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MYC/BCL2 protein co-expression contributes to the inferior survival of activated B-cell subtype of diffuse large B-cell lymphoma and demonstrates high-risk gene expression signatures: a report from The International DLBCL Rituximab-CHOP Consortium Program Study

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Running title: MYC/BCL2 co-expression in diffuse large B-cell lymphoma

Key words: double-hit, activated B-cell, MYC, BCL2, NF-κB, stromal signature, cell adhesion

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Key Points: DLBCL patients with MYC/BCL2 co-expression demonstrate inferior prognosis with high-risk gene expression signatures

ABSTRACT

Diffuse large B-cell lymphoma (DLBCL) is stratified into prognostically favorable germinal center B-cell (GCB)-like and unfavorable activated B-cell (ABC)-like subtypes according to their gene-expression signatures. In this study, we assessed a cohort of 893 *de novo* DLBCL patients treated with R-CHOP therapy. We show that MYC/BCL2 protein co-expression occurred significantly more commonly in the ABC subtype. The ABC and GCB subtypes had similar prognoses in DLBCL with MYC/BCL2 co-expression as well as in DLBCL without MYC/BCL2 co-expression. Consistent with the notion that the prognostic difference between the two subtypes was attributable to MYC/BCL2 co-expression, the difference in gene-expression signatures between the two subtypes was dramatically diminished in the absence of MYC/BCL2 co-expression. Furthermore, DLBCL with MYC/BCL2 co-expression demonstrated a signature of marked downregulation of genes encoding extracellular matrix proteins, those involving matrix deposition/remodeling and cell adhesion, and upregulation of proliferation-associated genes. We conclude that MYC/BCL2 co-expression in DLBCL is associated with an aggressive clinical course, is more common in the ABC subtype, and contributes to the overall inferior prognosis of patients with ABC-DLBCL. Furthermore, the data suggest that MYC/BCL2 co-expression, rather than cell of origin classification, is a better predictor of prognosis in patients with DLBCL treated with R-CHOP therapy.

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INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is the most common type of non-Hodgkin lymphoma and has heterogeneous clinicopathologic, immunophenotypic, and genetic features. According to the results of gene-expression profiling (GEP) studies, DLBCL can be stratified into germinal center B-cell (GCB)-like or activated B-cell (ABC)-like subtypes, and patients with the ABC subtype of DLBCL have an inferior prognosis.[1] The GCB and ABC subtypes have distinctive gene-expression signatures. GCB-DLBCL expresses many genes selectively and/or highly expressed by normal germinal center B-cells, such as *CD10* and *BCL6*. In contrast, ABC-DLBCL has a gene signature similar to peripheral blood B-cells activated *in vitro*. Notably, genes upregulated in ABC-DLBCL include *MYC*, *BCL2*, *MUM1*, *CD44, FLIP*, and *cyclin D2* as well as many other genes. It is believed that constitutive NF-κB activation in the ABC-DLBCL drives the expression of this array of genes and contributes to the ABC phenotype.[2] The high NF-κB activity is attributable to a variety of molecular and genetic mechanisms. Mutations of multiple genes have recently been identified that encode proteins involved in the signaling of B-cell receptor and members of the tumor necrosis factor receptor superfamily, as well as those involving NF-κB regulation.[2,3] Despite the identification of many deregulated target genes in ABC-DLBCL, it remains unknown which gene products at the protein level contribute most significantly to the inferior prognosis of patients with ABC-DLBCL.

Although the GCB and ABC subtypes convey general trends regarding clinical outcome, these subtypes do not reliably predict the prognosis of individual patients. Furthermore, it is impractical to routinely perform GEP in the clinical setting. Immunohistochemistry (IHC) studies using various antibody panels and algorithms have

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been proposed as surrogates for predicting the GCB versus non-GCB subtype.[4-10] The results, however, have been controversial as the concordance with GEP results is imperfect to varying degrees, and in some studies IHC results do not correlate with prognosis.[11-13] Furthermore, both the GCB and ABC subtypes of DLBCL as defined by GEP are heterogeneous and contain biological subgroups that have different prognoses and may require different therapeutic approaches. Therefore, a stratification of DLBCL patients into subgroups that are biologically similar and prognostically meaningful, and that are more predictive than the overall categories of GCB and ABC is needed, thereby facilitating therapeutic decisions.

Double-hit B-cell lymphoma is defined as a B-cell lymphoma associated with chromosomal breaks targeting the *MYC* gene located at chromosome 8q24 in combination with additional rearrangement affecting another gene, such as *BCL2* or *BCL6.*[14] By far, the most studied type of double-hit B-cell lymphoma has concurrent *MYC* and *BCL2* breaks (i.e. *MYC/BCL2* double-hit). There is a general consensus that patients with *MYC/BCL2* double-hit lymphomas have an extremely aggressive clinical course.[14-22] Despite their clinical aggressiveness, almost all cases of *MYC/BCL2* double-hit lymphoma are of the GCB subtype, a generally favorable prognostic group, illustrating an important discordance between clinical behavior and cell of origin subtypes.[14,16,17]

More recently, others have extended the concept of *MYC/BCL2* double-hit lymphoma by assessing for MYC and BCL2 protein expression by IHC, the logic being that protein expression, regardless of mechanisms, may have prognostic significance. In two studies, Green et al and Johnson et al showed that patients with DLBCL with MYC/BCL2 co-expression, with or without *MYC* or *BCL2* gene rearrangements, have a

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poorer prognosis.[23,24] These studies were possible because of the recent availability of anti-MYC antibodies suitable for IHC staining in paraffin-embedded tissues.

In this study, we used IHC to assess the prognostic value of MYC and BCL2 expression, and particularly MYC/BCL2 co-expression, in a large cohort of 893 *de novo* DLBCL patients treated with rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) therapy. Our results show that MYC/BCL2 co-expression is associated with a poor prognosis and is more common in the ABC-DLBCL. We further suggest that MYC/BCL2 co-expression explains the poorer prognosis of patients with ABC-DLBCL and may be a better predictor of prognosis than cell of origin classification.

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MATERIALS AND METHODS

Patient selection

We studied 893 cases of *de novo* DLBCL from patients who were treated with R-CHOP chemotherapy, including 466 cases in a training set and 234 cases in a validation set (validation set #1). These cases were collected as a part of the International DLBCL Rituximab-CHOP Consortium Program study.[10,25] All cases were diagnosed according to World Health Organization (WHO) classification criteria. Cases were excluded if patients had a history of low grade B-cell lymphoma, acquired immunodeficiency syndrome/HIV infection, primary cutaneous DLBCL, primary central nervous system DLBCL, and Epstein-Barr virus-positive DLBCL. Only cases with successful MYC and BCL2 stainings were included for further study. The patient treatment information was included in the in Supplemental Materials and Methods section. A separate validation set (validation set #2) of 193 cases of *de novo* DLBCL was previously reported.[23] The study was reviewed and approved by the Institutional Review Boards of each participating center.

Tissue microarray immunohistochemical studies

Hematoxylin-eosin stained slides from all DLBCL cases were reviewed and representative areas with the highest percentage of tumor cells were selected for tissue microarray (TMA) construction. IHC studies for a variety of markers were performed using a streptavidin-biotin complex technique on 4-µm TMA sections. MYC (clone Y69; Epitomics, Burlingame, CA) expression showed a distinct nuclear pattern and BCL2 (clone 124; DAKO, Denmark) expression exhibited a cytoplasmic pattern. A cutoff value for each marker was established from analysis of receiver-operating characteristic curves

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to achieve maximum specificity and sensitivity as described previously.[10] Cutoff values of 40% for MYC and 70% for BCL2 were established. These values were similar to those in a previous study, in which median values were used as the cutoff values.[23] For all other markers assessed in this study, cutoff values have been described previously.[10]

Fluorescence *in situ* hybridization for *MYC* and *BCL2* and sequencing of *TP53*

Fluorescence *in situ* hybridization (FISH) analysis was performed using formalin-fixed paraffin-embedded (FFPE) tissue sections of all 466 cases in the training set using *BCL2* dual-color break-apart probes (Vysis, Downers Grove, IL) as described previously.[10] *MYC* was assessed by FISH using locus-specific *IGH/MYC/CEP8* tri-color dual-fusion probes and locus-specific *MYC* dual-color break-apart probes (Vysis, Downers Grove, IL). Cases were considered for evaluation if at least 200 tumor cell nuclei per core displayed positive signals in the TMA sections. For *TP53* sequencing, genomic DNA and total RNA were extracted from FFPE tissue of all cases in the training set and processed as previously described.[25]

Gene expression profiling

Total RNA was extracted from FFPE tissue samples of 451 cases in the training set using the HighPure Paraffin RNA Extraction Kit (Roche Applied Science, Indianapolis, IN) and subjected to GEP as described previously.[10] For data analysis and classification, the microarray DQN signals were generated and normalized to the quantiles of beta distribution with parameters p=1.2 and q=3. A Bayesian model was also utilized to determine the classification probability.[26] The GEP classification method developed from this study was validated with an independent LLMPP dataset in the Gene

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Expression Omnibus database GSE#10846 [27] with 181 CHOP-treated and 233 R-CHOP-treated DLBCL patients and achieved over 97% concordance rate for the classification of two subtypes (GCB and ABC).

