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Research paper

Immunohistochemical distinction of ABC and GCB in extranodal DLBCL is not reflected in mutation patterns

Cora Hallas*, Michael Preukschas, Markus Tiemann

Institute for Hematopathology Hamburg, Fangdieckstr. 75a, 22547, Hamburg, Germany

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ABSTRACT

Gene expression profiling (GEP) separated diffuse large B-cell lymphoma (DLBCL) in two different entities, i.e. activated B cell-like (ABC) and germinal center B cell-like (GCB) lymphomas with ABC lymphomas demonstrating a less favorable outcome. NF-kB pathway activating mutations in MYD88, CD79A/B and CARD11 are predominantly found in ABC type lymphomas. Targeted therapies affecting NF-kB pathways have shown therapeutic potential in this subtype. Immunohistochemistry algorithms have been developed as a tool for distinguishing these entities in routine clinical diagnostics. To test whether this immunohistochemistry classifier would detect the biological differences between the entities 147 DLBCLs were subtyped into ABC and GCB using the Visco-Young algorithm. Mutation analysis demonstrated mutations in MYD88 or CD79 A/B in 21% (10/47) of non-GCB type but only in 3% (1/31) of GCB lymphomas ($p = 0.012$) in nodal lymphomas. In primary extra nodal lymphomas, however, 17.5% (4/23) of GCB type and 37.5% (15/40) of non-GCB lymphomas carried mutations in MYD88 and CD79 A/B. While the Visco-Young algorithm was sufficient to detect biological differences (i.e. mutation patterns) in nodal DLBCL it did not distinguish GCB and non-GCB type lymphomas of primary extranodal sites. Here, the morphological sites of the lymphomas seem to be more important for their molecular pattern than their immunohistochemical status.

1. Introduction

Diffuse large B cell lymphoma (DLBCL) is the most frequent form of adult lymphoma, accounting for 30–40% of diagnoses of Non Hodgkin-Lymphoma worldwide. The entity, however, is not homogenous and consists of biologically and clinically distinct subtypes. Gene expression profiling (GEP) has demonstrated at least two different types of DLBCL with distinct cells of origin. According to the gene expression patterns one type has been identified as activated B cell-like (ABC) and another as germinal center B cell-like (GCB) [1]. Both types show divergent prognoses with ABC type lymphomas demonstrating a significantly less favorable outcome. Although the difference in outcome was first described in the pre-rituximab era [2] it has been confirmed under immunochemotherapy [3]. Gene expression patterns and further functional studies revealed that ABC lymphomas rely on an activated B-cell receptor (BCR) pathway with activation of nuclear factor kappa B (NF-kB) for their survival [4]. This is evidenced by mutations in MYD88 [5], CD79 A and CD79B [6], and CARD11 [3] upstream of NF-kB. Therapies specifically targeting the BCR pathway have been used successfully in this type of DLBCL [7]. The background of GCB type lymphomas is less well defined and signature mutations have not been found. Although these lymphomas generally do better under standard chemotherapy [2] a specific therapy is not yet available.

Since ABC and GCB type lymphomas show marked differences in prognosis and therapy options it would be beneficial to differentiate these DLBCL types in routine diagnostics. Furthermore, studies using GEP for distinguishing ABC and GCB lymphomas have found MYD88 and CD79 A/B mutations nearly exclusively in ABC type lymphomas [8,9]. Gene expression profiling, however, is a complex technique fraught with many difficulties and not practical for routine use in standard clinical practice. Therefore various immunohistochemical (IHC) algorithms have been developed with the goal of providing a simpler tool for this task. Immunohistochemistry is well established and easily available in pathology. While there is reasonably good concordance between particular classifiers and GEP (88–95%, [10–12]) reported, other studies concluded that the IHC algorithms failed to detect the difference in prognosis between the subtypes [13,14]. Another study comparing various IHC classifiers found little concordance between them [15]. The arguably highest concordance between GEP data and IHC analysis was demonstrated for the Visco-Young algorithm. The algorithm is based on immunohistochemical staining of CD10, FOXP1, and BCL6 and was demonstrated to be an independent

* Corresponding author.
E-mail address: hallas@hp-hamburg.de (C. Hallas).

