Activating Mutations Affecting the NF-kappa B Pathway and *EZH2*-mediated Epigenetic Regulation are Rare Events in Primary Mediastinal Large B-cell Lymphoma

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Abstract. *Background: Primary mediastinal large B-cell lymphoma (PMBL) is a distinct subtype of diffuse large B-cell lymphoma (DLBCL) frequently observed in young patients. High-dose immunochemotherapy constitutes the current therapeutic gold-standard, despite significant toxicity and serious late effects. Several hotspots harboring oncogenic gain-of-function mutations were recently shown to pose vital hallmarks in activated B-cell like (ABC-) (CD79B, CARD11 and MYD88) and germinal center like (GCB-) DLBCL (EZH2), respectively. Several promising targeted-therapy approaches, derived from these findings, are currently under development. Materials and Methods: We thoroughly characterized a cohort of 25 untreated patients with de novo PMBL by immunohistochemical and cytogenetic means and assessed the prevalence of activating mutations affecting EZH2, CD79B and CARD11 utilizing a polymerase chain reaction (PCR)-based capillary sequencing approach. Moreover, the MYD88 p. L265P status was assessed by employing a pyrosequencing approach. Results: PMBLs included in this study did not harbor any of the reported hotspot mutations activating the nuclear factor (NF)-kappa B signaling cascade or the EZH2-mediated epigenetic deregulation of gene expression. Immunohistochemical characterization revealed an ABC phenotype in 44% (n=11) of cases. Conclusion: We report that genetic alterations of these genes are rare events in PMBL unlike other subtypes of DLBCL. Our findings suggest that a substantial subset of PMBL patients may benefit from treatment approaches targeting BCR-mediated activation of NF-kappa B.*

Primary mediastinal large B-cell lymphoma (PMBL) constitutes a distinct subtype of diffuse large B-cell lymphoma (DLBCL) accounting for approximately 10% of DLBCL cases with thymic origin (1-3). Its clinical course is aggressive and most patients present at a young age a bulky mediastinal mass. There appears to be a female predominance. Prominent pleural and pericardial effusions are common features (4). Extranodal involvement is common, yet extramediastinal tumor formation should also suggest reconsideration of PMBL diagnosis (5).

From both clinical and pathobiological perspectives, numerous aspects of PMBL closely resemble classical Hodgkin's lymphoma of nodular sclerosing type (6, 7). Moreover, these two conditions share the thymic B-cell as a putative cell of origin (3). The cytogenetic and molecular properties of PMBL and its close biological association with other sub-entities within the spectrum of DLBCL, especially with regard to its strong dependence on nuclear factor (NF-) kappa signaling, have been studied to some extent (2, 3, 8). Reported molecular features of PMBL include mutations affecting B-cell lymphoma 6 (*BCL6*), frequently concurring with prominent somatic mutations in the immunoglobulin heavy-chain gene, indicating a late-stage germinal-center B-cell as cell of origin (9).

Despite the most recent advances in the development of less cytotoxic treatment approaches obviating the need for supplementary radiotherapy in addition to immuno-chemotherapy, all established treatment regimens require high-dose cytoreductive treatment. Dunleavy *et al*. proposed a therapeutic concept of dose-adjusted etoposide, doxorubicin and cyclophosphamide with vincristine,

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Table I. *Primary antibodies, pre-treatments and dilutions for immunohistochemical investigations.*

Antibody	Manufacturer	Clone	Pre-treatment*	Dilution
BCL2	Lab Vision	100/D5	PC (pH 6.1)	1:100
BCL6	Lab Vision	GI191(EA8	MW (pH 6.1)	1:100
CD10	Menarini	56C6	ST (pH 9.0)	1:30
MUM-1 (Irf4)	DAKO	Mum 1P	MW (pH 6.1)	1:400
LMO2	Cell Marque	SP51	PC (pH 6.0)	1:2000
FOXP1	Lab Vision	polyclonal	PC (pH 6.0)	1:100
MIB-1	DAKO	MIB-1	PC (pH 6.0)	1:500

