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Deep Sequencing as a Diagnostic Tool in Patients with Suspected Primary Vitreoretinal Lymphoma

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Abstract

Purpose: To compare the diagnostic utility of metagenomic deep sequencing (MDS) to cytology, flow cytometry, and gene rearrangement by polymerase chain reaction (PCR) in ocular samples of patients with suspected vitreoretinal lymphoma (VRL).

Methods: Patients with suspected VRL underwent ocular sampling of one or both eyes at the Emory Eye Center from September 2017 to June 2022. Ocular samples were evaluated with MDS and conventional diagnostics. MDS was performed at the Ralph and Sophie Heintz Laboratory at the F.I. Proctor Foundation. Relevant demographic and clinical data were retrospectively collected from medical records. Patients were diagnosed with VRL based on clinical assessment and conventional diagnostic testing.

Results: This study included 13 patients with suspected VRL who underwent diagnostic vitrectomy, including 1 patient who had an additional subretinal biopsy. Six patients (46.2%) were diagnosed with VRL. Among patients diagnosed with VRL, MDS detected pathogenic mutations in 5 out of 6 patients (83.3%) while cytology was positive for VRL in 4 out of 6 patients (66.7%), flow cytometry in 4 out of 4 patients (100.0%), and PCR in 4 out of 4 patients (100.0%). MDS detected mutations in *MYD88* in 2 out of 6 patients diagnosed with VRL. In 7 patients (53.8%) not diagnosed with VRL, MDS detected pathogenic lymphoma mutations in 2 patients (28.6%).

Discussion: MDS detected pathogenic mutations in 5 out of 6 patients diagnosed with VRL, including in 2 patients with negative cytology, demonstrating its potential to improve diagnostic rates of VRL as an adjunctive test.

Synopsis/Precis:

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Ethical Statement: Informed consent was obtained from all human subjects according to the tenets of the Declaration of Helsinki

Competing Interests: None declared

MDS detected pathogenic mutations related to lymphoma in most patients who were diagnosed with VRL, highlighting its potential to improve VRL detection as an adjunctive diagnostic tool.

Introduction

Vitreoretinal lymphoma (VRL), also known as primary intraocular lymphoma, is a subset of primary central nervous system lymphoma (PCNSL) that has an annual incidence estimated at 0.4 per 100,000 persons.[1, 2] The majority of VRL is high grade and of non-Hodgkin's diffuse large B-cell type (DLBCL).[3] Patients with VRL frequently present with blurred or decreased vision and floaters and have vitritis, subretinal infiltrates, or panuveitis on exam.[3, 4] VRL often confers a guarded prognosis with a progression-free-survival of about 30 months and overall survival of 58 months after diagnosis. Central nervous system (CNS) involvement is the leading cause of mortality in patients with VRL and is found in 15% of patients at diagnosis and over 60% of patients with relapse.[5] However, the diagnosis of VRL is challenging and often delayed due to protean ocular manifestations that masquerade as chronic uveitis and the limitations of diagnostic testing on ocular fluid including limited sample volume and cellularity for cytopathology and flow cytometry.

Current diagnostic methods for VRL involve obtaining ocular fluid or tissue through anterior chamber (AC) paracentesis, pars plana vitrectomy, or chorioretinal biopsy, followed by cytology, which is often considered the gold standard for the diagnosis of VRL.[6] As most VRL is of DLBCL histologic subtype, lymphomatous cells that appear as large, atypical lymphocytes with minimal cytoplasm, large and irregular hyperchromic nuclei, and prominent nucleoli on cytologic evaluation confirm the diagnosis of VRL.[7–9] However, the absence of lymphomatous cells on cytology does not rule out VRL and requires further investigation with adjunctive tests. Flow cytometry can detect cell surface markers for monoclonal B- or T-cells and skewed expression of immunoglobulin kappa (IgK) or lambda (IgL) light chains that indicates a monoclonal B-cell population.[10, 11] The ratio of interleukin-10 (IL-10), a cytokine that promotes the proliferation of B-cell lymphoma, and IL-6, a proinflammatory cytokine, may help to differentiate VRL from an inflammatory process.[12, 13] Molecular testing with PCR can identify B-cell and T-cell receptor clonality using a lower number of cells compared to conventional diagnostic tools.[14] PCR can also detect the L265P mutation in the myeloid differentiation primary response 88 (MYD88) gene that is present in 69% to 82.4% in patients with VRL.[15, 16]