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predictor of progression-free and overall survival [12].

DLBCL mostly presents as a nodal neoplasia but up to 40% of cases are at least initially confined to extranodal sites (primary extranodal DLBCL). Primary extranodal lymphomas are most commonly found in the gastrointestinal tract but can occur in virtually any extranodal site. Although these lymphomas all share general biological and morphological characteristics at least DLBCL from some sites show distinct features separating them from the group. Among those are central nervous system lymphomas, testicular lymphomas, and primary cutaneous DLBCL (leg type) [16–18]. Lymphomas from other sites may have as yet undefined features demarcating them. Nevertheless studies concerning molecular subtyping so far have mostly not differentiated between nodal and primary extranodal DLBCL. This study, therefore, compared the mutational pattern regarding NF-kB activating genes (MYD88, CD79 A, CD79B, and CARD11) in nodal and primary extranodal DLBCL. The mutational status of both groups was correlated with ABC and GCB subtypes as determined by the Visco-Young IHC algorithm [12].

2. Material and methods

2.1. Material

147 formalin fixed paraffin embedded (FFPE) tissue blocks of tumor resection specimens of diffuse large B-cell lymphoma (DLBCL) were obtained from the archive of the Institute of Hematopathology, Hamburg, Germany. The samples were examined and diagnosed as DLBCL in routine diagnostics according to the WHO criteria. “Double hit” lymphomas, i.e. lymphomas carrying translocations involving BCL2 and MYC were excluded from the study. All tissue samples were obtained during standard diagnostical procedures at the Institute of Haematopathology, Hamburg, Germany, in accordance with the local ethic board requirements and the Declaration of Helsinki.

2.2. DNA-extraction and real time PCR or sequencing

Total DNA was extracted from FFPE tissue blocks using the Maxwell® 16 instrument (Promega, Madison, USA) according to the manufacturer’s instructions. Sufficient tumor content of samples was assured by preparing an additional HE slide from tissue directly adjacent to the tissue taken for DNA preparation and having it evaluated by a certified hematopathologist. CARD11 exons 5–9, EZH2 exon 15, CD79 A exon 4–5, and CD79B exon 5–6 were amplified using 5 units Hot Star Taq (Qiagen, Germany) in the buffer supplied by the manufacturer and a final concentration of 200 μM dNTPs each, and 0.2 μM (final concentration) of the respective primers. Primer sequences are given in Table 1. PCR cycles consisted of 40 cyles of 30 s. at 95 °C, 30 s. at 60 °C and 30 s. at 72 °C, preceded by a denaturation step of 15 min. at 95 °C and followed by 5 min at 72 °C. PCR products were purified using DyeEx 2.0 Spin Kit (Qiagen) and sequenced using Terminator v1.1 cycle sequencing kit (Applied Biosystems). Sequences were analyzed on an ABI 3130 Genetic Analyzer and evaluated manually. MYD88 exon 5 was amplified using the OneStep RT-PCR kit (Qiagen, Germany) according to the manufacturer’s instructions. Primers and MGB-probes are shown in Table 1. 50 PCR cycles were run consisting of 15 s. at 96 °C and 1 min at 60 °C following an initial step of 15 min at 96 °C.

2.3. Immunohistochemistry

Immunohistological staining according to the Visco-Young algorithm was carried out on BenchMark Ultra (Ventana Medical Systems, Roche, Tuscon, USA) automated staining systems according to the manufacturer’s instructions using antibodies against CD10 (clone 56C6, Novocastra, Leica Biosystems, Germany) in a 1:50 dilution, FOXP1 (clone SP133, ready to use, ROCHE, Germany), and BCL6 (clone Gl191E/A6, ready to use, Roche, Germany). Antibody incubation time was 32 min except for FOXP1 (16 min). The protocol included an antigen retrieval step in CC1 buffer (BenchMark Systems, ROCHE) at 90 °C for 64 min for CD10 and FOXP1 and 40 min for BCL6. The staining pattern was evaluated and scored by the same certified and experienced hematopathologist in all cases.

