MYC STATUS IN CONCERT WITH BCL2 AND BCL6 EXPRESSION
PREDICTS OUTCOME IN DIFFUSE LARGE B-CELL LYMPHOMA

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KEY POINTS

- outcome prediction in DLBCL
- MYC status in concert with BCL2 and BCL6

ABSTRACT

MYC rearrangements occur in 5-10% of diffuse large B-cell lymphomas (DLBCL) and confer an increased risk to CHOP and R-CHOP treated patients. We investigated the prognostic relevance of MYC-, BCL2- and BCL6-rearrangements and protein expression in a prospective randomized trial. Paraffin-embedded tumor samples from 442 de novo DLBCL treated within the RICOVER study of the German High Grade Lymphoma Study Group (DS HNHL) were investigated using immunohistochemistry and fluorescence in situ hybridisation to detect protein expression and breaks of MYC, BCL2 and BCL6. Rearrangements of MYC, BCL2 and BCL6 were detected in 8.8%, 13.5% and 28.7%, respectively. Protein overexpression of MYC (>40%) was encountered in 31.8% of tumors. 79.6% and 82.8% of tumors expressed BCL2 and BCL6, respectively. MYC translocations, MYC high, BCL2 high and BCL6 low protein expressions were associated with inferior survival. In multivariate Cox regression modelling, protein expression patterns of MYC, BCL2 and BCL6, and MYC rearrangements were predictive of outcome and provided prognostic information independent from the International Prognostic Index (IPI) for OS and EFS. A combined immunohistochemical or FISH/immunohistochemical score predicts outcome in DLBCL patients independent of the IPI and identifies a subset of 15% of patients with dismal prognosis in the high-risk IPI group following treatment with R-CHOP. (Registered at http://www.cancer.gov/clinicaltrials: RICOVER trial of the DSHNHL is NCT 00052936)
**INTRODUCTION**

Diffuse large B-cell lymphomas (DLBCL) constitute a heterogeneous category of aggressive lymphomas.¹ Gene expression profiling (GEP) has helped to stratify DLBCL into biologically meaningful and prognostically relevant subgroups.²,³ The translation of complex GEP predictors into e.g. immunohistochemistry (IHC)-based assays applicable to paraffin-embedded and formalin-fixed (FFPE) tumor specimens, however, a prerequisite for everyday clinical practice, has been difficult.⁴,⁵ Recurrent translocations targeting *BCL2*, *BCL6* and *MYC* have been described in DLBCL. While different results have been published regarding the impact of *BCL2* and *BCL6* breaks on prognosis in DLBCL⁶⁻¹², *MYC* translocations confer a worse prognosis in patient cohorts treated with CHOP-like¹⁰,¹¹ and CHOP plus Rituximab (R-CHOP) regimens.¹³,¹⁴ Of note, patients whose tumors present with “double hit” features, i.e. dual translocations involving both *MYC* and *BCL2* or *BCL6* have shown a dismal clinical course.¹⁵ However, some questions regarding the deregulation of *MYC* in DLBCL still exist. First, analyses targeting the importance of *MYC* in DLBCL have been carried out mostly in population-based cohorts, but rarely within randomized prospective clinical trials.¹³,¹⁴ Second, the prognostic relevance of *MYC* rearrangements in DLBCL has rarely been studied in the context of concomitant translocations involving *BCL2* and *BCL6* and expression of these two proteins. Third, the high-throughput analysis of fluorescence *in situ* hybridization (FISH) in the context of large case numbers and – especially – on tissue microarray (TMA) sections has not been convincingly shown to be both reproducible and feasible. Finally, MYC can also be activated and overexpressed by mechanisms other than translocations, e.g. by amplifications, mutations or by microRNA-dependent mechanisms.¹⁶⁻¹⁸ Recently, a novel monoclonal antibody has been validated for use
in FFPE tissues\cite{19}, and nuclear reactivity for this antibody was shown to be predictive of \textit{MYC} rearrangements\cite{20,21}.

The aim of the present study was, therefore, to comprehensively assess the prognostic impact of protein expression patterns of \textit{MYC}, \textit{BCL2} and \textit{BCL6} in concert with the recurrent chromosomal translocations targeting \textit{MYC}, \textit{BCL2} and \textit{BCL6} in DLBCL patients treated in a large, prospective randomized clinical trial.

