Biological characterization of adult MYC-translocation-positive mature B-cell lymphomas other than molecular Burkitt lymphoma

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A full list of MMML-members is provided in the Online Supplementary Appendix.

ABSTRACT

Chromosomal translocations affecting the MYC oncogene are the biological hallmark of Burkitt lymphomas but also occur in a subset of other mature B-cell lymphomas. If accompanied by a chromosomal break targeting the BCL2 and/or BCL6 oncogene these MYC translocation-positive (MYC+) lymphomas are called double-hit lymphomas, otherwise the term single-hit lymphomas is applied. In order to characterize the biological features of these MYC+ lymphomas other than Burkitt lymphoma we explored, after exclusion of molecular Burkitt lymphoma as defined by gene expression profiling, the molecular, pathological and clinical aspects of 80 MYC-translocation-positive lymphomas (31 single-hit, 46 double-hit and 3 MYC+-lymphomas with unknown BCL6 status). Comparison of single-hit and double-hit lymphomas revealed no difference in MYC partner (IG/non-IG), genomic complexity, MYC expression or gene expression profile. Double-hit lymphomas more frequently showed a germinal center B-cell-like gene expression profile and had higher IGH and MYC mutation frequencies. Gene expression profiling revealed 130 differentially expressed genes between BCL6+/MYC+ and BCL2+/MYC+ double-hit lymphomas. BCL2+/MYC+ double-hit lymphomas more frequently showed a germinal center B-like gene expression profile. Analysis of all lymphomas according to MYC partner (IG/non-IG) revealed no substantial differences. In this series of lymphomas, in which immunochemo therapy was administered in only a minority of cases, single-hit and double-hit lymphomas had a similar poor outcome in contrast to the outcome of molecular Burkitt lymphoma and lymphomas without the MYC break. Our data suggest that, after excluding molecular Burkitt lymphoma and pediatric cases, MYC+ lymphomas are biologically quite homogeneous with single-hit and double-hit lymphomas as well as IG-MYC and non-IG-MYC lymphomas sharing various molecular characteristics.

Introduction

Approximately 40% of B-cell lymphomas display recurrent chromosomal translocations and most of them can be readily detected using conventional cytogenetics (karyotyping) or molecular cytogenetics (fluorescent in situ hybridization, FISH). They may act as cancer-initiating events or may be involved in tumor progression.1 The presence, or absence, of chromosomal translocations can be of pivotal importance in establishing the correct diagnosis and in predicting the course of the disease. Well-known translocations in B-cell lymphomas are those involving chromosomal bands/gene loci 18q21/BCL2, 3q27/BCL6 and 8q24/MYC.

MYC translocations, a biological hallmark of Burkitt lymphoma (BL), can also be detected, albeit at relatively lower frequencies, in other B-cell lymphomas including follicular lymphoma (FL), diffuse large B-cell lymphoma (DLBCL) and “B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and Burkitt lymphoma” (BCLU).1,2 In consequence, due to the high incidence of these lymphomas as compared to BL, the absolute number of MYC breaks in these lymphomas outnumbers that in BL. However, there are some fundamental differences between the MYC translocation in BL and in other mature B-cell lym-
phomas. In BL, the MYC translocation always involves one of the immunoglobulin loci (most commonly IGH, alternatively IGL or IGG) and is considered a disease-initiating event which occurs in the context of a rather simple karyotype. Indeed, the genomic complexity in BL is, overall, low.\textsuperscript{6-8} In contrast, MYC translocations in other mature B-cell lymphomas frequently involve non-IG partners and are mostly found in complex karyotypes, often in addition to well-known primary aberrations including the IGH-BCL2 translocation.\textsuperscript{6,9-11} Consequently, they likely occur during disease progression rather than disease initiation. Indeed, in 20-80\% of cases of DLBCL and BCLU with a MYC breakpoint, there is an accompanying BCL2 and/or BCL6 breakpoint.\textsuperscript{12-16}