Antibodies and preparations. Immunohistochemical studies were performed on formalin-fixed paraffin-embedded (FFPE) sections according to a standard three-step immunoperoxidase technique using the automated TechMate system and the BrightVision Kit according to the manufacturer's instructions. *Pre-treatment with PC=pressure cooker, ST=steamer, MW=microwave.

prednisone, and rituximab (DA-EPOCH-R) (10). An impressive outcome was achieved with overall survival rates of 97-100% and event-free survival of 93-100% in a five-year follow-up. Although several doubts regarding these highly promising results and their superiority to cyclophosphamide, doxorubicin, vincristine and prednisone combined with rituximab (R-CHOP) were subsequently raised, DA-EPOCH-R has emerged as a promising approach for the consolidation of PMBL (11, 12). In the light of young median patient age at diagnosis and thus, the time span for serious late effects to occur following high dose treatment with DA-EPOCH-R, such an approach has been discussed controversially for its predicted cardiotoxic effects as well as subsequent impairment of fertility (11).

These conditions strongly highlight the need for an increased effort in delivering more individualized treatment strategies. Over the past decade, investigations regarding molecular mechanisms causing inferior prognosis and higher risk for treatment, failure and/or early recurrence in activated B-cell type (ABC) compared to germinal center like (GCB) DLBCL have been uncovered, such as multiple mutational hotspots that promote B-cell receptor (BCR) and/or Toll-like receptor (TLR)-mediated activation of NF-kappa B signaling. Ngo *et al*. were the first to describe an oncogenic mutation (most commonly p. L265P) in *MYD88*, encoding an adaptor protein in the TLR signaling cascade in approximately 30% of ABC but not GCB DLBCL (13). Similar oncogenic driver mutations have subsequently been described in the B-cell receptor (BCR)-mediated axis of NF-kappa B activation. Most recurrently affected targets were the ITAM domain within *CD79B* as well as the coiled-coil domain spanning exons 4 through 9 of *CARD 11* (14, 15).

Gene expression signature (GES) experiments found PMBL to be derived from late-stage germinal center B-cells sharing significant features with ABC DLBCL such as strong activation of NF-kappa B signaling (2). Thus, modern targeted-treatment approaches, *e.g*. ibrutinib, an oral inhibitor of Bruton's tyrosine kinase (BTK), may potentially be applied (15, 16). The efficiency of ibrutinib treatment is closely associated with the mutational profiles of *CD79B, CARD11* and *MYD88*. A central aim of our study was therefore to assess the prevalence of oncogenic mutations affecting these hotspots.

Additionally, activating mutations in the SET domain of *EZH2* are becoming a therapeutic target (17). Thus, we further approached the role of these novel developments in the context of PMBL treatment.

Materials and Methods

Patients. Formalin-fixed and paraffin-embedded (FFPE) tissue biopsy samples from 25 patients with primary mediastinal large B-cell lymphoma were retrieved from the registry of the Reference Center for Lymph Node Pathology and Hematopathology, University Hospital of Schleswig-Holstein, Campus Luebeck.

All samples, including controls, 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. All cases were reassessed for independent pathology review by two experienced Haematopathologists without knowledge of the mutation status.

Tissue microarray construction. Two representative tissue cores (1.5 mm) per sample were dissected and mounted onto a tissue microarray employing the Unitma QuickRay® kit (Seoul, Korea) according to manufacturer's instructions.

Immunohistochemistry. Immunohistochemical studies (Table I) were performed on 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).

Fluorescence in situ hybridization (FISH) for c-MYC, 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*) (Abbott Vysis, Des Plaines, IL, USA) according to the manufacturer's instructions.

