

# Correlations between *BCL6* rearrangement and outcome in patients with diffuse large B-cell lymphoma treated with CHOP or R-CHOP

Jesse Shustik,<sup>1</sup> Guangming Han,<sup>2</sup> Pedro Farinha,<sup>2</sup> Nathalie A. Johnson,<sup>2</sup> Susana Ben Neriah,<sup>2</sup> Joseph M. Connors,<sup>1</sup> Laurie H. Sehn,<sup>1</sup> Douglas E. Horsman,<sup>2</sup> Randy D. Gascoyne,<sup>2</sup> and Christian Steidl<sup>2</sup>

<sup>1</sup>Division of Medical Oncology and <sup>2</sup>Department of Pathology and Laboratory Medicine, BC Cancer Agency, University of British Columbia, Vancouver, Canada

## ABSTRACT

### Background

*BCL6* gene rearrangement is the most frequent chromosomal abnormality in diffuse large B-cell lymphoma, a malignancy characterized by genetic heterogeneity and wide variability in clinical outcome. The prognostic significance of *BCL6* rearrangement has not been evaluated in the context of rituximab therapy for diffuse large B-cell lymphoma. We analyzed the effect of the *BCL6* rearrangement on survival in patients with diffuse large B-cell lymphoma treated with CHOP and CHOP plus rituximab (R-CHOP).

### Design and Methods

*BCL6* rearrangement status was analyzed by fluorescence *in situ* hybridization with break-apart probes in 164 patients with diffuse large B-cell lymphoma treated with CHOP (n=65) or R-CHOP (n=99). Cell-of-origin immunophenotype including *BCL6* protein expression were determined by immunohistochemistry on a tissue microarray.

### Results

*BCL6* rearrangement was detected in 19.5% of cases. The presence of the gene rearrangement was associated with a non-germinal center B-cell immunophenotype ( $P=0.006$ ), and showed no correlation with *BCL6* protein expression. A trend toward inferior overall survival was observed in association with the *BCL6* rearrangement among patients treated with R-CHOP ( $P=0.08$ ), but not among patients treated with CHOP ( $P=0.64$ ). However, *BCL6* rearrangement also correlated with a high International Prognostic Index score ( $P=0.02$ ), and did not demonstrate independent prognostic value by multivariate analysis.

### Conclusions

The introduction of rituximab may have altered the prognostic impact of *BCL6* gene rearrangement in patients with diffuse large B-cell lymphoma. However, prospective analysis within large randomized clinical trials will be needed to clarify the prognostic significance of this biomarker in the rituximab era.

Key words: diffuse large B-cell lymphoma, biomarkers, *BCL6*, FISH, rituximab.

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Correspondence: Christian Steidl, Department of Pathology, British Columbia Cancer Agency, 600 West 10th Avenue, Vancouver, BC V5Z 4E6, Canada. E-mail: csteidl@bccancer.bc.ca

## Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common subtype of non-Hodgkin's lymphoma, accounting for 30-40% of newly-diagnosed cases.<sup>1</sup> Although curable in the majority of cases with anthracycline-based combination chemotherapy and the monoclonal antibody rituximab, approximately 30-40% of patients with DLBCL will relapse after standard first-line therapy.<sup>2,3</sup> This variability in clinical outcome likely relates to genetic heterogeneity within DLBCL, reflected in a wide array of cytogenetic and molecular abnormalities. Recently, microarray gene expression studies have identified multiple genes of potential prognostic significance in DLBCL, and have led to the subdivision of DLBCL into two major biological categories based on their presumed cell of origin: germinal center B-cell (GCB), and activated B-cell (ABC).<sup>4</sup> However, the prognostic value of genetic markers in DLBCL remains controversial, and these have not yet been incorporated into routine clinical practice.<sup>5</sup>

Rearrangement of the *BCL6* proto-oncogene at chromosome band 3q27 is the most frequent cytogenetic abnormality in DLBCL, occurring in up to 35% of cases.<sup>6-8</sup> The *BCL6* gene, a zinc-finger transcription factor, may be translocated with diverse partners in DLBCL, including both immunoglobulin (*IGH*, *IGK*, *IgL*) and non-*IG* loci.<sup>9,10</sup> Clinical studies investigating the prognostic impact of *BCL6* rearrangement in DLBCL have yielded contradictory results, variably demonstrating favorable,<sup>6</sup> intermediate,<sup>7,8,11</sup> and adverse outcomes<sup>12,13</sup> in association with this abnormality.

