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Immunohistochemical detection of inhibitor of DNA binding 3 mutational variants in mature aggressive B-cell lymphoma

In addition to the hallmark translocations involving the *MYC* oncogene and immunoglobulin loci,[1] Burkitt lymphomas (BL) frequently carry mutations in the *inhibitor of DNA binding 3 (ID3)* gene.[2,3] Genetic aberrations of *ID3* in BL comprise a spectrum of mono- and bi-allelic structural and point mutations.[3] ID3 acts as negative transcriptional regulator by sequestering transcription factors with basic helix-loop-helix motifs. Mutated ID3 attenuates this regulatory interaction.[4,5] ID3 and its interaction partner, TCF3, are involved in controlling cell cycle progression and survival pathways through tonic B-cell signaling.[6,7]

ID3 mutations occur in 34-68% of BL but are rare in diffuse large B-cell lymphomas (DLBCL).[2,3,7] Interestingly, the incidence of *ID3* mutations was reported to be higher in B-cell lymphomas, unclassifiable, with features intermediate between DLBCL and BL,[8] than in DLBCL. However, in the quoted study a molecular diagnosis was not available and the diagnosis of an "intermediate" lymphoma was based on histopathological features only.[8,9]

Mutation-specific immunohistochemistry is a valuable diagnostic tool,[10] and we used it to test six anti-ID3 antibodies for their ability to detect *ID3* mutational variants in molecularly defined BL (mBL), "intermediate" lymphomas and non-mBL lymphomas[11] (*Online Supplementary Table S1*).

First, we tested all six antibodies on formalin-fixed and paraffin-embedded tonsil tissue using immunohistochemistry. ID3 has been reported to be strongly expressed in the dark zone and less intensively in the light zone of germinal centers.[12] The expected staining pattern was only observed with clone 17-3 (BioCheck Inc., Foster City, USA) (Figure 1), but not for the other antibodies tested (*Online Supplementary Figure S1*). To determine whether clone 17-3 shows a mutation-specific staining pattern, selected wild-type (wt) and point-mutated *ID3* cell lines and lymphoma specimens were tested by immunohistochemistry (*Online Supplementary Figure S1*) and by western blotting (*Online Supplementary Figure S2*). As expected, clone 17-3 showed no reactivity in BL cell lines or mBL biopsies with homozygous loss of *ID3* (Figure 1; *Online Supplementary Figures S1 and S2; Online Supplementary Table S2*). The other five antibodies positively stained cell lines and/or biopsies by immunohistochemistry despite a homozygous deletion of the *ID3* locus and were not, therefore, used further (*Online Supplementary Figure S1; Online Supplementary Table S2*).

The *Online Supplementary Data* contains more detailed information on the materials and methods, cell lines and cases.

Mutation-sensitive ID3 immunohistochemistry using clone 17-3 was performed on 89 formalin-fixed, paraffin-embedded lymphoma biopsies. The conventionally assigned diagnoses based on histomorphological and immunophenotypic features according to the current World Health Organization classification[8] were as follows: BL (23/89), Burkitt leukemia (1/89), atypical BL (15/89), DLBCL (27/89), high-grade B-cell non-Hodgkin lymphoma (3/89), B-cell lymphomas, unclassifiable (2/89), follicular lymphoma grade 1-3a (10/89), transformed follicular lymphoma grade 3a/b/DLBCL (4/89), primary mediastinal B-cell lymphoma (2/89), primary central nervous system DLBCL (1/89), and post-transplant lymphoproliferative disease with features of DLBCL (1/89) (*Online Supplementary Table S3*). All cases were molecularly studied in either the MMML (n=41) or ICGC MMML-Seq (n=43) projects or both (n=5). Thus, the molecular classification based on gene expression analysis as well as the *ID3* mutation status based on whole genome and/or Sanger sequencing were available.[3,11,13,14] In detail, according to a defined gene expression signature, the so-called mBL signature index, which reflects the probability that a case resembles a BL, all cases were assigned their specific molecular diagnosis. In accordance with Hummel *et al.*[11] cases with a mBL signature index score higher than 0.95 were classified as mBL (38/89), cases with an intermediate mBL signature index score between 0.05 and 0.95 as "intermediate" lymphomas (14/89), and cases with a mBL signature index score lower than 0.05 as non-mBL (36/89); one nodal manifestation of BL leukemia was not assigned a molecular diagnosis (*Online Supplementary Table S3; Online Supplementary Methods*).