\section*{MATERIAL AND METHODS}

\subsection*{Study cohort}

All cases analyzed in this study were enrolled in the prospective randomized multicenter clinical trial RICOVER-60 of the German High Grade Lymphoma Study Group (DSHNHL)\cite{22}, in which patients over 60 years of age with CD20-positive aggressive B-cell lymphomas had been randomly assigned to 6 or 8 cycles of CHOP-14 treatment with or without 8 applications of rituximab. Diagnostic samples from the study patients were reviewed by expert hematopathologists according to the 2008 WHO classification\cite{1}. Assembly of tissue microarrays was performed as described previously\cite{4}. Altogether, 506 DLBCL specimens were represented on the TMAs. This clinical trial was conducted in accordance with the Helsinki declaration and the protocol had been approved by the ethics review committee of each participating center.

\subsection*{Fluorescence \textit{in situ} hybridisation (FISH)}

Interphase FISH was performed on standard tissue sections according to described protocols\cite{23,24}. Vysis LSI\textsuperscript{\copyright} \textit{BCL2}-, Vysis LSI\textsuperscript{\copyright} \textit{BCL6}- and Vysis LSI\textsuperscript{\copyright} \textit{MYC}-dual color break-apart rearrangement probes (Vysis/Abbott Molecular Diagnostics, Wiesbaden-Delkenheim, Germany) were used. The cut-off levels for the break-apart probes were
established by evaluating the split-signal distribution in samples of reactive lymphoid tissues, calculating the mean number of split signals plus three times the standard deviation. The cut-off levels were 13%, 12% and 15% for the BCL2, BCL6 and MYC break-apart probes, respectively. To validate the hybridization efficiency of TMA-spotted samples, a series of ten cases with different hybridization results for each probe was re-analyzed by performing FISH with BCL2, BCL6 and MYC probes, respectively, on sections of the original formalin-fixed paraffin-embedded (FFPE) tumor block. Evaluation of the signal distributions in the TMAs was carried out by two independent experienced observers in two institutions. In case of discordant results between the two observers, the hybridization was repeated using sections of the original FFPE tumor block, and a third investigator was involved. Cases judged as not evaluable by one of the investigators were excluded from statistical analyses.

**Immunohistochemical staining and scoring**

Immunohistochemistry with antibodies against CD20, CD10, BCL2, BCL6, IRF4/MUM1, and Ki67 had previously been performed.4 MYC immunohistochemistry was done on TMA sections using the antibody clone Y69 (Epitomics, CA, USA) at a 1:50 dilution and resulted in nuclear staining of variable intensity in the positive cases.19 After applying pressure cooking in citric acid (pH 6.0) for retrieval, detection was accomplished with Dako Advance reagents (K4068). Due to exhaustion of material, MYC was stained in only 310 cases. Immunohistochemistry was evaluated by two experienced hematopathologists on a multihead microscope and results were recorded as the percentage of positive tumor cells in increments of 10% regardless of the staining intensity.25,26 BCL2 and BCL6 stainings had been evaluated previously and had been recorded in steps of 25%. Lymphoma cases were judged as positive or overexpressed for these antigens (BCL2high, BCL6high) if positive in >0% and >25%
of cells, respectively (as extensively discussed in Ott et al., and Bernd et al.,\textsuperscript{4,27}). CD10 was judged as positive, if 1-100% of tumor cells were positive, and IRF4/MUM1 was rated as positive, if >5% of tumor cell nuclei were stained. Determination of these – different - cut points had also been accomplished by taking into account the highest level of reproducibility among different hematopathologists evaluating the initial stainings.\textsuperscript{4}

**Statistical evaluation**

Event-free survival (EFS) was the main endpoint of the RICOVER-60 trial and is defined as the time from randomization to disease progression, start of salvage treatment, additional (unplanned) treatments, relapse, or death from any cause. Overall survival (OS) was defined as the time from randomization to death of any cause. EFS and OS were estimated according to Kaplan and Meier.\textsuperscript{28}

To determine the cutpoint for statistical analysis discriminating BCL2, BCL6 and MYC staining positive and negative cases, the sample size within score groups and the effect sizes regarding EFS and OS for different scenarios with different definitions of positivity were taken into account. The most appropriate cutpoints were 0% (=BCL2low) vs. 1-100% (=BCL2high) positive cells for BCL2, 0-25% (=BCL6low) vs. 26-100% (=BCL6high) for BCL6 and 0-40% (MYClow) vs. 41-100% (=MYChigh) for MYC. Interestingly, the MYC cutoff point was very similar to that chosen in two recent publications by Johnson et al. and Green and colleagues.\textsuperscript{4,25-27}

