Whole exome sequencing identifies mutational signatures of vitreoretinal lymphoma

Primary vitreoretinal lymphoma (VRL) is a rare intraocular malignancy that is closely related to primary central nervous system lymphoma (PCNSL). Most cases of VRL are comprised of high-grade diffuse large B-cell lymphoma (DLBCL), which is aggressive and has a poor prognosis.[1] Confirming the diagnosis of VRL is quite difficult. Cytopathologic examination, the standard diagnostic procedure, suffers from very low sensitivity due to the limited number of cells, preceding corticosteroid therapy, and the rapid degeneration of lymphoma cells.[1] The biological background of VRL is largely unknown, therefore, understanding its genomic landscape could be the first step in clarifying its pathogenesis, diagnosis, prognosis and treatment. We investigated the mutational profile of VRL using whole exome sequencing (WES) and evaluated the diagnostic value of WES in patients with suspected VRL.

Vitreous samples from nine patients with VRL (with six samples of matched blood), and from four patients with intermediate or posterior uveitis were obtained consecutively between June 2018 and April 2019 at the Severance Hospital. All vitreous samples were collected prospectively by *pars plana* vitrectomy before chemotherapy. The diagnosis of VRL was made comprehensively based on cytology of the vitreous or central nervous system (CNS) samples, laboratory results of the vitreous or CNS samples and vitreoretinal findings. This prospective study was approved by the Severance Hospital Institutional review board (4-2018-0389). It was conducted in accordance with the tenets of the Declaration of Helsinki. All samples on which WES was performed had also been examined using standard cytology, interleukin (IL)-6 and IL-10 assays, and clonality assays for immunoglobulin heavy chain (*IGH*) and kappa light chain (*IGK*). The WES was performed using the Twist Human Core Exome kit (Twist Bioscience, San Francisco, CA, USA) and NovaSeq system (Illumina, San Diego, CA, USA). Other experimental details are described in the *Online Supplementary Materials and Methods*.

All enrolled patients were human immunodeficiency viruses-negative and immunocompetent. The specific etiologies of uveitis included cytomegalovirus (CMV) retinitis (Control 1 and 3), acute retinal necrosis (Control 2), and sarcoidosis (Control 4). Detailed clinical information of the VRL patients is summarized in Table 1. In two patients (Case 8 and 9), PCNSL invaded into the vitreo-retina. In the remaining seven patients, lymphoma occurred in the vitreo-retina primarily without CNS involvement at the time of diagnosis. Three of the nine patients was diagnosed by cytopathologic examination. In all patients, the IL-10 to IL-6 ratios were greater than 1, and the absolute IL-10 levels were greater than 150 pg/mL. Monoclonal patterns were detected in the *IGH* or *IGK* clonality assay in eight patients.

Table 1. Demographics, clinical findings, and results of diagnostic vitrectomy in patients with vitreoretinal lymphoma.

No	Sex	Age (years)	Previous diagnosis of PCNSL	Germline-Somatic paired sample	Right/ Left eye	Vitreoretinal finding	Pathology (Vitreous)	IL-6 (pg/mL)	IL-10 (pg/mL)	Ig H clonality	Ig K clonality
1	F	53	No	(+)	Left	Vitreous opacity	Atypical large lymphoid cells, suspecting lymphoma	12.3	290.0	Monoclonal	Polyclonal
2	F	63	No	(+)	Right	Vitreous opacity	Atypical large lymphoid cells, suspecting lymphoma	128.6	2,606.9	Monoclonal	Monoclonal
3	M	66	No	(+)	Left	Vitreous opacity	Atypical large lymphoid cells, suspecting lymphoma	6.4	738.0	Inconclusive	Inconclusive
4	M	67	No	(+)	Right	Sub-RPE infiltration	Unsatisfactory	376.4	2,780.0	Monoclonal	Monoclonal
5	M	85	No	(+)	Left	Sub-RPE infiltration	Negative for malignancy	233.3	864.4	Inconclusive	Monoclonal
6	F	53	No	(-)	Right	Vitreous opacity	Unsatisfactory	14.6	521.9	Monoclonal	Monoclonal
7	M	77	No	(-)	Right	Vitreous opacity	Negative for malignancy	24.2	798.8	Polyclonal	Monoclonal
8	M	81	Yes	(+)	Right	Vitreous opacity	Unsatisfactory	72.5	251.2	Monoclonal	Inconclusive
9	F	48	Yes	(-)	Left	Vitreous opacity	Negative for malignancy	232.5	1,411.6	Polyclonal	Monoclonal