Metagenomic deep sequencing (MDS) is an unbiased high-throughput deep sequencing, or next-generation sequencing (NGS) method that utilizes techniques of metagenomics to characterize the host and microbial genome.[17] MDS has been used to successfully detect pathogens in various ocular inflammatory diseases, such as conjunctivitis, endophthalmitis, and uveitis.[18–20] Recently, MDS has been used not only to detect pathogens but also mutations associated with lymphoma in patients with VRL, demonstrating its potential as an adjunctive test to improve diagnostic rates in VRL.[21]

In this study, patients with suspected VRL underwent ocular sampling with diagnostic vitrectomy, including 1 patient who underwent a subretinal biopsy. The ocular samples were evaluated with MDS, as well as traditional testing including cytopathology, flow cytometry

and/or PCR when clinically indicated. The purpose of this study was to compare the results and diagnostic rates of MDS to that of existing diagnostic tests and the potential utility of MDS for the diagnosis of VRL. We hypothesized that MDS would improve the diagnostic rates in patients suspected with VRL.

Methods

Patient recruitment and data collection

Institutional review board approval for this study was obtained at Emory University (IRB#00107236). Our research adhered to the tenets of the Declaration of Helsinki. Eligibility criteria included the diagnosis of intermediate, posterior, or panuveitis of unknown etiology requiring ocular sampling for suspected VRL. Patients from the Emory Eye Center were identified based on their clinical presentation, consecutively selected, and prospectively enrolled for assessment of ocular sample with conventional diagnostics and MDS from September 2017 to June 2022. All patients consented and underwent diagnostic vitrectomy, including 1 patient who had an additional subretinal biopsy.

Data were retrospectively collected and included demographics, medical history, ophthalmic exam, relevant laboratory tests and imaging and ophthalmic diagnostic tests, including MDS, cytology, flow cytometry, *immunoglobulin heavy chain (IgH/IgK)* and *T-cell receptor (TCR)* gene rearrangement on PCR. Treatment and long-term follow-up data were collected in patients who were diagnosed with VRL until their last known survival date.

Diagnosis of vitreoretinal lymphoma

All patients underwent comprehensive work-up to rule out infectious and noninfectious causes of uveitis. PCR was performed on vitreous samples obtained during vitrectomy if there was suspicion for infectious uveitis caused by cytomegalovirus, herpes simplex virus, varicella zoster virus or toxoplasmosis.

All ocular samples included in the study underwent cytologic evaluation. Adjunctive testing, including flow cytometry and/or PCR, was ordered in consultation with the ocular pathologist who had access to the patient's clinical information. MDS was performed in leftover ocular samples not used for cytology and/or adjunctive testing (approximately 20–50 μL). Patients were diagnosed with VRL based on their past medical history, clinical presentation, ophthalmic examination, and results of cytology with or without adjunctive testing. The positivity rate for each diagnostic test was calculated in patients diagnosed with VRL and not diagnosed with VRL. Indeterminate or missing data on diagnostic testing were excluded from calculations of diagnostic rates.

Metagenomic Deep Sequencing (MDS)

De-identified ocular samples were sent for MDS to the Ralph and Sophie Heintz Laboratory at F.I. Proctor Foundation, University of California San Francisco. Samples were prepared for pathogen identification as there was a concern for infectious etiology. Instead of interrogating the non-host reads (discarding host-reads) for pathogens, the host-reads (discarding non-host reads) were interrogated for potential lymphomatous mutations.

