ID3 Mutations Are Recurrent Events in Double-hit B-Cell Lymphomas

NIKLAS GEBAUER, VERONICA BERNARD, ALFRED C. FELLER and HARTMUT MERZ

Department of Pathology, Reference Centre for Lymph Node Pathology and Hematopathology, University Hospital of Schleswig-Holstein, Luebeck, Germany

Abstract. *Background: Double-hit lymphomas (DHL) with chromosomal rearrangements affecting the avian myelocytomatosis viral oncogene homolog (cMYC) and either the B-cell lymphoma-2 (BCL2) or -6 (BCL6) locus are uncommon neoplasms with an aggressive clinical course and dismal prognosis. Most cases exhibit a phenotype intermediate between diffuse large B-cell lymphoma (DLBCL) and Burkitt lymphoma. Recently mutations affecting the inhibitor of DNA binding 3 (ID3), a helix-loop-helix protein regulating cell cycle progression and B-cell differentiation, were identified as being molecular hallmarks in Burkitt lymphoma, with only rare mutations being found in other lymphomas with translocations affecting cMYC. Materials and Methods: In the present study, we evaluated the mutational status of ID3 in 37 cases of DHL and 16 cases of sporadic Burkitt lymphoma in order to identify a possible association of this new found hallmark with the rare and insufficiently-defined entity of DHL, seeking to broaden the understanding of these lymphomas at a molecular level. Results: We identified ID3 mutations in lymphomas with chromosomal aberrations at cMYC and either BCL2 or BCL6 at a frequency intermediate between that of DLBCL and Burkitt lymphoma, hinting at a common pathway in lymphomagenesis for a subset of patients with DHL. Conclusion: The results of this study assist in the molecular characterization of these highly aggressive lymphomas, potentially giving rise to novel therapeutic approaches.*

Reciprocal chromosomal translocations are common genetic hits in B-cell non-Hodgkin's lymphomas; in particular chromosomal translocations involving the immunoglobulin genes, resulting in the juxtaposition of an oncogene to an enhancing locus, are common (14, 22). For certain types of lymphomas, the occurrence of such a chromosomal aberration is considered to be the moment of lymphomagenesis (*e.g.* t(8;14)(q24;q34) or variant translocations involving the avian myelocytomatosis viral oncogene homolog (*cMYC*) and immunoglobin (IG) loci in Burkitt lymphoma (25). Mature B-cell lymphomas carrying two or more activating chromosomal breakpoints, one of which affecting the cMYC locus, are regularly referred to as double/triple-hit lymphomas (DHL) (2). Morphologically and by means of immunohistochemistry, these cases often exhibit aspects resembling both diffuse large B-cell lymphoma (DLBCL) and Burkitt lymphoma, giving rise to the artificial and temporary entity of B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and Burkitt lymphoma (B-UCL) as defined by the World Health Organization (WHO) (17, 24). Both progression of Burkitt lymphoma, primarily carrying the t(8;14)(q24;q34) translocation and secondarily acquiring a chromosomal hit affecting the *B-cell lymphoma-2* (*BCL2*) or -6 (*BCL6*) locus, and indolent lymphomas with pre-existing translocations involving *BCL2* or *BCL6* subsequently attaining translocations affecting cMYC and an IG locus during high-grade transformation have been reported (11, 18, 23). The latter concept explains the existence of high-grade follicular lymphomas with a double-hit karyotype. From a clinical perspective, DHL patients recurrently present with advanced stage disease, with higher International Prognostic Index (IPI) at diagnosis and a frequent tendency for bone marrow and central nervous system (CNS) involvement. Patients treated with either (R-)CHOP (rituximab-), cyclophosphamide, doxorubicin, vincristine and prednisone, or high-dose chemotherapy followed by stem cell transplantation have a dismal outcome (1, 3). Possible explanations for the worse prognosis of patients with DHL compared to both *cMYC*+ Burkitt lymphoma and DLBCL with *BCL2* and/or *BCL6* translocations include both synergistic action of *cMYC* and *BCL2*/*BCL6*, as well as other molecular features originating from the often complex

Correspondence to: Niklas Gebauer, Institut für Pathologie, Referenzzentrum für Lymphknotendiagnostik und Hämatopathologie, Universität zu Luebeck, 23538 Lübeck, Germany. Tel: +49 4515003728, Fax: +49 4515003328, e-mail: niklas.gebauer@medizin.uni-luebeck.de

Key Words: ID3, double-hit lymphomas, unclassifiable B-cell lymphoma.