No: number; PCNSL: primary central nervous system lymphoma; IL: interleukin; Ig H: immunoglobulin heavy chain; Ig K: immunoglobulin kappa light chain; F: female; M: male; RPE: retinal pigment epithelium.

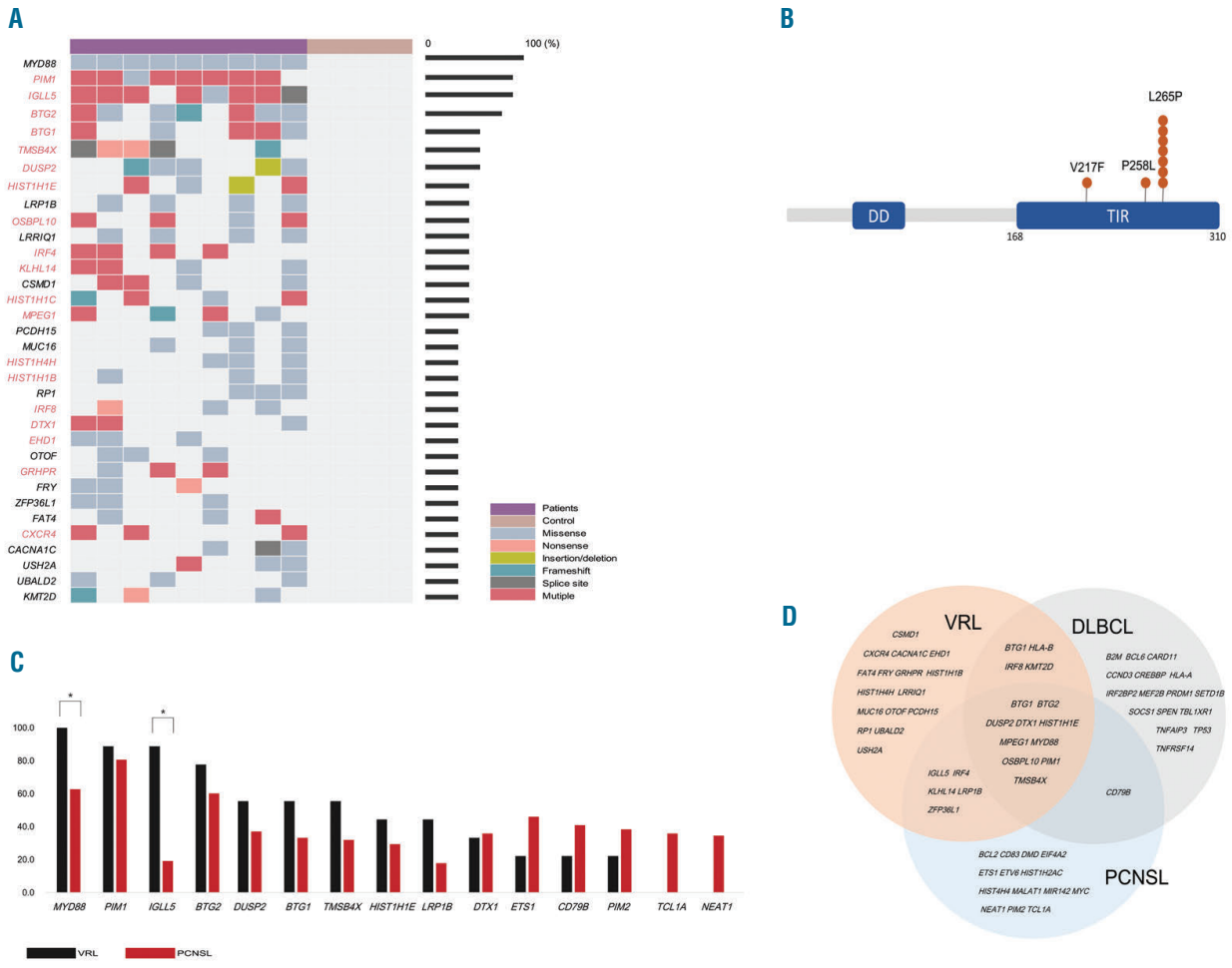

Figure 1. Analysis of whole exome sequencing data in patients with vitreoretinal lymphoma. (A) Recurrently mutated genes identified more than three patients. Columns represent patients (VRL: n=9; uveitis: n=4) and rows represent genes. Box color-coding indicates the type of mutation or genomic alteration. Known somatic hypermutation associated genes are displayed in red. The bar graph on the right represents the proportion of mutated cases in this study. (B) Somatic mutations in *MYD88*. The relative positions and case number of somatic mutations are shown in the predicted protein sequences of *MYD88*. All mutations were located in the Toll/interleukin-1 receptor homology (TIR) domain. (C) The percent of mutated cases in VRL (black bars) and in PCNSL cohorts from four different studies (red bars; data from ref. 2, 5-7). Asterisks indicate statistical significance. (D) Overlaps in recurrently mutated genes are detected in total DLBCL, PCNSL, and our VRL cohort. The Venn diagram depicts the top 30 frequently mutated genes from five other DLBCL or PCNSL studies (ref. 2, 5-7 and 12), and the present study. VRL: vitreoretinal lymphoma; DD: death domain; PCNSL: primary central nervous system lymphoma; DLBCL: diffuse large B-cell lymphoma.

The overall frequency of the identified somatic mutations are depicted in Figure 1A. Mutations in the *MYD88* gene were detected in all nine patients. The known somatic hypermutation (SHM) associated genes, including *PIM1* and *IGLL5*, were also frequently mutated.