Briefly, RNA was extracted from 20 to 50μL of ocular fluid with the ZR-Duet extraction kit (Zymo Research, California, USA)and eluted in 20 μL of nuclease-free water per manufacturer's instructions. Sequencing libraries were prepared using the NEBNext RNA Ultra II kit (New England Biolabs) and then amplified with 20 cycles. Pooled libraries were sequenced on the Illumina sequencing platform (HiSeq 4000 or NovaSeq 6000) 150-nucleotide (nt) paired-end sequencing and analyzed using the RNAseq short variant discovery (SNP and Indels) pipeline using GATK tools and GATK best practices to call variants related to lymphoma against the Curated Online Somatic Mutations in Cancer Database version 89 (downloaded August 2019).[22, 23] In an additional step, filtered human sequences that aligned to the *MYD88* gene (NC_000003.12) using HISAT2 (V.2.0.5) were visually inspected and verified using Geneious (Auckland, New Zealand). A mutation is labeled as "pathogenic" based on the FATHMM prediction score as documented by the COSMIC database.

Results

Demographic and clinical characteristics

Diagnostic vitrectomy was performed in 17 eyes of 13 patients with suspected VRL, including 1 patient who underwent vitrectomy with concomitant subretinal biopsy (Table 1). The cohort included 7 females (53.8%) and the mean age was 69.5 years (SD = 9.4). Common presenting ocular symptoms included blurred or decreased vision (70.6%), floaters (47.1%) and eye pain or soreness (29.4%). The most common type of ocular inflammation was panuveitis (58.8%), followed by intermediate uveitis (29.4%) and anterior/intermediate uveitis (11.8%). One patient presented with an exudative retinal detachment. Notably, 8 patients had a history of B- or T-cell lymphoma, including 4 patients with PCNSL. One patient had a history of HIV/AIDS. Serologic work-up for infectious and noninfectious causes of uveitis was negative in all patients. MDS was also unable to identify an infectious etiology for all enrolled patients.

Diagnostic testing results in patients diagnosed with VRL

Six out of 13 patients (46.2%) were diagnosed with VRL based on clinical evaluation and diagnostic testing (Table 2). Four out of 6 patients (66.7%) diagnosed with VRL had a history of PCNSL, and 1 patient was diagnosed with DLBCL on a submandibular biopsy while undergoing workup for VRL. All 8 ocular samples from 6 patients with VRL underwent cytologic evaluation and MDS, but flow cytometry and PCR were each performed in 4 samples from 4 patients after consultation with the ocular pathologist.

Cytologic evaluation was positive for VRL in 4 out of 6 patients (66.7%), flow cytometry in 4 out of 4 patients (100.0%) and PCR for gene rearrangement in 4 out of 4 patients (100.0%). MDS detected pathogenic mutations in 5 out of 6 patients (83.3%) diagnosed with VRL. This included patient 2 who had negative cytology tests but was diagnosed with VRL based clinical suspicion and a history of PCNSL. Patient 5 also had negative cytology but was positive on PCR for gene rearrangement. Flow cytometry was not performed in either patient due to limited quantity of sample with cells.

Diagnostic testing results in patients not diagnosed with VRL

Seven patients (53.8%) were not diagnosed with VRL (Table 2). A total of 9 ocular samples from 7 patients who were not diagnosed with VRL underwent cytologic evaluation and MDS. After discussion with the ocular pathologist, PCR was only performed in 2 samples from 2 patients and flow cytometry was not performed in any samples. Cytology did not detect lymphomatous cells in any patients. PCR detected gene rearrangement in 1 out of 2 patients (50.0%) and MDS detected pathogenic mutations in 2 out 7 patients (28.6%).

Patient 7 had pathogenic mutations on MDS and was positive for *TCR* gene rearrangement on PCR, but this was determined to be related to the patient's history of T-cell lymphoma as she responded to topical corticosteroid treatment without further ocular disease. Patient 8 had pathogenic mutations on MDS but was not diagnosed with VRL due to low clinical suspicion and negative cytology.

