Next-Generation Sequencing of Vitreoretinal Lymphoma by Vitreous Liquid Biopsy: Diagnostic Potential and Genotype/Phenotype Correlation

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Purpose. To determine the diagnostic potential of next-generation sequencing (NGS) in vitreous samples, analyze genotype-phenotype characteristics, and compare NGS of matched vitreous and brain samples in patients with associated central nervous system lymphoma (CNSL).

Methods. A total of 32 patients suspected of vitreoretinal lymphoma (VRL) who underwent diagnostic vitrectomy and NGS were included in this retrospective observational case-series. Fresh vitreous specimens from diagnostic vitrectomy of VRL-suspected patients underwent NGS using a custom panel targeting 747 candidate genes for lymphoma. They also underwent malignancy cytology, interleukin (IL)-10/IL-6, immunoglobulin heavy chain (IGH)/immunoglobulin kappa light chain (IGK) monoclonality testing. *MYD88 L265P* mutation was examined from anterior chamber tap samples. The diagnosis of VRL was made based on typical clinical characteristics for VRL, as well as malignant cytology, IGH/IGK clonality, or IL-10/IL-6 > 1. Sensitivity and specificity of NGS were compared with conventional diagnostic tests. Brain tissues suspected of lymphoma were collected by stereotactic biopsy and underwent NGS. Genetic variations detected in NGS of vitreous and brain tissue specimens were compared.

Results. The sensitivity values for cytology, IL-10/IL-6 > 1, clonality assays for IGH and IGK, *MYD88 L265P* detection in anterior chamber tap samples, and vitreous NGS were 0.23, 0.83, 0.68, 0.79, 0.67, and 0.85, with specificity values of 1.00, 0.83, 0.50, 0.25, 0.83, and 0.83, respectively. The sensitivity (0.85) of vitreous NGS was the highest compared to other conventional diagnostic tests for VRL. The most common mutations were *MYD88* (91%), *CDKN2A* (36%), *PIM1* (32%), *IGLL5* (27%), and *ETV6* (23%). Although several gene alterations demonstrated heterogeneity between the brain and eyes, some common mutational profiles were observed in matched vitreous and brain samples.

Conclusions. Overall, NGS of the vitreous demonstrated high sensitivity among conventional diagnostic tests. VRL and CNSL appeared to have both shared and distinct genetic variations, which may suggest site-specific variations from a common origin.

Keywords: vitreoretinal lymphoma, next-generation sequencing, diffuse large B-cell lymphoma

Vitreoretinal lymphoma (VRL) is a rare, but most common form of intraocular lymphoma that is considered a variant of central nervous system lymphoma (CNSL). Approximately 65% to 90% of VRL eventually develop central nervous system (CNS) dissemination.[1] Conversely, 15% to 25% of patients with CNSL show intraocular involvement at the time of diagnosis, and 25% of patients without ocular involvement will eventually develop VRL.[2,3] The overall five-year survival rate of VRL with CNS involvement ranges from 19% to 61.1%.[4,5] Secondary VRL from non-CNS lymphomas is rare. One study showed that 90% of VRLs are B-cell lymphoma and only 10% are T-cell lymphoma.[6]

Cytologic confirmation is the standard diagnostic procedure for VRL; however, it suffers from the low yield of high-quality material for pathological evaluation owing to an insufficient number of tumor cells, cytolytic effects of preceding corticosteroid therapy from misdiagnosis as intraocular inflammation, and rapid degeneration of lymphoma cells.[7,8] Thus many efforts have been made to aid the diagnosis, including interleukin (IL) ratio (IL-10/IL-6 > 1), IL-10 level (>50 pg/mL) from anterior chamber

tap samples, and clonality assays for immunoglobulin heavy chain (IGH) and kappa light chain (IGK).[9] Recent findings showed that *MYD88 L265P* mutation is detected in vitreous samples in approximately 66% to 82% of VRL patients.[10,11] *MYD88 L265P* can be detected in even smaller volumes from anterior chamber tap samples repeatedly, making it a useful method for disease monitoring.[12] Recent gene expression profiling studies have demonstrated additional molecular alterations that are commonly detected in VRL, which could be useful in the current diagnostic workup of VRL.[13,14]

In the present study, we used next-generation sequencing (NGS) with an in-house lymphoma panel to analyze genetic alterations using a fresh vitreous specimen from patients who had undergone diagnostic vitrectomy suspected of VRL. Whole exome sequencing (WES) data of VRL patients from our previous study was included for analysis.[14] The diagnostic capability of NGS in comparison to other conventional diagnostic tools for VRL was evaluated. Additionally, genotype-phenotype characteristics and NGS results of matched vitreous and brain samples in selected patients were analyzed.