Cell of origin classification

Cell of origin (COO) classification was achieved by combining GEP and IHC data, with the GEP data considered the “gold standard”. Briefly, IHC was performed in all cases in the training set and validation set #1. GEP was performed in 451 cases in the training set and 411 were classified as GCB or ABC; 40 (9%) were unclassifiable. The classification of these 411 cases was based on the GEP results regardless IHC results. The 40 cases not classifiable by GEP and 15 additional cases in the training set, for which GEP was not performed, as well as all cases in validation set #1 were classified by IHC methods according to both the Visco-Young and William Choi algorithms (Supplemental Figure S6).[10] The COO classification of the validation set #2 was previously reported.[23]

Statistical analysis

Statistical analyses were performed as described in Supplemental MATERIALS AND METHODS section. The outcome analyses were based on the entire training set of 466 cases. The outcome analysis results limited to the 411 cases stratified by GEP were strikingly similar to those derived from the entire training set and were shown in Supplemental Figure S1 through S5.

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RESULTS

The clinical and pathologic features of 466 cases in the training set are listed in Table 1. Two hundred forty-one (52%) cases were classified as the GCB subtype and 225 (48%) the ABC subtype (Table 1). The median follow-up time for this study cohort was 57 months.

MYC/BCL2 protein co-expression predicts poor prognosis in DLBCL

Using cutoff values of 40% and 70% positive tumor cells for MYC and BCL2, respectively, 300 (64%) were positive for MYC and 233 (50%) cases were positive for BCL2. One-hundred fifty-seven (34%) were positive for both MYC and BCL2 and 90 (19%) were negative for both.

MYC and BCL2 protein co-expression in DLBCL had significant adverse impact on patient survival (Figure 1A-B). The 5-year OS of patients with DLBCL with MYC/BCL2 co-expression versus all other patients was 30% vs 75% ($p<.0001$); the 5-year PFS was 27% vs 73% ($p<.0001$). When assessed separately, patients with MYC+ or $BCL2^{+}$ DLBCL had significantly inferior OS (Figure 1C, E) and PFS (data not shown) compared with patients with MYC-negative or BCL2-negative DLBCL respectively. However, the prognostic impact of MYC or BCL2 protein expression was apparently due to the confounding effect of cases with MYC/BCL2 co-expression. When all cases with MYC/BCL2 co-expression were excluded, neither MYC nor BCL2 protein expression significantly impacted OS (Figure 1D, F) and PFS overall or in COO subtypes (data not shown).

Stratifying all patients into GCB and ABC subtypes, patients with DLBCL with MYC/BCL2 co-expression had significantly worse OS and PFS within both COO

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subtypes (Figure 2A-D). The prognostic impact of MYC/BCL2 co-expression in DLBCL was further assessed according to various clinical parameters. The significantly worse OS and PFS conferred by MYC/BCL2 co-expression were observed in both low- and high-risk subgroups of DLBCL stratified by IPI scores (Figure 2E-F) and other individual clinical parameters (Supplemental Figure S7).

The adverse prognostic impact of MYC/BCL2 co-expression in DLBCL and its COO subtypes was validated in an independent set of 234 cases of *de novo* DLBCL treated with R-CHOP (Supplemental Figure S8). In multivariate analysis, controlling for other clinicopathologic parameters, MYC/BCL2 co-expression remained a strong independent predictor of OS (p<.0001) and PFS (p<.0001) in DLBCL patients (Table 2).

MYC/BCL2 co-expression is associated with high-risk clinical parameters

Various clinicopathologic parameters were compared between patients with DLBCL with or without MYC/BCL2 co-expression (Table 1). Patients with DLBCL with MYC/BCL2 co-expression had multiple adverse prognostic factors included in the IPI risk stratification, including advanced age (p=.0011), high-stage disease (p<.0001), poor performance status (p=.0453), and multiple extranodal sites of disease (p=.0160). Consequently, more patients with DLBCL with MYC/BCL2 co-expression had an intermediate-high to high IPI score (p=.0001). Patients with DLBCL with MYC/BCL2 co-expression were also associated with a lower rate of complete remission (p<.0001) and a higher proliferation index (p=.0086). There was no significant difference in gender, serum LDH level, tumor size, or frequency of *TP53* mutations between DLBCL patients with or without MYC/BCL2 co-expression.

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MYC/BCL2 co-expression shows ABC predominance and contributes to the inferior prognosis of ABC-DLBCL

The presence of MYC/BCL2 co-expression correlated significantly with the ABC subtype ($p<.0001$) (Table 1). Of total 157 cases of DLBCL with MYC/BCL2 co-expression, 104 (66%) were of the ABC-DLBCL (Figure 3A and Table 1). By contrast, only 121 of 309 (39%) of DLBCL without MYC/BCL2 co-expression were of the ABC-DLBCL (Table 1). Approximately 46% (104/225) of the ABC-DLBCL had MYC/BCL2 co-expression compared with 22% (53/241) of the GCB-DLBCL with MYC/BCL2 co-expression ($p<.0001$) (Figure 3B). In cases only stratified by GEP, 49% of the ABC-DLBCL and 19% of the GCB-DLBCL showed MYC/BCL2 co-expression (Supplemental Figure S3). Considering BCL2 and MYC protein expression individually, the ABC-DLBCL had a significantly higher frequency of BCL2 (61% vs 40%, $p<.0001$) and MYC (72% vs 57%, $p=.0009$) expression than the GCB-DLBCL (Figure 3B).

In the training set, the ABC-DLBCL was associated significantly with inferior OS ($p=.0080$) and PFS ($p=.0075$) (Figure 4A-B). However, after excluding all cases with MYC/BCL2 co-expression, the prognosis of patients with ABC-DLBCL was similar to that of patients with GCB-DLBCL (OS: $p=.3163$; PFS: $p=.4291$) (Figure 4C-D). This result was validated in a previously reported independent cohort (Supplemental Figure S9). Considering only patients with DLBCL with MYC/BCL2 co-expression, there was no significant difference in OS ($p=.4114$) or PFS ($p=.7020$) between the ABC and GCB subtypes (Figure 4E-F).

When analysis was limited to the 411 cases classified by GEP data, similar results were observed (Supplemental Figure S4). Consistent with these results, in multivariate analysis, after controlling for MYC/BCL2 co-expression, the ABC subtype was not a

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significant prognostic predictor of OS (p=.4329) or PFS (p=.3750) (Table 2). These data support the notion that the inferior clinical outcome of patients with ABC-DLBCL is attributable to a significantly higher frequency of cases with MYC/BCL2 co-expression.

MYC/BCL2 co-expression confers an adverse prognostic impact independent from *MYC/BCL2* co-rearrangement and *TP53* mutation status

Approximately 3% (10/394) of DLBCL cases in the training set had concurrent *MYC* and *BCL2* co-rearrangements; 9 of them were of the GCB subtype. DLBCL with *MYC/BCL2* co-rearrangement was associated with markedly poorer OS (p<.0001) and PFS (p<.0001) (Figure 5A-B). By IHC, 8 of these cases exhibited MYC/BCL2 co-expression and the other two cases were positive for either MYC or BCL2.

The remaining 384 cases (199 ABC and 185 GCB) in the training set lacked concomitant *MYC/BCL2* co-rearrangements; 124 (32%) had MYC/BCL2 co-expression. In the absence of *MYC/BCL2* co-rearrangement, MYC/BCL2 co-expression showed a more marked predilection for the ABC subtype: 47% (93/199) vs 17% (31/185) in the GCB subtype (p<.0001) (Figure 3B) and remained a significant predictor of inferior OS (p<.0001) and PFS (p<.0001) (Figure 5C-D). Similarly, MYC/BCL2 co-expression predicted inferior survival in the absence of *TP53* mutations (Figure 5E). However, *TP53* mutation remained a significant prognostic factor in patients with DLBCL with MYC/BCL2 co-expression (Figure 5F).