karyotype and more numerous genetic aberrations in DHL (7, 13, 21). Most cases of B-UCL are morphologically composed of architectural and cytological features intermediate between DLBCL and Burkitt lymphoma. Predominantly medium-sized, centroblast-like cells accompanied by numerous starry sky macrophages constitute the overall diffuse proliferation. The strongly elevated cellular turnover is underlined by high proliferation, as well as a significantly elevated number of apoptotic figures. Recurrent cases exhibiting classical morphology of Burkitt lymphoma or DLBCL display an atypical immunophenotype, preventing definite classification. Furthermore, there are cases with an immunophenotype consistent with a specific diagnosis but presenting with atypical morphology, warranting for specific classification. The most common immunophenotype among B-UCL resembles germinal center B-cells (GCB) with positivity for the common acute lymphoblastic leukemia antigen (CALLA/CD10), BCL6 and BCL2 (24). Inhibitors of DNA binding (ID) proteins, such as ID3 bind E proteins, such as Transcription factor-3 (TCF3) by means of their helix-loop-helix (HLH) motif and thus inhibit DNA binding (16). Through this function ID3 has been implicated in a variety of processes, including regulation of cell-cycle progression and B-cell differentiation. *ID3*-knockout mice show defects in humoral immunity and B-cell proliferation, and develop T-cell lymphomas (10, 15). The disruption of ID3 function was recently proposed to be a key mechanism in the pathogenesis of both sporadic and endemic Burkitt lymphoma independent of IG/*cMYC* translocation (19, 20). For Burkitt lymphoma, it has been shown that protein-damaging sequence variations impair the inhibitory function of ID3 on proteins of the TCF family. This regulatory defect is underlined by the fact that most *ID3* missense mutations in cases of Burkitt lymphoma affect conserved protein residues in the HLH motif (19, 20). It has been reasoned that recurrent mutations affecting the ID3-TCF3 regulatory loop with consecutive tonic B-cell signaling pose targets for novel therapeutic approaches, especially for older patients or patients in developing countries for whom the established therapeutic protocols cannot be effectively applied. In the present study, we aimed to elucidate whether or not this oncogenic mechanism is of relevance in the different subtypes of Double-hit lymphoma as current therapeutic strategies for this entity remain widely insufficient, necessitating the search for targeted therapy regimens.

Materials and Methods

Patients. Formalin-fixed and paraffin-embedded (FFPE) tissue biopsy samples from 16 patients with sporadic Burkitt Lymphoma and 37 patients with at least a chromosomal double-hit constellation were retrieved from the registry of the Reference Center for Lymph Node Pathology and Hematopathology, University Hospital of Schleswig-Holstein, Campus Luebeck. Of the latter 17 cases presented with both *cMYC* and *BCL2* rearrangement and 16 cases showed *cMYC* and *BCL6* rearrangement. Four cases revealed aberrations in all three loci (triple-hit).

All samples were collected as part of standard clinical care, and all studies were approved by the Ethics Commission at the University of Luebeck and are in accordance with the Declaration of Helsinki (file no. 13-077A). All cases were re-assessed for independent pathology review by two experienced hematopathologists without knowledge of the mutation status.

Immunohistochemistry. Immunohistochemical studies were performed on FFPE sections according to a standard, three-step immunoperoxidase technique using AN automated TechMate system (DAKO, Glostrup, Denmark) and the BrightVision Kit (ImmunoLogic, Duiven, the Netherlands). In addition to hematoxylin and eosin (HE), Giemsa, Gomori and periodic acid-Schiff (PAS) stains immunohistochemical stains for CD3, CD5, CD10, CD20, CD23, BCL2, BCL6, Interferon regulatory factor 4 (IRF4/MUM1) and Ki67 (MIB1) were evaluated when available. Staining results were evaluated qualitatively for CD3, CD10, CD20, CD23, BCL2, BCL6 and IRF4, whereas Ki67 and CD5 (in cases of coexpression) staining was detected quantitatively.

Fluorescence in situ hybridization (FISH) for cMYC, BCL2 and BCL6. Chromosomal breakpoints were analyzed by means of FISH using dual-color break-apart probes for 8q24 (*cMYC*), 18q21 (*BCL2*) and 3q27 (*BCL6*) (Abott Vysis, Des Plaines, IL, USA) according to the manufacturer's instructions.

Sequencing of ID3. Genomic DNA was obtained from FFPE specimens using the QiaAmp mini kit 250 (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Following DNA extraction, the coding exons of *ID3* were amplified by polymerase chain reaction (PCR) using primers as follows:
ID3 exon1-F: caggcaggctctataagtgac, ID3 exon1-R: ctgcaggtc gagaatgtagtc, ID3 exon2-F: cgagaggcactcagcttagc, ID3 exon2-R: ctgccaactccaggacttgcc.

Success of the amplification was assessed by gel electrophoresis and subsequently bidirectional Sanger sequencing was performed using a CEQ 8800 platform (Beckman & Coulter, Pasadena, CA, USA). Known single-nucleotide polymorphisms were excluded from further analysis.

Statistical analysis. Statistical data analyses were performed using GraphPad Prism 5. In order to determine statistically significant differences in mutation frequency between different subsets of patients the Fisher's exact test was applied. All analyses were two-sided and the statistical significance level was set to 5% ($\alpha \leq 0.05$).

Results

Histopathological and immunophenotypic features of the study group. The majority of the DHL cases shared similar morphological features in both nodal and extranodal sites. By means of morphological and immunohistochemical investigations and in accordance with the WHO Classification of lymphoid malignancies 20 cases (66%) were classified as B-UCL and 10 cases had a DLBCL

Table I. *Immunohistochemical features of double-hit lymphomas included in the study group. Immunohistochemical studies were performed on formalin-fixed paraffin-embedded (FFPE) sections according to a standard, three-step immunoperoxidase technique using the automated TechMate system (DAKO, Glostrup, Denmark) and the BrightVision Kit (ImmunoLogic, Duiven, the Netherlands).*