[2] In contrast, genes that are known to be frequently mutated in PCNSL, such as *CD79B*, were not frequently mutated in our cohort. In *MYD88* gene, the L265P mutation was detected in seven patients (7 of 9, 77.8%; see the *Online Supplementary Figure S1*) and P258L and V217F were found in the other two patients (Figure 1B), with a variant allele fraction ranging from 0.28 to 0.89. The L265P mutation was the most common single mutation in our cohort with a similar frequency to that in previous report (69%).[3] P258L and V217F are somatic mutations that have been previously reported in cancers including VRL and DLBCL.[3,4] All of the mutations were located in the known mutational hot spot, Toll/IL-1 receptor (TIR) domain, which interacts with the Toll-like receptor (TLR) (Figure 1B). Although the characteristic *MYD88* mutations were shared between VRL and PCNSL patients, the mutation was significantly more frequent in our VRL patients than it was in PCNSL (100% *vs.* 62.8%; *P*=0.0263).[2,5-7] The *CD79B* mutation is another hallmark of PCNSL.[2,5,6] The mutation in *CD79B* was less frequently identified in our VRL patients than it was in the PCNSL cohort (2 of 9; 22.2%) (22.2% *vs.* 40.0%; *P*=0.473). The Y197D and L200Q mutations in *CD79B* were identified. All detected mutations were located on the immunoreceptor tyrosine-based activation motif (ITAM) domain. *PIM1* point mutations were identified in eight patients. Each patient harbored 1 to 22 point mutations in the *PIM1* gene (*Online Supplementary Table S1*). Point mutations, as well as increased expression, of *PIM1* gene were associated with adverse effects in patients with the activated B-cell (ABC) type DLBCL.[8] We also identified specific point mutations of *PIM1* gene that were associated with drug resistance in our patients (*Online Supplementary Table S1*).[8] Historically, *IGLL5* had not been described as being frequently mutated in B-cell lymphomas. However, several recent studies have suggested a relative high frequency of *IGLL5* mutation, with associated biological importance, in DLBCL and