In the univariate outcome analyses, logrank tests were performed. The International Prognostic Index (IPI, i.e., age > 60, LDH > normal, ECOG > 1, stage III/IV, and extranodal involvement > 1) is the current gold standard for prognostic stratification in DLBCL also in the rituximab era\textsuperscript{29}, and proportional hazard models for each of the selected parameters were separately adjusted for the factors of the IPI. Relative risks
with 95% confidence intervals (CI) and p-values are presented. For the correlation of
translocation and marker expression with qualitative data (morphology, GCB/non-GCB -
subtypes) and for differences regarding patient characteristics, we used chi-square and,
if necessary, Fisher’s exact test. Because of the descriptive nature of these
comparisons, the p-values were not adjusted for multiple comparisons and the
significance level was p=0.05. For many of the IHC/translocation markers we observed
a variable number of cases failing to yield reliable staining/FISH results and,
accordingly, the latter were excluded from analysis. Thus, the number of evaluable
cases varied from one marker to another. Cohens kappa statistic was used to assess
the concordance between two different observers. Statistical analyses were done with
SPSS PASW 18 and IBM SPSS Statistics 20.

RESULTS
Clinical and Sample Characteristics
The clinical features of the DLBCL groups with FISH results are provided in Table 1.
Tissue cores from 506 patients were represented on the TMAs, and 442 tumors were
amenable to FISH analysis (87.4%). 274/442 tumors (62.0%) had been classified as
centroblastic (CB) and 29/442 (6.6%) as immunoblastic (IB) DLBCL. In 139 (31.5%)
lymphomas, no subclassification had been rendered (DLBCL NOS). According to the
Hans classifier\(^3^0\), 157 DLBCL (47.0%) were of GCB-type, and 177 (53.0%) of non-
GCB-type.\(^4\)

FISH analyses
Initially, we created a pilot series of 46 B-NHL tumor samples spotted on TMAs and
with available classical chromosome banding data (at least for \(BCL2, BLC6,\) and
MYC translocations). This series was studied by FISH on the TMA using break-apart probes for BCL2, BCL6, MYC and IGH. In addition, 25 samples were also studied on whole tissue sections. Of these 100 hybridization events, all 87 hybridizations evaluable (87%) showed concordant results on the TMA and the whole tissue sections, while 13 hybridizations (13%) were not evaluable on the TMA. The results obtained by FISH on the TMA showed a correlation of 94% to the chromosome banding approaches (data not shown). Of 442 DLBCL specimens hybridized, 384 (86.9%), 404 (91.4%) and 407 (92.1%) samples were interpretable by two independent observers for the BCL2, BCL6 and MYC break-apart probes used, respectively. Comparison of the hybridization results for BCL2, BCL6 and MYC break-apart probes in ten TMA-spotted samples and in the corresponding whole-tissue sections from the original FFPE tumor blocks (30 hybridizations) yielded concordant results in all hybridizations covering samples with different translocation events. With regard to the independent evaluation of FISH signal distributions by two observers in two institutions, highly satisfactory kappa-values of 0.92, 0.97 and 0.94 for BCL2, BCL6 and MYC rearrangements, respectively, were achieved. Discordant results between the initial two investigators were observed in six (6/384, 1.6%), five (5/404, 1.2%) and four (4/407, 1.0%) samples for BCL2, BCL6 and MYC translocation probes, respectively, which all could be resolved after repeating the hybridizations with whole sections from the original FFPE tumor block.

BCL2 breaks were detected in 52/384 (13.5%) DLBCL. BCL6 was rearranged in 116/404 (28.7%) samples, and 11/367 DLBCL harbored dual translocations involving BCL2 and BCL6 (3.0%). Rearrangements of MYC were found in 36/407 specimens (8.8%). Analyzing patients with measurements for each of the three breaks, the MYC rearrangement was the sole translocation in 14/35 (40.0%) tumor samples, while 21
cases (60.0%) harbored additional breaks in either BCL2, BCL6, or both. 17/350 DLBCL (4.9%) had breaks of MYC and BCL2, and 8/350 (2.3%) of MYC and BCL6. A triple hit constellation targeting BCL2, BCL6 and MYC simultaneously was detected in 4/350 samples (1.1%).

**Immunophenotypes and cell of origin classification**

DLBCL specimens with MYC translocations were more often CD10 positive and IRF4/MUM1 negative than their MYC non-rearranged counterparts (84.4% vs. 30.7% and 57.6% vs. 14.7%, respectively) (p<0.001 for both). A higher proportion of MYC break positive tumors had a Ki67 index >90% in comparison to MYC break-negative tumors [15/34 (44.1%) vs. 68/305 (22.3%), p=0.005]. For the cutpoint of 75% investigated for EFS and OS, we did not observe a significant result regarding MYC-break (p=0.468). BCL2 (32.4% vs. 3.1%) and MYC breaks (18.9% vs. 2.4%) were more frequent in GCB-DLBCL (p<0.001 for both). In contrast, BCL6 breaks were more often observed in non-GCB DLBCL (34.7% vs. 21.9%; p=0.012). No significant differences in the distribution of breaks were observed between centroblastic and immunoblastic DLBCL, and/or DLBCL NOS.