Case no.	Histology	Hans	CD20	CD10	BCL6	IRF-4/MUM-1	BCL2	CD23	Cyclin D1	Ki67 (%)	CD5	EBER
17	DLBCL	GCB	+	+	ND	+	+	–	–	95	–	ND
18	B-UCL	GCB	+	+	Weak+	ND	+	+	ND	90	ND	ND
19	B-UCL	GCB	+	+	+	–	+	–	ND	90	Only T-cells/no co-expression	ND
20	DLBCL	GCB	+	+	ND	ND	+	–	ND	90	Only T-cells/no co-expression	ND
21	DLBCL	GCB	+	+	ND	ND	+	ND	–	90	ND	ND
22	B-UCL	GCB	+	+	ND	ND	+	Weak+	ND	80	ND	ND
23	B-UCL	GCB	+	+	+	–	+	ND	ND	90	ND	ND
24	DLBCL	GCB	+	+	ND	ND	+	Weak+	ND	60	Only T-cells/no co-expression	ND
25	B-UCL	GCB	+	+	Weak+	+	+	ND	ND	90	ND	ND
26	B-UCL	GCB	+	+	+	ND	+	Weak+	ND	50	Only T-cells/no co-expression	ND
27	B-UCL	GCB	+	+	+	+	+	Weak+	–	90	partial co-expression (15%)	ND
28	DLBCL	GCB	+	–	+	–	+	ND	ND	90	ND	ND
29	B-UCL	GCB	+	+	ND	ND	+	ND	–	95	–	ND
30	B-UCL	GCB	+	+	+	ND	+	–	ND	85	Only T-cells/no co-expression	–
31	B-UCL	GCB	Weak+	+	ND	+	+	ND	ND	90	ND	–
32	B-UCL	GCB	+	+	Weak+	ND	+	–	Weak+	60	Only T-cells/no co-expression	ND
33	B-UCL	GCB	+	Weak+	+	ND	–	ND	ND	95	ND	ND
34	DLBCL	GCB	+	+	+	ND	+	–	ND	90	+	ND
35	DLBCL	GCB	+	+	+	ND	–	–	–	70	Only T-cells/no co-expression	ND
36	B-UCL	GCB	+	+	+	ND	+	–	Weak+	50	Only T-cells/no co-expression	ND
37	B-UCL	GCB	+	+	+	ND	+	ND	ND	90	ND	–
38	B-UCL	GCB	+	Weak+	+	ND	Weak+	ND	ND	90	Only T-cells/no co-expression	ND
39	DLBCL	GCB	+	–	+	–	+	ND	ND	95	ND	–
40	DLBCL	GCB	+	+	+	ND	–	+	–	95	ND	–
41	B-UCL	GCB	+	+	+	ND	–	–	ND	98	–	–
42	B-UCL	GCB	+	–	+	–	+	ND	ND	90	ND	ND
43	B-UCL	GCB	+	+	+	ND	–	ND	ND	95	Weak+/no co-expression	ND
44	B-UCL	GCB	+	+	Weak+	ND	+	ND	ND	90	ND	ND
45	DLBCL	GCB	+	+	Weak+	ND	Weak+	Weak+	ND	95	partial co-expression (10%)	ND
46	B-UCL	GCB	+	–	+	–	Weak+	–	ND	95	Only T-cells/no co-expression	–

DLBCL, Diffuse large B-cell lymphoma; B-UCL, B-cell lymphoma unclassifiable with features intermediate between Burkitt lymphoma and diffuse large B-cell lymphoma; GCB, germinal center B-cell as cell of origin according to the classification algorithm established by Hans *et al*. (6). ND=Not done; EBER, Epstein-Barr virus–encoded small RNA detected by chromogenic *in situ* hybridization;+, positive; –, negative.

phenotype with no trend for predominance of the former or the latter with regard to the different types of double-hit constellations (24). Curiously, all DHL appeared to be of germinal center origin as assessed in accordance with the classification algorithm proposed by Hans *et al*. (6). Due to highly recurrent tingible-body macrophages a 'starry sky' pattern could be observed in most of the cases which were composed of diffuse sheets of predominantly medium-sized, centroblast-like B-cells with multiple small nucleoli (Figure 1a and 2a). All cases showed immunoreactivity for CD20. Compared to cases of sporadic Burkitt lymphoma, we observed a highly elevated degree of pleomorphism and variation in size and nuclear shape (Figure 1b and c; Figure 2b and c). Symbolizing the high cellular turnover and the highly aggressive clinical behavior, mitosis and apoptotis were frequent, correlating with a high expression of Ki67 (mean expression level 84% for *cMYC+/BCL2+* and 86% for *cMYC+/BCL6+* DHL) (Figure 1c and d; Figure 2c and d). All cases, in which a *BCL2* gene rearrangement was detected by means of flourescence *in situ* hybridization, showed immunohistochemical reactivity for BCL2, which was elevated in all but two cases. Furthermore, BCL6 immunohistochemical expression was found to be elevated in 12/15 cases with chromosomal imbalances affecting the 3q27 region. All cases investigated for the presence of Epstein-Barr virus by means of chromogene *in situ* hybridization tested negatively (0/6). Immunohistochemical findings are briefly summarized in Table I.

Mutations of the ID3 gene are recurrent events in DHL. ID3 was strongly expressed in all samples investigated in the current study and PCR products for both exons were successfully amplified in every case. Moreover, bi-directional sequencing was successful in all cases. *ID3* was shown to