2.4. Statistical analysis

The mutation frequency of primary nodal and extranodal DLBCL and various subgroups was compared using Fisher’s exact test.

3. Results

78 nodal and 69 primary extranodal DLBCL were investigated for mutations in MYD88, CD79 A and B, CARD11. The extranodal cases consisted of 32 intestinal lymphomas, 13 primary bone lymphomas, 7 testicular lymphomas, 6 primary liver tumors, 4 leg type DLBCL and 7 lymphomas from various other sites. The Visco-Young immunohistochemical algorithm [12] was used to subtype the primary extranodal lymphomas into 23 germinal center B-cell (GCB) type and 40 non-GCB type lymphomas. 6 samples were not classifiable. GCB type lymphomas were found mainly among the ossary tumors (7 of 12) and the intestinal lymphomas (11 of 32). Only one of the testicular and liver lymphomas each was classified as GCB. The remaining 3 GCB were from other sites. The nodal samples consisted of 31 GCB type and 47 non-GCB type lymphomas.

Only 4 (12.9%) of the 31 nodal GCB type lymphomas had mutations in the NF-kB activating genes MYD88, CARD11, and CD79 A and

Table 1
Primers.

Name	forward	reverse
CARD-e5-1	CAGTCCTCAGGCCTGACTG	GTAGTACCGCTCCTGGAAGG
CARD-e5-2	GAAGAAGCAGATGACGCTGAC	GAGAATTGAGCCCTGGTGAC
CARD-e6	GTTTCTTGCCACCTGCCTTC	CACACCCTGGCAGGTTCATC
CARD-e7	GACCCTGACCCTCTGAAACC	GTCCCTGGAAAGGAGTGTGG
CARD-e8	GTCCTGACCTATTGATTGCTC	TCAGTGATGTTGCACTGGAC
CARD-e9	AGCCTTCGTCCCGCTTCTC	CTGGCCACAGCCCTCAGTG
CD79A	CAGAAACGATGGCAGAACGAG	CTGGACATCTCCTATGTTGAG
CD79B	GGACACTAACACTCTGATCTC	CTGGGTGCTCACCTACAGAC
MYD88-e5	GGTTGAAGACTGGGCTTGTC	GTGTAGTCGCAGACAGTGATG
EZH2-e15	CATCTATTGCTGGCACCATC	CCTGAATACAGGTTATCAGTGC
MYD88 probe wt:	VIC-AAGCGACTGATCC - MGB	
MYD88 probe mut:	6FAM-AAGCGACCGATCC - MGB	

Primers used for amplification and sequencing of CARD11, exons 5, 6, 7, 8, and 9, CD79A exons 4–5, CD79B exons 5–6, MYD88 exon 5, and EZH2 exon 15. MYD88 was not sequenced but the L265P mutation was detected by real time PCR using mutation specific probes.