**MYC, BCL2 and BCL6 immunohistochemistry**

MYC stainings were reliably interpretable in 283 samples. To ensure the quality of MYC staining evaluation, a multicenter trial had been performed, involving seven diagnostic centers for hematopathology in Germany and Switzerland. Analyzing the number of positive cells in six B-NHL samples, the results were highly concordant (kappa-value of 0.69 for MYC overexpressed or not overexpressed tumors, data not shown). 90 tumors (31.8%) showed overexpression (MYChigh) with samples harboring between 41% and 100% stained nuclei. The numbers of MYChigh cases
were 18/26 (69.2%) in the MYC translocation-positive group, and 67/241 (27.8%) in the translocation-negative group, (p<0.001). Eight translocation positive DLBCL, therefore, were MYClow. MYChigh samples harbored more tumors with a Ki67 index >75% in comparison to MYClow tumors [73/83 (88.0%) vs. 114/169 (67.5%), p<0.001]. No significant differences with respect to MYChigh protein expression was noted between GCB-DLBCL and non-GCB-DLBCL, and between centroblastic and immunoblastic DLBCL. Out of 442 patients with available FISH data, 313/393 (79.6%) were BCL2high (>0%), while 284/343 (82.8%) tumors were BCL6high (>25%). BCL2high was more frequent in non-GCB (154/177; 87.0%) than GCB-DLBCL (107/152; 70.4%) (p<0.001), and BCL6high was observed in 130/177 non-GCB (73.4%) and in 137/143 (95.8%) GCB (p<0.001). Out of 8 MYC break positive patients with MYClow protein expression, 7/7 with IHC data were BCL2high and 6/7 were BCL6high. Within this group of patients, only three events occurred in EFS or OS.

Survival analysis

In the entire patient cohort, the presence of MYC translocations constituted a significant risk factor in univariate analysis (EFS: p=0.025 (log rank), OS: p=0.001 (log rank) and in multivariate analyses adjusted for IPI factors (EFS: RR=1.5 (95% CI: 0.9-2.4) p=0.100 and OS: RR=2.1 (95% CI: 1.3-3.5) p=0.003). Inclusion of an interaction term (Rituximab*MYC break) showed the difference in EFS/OS between MYC break-positive and -negative tumors within subgroups treated with or without rituximab to be in similar order (EFS: RR=1.0, OS: RR=0.7). Therefore, we observed relevant EFS and OS differences for patients with and without MYC translocation. Due to the small sample size especially of patients with MYC translocations in the
rituximab-treated group these differences did not reach statistical significance. The results for rituximab treated patients are shown in Figure 1A/B.

In contrast to MYC translocations, breaks in BCL2 (Figure 1C,D) or BCL6 (Figure 1E,F) genes did not predict EFS and OS in univariate and multivariate analyses adjusted for the IPI factors in rituximab treated patients (Table 2). We observed similar results for patients treated without rituximab (data not shown). In a multivariate Cox regression model for patients treated with rituximab including MYC, BCL2 and BCL6 breaks adjusted for IPI factors, neither BCL2 nor BCL6 breaks were independent prognostic factors for EFS (BCL2: RR=0.8 p=0.505, BCL6: RR=1.0 p=0.947) and OS (BCL2: RR=1.3 p=0.553, BCL6: RR=0.7 p=0.296). In addition, in our cohort, there were only few double hit lymphomas (with simultaneous breaks in MYC and BCL2 or BCL6 genes) among rituximab-treated patients, and, therefore, the confidence intervals for the 3-year OS and EFS rates were broad for these double-hit positive cases (MYC+/BCL2+ and MYC+/BCL6+) (EFS: 38.1% and 50.0%; 95% CI: 0.0-77.1 and 1.0-99.0; OS: 35.7% and 75.0%; 95% CI: 0.0-74.5 and 32.5-100.0; n=7 and n=4) in comparison to double-hit negative cases (MYC+/BCL2- and MYC+/BCL6-) (EFS: 58.3% and 53.3%; 95% CI: 30.5-86.1 and 28.0-78.6; OS: 75.0% and 59.3%; 95% CI: 50.5-99.5 and 34.0-84.6; n=12 and n=15). Based on the data obtained in the present patient cohort, therefore, no meaningful statement as to the risk impact of the double hit feature could be made. In addition, Cox regression models including MYC break-only tumors, without additional breaks in BCL2 or BCL6, were unstable because of small sample size (data not shown).