According to the World Health Organization (WHO) classification, lymphomas in which such a combination of a MYC break with a BCL2 break and/or a BCL6 break (further indicated as BCL2+/MYC, BCL6+/MYC or BCL2+/BCL6+/MYC) occurs are called double-hit lymphomas (DHL).\textsuperscript{1} All other lymphomas with a MYC breakpoint, irrespective of the presence of other aberrations, are called “single-hit” lymphomas (SHL). MYC breaks are seen in approximately 10\% (3-17\%) of all DLBCL and 15-20\% of FL grade 3B,\textsuperscript{17,18} representing on average a DHL in 50-60\%.\textsuperscript{14,15,20} This also implies that the remaining 40-50\% of MYC lymphomas are “single-hit” and that their importance, despite this high percentage, might have been underappreciated. These lymphomas with MYC translocations, including DHL, have received increased attention because several studies showed them to run an aggressive clinical course.\textsuperscript{5,11,12} However, gene expression and other molecular genetic data are scarce\textsuperscript{12} and, consequently, the molecular make up of DHL and SHL other than BL remains largely unknown. Moreover, it is unclear in which pathological and molecular aspects DHL differs from SHL other than molecular Burkitt lymphoma (mBL).

In that respect it should be noted that, in the presence or absence of a MYC break, oncogenes other than BCL2 and BCL6, including BCL3, chromosomal locus 9p13 (potentially affecting PAX5), CCNE1, as well as unknown partners involved in IGH breaks, can be deregulated through juxtaposition to one of the IG-loci.\textsuperscript{16,21-23} Breakpoints affecting both MYC and these genes might, therefore, also point to a DHL, although according to the WHO classification they are defined as SHL.\textsuperscript{4}

To investigate differences and similarities between SHL and DHL as well as between BCL2+/MYC\textsuperscript{+} and BCL6+/MYC\textsuperscript{+} DHL we explored the morphological, immunohistochemical, genetic and gene expression features of 80 adult MYC\textsuperscript{+} mature aggressive B-cell lymphomas other than mBL.

**Methods**

**Sample selection and pathology review**

All lymphomas were investigated as part of the Molecular Mechanisms in Malignant Lymphomas (MMML) network project. The MMML protocols have been approved centrally by the institutional review board of the coordination center in Göttingen, Germany. All cases with an mBL gene expression signature (see Bioinformatical and statistical analysis), were excluded. Similarly, no pediatric cases (age ≤18 years) were included as many MYC lymphomas in children have been shown to represent biological BL.\textsuperscript{2} For a complete description, see the Online Supplementary Appendix. Due to the retrospective nature of the study patients had been treated with a variety of treatment regimens, which included immunotherapy (rituximab) in only a minority of cases.

**Molecular cytogenetics (fluorescence in situ hybridization)**

Cases positive for IGH-MYC, ICK-MYC, or IGL-MYC fusion were assigned as “IG-MYC”; all cases lacking such fusions were assigned “non-IG MYC”. An overview of the algorithm to identify MYC partners and details on all FISH probes are provided in the Online Supplementary Materials and Methods and Online Supplementary Figure S1.

**Immunohistochemistry**

Immunohistochemical studies were performed as previously described with antibodies against CD20, CD10, BCL2, BCL6, MUM1/IRF4, and Ki67.\textsuperscript{7} As previously published, a quantitative approach (in quartiles) was applied with the following cut-offs:\textsuperscript{24} CD10 (>0\% = positive), BCL2 (>25\% = positive), BCL6 (>25\% = positive), MUM1/IRF4 (>25\% = positive).

**Mutational analysis**

Mutational screening for MYC, BCL6 and IGHV and somatic hypermutation analysis were performed and analyzed as previously described.\textsuperscript{25-27}

**Bioinformatical and statistical analysis**

**Gene expression analysis**

Gene expression data were generated on Affymetrix U133A gene expression arrays.\textsuperscript{1} Based on gene expression a “molecular BL index”\textsuperscript{26} was calculated for each individual sample and was assigned one of the following molecular diagnoses; mBL (index ≥0.95), non-mBL (index score ≤0.05), or molecular intermediate (remaining cases).\textsuperscript{1} The lymphomas were also stratified according to their “pathway activation patterns”.\textsuperscript{3} The cell of origin was classified according to the methods described by Wright et al.\textsuperscript{28} using a modified classifier.\textsuperscript{1} MYC expression was measured from Affymetrix probe set 202431_s_at.\textsuperscript{24} Differences in MYC expression between groups were compared by the Mann–Whitney U test. A full description of the methods used for gene expression analysis is provided in the Online Supplementary Materials and Methods.