ID3 expression in mBL showed a biphasic pattern. Almost all BL displayed either high ID3 expression scores (>50% positive lymphoma cells) or no expression (Figure 1h and *Online Supplementary Table S3*). mBL with wt, monoallelic point or monoallelic structural *ID3* mutations (deletions, insertions or frameshifts) displayed ID3 immunoreactivity by immunohistochemistry (27/27, 100%; interpretable staining failed in 2 mBL). All mBL in our series with lack of ID3 immunoreactivity (10/10, 100%) harbored complex biallelic structural *ID3* mutations (e.g. biallelic frameshifts). Sequence analyses predicted a loss of the C-terminal ID3 epitope of clone 17-3 (Figure 1; *Online Supplementary Table S3*).

We detected a broad spectrum of ID3 immunoreactivity in non-mBL, ranging from no expression to high expression (Figure 1h and *Online Supplementary Table S3*). However, a high level of ID3 expression (>50% positive lymphoma cells) was rare in non-mBL (6/36, 17%). *ID3* expression in non-mBL seems to be independent of the mutational status, since none of the ID3-negative non-mBL harbored biallelic structural *ID3* variants (0/8, 0%). Monoallelic mutations of *ID3* were detected in only two cases of non-mBL (2/36, 6%) and neither of these lymphomas showed ID3 immunoreactivity (*Online Supplementary Table S3*).

Like non-mBL, molecularly defined "intermediate" lymphomas showed a broad spectrum of ID3 expression (Figure 1 and *Online Supplementary Table S3*). High ID3 immunoreactivity (>50% positive lymphoma cells) was as frequent as in mBL (9/14, 64% *versus* 27/37, 73%, respectively) and more frequent than in non-mBL (6/36, 17%). Complete lack of ID3 immunoreactivity was observed in only 2/14 "intermediate" lymphomas which both harbored *ID3* structural mutations. One was a pediatric case with a biallelic *ID3* frameshift insertion and a splice site alteration. Unfortunately, we were not able to assess whether the lesion is biallelic in the second case (*Online Supplementary Table S3*). Interestingly, none of the four double- or triple-hit lymphomas with *MYC* and either *BCL2* and/or *BCL6* translocations in our series had mutated *ID3* nor lacked ID3 immunoreactivity (*Online Supplementary Table S3*).

Here we describe an anti-ID3 antibody which was the only one of six antibodies tested with high specificity for ID3 in immunohistochemistry and western blots. Clone 17-3 showed highly specific immunoreactivity for wt and point-mutated *ID3* in mature aggressive B-cell lymphomas. We found that ID3 is highly expressed in mBL and the "intermediate" group of lymphomas, whereas it is not expressed, or only moderately expressed in non-mBL. Furthermore, both mBL and "intermediate" lymphomas are characterized by a high frequency of *ID3* mutations whereas non-mBL are not. Moreover, mBL and "intermediate" lymphomas,[11] mainly resembling BL, atypical BL and B-cell lymphomas, unclassifiable,[8] show a complete lack of ID3 staining only when biallelic structural aberrations causing a