An association has recently been reported between *BCL6* rearrangement and ABC phenotype.<sup>14</sup> This cell-of-origin profile was initially demonstrated as an adverse biological marker in DLBCL patients treated with CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone), and has been shown to retain its predictive impact in patients treated with CHOP plus rituximab (R-CHOP).<sup>15</sup>

The introduction of rituximab to standard first-line therapy has significantly improved clinical outcome in DLBCL, and may alter the prognostic impact of both clinical and biological markers in this disease.<sup>2,3</sup> The prognostic significance of *BCL6* rearrangement has not been re-evaluated since the introduction of rituximab into DLBCL therapy. In this study we used tissue microarray-based fluorescence *in situ* hybridization (FISH) to analyze *BCL6* rearrangement status in a retrospective cohort of patients with DLBCL. The objectives of this study were to: (i) compare the effect of *BCL6* rearrangement on survival in DLBCL patients treated with CHOP and R-CHOP, and (ii) evaluate the relationship between *BCL6* rearrangement and other clinical and biological prognostic variables in this disease, including cell-of-origin phenotype.

## Design and Methods

### Patients' samples

We included all patients identified through the Centre for Lymphoid Cancer database of the British Columbia Cancer Agency (BCCA) who met the following criteria: (i) confirmed diagnosis of DLBCL (excluding primary mediastinal B-cell lymphoma) following a pathology review; (ii) treated with either CHOP alone or in combination with rituximab immunotherapy (R-CHOP); (iii) available diagnostic paraffin material on a tissue microarray; (iv) negative for human immunodeficiency virus; (v) treated at the BCCA between 1999 and 2007. Ethical approval for this study was obtained from the University of British Columbia – BCCA Research Ethics Board.

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### Fluorescence *in situ* hybridization and immunohistochemistry on tissue microarrays

Archived, formalin-fixed, paraffin-embedded, diagnostic biopsy specimens were selected for construction of the tissue microarrays and 0.6 mm duplicate cores were obtained from representative areas containing large B cells with typical morphology. FISH was performed according to a standard protocol for paraffin-embedded material, described elsewhere,<sup>14,16</sup> using commercially available Vysis LSI *BCL6* Dual Color, Break-Apart Rearrangement Probes (Abbott Molecular, IL, USA). Cases were recorded as having *BCL6* rearrangement (*BCL6*<sup>+</sup>) if break aparts occurred in at least one signal of more than 5% of nuclei. All other signal constellations were regarded as negative (*BCL6*<sup>-</sup>). Immunohistochemical analysis was performed using monoclonal antibodies for BCL2 (Dako, clone 124, Denmark), BCL6 (Ventana Medical Systems, Tucson, Arizona), CD10 (Ventana Medical Systems, Tucson, Arizona), and MUM1 (Dako, Denmark), following routine protocols for automated immunohistochemistry on the Ventana Benchmark XT (Ventana Medical Systems, Tucson, Arizona). Cases were then categorized as GCB-like or non-GCB using a standard algorithm.<sup>17</sup> Accordingly, for each case, the core with the highest percentage of tumor cells stained was used for analysis. Cases were considered positive if 30% or more of the tumor cells were stained with an antibody.