CLL.[9] Although *IGLL5* is known to play a critical role in B-cell development, little else is known about its function. Therefore, further studies regarding its potential role in lymphomagenesis would be needed. A germline heterozygous *ERCC6* splice-site mutation (c.1527-2A>G) was identified in one patient (Case 8, *Online Supplementary Figure S2*). Germline genes that may predispose a patient to lymphoma include DNA repair genes.[10] *ERCC6* is known to play a critical role in DNA repair and prior studies found that disruption of the function of *ERCC6* increases cancer susceptibility.[11] As DNA repair genes, such as *BRCA1* and *PALB2*, are related to cancer predisposition even in the presence of a mono-allelic mutation, *ERCC6* germline mutations may be associated with malignancy. Regardless, this hypothesis requires further investigation.

The mutated genes in patients with VRL were similar to those in patients with PCNSL. Still there were some differences in specific mutation frequencies. For instance, VRL had a significantly higher rate of *MYD88* and *IGLL5* mutations than PCNSL (P=0.0263 and P=0.0001, respectively; Figure 1C). Among the list of frequently mutated genes in VRL and PCNSL, SHM-associated genes such as *PIM1, IGLL5, BTG1*, and *BTG2* were included.[2,12] Using copy-number variation (CNV) analysis, we identified biallelic or monoallelic deletion of *CDKN2A* in 6 of the 9 patients (66.7%; *Online Supplementary Figure S3*). In contrast, we did not detect the *TP53* sequence variation or CNV, which resembled the reported features of PCNSL and primary testicular lymphomas.[13] As a subtype of DLBCL, most of the frequently mutated genes in VRL were identical to those of DLBCL. These mutations included MYD88 and SHM-associated genes (Figure 1D). However, there were larger differences in the frequencies of mutations between DLBCL and VRL than there were between VRL and PCNSL. According to the genetic subtypes of DLBCL reported by Schmitz *et al.*,[12] it could be speculated that VRL could be classified into the MCD subtype (defined based on co-occurrence of *MYD88* L265P and *CD79B* mutations) based on a high frequency of the characteristic *MYD88* mutation and similarity in pattern of other gene mutations (*Online Supplementary Figure S4*).

The genetic profiling of VRL can facilitate precise therapy. For instance, Ibrutinib is a molecular targeted drug for *BTK* that has been used in the treatment of *MYD88*-postive B-cell lymphomas. However, the primary sensitivity or resistance to this drug is influenced by the presence of mutations beyond *MYD88* alone and concurrent mutations in *CXCR4, CD79A, CD79B*, or *PIM1* have been associated with drug sensitivity and resistance.[8,14] In addition, an alternative type of *MYD88* can serve as a predictive marker of BTK inhibition.[13] Therefore, it is clinically important not only to evaluate well-known genes, but also the whole exome to optimize precise therapy. VRL typically masquerades as uveitis. Its diagnosis remains difficult, and is often delayed for several months.[1] However, rapid diagnosis of VRL is critical for its appropriate treatment. The standard diagnosis of VRL involves the cytologic evaluation of malignant lymphoma cells, however, false-negatives are common.[1] Therefore, complementary diagnostic tools evaluating the IL-10 to IL-6 ratio or immunoglobulin clonality have also been used.[1] Recently, the allele specific PCR for the *MYD88* L256P mutation and specific microRNA have demonstrated high diagnostic power.[3,15] However, evaluating proteins such as IL-10 or microRNA is not a confirmatory diagnostic tool because they illustrate the clinical phenomenon rather than identifying the cancer itself. In addition, the immunoglobulin clonality assay is susceptible to false positives due to the difficulty of acquiring sufficient DNA and the possibility of contaminating reactive cells. Given the existing diagnostic challenges, we confirmed the utility of WES in making the diagnosis of VRL and in mutational profiling. Considering feasibility and cost-effectiveness in routine clinical practice, however, a smaller targeted gene panel testing based on frequently mutated genes in this WES study would be easier, cheaper, and a more readily available way to allow a rapid diagnosis and a higher diagnostic yield.

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