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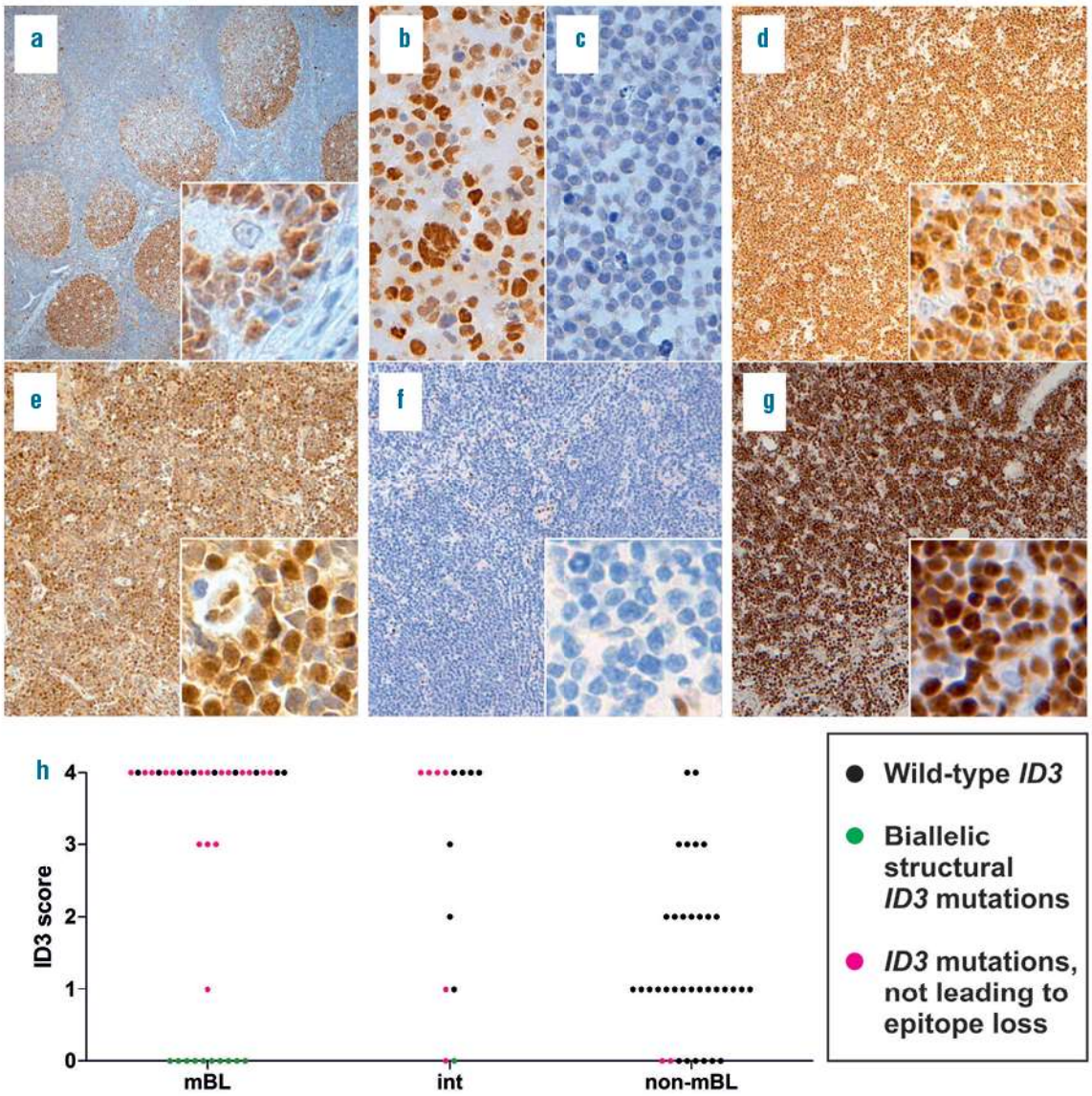

Figure 1. Immunohistochemistry for ID3 and *ID3* mutation distribution among mBL, "intermediate" lymphomas, and non-mBL. (a-g). ID3 immunohistochemistry of formalin-fixed, paraffin-embedded sections of tonsil, BL and BL cell lines; original magnification of tonsil 50x, inlet 400x, cell lines 400x, cases 100x, inlets 400x. (a-g) were stained with clone 17-3. (a) Reactive tonsil, pronounced ID3 distribution in the dark zone of germinal centers; (b) BL cell line EB-1, wt *ID3*; (c) BL cell line BL-41, biallelic stop gain, loss of ID3 amino acids 69-109; (d) case 34, mBL, wt *ID3*; (e) case 17; mBL, two *ID3* point mutations; (f) case 2, mBL, homozygous loss of ID3 C-terminal domains; (g) case 15, mBL, stop gain and splice site mutation, the latter without structural consequences; (h) Scatter plot of the ID3 immunohistochemical scoring based on percentages of ID3-positive tumor cells: 0=0%, 1=1-25%, 2=26-50%, 3=51-75%, and 4=76-100%. Each point is a case and the color codes illustrate the mutational status of *ID3*. Interpretable ID3 staining failed in two cases which are not included in the plot. BL: Burkitt lymphoma; int: intermediate; mBL: molecular Burkitt lymphoma; non-mBL: non-molecular Burkitt lymphoma; wt: wild-type.

loss of C-terminal domains of *ID3* are present. In contrast, none of the non-mBL cases in this study, mainly resembling DLBCL, harbored biallelic structural *ID3* mutations. ID3-negative non-mBL had either wt *ID3* or harbored monoallelic *ID3* locus deletions, so the lack of ID3 expression was not associated with a genetic loss of *ID3* domains but probably due to transcriptional regulation. Thus, lack of ID3 staining in a mature aggressive B-cell lymphoma with features of BL can be regarded as an indicator of biallelic loss of *ID3*. Since lack of ID3 immunoreactivity also occurs in a small subset of non-mBL with wt or monoallelic structural *ID3* aberrations, staining for ID3 currently seems to be of limited value in the differential diagnosis of lymphoma. A potential use in combination with other biomarkers needs to be determined in future studies.

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