Protein overexpression of MYC (MYChigh) was an adverse prognostic factor for R-CHOP (EFS p=0.004; OS p=0.005; Figure 2A,B), but not for CHOP-treated patients (EFS p=0.140; OS p=0.941; data not shown). MYC overexpression retained its
importance in R-CHOP treated patients after adjusting for the IPI factors (EFS: RR=2.2 (95% CI: 1.3-3.9) p=0.005 and OS: RR=2.5 (95% CI: 1.3-4.8) p=0.005) and after adjusting for the IPI factors and the presence of MYC breaks (EFS: RR=2.0 (95% CI: 1.1-3.7) p=0.030 and OS: RR=2.1 (95% CI: 1.0-4.4) p=0.063) (Table 2). In accordance with our previously published results, expression patterns of BCL2 (Figure 2C,D) and BCL6 (Figure 2E,F) predicted EFS and OS in R-CHOP treated patients within the RICOVER study. In Cox models adjusted for the IPI factors (Table 2), MYChigh, BCL2high, and BCL6low had prognostic relevance with relative risks greater or equal than 1.5 across all models tested (IHC models 1-3). Most notably, inclusion of MYC, BCL2 and BCL6 expression in a multivariate Cox regression model adjusted for the IPI factors revealed all three factors as significant independent risk indicators in R-CHOP treated patients (IHC model 4). Given that all three variables contributed in similar order in the multivariate cox regression analyses, an IHC sum score of 0-3 was created, assigning an individual risk score of 0, 1, 2, or 3 for each patient according to the number of adverse features (MYChigh, BCL2high, BCL6low). Figure 3 illustrates the prognostic impact of the score for four groups (0, 1, 2, 3 adverse features), and for two groups (pooling the scores 0,1 and 2,3). Patients with a higher score had poorer survival. Within the IPI risk groups 1,2 and 3-5 the dichotomized IHC-score separated patient groups with significant survival differences. Of pivotal importance, we identified a poor prognosis group (15% of patients) within the IPI risk group 3-5, with a 3-year EFS and OS of only 15.6% and 41.6%, respectively (Figure 3E,F). Cox regression analyses also including the morphological classifier (immunoblastic vs. centroblastic) could not be undertaken due to the small sample size for patients with immunoblastic subtype within the Cox model (n=9).
In view of our observation that not all tumors with detectable \textit{MYC} breaks also showed MYChigh expression, we also tested Cox models including the variable ‘\textit{MYC} breakage’ (Table 2). In these models, the three immunohistochemical markers (MYChigh, BCL2high and BCL6low) remained significant independent risk indicators. Relative risks in relevant order (EFS RR=1.5, OS RR=1.8) were also observed for \textit{MYC} breaks (FISH/IHC model 5). In a combined algorithm including MYC, BCL2 and BCL6 protein expression patterns and \textit{MYC} breakage (sum score ranging from 0-4 for each patient), patients with an index of >1 again had a significantly poorer EFS and OS. Figure 4 illustrates the prognostic impact of the FISH/IHC score for five groups (0, 1, 2, 3, 4; 4A,B), and in a dichotomized approach (groups 0, 1 and 2, 3, 4; 4C,D), and within IPI groups (4E,F). The survival differences regarding EFS and OS as evident in the IHC or FISH/IHC score could also be observed within each of the IPI score groups 1,2,3,4,5, but suffered from small sample size within the single IPI groups (data not shown). The goodness of fit was comparable for both the IHC model and the FISH/IHC model (-2 log Likelihood for EFS: 362.928 and 362.259). Chi square tests were performed in order to test whether the cohort included either in the final IHC-model, or in the final FISH/IHC-model is representative of the entire study population. No significant differences were observed when comparing relevant clinical characteristics of the IHC-score and the FISH/IHC-score patient samples to the entire RICOVER-60 cohort.