Pathogenic mutations detected by MDS

MDS identified 27 pathogenic mutations in 23 genes in patients with suspected VRL. Out of 27 mutations, 25 mutations were identified as originating from tissue with DLBCL histology (Supplementary Table 1). Pathogenic mutations identified by MDS in two or more patients with suspected VRL are listed in Table 3. Three patients had mutations in *SMG6* and *SNW1* and 2 patients in *MYD88, PDHX, SDHA, TMEM214,* and *TNFAIP3*. Mutations in *MYD88* were only identified from two samples and included c.794T>C (p.Leu265Pro) in Patient 1 (Figure 1) and c.2369C>T (p.Thr790Met) in Patient 4.

Visual acuity in the entire cohort

At the baseline visit, the median visual acuity (VA) in the biopsied eye was 0.30 logMAR (Snellen VA 20/40) in the entire cohort, 0.24 logMAR (Snellen VA 20/35) in patients diagnosed with VRL, and 0.30 logMAR (Snellen VA 20/40) in patients not diagnosed with VRL (Table 4). Follow-up visit at 4 −7 months was available in 7 patients from the entire cohort, in which median VA was logMAR 0.30 (Snellen 20/40). The median VA was 0.30 logMAR (Snellen VA 20/40) in 4 patients diagnosed with VRL and 0.54 logMAR (Snellen VA 20/70) in 3 patients not diagnosed with VRL. Follow-up visit at 10–14 months was available in 8 patients from the entire cohort, in which median VA was 0.30 logMAR (Snellen VA 20/40). The median VA was 0.1 logMAR (Snellen VA 20/50) in 4 patients diagnosed with VRL and 0.48 logMAR (Snellen VA 20/60) in 4 patients not diagnosed with VRL.

Treatment and clinical outcomes in patients diagnosed with VRL

Four patients received systemic chemotherapy, 1 patient received local therapy only, and 1 patient was lost to follow-up. Systemic chemotherapy included high-dose methotrexate and rituximab in all 4 patients. Three patients additionally received temozolomide and 1 patient additionally received cyclophosphamide, doxorubicin, and vincristine. Local therapy included prednisolone or difluprednate drops and intravitreal methotrexate. In 5 patients who had follow-up, all patients received local therapy with intravitreal methotrexate, which were administered an average of 5 times (SD = 3.20) per eye. In the patient that received only local therapy, bilateral injections were given.

Long term, one patient was lost to follow-up. Patients 1 and 4 followed at the Emory Eye Center and had overall survivals of 4.3 years and 2.5 years, respectively. Recurrence of VRL and/or CNS involvement occurred in 4 out of 6 patients (66.7%) who had a progression-free survival of 13.5 months (SD = 5.0).

Discussion

This study showed that MDS can detect pathogenic mutations associated with VRL in patients using vitreous and subretinal biopsy samples. MDS notably detected pathogenic mutations in two patients with negative cytology. PCR detected VRL from the sample of one of these patients, but no adjunctive testing was ordered for the sample from the second patient, which made MDS the only adjunctive test that supported the clinical diagnosis of VRL.

In patient 4, cytology and MDS detected pathogenic mutations in the subretinal biopsy but not from the vitreous in either eye. This case illustrates how the sparsity of lymphomatous cells in the vitreous may lead to negative results on cytology. Cytologic evaluation is often limited by the amount of well-preserved lymphoma cells in the ocular sample. In addition, the sensitivity of cytologic evaluation varies widely with the training of the cytopathologist and has been reported to be as low as 31% when performed by a general pathologist instead of an ocular pathologist.[24] Adjunctive testing is often used to increase the diagnostic yield, but is often not possible due to the limited quantity of ocular sample.

