

Patients with diffuse large B-cell lymphoma of germinal center origin with *BCL2* translocations have poor outcome, irrespective of *MYC* status: a report from an International DLBCL rituximab-CHOP Consortium Program Study

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ABSTRACT

Diffuse large B-cell lymphoma can be classified by gene expression profiling into germinal center and activated B-cell subtypes with different prognoses after rituximab-CHOP. The importance of previously recognized prognostic markers, such as Bcl-2 protein expression and *BCL2* gene abnormalities, has been questioned in the new therapeutic era. We analyzed Bcl-2 protein expression, and *BCL2* and *MYC* gene abnormalities by interphase fluorescence *in situ* hybridization in 327 patients with *de novo* disease treated with rituximab-CHOP. Isolated *BCL2* and *MYC* rearrangements were not predictive of outcome in our patients as a whole, but only in those with the germinal center subtype of lymphoma. The prognostic relevance of isolated *MYC* rearrangements was weaker than that of *BCL2* isolated translocations, but was probably limited by the rarity of the rearrangements. Seven of eight patients with double hit lymphoma had the germinal center subtype with poor outcome. The germinal center subtype patients with isolated *BCL2* translocations had significantly worse outcome than the patients without *BCL2* rearrangements ($P=0.0002$), and their outcome was similar to that of patients with the activated B-cell subtype ($P=0.30$), but not as bad as the outcome of patients with double hit lymphoma ($P<0.0001$). Bcl-2 protein overexpression was associated with inferior outcome in patients with germinal center subtype lymphoma, but multivariate analysis showed that this was dependent on *BCL2* translocations. The gene expression profiling of patients with *BCL2* rearrangements was unique, showing activation of pathways that were silent in the negative counterpart. *BCL2* translocated germinal center subtype patients have worse prognosis after rituximab-CHOP, irrespective of *MYC* status, but the presence of combined gene breaks significantly overcomes the prognostic relevance of isolated lesions.

Introduction

Diffuse large B-cell lymphoma (DLBCL) is a heterogeneous group of tumors with different clinical, immunophenotypic, and genetic abnormalities as well as highly variable prognosis.¹ Identifying distinctive subgroups within the DLBCL category can assist with prognostication and therapeutic strategy. According to gene expression profile (GEP), DLBCL can be

divided into germinal center B-cell (GCB), activated B-cell (ABC), and unclassified subtypes,²⁻⁴ with different outcomes after treatment with rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP).^{3,5}

The t(14;18)(q32;q21) has been identified in 18–20% of patients with *de novo* DLBCL.⁶⁻⁹ Rarely, *BCL2* is translocated to Ig light chain (*IgK*, *IgL*) loci, as a part of t(2;18)(p11;q21.3) or t(18;22)(q21.3;q11), similarly resulting in Bcl-2 overexpression.

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BCL2 translocations are more frequently found in the GCB subtype, whereas 18q21 locus amplification is more common in the ABC subtype of DLBCL.^{3,8,10}

The prognostic significance of *BCL2* amplification or translocations in *de novo* DLBCL in the era of CHOP therapy alone, without rituximab, was controversial.¹¹⁻²⁰ Some data on the prognostic significance of *BCL2* aberrations in patients treated with R-CHOP have recently become available, with two studies reporting no influence of *BCL2* gene rearrangements on the survival of DLBCL patients.^{21,22} On the other hand, the concomitant presence of t(14;18) or variants and *MYC* rearrangements, referred to as double hit lymphomas, has consistently been associated with adverse outcome in DLBCL patients treated with R-CHOP.²³⁻²⁵

Bcl-2 protein expression seems only partially related to *BCL2* gene abnormalities as analyzed by fluorescence *in situ* hybridization (FISH), as Bcl-2 is expressed in a greater number of DLBCL cases than in those tumors carrying t(14;18)(q32;q21).¹⁰⁻¹² Indeed, in the absence of *BCL2* translocations, amplification of 18q21 and/or activation of the nuclear factor κ B (NF- κ B) pathway can cause Bcl-2 protein overexpression.²⁶ The prognostic significance of Bcl-2 expression is also controversial, and comparison between different studies is hampered by the choice of different cut-offs of positive cells, and by the variability of treatments. In patients treated with R-CHOP, Bcl-2 protein did not correlate with outcome,^{5,27} since the addition of rituximab seemed to improve survival of Bcl-2-positive patients,²⁸⁻³⁰ apparently eliminating the gap between Bcl-2-positive and Bcl-2-negative patients found in the pre-rituximab era. This result does, however, appear to be contradicted in a very recent study in which Bcl-2 expression in GCB-DLBCL was associated with poorer outcome.²²

The goal of this study was to investigate the prognostic value of *BCL2* gene aberrations and Bcl-2 expression in a large number of patients with *de novo* DLBCL, uniformly treated with R-CHOP, for whom *MYC* and GEP characterization was available.

Design and Methods

Patients

We studied 327 cases of previously untreated *de novo* DLBCL, diagnosed between January 2002 and October 2009, and collected as part of the International DLBCL Rituxan-CHOP Consortium Program Study. These cases were analyzed for Bcl-2 protein expression, and *BCL2* and *MYC* gene abnormalities, and gene expression profiling (GEP) was performed. All cases were reviewed by a group of hematopathologists (SMM, MAP, MBM, AT, and KHY), and the diagnoses were confirmed based on World Health Organization classification criteria. Patients with transformation from low grade lymphoma, those with composite follicular lymphoma, primary mediastinal large B-cell lymphoma, primary cutaneous and primary central nervous system DLBCL were excluded from the analysis due to the unique biological features of these types of lymphoma. All patients were adults who were negative for human immunodeficiency virus and had sufficient clinical data and clinical follow-up. Patients in this study were treated with R-CHOP (n=291, 89%) or R-CHOP-like regimens (n=36, 11%; CHOP scheme adopting different anthracyclines i.e. novantrone or epirubicin). All patients with advanced stage disease received six (92%) or eight (8%) cycles, every 21 days, with or without radiotherapy for residual disease or initial bulky dis-

ease; localized cases received at least three cycles followed by radiotherapy or six cycles without radiotherapy. The current study was approved by each of the participating centers' Institutional Review Boards, and the overall collaborative study was approved by the Institutional Review Board at The University of Texas MD Anderson Cancer Center in Houston, Texas, USA.