**Copy number analysis**

Chromosomal imbalances were detected using array-comparative genomic hybridization and analyzed as previously described.\textsuperscript{29} For a complete description, see the Online Supplementary Materials and Methods.

**Survival analyses**

Overall survival was defined as the time from the date of diagnosis to death from any cause. Patients without an event were censored at the last day with valid information. Overall survival was estimated by the Kaplan-Meier method and differences were compared using the log-rank test.

**Molecular and clinical characterization**

Age at diagnosis, IGH mutation frequency, number of BCL6 and MYC mutations and percentage of Ki67-positive cells were compared between lymphoma groups by the Mann–Whitney U test. Gender of patients, immunohistochemical staining, FISH data for selected chromosomal aberrations and cell of origin signature (activated B-cell-like (ABC), germinal center B-cell-like (GCB)), mBL signature and pathway activation patterns were compared using the Fisher exact test.
Results

Description of the cohort and case selection
At the point of analysis, the MMML-study cohort consisted of 363 lymphomas of which 168 showed a MYC break with the MYC BAP and/or fusion with the IGH-MYC fusion probe (MYC+ lymphomas) (Online Supplementary Figure S2). To exclude all cases of biological BL, all pediatric cases (age ≤18 years) and all adult lymphomas with a BL index score ≥0.95, and thus representing mBL, were excluded. In consequence, 88 MYC+ lymphomas were excluded, leaving 80 MYC+ lymphomas, having either a “molecular intermediate” (n=48) or a “non-mBL” (n=52) gene expression profile, available for further analysis (Online Supplementary Figure S2). The baseline histopathological and genetic characteristics of the 80 MYC+ lymphomas included in our study are shown in Table 1. For a complete description, also regarding the treatment regimens, see the Online Supplementary Appendix and Online Supplementary Table S1.

These 80 MYC+ lymphomas were classified as DHL (n=47; 60%) according to the definition of the present WHO classification, i.e. by the presence of IGH-BCL2 juxtaposition (BCL2+) and/or BCL6 breaks (as determined with BCL6 BAP) in addition to MYC+ cases lacking these breaks were classified as SHL (BCL2+/BCL6+/MYC+). The 47 DHL consisted of 26 BCL2+/BCL6+/MYC+ DHL (called BCL2+/MYC+ DHL, 57%), 14 BCL2+/BCL6−/MYC+ DHL (called BCL6−/MYC− DHL; 30%), and six DHL with both BCL2 and BCL6 breaks (called BCL2+/BCL6+/MYC− “triple-hit” lymphomas, 13%). BCL6 rearrangement status was not available for three MYC cases (two BCL2+/MYC+ and one BCL2+/MYC−) and these cases were not, therefore, assigned to the MYC SHL or BCL2+/MYC+ DHL groups, respectively.

As an indicator of MYC activation we compared MYC transcription expression in the 80 MYC+ lymphomas with that of their counterparts without a MYC break (n=574); MYC transcript expression was significantly higher in MYC+ lymphomas than in MYC− ones, but lower than in IG− MYC mBL (Online Supplementary Figure S3).

Comparison of MYC “single-hit” versus MYC “double-hit” mature B-cell lymphoma other than molecular Burkitt lymphoma
As a first step we compared the molecular, pathological and clinical features of the 31 SHL with those of the 47 DHL (26 BCL2+/BCL6+/MYC+ DHL, 14 BCL2+/BCL6−/MYC+ DHL and 6 BCL2+/BCL6−/MYC− DHL and 6 BCL2+/BCL6+/MYC− “triple-hit” lymphomas). The single BCL2+/BCL6−/MYC− lymphoma with unknown BCL6 status was included in the DHL group as it represents, irrespective of BCL6 status, a DHL.

