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 immunochemotherapy 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).[1] They may act as cancer-initiating events or may be involved in tumor progression.[2] 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).[3-5] 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-

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phomas. In BL the *MYC* translocation always involves one of the immunoglobulin loci (most commonly *IGH,* alternatively *IGL* or *IGK*) 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.[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.[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.[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).[4] 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,[17,18] representing on average a DHL in 50-60%.[14,16-20] 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.[9,11,21] However, gene expression and other molecular genetic data are scarce[3,22] 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.[16,23-26] 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.[4]

To investigate differences and similarities between SHL and DHL as well as between *BCL2*$^+$/*MYC*$^+$ and *BCL6*$^+$/*MYC*$^+$ DHL we explored the morphological, immunohistochemical, genetic and gene expression features of 80 adult *MYC*$^+$ 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.[27] 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*, *IGK-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.[3] As previously published, a quantitative approach (in quartiles) was applied with the following cut-offs:[28] 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.[29-31]

Bioinformatical and statistical analysis

Gene expression analysis

Gene expression data were generated on Affymetrix U133A gene expression arrays.[3] Based on gene expression a "molecular BL index"[3] 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).[3] The lymphomas were also stratified according to their "pathway activation patterns".[32] The cell of origin was classified according to the methods described by Wright *et al.*[33] using a modified classifier.[3] *MYC* expression was measured from Affymetrix probe set 202431_s_at.[34] 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.[28] 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 863 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=32) 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,[4] i.e. by the presence of *IGH-BCL2* juxtaposition (*BCL2*$^+$) and/or *BCL6* breaks (as determined with *BCL6* BAP) in addition to *MYC*. *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* transcript 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*$^+$ triple-hit lymphomas). The single *BCL2*$^+$/*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*=0.638). 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 3A-C).

Gene-expression profiling

All but one of the *BCL2*$^+$/*MYC*$^+$ DHL (25/26; 96%) were

classified as GCB-like while the pattern was much more heterogeneous for the *BCL6*$^+$/*MYC*$^+$ DHL group with 7/14 (50%) GCB-like, 4/14 (29%) ABC-like and 3/14 (21%) unclassified (*P*=0.001, Table 1).

Supervised comparison of the gene expression profiles of the *BCL2*$^+$/*MYC*$^+$ and *BCL6*$^+$/*MYC*$^+$ DHL revealed that 130 tags (representing 120 different genes) were differentially expressed (Figure 3D; *Online Supplementary Table S2*).

Table 1. Overview of histopathological, genetic and molecular characteristics of *MYC*$^+$ lymphomas.