### Statistical analysis

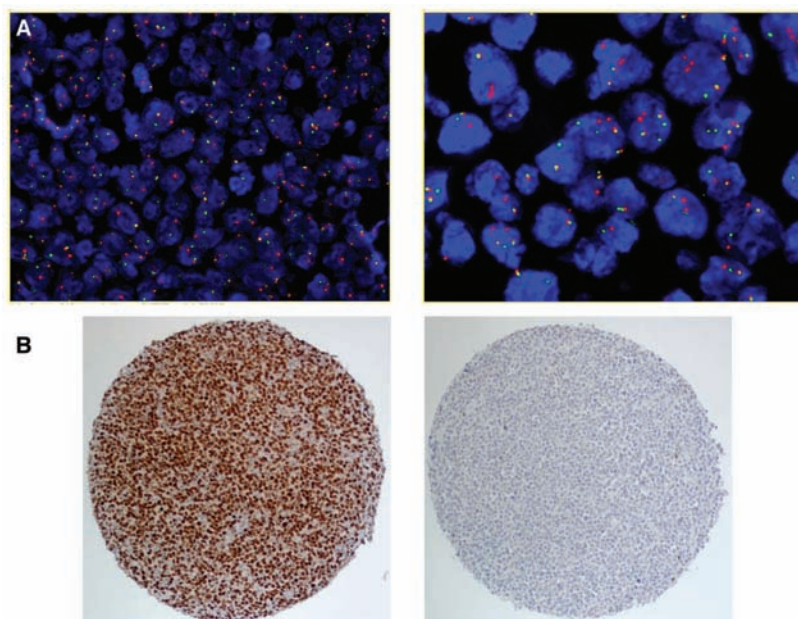
Group comparisons were performed by means of  $\chi^2$  and Student's *t* tests. For time to event analyses we used SPSS software version 11.0.0, applying Kaplan-Meier survival estimates and univariate and multivariate Cox proportional hazard models with the end-point of overall survival, defined as the time from initial diagnosis to death from any cause. *P* values less than 0.05 were considered statistically significant.

## Results

### Patients' characteristics

A total of 174 patients with DLBCL who met all inclusion criteria were identified. FISH was successfully performed in 164 out of these 174 cases (94.3%). *BCL6* rearrangement was detected in 32 out of the 164 cases (19.5%). Representative *BCL6*<sup>+</sup> FISH images of the tissue microarray sections are shown in Figure 1A.

The baseline clinical characteristics of the patients, divided according to *BCL6* rearrangement status, are listed in Table 1. There were no significant differences in initial treatment regimen between the two subgroups, with similar proportions of *BCL6*<sup>+</sup> (15/32) and *BCL6*<sup>-</sup> (84/132) patients receiving rituximab as part of their initial therapy. A higher proportion of patients in the *BCL6*<sup>+</sup> subgroup had a high-intermediate or high-risk International Prognostic Index (IPI) score (IPI 3-5) (62.5% versus 40.2%, *P*=0.02). Stage IE/IIIE cases were designated as 'primary extranodal



**Figure 1.** (A) Fluorescence *in situ* hybridization on formalin-fixed, paraffin-embedded tissue sections on a tissue microarray. Left panel: interphase nuclei with *BCL6* break apart (*BCL6*<sup>+</sup>). The most common signal pattern is one fusion signal (yellow) and two split signals (1 red, 1 green) visualized at 200x magnification. Right panel: *BCL6*<sup>+</sup> interphase nuclei with break apart (split signals) and polyploidy (multiple fused signals) of the *BCL6* locus (high power field). (B) Immunohistochemistry for BCL-6 on tissue microarray cores. Left panel: positive staining in virtually all large B cells. Right panel: negative staining.

disease', while all other cases were considered as 'primary nodal disease'. The frequency of primary extranodal disease was not significantly different between the *BCL6*<sup>+</sup> and *BCL6*<sup>-</sup> subgroups (13% versus 25%, *P*=NS).

Table 2 shows the patients' immunohistochemical characteristics according to *BCL6* rearrangement status. *BCL6* staining and cell-of-origin classification<sup>17</sup> was successfully performed in 162 of the 164 patients. Representative *BCL6* staining results are shown in Figure 1B. Results of *BCL2* staining were available for 115 of the 164 (70%) patients. The presence of *BCL6* rearrangement was significantly correlated with cell-of-origin; 68% of *BCL6*<sup>+</sup> patients had a non-GCB subtype, as compared with 48% of *BCL6*<sup>-</sup> patients (*P*=0.006). No association was found between *BCL6* rearrangement and either *BCL6* (*P*=0.78) or *BCL2* (*P*=0.95) protein expression.

### Survival

The median duration of follow-up was 2.9 years (range, 0.3-8.7). Rituximab use was associated with a significant survival improvement among *BCL6*<sup>-</sup> patients (5-year overall survival, 70.1% versus 45.0%, *P*=0.009), but not among *BCL6*<sup>+</sup> patients (5-year overall survival, 45.3% versus 41.2%, *P*=0.70). Kaplan-Meier analysis of the entire cohort revealed a trend toward decreased overall survival in patients with a *BCL6* rearrangement, though this did not reach statistical significance (5-year overall survival, 59.4% versus 43.4%, *P*=0.07). Within treatment groups, *BCL6* rearrangement was associated with a trend toward inferior overall survival among patients treated with R-CHOP (5-year overall survival, 45.3% versus 70.1%; *P*=0.08), but not among patients treated with CHOP (5-year overall survival, 41.2% versus 45.0%; *P*=0.64) (Figure 2). No significant association was found between *BCL6* protein expression and overall survival in the whole cohort (5-year overall survival, *BCL6*<sup>+</sup> versus *BCL6*<sup>-</sup>, 52.2% versus 31.8%, *P*=0.11).<sup>18</sup>