Molecular cytogenetics and array comparative genomic hybridization
There were no significant differences between SHL and DHL in the types of the MYC partners (i.e. IG or non-IG partner) (Table 1) or genomic complexity (median 8 aberrations for SHL versus median 10 for DHL, P=0.255, Figure 1A). Array comparative genomic hybridization showed similar patterns of gains and losses in SHL and DHL with only minor quantitative differences (Online Supplementary Figure S4). SHL displayed higher proportions of gains of chromosome 6p and losses of 6q. DHL showed higher proportions of gains of chromosomes 8q and 12q.

Mutational analysis
DHL showed a significantly higher IGH mutational frequency (P<0.001, Figure 1B) and number of MYC mutations (P=0.048, Figure 1C) with no significant difference for number of BCL6 mutations (P=0.106; data not shown).

Gene-expression profiling
No significant differences were seen in molecular diagnosis or pathway activation patterns (Table 1). Significant differences were seen in the cell of origin classification with the vast majority (39/47, 83%) of DHL being classified as GCB-like. The pattern was much more heterogeneous among the SHL with 16/31 (52%) classified as GCB-like, 9/31 (29%) ABC-like and 6/31 (19%) unclassified (P=0.01, Table 1). SHL were a minority among both the “molecular intermediate” lymphomas as well as the “non-mBL” (20/47, 43% and 11/31, 35%, respectively; P=0.658). In a supervised gene expression analysis no genes were differentially expressed between SHL and DHL at a false discovery rate ≤0.05. Figure 1D supports these findings, with the resulting permutation scores also revealing no differentially expressed genes. No differences were seen in MYC transcript levels between SHL and DHL (P=0.490, Figure 1E).

Pathology
In both groups the most common morphological diagnosis was DLBCL (71% and 72% for SHL and DHL, respectively). DHL included a higher number of cases classified as FL (DHL n=7, 15% versus SHL n=1, 3%). No significant differences were seen for CD10 and BCL6 expression but SHL more frequently expressed MUM1 (P=0.018) (Table 1). Using a cut-off of 90% no significant difference in Ki67 staining was seen, but when Ki67 staining was analyzed as a continuous variable SHL showed a trend towards having higher Ki67 levels (P=0.088, data not shown).

Clinical aspects
There was no difference in survival between patients with DHL or SHL (P=0.690, Figure 2A). Importantly, this was also the case when the overall survival analysis was restricted to morphologically diagnosed DLBCL (without any FL-component) (P=0.586, Figure 2B).

Comparison of BCL2 + MYC− versus BCL6 + MYC− “double-hit” mature B-cell lymphoma other than molecular Burkitt lymphoma
In a second step we compared the molecular, pathological and clinical features of the BCL2+/MYC− DHL (n=26) with those of BCL6+/MYC− DHL (n=14). To avoid any overlap in analyses, triple-hit lymphomas (n=6) and the BCL2+/MYC− case with missing data for BCL6 rearrangement status were excluded from this analysis.

Molecular cytogenetics, array comparative genomic hybridization and mutational analysis
There was no significant difference between BCL2+/MYC− and BCL6+/MYC− DHL with respect to the usage of IG versus non-IG MYC partners, genomic complexity, or the mutation frequency of IGH, BCL6 and MYC genes (Table 1, Figure S5A-C).

Gene-expression profiling
All but one of the BCL2+/MYC− DHL (25/26; 96%) were
Table 1. Overview of histopathological, genetic and molecular characteristics of MYC lymphomas.