MATERIAL AND METHODS

In this retrospective observational study, patients suspected of VRL underwent diagnostic vitrectomy in the Department of Ophthalmology, Severance Eye Hospital, Yonsei University College of Medicine, between January 2017 and November 2021. This study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and was approved by the Institutional Review Board of the Yonsei University College of Medicine (IRB no. 4-2021-1352). The requirement for informed consent was waived. We performed diagnostic vitrectomies for patients suspected of VRL. The diagnosis for VRL was based on the following criteria, which was adapted from previous reports:[13,15] (1) characteristic ocular findings consistent with VRL (vitreous opacities and/or subretinal infiltrates) with negative results from infectious and noninfectious uveitis tests, (2) malignant cytology in the vitreous, (3) clonality of the infiltrating lymphoma cells in the vitreous using either PCR analysis of IGH or IGK gene rearrangements, and (4) IL-10/IL-6>1.[16] Patients who had (1) accompanied by either (2), (3), or (4) were diagnosed with VRL.

Optical coherence tomography, wide-field fundus photography, and wide-field fundus autofluorescence images were used to evaluate any subtle features of VRL in both eyes. AC tap samples (∼0.1 mL) were collected at the beginning of the surgery before vitrectomy. DNA extracts were subjected to mutant enrichment with 3′-modified oligonucleotides-PCR[17] for detection of *MYD88 L265P* mutation. Undiluted and diluted vitreous samples were collected from 25-gauge vitrectomies and underwent NGS using a custom panel targeting 747 candidate genes for lymphoma (Supplementary Appendix A). We collected approximately 2 mL of undiluted samples and analyzed them for IGH/IGK, NGS, and IL-6/10 tests. The remaining undiluted samples and diluted samples were mixed and sent for cytology. Massively parallel sequencing was done on the NextSeq 550Dx System (Illumina, San Diego, CA, USA). Quality control and sequence analysis were performed using our custom analysis pipeline. Databases used for analysis and variant annotation include Online Mendelian Inheritance in Man, Human Gene Mutation Database (subscription-only), ClinVar, dbSNP, 1000 Genome, Exome

Aggregation Consortium, Exome Sequencing Project, and Korean Reference Genome Database. All pathogenic and likely pathogenic variants were further confirmed by Sanger sequencing. All variants were classified by the American College of Medical Genetics and Genomics guidelines and benign and likely benign variants were filtered out. Variants are classified into four tiers based on their level of clinical significance in cancer diagnosis, prognosis, and/or therapeutics following the standards and guidelines established by the Association for Molecular Pathology, American Society of Clinical Oncology, and College of American Pathologists (Tier I, variants with strong clinical significance; Tier II, variants with potential clinical significance; Tier III, variants of unknown clinical significance; and Tier IV, variants deemed benign or likely benign).[18] Experimental details for WES were described in the previous study.[14] Genes that were covered in the WES but were not covered in the NGS panel were not included in this study.

Brain tissues suspected of lymphoma were collected by stereotactic biopsy. For NGS of brain tissue, formalin-fixed and paraffin-embedded samples were sequenced with TruSight Oncology 500 panel (Illumina) on NextSeq 550Dx System. The Trusight Tumor 500 panel comprises a panel of 523 genes for the identification of single-nucleotide variants, small insertions and deletions, and copy-number variation, as well as a panel of 55 genes for an RNA workflow for the identification of splice variants and gene fusions.[19] The complete list of genes for TruSight Oncology 500 is listed on Illumina's website. Variants were classified into four tiers as in vitreous NGS.[18]

All cytological preparations were reviewed by experienced cytopathologists. An IL-10/IL-6 ratio > 1.0 was considered suggestive of a diagnosis of B-cell VRL. In cases of suspected infectious uveitis, the vitreous specimen was sent to a laboratory for culture and comprehensive polymerase chain reaction analysis for agents to known to cause infectious uveitis. We examined the sensitivity and specificity of cytology, IL-10/IL-6 > 1.0, IGH/IGK monoclonality, *MYD88 L265P* mutation in AC tap samples, and NGS results. We used OncoPrinter, a tool provided by the cBioPortal for Cancer Genomics (cbioportal.org/oncoprinter), to visualize and analyze our data.

The data are presented as the mean ± standard deviation (SD) unless otherwise stated. All statistical analyses were performed using SPSS for Windows (version 22.0; SPSS Inc., Chicago, IL, USA). A *P* value < 0.05 was considered statistically significant.