Subgroup analysis of overall survival was performed according to the site of presentation of disease and the patients' age. Survival analysis limited to patients with primary nodal disease showed that the 5-year overall survival among *BCL6*<sup>+</sup> patients was 39.7% compared with 54.7% in *BCL6*<sup>-</sup> patients (*P*=0.09). Among patients who presented with primary extranodal disease, no difference in overall survival was found between *BCL6*<sup>+</sup> and *BCL6*<sup>-</sup> patients (*P*=0.95). Subgroup analysis by patients' age did not reveal statistically significant associations between gene rearrangement status and overall survival in patients under 65 (*P*=0.38) or those over 65 years of age (*P*=0.24).

In R-CHOP-treated patients, high IPI score (IPI 3-5) predicted a significantly worse overall survival (5-year overall survival, 46.1% versus 77.8%, *P*=0.002), while non-GCB immunohistochemical profile was associated with a trend toward inferior outcome (5-year overall survival, 50.8% versus 76.8%, *P*=0.07). A Cox regression analysis was performed for the R-CHOP-treated cohort using the following four variables: IPI score, cell-of-origin immunophenotype, *BCL2* protein expression, and *BCL6* gene rearrangement status. In multivariate analysis, only high IPI score was found to be an independent adverse prognostic factor for overall survival (*P*=0.002).

### Discussion

This study analyzed the clinical impact of *BCL6* gene rearrangement in a retrospective cohort of DLBCL patients, treated in both the pre- and post-rituximab eras. Our data suggest an association between *BCL6* rearrangement and inferior outcome in patients treated with R-CHOP, but not in patients treated with CHOP. However, *BCL6* rearrangement also correlated with the presence of high-risk clinical features and was not demonstrated to have a prognostic significance independent of the IPI score.



The effect of the *BCL6* rearrangement on outcome in DLBCL has remained uncertain despite previous clinical analyses. In a seminal study by Offit *et al.*, *BCL6* rearrangement was found to be a favorable prognostic marker in DLBCL, possibly due to an association with limited extranodal disease.<sup>6</sup> Most subsequent investigators failed to demonstrate any influence of *BCL6* rearrangement on prognosis in DLBCL, and similarly our analysis did not find that this marker had predictive value in patients treated with CHOP alone.<sup>7,8,11,14</sup> A more recent analysis, restricted to DLBCL patients with primary nodal disease, reported inferior survival in patients with *BCL6* rearrangement, as well as a correlation between this marker and non-GCB immunophenotype.<sup>12</sup> In agreement with our results, Vitolo *et al.* reported a correlation between *BCL6* rearrangement and adverse clinical features,<sup>11</sup> although this has not been a uniform finding in previous studies. These discrepancies may be explained in part by heterogeneity between study populations, due to concomitant genetic alterations, selection biases, and differences in molecular diagnostic techniques. Specifically, rearrangement of the *MYC* oncogene has been found by several authors to impart an inferior prognosis in CHOP-treated DLBCL patients, although this abnormality occurs in only 5-10% of patients<sup>19,20</sup> and *MYC* and *BCL6* rearrangement rarely coexist.<sup>6,8,11</sup>

The addition of rituximab to first-line combination ther-

apy has led to a significant improvement in survival in patients with DLBCL, requiring a re-evaluation of established prognostic markers.<sup>3</sup> Biological prognostic markers in particular may be influenced by changes in standard therapy, because of the differential effects of new agents within biological subgroups. It has recently been reported that *BCL2* protein expression, an adverse prognostic marker in CHOP-treated patients, loses its prognostic effect in the rituximab era, due to a disproportionate benefit from rituximab in *BCL2*<sup>+</sup> cases.<sup>21</sup> In our analysis, the addition of rituximab appeared primarily to benefit patients without *BCL6* rearrangement, and was associated with significantly prolonged overall survival in this subgroup. In contrast, little improvement in outcome was observed with the addition of rituximab among *BCL6*<sup>+</sup> patients, in whom long-term survival remained less than 50% even with R-CHOP. These data suggest a potential value for *BCL6* rearrangement as an adverse predictive biomarker in DLBCL in the rituximab era.