MYC/BCL2 co-expression contributes to different gene expression signatures of GCB and ABC-DLBCL

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The above data show that the poorer prognosis of patients with ABC-DLBCL can be attributed, in large part, to the higher frequency of cases with MYC/BCL2 co-expression in the ABC subtype. We compared GEP results between the ABC and GCB subtypes with MYC/BCL2 co-expression (Figure 6 and Supplemental Table S1). A total of 208 genes were differentially expressed (p<.001), including 121 genes highly expressed in the GCB subtype and 87 genes highly expressed in the ABC subtype. As expected, the gene signatures of the ABC vs GCB subtype of DLBCL with MYC/BCL2 co-expression largely reflected those reported by Alizadeh et al.[1] The notable genes included *CD10*, *BCL6*, *MYBL1*, and *PI3KCG* in the GCB subtype versus *MUM1*, *cyclin D2*, *FLIP*, *CD44*, and *SLAP* in the ABC subtype. Additionally, we also identified many genes that have been shown to be differentially expressed between the two COO subtypes and confer prognostic impact by others, such as *REL* and *CIITA* in GCB-DLBCL, and *CARD11*, *IGHM*, *FOXP1*, and *SPIB* in ABC-DLBCL (Supplemental Table S1). [2,28-32]

We further compared GEP results between the ABC and GCB subtypes in cases negative for both MYC and BCL2 expression (Figure 6B and Supplemental Table S2). Surprisingly, there were no genes differentially expressed between the two subtypes at the same significance level of p<.001 and only a few genes at a significance level of p<.01. A total of 20 genes were differentially expressed between the two subtypes, including 12 highly expressed in the GCB and 8 highly expressed in the ABC subtype. Thirteen of these 20 genes were also differentially expressed between the two subtypes with MYC/BCL2 co-expression. However, only few genes (*AFF2/FMR2*, *FOXP1* and *PIM2*) were among those previously found to be differentially expressed between the two COO subtypes.[1,32]

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Gene expression signature of DLBCL with MYC/BCL2 co-expression

To elucidate the potential molecular basis behind the aggressive clinical course of patients with DLBCL with MYC/BCL2 co-expression, we compared GEP results of DLBCL with MYC/BCL2 co-expression with those of DLBCL negative for both MYC and BCL2 expression (Figure 7 and Supplemental Table S3). A total of 153 genes were differentially expressed, including 65 genes up-regulated and 88 genes down-regulated in DLBCL with MYC/BCL2 co-expression ($p<.001$).

The most striking finding in DLBCL with MYC/BCL2 co-expression was the down-regulation of a large number of genes (33/88 or 38%) encoding various extracellular matrix (ECM) proteins or those involving the ECM deposition and remodeling (Table 3 and Supplemental Table S3). The ECM-encoding genes included those encoding various subtypes of collagen, fibronectin, versican, thrombospondin, SPARC, and biglycan. The ECM remodeling genes included metallopeptidase/serine proteases and their inhibitors, and matrix associated proteins. Those genes involving the production of ECM included FGFR1 and FAP. Another prominent feature was the down-regulation of genes (21/88, 24%) involved in cell adhesion, motility and cytoskeletal organization, such as integrins, CD58, cortactin, caldesmon, calponin, and tropomysosin.

Of the genes up-regulated in DLBCL with MYC/BCL2 co-expression, 20 encoded proteins involved in cell proliferation, including MYC and BCL2 as expected, as well as *TCL1A, MLL, FOXP1, SP1B, TCF4, TNFRSF13B*, and those promoting DNA and protein synthesis (Table 3 and Supplemental Table S3). Chromosomal breakpoints involving the *TCL1A* and *MLL* genes define T-prolymphocytic leukemia and a subtype of acute myeloid leukemia, respectively. *FOXP1* and *SPIB* are transcriptional factors amplified in the ABC-DLBCL.[32] Mutations of *TCF4*, a component of Wnt pathway, have

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been shown in various types of solid tumors and lymphoma/leukemia. TNFRSF13B signaling activates NF-κB, NF-AT and AP1. POLR3G and POLR1B were DNA-dependent RNA polymerases.

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Discussion

In this study we show that patients with DLBCL characterized by MYC/BCL2 co-expression have a poor clinical outcome with a 5-year OS and PFS of less than 30%. MYC/BCL2 co-expression in DLBCL is also a strong predictor of poor prognosis in the two COO subtypes. Patients with DLBCL with MYC/BCL2 co-expression have many clinicopathologic features associated with adverse prognosis, including older age, advanced stage of disease, multiple extranodal sites of involvement, high IPI score, high proliferation index, and poor treatment response. Approximately one-third of DLBCL demonstrate MYC/BCL2 co-expression, in keeping with the 29% frequency reported in an earlier study by Green et al.[23] By contrast, *MYC/BCL2* double-hit B-cell lymphoma characterized chromosomal breaks involving *MYC* and *BCL2* is a rare disease, representing approximately 3% of all DLBCL cases in our study. Thus, the findings in this study expand the spectrum of aggressive DLBCL, defined previously at the genetic level, by using IHC. In our study, we observed an overall MYC$^+$ rate of 64% and MYC$^+$BCL2$^+$ 34% in our training set and an overall MYC$^+$ rate of 54% and MYC$^+$BCL2$^+$ 32% in our validation set, which were in line with the overall MYC$^+$ rate of 54% and MYC$^+$BCL2$^+$ 29% observed by Green et al [23](Personal communication and Supplemental legends of Figure S8 and S9), but higher than those reported by Johnson et al.[24] The cause of the discrepancy is not known.

ABC-DLBCL, as stratified by gene-expression signatures, is associated with a poorer prognosis compared with GCB-DLBCL.[1] The inferior prognosis of patients with the ABC-DLBCL has been attributed to constitutive NF-κB activity, leading to the up-regulation of a number of NF- κB target genes, including *MYC* and *BCL2*.[2] At the protein level, we show here that MYC/BCL2 co-expression is more frequently observed in the

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ABC subtype. We suggest that the high frequency of MYC/BCL2 co-expression in ABC-DLBCL greatly contributes to the overall poorer prognosis of this patient subset. Our data support this statement in three ways. First, there was no significant prognostic difference between DLBCL patients with the GCB vs ABC subtype when all cases with MYC/BCL2 co-expression were excluded. Secondly, when only patients with DLBCL with MYC/BCL2 co-expression were considered, the two subtypes did not confer significant prognostic difference either. Thirdly, although there was a striking difference in gene signatures between the ABC and GCB subtypes with MYC/BCL2 co-expression, the difference in gene signatures between the two subtypes was minimal in MYC/BCL2 double-negative DLBCL. We found this result somewhat surprising given that the GCB and ABC subtypes of DLBCL are assumed to be derived from B-cells at different differentiation stages. We found that MYC/BCL2 double-negative DLBCL had a higher percentage of cases unclassifiable by GEP or that showed a discordance between GEP and IHC-based classification (30% in MYC/BCL2 double-negative group versus 19% in DLBCL with MYC/BCL2 co-expression, p=.07). These borderline cases may blur the boundary between the GCB and ABC signatures. There are at least five distinct subsets of mature B-cells corresponding to different B-cell differentiation stages[33] and it is possible that DLBCL could be derived from each of these subsets. Our results raise the issue as to whether it is appropriate to "force" DLBCL cases in a binary and perhaps too simplistic classification.

Our results show that DLBCL with MYC/BCL2 co-expression is a unique subset of DLBCL with dismal clinical outcome. The potential molecular basis behind the dismal outcome is seen through the gene-expression profiles of cases with MYC/BCL2 co-expression: stromal, adhesion, and proliferation signatures. This stromal signature is

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similar to what was described by Lenz et al (stromal signature 1) although it is more striking in DLBCL with MYC/BCL2 co-expression.[27] Both stroma-poor and proliferation signatures are associated with poor prognosis in DLBCL.[27,28,34,35] A cell adhesion signature has not been described in lymphoma to date although its role in the invasion of solid tumors is well-established.[36] Presumably, the lack of cell-cell and cell-matrix adhesions might play a role in the high frequency of advanced stage of disease and involvement of multiple extranodal sites in DLBCL with MYC/BCL2 co-expression.

However, we do not wish to imply that there are no other pathogenetic factors that contribute to the inferior prognosis of patients with ABC-DLBCL, nor are we suggesting that the ABC and GCB subtypes of DLBCL with MYC/BCL2 co-expression are biologically homogenous. Each of these subtypes might include additional subsets with additional predictive prognostic factors that deserve further investigation. Our GEP studies clearly show heterogeneity in the group of DLBCL with MYC/BCL2 co-expression. Instead, we only wish to emphasize that MYC/BCL2 co-expression has significant prognostic value for DLBCL patients and these tumors represent almost half of the ABC-DLBCL cases. In addition, the recognition of DLBCL with MYC/BCL2 co-expression expands the spectrum of aggressive B-cell lymphomas for which novel therapies are needed, and IHC assessment for MYC and BCL2 expression provides a practical approach to effectively stratify DLBCL into prognostically relevant subgroups.