Adjunctive testing was ordered in consultation with the ocular pathologist was based on the cellularity and quantity of the ocular sample and clinical suspicion for detecting VRL. Flow cytometry detected monoclonal B-cells in all the samples that were tested (100.0%) but was unable to be performed in 9 out of 13 patients (69.2%) due to insufficient quantity of cells in the ocular samples. A relatively large volume of ocular sample is required for flow cytometry, which is a major limitation in diagnosing VRL. PCR requires a lower number of cells than cytology or flow cytometry but was not ordered in patients with lower clinical suspicion for VRL. Although PCR detected VRL in 4 out of 4 patients (100.0%) diagnosed with VRL, but the true diagnostic rate is unclear since it was not performed in all patients.

After a comprehensive work-up, 7 patients were not diagnosed with VRL. MDS detected pathogenic mutations in 2 out 7 patients who were not diagnosed with VRL. The pathogenic mutations detected by MDS in one of the patients was likely related to a previously diagnosed and treated T-cell lymphoma, consistent with a TCR gene rearrangement identified by PCR. This highlights the importance of interpreting MDS results in the context of the patient's entire clinical history as pathogenic mutations may be associated with previously treated or non-ocular malignancies. MDS detected mutations of unclear significance in a second patient who did not have a history of malignancy and had negative conventional diagnostic testing. Although a diagnosis of VRL could not be made due insufficient data, it is possible that this patient had VRL that was below the threshold of detection by clinical examination and conventional diagnostics. The implications of the mutations in genes other than in *MYD88* are not yet clear. More studies are required to

understand whether pathogenic mutations in patients without clinical manifestations of VRL represent occult or impending development of VRL.

Limitations of this study include the small sample size, enrollment from a single study site, and the lack of uniform work-up for all patients. Due to the retrospective design of the study, follow-up in this patient population was variable. In addition, variant calling analysis was performed on RNA-seq results from sequencing libraries optimized for pathogen detection which then was leveraged for mutation analysis. Thus, it is likely that future laboratory, sequencing, and analysis protocols oriented to mutation identification will only improve detection.

MDS requires only 20 to 50 μL of ocular fluid or tissue without whole cells and can concurrently detect infectious causes of uveitis that can present similarly to VRL.[6] This was demonstrated in our previous study that detected Epstein-Barr virus (EBV) and human herpes virus 8 (HHV-8) and a mutation in *MYD88* from the aqueous fluid of patients with VRL.[21] Another advantage of MDS is that it can be performed on aqueous fluid, which was found to have genomic profiles that are highly concordant to vitreous fluid in patients with VRL based on targeted next-generation sequencing.[25] Although MDS is not sufficient currently as a standalone test and may require several years to become widely accessible and affordable, it is a useful adjunctive test that detected VRL in patients missed by cytology. In addition, MDS has the potential to lower the barrier to diagnostic testing by eliminating the need for surgical ocular sampling in the future and provides genetic information that could have implications for disease classification, risk stratification, and treatment response and outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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What is already known on this topic –

One of the reasons the diagnosis of VRL is challenging is the lack of a true gold standard diagnostic testing. Cytology is highly specific for VRL but has low or variable sensitivity, which necessitates adjunctive testing, such as flow cytometry or PCR to improve diagnostic rates in VRL.

What this study adds –

In this study, MDS detected pathogenic mutations in 5 out 6 patients diagnosed with VRL, including in 2 patients who were clinically diagnosed but had negative cytology. This shows that MDS has the potential to capture patients with VRL who may have negative results on conventional testing. The study also demonstrates the application of MDS beyond that of identifying pathogens in infectious uveitis.

How this study might affect research, practice, or policy –

The promising results of MDS in patients diagnosed with VRL in this study may influence other studies to investigate deep sequencing as an adjunctive diagnostic tool for VRL.

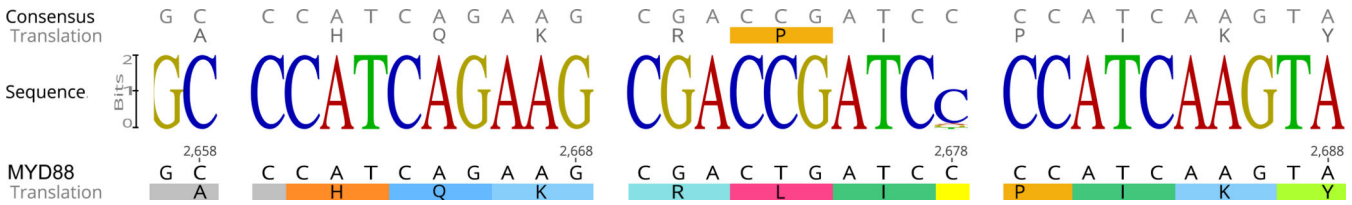

Figure 1.