Immunohistochemistry for Bcl-2 and cut-off determination

Bcl-2 protein expression was evaluated in all patients using a monoclonal anti-Bcl-2 antibody (Clone-124, Dako, Carpinteria, CA, USA) and standard immunohistochemical methods. The formalin-fixed, paraffin-embedded tissue slides underwent deparaffinization and heat-induced antigen retrieval techniques. An endogenous biotin-blocking kit (Ventana) was used to decrease background staining. Following antigen retrieval and primary antibody incubation, the reaction was completed in a Ventana ES instrument using a diaminobenzidine immunoperoxidase detection kit (Ventana). Immunoreactivity was determined without knowledge of the patients' survival, clinical data, or GEP data. The samples were analyzed independently by a group of four hematopathologists/pathologists in addition to the hematopathologist of each of the contributing centers, and disagreements were resolved by joint review at a multi-headed microscope. An average of 300-400 cells in four to five fields were counted in the tissue microarray cores. A percentage of tumor cell staining $\geq 50\%$ was considered positive after receiver operating characteristic (ROC) curve analysis was implemented to assess the discriminatory accuracy of Bcl-2 protein in recognizing patients with different overall survival (OS) and progression-free survival (PFS). The 50% value was established from the analysis of the area under the ROC curve (AUROC) and had the maximum specificity and sensibility for OS and PFS discrimination in our patients (AUROC=0.564, $P=0.017$ for OS and AUROC=0.564, $P=0.015$ for PFS).³¹

Gene expression profiling analysis

RNA was extracted from 327 formalin-fixed, paraffin-embedded tissue samples using a HighPure Paraffin RNA Extraction Kit (Roche Applied Science). Fifty nanograms of RNA were transcribed into cDNA, linearly amplified using the WT-OvationTM FFPE System (Nugen), and biotin-labeled using FL-OvationTM cDNA Biotin Module V2 (Nugen) in all cases. For GeneChip hybridization, 5 μ g of WT-Ovation amplified cDNA were applied to HG-U133 Plus 2.0 GeneChips (Affymetrix) and hybridized overnight. GeneChips were washed, stained, and scanned using the Fluidic Station 450 and GeneChip Scanner 3000 (Affymetrix) according to the manufacturer's recommendations. For data analysis and classification, the microarray DQN (trimmed mean of differences of perfect match and mismatch intensities with quantile normalization) signals were generated and normalized to the quantiles of beta distribution with parameters $p=1.2$ and $q=3$ as previously described.³² A Bayesian model was also utilized to determine the class probability. The classification model was built on the 47 paired formalin-fixed, paraffin-embedded tissue sample dataset previously generated with a confidence rate of 90-100% in fresh frozen tissue and 92-100% in formalin-fixed, paraffin-embedded tissue. The same methodology developed during this study has been validated and demonstrated to be applicable by using the LLMP dataset in the Gene Expression Omnibus (GEO) database GSE#10846 that has 181 CHOP-treated and 233 R-CHOP-treated DLBCL patients with fresh-frozen samples.^{3,4}

Fluorescence *in situ* hybridization for BCL2 and MYC gene rearrangements

FISH was performed using a locus-specific identifier *BCL2* dual-

color, break-apart probe (07J75-001 from Vysis, Downers Grove, IL, USA) on paraffin-embedded tissue sections according to the Vysis protocol. FISH for the *MYC* gene was performed with a locus-specific identifier *IGH/MYC/CEP 8* tri-color, dual fusion probe (DFP, 05J75-001 from Vysis) and, due to shortcomings of the former in identifying alternative (non-*IGH*) *C-MYC* rearrangement partners, a locus-specific identifier *MYC* dual-color, break-apart probe (BP, 05J91-001 from Vysis). Abnormal FISH signals were recorded as percentage of cells showing an abnormality. The cutoff score to consider a case rearranged ("breaks") was the mean + 3 SD of split nuclei in reference cases (i.e., >3%). A high-level of 18q21 amplification was defined as the presence of either more than ten gene signals or tight clusters of at least five gene signals. Sections of five tonsils were used as controls. To assess reproducibility, the results obtained by two observers were compared in a blinded fashion in 80 cases, showing a perfect agreement for breaks ($\kappa=1$) and an excellent agreement for gains ($\kappa=0.91$).

Validation set

To validate our observations in predicting survival in an independent series of cases, we analyzed a second group of 120 archival DLBCL cases studied similarly to the first cohort except for *MYC* analysis that was not available (GCB 49%, ABC 40%, unclassified 11%; *BCL2* translocations in 18%; Bcl-2 overexpression in 54%). All these patients had been treated with R-CHOP and the same selection criteria as those for the first cohort were applied. The clinical characteristics at presentation of the patients in the validation set were not significantly different from those of the patients in the test set.

Statistical analysis

Following pre-defined criteria,³³ PFS was measured from the time of diagnosis to the time of progression or death from any cause. OS was measured from the time of diagnosis to last follow-up or death from any cause. Only patients with a follow-up of longer than 12 months were included in the survival analysis. The actuarial probabilities of PFS and OS were determined using the Kaplan-Meier method, and differences were compared using the log-rank test. A Cox proportional-hazards model was used for multivariate analysis. The χ^2 test or Mann-Whitney test was applied to assess differences between variables. The interobserver agreement for FISH was assessed using the κ statistic; a κ value of >0.75 implied excellent agreement. All statistical calculations, except for ROC and the κ statistic which were performed with SPSS 18.0 (SPSS Inc., Chicago, IL, USA), were conducted using StatView (Abacus Concepts, Berkeley, CA, USA).

Results

Patients' characteristics and outcome

The median age of the patients at diagnosis was 62 years (range, 18–86). Their clinical characteristics are reported in Table 1. The average follow-up time for survivors was 48 months (median 46; range, 12–113). Only 322 of the 327 patients were included in the survival analysis, since a minimum follow-up of 12 months was required by the study criteria. The 3-year OS rate of the 322 patients from this study cohort was 70%±3%, and the 3-year PFS rate was 58%±3%. Of the common, clinically relevant prognostic variables, International Prognostic Index (IPI) risk group 0–2 versus 3–5 ($P<0.0001$), and all the single variables included in the IPI were significantly associated with OS and PFS. Patients with the GCB subtype had significantly better 3-year OS and PFS com-

pared to patients with ABC subtype lymphoma (OS: 75%±4% versus 65%±5%, respectively, $P=0.003$; PFS: 65%±4% versus 52%±4%, respectively, $P=0.003$).

BCL2 and MYC genes in the subgroups defined by gene expression profiling

Sixty patients (18.3%) had DLBCL with *BCL2* gene translocations, and 50 (15.3%) had *BCL2* gene amplifications. The presence of *BCL2* translocations was not associated with any clinical prognostic variable at diagnosis, except for Ann Arbor Stage (70% versus 49% with stage III–IV, $P=0.004$), as shown in Table 1. As expected, *BCL2* translocations were significantly associated with the GCB subtype (84%, $P<0.0001$), while gains were present mostly in the ABC subtype (70%, $P<0.0001$). *MYC* rearrangements were identified in 27 patients (8%), and eight of them had a concomitant *BCL2* translocation, representing double hit lymphomas. Seven of these eight (88%) patients with double hit lymphoma had GCB-DLBCL, and all progressed or relapsed within 12 months from start of therapy (Figure 1A). Patients with *MYC* breaks were equally distributed among patients with or without *BCL2* translocations ($P=0.11$), but were more frequent (16 cases, 70%) within the GCB subtype group.