**DISCUSSION**

The poor prognosis of CHOP-treated DLBCL patients whose tumors carry \textit{MYC} rearrangements is well established.\textsuperscript{11,12,31} More recently, the negative prognostic impact of \textit{MYC} translocations has also been confirmed in patients treated with R-CHOP. In these studies, \textit{MYC} breaks have been encountered in roughly 10% of
We have confirmed these data in the largest series analyzed to date comprising DLBCL samples from 442 patients treated in the prospective randomized RICOVER-60 study of the DSHNHL. Moreover, we are able to convincingly demonstrate that the analysis of translocations by FISH is feasible on TMAs in a highly reliable and reproducible manner. A total of 36/407 tumors (8.8%) had a MYC break, similar to the findings of Barrans and associates. Multivariate analysis disclosed MYC rearrangement as the sole significant genetic factor influencing prognosis in the RICOVER-60 trial, whereas breaks in BCL2 and BCL6 had no impact on survival.

MYC is a global transcription factor that has been reported to regulate up to 10% of genes within the human genome (reviewed in ). Deregulation of MYC has been described to produce an “avalanche” effect upon gene expression, thus further underlining its central role in cellular processes such as proliferation, differentiation and metabolism. It is important to note, however, that deregulation of MYC is not solely based on translocations of the gene. The study of the German Molecular Mechanisms in Malignant Lymphoma Network described deregulated expression of MYC in a sizeable number of tumors with a Burkitt lymphoma expression profile lacking MYC translocation by FISH. Furthermore, these findings were also confirmed in recent studies showing significantly elevated MYC protein expression in tumors lacking translocations of the MYC gene. Interestingly enough, recent publications have suggested alternative mechanisms equally leading to upregulation of MYC. For example, microRNAs may regulate MYC expression and, accordingly, microRNA profiles have been found to be different between MYC-rearranged and non-rearranged Burkitt lymphomas. Amplification of MYC has been shown to be associated with MYC overexpression and poorer prognosis.
above-mentioned findings, it is not surprising that we also encountered MYChigh protein expression in the obvious absence of detectable MYC rearrangements in a subset of DLBCL using a newly described monoclonal antibody.\textsuperscript{21,21,25,26,34} MYChigh protein expression was associated with inferior outcome both in the entire DLBCL cohort as well as in the R-CHOP treated subgroup, in accordance with its central role in the regulation of thousands of genes in the genome, as also reported recently.\textsuperscript{25,26,34} Moreover, MYC protein expression retained its prognostic significance in R-CHOP treated patients after adjusting for the IPI factors and – most notably – also after adjusting for the presence of MYC breaks. These data imply that MYC activation and deregulation represents a significant adverse prognostic factor in DLBCL irrespective of the underlying cause. Interestingly, protein overexpression of MYC (MYChigh) was an adverse prognostic factor in R-CHOP but not in CHOP-treated patients. The mechanism, by which MYC as well as BCL2 and BCL6 overexpression selectively influences the outcome of Rituximab-CHOP treated patients in contrast to patients treated with CHOP only, is unknown. However, some possible links of Rituximab and MYC have been described in the literature. Jazirehi and colleagues reported the Rituximab-dependent inhibition of Raf-MEK1/2-ERK1/2 signaling which sensitizes B-cells to Rituximab-treatment.\textsuperscript{36} The activation of MYC is functionally linked to the ERK1/2 pathway\textsuperscript{37,38}, and very interestingly, it has been shown that the inactivation of ERK1/2 signaling results in dephosphorylation and inhibition of MYC.\textsuperscript{39} We have previously shown that the use of the Hans classifier\textsuperscript{30} failed to identify patients at risk in the R-CHOP arm of the RICOVER-study, while immunoblastic morphology had emerged as a robust negative prognostic factor.\textsuperscript{4} We here show that the integration of MYC protein expression and the MYC translocation status into existing survival predictors in DLBCL are valuable adjunct markers. In our analysis,
MYChigh protein expression (and/or MYC rearrangement status) in concert with BCL2high and BCL6low status emerged as critical prognostic variables with comparable relative risks for both EFS and OS in DLBCL. A simple scoring approach that sums up combinations of these biological features was able to predict survival of DLBCL under R-CHOP treatment in addition to the well-established IPI factors. Specifically, patients with a sum score of >1 had an inferior survival both in the low-risk and high-risk IPI groups. Of note, the prognostic value of this combined IHC or IHC/FISH approach was particularly pronounced in the clinically adverse IPI 3-5 group, in which the 3-year EFS of the patient group identified at risk (IHC score 2,3 or IHC/FISH score 2-4) was 15% only. For this patient population, treatment strategies other than conventional R-CHOP are warranted. It is highly interesting to note that two recent studies also came to the conclusion that the dual deregulation of MYC and BCL2 evidenced by protein overexpression does potentiate the negative prognostic impact of the two factors, identifying cases of inferior OS and EFS when both proteins are overexpressed. Most notably, the study by Johnson and colleagues showed that this effect was also significant on the gene expression level, and in both studies a number of MYC protein expression positive cases without MYC translocation were identified.25,26 The importance of immunoblastic morphology could not be tested due to the small sample size for patients with immunoblastic subtype within the Coxmodel (n=9). In the RICOVER-60 study, inclusion of MYC break analysis via FISH to the MYC/BCL2/BCL6 protein expression model added little to its predictive value. As detailed above, the populations at risk identified with use of the IHC or the IHC/FISH classifier are greatly overlapping and the goodness of fit was comparable for the IHC model and the FISH/IHC model (-2 log Likelihood for EFS: 362.928 and 362.259). However, not all MYC translocated DLBCL samples showed a significant (>40%) MYC protein staining pattern. Paying tribute to the fact that only
recent and as yet incomplete experience has been gained for immunohistochemical stainings with the anti-MYC antibody, at the moment we strongly advise the usage of a combined MYC-FISH and IHC model. Nevertheless, further validation studies need to be performed to get insights into the applicability and practicability of MYC immunohistochemistry in the future. A possible compromise for the moment might be to use MYC staining as a screening tool first, and then hybridize all cases with MYC protein expression >20%. Taking this into account and admitting that FISH still represents a time-consuming and expensive method, and since the analysis of MYC protein expression by immunohistochemistry is easily performed in a routine clinical setting, its continued value as a predictive marker will have to be established. Nevertheless, screening for MYC deregulation via immunohistochemistry for MYC protein expression represents an attractive tool in order to identify patients at risk in everyday practice. On the other hand, the sensitivity of MYC staining in recognizing a breakpoint of MYC, according to our data, would be only high, if a threshold of 20% was chosen. Using this cutoff, FISH analysis for MYC could have been ‘safely’ omitted in 90/241 (37.3%) patients of our study with a sensitivity of 92.3%. It has to be kept in mind, however, that immunohistochemistry, especially regarding the assessment of BCL6 expression, has recently been shown to be difficult to reproduce arguing for the addition of MYC FISH to the model resulting in a more robust prognostic score. Generally, our results had been generated in patients aged 61-80 years. Given the lack of comparable investigations reported to date in younger as well as in older patients beyond 80 years, it remains to be clarified if the prognostic implications of chromosomal aberrations and protein expression patterns reported here will apply to these age groups as well.
ACKNOWLEDGEMENTS

