have been variable with various studies.[8,9] This study showed that the OS for patients with DLBCL as a single entity, had a poorer survival when there was BCL2 overexpression, with poorer survival seen in both ABC and GCB subtypes when BCL2 positive. This is in concordance with the study by Rantanen *et al*.[8] which showed that BCL2 positivity had a bearing on survival and was associated with poor outcomes, especially in those who had received anthracycline-based chemotherapy. This study, however, had not studied the subtypes separately, and none had received rituximab-based chemotherapy. Results different from these were seen in the study by Iqbal *et al*.[1] which showed that BCL2 overexpression was associated with poor survival in the non-GCB and not the GCB subtype. However, BCL2 overexpression as such was not associated with a poor survival in DLBCL as a whole. These studies were in the prerituximab era.

In DLBCL, the inhibitory action of BCL2 on apoptosis is hypothesized as a cause of chemotherapy resistance and this notion was supported by several clinical studies in the prerituximab era demonstrating an inverse correlation between BCL2 protein expression and survival. Studies carried out in the postrituximab era, however, raise the question of whether BCL2 remains a biomarker of treatment failure and many studies have demonstrated that this is no longer the case.[2]

The significance of BCL2 overexpression was re-evaluated in patients treated with R-CHOP in the GELA trial. In contrast to patients treated with CHOP alone, no correlation between BCL2 overexpression and survival was seen in patients treated with R-CHOP, implying that the addition of rituximab had overcome its negative influence. The article by Sehn *et al*. also studied the influence of BCL6 in the rituximab era and concluded that there are no prognostic molecular markers in the rituximab era for patients treated with R-CHOP.[10] The study showed that expression of the BCL2 protein was not predictive of OS in either group treated with rituximab, or the group treated with standard chemotherapy alone. In addition, we confirmed that rituximab significantly benefited BCL2 positive but not BCL2 negative cases, which is in concordance with recently published data by Jovanovic *et al*.[11]

The study showed that BCL2 had an overbearing on the survival in all patients irrespective of the IPI scores of these patients, with lower IPI score patients having better OS as a whole. The effect of chemotherapy on these patients showed that addition of rituximab increased the OS in all stages of disease and in those with poor performance status too.

Patients in both CHOP and R-CHOP arms had a better survival when BCL2 negative. Analysis with respect to subtype, however, showed that rituximab reduced the difference in outcome in the ABC subtype, seconding the fact that NF-kβ pathway overactivation is a primary pathway seen in the ABC subtype, and that rituximab does, in fact, nullify the poor prognosis seen in this subset by reducing the NF-Kβ overactivation. This hypothesis was stated in the study by Iqbal *et al*.[12] who studied the outcomes of these patients with the addition of rituximab chemotherapy since all the previous studies had conflicting results on the prognostic influence of rituximab on survival outcomes of patients with BCL2 positivity. This study showed that addition of rituximab nullified the poor prognosis associated with the ABC subtype, however there was no improvement in the survival in the GCB subtype. The proposed explanation was that rituximab downregulated the NF-kB pathway overactivation that is responsible for BCL2 overexpression the non-GCB subtype of DLBCL. This was thought to increase the susceptibility to chemotherapy in this subset of patients. Different mechanisms by which BCL2 suppresses apoptosis have been reported. Among them are the ability to act as an antioxidant, block caspase activity, and regulate calcium flux. In addition, interleukin 10 (IL-10) is a known promoter of BCL2 expression in hematopoietic cells, and *in vitro* studies have shown that the rituximab downregulates IL-10 expression and consequently, BCL2 protein expression. These features common to BCL2 function, chemotherapeutic drugs, and CD20 signaling suggest the mechanisms that might be involved in the reversal of resistance to chemotherapeutic agents.[13]

When the analysis was performed for whether the expression of BCL2 (positive/negative) was of prognostic significance in those who had received CHOP/R-CHOP chemotherapy as separate subgroups, it was seen that BCL2 overexpression had no bearing on outcomes of patients in both these treatment groups. The discrepancy seen in outcomes between BCL2 overexpression and survival in DLBCL as a whole as against specific subtypes which were BCL2 positive could be due to the small patient population in our study.

The reason for varied results between our and other studies could be due to the following, which are also the limitations of our study:

1. Factors that could be contributory to modifying survival, such as CD10, BCL6,[14] MUM-1,[15] bak,

bax, bad, BCL-Xs,[8] which are all associated with poorer outcomes in DLBCL have not be studied along with BCL2 overexpression in the study and could be confounding factors for survival
2. The varied techniques to identify BCL2 expression in various studies could also be a factor causing inconsistent survival results in studies. The study by Rantanen *et al*.[8] had found that BCL2 positivity conferred poor survival outcomes in those detected by IHC and not by western blot technique. Hence, whether IHC alone (used in our study) without further confirmation of BCL2 overexpression could yield reliable results with respect to survival outcomes have to be researched further
3. The study population was a small number.

Conclusions

BCL2 as a prognostic marker although an indicator of poor outcome in those with DLBCL, its use to predict outcomes alone in the absence of study of the expression of concomitant markers such as myc/BCL6 would cause a bias in results. Furthermore, its relevance in the rituximab era needs further validation.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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