PCR-based capillary sequencing of CD79B, CARD11 and EZH2 hotspots. Genomic DNA was obtained from FFPE specimen using the

Table II. *Clinical, immunohistochemical and cytogenetic features of the study group.*

Case	Gender	Age	MIB1*	CD10[1]	BCL2[2]	BCL6[3]	MUM1[4]	FOXP1[5]	Hans**	FISH		
										BCL2	*BCL6*	*cMYC*
1	m	43	50%	+	+	+	–	–	GCB	–	–	–
2	m	24	50%	+	+	+	–	–	GCB	–	+	–
3	f	24	60%	–	–	+	–	–	GCB	–	–	–
4	f	28	70%	–	+	+	–	–	GCB	–	–	–
5	m	49	40%	–	+	–	–	–	nGCB	–	–	–
6	m	18	70%	–	+	+	–	–	GCB	–	–	–
7	m	22	50%	–	–	–	–	–	nGCB	–	–	–
8	f	24	80%	–	+	–	–	–	nGCB	–	–	–
9	f	57	20%	–	+	–	–	–	nGCB	–	–	–
10	f	46	80%	–	+	+	+	–	nGCB	–	–	–
11	f	35	85%	–	+	+	–	–	GCB	–	–	–
12	f	42	75%	–	+	–	–	–	nGCB	–	–	–
13	f	48	40%	–	+	+	–	–	GCB	–	–	–
14	m	80	90%	–	+	+	+	–	nGCB	–	–	–
15	f	32	50%	–	+	+	–	–	GCB	–	–	–
16	f	62	30%	–	+	–	–	–	nGCB	–	–	–
17	f	25	60%	–	+	+	–	–	GCB	–	–	NA
18	m	48	60%	+	+	+	–	–	GCB	+	–	–
19	m	43	65%	–	–	+	–	–	GCB	–	–	–
20	f	29	50%	–	–	+	–	–	GCB	–	+	–
21	m	20	40%	–	+	+	+	–	nGCB	–	–	NA
22	m	59	70%	–	–	+	–	–	GCB	–	–	–
23	f	49	30%	–	+	–	–	–	nGCB	–	–	–
24	f	21	70%	–	–	+	–	–	GCB	–	–	–
25	f	62	30%	–	+	–	–	–	nGCB	–	–	–

*Percentage of MIB1 (Ki-67)-positive stained cells. **Classification according to the immunohistochemical algorithm proposed by Hans *et al*. (19). [1]CD10 expression – cut-off: 30% positive stained cells. [2]BCL2 expression – cut-off: 50% positive-stained cells. [3]BCL6 expression – cut-off: 30% positive stained cells. [4]MUM1 expression – cut-off: 30% positive stained cells. [5]FOXP1 expression – cut-off: 80% positive stained cells. NA, Not available. M: Male. F: Female.

QiaAmp mini kit 250 (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Following DNA extraction, the PCR based amplification of mutational hotspots in *CD79B* (exons 5, 6), *CARD11* (exons 4-9) and *EZH2* (exons 16, 18) was performed. Successful amplification was verified by gel electrophoresis and subsequently bidirectional Sanger sequencing was performed employing a CEQ 8800 platform (Beckman & Coulter, Pasadena, CA, USA).

Known single-nucleotide polymorphisms, as well as silent mutations were excluded from further analysis.

Myd88 p. L265P mutation analysis and quantification of allele burden. Initially, a short biotinylated PCR product covering the mutation site was amplified. In a second step, pyrosequencing was performed using streptavidin-coated sepharose® beads for single strand preparation and a biotin-tagged sequencing primer, as previously described (18). Allele frequency was quantified using the PyroMark Software (Qiagen, Hilden, Germany).

Results

Clinical, histopathological and cytogenetic features of the study group. All patients presented with bulky mediastinal disease histopathologically consistent with the diagnosis of PMBL. Staging revealed no significant extramediastinal tumor formation. Mean patient age at presentation was 39.6 (range=18-80) years with a male:female ratio of 1:1.5. Immunohistochemical staining for CD10, BCL6 and MUM1, according to the classification algorithm proposed by Hans *et al*., revealed 14 (56%) cases of PMBL to be of germinal center origin, whereas 11 (44%) cases grouped within the non-germinal center entity (19). FISH analysis revealed low frequency of aberrations affecting the BCL2 (n=1), BCL6 (n=2) and cMYC (n=0) loci.

Clinical, immunohistochemical and cytogenetic features of the study group are summarized in Table II.

