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ORIGINAL ARTICLE

High prevalence of oncogenic *MYD88* and *CD79B* mutations in diffuse large B-cell lymphomas presenting at immune-privileged sites

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Activating mutations in *CD79* and *MYD88* have recently been found in a subset of diffuse large B-cell lymphoma (DLBCL), identifying B-cell receptor and MYD88 signalling as potential therapeutic targets for personalized treatment. Here, we report the prevalence of *CD79B* and *MYD88* mutations and their relation to established clinical, phenotypic and molecular parameters in a large panel of DLBCLs. We show that these mutations often coexist and demonstrate that their presence is almost mutually exclusive with translocations of *BCL2*, *BCL6* and *cMYC*, or Epstein–Bar virus infection. Intriguingly, *MYD88* mutations were by far most prevalent in immune-privileged site-associated DLBCL (IP-DLBCL), presenting in central nervous system (75%) or testis (71%) and relatively uncommon in nodal (17%) and gastrointestinal tract lymphomas (11%). Our results suggest that *MYD88* and *CD79B* mutations are important drivers of IP-DLBCLs and endow lymphoma-initiating cells with tissue-specific homing properties or a growth advantage in these barrier-protected tissues.

INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is a heterogeneous diagnostic class of lymphomas comprising molecularly distinct subtypes that differ in gene expression profile, oncogenic aberrations, clinical presentation and outcome.[1,2] A current gene expression profile-based molecular classification of DLBCL distinguishes two main subtypes: activated B-cell-like (ABC) lymphoma and germinal centre B-cell-like lymphoma. The antiapoptotic nuclear factor-kB signalling pathway is constitutively active in ABC-type DLBCLs as a result of oncogenic *CARD11* mutations and/or of *CD79* mutations causing chronic active B-cell receptor (BCR) signalling enhanced by inactivation of A20.[3–6] In addition, somatically acquired mutations in *MYD88*, an adaptor protein that mediates toll-like receptor and interleukin-1 receptor signalling were recently shown to control cell survival in this lymphoma type by promoting NF-κB and Janus Kinase (JAK)/Signal transducer and activator of transcription 3 (STAT3) signalling. These mutations were present in approximately one-third of ABC DLBCLs. The *MYD88* L265P was biologically the most potent mutant and was unique in its ability to organize a stable signalling complex containing phosphorylated IRAK1, which was suggested to account for its high prevalence in lymphomas.[7] From a therapeutic perspective, the above findings are of great interest as they not only identify BCR and MYD88 signalling as potential therapeutic targets but also provide a genetic tool to identify patients that may benefit from personalized treatment targeting these pathways.

These notions prompted us to explore the prevalence of *CD79B* and *MYD88* mutations and their relation to established clinical, phenotypic and molecular parameters in a large panel of DLBCL patients.

MATERIALS AND METHODS

Patient samples

The study comprises a panel of 177 DLBCLs diagnosed according to the WHO (World Health Organization) classification. All tissue samples were obtained during standard diagnostic procedures at the Academic Medical Center Amsterdam, The Netherlands and affiliated hospitals, and the University Medical Center Groningen, The Netherlands in accordance with the local institutional board requirements and the Declaration of Helsinki.

Immunohistochemical studies and fluorescence *in situ* hybridization

Immunohistochemical stainings were performed on formalin-fixed paraffin-embedded sections with the following antibodies: CD10 (Thermo Fisher Scientific, Rockford, IL, USA clone 56C6), MUM1 (clone MUM1p, DAKO), BCL2 (clone 124, DAKO, Glostrup, Denmark), BCL6 (clone PG-B6p, DAKO) using a Labvision Autostainer 480S (Thermo Fisher Scientific).

Expression of the Epstein–Barr virus (EBV) in tumour was determined by EBV-encoded RNA *in situ* hybridization probes (Biogenex, Fremont, CA, USA).