	MYC$^+$	*MYC*$^+$ SHL	*BCL2*$^+$/*MYC*$^+$	*BCL6*$^+$/*MYC*$^+$	*MYC* THL	*MYC* DHL/THL	*IG-MYC*	Non-*IG MYC*	*MYC* SHL vs. *MYC* DHL/THL	*BCL2*$^+$/*MYC*$^+$ vs. *BCL6*$^+$/*MYC*$^+$	*IG-MYC* vs. non-*IG-MYC*
Total	80 (100)	31 (100)	26 (100)	14 (100)	6 (100)	47 (100)	47 (100)	33 (100)			
Sex											
Female	41 (51)	14 (45)	11 (42)	10 (71)	4 (67)	26 (55)	22 (47)	19 (58)	0.488	0.105	0.372
Male	39 (49)	17 (55)	15 (58)	4 (29)	2 (33)	21 (45)	25 (53)	14 (42)			
Morphological diagnosis											
Unclassifiable	4 (5)	3 (10)	0 (0)	0 (0)	1 (17)	1 (2)	2 (4)	2 (6)			
DLBCL / BL[2]											
High-grade B-cell, NOS[3]	3 (4)	3 (10)	0 (0)	0 (0)	0 (0)	0 (0)	1 (2)	2 (6)			
DLBCL	58 (73)	22 (71)	16 (62)	13 (93)	4 (67)	34 (72)	36 (77)	22 (67)			
DLBCL from FL[4]	6 (8)	2 (6)	3 (12)	1 (7)	0 (0)	4 (9)	3 (6)	3 (9)			
FL	8 (10)	1 (3)	6 (23)	0 (0)	1 (17)	7 (15)	4 (9)	4 (12)			
Other[5]	1 (1)	0 (0)	1 (1)	0 (0)	0 (0)	1 (2)	1 (2)	0 (0)			
CD10											
Absent	25 (32)	10 (34)	1 (4)	11 (79)	1(17)	13 (28)	15 (33)	10 (31)	0.610	**<0.001**	1
Present	53 (68)	19 (66)	25 (96)	3 (21)	5 (3)	34 (72)	31 (67)	22 (69)			
BCL6											
Negative	15 (20)	8 (28)	3 (12)	4 (31)	0 (0)	7 (16)	13 (30)	2 (6)	0.250	0.203	**0.018**
Positive	60 (80)	21 (72)	22 (88)	9 (69)	5 (100)	37 (84)	31 (70)	29 (94)			
MUM1											
Negative	44 (63)	12 (46)	20 (87)	6 (46)	6 (100)	32 (76)	25 (62)	19 (63)	**0.018**	**0.018**	1
Positive	26 (37)	14 (54)	3 (13)	7 (54)	0 (0)	10 (24)	15 (38)	11 (37)			
ABC-GCB signature[6]											
ABC	14 (18)	9 (29)	0 (0)	4 (29)	0 (0)	4 (9)	9 (19)	5 (15)	**0.010**	**0.001**	0.831
GCB	55 (69)	16 (52)	25 (96)	7 (50)	6 (100)	39 (83)	31 (66)	24 (73)			
Unclassifiable	11 (14)	6 (19)	1 (4)	3 (21)	0 (0)	4 (9)	7 (15)	4 (12)			
BCL2											
Negative	15(19)	7 (23)	1 (4)	5 (36)	2 (33)	8 (17)	12 (26)	3 (9)	0.569	**0.014**	0.084
Positive	65 (81)	24 (77)	25 (96)	9 (64)	4 (67)	39 (83)	35 (74)	30 (91)			
Ki67											
<90%	55 (71)	20 (65)	18 (75)	10 (71)	5 (83)	34 (76)	30 (65)	25 (78)	0.316	1	0.313
≥90%	23 (29)	11 (35)	6 (25)	4 (29)	1 (17)	11 (24)	16 (35)	7 (22)			
MYC status											
IG-MYC	47 (59)	22 (71)	15 (58)	8 (57)	2 (33)	25 (53)	47 (100)	0 (0)	0.157	1	**<0.001**
Non-*IG-MYC*	33 (41)	9 (29)	11 (42)	6 (43)	4 (67)	22 (47)	0 (0)	33 (100)			
IGH-BREAK[7]											
Negative	12 (16)	10 (32)	0 (0)	2 (18)	0 (0)	2 (5)	4 (9)	8 (29)	**0.003**	0.083	**0.047**
Positive	63 (84)	21 (68)	26 (100)	9 (82)	6 (100)	42 (95)	43 (91)	20 (71)			
Molecular diagnosis											
Intermediate	48 (60)	20 (65)	16 (62)	5 (36)	5 (83)	27 (57)	29 (62)	19 (58)	0.638	0.186	0.818
Non-mBL	32 (40)	11 (35)	10 (38)	9 (64)	1 (17)	20 (43)	18 (38)	14 (42)			
COMAP[8]											
BL-PAP	4 (5)	2 (6)	2 (8)	0 (0)	0 (0)	2 (4)	4 (9)	0 (0)	0.390	0.603	0.240
Mind-L	45 (56)	17 (55)	14 (54)	6 (43)	6 (100)	26 (55)	25 (53)	20 (61)			
PAP-1	15 (19)	5 (16)	6 (23)	4 (29)	0 (0)	10 (21)	10 (21)	5 (15)			
PAP-2	5 (6)	4 (13)	1 (4)	0 (0)	0 (0)	1 (2)	2 (4)	3 (9)			
PAP-3	9 (11)	2 (6)	3 (12)	3 (21)	0 (0)	7 (15)	6 (13)	3 (9)			
PAP-4	2 (3)	1 (3)	0 (0)	1 (7)	0 (0)	1 (2)	0 (0)	2 (6)			