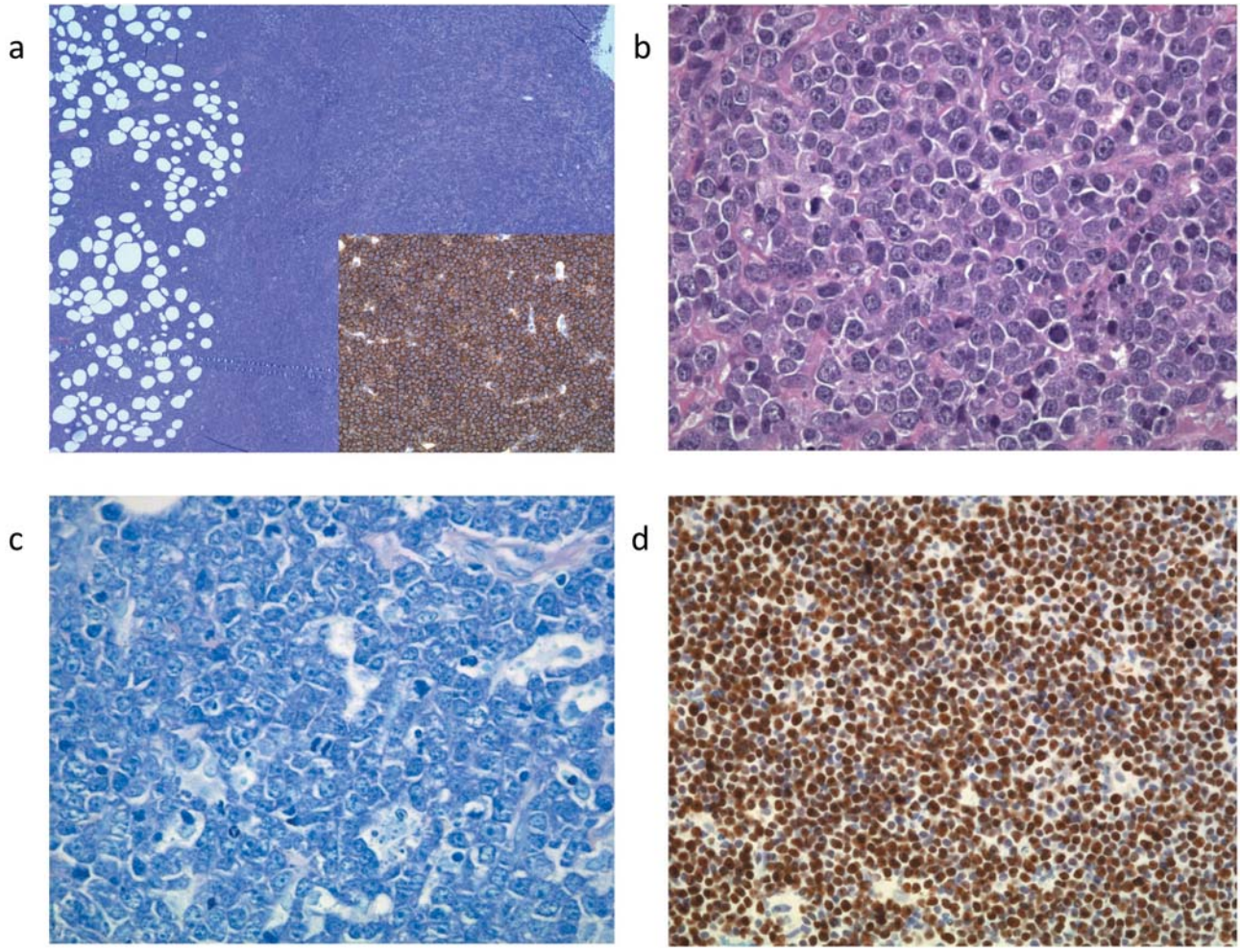

Figure 1. *Morphological and immunohistochemical findings in a avian myelocytomatosis viral oncogene homolog translocated (cMYC+) and B-cell lymphoma-2 translocated (BCL2+) double-hit lymphoma: The retroperitoneal biopsy reveals a dense lymphatic infiltrate with homogeneously high expression of CD20. The tumor is predominantly composed of blast-like B-cells exhibiting a high degree of pleomorphism and frequently intermingled macrophages forming a starry sky pattern. Cellular turnover is strongly elevated with recurrent mitotic and apoptotic figures and immunohistochemical staining for Ki67 (MIB1) reveals nuclear positivity in more than 95% of the neoplastic cells. a: Hematoxylin & Eosin staining ×25; a inset: CD20 ×200; b: Hematoxylin & Eosin stain ×400; c: Giemsa stain ×400; d: Ki67 (MIB1) ×200.*

harbor mutations at the highest frequency in cases of Burkitt lymphoma (7/16; 44%) We recorded two non-sense mutations and five missense mutations. Furthermore, we demonstrated that *ID3* mutations are frequent events in both types of DHL (*cMYC* and *BCL2* translocated, *cMYC* and *BCL6* translocated) with 3/14 and 4/16 mutations respectively (21%/25%). Location and type of mutation are summarized in Figure 3. The singular case of triple-hit lymphoma showed wild-type ID3. There was no significant association between mutational status and dissemination of the disease with regard to nodal/extranodal involvement. Moreover, no statistically significant association between mutational status and history of prior or concurrent low-grade lymphoma was observed. Cytogenetic and morphological features, as well as *ID3* mutation status and epidemiological data for the study group are summarized in Table II.

Discussion

DHL are uncommon, representing about 1% of all lymphomas and approximately 4% of high-grade B-cell lymphomas (5, 12). Most studies regarding this entity have focused on B-cell lymphomas harboring both *cMYC* re-arrangement as well as chromosomal abnormalities affecting *BCL2* and only few cases of *cMYC+/BCL* 6+ DHL have been published to date (18). For DHL, especially those harboring *cMYC+/BCL2+*, a broad spectrum of