The OS and PFS rates of patients with *BCL2* translocations were similar to those of patients without *BCL2* translocations, irrespectively of *MYC* status. When we restricted the analysis to the GCB subtype, patients with *BCL2* translocations alone, in the absence of *MYC* breaks, had a significantly worse outcome than GCB patients without *BCL2* translocations (3-year PFS of 53% versus 76%, respectively; $P=0.0002$). The outcome of patients with *BCL2* rearranged GCB subtype was similar to that of the patients with the ABC subtype of DLBCL (52%, $P=0.30$), but still better than that of the patients with double hit lymphomas ($P<0.0001$, Figure 1A). This finding was confirmed in our validation set of 120 patients, in which *BCL2* translocations, while not being predictive in the cohort as a whole ($P=0.05$ for OS; $P=0.11$ for PFS), was an adverse variable in the GCB subtype ($P=0.01$ for both OS and PFS, Figure 1B).

The presence of *MYC* breaks alone in the 19 patients without concomitant *BCL2* translocations was not associated with impaired PFS ($P=0.70$) or OS ($P=0.66$) in the whole cohort, but was associated with inferior OS ($P=0.03$), but not PFS ($P=0.22$), in patients with GCB-DLBCL (only 9 with isolated *MYC* breaks). As shown in Figure 1A, *BCL2* and *MYC* rearrangements each contributed with a cumulative pejorative hit to the outcome of GCB-DLBCL patients, while they had no prognostic impact in patients with ABC-DLBCL.

BCL2 gains were not prognostic in any of the subgroups of patients. Particular consideration of high-level amplifications was of no additional prognostic value.

Bcl-2 protein expression, clinical characteristics, fluorescence in situ hybridization and gene expression profiling

None of the common clinical characteristics of our patients at the time of presentation was significantly associated with Bcl-2 protein expression except age, with older patients more often being Bcl-2 positive (≥ 60 years old, $P=0.02$). Bcl-2 protein expression in GEP- and FISH-defined subgroups is shown in Figure 2. The number of positive cells in patients with the *BCL2* translocation was distinctly higher (range, 10–100%; median 90%) than in

Table 1. Clinical characteristics and treatment response of 327 DLBCL patients treated with R-CHOP, divided according to the presence of *BCL2* gene rearrangements [t(14;18) or its variants].

	Overall		t(14;18) or variants		No translocations		P value
	N.	%	N.	%	N.	%	
Patients	327	100	60	100	267	100	
Median age	62						
≥60	190	58	34	57	156	58	0.80
<60	137	42	26	43	111	42	
Gender							
Female	140	43	32	53	108	41	
Male	187	57	28	47	159	59	0.07
Stage							
I-II	154	47	18	30	136	51	0.004
III-IV	173	53	42	70	131	49	
Lactate dehydrogenase							
Normal	103	36	19	37	84	36	0.94
Elevated	182	64	33	63	149	64	
Extranodal sites*							
0-1	243	79	43	77	200	79	0.66
>1	65	21	13	23	52	21	
Performance Status							
0-1	204	86	37	88	167	86	0.62
>1	34	14	5	12	29	14	
IPI risk group							
0-2	176	64	33	65	143	64	0.90
3-5	99	36	18	35	81	36	
Gene expression profiling							
GCB	139	47	48	82	91	38	<0.0001**
ABC	138	46	9	16	129	54	
Unclassified	21	7	1	2	20	8	
Bcl-2 protein							
Negative	116	39	10	18	106	45	0.0002
Positive	178	61	47	82	131	55	
MYC gene abnormalities							
No break	300	92	52	87	248	93	0.11
Break	27	8	8	13	19	7	

Data regarding lactate dehydrogenase (n=285), involvement of extranodal sites (n=308), Performance Status (n=238), IPI risk group (n=275), GEP (n=298), and Bcl-2 protein expression (n=294), were not available for all patients; *Primary nodal DLBCL (no extranodal site involved, 42% of patients) had no significant association with BCL2 translocations compared to patients with one or more extranodal sites involved (P=0.43); **refers to the comparison between GCB and ABC. Clinical and biological characteristics of 327 DLBCL patients treated with R-CHOP. The patients were stratified according to the presence of BCL2 translocations.

patients without the *BCL2* translocation (range, 0-100%; median 60%).

Bcl-2 protein expression was significantly associated with worse PFS ($P=0.01$) and OS ($P=0.02$) in the whole cohort, but when patients were divided according to GEP-defined subtypes, we observed that higher Bcl-2 expression was associated with significantly inferior PFS in the GCB subgroup ($P=0.04$), but not in the ABC subgroup ($P=0.57$), as shown in Figure 3.

Figure 4 illustrates that Bcl-2 protein expression was not prognostic in patients with or without *BCL2* translocations, indicating that Bcl-2 expression cannot be used as a surrogate of t(14;18) and its variants in assessing prognosis (see also multivariate analysis). These curves were obtained after excluding patients with double hit lymphomas from the analysis, since these are associated with Bcl-2 overexpression (Figure 2), and might have represented a confounding variable. When we tested this finding in the validation set of patients, for whom *MYC* analysis was not available, the prognostic relevance of Bcl-2 pro-

tein expression appeared again dependent of *BCL2* gene status, even though statistical significance between subgroups was not reached due to low numbers (58 patients with GCB-DLBCL, 20 with *BCL2* translocations, only 3 not expressing Bcl-2).

Multivariate analysis

Multivariate analysis of all 137 patients with the GCB subtype of DLBCL showed that *BCL2* translocations (HR 0.40, 95% CI: 0.18-0.89; $P=0.02$), but not Bcl-2 expression (HR 1.01, 95% CI: 0.45-2.21; $P=0.98$), *MYC* breaks (HR 0.25, 95% CI: 0.10-0.59; $P=0.001$), and IPI score (HR 0.41, 95% CI: 0.20-0.84; $P=0.01$), were independently associated with patients' outcome. Results were not modified after each molecular feature was computed with age as a continuous parameter.³⁴ However, both *BCL2* and *MYC* rearrangements lost their predictive power when patients with double hit lymphomas were excluded, signifying that the presence of double gene breaks overcomes the significance of the isolated lesions in GCB-DLBCL.