With regard to the prognostic impact of BCL2 expression alone in DLBCL in the era of R-CHOP chemotherapy, previous studies reported inconsistent results.[21,23,24,38-45] Most of these studies had small patient cohorts and/or also did not address the confounding effects of other factors, such as MYC expression. In this study we found that BCL2 expression predicted survival in DLBCL in the overall patient cohort and in the

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COO subtypes. However, the observed prognostic impact of BCL2 expression was attributable to the subset of DLBCL cases with MYC/BCL2 co-expression. Similarly, MYC protein expression affected prognosis only in the presence of BCL2 co-expression, consistent with the studies by Johnson et al.[24] Approximately 60% of cases with *BCL2* but without concurrent *MYC* rearrangement demonstrated MYC protein expression in our cohort. This may explain our previous observation that DLBCL with *BCL2* rearrangement showed worse prognosis irrespective of *MYC* rearrangement status.[37]

In summary, DLBCL with MYC/BCL2 co-expression characterizes a subset of DLBCL patients with high-risk gene signatures, high-risk clinicopathologic features and poor prognosis. DLBCL with MYC/BCL2 co-expression apparently expands the spectrum of *MYC/BCL2* double-hit B-cell lymphoma defined genetically and well recognized to have a poor prognosis. However, it should be emphasized that our data do not show that *MYC/BCL2* double-hit B-cell lymphoma and DLBCL with MYC/BCL2 co-expression are equivalent. It is possible that additional molecular abnormalities or levels of MYC and BCL2 protein expression may distinguish these two groups. We further show that DLBCL with MYC/BCL2 co-expression occurs in almost half of ABC-DLBCL cases and appears to account, in large part, for the inferior prognosis of patients with ABC-DLBCL. These data also suggest that assessment for MYC and BCL2 protein expression more reliably predicts prognosis than the cell of origin classification.

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AUTHOR CONTRIBUTION

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Research performing: SH, KHY

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Manuscript writing: SH, LJM, KHY

Final approval of manuscript: All authors

DISCLOSURE OF CONFLICTS OF INTEREST
The authors declare no conflicts of interest.

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REFERENCES

1. Alizadeh AA, Eisen MB, Davis RE, et al. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature.* 2000;403(6769):503-511.
2. Lim KH, Yang Y, Staudt LM. Pathogenetic importance and therapeutic implications of NF-kappaB in lymphoid malignancies. *Immunological reviews.* 2012;246(1):359-378.
3. Compagno M, Lim WK, Grunn A, et al. Mutations of multiple genes cause deregulation of NF-kappaB in diffuse large B-cell lymphoma. *Nature.* 2009;459(7247):717-721.
4. Hans CP, Weisenburger DD, Greiner TC, et al. Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. *Blood.* 2004;103(1):275-282.
5. Muris JJ, Meijer CJ, Vos W, et al. Immunohistochemical profiling based on Bcl-2, CD10 and MUM1 expression improves risk stratification in patients with primary nodal diffuse large B cell lymphoma. *J Pathol.* 2006;208(5):714-723.
6. Natkunam Y, Farinha P, Hsi ED, et al. LMO2 protein expression predicts survival in patients with diffuse large B-cell lymphoma treated with anthracycline-based chemotherapy with and without rituximab. *J Clin Oncol.* 2008;26(3):447-454.
7. Nyman H, Jerkeman M, Karjalainen-Lindsberg ML, Banham AH, Leppa S. Prognostic impact of activated B-cell focused classification in diffuse large B-cell lymphoma patients treated with R-CHOP. *Mod Pathol.* 2009;22(8):1094-1101.
8. Choi WW, Weisenburger DD, Greiner TC, et al. A new immunostain algorithm classifies diffuse large B-cell lymphoma into molecular subtypes with high accuracy. *Clin Cancer Res.* 2009;15(17):5494-5502.
9. Meyer PN, Fu K, Greiner TC, et al. Immunohistochemical methods for predicting cell of origin and survival in patients with diffuse large B-cell lymphoma treated with rituximab. *J Clin Oncol.* 2011;29(2):200-207.
10. Visco C, Li Y, Xu-Monette ZY, 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;Prepublished on Mar 22, 2012 as DOI 10.1038/leu.2012.83.
11. Gutierrez-Garcia G, Cardesa-Salzmann T, Climent F, et al. Gene-expression profiling and not immunophenotypic algorithms predicts prognosis in patients with diffuse large B-cell lymphoma treated with immunochemotherapy. *Blood.* 2011;117(18):4836-4843.
12. Thieblemont C, Briere J, Mounier N, et al. The germinal center/activated B-cell subclassification has a prognostic impact for response to salvage therapy in relapsed/refractory diffuse large B-cell lymphoma: a bio-CORAL study. *J Clin Oncol.* 2011;29(31):4079-4087.
13. Gu K, Weisenburger DD, Fu K, et al. Cell of origin fails to predict survival in patients with diffuse large B-cell lymphoma treated with autologous hematopoietic stem cell transplantation. *Hematol Oncol.* 2011;Prepublished on Oct 18, 2011 as DOI: 10.1002/hon.1017.

From bloodjournal.hematologylibrary.org at M D ANDERSON HOSP on March 6, 2013. For personal use only.

14. Aukema SM, Siebert R, Schuuring E, et al. Double-hit B-cell lymphomas. *Blood.* 2011;117(8):2319-2331.
15. Kanungo A, Medeiros LJ, Abruzzo LV, Lin P. Lymphoid neoplasms associated with concurrent t(14;18) and 8q24/c-MYC translocation generally have a poor prognosis. *Mod Pathol.* 2006;19(1):25-33.
16. Lin P, Medeiros LJ. High-grade B-cell lymphoma/leukemia associated with t(14;18) and 8q24/MYC rearrangement: a neoplasm of germinal center immunophenotype with poor prognosis. *Haematologica.* 2007;92(10):1297-1301.
17. Le Gouill S, Talmant P, Touzeau C, et al. The clinical presentation and prognosis of diffuse large B-cell lymphoma with t(14;18) and 8q24/c-MYC rearrangement. *Haematologica.* 2007;92(10):1335-1342.
18. Niitsu N, Okamoto M, Miura I, Hirano M. Clinical features and prognosis of de novo diffuse large B-cell lymphoma with t(14;18) and 8q24/c-MYC translocations. *Leukemia.* 2009;23(4):777-783.
19. Tomita N, Tokunaka M, Nakamura N, et al. Clinicopathological features of lymphoma/leukemia patients carrying both BCL2 and MYC translocations. *Haematologica.* 2009;94(7):935-943.
20. Johnson NA, Savage KJ, Ludkovski O, et al. Lymphomas with concurrent BCL2 and MYC translocations: the critical factors associated with survival. *Blood.* 2009;114(11):2273-2279.
21. Li S, Lin P, Fayad LE, et al. B-cell lymphomas with MYC/8q24 rearrangements and IGH@BCL2/t(14;18)(q32;q21): an aggressive disease with heterogeneous histology, germinal center B-cell immunophenotype and poor outcome. *Mod Pathol.* 2012;25(1):145-156.
22. Snuderl M, Kolman OK, Chen YB, et al. B-cell lymphomas with concurrent IGH-BCL2 and MYC rearrangements are aggressive neoplasms with clinical and pathologic features distinct from Burkitt lymphoma and diffuse large B-cell lymphoma. *Am J Surg Pathol.* 2010;34(3):327-340.
23. Green TM, Young KH, Visco C, et al. Immunohistochemical double-hit score is a strong predictor of outcome in patients with diffuse large B-cell lymphoma treated with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone. *J Clin Oncol.* 2012;30(28):3460-3467.
24. Johnson NA, Slack GW, Savage KJ, et al. Concurrent expression of MYC and BCL2 in diffuse large B-cell lymphoma treated with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone. *J Clin Oncol.* 2012;30(28):3452-3459.
25. Xu-Monette Z, Wu L, Visco C, et al. . Mutational profile and prognostic significance of TP53 in diffuse large B-cell lymphoma patients treated with rituximab-CHOP: A Report from an International DLBCL Rituximab-CHOP Consortium Program Study. . *Blood.* 2012; In press.
26. 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 U S A.* 2003;100(17):9991-9996.
27. Lenz G, Wright G, Dave SS, et al. Stromal gene signatures in large-B-cell lymphomas. *N Engl J Med.* 2008;359(22):2313-2323.
28. Rosenwald A, Wright G, Chan WC, 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):1937-1947.

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29. Roberts RA, Wright G, Rosenwald AR, et al. Loss of major histocompatibility class II gene and protein expression in primary mediastinal large B-cell lymphoma is highly coordinated and related to poor patient survival. *Blood.* 2006;108(1):311-318.

30. Rimsza LM, Roberts RA, Campo E, et al. Loss of major histocompatibility class II expression in non-immune-privileged site diffuse large B-cell lymphoma is highly coordinated and not due to chromosomal deletions. *Blood.* 2006;107(3):1101-1107.

31. Ruminy P, Etancelin P, Couronne L, et al. The isotype of the BCR as a surrogate for the GCB and ABC molecular subtypes in diffuse large B-cell lymphoma. *Leukemia.* 2011;25(4):681-688.

32. Lenz G, Wright GW, Emre NC, et al. Molecular subtypes of diffuse large B-cell lymphoma arise by distinct genetic pathways. *Proc Natl Acad Sci U S A.* 2008;105(36):13520-13525.

33. Kjeldsen MK, Perez-Andres M, Schmitz A, et al. Multiparametric flow cytometry for identification and fluorescence activated cell sorting of five distinct B-cell subpopulations in normal tonsil tissue. *Am J Clin Pathol.* 2011;136(6):960-969.

