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

Carlo Visco,1,2 Alexander Tzankov,2 Zijun Y. Xu-Monette,1 Roberto N. Miranda,1 Yu Chuan Tai,1 Yan Li,4 Wei-min Liu,4 Emanuele S. G. d’Amore,2 Yong Li,6 Santiago Montes-Moreno,6 Karen Dybkær,19 April Chiu,8 Attilio Orazi,9 Youli Zu,10 Govind Bhagat,11 Huan-You Wang,12 Cherie H. Dunphy,13 Eric D. His,14 X. Frank Zhao,15 William WL. Choi,16 Xiaoying Zhao,17 J. Han van Krieken,18 Qin Huang,19 Weiyan Ai,20 Stacey O’Neill,13 Maurilio Ponzi,21 Andres JM. Ferreri,21 Brad S. Kahl,22 Jane N. Winter,23 Ronald S. Go,24 Stephan Dirnhofer,2 Miguel A. Piris,4 Michael B. Møller,25 Lin Wu,4 Xiaoying Zhao,17 J. Han van Krieken,18 Qin Huang,19 Weiyan Ai,20 Stacey O’Neill,13 Maurilio Ponzi,21 Andres JM. Ferreri,21 Brad S. Kahl,22 Jane N. Winter,23 Ronald S. Go,24 Stephan Dirnhofer,2 Miguel A. Piris,4 Michael B. Møller,25 Lin Wu,4

1Department of Hematopathology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA; 2San Bortolo Hospital, Vicenza, Italy; 3University Hospital, Basel, Switzerland; 4Roche Molecular Systems, Inc., Pleasanton, CA, USA; 5University of Louisville School of Medicine, Louisville, KY, USA; 6Hospital Universitario Marques de Valdecilla, IFIMAV, Santander, Spain; 7Aalborg Hospital, Aarhus University Hospital, Aalborg, Denmark; 8Memorial Sloan-Kettering Cancer Center, New York, NY, USA; 9Well Medical College of Cornell University, New York, NY, USA; 10The Methodist Hospital, Houston, TX, USA; 11Columbia University Medical Center and New York Presbyterian Hospital, New York, NY, USA; 12University of California San Diego School of Medicine, San Diego, CA, USA; 13University of North Carolina School of Medicine, Chapel Hill, NC, USA; 14Cleveland Clinic, Cleveland, OH, USA; 15University of Maryland School of Medicine, Baltimore, MD, USA; 16University of Hong Kong Li Ka Shing Faculty of Medicine, Hong Kong, China; 17Zhejiang University School of Medicine, Second University Hospital, Hangzhou, China; 18Radboud University Nijmegen Medical Centre, Nijmegen, the Netherlands; 19City of Hope National Medical Center, Los Angeles, CA, USA; 20University of California San Francisco School of Medicine, San Francisco, CA, USA; 21San Raffaele H. Scientific Institute, Milan, Italy; 22University of Wisconsin Hospital and Clinic, Madison, WI, USA; 23Feinberg School of Medicine, Northwestern University, Chicago, IL, USA; 24Gundersen Lutheran Health System, La Crosse, WI, USA, and 25Odense University Hospital, Odense, Denmark

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).5,5

The t(14;18)(q32;q21) has been identified in 18–20% of patients with de novo DLBCL.6 Rarely, BCL2 is translocated to lg light chain (lgK, lgL) loci, as a part of t(2;18)(p11;q21.3) or t(18;22)(q21.3;q11), similarly resulting in Bcl-2 overexpression.
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).16,17 Indeed, in the absence of BCL2 translocations, amplification of 18q21 and/or activation of the nuclear factor-kB (NF-κB) 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,27,28 since the addition of rituximab seemed to improve survival of Bcl-2-positive patients,29,30 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.31

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 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 dianmonobenzidine 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 ≥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 sensitivity 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-Ovation™ FFPE System (Nugen), and biotin-labeled using FL-Ovation™ cDNA Biotin Module V2 (Nugen) in all cases. For GeneChip hybridization, 5 μg 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 LLMPP 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.33

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

FISH was performed using a locus-specific identifier BCL2 dual-
The log-rank test. A Cox proportional-hazards model was used for actuarial probabilities of PFS and OS were determined using the longer than 12 months were included in the survival analysis. The time of diagnosis to the time of progression or death from any cause. Only patients with a follow-up of years (range, 18–86). Their clinical characteristics are defined subgroups is shown in Figure 2. The number of patients with the ABC subtype of DLBCL (52%, 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
patients without the \textit{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 \textit{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 \textit{BCL2} 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 protein expression appeared again dependent of \textit{BCL2} gene status, even though statistical significance between subgroups was not reached due to low numbers (58 patients with GCB-DLBCL, 20 with \textit{BCL2} translocations, only 3 not expressing Bcl-2).

**Multivariate analysis**

Multivariate analysis of all 137 patients with the GCB subtype of DLBCL showed that \textit{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. However, both \textit{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.
BCL2 translocations in diffuse large B-cell lymphoma

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.

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. UC: unclassified; DHL: double hit lymphoma.
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 (AIMP1, PPIA, and ALOX), while others are involved in promoting apoptosis or regulating B-cell signaling (STK17A, RAL-GDS2, NCOA3, STRBP, and ZNF117).35-37

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**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; \(P=0.04\) 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 probe 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. 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.
with isolated BCL2 or MYC lesions. Confirming previous findings, 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. However, Bcl-2 overexpression had prognostic value only in the GCB subtype, as already observed by others in the era of R-CHOP therapy.

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. recently published a similar analysis to our study in patients classified by GEP analysis, confirming 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 concomitant presence of BCL2 translocations.

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 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, but without further characterization. These cases might correspond to our subgroup of GCB-DLBCL with BCL2 translocations overexpressing BCL11A. The c-Rel/NF-κB pathway can also be constitutively activated in the ABC subgroup, 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-κB signaling pathway. Hence, the activation of the NF-κB 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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**Authorship and Disclosures**

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