Split-fluorescence *in situ* hybridization for *BCL2*, *BCL6* and *cMYC* was performed using probes and a fluorescence *in situ* hybridization accessory kit according to the manufacturer's recommendations (DAKO).

Mutation analysis

DNA was isolated with the QIAamp DNA Micro kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. Screening

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for *MYD88* and *CD79B* mutations was performed with allele-specific PCR assays, employing primers that were designed (Table 1) to specifically anneal with their 3′-terminal nucleotide to either the mutated or wild-type base. This technique allows reproducible detection of as little as 1% tumour DNA diluted in wild-type DNA (Supplementary Figure 1). To avoid aspecific binding of the primer pairs designed for the mutated to the wild-type allele, and *vice versa*, every set was tested at temperatures ranging from 60 to 64 °C and with different concentrations of MgCl₂. To avoid underdetection of mutations in samples with low tumour load, 100 ng of DNA was used in each PCR reaction to obtain sufficient DNA originating from the tumour to detect a potential mutation. In all samples in which a PCR product was obtained using the mutation-specific primers, the region surrounding the mutation was analysed by Sanger sequencing to verify the presence of a mutation. For cases with low tumour load, PCR products were cloned before sequencing.

RESULTS

The lymphomas included in this study were diagnosed as DLBCL according to the WHO criteria. As part of the routine diagnostic workup, all cases were subclassified as either ABC- or GC-like by employing the immunohistochemical algorithm of Hans *et al.*[8]

In addition, a systematic molecular characterization consisting of *cMYC*, *BCL2* and *BCL6* translocation analysis employing fluorescence *in situ* hybridization, and assessment of EBV status by EBV-encoded RNA *in situ* hybridization, were routinely performed. To detect somatic mutations in *MYD88* or *CD79B*, we designed a panel of allele-specific PCRs covering all major mutation (hot)spots previously reported[6,7] (Table 1). This strategy permits efficient and sensitive detection of mutations using DNA extracted from paraffin-embedded tissue, even in samples with relatively low tumour load (Supplementary Figure 1). The detected mutations were verified by Sanger sequencing.

Of the total group of 177 DLBCL, 43 (24.3%) were found to contain a *MYD88* mutation (Figure 1a). In accordance with the study of Ngo *et al.*,[7] these mutations were predominantly present in ABC-type DLBCL (Figure 1b), occurring in 32.5% of ABC DLBCLs (Figure 1b) as opposed to only 9.5% of GBC DLBCL, and the L265P mutation was by far the most common mutation. Similarly, *CD79B* mutations were also predominantly found in ABC DLBCL (12.2% in ABC DLBCL vs 0% in germinal centre B-cell-like DLBCL); in more than half of these tumours, a coexisting *MYD88* mutation was found. Molecular correlation revealed that the presence of *MYD88*