34. Meyer PN, Fu K, Greiner T, et al. The stromal cell marker SPARC predicts for survival in patients with diffuse large B-cell lymphoma treated with rituximab. *Am J Clin Pathol.* 2011;135(1):54-61.

35. Perry AM, Cardesa-Salzmann TM, Meyer PN, et al. A new biologic prognostic model based on immunohistochemistry predicts survival in patients with diffuse large B-cell lymphoma. *Blood.* 2012;120(11):2290-2296.

36. Friedl P, Alexander S. Cancer invasion and the microenvironment: plasticity and reciprocity. *Cell.* 2011;147(5):992-1009.

37. Visco C, Tzankov A, Xu-Monette ZY, et al. 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. *Haematologica.* 2013;98(2):255-263.

38. Mounier N, Briere J, Gisselbrecht 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):4279-4284.

39. Iqbal J, Neppalli VT, Wright G, et al. BCL2 expression is a prognostic marker for the activated B-cell-like type of diffuse large B-cell lymphoma. *J Clin Oncol.* 2006;24(6):961-968.

40. Shivakumar L, Armitage JO. Bcl-2 gene expression as a predictor of outcome in diffuse large B-cell lymphoma. *Clinical lymphoma & myeloma.* 2006;6(6):455-457.

41. Amen F, Horncastle D, Elderfield K, et al. Absence of cyclin-D2 and Bcl-2 expression within the germinal centre type of diffuse large B-cell lymphoma identifies a very good prognostic subgroup of patients. *Histopathology.* 2007;51(1):70-79.

42. Wilson KS, Sehn LH, Berry B, 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):1102-1109.

43. 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-

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treated non-germinal center diffuse large B-cell lymphomas. *Eur J Haematol.* 2009;82(5):364-372.

44. Iqbal J, Meyer PN, Smith LM, 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):7785-7795.

45. Horn H, Ziepert M, Becher C, et al. MYC status in concert with BCL2 and BCL6 expression predicts outcome in diffuse large B-cell lymphoma. *Blood.* Jan 18 2013.

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Table 1. Clinicopathologic characteristics and outcome of DLBCLs treated with R-CHOP

	Overall			DP	Non-DP	
	N (%)	OS (p-value)	PFS (p-value)	N (%)	N (%)	p-value
Patients	466 (100%)			157 (100%)	309 (100%)	
Gender						
Male	272 (58%)	0.7477	0.4730	90 (57%)	182 (59%)	0.7445
Female	194 (42%)			67 (43%)	127 (41%)	
Age						
≤60	194 (42%)	0.0004	0.0016	49 (31%)	145 (47%)	0.0011
>60	272 (58%)			108 (69%)	164 (53%)	
B symptoms*						
Absence	276 (68%)	0.0015	0.0014	88 (62%)	188 (72%)	0.0541
Presence	127 (32%)			53 (38%)	74 (28%)	
ECOG performance status*						
<2	350 (88%)	<0.0001	<0.0001	111 (83%)	239 (90%)	0.0453
≥2	50 (12%)			23 (17%)	27 (10%)	
Stage*						
I-II	219 (49%)	<0.0001	<0.0001	50 (33%)	169 (57%)	<0.0001
III-IV	228 (51%)			100 (67%)	128 (43%)	
Extranodal Sites*						
<2	346 (78%)	<0.0001	<0.0001	106 (72%)	240 (82%)	0.0160
≥2	96 (22%)			42 (28%)	54 (18%)	
LDH*						
Normal	168 (40%)	0.0003	<0.0001	51 (36%)	117 (42%)	0.2908
Elevated	252 (60%)			89 (64%)	163 (58%)	
IPI risk group*						
0-2	263 (64%)	<0.0001	<0.0001	70 (51%)	193 (70%)	0.0001
3-5	148 (36%)			67 (49%)	81 (30%)	
Tumor size (cm)*						
<7.5	253 (77%)	0.0100	0.0172	81 (73%)	172 (79%)	0.2587
≥7.5	77 (23%)			30 (27%)	47 (21%)	
Treatment response						
CR	354 (76%)	<0.0001	<0.0001	103 (66%)	251 (84%)	<0.0001
Others	112 (24%)			54 (34%)	48 (16%)	
COO Classification						
GCB	241 (52%)	0.0080	0.0075	53 (34%)	188 (61%)	<0.0001
ABC	225 (48%)			104 (66%)	121 (39%)	
Ki-67*						
<70%	158 (34%)	0.2998	0.3434	41 (26%)	117 (38%)	0.0086
≥70%	304 (66%)			116 (74%)	188 (62%)	
TP53 mutations						
Absence	357 (77%)	0.0005	0.0004	117 (75%)	240 (78%)	0.4480
Presence	109 (23%)			40 (25%)	69 (22%)	

Abbreviations: DLBCL: diffuse large B-cell lymphoma; OS: overall survival; PFS: progression-free survival; DP: MYC/BCL2 double-positive by immunohistochemistry; Non-DP: non MYC/BCL2 double-positive; LDH: lactate dehydrogenase; IPI: International Prognostic Index; CR: complete remission; COO: cell of origin; GCB: germinal center cell; ABC: activated B-cell. *Information not available in some cases.

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Table 2. Multivariate analysis of clinicopathologic parameters in DLBCLs treated with R-CHOP

	Overall Survival			Progression-free Survival		
	HR	95% CI	p-value	HR	95% CI	p-value
B-symptoms	1.47	1.04-2.09	0.0310	1.45	1.03-2.03	0.0314
Tumor size (≥7.5 cm)	1.22	0.87-1.71	0.2467	1.21	0.86-1.69	0.2708
IPI risk (>2)	2.38	1.67-3.38	<0.0001	2.22	1.59-3.11	<0.0001
COO classification (ABC)	1.17	0.79-1.72	0.4329	1.18	0.82-1.71	0.3750
TP53 mutation	1.72	1.17-2.52	0.0057	1.63	1.12-2.37	0.0105
MYC/BCL2 co-expression	2.52	1.73-3.67	<0.0001	2.45	1.71-3.51	<0.0001

Abbreviations: DLBCL: diffuse large B-cell lymphoma; HR: hazard ratio; CI: confidence interval; IPI: International Prognostic Index; COO: cell of origin; ABC: activated B-cell.

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Table 3. Differentially expressed genes in MYC^{+}BCL2^{+} *de novo* DLBCL

A. Downregulated genes

Gene functional categories	No. of genes	Representative genes
ECM, ECM production and remodeling	33	COL3A1, VCAN, TNS1, FN1, THBS2, TIMP3, SPARC, SULF1, SPINK2, MMP2, ADAM12, FGFR1, FAP
Cell adhesion and cytoskeletal organization	21	CD11A/CD11B, CD58, THY1, RFTN1, ANTXR1, RHOB, MICAL2
Cell growth regulation	16	LMO2, TRAF1, CDK14, SGK1, RGS1, NBL, PDE4D
Others, including unknown	18	PSAP, LYZ, LOC115110, ZNF662

B. Upregulated genes

Gene functional categories	No. of genes	Representative genes
Cell proliferation	20	MYC, BCL2, TCL1A, MLL, FOXP1, SPIB, TCF4, TNFRSF13B, PMDAIP1, GAB1, PLOR3G
Cell metabolism	5	DCTPP1, CYB5R2, HK2, TMEM97, CYB5R2
Miscellaneous cell functions	13	PPIL1, PIGW, FUT8, SPINK5
Unknown	27	KIAA0664, C9orf91, ZNF107

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Figure Legends

Figure 1. Prognostic impact of MYC/BCL2 co-expression in DLBCL

(A, B) OS (A) and PFS (B) of patients with DLBCL with MYC/BCL2 co-expression (MYC^+BCL2^+) in the training set. **(C, D)** OS of patients with MYC^+ DLBCL in the presence (C) or absence (D) of BCL2 co-expression in the training set. **(E, F)** OS of patients with $BCL2^+$ DLBCL in the presence (E) or absence (F) of MYC co-expression in the training set.

Figure 2. Prognostic impact of MYC/BCL2 co-expression in DLBCL risk-stratified according to clinicopathologic parameters

(A, B) OS (A) and PFS (B) of patients with MYC^+BCL2^+ DLBCL of the GCB subtype in the training set. **(C, D)** OS (C) and PFS (D) of patients with MYC^+BCL2^+ DLBCL of the ABC subtype in the training set. **(E, F)** OS (E) and PFS (F) of patients with MYC^+BCL2^+ DLBCL risk-stratified according to IPI risk scores in the training set. DP: MYC/BCL2 double-positive; Non-DP: non-double positive.

Figure 3. Frequency of BCL2 and MYC expression in COO subtypes of DLBCL

(A) Relative frequency of the ABC vs GCB subtype in DLBCL positive for BCL2 expression, MYC expression, or MYC/BCL2 co-expression in the training set. **(B)** Frequency of BCL2 expression, MYC expression, or MYC/BCL2 co-expression (in the presence or absence of *MYC*/*BCL2* DH) in DLBCL of the ABC and GCB subtypes in the training set. DH: double-hit.