Percentages are provided between parentheses ( ) and may not be equal to 100 as a result of rounding. Percentages refer to analyzed cases. [1]For two BCL2/MYC$^+$ *and one* BCL2$^+$/MYC$^+$ *the* BCL6 *status was not available. The former two cases were therefore excluded from the* MYC *SHL vs. DHL/THL comparison (but remained included in the* IG-MYC vs. *non-*IG-MYC *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* IG-MYC vs. *non-*IG-MYC *comparison); [2]At the time of panel diagnosis cases were classified as atypical Burkitt or Burkitt-like lymphoma; [3]High-grade B-cell, not otherwise specified (NOS); aggressive B cell lymphomas with morphologies different from other categories, also including cases with poor morphology; [4]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. [5]Includes one* BCL2$^+$/MYC$^+$ *low-grade B-cell lymphoma, not otherwise specified; [6]Cell-of-origin signature determined by a modified classifier[3] according to the method described by Wright* et al.[33] *and was applied on all morphologies. [7]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. [8]PAP: pathway activation pattern;[32] 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 *BCL6*$^+$/*MYC*$^+$ DHL although *BCL6*$^+$/*MYC*$^+$ DHL showed a trend towards higher expression (P=0.130, Figure 3E). These levels were higher in both *BCL2*$^+$/*MYC*$^+$ and *BCL6*$^+$/*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 *BCL6*$^+$/*MYC*$^+$ DHL (13/14, 93%) were DLBCL. In agreement with the gene-expression profiling data, *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 *BCL6*$^+$/*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 *BCL6*$^+$/*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[16,35] 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*

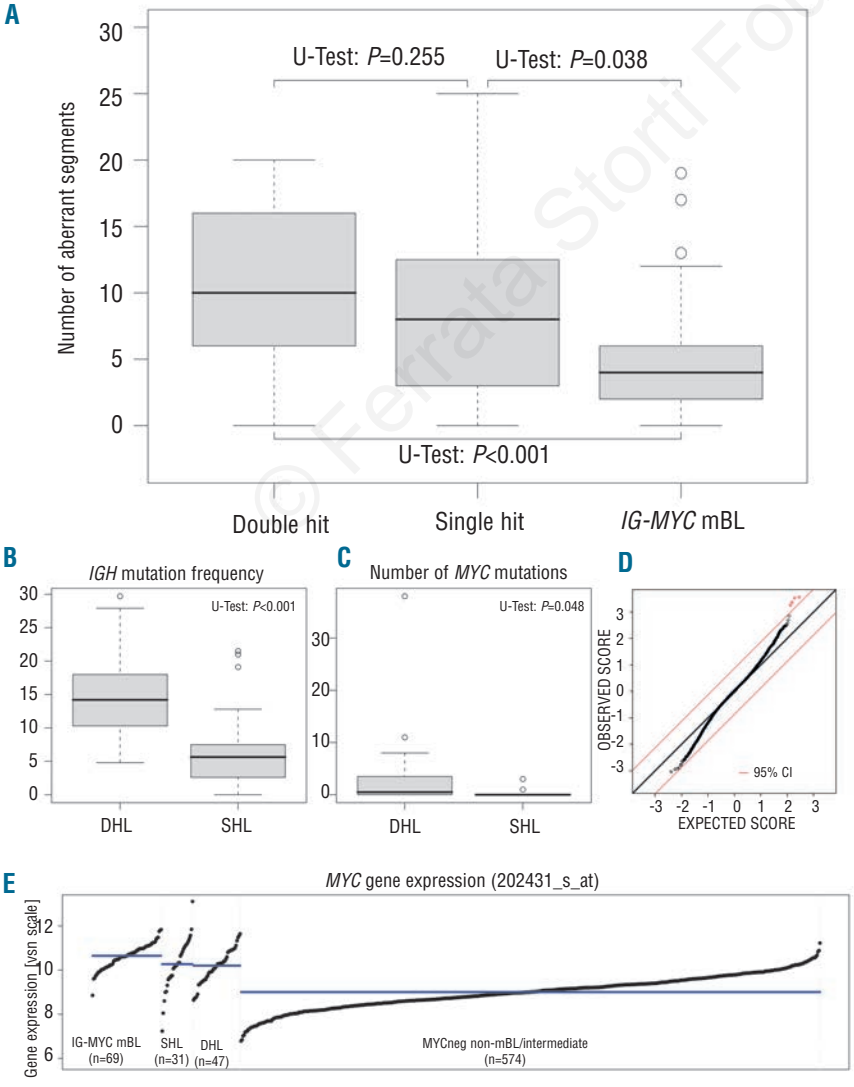

Figure 1. (A) Genomic complexity as assessed by array-comparative genomic hybridization. There is no significant difference in the number of aberrant segments between DHL and SHL (P=0.255). Both SHL as well as DHL show a significantly higher genomic complexity than *IG-MYC* mBL (P=0.038 for SHL and P<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=0.048). (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 permutated 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.*[55] (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,[3,22,36,37] 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*$^+$ non-mBL/intermediate cases had a worse outcome than that of the *MYC*$^-$ cases (Figure 2C). The survival data for this group of patients are, therefore, well in line with those in many other studies.[3,9,12-15] In addition, whereas the majority of mBL has *ID3* mutations,[38-40] only two (6%) of the currently 33 analyzed *IG-MYC* (15 SHL, 18 DHL) lymphomas (representing 70% of all *IG-MYC* cases in our current study) carried an *ID3* mutation.[38]