FIGURE A COLORI SOLO ONLINE

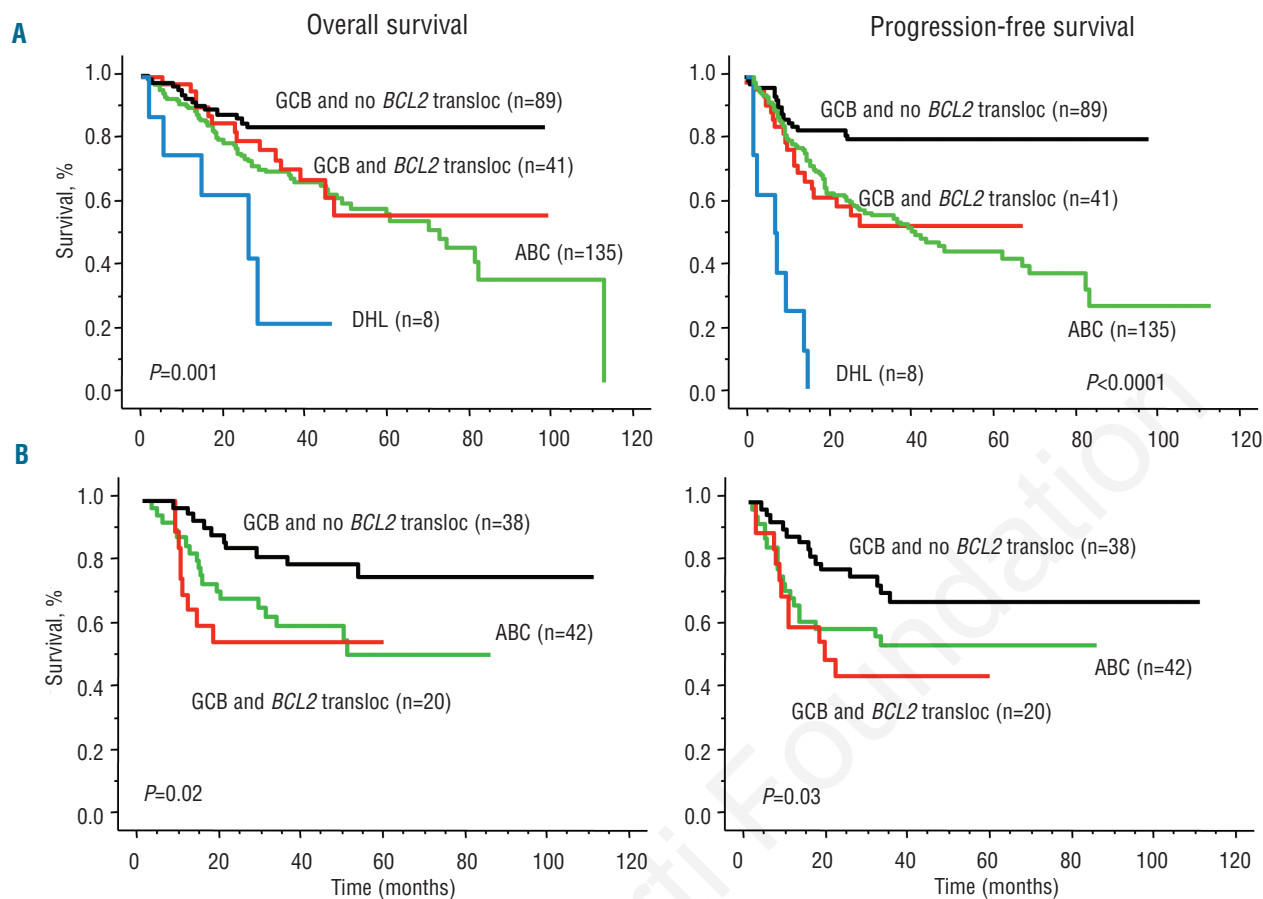
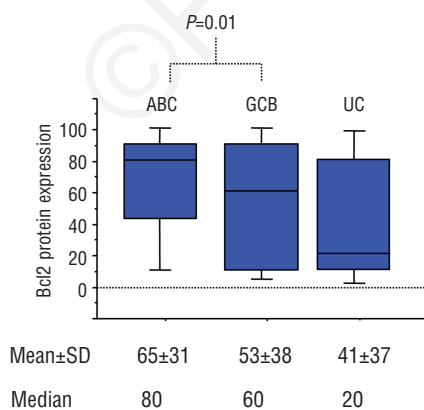


Figure 1. Overall survival and progression-free survival of patients with DLBCL according to (A) the presence of *BCL2* translocations alone or concomitant *MYC* breaks stratified by GEP-defined subgroups; (B) *BCL2* translocations stratified with GEP subgroups in the validation set. DHL: double hit lymphoma.

A Bcl-2 protein expression in GEP-defined subgroups

FIGURE A COLORI SOLO ONLINE



B Bcl-2 protein expression in correlation with *BCL2* and *MYC* gene aberrations

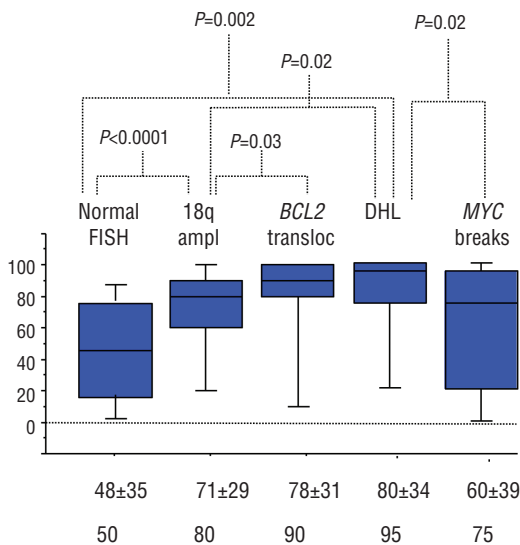


Figure 2. Box plot graphs for the distribution of Bcl-2 expression in 327 patients with DLBCL according to GEP and FISH analysis. Reported P values were calculated with the Mann-Whitney test. Only significant differences are reported. SD= standard deviation. (A) Bcl-2 protein expression in GEP-defined subgroups; (B) Bcl-2 protein expression in correlation with *BCL2* and *MYC* gene aberrations in GEP-defined subgroup. UC: unclassified; DHL: double hit lymphoma.

- Reported P values were calculated with the Mann-Whitney test
 - Bcl-2 protein expression: mean 57, SD 35, and median 70. SD: standard deviation.

Differential gene expression between patients with germinal center B-cell lymphoma with or without t(14;18)

Four-hundred and forty-four genes were found to be differentially expressed (>1.5 fold and $P < 0.005$) in DLBCL patients with or without *BCL2* translocations including both GCB and ABC subtypes. In the GCB group, however, only 43 genes were differentially expressed among patients with and without *BCL2* translocations (Figure 5A). Most of these genes (72%) were informative. As expected, *BCL2* was highly expressed in the translocated cases, as was the *MME*

(CD10) gene. *BCL11A*, an oncogene that is located at 2p16.1 beside the *c-REL* locus and that functions as a transcriptional repressor, was highly expressed in patients with *BCL2* translocated tumors. Neither *BCL11A* nor *c-REL* was increased in the ABC subgroup with *BCL2* translocations (Figure 5B).