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AUTHOR CONTRIBUTIONS

Conception and design: H.H., M.Z., M.L., A.R., G.O.


Biometry: M.Z., M.L.


CONFLICT OF INTEREST DISCLOSURES

The authors have no conflicts of interest and no competing financial interests.
REFERENCES


TABLES

Table 1. Clinical and morphological characteristics of DLBCL patients

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<tr>
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<th>RICOVER-60</th>
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<td>all DLBCL patients</td>
<td>n=442</td>
<td>n=949</td>
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<tr>
<td>Male</td>
<td>240 (54.3%)</td>
<td>511 (53.8%)</td>
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<tr>
<td>Female</td>
<td>202 (45.7%)</td>
<td>438 (46.2%)</td>
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<td>Age, median (range)</td>
<td>68 (61,80)</td>
<td>69 (61,80)</td>
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<td>LDH &gt; UNV</td>
<td>200 (45.2%)</td>
<td>467 (49.2%)</td>
</tr>
<tr>
<td>ECOG &gt; 1</td>
<td>53 (12.0%)</td>
<td>134 (14.1%)</td>
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<td>stage III / IV</td>
<td>192 (43.4%)</td>
<td>462 (48.7%)</td>
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<tr>
<td>E &gt; 1</td>
<td>64 (14.5%)</td>
<td>171 (18.0%)</td>
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<td>IPI score 1</td>
<td>157 (35.5%)</td>
<td>300 (31.6%)</td>
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<tr>
<td>2</td>
<td>126 (28.5%)</td>
<td>258 (27.2%)</td>
</tr>
<tr>
<td>3</td>
<td>104 (23.5%)</td>
<td>235 (24.8%)</td>
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<tr>
<td>4,5</td>
<td>55 (12.4%)</td>
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<td>bulky disease</td>
<td>154 (34.8%)</td>
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<td>B-symptoms</td>
<td>134 (30.3%)</td>
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<td>E-involvelement</td>
<td>228 (51.6%)</td>
<td>543 (57.2%)</td>
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<td>BM involvement</td>
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<td>anaplastic large cell</td>
<td>8 (1.8%)</td>
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<td>T-cell-rich B-cell</td>
<td>14 (3.2%)</td>
<td>18 (1.9%)</td>
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<td>NOS</td>
<td>111 (25.1%)</td>
<td>313 (33.0%)</td>
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<td>Primary mediastinal B-cell</td>
<td>4 (0.9%)</td>
<td>13 (1.4%)</td>
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Table 2. Cox models adjusted for IPI factors for EFS and OS for patients treated with rituximab