Figure 4. MYC/BCL2 co-expression contributes to the inferior prognosis of ABC-DLBCL

(A, B) OS (A) and PFS (B) of the ABC vs GCB subtype of DLBCL in the entire training set. Cell of origin classification of 411 cases was based on GEP results and 55 cases based on IHC results. **(C, D)** OS (C) and PFS (D) of the ABC vs GCB subtype of DLBCL after all MYC^+BCL2^+ cases were excluded. **(E, F)** OS (E) and PFS (F) of the ABC vs GCB subtype in MYC^+BCL2^+ DLBCL.

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Figure 5. Prognostic impact of MYC/BCL2 co-expression in DLBCL is independent of *MYC/BCL2* co-rearrangement and *TP53* mutation status

(A, B) OS (A) and PFS (B) of patients with *MYC/BCL2* double-hit DLBCL. (**C, D**) OS (C) and PFS (D) of patients with MYC^{+}BCL2^{+} DLBCL in the absence of *MYC/BCL2* double-hit. **(E)** OS of patients with MYC^{+}BCL2^{+} DLBCL in the absence of *TP53* mutation. **(F)** Prognostic impact of *TP53* mutation in MYC^{+}BCL2^{+} DLBCL. DH: double-hit.

Figure 6. MYC/BCL2 co-expression contributes to the different gene expression profiles between GCB and ABC subtypes of DLBCL

(A) GEP comparison between the ABC vs GCB subtype of DLBCL with MYC/BCL2 co-expression. Of 157 cases of MYC^{+}BCL2^{+} DLBCL, GEP was successfully performed in 149 cases (ABC: 102; GCB: 47). DP: MYC/BCL2 double-positive. A total of 208 genes corresponding to 365 probe sets were differentially expressed ($p<.001$). **(B)** GEP comparison between the ABC (30 cases) vs GCB (58 cases) subtype of DLBCL negative for both MYC and BCL2 protein expression. A total of 20 genes corresponding 30 probe sets were differentially expressed between the two COO subtypes ($p<.01$). DN: MYC/BCL2 double-negative.

Figure 7. Gene expression signature of DLBCL with MYC/BCL2 co-expression

Comparison of GEPs of DLBCL with MYC/BCL2 co-expression (149 cases) vs. DLBCL negative for MYC and BCL2 expression (88 cases). A total of 153 genes corresponding to 219 probe sets were differentially expressed ($p<.001$).

Figure 1

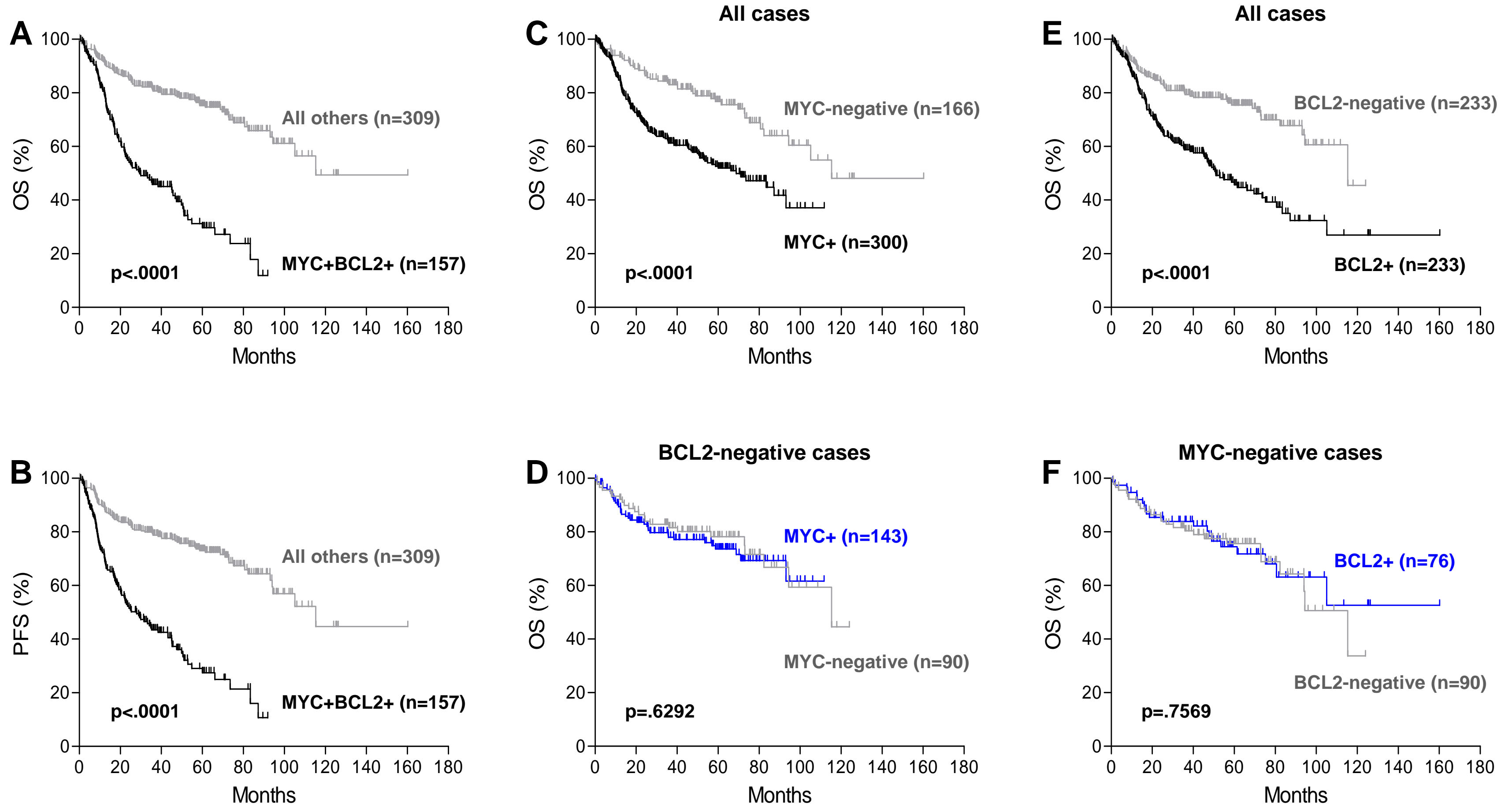
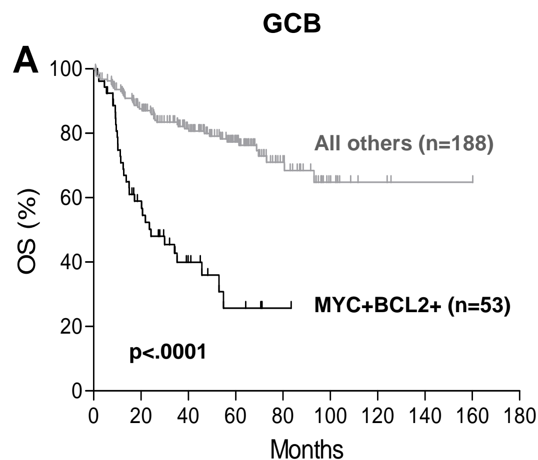
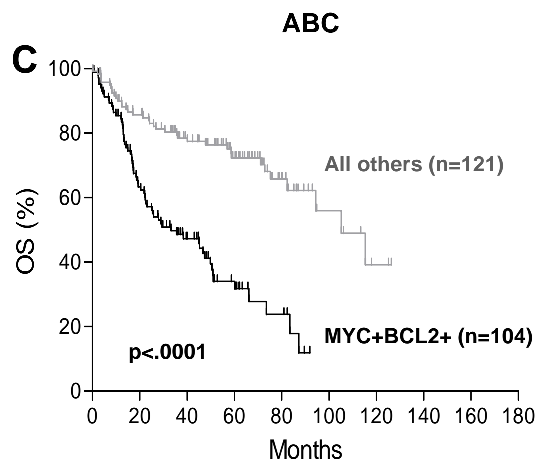
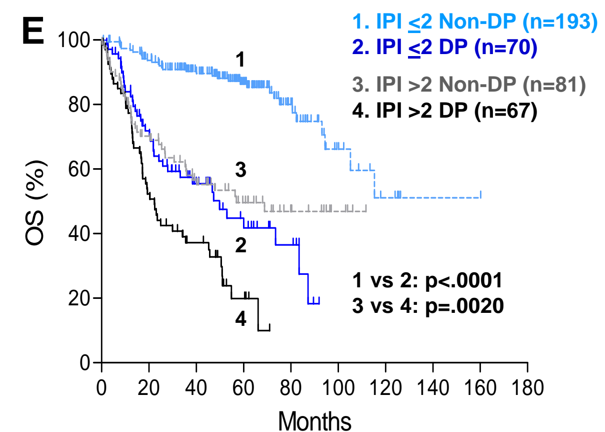
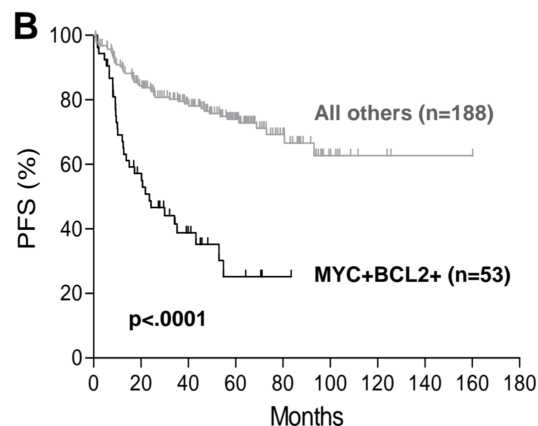