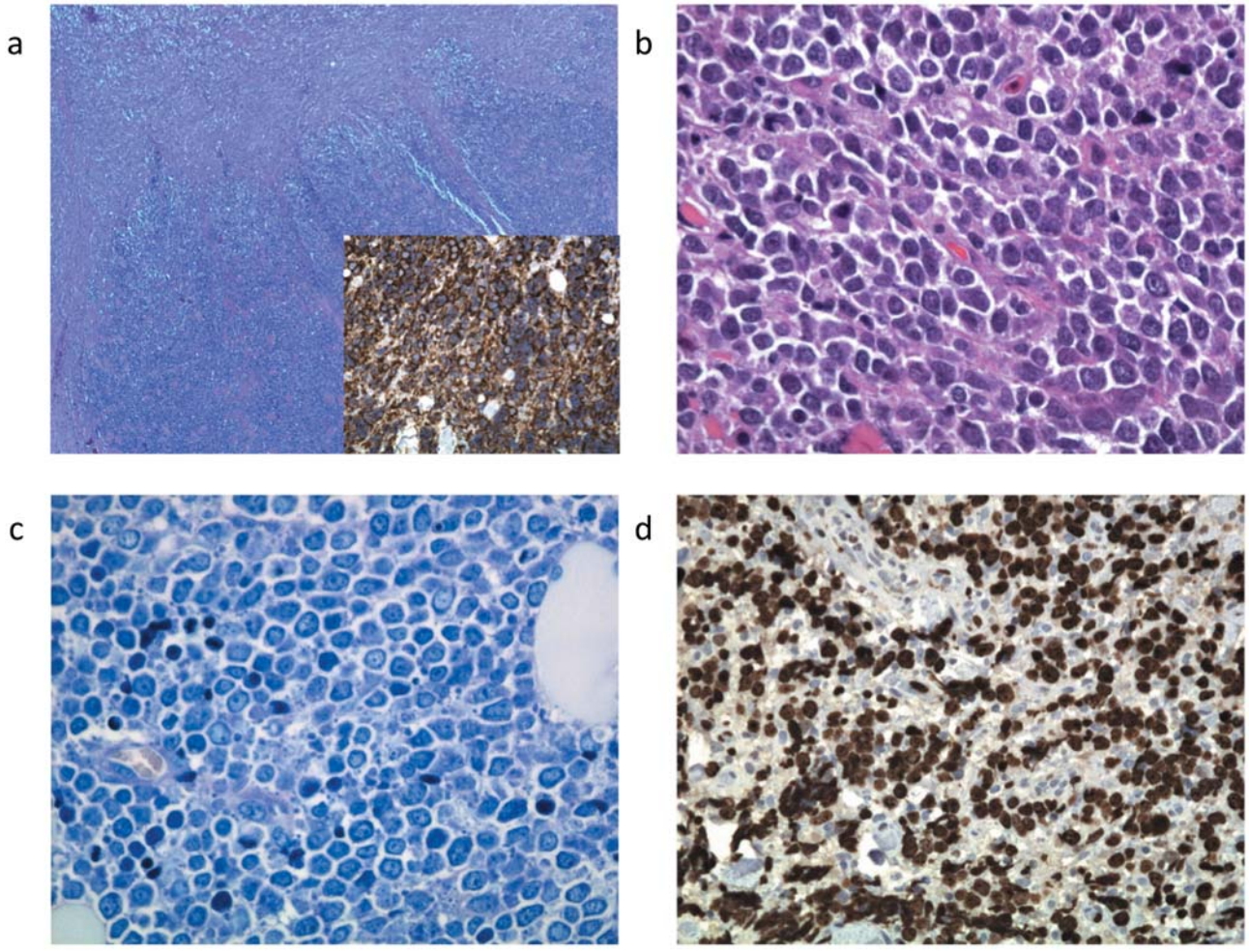

Figure 2. *Morphological and immunohistochemical findings in a avian myelocytomatosis viral oncogene homolog translocated (cMYC+) and B-cell lymphoma-6 translocated (BCL6+) double-hit lymphoma: The histological architecture of the lymph node is entirely dissolved by a dense lymphatic infiltrate with strong reactivity for CD20 (a and inset). A starry sky pattern due to frequently intermingled macrophages can be seen as well as a high degree of pleomorphism in the neoplastic B-cell population with frequent nucleoli and strong variations in nuclear size. Cellular turnover is elevated with numerous mitotic figures and apoptotic bodies. Immunohistochemical staining for Ki67 (MIB1) reveals nuclear positivity in about 90% of the malignant B-cells. a: Hematoxylin & Eosin stain ×25; a inset: CD20 ×200; b: Hematoxylin & Eosin stain ×400; c: Giemsa stain ×400; d: Ki67 (MIB1) ×200.*

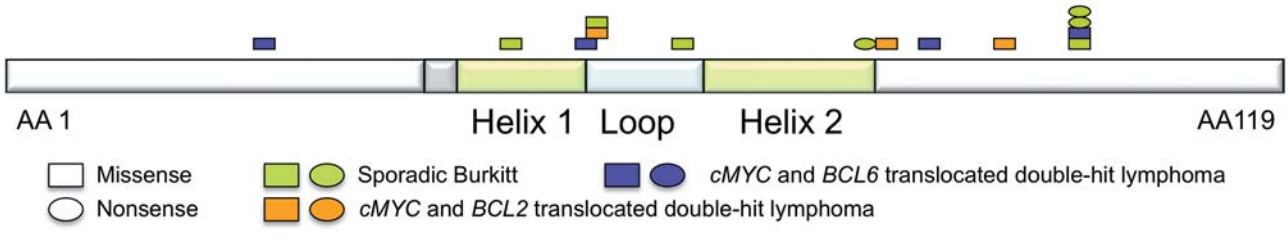

Figure 3. *Spectrum and distribution of Inhibitor of DNA binding 3 (ID3) mutations in Burkitt lymphomas and double-hit B-cell lymphomas. AA, Amino acid.*