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<th>95% CI</th>
<th>p-value</th>
<th>RR</th>
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<td>MYC break +</td>
<td>19</td>
<td>185</td>
<td>1.6</td>
<td>(0.8 - 3.2)</td>
<td>0.162</td>
<td>1.8</td>
<td>(0.8 - 3.9)</td>
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<td>MYC IHC (41-100%)</td>
<td>43</td>
<td>98</td>
<td>2.2</td>
<td>(1.3 - 3.9)</td>
<td>0.005</td>
<td>2.5</td>
<td>(1.3 - 4.8)</td>
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<td>158</td>
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<td>0.064</td>
<td>2.4</td>
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<td>34</td>
<td>2.2</td>
<td>(1.3 - 3.8)</td>
<td>0.003</td>
<td>2.1</td>
<td>(1.1 - 3.8)</td>
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<td>MYC IHC (41-100%)</td>
<td>138</td>
<td>95</td>
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<td>BCL2 IHC (1-100%)</td>
<td>170</td>
<td>38</td>
<td>1.5</td>
<td>(0.8 - 3.1)</td>
<td>0.228</td>
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<tr>
<td>MYC IHC (41-100%)</td>
<td>135</td>
<td>92</td>
<td>2.2</td>
<td>(1.2 - 3.9)</td>
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<td>2.3</td>
<td>(1.2 - 4.7)</td>
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<td>0.016</td>
<td>1.8</td>
<td>(0.8 - 4.0)</td>
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* sample sizes differ due to complete data set per cox model
FIGURE LEGENDS

Figure 1:
Event-free survival (EFS) and overall survival (OS) in R-CHOP treated DLBCL patients whose tumors were hybridized for breaks in MYC (A,B), BCL2 (C,D) and BCL6 (E,F). The numbers of patients showing breakage within these genes and p-values are indicated.

Figure 2:
Event-free survival (EFS) and overall survival (OS) in R-CHOP treated DLBCL patients whose tumors were stained for MYC (A,B), BCL2 (C,D) and BCL6 (E,F). The numbers of patients showing negative or positive stainings (MYC>40%, BCL2>0%, BCL6>25%), cutoff values, and p-values are indicated.

Figure 3:
Event-free survival (EFS) and overall survival (OS) in R-CHOP treated DLBCL patients whose tumors were stratified according to the number of adverse features in immunohistochemical stainings for MYChigh, BCL2high and BCL6low. (A,B) Survival curves of patients stratified according to the presence of 0, 1, 2, or 3 adverse features. (C,D) Survival curves of patients who were stratified according to the presence of 0 or 1 and >1 adverse features. (E,F) Survival curves of patients stratified according to the presence of 0 or 1 and >1 adverse features and IPI groups 1,2 and 3-5. The numbers of patients within the respective groups and p-values are indicated.
Figure 4:

Event-free survival (EFS) and overall survival (OS) in R-CHOP treated DLBCL patients whose tumors were stratified according to the number of adverse features in immunohistochemical stainings for MYChigh, BCL2high and BCL6low and chromosomal translocations of MYC. (A,B) Survival curves of patients stratified according to the presence of 0, 1, 2, 3 or 4 adverse features. (C,D) Survival curves of patients who were stratified according to the presence of 0 or 1 and >1 adverse features. (E,F) Survival curves of patients stratified according to the presence of 0 or 1 and >1 adverse features and IPI groups 1,2 and 3-5. The numbers of patients within the respective groups and p-values are indicated.
MYC break - (n=185)  MYC break + (n=19)  p=0.072

BCL2 break - (n=166)  BCL2 break + (n=28)  p=0.750

BCL6 break - (n=144)  BCL6 break + (n=55)  p=0.687

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MYC status in concert with BCL2 and BCL6 expression predicts outcome in diffuse large B-cell lymphoma

Heike Horn, Marita Ziepert, Claudia Becher, Thomas F.E. Barth, Heinz-Wolfram Bernd, Alfred C. Feller, Wolfram Klapper, Michael Hummel, Harald Stein, Martin-Leo Hansmann, Christopher Schmelter, Peter Möller, Sergio Cogliatti, Michael Pfreundschuh, Norbert Schmitz, Lorenz Trümper, Reiner Siebert, Markus Loeffler, Andreas Rosenwald and German Ott