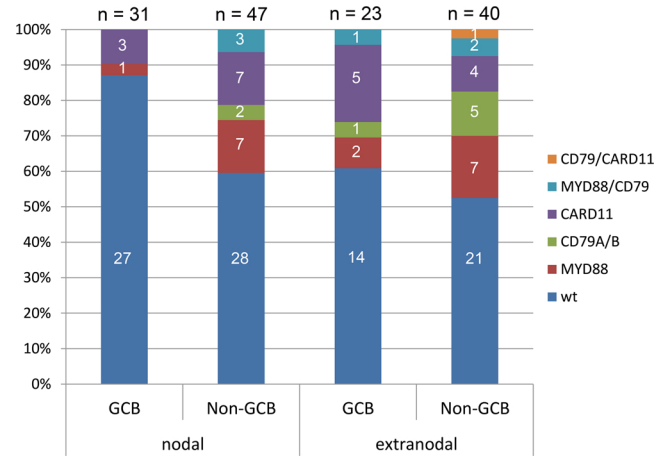

Fig. 1. Mutations in extranodal and nodal GCB and non-GCB DLBCL. Distribution of mutations between GCB and non-GCB type lymphomas (as determined using the Visco-Young immunohistochemical algorithm). Numbers of mutated samples are given in the bars. While nodal GCB type lymphomas carry few mutations primary extranodal GCB show no visible difference to non-GCB type DLBCL. MYD88/CD79 = Mutations in both MYD88 and CD79; CD79/CARD11 = mutations in both CD79 and CARD11.

Table 2
Mutations in extranodal DLBCL according to site.

	wt	MYD88	CD79	CARD11	MYD88/ CD79	CD79/ CARD11	n
GI	22	1	5	4	0	0	32
Bone	7	2	2	2	0	0	13
Testicle	1	1	1	0	4	0	7
Liver	2	1	0	2	0	1	6
Leg-type	3	1	0	0	0	0	4
Other	4	1	0	2	0	0	7

Mutations in MYD88, CD79A/B, and CARD11 in primary extranodal DLBCL derived from different anatomical locations. GI = gastrointestinal; MYD88/CD79 = Mutations in both MYD88 and CD79; CD79/CARD11 = mutations in both CD79 and CARD11.

CD79B, including one mutation in MYD88 (L265 P) and three in CARD11. Mutations in CD79 A/B were not found (Fig. 1). However, 19 (40.4%) of the 47 nodal non-GCB type lymphomas carried Mutations, including 7 (14.9%) in MYD88 (L265 P), 7 (14.9) in CARD11 and one (2.1%) in CD79 A and B, each (Fig. 1). The remaining three samples (6.4%) carried mutations in both MYD88 and CD79B. Overall, in nodal lymphomas GCB type samples had significantly fewer mutations than non-GCB type samples ($p < 0.012$). This was mainly due to a significant difference in the number of MYD88 mutations ($p < 0.05$), whereas no difference was detected in the number of CARD11 mutations. The overall number of CD79 mutations was too small for a meaningful statistical analysis.

However, these differences were not replicated in the primary extranodal lymphomas. Here, the GCB type lymphomas, as classified by the Visco Young algorithm, carried 9 (39.1%) mutations in 23 cases, consisting of 2 (8.7%) in MYD88 (L265 P), 1 (4.3%) in CD79B, 5 (21.7%) in CARD11, and one case with a mutation in both MYD88 and CD79B mutation (Fig. 1). The 40 samples classified as non-GCB type lymphomas showed mutations in 19 (47.5%) cases. These included 7 (17.5%) mutations in MYD88 (L265 P), 4 (10%) in CARD11, 4 (10%) in CD79B, 1 (2.5%) in CD79 A, 2 (5%) samples with mutations in MYD88 and CD79B, and one surprising sample with mutations in both CD79B and CARD11 (Fig.1). Significant differences between GCB type and non-GCB type lymphomas from primarily extranodal sites were not detected. 6 further cases could not conclusively be classified. 3 of these contained mutations, 2 in MYD88 and one in both MYD88 and CD79B.

Interestingly, all 4 cases with mutations in MYD88 and CD79B were primary testicular lymphomas. One other testicular lymphoma carried a MYD88 mutation alone and one a CD79B mutation. Only one of the 7 testicular lymphomas had no mutation in any of the genes analyzed. Mutations in the other entities of primary extranodal lymphomas are shown in Table 2 and Fig. 2. Despite the small number of testicular lymphomas tested the difference between testicular lymphomas and all other primary extranodal lymphomas regarding overall number of mutations was striking enough to be statistically significant ($p < 0.04$). Since among the primary extranodal lymphomas only testicular lymphomas carried mutations in MYD88 and CD79B combined the difference was obvious ($p < 0.0005$). Only one of the testicular lymphomas was classified as a GCB type lymphoma using the Visco Young algorithm and this sample carried mutations in both MYD88 and CD79B.