Mutation	Number	Sequence	Primer pairs	Length	Temperature	MgCl₂ concentration
Table 1. Primer sequences and PCR conditions					*Optimal conditions*	
MYD88						
L265	WTª	278F	5′-GTGCCCATCAGAAGCGACT-3′	172 bp	64 °C	1.0 mM
		277R	5′-GGGCCTCAGAACAGTCTTCA-3′			
	Mutᵇ	279F	5′-TGCCAGGGGTACTTAGATGG-3′	152 bp		
		276R	5′-CCTTGTACTTGATGGGGATCG-3′			
	Seqᶜ	279F	5′-TGCCAGGGGTACTTAGATGG-3′	285 bp		
		277R	5′-GGGCCTCAGAACAGTCTTCA-3′			
S219	WT	286F	5′-CTGGCACCTGTGTCTGGTC-3′	184 bp	61 °C	1.5 mM
		285R	5′-ATCCCCTGGGAATAGCTTCA-3′			
	Mut	284F	5′-ATATGCCTGAGCGTTTCGAT-3′	172 bp		
		283R	5′-TCGATGAGCTCACTAGCAATAC-3′			
	Seq	284F	5′-ATATGCCTGAGCGTTTCGAT-3′	316 bp		
		285R	5′-ATCCCCTGGGAATAGCTTCA-3′			
S243	WT	282F	5′-GGCATCTCCTCCTAGCTGTG-3′	188 bp	64 °C	1.0 mM
		281R	5′-GTCTGGAAGTCACATTCCTTGC-3′			
	Mut	282F	5′-GGCATCTCCTCCTAGCTGTG-3′	189 bp		
		280R	5′-GGTCTGGAAGTCACATTCCTTGT-3′			
	Seq	282F	5′-GGCATCTCCTCCTAGCTGTG-3′	285 bp		
		291R	5′-GGAAGGCTGACAATCCAGAG-3′			
V217	WT	287F	5′-ATATGCCTGAGCGTTTCGAT-3′	167 bp	62 °C	1.5 mM
		288R	5′-GAGCTCACTAGCAATAGACCAGAC-3′			
	Mut	287F	5′-ATATGCCTGAGCGTTTCGAT-3′	167 bp		
		290R	5′-GAGCTCACTAGCAATAGACCAGAA-3′			
	Seq	287F	5′-ATATGCCTGAGCGTTTCGAT-3′	265 bp		
		289R	5′-GCACAGCTAGGAGGAGATGC-3′			
CD79B						
Y196	WT	292F	5′-CATGGAGGAAGATCACACCT-3′	183 bp		
		293R	5′-GCAGCGTCACTATGTCCTCA-3′			
	Y196C	296F	5′-ATGGAGGAAGATCACACCTG-3′	182 bp	63 °C	1.0 mM
		293R	5′-GCAGCGTCACTATGTCCTCA-3′			
	Y196D	297F	5′-TCTTGCAGAATGCACCTCAC-3′	178 bp	63 °C	1.0 mM
		298R	5′-CCCCTCTCCTTACCTCGTC-3′			
	Y196F	299F	5′-CATGGAGGAAGATCACACCTT-3′	183 bp	64 °C	1.5 mM
		293R	5′-GCAGCGTCACTATGTCCTCA-3′			
	Y196H	297F	5′-TCTTGCAGAATGCACCTCAC-3′	176 bp	64 °C	1.0 mM
		294R	5′-CCCCTCTCCTTACCTCGTG-3′			
	Y196N	295F	5′-CATGGAGGAAGATCACACCA-3′	183 bp	61 °C	1.5 mM
		293R	5′-GCAGCGTCACTATGTCCTCA-3′			
	seq	297F	5′-TCTTGCAGAATGCACCTCAC-3′	323 bp		
		293R	5′-GCAGCGTCACTATGTCCTCA-3′			