Interestingly, a number of genes overexpressed in the *BCL2* translocated group are involved in the control of angiogenesis and the inflammatory response (*ALMP1*, *PPIA*, and *ALOX*), while others are involved in promoting apoptosis or regulating B-cell signaling (*STK17A*, *RALGPS2*, *NCOA3*, *STRBP*, and *ZNF117*).³⁵⁻³⁷

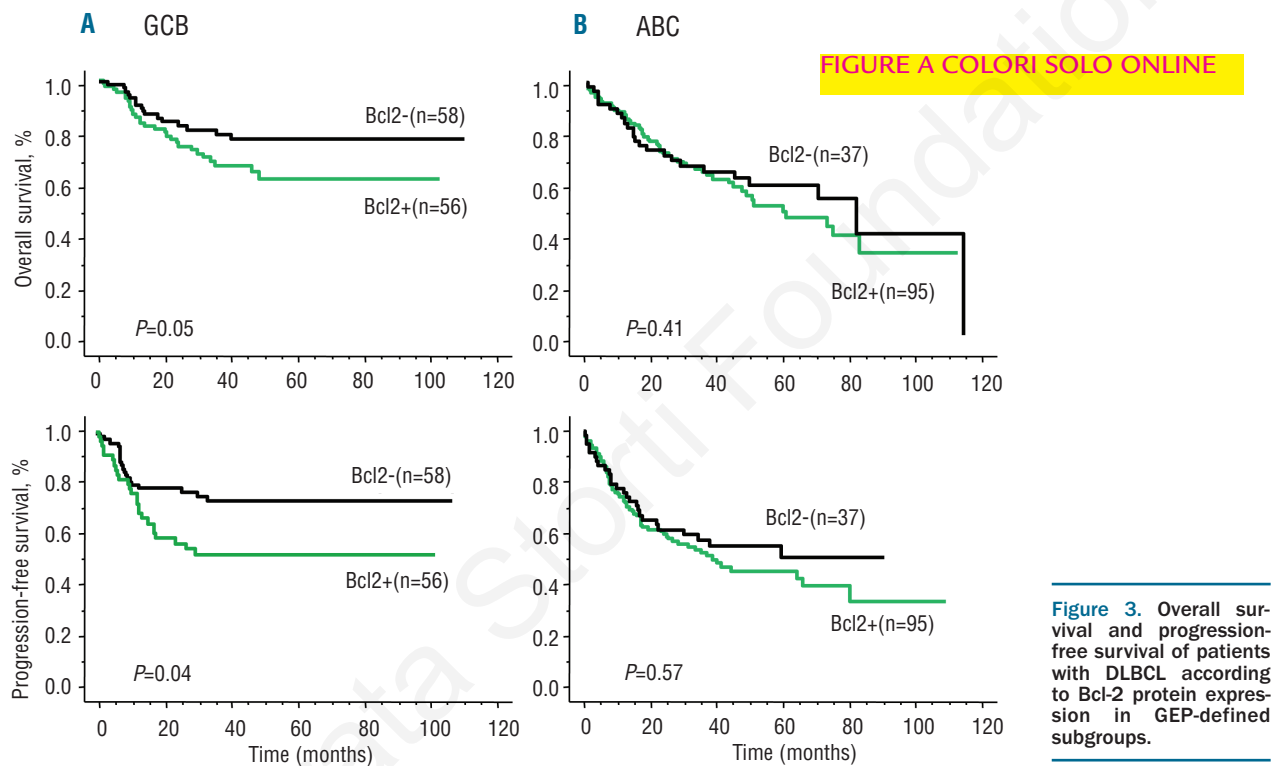


Figure 3. Overall survival and progression-free survival of patients with DLBCL according to Bcl-2 protein expression in GEP-defined subgroups.

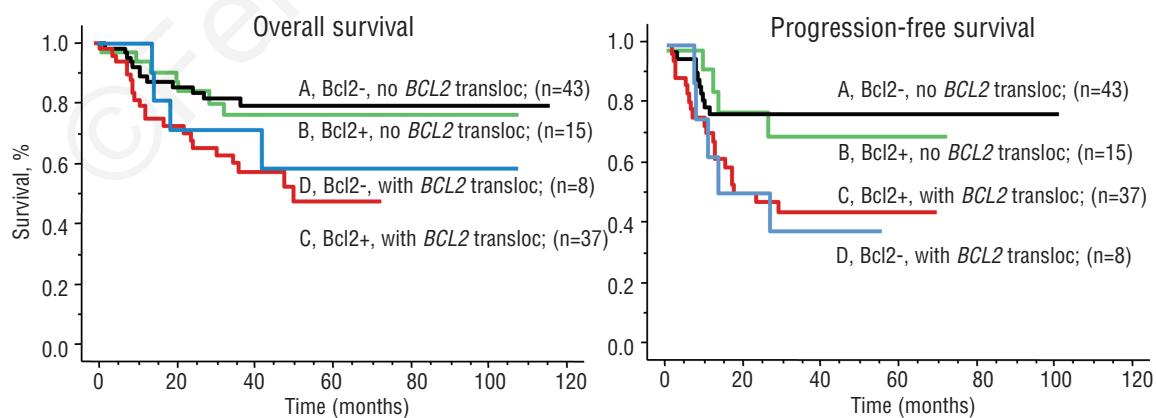


Figure 4. Overall survival and progression-free survival of 103 patients with GCB-DLBCL, stratified according to Bcl-2 protein expression and *BCL2* translocations. Patients with double hit lymphoma were excluded from this analysis. For OS: $P = 0.79$ and $P = 0.58$ between A and B, and C and D, respectively; $P = 0.23$ and $P = 0.04$ between A and D, and B and C, respectively. For PFS: $P = 0.61$ and $P = 0.94$ between A and B, and C and D, respectively; $P = 0.01$ and $P = 0.01$ between A and D, and B and C, respectively.

Discussion

We addressed the clinical impact of *BCL2* aberrations and their relationship to Bcl-2 protein expression in a large series of patients with DLBCL homogeneously treated with R-CHOP, with known *MYC* gene status and molecularly characterized according to GEP analysis. We were able to establish the role of the *BCL2* gene in different subtypes of DLBCL, irrespectively of concomitant *MYC* aberrations. We found that isolated *BCL2* translocations, in the absence of *MYC* breaks, were associated with a poor outcome in the subset of patients with GCB-DLBCL, and that the prognosis of these patients was similar to that of patients with ABC-DLBCL. The concomitant presence of *MYC* breaks (double hit lymphoma) further worsened the outcome of these patients. The role of Bcl-2 protein expression appeared dependent on its association with *BCL2* translocations, as outlined by multivariate analysis and survival curves (Figure 4). Finally, *BCL2* gains, more common in ABC-DLBCL, did not affect prognosis.

As determined by FISH break apart analysis, the overall frequency of *BCL2* translocations in *de novo* DLBCL was 18.3%. The *BCL2* translocations were almost exclusively associated with GCB-DLBCL, found in 34.5% of

cases (Table 1), similarly to other reports.^{3,4,8,88} Nine cases of ABC-DLBCL harbored *BCL2* translocations (one was a double hit lymphoma), and the presence of *BCL2* translocations in ABC DLBCL did not influence the PFS of these patients. The preferential occurrence of *BCL2* rearrangements in the GCB subgroup, and its impact on survival of this subgroup, suggests that *BCL2* translocations might be an important and initial event in the pathogenesis of GCB DLBCL.