RESULTS

Patient Characteristics

This study includes 32 patients initially suspected of VRL. Vitreous samples from 23 patients underwent targeted NGS, whereas nine patients underwent WES. Twenty-six patients were eventually diagnosed with VRL (Table 1A). Six patients did not meet the diagnostic criteria for VRL. Five of these six patients showed negative NGS results from vitreous specimens. However, one patient (Patient 17) showed positive NGS result including mutations in *MYD88, CD79B,* and *SETD1B*. The clinical characteristics of 26 VRL patients are shown in Table 1B. The mean ± SD age was 64 ± 11 years. Thirteen (50%) patients were men. Except for two patients (cases 7 and 13) who had undergone vitrectomies at a different hospital, no other patients had previous vitrectomy or

TABLE 1A. Demographics and Clinical Findings for Patients With Vitreoretinal Lymphoma

Case No.	Age (Yr)/Sex	Laterality	Origin Of Lymphoma	Primary Ocular Site	Extraocular Lesions and Tumor Type During Follow-Up	Months to Distant Sites	Treatment	Malignancy Cytology	IGH/IGK Clonality	IL-10/IL-6 Ratio	MYD88 mutation in AH	Lymphoma Genes on NGS/WES	Clinical Course, Follow-Up Months
1	64/M	OU	CNSL	V	Brain, DLBCL	10	ivMTX, sCTx, RT	Y	NC/C	2.65	Y	Y	Surviving, 31
2	45/M	OU	CNSL	V	Brain, DLBCL	19	ivMTX, sCTx, RT	Y	NC/C	8.75	Y	Y	Surviving, 12
3	77/M	OU	Lung DLBCL	SR	Lung, DLBCL	10	ivMTX, sCTx	N	C/C	0.17	NA	N	Surviving, 37
4	62/M	OU	VRL	SR	Brain, DLBCL	21	ivMTX, sCTx	N	C/NA	4.54	Y	Y	Surviving, 34
5	61/M	OU	VRL	SR	LN, lung, spleen, neck, peritoneum, DLBCL	10	ivMTX, sCTx	N	C/C	11.67	Y	Y	Surviving, 12
6	56/F	OD	VRL	V		—	ivMTX	N	C/C	11.85	N	Y	Surviving, 24
7	62/F	OU	VRL	V+SR	Brain, DLBCL	15	ivMTX, sCTx	Y	NA/NA	16.46	N	N	Surviving, 24
8	63/F	OU	VRL	SR	Brain, DLBCL	12	ivMTX	N	C/C	0.30	NA	N	Surviving, 23
9	85/F	OS	CNSL+VRL	V	Brain, DLBCL	Concurrent	ivMTX, sCTx, RT	Y	C/C	7.31	Y	Y	Surviving, 24
10	51/F	OU	VRL	V+SR		—	ivMTX	N	C/C	0.08	Y	Y	Surviving, 20
11	64/F	OU	VRL	V+SR		—	ivMTX	N	C/C	3.72	Y	Y	Surviving, 19
12	67/M	OU	VRL	V	Brain, DLBCL	11	ivMTX	N	C/C	NA	N	Y	Surviving, 17
13	70/F	OS	VRL	V+SR		—	ivMTX	N	C/C	0.54	N	N	Surviving, 6
14	68/M	OU	VRL	V+SR		—	ivMTX, ivRTX, RT	N	C/C	6.11	Y	Y	Surviving, 11
15	77/F	OD	CNSL+VRL	V+SR	Brain, DLBCL	Concurrent	ivMTX, sCTx	N	C/NC	NA	N	Y	Surviving, 8
16	73/M	OD	CNSL+VRL	V	Brain, DLBCL	Concurrent	ivMTX, sCTx	N	NC/C	23.4	Y	Y	Surviving, 12
18	58/F	OU	CNSL+VRL	V	Brain, DLBCL	Concurrent	ivMTX	Y	NC/NC	2.81	Y	Y	Surviving, 3
20	47/F	OU	VRL	V	Brain, DLBCL	24	ivMTX, sCTx	N	C/NC	23.46	NA	Y	Surviving, 96
21	61/F	OU	VRL	V	Brain, DLBCL	14	ivMTX, sCTx	N	C/C	20.27	NA	Y	Surviving, 30
22	64/M	OS	VRL	V	Brain, DLBCL	36	ivMTX, sCTx	Y	NC/NC	115.3	NA	Y	Deceased, 40
23	64/M	OU	VRL	SR		—	ivMTX	N	C/C	7.39	NA	Y	Surviving, 49
24	84/M	OS	VRL	SR		—	ivMTX	N	NC/C	3.70	NA	Y	Surviving, 2
25	49/F	OD	VRL	V		—	Systemic steroid	N	C/C	35.75	NA	Y	Surviving, 13
26	74/M	OU	VRL	V		120	ivMTX	N	NC/C	33.01	NA	Y	Surviving, 19
27	81/M	OU	CNSL	V	Brain, DLBCL	120	ivMTX,	N	C/NC	3.46	NA	Y	Surviving, 18
28	45/F	OS	CNSL	V	Brain, DLBCL	24	ivMTX,	N	NC/C	6.07	NA	N	Surviving, 16