Table II. *Epidemiological and cytogenetical features of the study group.*

Case	Age, years	Gender	Diagnosis	Translocations			Sample localization	ID3
				cMYC	*BCL2*	*BCL6*		
1	88	F	Burkitt	+	–	–	Nasal mucosa	WT
2	3	M	Burkitt	+	–	–	Thyroid	p.64 leu>phe
3	26	M	Burkitt	+	–	–	Small intestine	p.100 gln>his
4	59	M	Burkitt	+	–	–	*M. supraspinatus*	p.55 val>glu
5	22	M	Burkitt	+	–	–	Appendix	WT
6	85	F	Burkitt	+	–	–	Nasal mucosa	WT
7	68	F	Burkitt	+	–	–	Peritoneum	WT
8	12	M	Burkitt	+	–	–	Colon	p.47 cys>arg
9	52	M	Burkitt	+	–	–	Colon	WT
10	69	F	Burkitt	+	–	–	Liver	WT
11	26	M	Burkitt	+	–	–	Lymph node	p.81 gln>X
12	73	M	Burkitt	+	–	–	Colon	WT
13	6	M	Burkitt	+	–	–	Lymph node	WT
14	49	F	Burkitt	+	–	–	Soft tissue	WT
15	70	F	Burkitt	+	–	–	Pleura	p.100 gln>X
16	7	M	Burkitt	+	–	–	Soft tissue	p.100 gln>X
17	76	M	DLBCL	+	+	+	Stomach	WT
18	72	M	B-UCL	+	+	–	*M. gluteus* max.	WT
19	45	F	B-UCL	+	+	–	unkown	WT
20	62	F	DLBCL	+	+	–	Lymph node	p.82 val>leu
21	61	M	DLBCL	+	+	–	Stomach	WT
22	60	M	B-UCL	+	+	–	Soft tissue	WT
23	83	F	B-UCL	+	+	–	Soft tissue	WT
24	74	F	DLBCL	+	+	–	Lymph node	WT
25	74	M	B-UCL	+	+	–	*M.vastus* med.	p.55 val>glu
26	62	M	B-UCL	+	+	–	Unknown	p.93 asp>asn
27	35	M	B-UCL	+	+	–	Retroperitoneal	WT
28	66	F	DLBCL	+	+	–	Bone marrow	WT
29	58	M	B-UCL	+	+	–	*M. erector spinae*	WT
30	76	M	B-UCL	+	+	–	Lymph node	WT
31	67	M	B-UCL	+	–	+	Kidney	WT
32	73	M	B-UCL	+	–	+	Bone	WT
33	77	M	B-UCL	+	–	+	Stomach	p.54 leu>val
34	73	M	DLBCL	+	–	+	Small intestine	WT
35	52	M	DLBCL	+	–	+	Stomach	WT
36	60	F	B-UCL	+	–	+	Lymph node	p.24 ile>ser
37	70	M	B-UCL	+	–	+	Oral mucosa	WT
38	76	F	B-UCL	+	–	+	Tonsill	p.100 gln>pro
39	61	F	DLBCL	+	–	+	Stomach	WT
40	80	M	DLBCL	+	–	+	Stomach	WT
41	75	F	B-UCL	+	–	+	Bladder	WT
42	67	M	B-UCL	+	–	+	Unknown	WT
43	67	M	B-UCL	+	–	+	Lymph node	WT
44	77	M	B-UCL	+	–	+	Skin	WT
45	69	F	DLBCL	+	–	+	Skin	p.86 glu>gln
46	82	M	B-UCL	+	–	+	Lymph node	WT

F, Female; M, male; cMYC, avian myelocytomatosis viral oncogene homolog; BCL, B-cell lymphoma; ID3, Inhibitor of DNA binding 3, WT, wild-type, DLBCL, diffuse large B-cell lymphoma, B-UCL, B-cell lymphoma unclassifiable with features intermediate between Burkitt lymphoma and DLBCL.

morphological findings has been reported, with the majority of cases being classified as B-UCL (2, 24). With regard to this morphological and immunophenotypic spectrum, recognition and classification of DHL has been difficult and it is currently believed that they may have been underdiagnosed in the past (4, 12). A substantial subset of cases can be classified as DLBCL and rare cases have been classified as (high-grade) follicular lymphoma, B-lymphoblastic lymphoma, and composite lymphoma (11). Previous studies uniformly revealed patients with *cMYC+/BCL2+* lymphomas to have a number of poor prognostic features and a more recent study extended most of these findings to *cMYC+/BCL6+* lymphomas (8, 9, 18, 23, 26). These prognostic features frequently included a high serum lactate dehydrogenase level at diagnosis, a high frequency of bone marrow involvement and multiple extranodal sites of involvement. Additionally CNS disease was also common and most patients had a high IPI. The presence of previous or concurrent low-grade follicular lymphomas constituted an independent marker of poor prognosis, wheras stratification into WHO classification categories did not correlate significantly with prognosis. In a recently published series of six cases, *cMYC+/BCL6+* double-hit lymphomas were reported to have morphologically uniform features consistent with the WHO entity B-UCL, although the authors recognized a bias that may be of relevance to the study presented here, as well as the cases selected for these studies were more likely to have cytogenetic FISH studies conducted due to their morphologically- and/or immunohistochemically-suspicious presentation compared with cases of classical DLBCL or other defined entities (18). Pan *et al*. first proposed a role for *ID3* in mediating signals from the B-cell receptor to cell-cycle progression during humoral immune responses (15). Given this key regulatory function, it was later concluded that mutations affecting *ID3* may result in tonic B-cell receptor signaling, leading to a *cMYC*-independent mechanism of lymphoid proliferation in Burkitt lymphoma (19, 20). These observations were further in accordance with a predisposition of *ID3* knock-out mice for lymphomagenesis as described by Li *et al*. (10). In this study, we report that ID3 mutations are recurrent events in both *cMYC+/BCL2+* as well as *cMYC+/BCL6+* DHL adding further evidence to the hypothesis that the biological behaviour of this rare entity has features intermediate between Burkitt lymphoma and DLBCL. Our data may hint at a subset of DHL sharing a molecular hallmark of Burkitt lymphoma in lymphomagenesis. We observed a trend towards patients with Burkitt lymphoma with *ID3* mutations being younger than patients with wild-type *ID3*. This trend, however, failed to reach statistical significance due to the small size of our study group (p=0.1120). No such correlation was found for DLBCL and/or B-UCL. In accordance with the literature, the frequency of missense mutations was highest within the evolutionarily-conserved regions containing the HLH domain. Considering the dismal prognosis of DHL and the potential therapeutical options targeting ID3 or consecutive tonic B-cell signaling, we believe that the involvement of *ID3* mutations may be of interest for future, targeted therapy approaches. In summary, our findings, alltough limited by a small sample size, indicate an oncogenic relevance of *ID3* mutation in a subset of DHL (both *cMYC+/BCL2+* and *cMYC+/BCL6+*) patients and characterize the ID3 TCF3 regulatory loop as a potential therapeutic target in this highly aggressive entity. Moreover we described and characterized 16 new cases of *cMYC+/BCL6+* DHL.