<table>
<thead>
<tr>
<th></th>
<th>MYC vs. Non-MYC</th>
<th>MYC vs. Non-MYC</th>
<th>BCL2/MYC vs. BCL6/MYC</th>
<th>MYC vs. Non-MYC</th>
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<tbody>
<tr>
<td>Total</td>
<td>80 (100)</td>
<td>31 (100)</td>
<td>26 (100)</td>
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<tr>
<td>Sex</td>
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<td>41 (51)</td>
<td>14 (45)</td>
<td>11 (42)</td>
<td>10 (71)</td>
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<td>15 (58)</td>
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<tr>
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<td>3 (10)</td>
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<td>2 (6)</td>
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<tr>
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<td>8 (26)</td>
<td>3 (12)</td>
<td>4 (31)</td>
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<tr>
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<td>22 (72)</td>
<td>9 (32)</td>
<td>5 (41)</td>
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<td>20 (71)</td>
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<td>14 (51)</td>
<td>7 (25)</td>
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<td>22 (71)</td>
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<td>PAP-4</td>
<td>2 (3)</td>
<td>1 (3)</td>
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<td>1 (7)</td>
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</table>

Percentages are provided between parentheses ( ) and may not be equal to 100 as a result of rounding. Percentages refer to analyzed cases. *For two BCL2/MYC and one BCL2-MYC the BCL2 status was not available. The former two cases were therefore excluded from the MYC SHL vs. DHL/THL comparison (but remained included in the IGMYC vs. non-IGMYC comparison). The latter case was excluded from the BCL2/MYC vs. BCL6-MYC DHL comparison (but, being a DHL, remained included in the MYC SHL vs. DHL/THL comparison as well as the IGMYC vs. non-IGMYC comparison). At the time of panel diagnosis cases were classified as atypical Burkitt or Burkitt-like lymphoma. High-grade B-cell, not otherwise specified (NOS), aggressive B cell lymphomas with morphologies different from other categories, also including cases with poor morphology; DLBCL from FL is defined as DLBCL with a low grade (grade 1-3A) FL component. DLBCL with FL grade 3B (n=3) component was classified as DLBCL. Includes one BCL2/MYC low-grade B-cell lymphoma, not otherwise specified, "Cell-of-origin signature determined by a modified classifier" according to the method described by Wright et al. and was applied on all morphologies. All cases positive for IGH-BCL2 and/or IGH-MYC fusion were assigned IGH break positive. In all other cases an IGH BAP probe was applied. PAP: pathway activation pattern; THL: triple hit lymphoma.
Interestingly, among these there was enrichment for genes also included in the modified cell-of-origin gene expression classifier (7 out of 15). No difference in MYC transcript expression was seen between BCL2+/MYC- and BCL2+/MYC+ DHL although BCL2+/MYC+ DHL showed a trend towards higher expression (P=0.130, Figure 3E). These levels were higher in both BCL2+/MYC- and BCL2+/MYC+ DHL than in MYC-negative lymphomas (P<0.001; Figure 3E).

Pathology

BCL2+/MYC- DHL were morphologically heterogeneous with only 16/26 (62%) being DLBCL and 9/26 (35%) of the cases being FL or transformed FL (to DLBCL). In contrast, almost all BCL2+/MYC+ DHL (13/14, 93%) were DLBCL. In agreement with the gene-expression profile, BCL2+/MYC- DHL more often expressed CD10 and BCL2 (P<0.001 and P=0.014, respectively) while fewer cases expressed MUM1 (P=0.018, Table 1).

Clinical aspects

Patients with BCL2+/MYC- DHL showed a trend towards being older at diagnosis (median age at diagnosis 68 versus 58 years; P=0.084). Survival analysis showed a significant trend towards an unfavorable outcome for patients with BCL2+/MYC+ DHL (P=0.040, Online Supplementary Figure S5).

Comparison of IG-MYC versus non-IG-MYC mature B-cell lymphomas other than molecular Burkitt lymphoma

Thirdly, we compared the molecular, pathological and clinical features of lymphomas according to their MYC partner, either “IG-MYC” or “non-IG-MYC”, since an IG-MYC configuration might point to a different origin and juxtaposition to an IG enhancer might result in a different type and level of MYC activation.