AC, anterior chamber; C, monoclonal; ivMTx, intravitreal methotrexate injection; ivVanc, intravitreal vancomycin injection; NA, not available; NC, not monoclonal; OD, oculus dexter (right eye); OS, oculus sinister (left eye); OU, oculus uterque (both eyes); sCTx, systemic chemotherapy; SL, systemic lymphoma; SR, subretinal infiltration; V, vitreous opacity.

TABLE 1B. Demographics and Clinical Findings for Control Group

Case No.	Age (Yr)/Sex	Laterality	Origin of Uveitis/Relevant Past History	Primary Ocular Site	Extraocular Lesions and Tumor Type During Follow-Up	Months to Distant Sites	Treatment	Malignancy Cytology	IGH/IGK Clonality	IL-10/IL-6 Ratio	Myd88 Mutation In Ac Tap Samples	Lymphoma Genes On Ngs/Wes	Clinical Course, Follow-Up Months
17	61/M	OS	Uveitis, possible VRL	SR	—	—	ivMTX	N	NA/NA	0.41	Y	Y	Surviving, 5
19	71/M	OU	Uveitis (Tonsil DLBCL)	V	Tonsil, DLBCL	—	ivMTX, sCTx	N	NC/NC	0.57	N	N	Surviving, 2
29	90/F	OU	Uveitis (Sarcoidosis)	V	—	—	Systemic corticosteroids	N	NC/C	0.003	N	N	Surviving, 12
30	71/F	OS	Uveitis (Bacterial endophthalmitis)	V+SR	—	—	ivVanc	N	C/C	0.81	N	N	Surviving, 8
31	80/M	OD	Hemorrhage	V	—	—	None	N	C/C	1.97	N	N	Surviving, >24
32	72/F	OU	Uveitis (Sarcoidosis)	V	—	—	Systemic corticosteroids	N	NA/NA	0.12	N	N	Surviving, 3

AC, anterior chamber; C, monoclonal; ivMTx, intravitreal methotrexate injection; ivVanc, intravitreal vancomycin injection; NA, not available; NC, not monoclonal; OD, oculus dexter (right eye); OS, oculus sinister (left eye); OU, oculus uterque (both eyes); sCTx, systemic chemotherapy; SL, systemic lymphoma; SR, subretinal infiltration; V, vitreous opacity.

intravitreal injection of the chemotherapeutic agent. Seventeen (65%) of 26 VRL patients had bilateral ocular involvement. Fourteen (54%) patients showed vitreous opacification, and six (23%) showed subretinal infiltration as the main clinical manifestation. Six (23%) patients demonstrated both vitreous opacification and subretinal infiltration.

Fifteen patients (58%) were eventually diagnosed with both VRL and CNSL. Four (15%) patients (cases 9, 15, 16, and 18) were diagnosed with VRL and CNS lymphoma concurrently. Four (15%) patients (cases 1, 2, 27, and 28) were diagnosed with VRL 10, 19, 120, and 24 months after diagnosis with CNSL, respectively. Seventeen (65%) patients had VRL without CNS involvement at the time of initial ophthalmic evaluation and seven (41%) of them developed CNS disease during the mean follow up of 15 ± 5 months (range 10-24 months), and one of them developed multiple non-CNS involvement after 10 months (case 5). One (4%) patient had secondary VRL from DLBCL of lung. The survival rate was 96% (25 out of 26 patients) with a mean follow-up of 23 ± 18 (range 2-96 months). The cause of death was hepatic veno-occlusive disease and septic shock.

Results of Conventional Diagnostic Tests

Six of 26 (23%) VRL patients showed positive malignant cytology. The yield of identifying cells in the mixed undiluted and diluted samples was 77%. Twenty of 24 (83%) patients had IL-10/IL-6 ratio greater than 1. Positivity for IGH and IGK monoclonality tests were detected in 17 (68%) of 25 patients and 19 (79%) of 24 patients, respectively. Ten (67%) of 15 patients showed *MYD88 L265P* mutation in AC tap samples. The sensitivity and specificity of each diagnostic test are shown in Table 2.