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

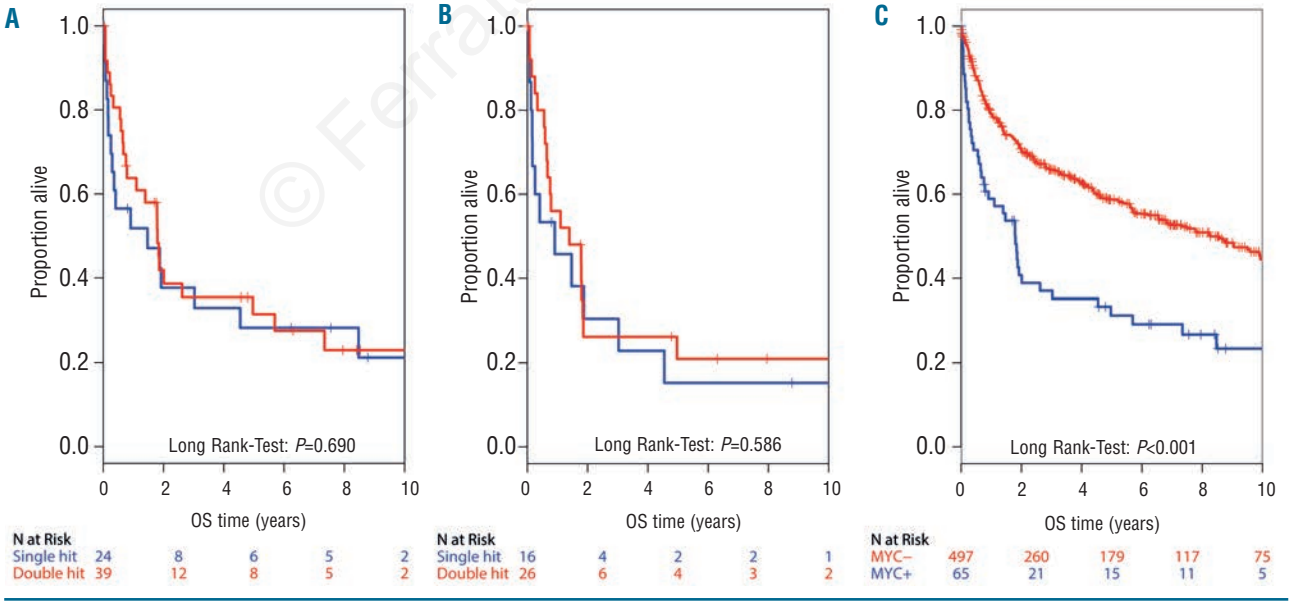

Figure 2. (A) Comparison of survival between DHL and SHL shows no significant differences between the two groups (*P*=0.690). The blue line represents SHL, the red line represents DHL. (B) Comparison of survival between DHL and SHL restricted to lymphomas with a morphological diagnosis of DLBCL (without 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*$^+$ and *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.[27] 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.[16] 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.[16,27,41-43]