Regarding all cases patients carrying MYD88 mutations showed a tendency to be older than patients without mutations (mean: 75.3 years versus 68.5 years, $p = 0.057$) and they are possibly older than patients carrying CARD11 mutations (mean: 66.2 years. $p = 0.072$), although the number of patients carrying mutations was not high enough for a meaningful statistical analysis.

4. Discussion

Based on GEP data DLBCL can be grouped into two subtypes with significantly different prognoses [1]. ABC DLBCL is characterized by constitutive activation of NF-kB. In a number of cases this activation is achieved via mutations in MYD88, CD79, or CARD11 [3,5,6]. GCB

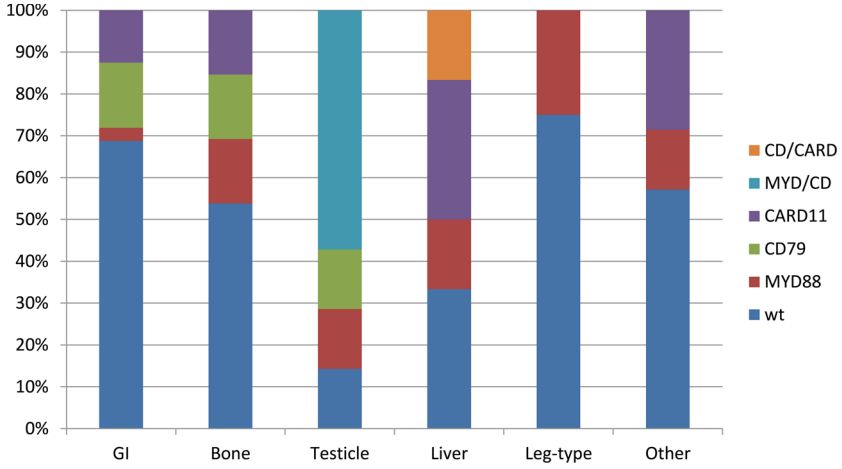

Fig. 2. Mutations in extranodal DLBCL according to site. Mutations in MYD88, CD79 A/B, and CARD11 in primary extranodal DLBCL derived from different anatomical locations. GI = gastrointestinal; MYD/CD = Mutations in both MYD88 and CD79; CD/CARD = mutations in both CD79 and CARD11. Only testicular lymphomas carry mutations in both MYD88 and CD79 in this cohort.

DLBCL is genetically less well defined, but mutations in CARD11 have been found in this subgroup, too [3]. Since GEP is not a practical method in routine clinical diagnostics various IHC classifiers have been developed to create a tool for DLBCL subtyping that is easily accessible to pathologists. Different algorithms have been proposed based on the sequential interpretation of well established antigens. However, these IHC algorithms have been the subject of much controversy regarding their specificity and reliability. This study used the Visco-Young [12] algorithm to subtype 147 nodal and primary extranodal DLBCL. In the original article establishing the Visco-Young algorithm no distinction was made between primary nodal and primary extranodal lymphomas. While differential statistics was done for lymphomas with extranodal involment (0–1 versus 2 and more extranodal sites) this seemed to be used as a marker for advanced disease and it remains unclear whether any primary extranodal lymphomas were included. If the Visco-Young test reliably characterizes the subtypes according to their biological differences, mutations in NF-kB activating genes like MYD88 and CD79 should be found in ABC DLBCL and not, or only very rarely, in GCB DLBCL. This distribution of mutations was found in nodal DLBCL with only one MYD88 mutation detected in 28 cases of GCB type lymphoma. In extranodal DLBCL, however, the pattern was not as obvious. Out of 24 GCB DLBCL 4 (16.7%) carried mutations in either MYD88, CD79, or even in both and 3 (12.5%) of those cases carried the MYD88 L265 P Mutation. Compared to the literature (and compared to the nodal cases evaluated here) this number seems high. Up to 30% of ABC DLBCL have been reported to show MYD88 mutations, but studies relying on GEP for DLBCL subtyping have only rarely detected this mutation in GCB DLBCL (less than 3%, 5, 8). When IHC classifiers were used, however, up to 10% of GCB samples carried MYD88 mutations [20], fitting well with the data presented here (7.7% of all GCB samples). The picture concerning CD79 is even more striking. Mutations are detected exclusively, or nearly exclusively, in ABC DLBCL [6,8,19,20]. Compared to these data the 2 (8%) CD79 mutations in 23 cases of extranodal GCB type lymphomas found in this study are rather surprising.