Results of NGS

The sensitivity of targeted NGS and WES together was 0.82 and the specificity was 1.00. Sensitivities of NGS and WES were 0.76 and 1.00, respectively and specificities were both 1.00.[14] Specific gene mutations from each NGS positive patient are shown in Supplementary Table S1. Patient 17 did not meet diagnostic criteria for VRL; however, the patient showed *MYD88* mutation from anterior chamber tap sample, and mutations in *MYD88*, *CD79B*, and *SETD1B* genes. Four patients with VRL did not show genetic variations significant for VRL. Three of the NGS-negative VRL patients and 9 of the 22 NGS-positive VRL patients had taken systemic or topical steroid medication for more than one month before diagnostic vitrectomy. Of the three NGS-negative patients, one patient had undergone a diagnostic vitrectomy one day before vitreous sampling in our hospital. Among 747 candidate genes, common mutations (number of patients, sensitivity value, and specificity value) found among 26 VRL patients were MYD88 (20, 0.77, 0.83), CDKN2A (8, 0.31, 1), PIM1 (7, 0.27, 1), IGLL5 (6, 0.23, 1), ETV6 (5, 0.19, 1), BTG1 (4, 0.15, 1), KMT2D (4, 0.15, 1), and SETD1B (4, 0.15, 1) (Fig. 1). All 11 patients who were positive for *MYD88* mutation in AC tap samples showed *MYD88* mutation in vitreous NGS.

VRL-Only Versus CNS-Involved VRL

Among 22 patients with positive NGS results, eight had ocular-only VRL, 13 had CNS-involvement, and one had a VRL with non-CNS spread. Eleven (85%) of 13 CNS-involving cases showed vitreous opacity as a major clinical

TABLE 2. Sensitivity and Specificity of Various Diagnostic Procedures

Diagnostic Test	VRL Group			Non-VRL Group			Sensitivity	Specificity
	Yes	No	Percent	Yes	No	Percent		
Malignancy cytology	6	20	23	0	6	0	0.23	1.00
IL-10/IL-6 > 1[*]	20	4	83	1	5	17	0.83	0.83
IGH monoclonality[†]	17	8	68	2	2	50	0.68	0.50
IGK monoclonality[‡]	19	5	79	3	1	75	0.79	0.25
MYD88 mutation in AC tap samples[§]	10	5	67	1	5	17	0.67	0.83
NGS+WES[‖]	22	4	85	1	5	17	0.85	0.83

AC, anterior chamber.
[*] Two patients in the VRL group did not undergo IL-10/IL-6 testing
[†] One patient in the VRL group and two patients in the non-VRL group did not undergo IGH monoclonality testing.
[‡] Two patients in the VRL group and two patients in the non-VRL group did not undergo IGK monoclonality testing
[§] 11 patients in the VRL group did not undergo *MYD88* mutation testing from AC tap samples.
[‖] Includes eight patients with whole-exome sequencing data

feature, while VRL-only cases demonstrated a more heterogeneous pattern (three cases with both vitreous opacity and subretinal infiltration, three with vitreous opacity, and two with subretinal infiltration). Bilateral ocular involvement was observed in eight (62%) CNS-involving cases and four (50%) VRL-only cases.

MYD88 mutation was detected in 11 (85%) CNS-involving cases and in eight (100%) VRL-only cases. For CNS-involving cases, *PIM1* (36%), *CDKN2A* (29%), *IGLL5* (29%), *B2M* (14%), *BTG1* (14%), *CD79B* (14%), *CREBBP* (14%), *ETV6* (14%), *GNA13* (14%), *KLHL14* (14%), *KMT2D* (14%), and *SETD1B* (14%) were next common mutations (Supplementary Fig. S1). For VRL-only cases, *CDKN2A* (38%), *BTG1* (25%), *CD58* (25%), *CIITA* (25%), *ETV6* (25%), *HLA-B* (25%), *IGLL5* (25%), and *PIM1* (25%) were next common mutations (Supplementary Fig. S2).

Comparison With NGS From Brain Biopsy

Four patients (cases 4, 15, 16, and 18) had NGS results from both vitreous and brain biopsies (Fig. 2, Supplementary Table S2). Case 4 was diagnosed with VRL before being diagnosed with CNS lymphoma (CNSL). Cases 15, 16, and 18 were concurrently diagnosed with VRL and CNSL. Three (cases 4, 15, and 16) of the four cases showed *MYD88* mutation from the brain. Of these three, two (cases 4 and 16) cases demonstrated *MYD88* mutation from the vitreous. Case 4 demonstrated *MYD88* and *IRF4* mutation from both brain and vitreous biopsies. Case 16 showed *MYD88, CD79B, B2M*, and *GNA13* mutations from both brain and vitreous biopsies. Case 18 demonstrated *CREBBP* and *ETV6* mutations from both brain and vitreous biopsies. *CD58, ACTB, IGLL5, KLHL14, BTG2*, and *SETD1B* mutations were only detected in the vitreous, while *BCOR, HIST1H3C, KLHL6, EGFR*, and *CDKN1B* mutations were only detected in the brain samples.