Molecular genetics, array comparative genomic hybridization and mutational analysis

Of all lymphomas, 47/80 (59%) were classified as IG-MYC and 33/80 (41%) as non-IG-MYC. As reported previously,26 chromosomal locus 9p13 was the most common MYC non-IG partner (7/33, 21%) while BCL6 was partnered to MYC in four cases (4/32, 13%). All four cases were females and were positive for BCL6 but negative for MUM1/IRF4 expression and had a GCB-like gene expression profile. One of these four cases with MYC-BCL6 fusion did not have a detectable BCL6 break with BCL6.

Figure 1. (A) Genomic complexity as assessed by array-comparative genomic hybridization. There is no significant difference in the number of aberrant segments between DHL and SHL (P=0.255). Both SHL as well as DHL show a significantly higher genomic complexity than IG-MYC mBL (P=0.038 for SHL and P<0.001 for DHL versus IG-MYC mBL). (B) IGH mutational frequency in SHL and DHL. DHL shows a significantly higher mutational frequency (P<0.001). (C) Number of MYC mutations in DHL and SHL. DHL show a significantly higher number of MYC mutations (P=0.046). (D) Expected (X-axis) versus observed (Y-axis) test scores between SHL and DHL. The distribution of the expected scores is estimated by repeatedly computing test scores from the same SHL/DHL data with randomly permuted class labels. Observed scores were computed by genewise analysis taking the log ratios of the SHL and DHL collective. The red lines mark the 95% confidence intervals on the absolute difference between observed and expected scores. Colored dots represent genes whose observed score exceeds the confidence bounds, whereas this does not directly imply differential expression as the false discovery rate is defined to be ≤0.05. The permutation approach is described in detail in Scheid et al.27 (E) MYC transcript expression. No difference in MYC expression was seen between SHL and DHL (P=0.490). Both grouped together as well as individually, SHL and DHL have higher MYC transcript expression compared with MYC-negative lymphomas (P<0.001) but lower expression than IG-MYC mBL (P=0.01 for SHL and P<0.001 for DHL).
BAP and could therefore have an atypical (ABR) BCL6 break or insertion of MYC into BCL6.

No differences were seen for IGH, BCL6 and MYC mutations between IG-MYC and non-IG-MYC lymphomas as well for genomic complexity (P=0.472, data not shown).

**Gene-expression profiling**

No significant differences were seen in frequency of molecular diagnosis, cell-of-origin subtypes and pathway activation patterns. In a supervised gene expression comparison between both groups only one gene was differentially expressed. IG-MYC positive lymphomas showed significantly higher MYC transcript levels compared with those with a non-IG-MYC translocation (P=0.040), however, transcript levels in these IG-MYC positive lymphomas (i.e., with a non-mBL or intermediate gene-expression profile) were still significantly lower than in IG-MYC-positive lymphomas with an mBL gene-expression profile (P<0.003; Online Supplementary Figure S6).

**Pathology**

No differences were seen in distribution of morphologies between the two groups. Non-IG-MYC lymphomas significantly more often had BCL6 expression and tended to have more frequent BCL2 expression. No differences were seen for the frequency of t(14;18)/IGH-BCL2 fusions and BCL6 breaks.

**Clinical aspects**

No significant difference in survival was seen between the two groups (Online Supplementary Figure S7, P=0.574). Non-IG-MYC cases showed a trend towards older age (median age at diagnosis 64 years versus 52; P=0.061). In addition, when an IG versus non-IG subgroup analysis was performed within the BCL2+/MYC- and BCL6+/MYC-DHL groups, no differences in survival were seen.

**Discussion**

Many recent studies have emphasized the importance of the assessment of MYC breakpoints in aggressive B-cell lymphomas, mainly DLBCL, as well as the detection of other breakpoints in so-called DHL. We evaluated the pathological, molecular genetic and clinical aspects of 80 adult B-cell lymphomas with a MYC break that did not represent mBL as defined by gene expression profiling. We excluded mBL since mBL represents a well-defined entity with very distinctive clinical and biological features which is well characterized by gene expression analysis.3,12,36,37 Even if morphological features are inconsistent with BL,3,37 however, how far subsets of MYC-positive lymphomas with an mBL signature overlap with the group of “discrepant BL” according to Salaverria et al.7 needs to be addressed in future studies. The validity of this selection is supported by the very favorable outcome of mBL patients in our series (Online Supplementary Figure S8), while the 80 MYC-negative cases in our current study carried an ID3 mutation.28