Prevalence of oncogenic mutations affecting MYD88, CD79B, CARD11 and EZH2. Conventional capillary sequencing of *CD79B, CARD11* and *EZH2* mutational hotspots revealed wild type sequences in all investigated amplicons. No mutations affecting codon 265 of *MYD88*, by means of pyrosequencing, were detected.

Discussion

PMBL constitutes a high-grade B-cell malignancy that predominantly occurs in young adults for whom combination immunochemotherapy currently poses the gold-standard of treatment. Although a vast majority of patients are cured by current therapeutic regimens, patients are subjected to high doses of cytotoxic agents.

Therefore, the next major goal has to be the reduction of toxicity administered to patients with regard to the expectedly long remaining lifespan and the significant risk for long term effects following therapeutic concepts such as DA-EPOCH-R or R-CHOP.

BCR signaling constitutes a complex process of multiple interconnected pathways ultimately resulting in transcriptional and translational control of gene expression in B-cells (20). In the light of increasingly sophisticated means of therapeutic knockdown of specific cellular effectors, we sought to assess the frequency of hotspot mutations indicating applicability of novel therapeutic agents including the BTK inhibitor ibrutinib (21).

PMBL molecular characteristics display a significant overlap with ABC DLBCL with both entities strongly exhibiting BCR-mediated activation of NF-kappa B signaling (22). A concurrent therapeutic targetability might be deduced from these observations. Moreover, BTK knockdown appears to exhibit a high degree of cytotoxicity in DLBCL. The absence of oncogenic mutations affecting the coiled-coil domain (exons 4-9) in *CARD11*, an effector of BCR signaling downstream of BTK-constitutes a vital criterion for efficiency of BTK inhibitive therapy (14, 16). We reason that our current observations regarding the lack of *CARD11* mutations in the pathogenesis of PMBL may predispose PMBL patients for BTK inhibitive treatment. Despite the interesting absence of *CD79B* mutations identified in our current study we reason that, most likely due to its vantage point downstream of *CD79*, ibrutinib might still prove effective in PMBL much as it did in ABC DLBCL regardless of the *CD79* mutation status (14, 15).

With the absence of mutations affecting *CD79B* and *CARD11*, genetic aberrations of non-canonical NF-kappa B-associated genes or epigenetic deregulation affecting relevant genes might be implicated in the pathogenesis of PMBL.

Altered TLR signaling offers a promising target for pharmacological intervention as both *in vitro* as well as in vivo inhibitors of TLR downstream effector IRAK-4 exhibited a significant synergy with the Bruton's tyrosine kinase (BTK) inhibitor ibrutinib. Similar synergisms were described for combination treatment with ibrutinib and lenalidomide and/or the proteasome inhibitor bortezomib (15, 16, 23). In keeping with observations by Ngo *et al*., who investigated a small cohort of PMBL cases in the context of their initial identification of *MYD88* mutations in high-grade B-cell neoplasms, we did not detect oncogenic mutations affecting *MYD88* in PMBL. Unlike promising treatment approaches with lenalidomide and/or bortezomib, inhibitive targeting of IRAK-4 may therefore not represent a viable option in PMBL therapy.

Taking into account the strong association of PMBL and late germinal center B-cells we assumed that recent discoveries regarding the transcriptional repressor EZH2 and its high frequency of harboring activating mutations might also apply to PMBL.

Based on *in vitro* studies revealing *EZH2* inhibition to result in decreased proliferative activity and induction of cell-cycle arrest in DLBCL and the development of an oral EZH2 histone methyltransferase inhibitor for relapsed DLBCL, we investigated the *EZH2* mutation frequency (17, 24, 25).

However, our observations may not fully support therapeutic targeting of *EZH2* as a viable option in PMBL.

With regard to the well-established role of NF-kappa B signaling in PMBL, future approaches in developing more targeted and less toxic treatment strategies should center on the latter. Since *CARD11* mutations are unlikely to affect BCR signaling downstream BTK and do not represent a significant factor in PMBL pathogenesis, ibrutinib may constitute a promising substance. Moreover, downstream effectors of NF-kappa B signaling, including proteasome inhibitors (*e.g.* bortezomib) and modulators of immune response (*e.g.* lenalidomide), will also have to be considered.

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