DISCUSSION

In this study, we explored the results from the targeted NGS gene panel and WES for patients suspected of VRL along with previously established diagnostic tests. Vitreous NGS and WES showed the highest sensitivity (0.85) among previously proposed diagnostic criteria. IL-10/IL-6 ratio showed the next highest sensitivity (0.83), which was comparable to some studies[16,20] but was lower compared to others with sensitivity over 0.90.[4,21,22] One possible explanation for lower sensitivity is that patients who were in the early stage of lymphoma or patients with T-cell lymphoma can have low IL-10 profile.[21] Common mutations among 747 candidate genes include *MYD88* (91%), *CDKN2A* (36%), *PIM1* (32%), *IGLL5* (27%), and *ETV6* (23%) (Fig. 1). A Chinese study which analyzed vitreous of 23 VRL patients, showed mutations in *MYD88* (78%), *ETV6* (48%), *PIM1* (48%), *BTG2* (30%), *IRF4* (30%), *CD79B* (26%), and *LRP1B* (26%).[23] In one study that reported gene expression profiling of VRL from seven cases, the frequencies of *MYD88* mutation and *CD79B* mutation were 71.4% and 28.6%, respectively.[13] Although not identical, these results showed similar trend with our results, which show highest frequency for *MYD88* mutation and lower frequencies for *PIM1, ETV6* and *CD79B* mutations (Fig. 1). Based on the current study and previous studies, mutations in *MYD88, CDKN2A, PIM1, IGLL5*, and *ETV6* can be considered as important variations in the VRL family and would be recommended to be included in a gene panel for multiplex PCR test for VRL.[14,24–28]

MYD88 is a protein associated with the innate immune system and is a key mutation in VRL because it is found in 69% to 100% of the vitreous from VRL patients.[10,11,14] Virtually all mutations in *MYD88* including *L265P* occur in the Toll-like receptor domain and result in constitutive activation of NF-*k*B signaling. Another common gene mutation detected in NGS was *CDKN2A* mutation (35%). *CDKN2A* is a tumor suppressor gene and mutations in *CDKN2A* are known to be associated with various cancers, such as pancreatic, colon, and non-small cell lung cancers.[29–31] *CDKN2A* loss is predominantly observed in DLBCL with the activated B-cell (ABC) phenotype, which in general, confers a considerably poorer prognosis than the germinal center b-cell (GCB) phenotype.[32] In our data, of nine VRL cases with *CDKN2A* loss, five were diagnosed with DLBCL of the brain, three did not have any detectable extraocular involvement, and one was diagnosed with DLBCL in multiple lymph nodes. Although the association of *CDKN2A* loss with extraocular involvement of VRL or long-term poor prognosis of VRL is not conclusive from this study, *CDKN2A* loss in VRL calls for further investigation.

Other common mutations found in our study were *IGLL5, PIM1, and ETV6*. *IGLL5* is known to play a critical role in B-cell development, though its specific function is not well known.[14] Prevalence of *IGLL5* mutation in our study was 27%, which was comparable to other targeted genetic sequencing studies on different types of DLBCL (13%,[33] 40%,[34] and 43%[35]). *PIM1* (32%) is commonly overexpressed
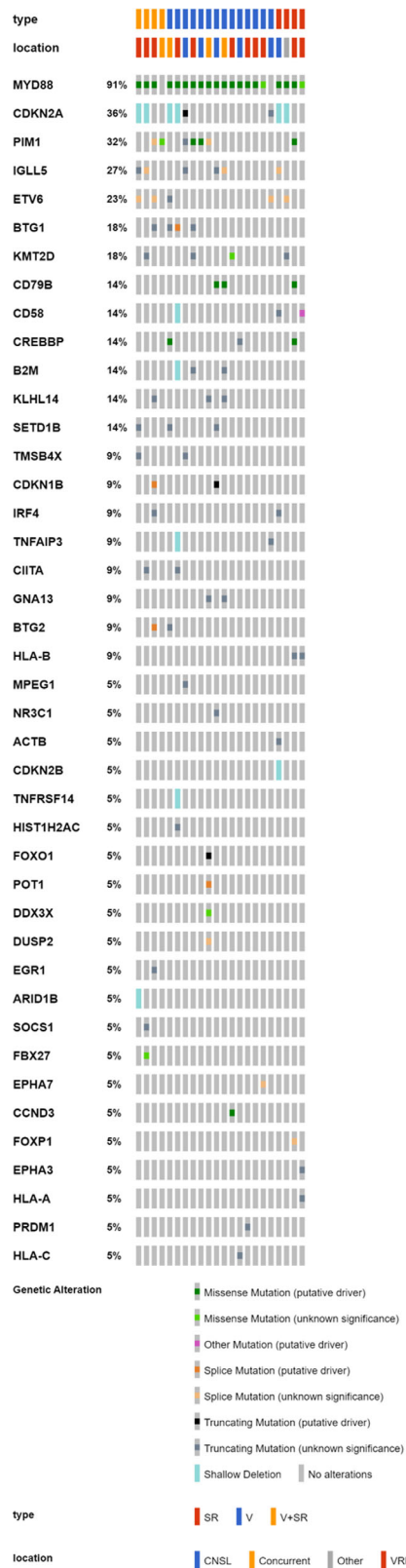
FIGURE 1. Frequencies of genetic alterations identified by next-generation sequencing of vitreous specimens from patients with vitreoretinal lymphoma. SR, subretinal infiltration; V, vitreous opacity.