Acknowledgements

We thank Tanja Oeltermann for her skilled and dedicated technical assistance.

References

1 Akyurek N, Uner A, Benekli M and Barista I: Prognostic significance of MYC, BCL2, and BCL6 rearrangements in patients with diffuse large B-cell lymphoma treated with cyclophosphamide, doxorubicin, vincristine, and prednisone plus rituximab. Cancer *118*: 4173-4183, 2012.

2 Aukema SM, Siebert R, Schuuring E, van Imhoff GW, Kluin-Nelemans HC, Boerma EJ and Kluin PM: Double-hit B-cell lymphomas. Blood *117*: 2319-2331, 2011.

3 Barrans S, Crouch S, Smith A, Turner K, Owen R, Patmore R, Roman E and Jack A: Rearrangement of MYC is associated with poor prognosis in patients with diffuse large B-cell lymphoma treated in the era of rituximab. J Clin Oncol *28*: 3360-3365, 2010.

4 Benz R and Tchinda J: Double hit, triple hit – look for it. Blood *121*: 2383, 2013.

5 Galteland E, Sivertsen EA, Svendsrud DH, Smedshammer L, Kresse SH, Meza-Zepeda LA, Myklebost O, Suo Z, Mu D, Deangelis PM and Stokke T: Translocation t(14;18) and gain of chromosome 18/BCL2: effects on BCL2 expression and apoptosis in B-cell non-Hodgkin's lymphomas. Leukemia *19*: 2313-2323, 2005.

6 Hans CP, Weisenburger DD, Greiner TC, Gascoyne RD, Delabie J, Ott G, Muller-Hermelink HK, Campo E, Braziel RM, Jaffe ES, Pan Z, Farinha P, Smith LM, Falini B, Banham AH, Rosenwald A, Staudt LM, Connors JM, Armitage JO and Chan WC: Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. Blood *103*: 275-282, 2004.

7 Hummel M, Bentink S, Berger H, Klapper W, Wessendorf S, Barth TF, Bernd HW, Cogliatti SB, Dierlamm J, Feller AC, Hansmann ML, Haralambieva E, Harder L, Hasenclever D, Kuhn M, Lenze D, Lichter P, Martin-Subero JI, Moller P, Muller-Hermelink HK, Ott G, Parwaresch RM, Pott C, Rosenwald A, Rosolowski M, Schwaenen C, Sturzenhofecker B, Szczepanowski M, Trautmann H, Wacker HH, Spang R, Loeffler M, Trumper L, Stein H and Siebert R: A biologic definition of Burkitt's lymphoma from transcriptional and genomic profiling. N Engl J Med *354*: 2419-2430, 2006.

8 Johnson NA, Savage KJ, Ludkovski O, Ben-Neriah S, Woods R, Steidl C, Dyer MJ, Siebert R, Kuruvilla J, Klasa R, Connors JM, Gascoyne RD and Horsman DE: Lymphomas with concurrent BCL2 and MYC translocations: the critical factors associated with survival. Blood *114*: 2273-2279, 2009.

9 Kanungo A, Medeiros LJ, Abruzzo LV, and Lin P: Lymphoid neoplasms associated with concurrent t(14;18) and 8q24/c-MYC translocation generally have a poor prognosis. Mod Pathol *19*: 25-33, 2006.

10 Li J, Maruyama T, Zhang P, Konkel JE, Hoffman V, Zamarron B and Chen W: Mutation of inhibitory helix-loop-helix protein Id3 causes gammadelta T-cell lymphoma in mice. Blood *116*: 5615-5621, 2010.

11 Li S, Lin P, Fayad LE, Lennon PA, Miranda RN, Yin CC, Lin E and Medeiros LJ: B-cell lymphomas with MYC/8q24 rearrangements and IGH@BCL2/t(14;18)(q32;q21): an aggressive disease with heterogeneous histology, germinal center B-cell immunophenotype and poor outcome. Mod Pathol *25*: 145-156, 2012.

12 Lin P and Medeiros LJ: High-grade B-cell lymphoma/leukemia associated with t(14;18) and 8q24/MYC rearrangement: a neoplasm of germinal center immunophenotype with poor prognosis. Haematologica *92*: 1297-1301, 2007.