Subtyping of DLBCL is supposed to reflect biological differences between ABC and GCB type lymphomas. ABC DLBCL are characterized by activation of NF-kB [4]. As a biological consequence of this the differentiation of the malignant cell is driven toward the plasma cell stage, but full plasmacytic differentiation is blocked [21], rev. in [22]). One way activation of NF-kB can be achieved is via activating mutations in the B-cell receptor (BCR) pathway upstream of NF-kB, namely in CD79 or CARD11. Another pathway leading to NF-kB starts with Toll-like receptors and uses MYD88 as a signal transmitter. Generally, activated NF-kB is not involved in GCB DLBCL [4], although CARD11 mutations have been found in both subtypes in significant numbers [3]. This makes the relatively high percentage of MYD88 or CD79 mutated cases in primary extranodal GCB DLBCL rather conspicuous. The most peculiar case is certainly one case of GCB lymphoma carrying a mutation both in MYD88 and in CD79. These double mutations have so far been considered a feature of ABC DLBCL only, creating doubt about the validity of the Visco-Young IHC algorithm used in this study when applied to primary extranodal DLBCL.

Furthermore, extranodal lymphomas do not look like a homogenous group of lymphomas regarding their mutation pattern. The most clearly different group evaluated here seem to be testicular lymphomas with more than half of them carrying mutations in both MYD88 and CD79 and two others showing MYD88 or CD79 mutations, respectively, alone. A high prevalence of MYD88 mutations in testicular B-cell lymphomas has been reported before [19,23–26], often combined with MYD88 mutations in 25%–75% of cases [19,24,24,25,26]. A similar picture was seen in other immune privileged sites like the CNS [19]. Other extranodal lymphomas, however, did not show any combined mutations of CD79 and MYD88. Testicular lymphomas further seem to lack mutations in CARD11 that were otherwise seen at all other extranodal sites, also seen by Chapuy et al. [26]. Gastrointestinal lymphomas, however, did carry CD79 mutations in a relatively high percentage of cases (15.6%) in contrast to other extranodal lymphomas. These results confirmed the data of Kraan et al, [19] but are contrasting with the study of Frick et al. [25] who reported a low frequency of CD79 mutations. Since the differences did not reach statistical significance in either study this discrepancy must be put down to normal variations in the populations tested and overall variability.

5. Conclusion

While immunohistochemistry algorithms show high concordance with GEP data concerning the differentiation of ABC and GCB type lymphomas, a high statistical correlation may not be enough to base a specific patients therapy decisions on. To be applicable to clinical decision making an algorithm needs to differentiate the different biological entities every time. Immunohistochemical algorithms, specifically the Visco-Young algorithm, seem to produce inconsistencies when applied to primary extra nodal DLBCL. Overall the distribution of mutations in extranodal lymphomas seems to indicate that the extranodal site itself and not the GCB/ABC differentiation determines the molecular basis and biology of the lymphomas in question. However, the overall number of cases investigated is too small for a definite, statistically validated answer to this question. Confirmation of the results on a larger, independent cohort is needed to verify the findings presented here.

Declaration of interest

Cora Hallas: None.
Michael Preukschas: None.
Markus Tiemann: None.

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We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

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