The impact of *BCL2* translocations on survival in our series could not be explained by differences in the clinical features of the patients because there was no association between the presence of *BCL2* translocations and IPI risk groups ($P=0.90$, Table 1). Patients with GCB-DLBCL and *BCL2* translocations had an outcome that was similar to that of patients with ABC-DLBCL, while the remaining patients with GCB-DLBCL without *BCL2* translocations had a very good PFS (Figure 1). The prognostic role of *BCL2* translocations appeared independent of the concomitant presence of *MYC* aberrations, being present also when patients with double hit lymphoma were excluded from the analysis (Figure 1A). However, the presence of double gene breaks (double hit lymphoma) conferred a significant further worsening to the outcome of patients

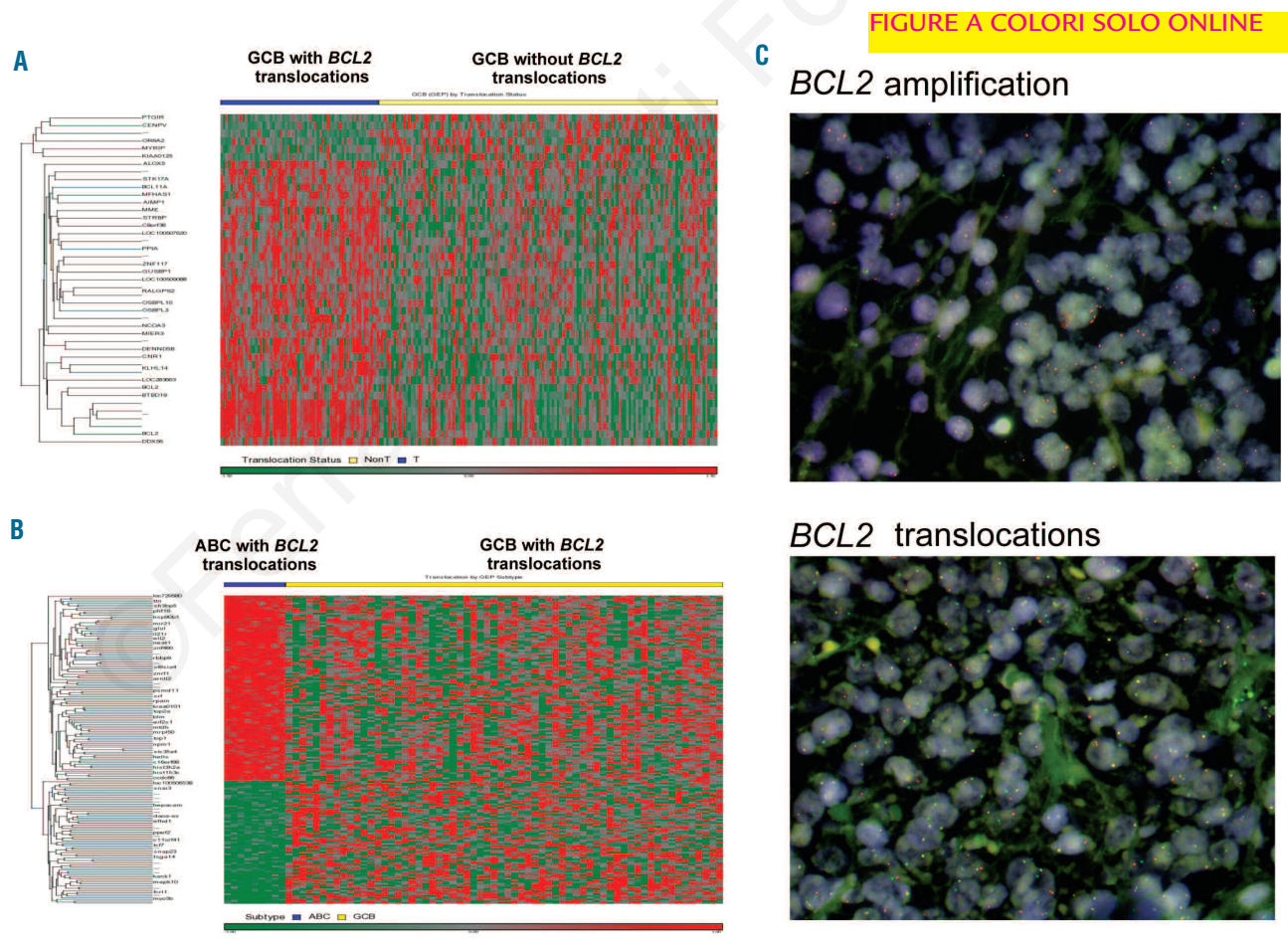


Figure 5. Gene expression profile and representative FISH analysis of DLBCL patients. (A). Gene expression profile of 139 GCB-DLBCL patients stratified according to the presence or absence of *BCL2* translocations; (B). Gene expression profile of 80 DLBCL patients with *BCL2* translocations stratified between GCB-DLBCL (n=78) and ABC-DLBCL (n=12); (C). Representative FISH of *BCL2* amplification (upper image) and translocation (lower image) using a locus-specific identifier *BCL2* dual-color break-apart probe.

with isolated *BCL2* or *MYC* lesions. Confirming previous findings,²³⁻²⁵ we were able to show in our series the synergistic clinical effect of combined activation of *MYC* (promoting cellular proliferation) and *BCL2* (blocking cellular death), which was associated with the GCB subtype, and was the strongest prognostic variable together with IPI risk group in this subgroup of patients, as outlined by our multivariate analysis and shown in Figure 1A.

In this series, Bcl-2 protein was overexpressed in half of the patients with GCB-DLBCL and in 72% of patients with ABC-DLBCL (Figure 2), consistent with other series.^{18,39} However, Bcl-2 overexpression had prognostic value only in the GCB subtype, as already observed by others in the era of R-CHOP therapy.^{22,40} In the present study we have shown that the prognostic effect of Bcl-2 expression within the GCB subgroup is related to the concomitant presence of *BCL2* translocations. No impact of Bcl-2 protein expression was present in patients with or without *BCL2* translocations (Figure 4), a finding obtained after excluding patients with double hit lymphoma.