in human cancers, and its expression is correlated with poor prognosis in leukemias,[36] mantle cell lymphoma,[37] and DLBCL.[38] Prevalence of *PIM1* mutation from previous studies showed 16.58%,[39] 40%,[40] and 23%.[33] *ETV6* is a strong transcriptional repressor and is recurrently mutated/deleted gene in DLBCL.[41,42] Though its function is unclear, ETV6 is upregulated during oncogenic transformation of germinal center B-cells and that it regulates DLBCL survival.[41] Recent genetic analysis of vitreous from VRL patients showed *ETV6* mutation to be found in 45% and 48% of patients, which is higher than our result of 23%.[23,43]

Whether VRL belongs to GCB or ABC subtypes has been controversial. The high frequency of t(14,18) translocation in VRL and a recent gene expression profiling study showed that the expression pattern of VRL was relatively closer to the GCB subtype than the ABC subtype.[13,44] In contrast, the immunophenotype of VRL tumor cells was MUM1/IRF4+; BCL-6+/−; CD10−, suggesting that VRL belongs to the ABC subtype of DLBCL.[45] Furthermore, *MYD88 L265P* mutation, a hallmark of VRL, is more frequently observed in ABC subtypes than in GCB subtypes.[27] More recent studies have proposed new genetic subtypes of DLBCL based on shared genomic abnormalities, rather than a cell-of-origin scheme that subdivides DLBCL into ABC and GCB subtypes. Among them, MCD and N1 subtypes are predominantly ABC subtypes and showed poorer outcomes than other subtypes.[27,46] Our findings suggest that mutational profiles of VRL partially resemble the MCD subtype according to the above DLBCL classification.

There were four cases with matched vitreous and brain samples. NGS of matched vitreous and CNS samples can be used to establish clonal relationships as well as to detect intra-patient tumor genomic heterogeneity between lymphomas involving the eye and the brain.[47] Clonal relationship was observed because some cases shared mutations including *MYD88, CD79B, B2M, GNA13,* and *CREBBP* (Fig. 2). *MYD88* mutation was the most common genetic variation noted in three of four (75%) cases for both brain and vitreous samples. For brain NGS, all other mutations were detected once among four cases, while *CDKN2A* loss and *IGLL5* mutation were detected in two of four (50%) vitreous samples, respectively. *CDKN2A* loss was not detected in any of the brain NGS samples. However, we cannot rule out the presence of *CDKN2A* loss in the brain samples because of the fact that gene panel for brain NGS was not designed to detect copy number alterations for *CDKN2A*. Nonetheless, some mutations were distinct in each tissue. *BCOR, KLHL6,* and *EGFR* mutations from brain NGS were not detected in any of the matched and unmatched vitreous NGS cases.

Within CNS-involving VRLs, the ocular manifestation may differ between CNSL-derived VRLs versus VRLs that later invaded the CNS. For four CNSL cases that later involved the eyes, all their main clinical manifestations were vitreous opacity without subretinal infiltration. A smaller study of two CNSL-derived VRL cases also reported vitreous lesions without retinal lesions.[47] However, for 18 VRLs that did not show CNS involvement at initial evaluation, a higher proportion of cases (11 of 18, 61%) demonstrated subretinal infiltration. Similar to our study, one study reported that retinal or subretinal infiltration was significantly more frequent in VRL-only eyes (68%) than in secondary VRLs from CNSL (44%).[48] We do not clearly understand the mechanism behind this difference. One report suggested the possibility of hematogenous spread for sub-retinal pigment epithelium (RPE) lymphoma deposits.[49] Lymphoma cells