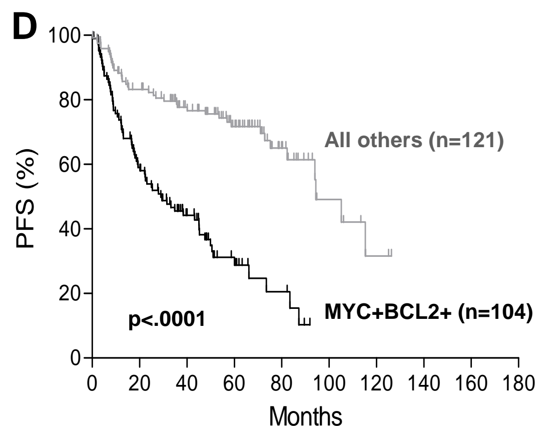
Figure 2











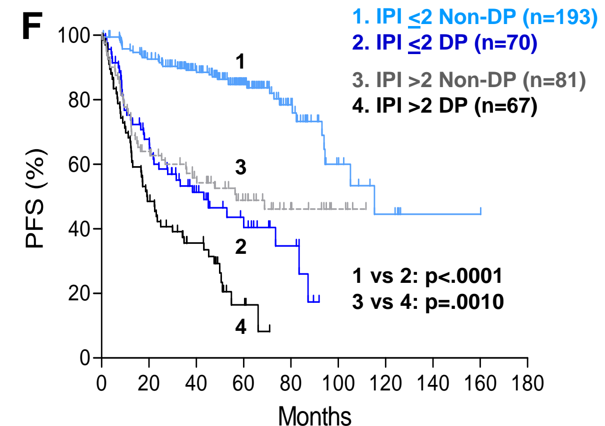
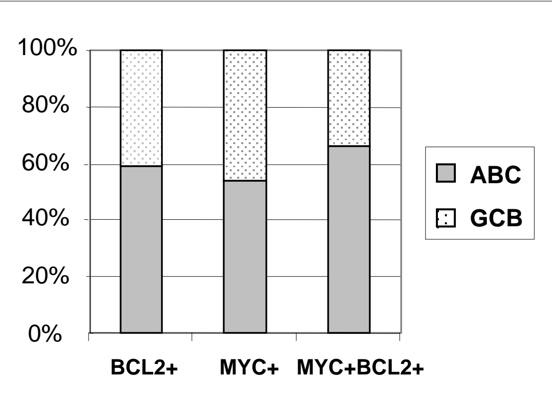


Figure 3

A



B

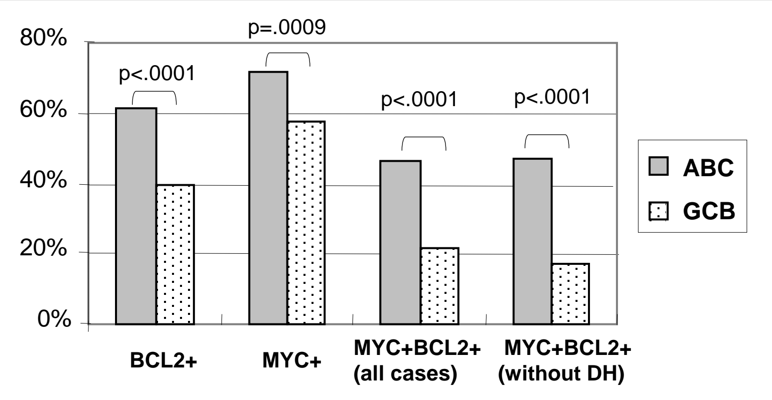
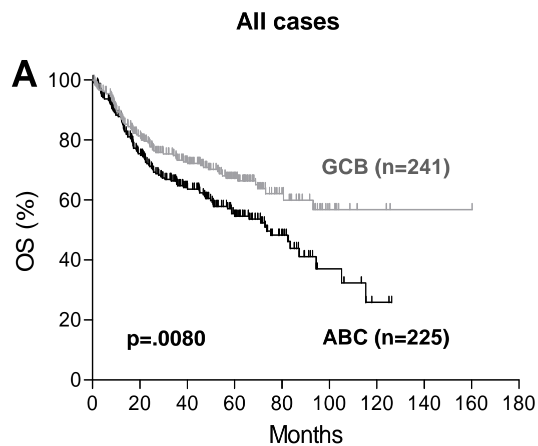
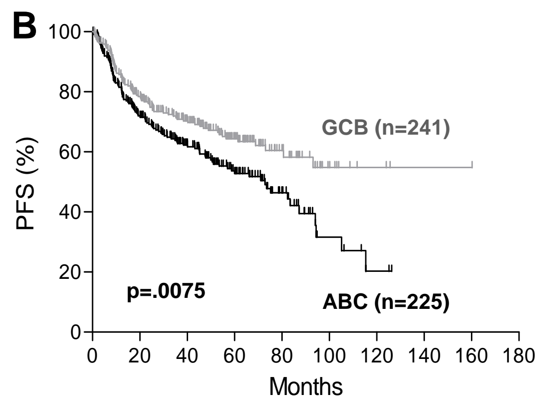