Iqbal *et al.*²² recently published a similar analysis to our study in patients classified by GEP analysis, confirming that the prognostic significance of Bcl-2 protein is observed in the GCB subset rather than in ABC-DLBCL. Differently from our findings, in their study *BCL2* translocations were not adverse in the GCB subset. We believe that several factors may have contributed to the different prognostic impact of *BCL2* translocations in our study compared to Iqbal's study. First, the number of patients with GCB-DLBCL and *BCL2* rearrangements in Iqbal's study was apparently low to drive conclusions on the independence of the association between *BCL2* translocations and Bcl-2 protein expression, similarly to what we observed in 20 such patients in our validation set.²² Secondly, Bcl-2 protein expression in Iqbal's study was significantly associated with adverse clinical prognostic factors (stage III-IV, elevated lactate dehydrogenase, high IPI risk group) in GCB-DLBCL, which was not the case in our study. Finally, no mention was made about exclusion of possibly confounding DLBCL subtypes such as double hit lymphoma, primary cutaneous or primary central nervous system DLBCL. We also acknowledge that different findings in the literature regarding *BCL2* rearrangements or protein expression could very well be related to lack of uniformity between different studies in terms of Bcl-2 staining and scoring. Moreover, patients' characteristics in the different series, differences in the management of the cases as they were not in clinical trials, data collection regarding outcome, and sometimes short follow-up times may also have contributed to different results.

Our GEP analysis revealed that patients with *BCL2* translocations substantially differed with respect to important recurrent oncogenic events, which may contribute to the adverse outcome of the subgroup of GCB-DLBCL patients with *BCL2* translocations. Up-regulation of the *BCL11A* gene occurred exclusively in the group of patients with *BCL2* translocations (Figure 5). *BCL11A* cooperates with *c-REL* in B-cell lymphomas,^{19,41} and amplification of

the *c-REL* locus has already been reported to be more common in the GCB-DLBCL subtype, in which it was found in 16% of cases,³ but without further characterization. These cases might correspond to our subgroup of GCB-DLBCL with *BCL2* translocations overexpressing *BCL11A*. The c-Rel/NF- κ B pathway can also be constitutively activated in the ABC subgroup,^{3,4,26} in which it is supposed to be responsible for the relatively poor outcome in this subgroup. On the other hand, the prognostically favorable GCB gene-expression signature is notable because the GCB subgroup otherwise has decreased activity of the NF- κ B signaling pathway. Hence, the activation of the NF- κ B pathway in our subgroup of GCB-DLBCL characterized by *BCL2* translocations and adverse outcome warrants further studies specifically addressing *BCL11A* amplifications in this subset of GCB-DLBCL.

We confirm that the outcome of GCB-DLBCL patients should be interpreted in the context of abnormalities of the *MYC* and *BCL2* genes. While the *MYC* rearrangement is quite rare, it is rarely found as the sole genetic abnormality, and its clinical relevance is mainly related to a double hit mechanism, *BCL2* rearrangements are present in a considerable fraction of patients with the GCB subtype who have similar outcomes to those of patients with the ABC subtype. Our results confirm that the GCB and ABC subtypes of DLBCL have distinct pathogeneses, and support the rationale for further classification of different subgroups.

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References

1. Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H. WHO Classification of Tumours of Haematopoietic and Lymphoid tissues. Lyon, France, IARC press. 2008.
2. Alizadeh AA, Eisen MB, Davis RE, Ma C, Lossos IS, Rosenwald A, et al. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature*. 2000;403(6769):503-11.
3. Rosenwald A, Wright G, Chan WC, Connors JM, Campo E, Fisher RL, et al. The use of molecular profiling to predict survival