13 Niitsu N, Okamoto M, Miura I and Hirano M: Clinical features and prognosis of de novo diffuse large B-cell lymphoma with t(14;18) and 8q24/c-MYC translocations. Leukemia *23*: 777-783, 2009.

14 Offit K, Wong G, Filippa DA, Tao Y and Chaganti RS: Cytogenetic analysis of 434 consecutively ascertained specimens of non-Hodgkin's lymphoma: clinical correlations. Blood *77*: 1508-1515, 1991.

15 Pan L, Sato S, Frederick JP, Sun XH and Zhuang Y: Impaired immune responses and B-cell proliferation in mice lacking the Id3 gene. Mol Cell Biol *19*: 5969-5980, 1999.

16 Perk J, Iavarone A and Benezra R: Id family of helix-loop-helix proteins in cancer. Nat Rev Cancer *5*: 603-614, 2005.

17 Perry AM, Crockett D, Dave BJ, Althof P, Winkler L, Smith LM, Aoun P, Chan WC, Fu K, Greiner TC, Bierman P, Gregory Bociek R, Vose JM, Armitage JO and Weisenburger DD: B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and burkitt lymphoma: study of 39 cases. Br J Haematol 2013.

18 Pillai RK, Sathanoori M, Van Oss SB and Swerdlow SH: Double-hit B-cell lymphomas with BCL6 and MYC translocations are aggressive, frequently extranodal lymphomas distinct from BCL2 double-hit B-cell lymphomas. Am J Surg Pathol *37*: 323-332, 2013.

19 Richter J, Schlesner M, Hoffmann S, Kreuz M, Leich E, Burkhardt B, Rosolowski M, Ammerpohl O, Wagener R, Bernhart SH, Lenze D, Szczepanowski M, Paulsen M, Lipinski S, Russell RB, Adam-Klages S, Apic G, Claviez A, Hasenclever D, Hovestadt V, Hornig N, Korbel JO, Kube D, Langenberger D, Lawerenz C, Lisfeld J, Meyer K, Picelli S, Pischimarov J, Radlwimmer B, Rausch T, Rohde M, Schilhabel M, Scholtysik R, Spang R, Trautmann H, Zenz T, Borkhardt A, Drexler HG, Moller P, MacLeod RA, Pott C, Schreiber S, Trumper L, Loeffler M, Stadler PF, Lichter P, Eils R, Kuppers R, Hummel M, Klapper W, Rosenstiel P, Rosenwald A, Brors B and Siebert R: Recurrent mutation of the ID3 gene in Burkitt lymphoma identified by integrated genome, exome and transcriptome sequencing. Nat Genet *44*: 1316-1320, 2012.

20 Schmitz R, Young RM, Ceribelli M, Jhavar S, Xiao WM, Zhang MZ, Wright G, Shaffer AL, Hodson DJ, Buras E, Liu XL, Powell J, Yang YD, Xu WH, Zhao H, Kohlhammer H, Rosenwald A, Kluin P, Muller-Hermelink HK, Ott G, Gascoyne RD, Connors JM, Rimsza LM, Campo E, Jaffe ES, Delabie J, Smeland EB, Ogwang MD, Reynolds SJ, Fisher RI, Braziel RM, Tubbs RR, Cook JR, Weisenburger DD, Chan WC, Pittaluga S, Wilson W, Waldmann TA, Rowe M, Mbulaiteye SM, Rickinson AB and Staudt LM: Burkitt lymphoma pathogenesis and therapeutic targets from structural and functional genomics. Nature *490*: 116-120, 2012.

21 Seegmiller AC, Garcia R, Huang R, Maleki A, Karandikar NJ and Chen W: Simple karyotype and bcl-6 expression predict a diagnosis of Burkitt lymphoma and better survival in IG-MYC rearranged high-grade B-cell lymphomas. Mod Pathol *23*: 909-920, 2010.

22 Siebert R, Rosenwald A, Staudt LM and Morris SW: Molecular features of B-cell lymphoma. Curr Opin Oncol *13*: 316-324, 2001.

23 Snuderl M, Kolman OK, Chen YB, Hsu JJ, Ackerman AM, Dal Cin P, Ferry JA, Harris NL, Hasserjian RP, Zukerberg LR, Abramson JS, Hochberg EP, Lee H, Lee AI, Toomey CE and Sohani AR: B-cell lymphomas with concurrent IGH-BCL2 and MYC rearrangements are aggressive neoplasms with clinical and pathologic features distinct from Burkitt lymphoma and diffuse large B-cell lymphoma. Am J Surg Pathol *34*: 327-340, 2010.

24 Swerdlow SHea: WHO Classification of Tumors of Haematopoetic and Lymphoid Tissues: WHO, 2008.

25 Taub R, Kirsch I, Morton C, Lenoir G, Swan D, Tronick S, Aaronson S and Leder P: Translocation of the c-myc gene into the immunoglobulin heavy chain locus in human Burkitt lymphoma and murine plasmacytoma cells. Proc Natl Acad Sci USA *79*: 7837-7841, 1982.

26 Tomita N, Tokunaka M, Nakamura N, Takeuchi K, Koike J, Motomura S, Miyamoto K, Kikuchi A, Hyo R, Yakushijin Y, Masaki Y, Fujii S, Hayashi T, Ishigatsubo Y and Miura I: Clinicopathological features of lymphoma/leukemia patients carrying both BCL2 and MYC translocations. Haematologica *94*: 935-943, 2009.

Received September 6, 2013
Revised October 11, 2013
Accepted October 15, 2013