Abbreviations: Mut, mutated; Seq, sequencing; WT, wild-type. ªPrimer pair recognizing the WT allele. ᵇPrimer pair recognizing Mut allele. ᶜPrimer pair used for Seq.
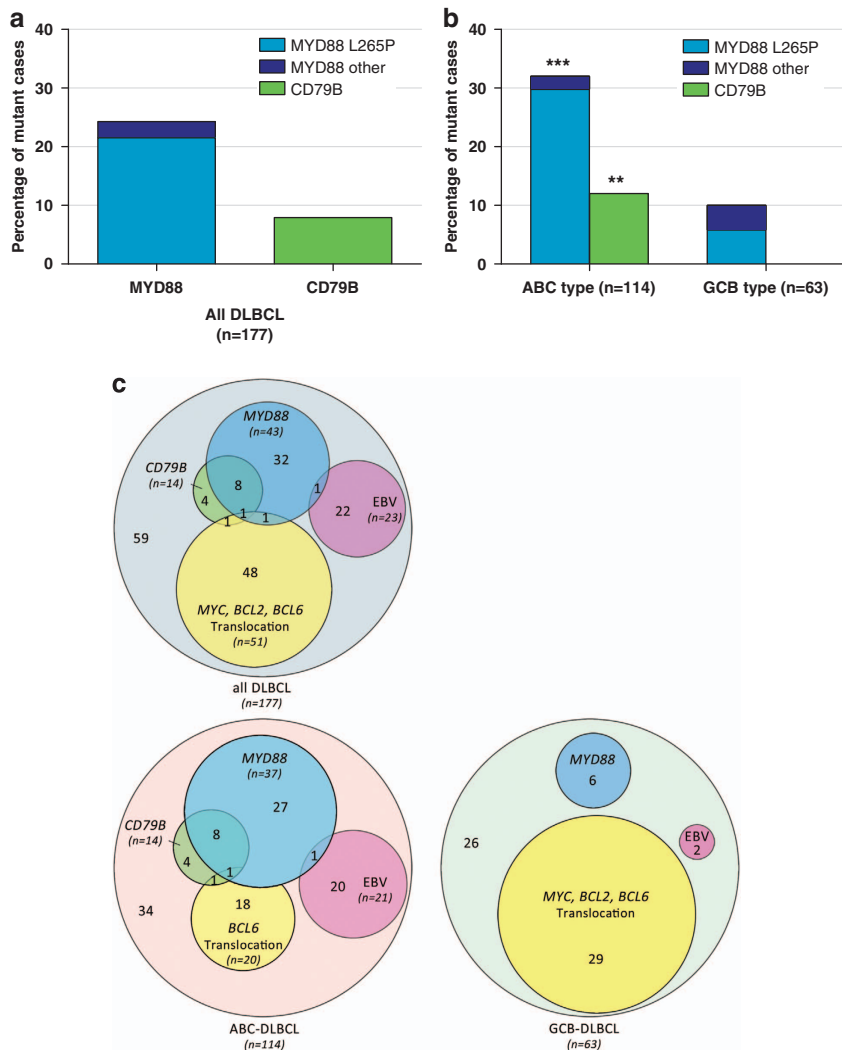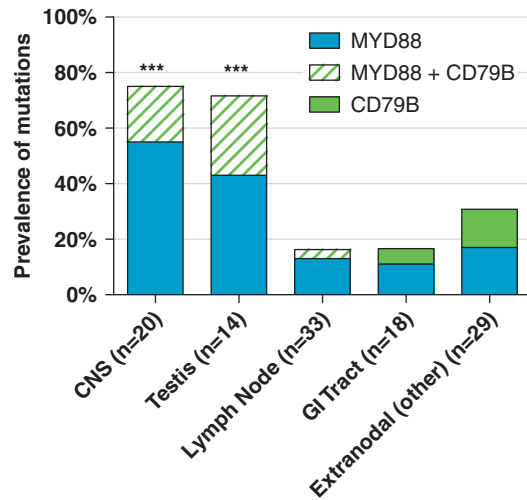
Figure 1. Prevalence of *MYD88* and *CD79B* mutations in DLBCL. (**a**) Percentage of DLBCL with *MYD88* and/or *CD79B* mutations in all DLBCLs tested ($n = 177$). (**b**) Percentage of tumours with *MYD88* and/or *CD79B* mutations in ABC-type and germinal centre B-cell (GCB)-type DLBCL. Prevalence of *MYD88* and *CD79* mutations in ABC DLBCL was significantly different from that in GC DLBCL (**$P < 0.01$; ***$P < 0.001$ by Fisher's exact test). (**c**) Overlap of *MYD88* and *CD79B* mutations with other recurrent oncogenic events in DLBCL.