The most important biological conclusion of our study is that, after exclusion of mBL, there are only few biological differences between SHL and DHL. First, the genomic

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**Figure 2.** (A) Comparison of survival between DHL and SHL shows no significant differences between the two groups (P=0.690). The blue line represents SHL, the red line represents DHL. (B) Comparison of survival between DHL and SHL restricted to lymphomas with a morphological diagnosis of DLBCL (without any follicular lymphoma component). No difference was seen in survival (P=0.586). The blue line represents SHL, the red line represents DHL. (C) Overall survival of patients with MYC + lymphomas with non-mBL or Intermediate gene-expression profile in the MMML cohort. Patients with MYC + lymphomas show markedly inferior survival compared to those with MYC lymphomas (P=0.001). The blue line represents MYC + lymphomas, the red line represents MYC lymphomas.
complexity was similar between the two groups but much higher than in mBL (Figure 1A). Array comparative genomic hybridization showed similar patterns of gains and losses between the two groups, with only minor quantitative differences (Online Supplementary Figure S4). These data suggest that, unlike in mBL which is characterized by a relatively simple karyotype, in both SHL and DHL the MYC translocation coexists with numerous other alterations. This implies that in (non-mBL) SHL other aberrations than translocations involving BCL2 and BCL6 may also be involved. Indeed, using additional FISH assays on the 31 cases classified as SHL according to the WHO definition, we identified four non-IG MYC+ lymphomas with a break at chromosomal locus 9p13 (all co-localizing with MYC) and three non-IG MYC+ lymphomas with an additional IGH break (one of which also showing 9p13-MYC co-localization). In analogy to BCL6/MYC DHL, these MYC+ lymphomas with breaks at 9p13/PAX5 could also be considered “double-hit” lymphoma. These cases accounted for six of nine (67%) of all non-IG MYC SHL. Most importantly, full karyotyping, array comparative genomic hybridization but also several next-generation sequencing studies suggest that the genomic landscape of lymphomas other than BL is much more complex than can be appreciated from BCL2 and BCL6 translocations alone.

Second, there was no difference between SHL and DHL with respect to the frequencies of IG or non-IG MYC partners. Third, gene expression profiling, too, revealed no differentially expressed genes between SHL and DHL. In addition, no significant difference in MYC expression was seen between SHL and DHL, the levels of expression...
being intermediate between IG-MYC mBL and the lymphomas without an MYC break that were studied (Figure 1E). For that reason we did not further explore the possibility that differences in gene expression between the currently studied subsets were caused by MYC as a transcriptional amplifier, more than activator of distinct target genes.\textsuperscript{44,45}

Finally, although it is generally thought that DHL/triple-hit lymphomas have a worse prognosis than SHL, we did not find such a difference, even when the survival analysis was restricted to DLBCL (Figure 2B). However, when mBL and pediatric lymphomas were included, patients with SHL had a much more favorable survival (\textit{data not shown}), underlining the importance of recognizing mBL and of distinguishing mBL from MYC- SHL non-mBL as defined in the present study. Of note, a detailed comparison with other studies of the impact on survival is difficult since other studies did not use gene-expression profiling and might have contained mBL-type lymphomas in the group of SHL. Furthermore, we are aware of the fact that our series included heterogeneously treated patients, with immunotherapy (e.g. rituximab) having been given to a minority of them, and we cannot exclude the possibility that administration of rituximab or an equivalent monoclonal antibody may have a different impact on the survival of patients with various subsets of MYC-translocation positive lymphomas. Two recent studies that both included cases of DLBCL treated with R-CHOP also investigated survival of patients with MYC-translocation positive lymphomas; in the study by Valera et al.\textsuperscript{19} a similarly poor outcome was observed for patients with SHL and DHL, while in the study by Johnson et al.\textsuperscript{20} MYC-translocation positive lymphomas without BCL2 protein expression did not have an aggressive clinical course.