The IPI remains the most successful clinical prognostic instrument in DLBCL.<sup>22</sup> However, the IPI score appears to lose discriminatory power in R-CHOP-treated patients, and in a recent analysis was unable to identify a subgroup with a less than 50% chance of long-term survival.<sup>3</sup> Biological markers may help to refine risk-stratification in DLBCL, and to identify high-risk subgroups of patients who might benefit from intensified or novel therapies. However, to be clinically useful these markers must provide prognostic information complementary to that provided by the IPI. In our study, the presence of a *BCL6* gene rearrangement correlated with high IPI score, and in multivariate analysis this biomarker did not show independent prognostic significance. *BCL6* rearrangement has also shown an association with non-GCB immunophenotype, previously demonstrated to be an unfavorable prognostic marker in CHOP-treated patients with DLBCL.<sup>17</sup> However, in our cohort of R-CHOP-treated patients, cell-of-origin phenotype did not emerge as an independent prognostic variable in multivariate analysis.

The clinical impact of oncogene rearrangements may also depend on the site of disease presentation. DLBCL arising from extranodal sites differs from nodal DLBCL in terms of pathogenesis and cell of origin, and several distinct subtypes of extranodal DLBCL are recognized in the

**Table 1. Clinical characteristics of 164 DLBCL patients, by *BCL6* rearrangement status.**

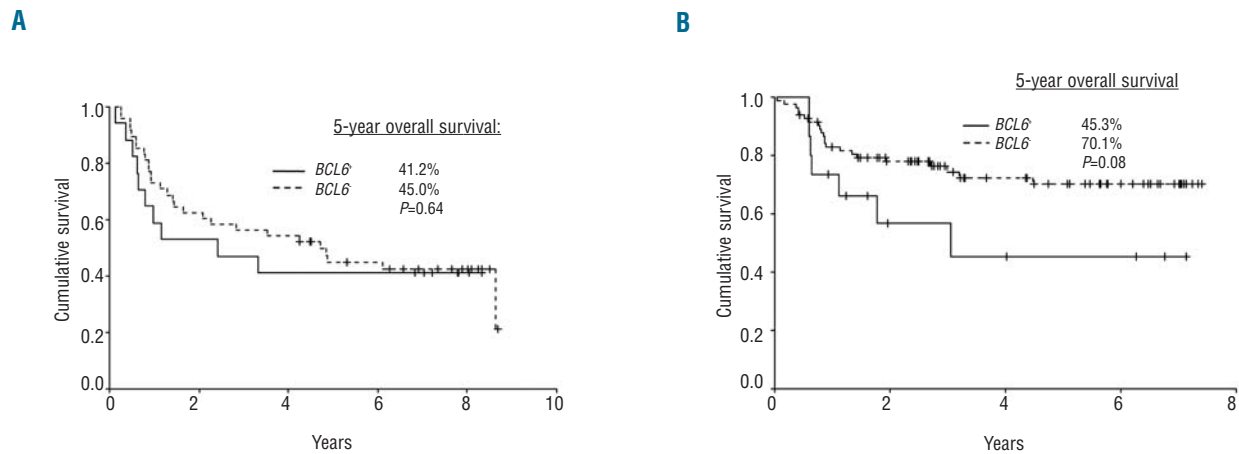
	<i>BCL6</i> <sup>+</sup> † (n=32)	<i>BCL6</i> <sup>-</sup> (n=132)	<i>P</i> value
Age (years), median (range, sd)	65.5 (35-85, 14.1)	63 (16-92, 14.8)	<i>P</i> =NS
<b>Gender (%)</b>			
Male	63	61	<i>P</i> =NS
Female	37	39	
Mass size (cm), median (range, sd)	10 (3-20, 5.4)	6 (1-25, 5.0)	<i>P</i> =NS
<b>Lactate dehydrogenase (%)</b>			
Normal	25	51	<i>P</i> =0.007
Elevated	75	49	
<b>Extranodal sites (%)</b>			
0-1	68	75	<i>P</i> =NS
>1	32	25	
<b>Site of presentation(%)</b>			
Primary nodal	87	75	<i>P</i> =NS
Primary extranodal	13	25	
<b>Stage, Ann Arbor (%)</b>			
I-II	31	43	<i>P</i> =NS
III-IV	69	57	
<b>IPI score (%)</b>			
0-2	38	60	<i>P</i> =0.022
3-5	62	40	

†*BCL6*<sup>+</sup>: *BCL6* gene rearrangement present; *BCL6*<sup>-</sup>: *BCL6* gene rearrangement absent; sd: standard deviation.