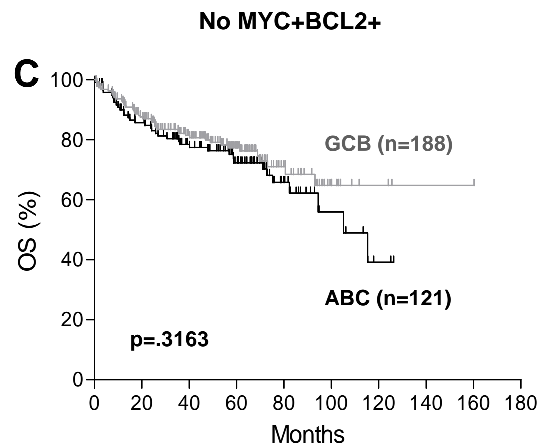
Figure 4

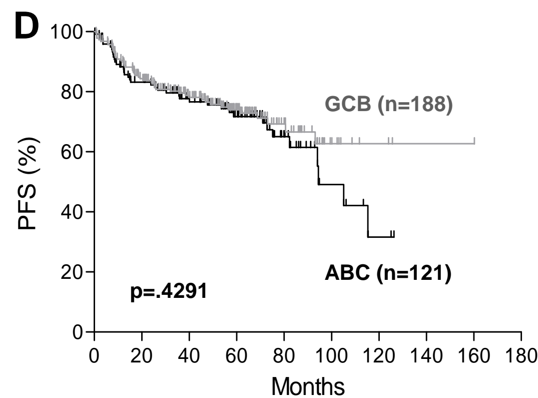
All cases



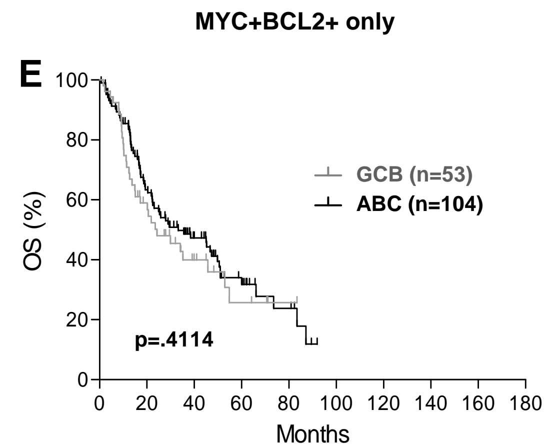


No MYC+BCL2+





MYC+BCL2+ only



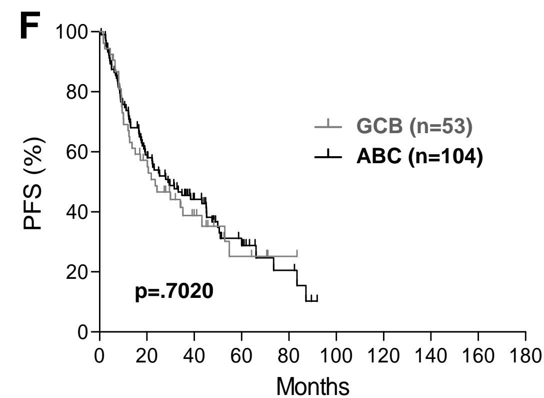


Figure 5

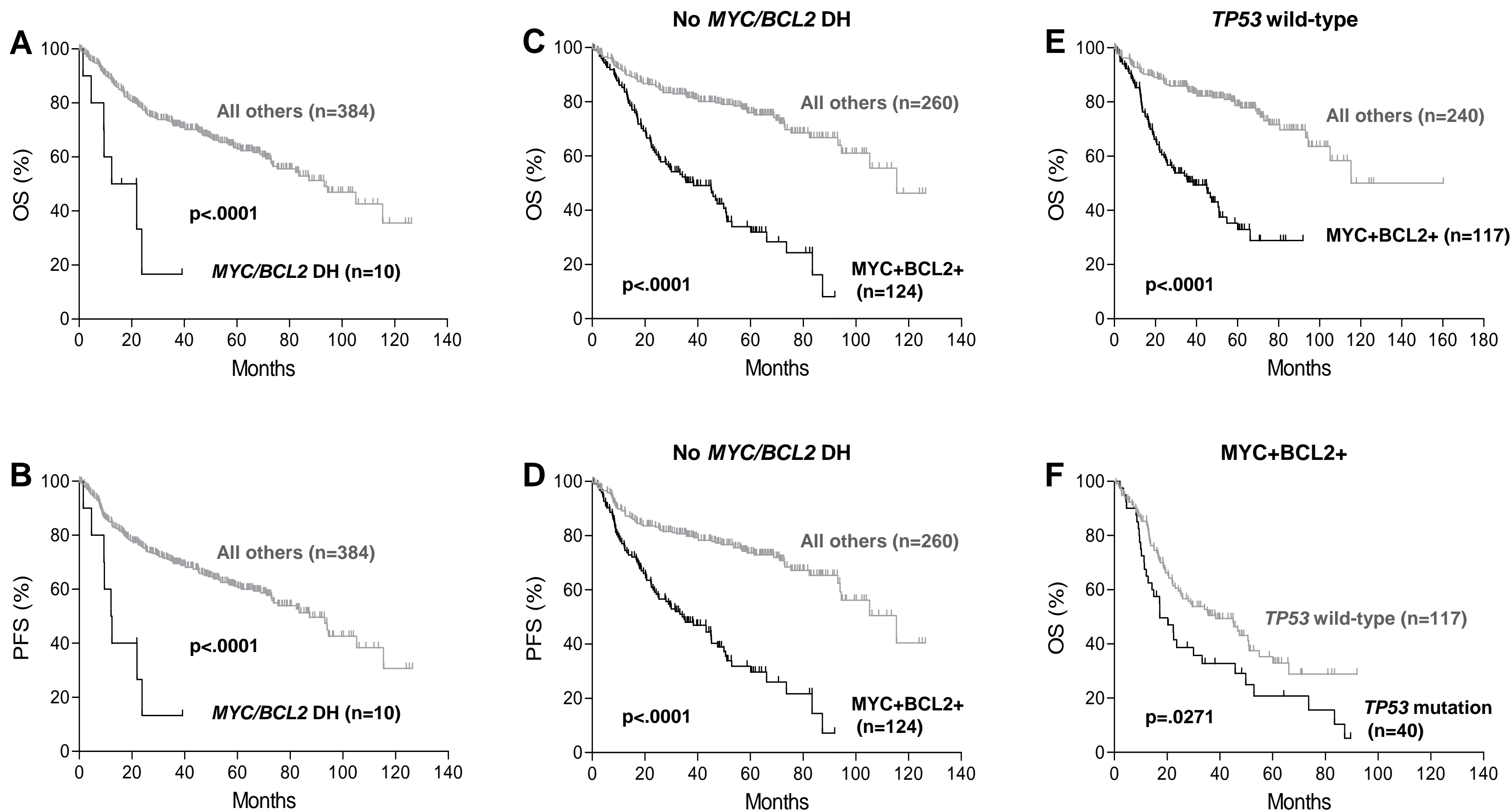


Figure 6A

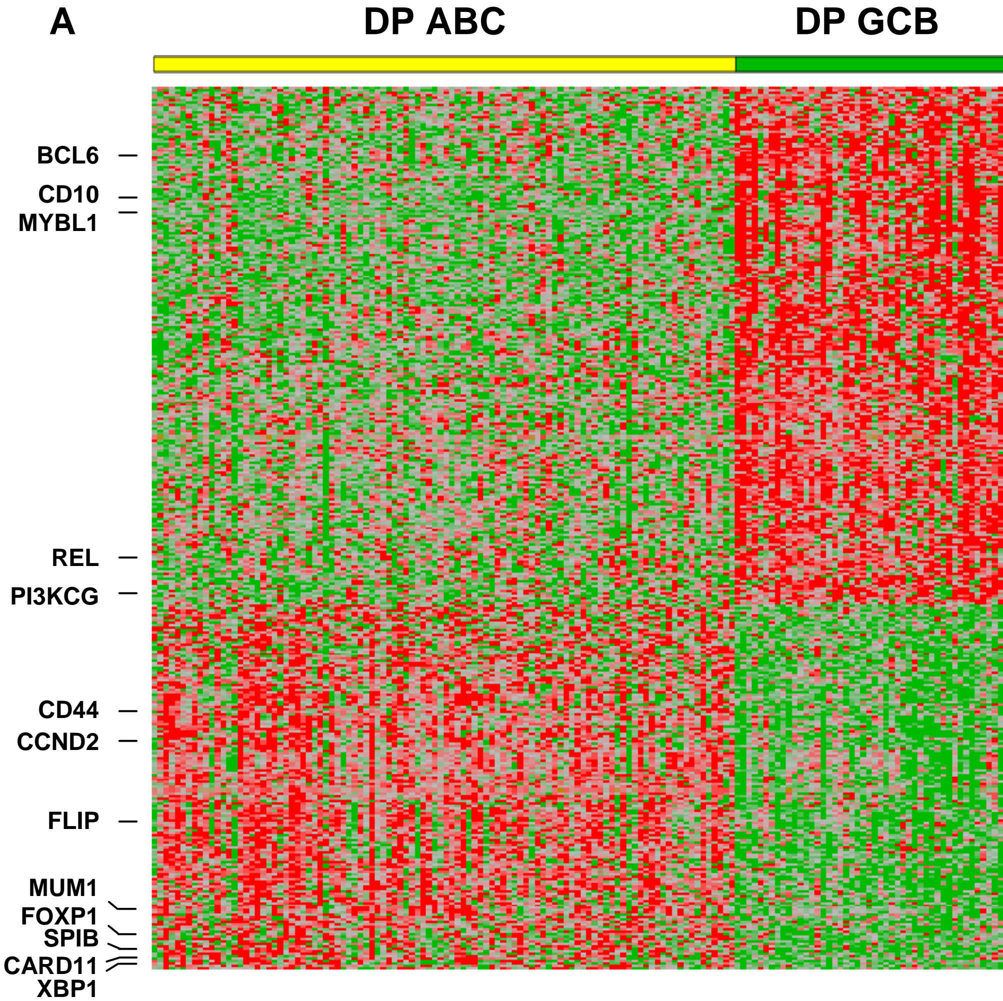


Figure 6B

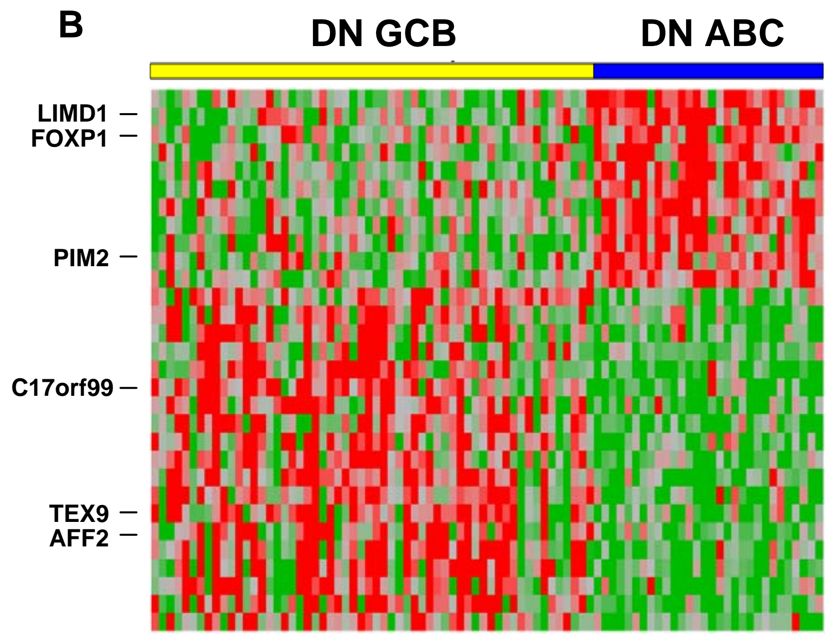


Figure 7