- after chemotherapy for diffuse large-B-cell lymphoma. *N Engl J Med*. 2002;346(25):1937-47.
4. Wright G, Tan B, Rosenwald A, Hurt EH, Wiestner A, Staudt LM. A gene expression-based method to diagnose clinically distinct subgroups of diffuse large B cell lymphoma. *Proc Natl Acad Sci USA*. 2003;100(17):9991-6.
 5. Fu K, Weisenburger DD, Choi WW, Perry KD, Smith LM, Shi X, et al. Addition of rituximab to standard chemotherapy improves the survival of both the germinal center B-cell-like and non-germinal center B-cell-like subtypes of diffuse large B-cell lymphoma. *J Clin Oncol*. 2008;26(28):4587-94.
 6. Weiss LM, Warnke RA, Sklar J, Cleary ML. Molecular analysis of the t(14;18) chromosomal translocation in malignant lymphomas. *N Engl J Med*. 1987;317(19):1185-9.
 7. Hill ME, MacLennan KA, Cunningham DC, Vaughan HB, Burke M, Clarke P, et al. Prognostic significance of BCL-2 expression and bcl-2 major breakpoint region rearrangement in diffuse large cell non-Hodgkin's lymphoma: a British National Lymphoma Investigation Study. *Blood*. 1996;88(3):1046-51.
 8. Huang JZ, Sanger WG, Greiner TC, Staudt LM, Weisenburger DD, Pickering DL, et al. The t(14;18) defines a unique subset of diffuse large B-cell lymphoma with a germinal center B-cell gene expression profile. *Blood*. 2002;99(7):2285-90.
 9. Tsujimoto Y, Cossman J, Jaffe E, Croce CM. Involvement of the bcl-2 gene in human follicular lymphoma. *Science*. 1985;228(4706):1440-3.
 10. Iqbal J, Sanger WG, Horsman DE, Rosenwald A, Pickering DL, Dave B, et al. BCL2 translocation defines a unique tumor subset within the germinal center B-cell-like diffuse large B-cell lymphoma. *Am J Pathol*. 2004;165(1):159-66.
 11. Gascoyne RD, Adomat SA, Krajewski S, Krajewska M, Horsman DE, Tolcher AW, et al. Prognostic significance of Bcl-2 protein expression and Bcl-2 gene rearrangement in diffuse aggressive non-Hodgkin's lymphoma. *Blood*. 1997;90(1):244-51.
 12. Kramer MH, Hermans J, Wijburg E, Philippo K, Geelen E, van Krieken JH, et al. Clinical relevance of BCL2, BCL6, and MYC rearrangements in diffuse large B-cell lymphoma. *Blood*. 1998;92(9):3152-62.
 13. Martinka M, Comeau T, Foyle A, Anderson D, Greer W. Prognostic significance of t(14;18) and bcl-2 gene expression in follicular small cleaved cell lymphoma and diffuse large cell lymphoma. *Clin Invest Med*. 1997;20(6):364-70.
 14. Vitolo U, Gaidano G, Botto B, Volpe G, Audisio E, Bertini M, et al. Rearrangements of bcl-6, bcl-2, c-myc and 6q deletion in B-diffuse large-cell lymphoma: clinical relevance in 71 patients. *Ann Oncol*. 1998;9(1):55-61.
 15. Offit K, Koduru PR, Hollis R, Filippa D, Jhanwar SC, Clarkson BC, et al. 18q21 rearrangement in diffuse large cell lymphoma: incidence and clinical significance. *Br J Haematol*. 1989;72(2):178-83.
 16. Yunis JJ, Mayer MG, Arnesen MA, Aepli DP, Oken MM, Frizzera G. bcl-2 and other genomic alterations in the prognosis of large-cell lymphoma. *N Engl J Med*. 1989;320(16):1047-54.
 17. Tang SC, Visser L, Hepperle B, Hanson J, Poppema S. Clinical significance of bcl-2-MBR gene rearrangement and protein expression in diffuse large-cell non-Hodgkin's lymphoma: an analysis of 83 cases. *J Clin Oncol*. 1994;12(1):149-54.
 18. Barrans SL, Evans PA, O'Connor SJ, Kendall SJ, Owen RG, Haynes AP, et al. The t(14;18) is associated with germinal center-derived diffuse large B-cell lymphoma and is a strong predictor of outcome. *Clin Cancer Res*. 2003;9(6):2133-9.
 19. Bea S, Colomo L, Lopez-Guillermo A, Salaverria I, Puig X, Pinyol M, et al. Clinicopathologic significance and prognostic value of chromosomal imbalances in diffuse large B-cell lymphomas. *J Clin Oncol*. 2004;22(17):3498-506.
 20. Obermann EC, Csato M, Dirnhofer S, Tzankov A. BCL2 gene aberration as an IPI-independent marker for poor outcome in non-germinal-centre diffuse large B cell lymphoma. *J Clin Pathol*. 2009;62(10):903-7.
 21. Copie-Bergman C, Gaulard P, Leroy K, Briere J, Baia M, Jais JP, et al. Immuno-fluorescence in situ hybridization index predicts survival in patients with diffuse large B-cell lymphoma treated with R-CHOP: a GELA study. *J Clin Oncol*. 2009;27(33):5573-9.
 22. Iqbal J, Meyer PN, Smith LM, Johnson NA, Vose JM, Greiner TC, et al. BCL2 predicts survival in germinal center B-cell-like diffuse large B-cell lymphoma treated with CHOP-like therapy and rituximab. *Clin Cancer Res*. 2011;17(24):7785-95.
 23. Barrans S, Crouch S, Smith A, Turner K, Owen R, Patmore R, et al. Rearrangement of MYC is associated with poor prognosis in patients with diffuse large B-cell lymphoma treated in the era of rituximab. *J Clin Oncol*. 2010;28(20):3360-5.
 24. Johnson NA, Savage KJ, Ludkovski O, Ben-Neriah S, Woods R, Steidl C, et al. Lymphomas with concurrent BCL2 and MYC translocations: the critical factors associated with survival. *Blood*. 2009;114(11):2273-9.
 25. Akyurek N, Uner A, Benekli M, Barista I. Prognostic significance of MYC, BCL2, and BCL6 rearrangements in patients with diffuse large B-cell lymphoma treated with cyclophosphamide, doxorubicin, vincristine, and prednisone plus rituximab. *Cancer*. 2012;118(17):4173-83.
 26. Davis RE, Brown KD, Siebenlist U, Staudt LM. Constitutive nuclear factor kappaB activity is required for survival of activated B cell-like diffuse large B cell lymphoma cells. *J Exp Med*. 2001;194(12):1861-74.
 27. Seki R, Ohshima K, Fujisaki T, Uike N, Kawano F, Gondo H, et al. Prognostic impact of immunohistochemical biomarkers in diffuse large B-cell lymphoma in the rituximab era. *Cancer Sci*. 2009;100(10):1842-7.
 28. Mounier N, Briere J, Gisselbrecht C, Emile JF, Lederlin P, Sebban C, et al. Rituximab plus CHOP (R-CHOP) overcomes bcl-2-associated resistance to chemotherapy in elderly patients with diffuse large B-cell lymphoma (DLBCL). *Blood*. 2003;101(11):4279-84.
 29. Wilson KS, Sehn LH, Berry B, Chhanabhai M, Fitzgerald CA, Gill KK, et al. CHOP-R therapy overcomes the adverse prognostic influence of BCL-2 expression in diffuse large B-cell lymphoma. *Leuk Lymphoma*. 2007;48(6):1102-9.
 30. Shivakumar L, Armitage JO. Bcl-2 gene expression as a predictor of outcome in diffuse large B-cell lymphoma. *Clin Lymphoma Myeloma*. 2006;6(6):455-7.
 31. Youden WJ. Index for rating diagnostic tests. *Cancer*. 1950;3(1):32-5.
 32. Visco C, Li Y, Xu-Monette ZY, Miranda RN, Green TM, Li Y, et al. Comprehensive gene expression profiling and immunohistochemical studies support application of immunophenotypic algorithm for molecular subtype classification in diffuse large B-cell lymphoma: a report from the International DLBCL Rituximab-CHOP Consortium Program Study. *Leukemia*. 2012;26(9):2103-13.
 33. Cheson BD, Pfistner B, Juweid ME, Gascoyne RD, Specht L, Horning SJ, et al. Revised response criteria for malignant lymphoma. *J Clin Oncol*. 2007;25:579-86.
 34. Klapper W, Kreuz M, Kohler CW, Burkhardt B, Szczepanowski M, Salaverria I, et al. Patient age at diagnosis is associated with the molecular characteristics of diffuse large B-cell lymphoma. Molecular Mechanisms in Malignant Lymphomas Network Project of the Deutsche Krebshilfe. *Blood*. 2012;119(8):1882-7.
 35. Mao P, Hever MP, Niemaszyk LM, Haghkerdar JM, Yanco EG, Desai D, et al. Serine/threonine kinase 17A is a novel p53 target gene and modulator of cisplatin toxicity and reactive oxygen species in testicular cancer cells. *J Biol Chem*. 2011;286(22):19381-91.
 36. Bodemann BO, White MA. Ral GTPases and cancer: linchpin support of the tumorigenic platform. *Nat Rev Cancer*. 2008;8(2):133-40.
 37. Anzick SL, Kononen J, Walker RL, Azorsa DO, Tanner MM, Guan XY, et al. AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. *Science*. 1997;277(5328):965-8.
 38. Chen Y, Han T, Iqbal J, Irons R, Chan WC, Zhu X, et al. Diffuse large B-cell lymphoma in Chinese patients: immunophenotypic and cytogenetic analyses of 124 cases. *Am J Clin Pathol*. 2010;133(2):305-13.
 39. Nyman H, Jerkeman M, Karjalainen-Lindsberg ML, Banham AH, Enblad G, Leppa S. Bcl-2 but not FOXP1, is an adverse risk factor in immunochemotherapy-treated non-germinal center diffuse large B-cell lymphomas. *Eur J Haematol*. 2009;92(5):364-72.
 40. Song MK, Chung JS, Shin DH, Seol YM, Shin HJ, Choi YJ, et al. Prognostic significance of the Bcl-2 negative germinal centre in patients with diffuse large B cell lymphoma treated with R-CHOP. *Leuk Lymphoma*. 2009;50(1):54-61.
 41. Satterwhite E, Sonoki T, Willis TG, Harder L, Nowak R, Arriola EL, et al. The BCL11 gene family: involvement of BCL11A in lymphoid malignancies. *Blood*. 2001;98(12):3413-20.