We also explored the similarities and differences between BCL2+/MYC and BCL6+/MYC DHL. BCL2+/MYC DHL more often expressed CD10 and BCL2 and less often MUM1/IRF4, similar to a very recent observation by Pillai et al.\textsuperscript{21} In accordance with published data\textsuperscript{18-23,28} almost all BCL2+/MYC lymphomas (96%) were assigned to the GCB-like group by gene-expression profiling. BCL6+/MYC DHL, on the other hand were classified as GCB in only half of the cases (Table 1). Gene-expression profiling confirmed the importance of this difference with enrichment of genes included in the cell-of-origin classifier BCL6+/BCL2+/MYC: triple-hit lymphomas clustered with BCL2+/MYC: DHL (Figure 3D), which fits with the phenotype of BCL6-/BCL2- FL resembling that of lymphomas with an isolated BCL2 rearrangement.\textsuperscript{47}

When analyzed according to MYC partner (IG or non-IG), very few differences were observed. Apparently, both the presence of an IG-MYC as well as a non-IG-MYC translocation resulted in deregulated MYC expression compared to MYC-translocation negative cases, with, as also found by Bertrand et al.\textsuperscript{22}, slightly higher MYC expression in IG-MYC lymphomas (Online Supplementary Figure S6). MYC expression in the MYC break-negative cases varies greatly and a subset of these lymphomas show high MYC expression. However, an analysis of the prognostic impact of this, as has been done for MYC immunohistochemistry in several recent studies,\textsuperscript{34,40} goes beyond the scope of the present study which focuses primarily on the biological characterization of lymphomas with MYC rearrangements.

From a diagnostic perspective (and in line with other recent reports\textsuperscript{32,33}), it is important to note that no differences in Ki67 proliferation rates, either using a cut-off of 90% or considered as a continuous parameter, were seen between MYC- and MYC- non-mBL/intermediate lymphomas (\textit{data not shown}). Another observation was that all DLBCL (with or without a FL component) with immunoblastic morphology, 13 cases (39%) had a MYC break, the vast majority (11/13, 85%) being IG-MYC (\textit{data not shown}). This is reminiscent of the inferior outcome of immunoblastic lymphomas with changes in chromosome 8q as shown by cytogenetics and the high percentage of MYC breaks and the predominance of IG-MYC translocations in plasmablastic lymphomas and could contribute to the inferior outcome of immunoblastic lymphomas.\textsuperscript{35-37}

Although our present study has the limitation of being retrospective and only a minority of the patients received immuno-chemotherapy, we would nevertheless recommend screening all patients with DLBCL and DLBCL/BL intermediate, irrespectively of immunophenotypic features including Ki67,\textsuperscript{12,15} and MYC expression, for MYC rearrangements in the diagnostic work up. Since an accompanying BCL3 and/or BCL6 breakpoint exists in 20-30% (in our study 60%) of MYC- lymphomas other than BL,\textsuperscript{46,48} FISH for these genes could still be used to identify the majority of MYC- lymphomas other than mBL. This result could be further improved by incorporating FISH assays for IGH and 9p13. In the (in daily practice probably very few) remaining cases in which, after taking into account all clinical, histopathological immunophenotypic and FISH data, there is still debate about a diagnosis of IG-MYC-SHL or mBL, dedicated gene expression profiling with selected genes from the mBL-classifier could reliably distinguish lymphomas with a favorable mBL signature from those with an intermediate or non-mBL signature.\textsuperscript{54} Finally, assays including Sanger sequencing to detect the recently identified ID3 and TCF3 mutations in BL may further help to discriminate these difficult-to-distinguish categories in future studies.\textsuperscript{38-40} The recently introduced alternative approaches using immunohistochemistry for MYC and BCL2 protein expression, which likely also cover alternative molecular mechanisms of MYC and BCL2 deregulation and other cellular pathways,\textsuperscript{44,45} are promising but need to be validated before they can replace the presently used genetic definitions for SHL and DHL.

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Authorship and Disclosures

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