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

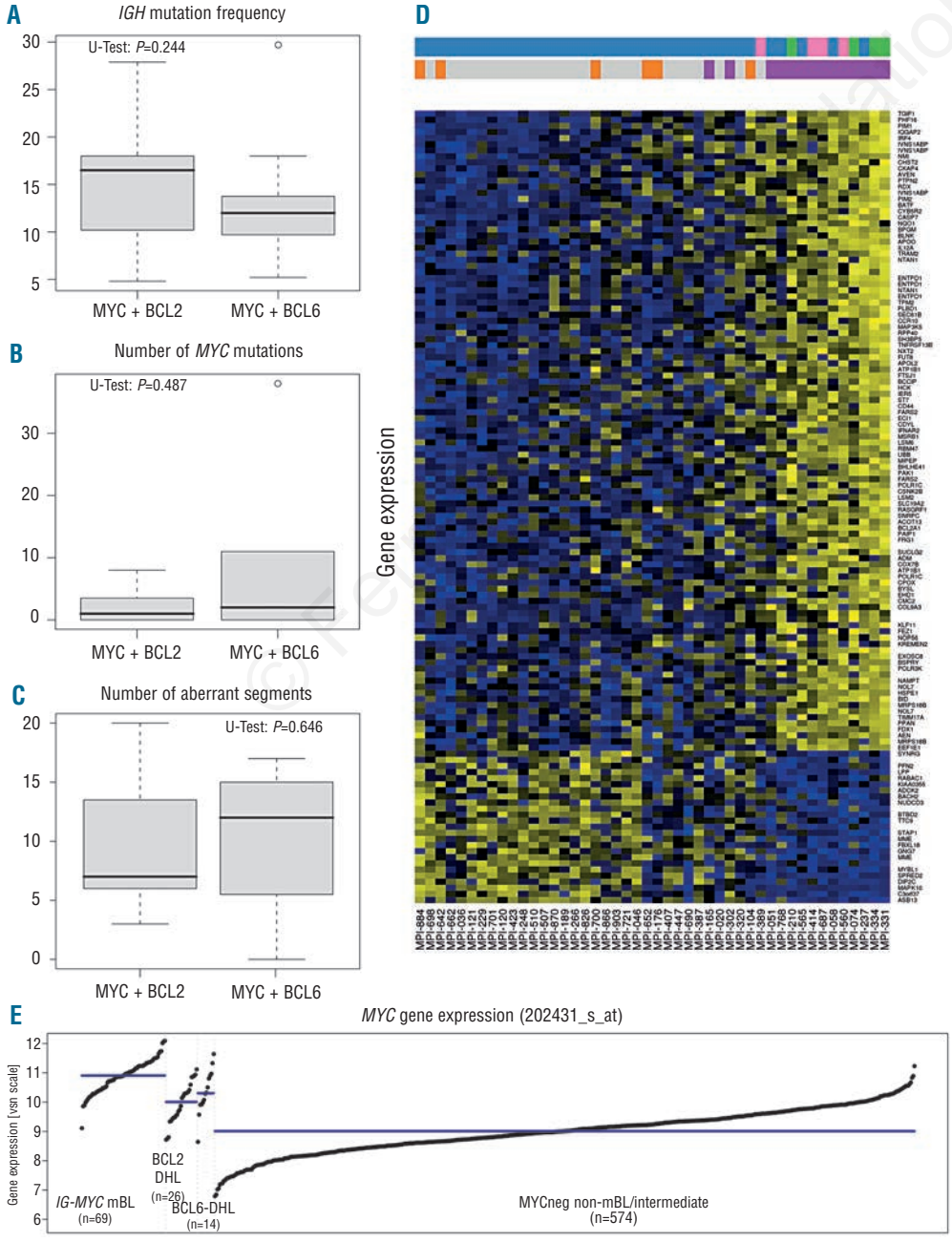

Figure 3. Biology of *BCL2*$^+$/*MYC*$^+$ *versus* *BCL6*$^+$/*MYC*$^+$ DHL. (A) *IGH* mutational frequency; (B) Number of *MYC* mutations; (C) Genomic complexity assessed by number of aberrant segments; (D) Comparison of gene expression profiles. One hundred thirty gene tags were differentially expressed between *BCL2*$^+$/*MYC*$^+$ and *BCL6*$^+$/*MYC*$^+$ DHL. Samples are ordered according to gene expression index calculated as described in the *Online Supplementary Materials and Methods* with the lowest index at the very left end. *BCL2*$^+$/*MYC*$^+$ (gray), *BCL6*$^+$/*MYC*$^+$ (purple) and *BCL2*$^+$/*BCL6*$^+$/*MYC*$^+$ (orange). Cell-of-origin labels are added at the very top of the figure (GCB-like, blue; ABC-like, green; unclassified, magenta). (E) *MYC* transcript expression in *BCL2*$^+$/*MYC*$^+$ and *BCL6*$^+$/*MYC*$^+$ DHL. *BCL6*$^+$/*MYC*$^+$ DHL showed a trend towards higher transcript expression (P=0.130). *MYC* transcript expression in both *BCL2*$^+$/*MYC*$^+$ and *BCL6*$^+$/*MYC*$^+$ DHL was higher compared to that in *MYC*-negative lymphomas (P<0.001).

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.[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 (*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.*[20] a similarly poor outcome was observed for patients with SHL and DHL, while in the study by Johnson *et al.*[19] *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.*[46] In accordance with published data[9,10,21] 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.[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.*,[25] 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,[19,48-50] 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[5,12,15]) 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 (*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* (*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.[51-53]

Although the 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[5,12,15] and MYC expression, for *MYC* rearrangements in the diagnostic work up. Since an accompanying *BCL2* and/or *BCL6* breakpoint exists in 20-80% (in our study 60%) of *MYC*$^+$ lymphomas other than BL,[12-16] 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.[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.[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,[19,48,49] 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

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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