Case	Genes	Brain	Eye
Case 4	MYD88 (p.L265P)	green	green
	CD58 (p.G88*)		blue
	IRF4 (p.W42*)		blue
	ACTB (p.Q41*)		blue
	IGLL5 (p.M1*)		blue
	IGLL5 (splice (c.206+1G>C))		blue
	CDKN2A (whole gene deletion)	gray	orange
	CDKN2B (whole gene deletion)	gray	orange
	BCOR (p.R1661*)	blue	
	BTG1 (p.Y67*)	blue	
	HIST1H3C (p.K28M)	green	
Case 15	MYD88 (p.L265P)	green	
	PIM1 (p.D128H)		green
Case 16	MYD88 (p.L265P)	green	green
	CD79B (p.Y197H)	green	green
	B2M (p.K26*)	blue	blue
	GNA13 (p.Q27*)	blue	blue
	ETV6 (p.R378*)	blue	
	PIM1 (p.K115N)	green	
	PIM1 (p.V187M)	green	
	PIM1 (p.S188N)	green	
	EGFR (p.R222C)	green	
	KLHL6 (p.R347H)	green	
	KLHL6 (p.L90F)	green	
	KLHL14 (p.Q90*)		blue
	IGLL5 (splice (c.206+1G>C))		blue
Case 18	MYD88 (p.L265P)		green
	CDKN2A (whole gene deletion)	gray	orange
	CREBBP (p.R1446C)	green	green
	ETV6 (p.K11*)	blue	blue
	BTG1 (p.Q38*)		blue
	BTG2 (p.E48*)		blue
	SETD1B (p.L85*)		blue
	CDKN1B (p.L144*)	blue	

Color	Meaning
green	Single nucleotide substitution
blue	Nonsense or frameshift
orange	Copy number variation
white	No alteration
gray	Not included in the gene panel

FIGURE 2. Comparative analysis of genetic alterations found in patients who had undergone both vitreous and brain biopsies. Gene panel for brain NGS was not designed to detect copy number alterations for *CDKN2A* and *CDKN2B* genes.

may take advantage of CXCR4 and CXCR5 and migrate to sub-RPE,[50] which may not be the case for those involved in vitreous opacities. Direct invasion of the optic nerve and meningeal infiltration are two possible routes of CNS dissemination,[51] which could be sources of vitreous opacity in CNSL-derived VRLs. Detection of *MYD88* mutation and increased IL-10 concentration in the cerebrospinal fluid of VRL patients support this hypothesis.[52] Our data support that CNSL-derived VRLs may take advantage of the optic nerve/meningeal route, which leads to a predominant vitre-

ous opacity phenotype for these cases. Further understanding of spatial and temporal differences in ocular phenotypes and genotypes may lead to effective tumor-specific treatment strategies.

This study has several limitations. One limitation was the low yield of lymphoma cells in the vitreous of VRL, which led to low sensitivity from malignant cytology, resulted in difficulty in setting the gold standard for VRL diagnosis. However, this was the reason we believe that genetic tests using NGS can aid in raising the diagnostic capability of VRL as was shown in this study. Patient 17, who did not meet the diagnostic criteria for VRL, showed the same *MYD88* mutations in both anterior chamber tap sample and vitreous, as well as other VRL-related mutations from the vitreous NGS. This case is an example that shows that genetic testing for VRL can aid the diagnosis in certain patients who are otherwise difficult to diagnose using traditional diagnostic tests. NGS-analysis result was reported in 32.3 ± 13.6 days, which can delay the diagnosis and treatment. However, we believe that the improvement in the speed of NGS-analysis, as well as more compact gene panel for VRL, would shorten the latency of diagnosis in the future. Another limitation is that we did not have sensitivity and specificity data for flow cytometry, as well as T cell receptor (TCR) clonality test. This was due to the fact that flow cytometry and TCR for VRL were not routinely done in our clinical setting. Absence of *CDKN2A* gene in the brain NGS is another weakness in our study that could be improved by including the panel in the future studies. Unlike tracking changes in genetic mutation using anterior chamber tap, serial follow-up of the genetic mutation using vitreal NGS analysis may be limited owing to the invasive nature of vitreous biopsy. In the future, a comparison of NGS data from vitreous, CNS, or other extraocular sites of a patient using the same NGS panel, could advance our knowledge regarding clonal diversification among anatomic regions and provide new information in overcoming potential sources of resistance in this disease.

To the best of our knowledge, this is the largest study for the detection of gene mutation using the NGS gene panel from the vitreous of patients with VRL. Among conventional diagnostic tests for VRL, NGS of the vitreous liquid biopsy demonstrated the highest sensitivity. VRL and CNSL appeared to have both shared and distinct genetic variations that may suggest site-specific variations from a common origin.

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