and/or *CD79B* mutations showed hardly any overlap with the occurrence of *cMYC* and *BCL2* translocations; as expected, these translocations were largely restricted to the germinal centre B-cell-like DLBCL subgroup (Figure 1c). Further, EBV infection, which activates nuclear factor-kB by signals through latent membrane protein-1 and -2, instigating an ABC phenotype, and translocation of *BCL6*, which is present in a subgroup of ABC DLBCLs, also hardly overlapped with the occurrence of *MYD88* and *CD79B* mutations (Figure 1c). Taken together, these observations imply that DLBCL with mutations in *MYD88* represent a separate subgroup of DLBCLs with a distinct molecular pathogenesis. Consistent with this notion, our study reveals a salient site-specific variation in the prevalence of *MYD88* mutations: they were relatively uncommon in ABC DLBCLs arising in lymph nodes (17%) or gut (11%), whereas tumours arising outside these 'professional' lymphoid tissues frequently contained these mutations, either with or without a coexisting *CD79B* mutation. Interestingly, these *MYD88* mutations were by far most prevalent in primary central nervous system (75%) and testicular lymphomas (71%), which represent the immune-privileged site-associated DLBCLs (IP-DLBCLs; Figure 2).

DISCUSSION

Our study confirms the previous finding that *MYD88* and *CD79B* mutations are predominantly present in ABC-type DLBCL and often coexist within the same tumour.[7] Moreover, it reveals that these mutations only rarely coexist with translocations of *BCL2*, *BCL6* and *cMYC* or with EBV infection, and that they intriguingly are strongly over-represented in lymphomas presenting outside 'professional' lymphoid tissues, particularly in so-called IP-DLBCL arising in the CNS and testis.

IP-DLBCLs have previously been shown to share a number of distinctive clinical and biological features. Besides having a relatively poor prognosis with preferential dissemination to other immune-privileged sites,[9–11] these tumours are predominantly of the ABC subtype[12,13] and show prominent loss of human leukocyte antigen class I and II expression, often caused by small interstitial deletions of chromosome 6p21.3.[14,15] This is associated with the downregulation of many immune-associated genes.[12] Our current finding that most IP-DLBCLs harbour a *MYD88* mutation, while these mutations are uncommon in the nodal and gastrointestinal tract DLBCLs, supports the concept that IP-DLBCLs present a pathogenetically distinct group of lymphomas.

Figure 2. Prevalence of mutations in *MYD88* and *CD79B* by tumour localization. Percentage of ABC DLBCL with *MYD88* and/or *CD79B* mutations at different anatomical sites. Prevalence of *MYD88* mutations in central nervous system (CNS) and testis was significantly different from that in the lymph node and gastrointestinal (GI) tract (***$P < 0.001$ by Fisher's exact test).

Consistent with this notion, two other studies have recently also reported a high frequency of *MYD88* mutations in primary central nervous systems.[16,17] Conceivably, mutational activation of toll-like receptor/MYD88 signalling endows lymphoma-initiating cells with a selective growth advantage at immune-privileged sites. These tissues are barrier-protected and immunologically silent, and, in marked contrast to lymph nodes and mucosa-associated lymphoid tissues, will presumably provide only limited stimulation by toll-like receptor ligands. The (concomitant) presence of *CD79B* (or other BCR pathway) mutations, causing chronic active BCR signalling, may further promote the selective outgrowth of the tumour cells within these relatively stimulus poor microenvironments. In view of the pivotal role of adhesion and chemokine receptors in tissue-specific lymphoma dissemination,[18,19] dysregulated 'homing' of lymphoma cells carrying oncogenic *MYD88* and/or *CD79* mutations could present an alternative mechanism underlying the observed site-specific differences in prevalence of these mutations in DLBCL.[18]

In conclusion, our results suggest that *MYD88* mutations, and to a lesser extent *CD79B* mutations, are important drivers of lymphoma-genesis in IP-DLBCLs. Hence, patients with these tumours may benefit from therapies targeting MYD88 signalling components like the IRAK4 kinase inhibitors, either alone or in combination with drugs blocking key mediators of BCR signalling such as Bruton's tyrosine kinase.[19,20] It will be of interest to explore whether or not patients with DLBCLs arising in lymph nodes and mucosa-associated lymphoid tissue and lacking these mutations, nevertheless, also show evidence of active MYD88 and/or BCR signalling. Such activation might be triggered by environmental ligands and be associated with non-oncogene addiction to these pathways.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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