**Table 2. Immunophenotypic characteristics of 164 DLBCL patients, by *BCL6* rearrangement status.**

	<i>BCL6</i> <sup>+</sup> (n=32)	<i>BCL6</i> <sup>-</sup> (n=132)	<i>P</i> value
<b>BCL2 protein expression (%)</b>			
Positive	78	71	<i>P</i> =NS
Negative	22	29	
<b><i>BCL6</i> protein expression (%)</b>			
Positive	69	71	<i>P</i> =NS
Negative	31	29	
<b>Cell-of-origin* (%)</b>			
GCB	32	59	<i>P</i> =0.006
Non-GCB	68	41	

\*According to Hans *et al.*<sup>17</sup>



**Figure 2.** Overall survival according to *BCL6* rearrangement status. (A) CHOP-treated patients (n=65). (B) R-CHOP-treated patients (n=99).

2008 revision of the WHO classification of lymphoid malignancies.<sup>1</sup> Unlike previous groups, we found no correlation between *BCL6* rearrangement and extranodal presentation,<sup>6,8</sup> and no statistically significant difference in overall survival between *BCL6*<sup>+</sup> and *BCL6*<sup>-</sup> patients was demonstrated in either the primary nodal or extranodal subgroup, although this analysis was limited by the small numbers in the two groups.

The *BCL6* proto-oncogene encodes a transcriptional repressor essential for normal germinal center formation. Its deregulated expression has been implicated as an important pathway in lymphomagenesis.<sup>25</sup> *BCL6* protein expression has been identified as a hallmark of GCB derivation in DLBCL, and has been associated with a favorable clinical outcome.<sup>17,24</sup> However, in keeping with prior studies, we did not find a correlation between *BCL6* rearrangement and *BCL6* protein expression, but demonstrated a relationship between *BCL6* rearrangement and non-GCB phenotype.<sup>12,14</sup> This seemingly paradoxical finding may be related to several factors. First, mechanisms other than gene rearrangement have been shown to deregulate *BCL6* expression in DLBCL, including targeted mutations which may interrupt negative gene autoregulation.<sup>25</sup> Second, the functional effect of *BCL6* rearrangement may vary depending on other biological factors, including the chromosomal translocation partner and cellular context. One group of investigators demonstrated significantly lower *BCL6* expression with non-*IGH/BCL6* than with *IGH/BCL6* rearrangements, and also reported inferior survival in DLBCL patients with non-*IGH/BCL6* translocations.<sup>13,25</sup> A

more recent study by the Leukemia/Lymphoma Molecular Profiling Project examined the effect of *BCL6* rearrangement on gene expression in patients with both GCB- and ABC-subtype DLBCL.<sup>14</sup> In this analysis, *BCL6* rearrangement was associated with increased *BCL6* mRNA levels in patients with ABC-subtype DLBCL, but not in patients with GCB-subtype DLBCL. The clinical significance of these biological subdivisions remains to be clarified.

In summary, the introduction of rituximab may have altered the prognostic impact of *BCL6* gene rearrangement in patients with DLBCL. However, to be incorporated into clinical practice as a useful prognostic biomarker, *BCL6* rearrangement must be shown to provide prognostic information additional to that afforded by the IPI. *BCL6* rearrangement also correlates strongly with non-GCB immunophenotype, and the interdependence of these two prognostic variables requires further study. Correlative biomarker analysis within prospective randomized clinical trials will be needed to determine the prognostic value of *BCL6* rearrangement in the rituximab era.

## Authorship and Disclosures

JS wrote the manuscript, GH and PF performed experiments and analyzed results, NAJ, JMC, LHS and DEH designed the research and approved the manuscript, SB analyzed and reviewed results, RDG and CS analyzed and reviewed results, designed the research and wrote the paper. The authors reported no potential conflicts of interest.

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