Deep sequencing on the vitreous sample from patient 1 revealed a pathogenic mutation in *MYD88* gene that replaces leucine with proline at codon 265.

Table 1.

Demographic and clinical characteristics of patients with suspected VRL

Characteristics	Patients (n = 13)
Age, years (mean ± SD)	69.5 ± 9.4
Female, n (%)	7 (53.8)
Race, n (%)	
Caucasian	7 (53.8)
Unknown	5 (38.5)
African American	1 (7.7)
Past medical history, n (%)	
PCNSL	4 (30.8%)
Other B-cell lymphoma	3 (23.1%)
T-cell lymphoma	1 (7.7%)
HIV/AIDS	1 (7.7%)
	Eyes (n = 17)
Initial eye symptoms *, n (%)	
Blurred/decreased vision	12 (70.6)
Floaters	8 (47.1)
Eye pain/soreness	5 (29.4)
Flashes	3 (17.6)
Type of uveitis, n (%)	
Panuveitis	10 (58.8)
Intermediate uveitis	5 (29.4)
Anterior/intermediate uveitis	2 (11.8)

VRL = vitreoretinal lymphoma, SD = standard deviation, PCNSL = primary central nervous system lymphoma, HIV = human immunodeficiency virus, AIDS = acquired immune deficiency syndrome

* Percentages do not add up to 100 as patient could have 1 or more eye symptoms

Table 2.

Diagnostic testing in patients with suspected VRL

Patient	Eye	PMH	Lymphoma cells *cytology*	Monoclonality *flow cytometry*	Rearrangement *PCR*	Number of pathogenic mutations *MDS*
Patients diagnosed with VRL						
1	OS	PCNSL	Positive	Positive	Positive	7
2	OS	PCNSL	Negative	Not performed	Not performed	6
3	OD	PCNSL	Positive	Positive	Positive	4
4	OD	None	Positive	Positive	Positive	2
4	OS		Negative	Not performed	Not performed	0
5	OD	PCNSL	Negative	Not performed	Positive	1
6	OD	None	Positive	Not performed	Not performed	0
6	OS		Negative	Positive	Not performed	0
Patients not diagnosed with VRL						
7	OD	T-cell lymphoma	Negative	Not performed	Positive	10
8	OD	None	Negative	Not performed	Not performed	2
8	OS		Negative	Not performed	Not performed	0
9	OS	None	Negative	Not performed	Negative	0
10	OD	Hodgkin's lymphoma, melanoma	Negative	Not performed	Not performed	0
11	OD	Tonsillar B-cell	Negative	Not performed	Not performed	0
11	OS	lymphoma	Negative	Not performed	Not performed	0
12	OD	None	Negative	Not performed	Not performed	0
13	OD	HIV/AIDS	Negative	Not performed	Not performed	0

VRL = vitreoretinal lymphoma, OD = right eye, OS = left eye, PCR = polymerase chain reaction, MDS = metagenomic deep sequencing

Table 3.

Genes with pathogenic mutations on MDS in 2 or more patients with suspected VRL

Gene	Official full name	Function
SMG6	SMG6 nonsense mediated mRNA decay factor	Encodes a protein involved in nonsense-mediated mRNA decay pathway, a post-transcriptional surveillance process that degrades mRNA with premature termination codons
SNW1	SNW domain containing 1	Encodes a protein that is transcriptional coactivator and a splicing factor. The protein is known to interact with the SKI oncogene and may be involved in oncogenesis
MYD88	Myeloid differentiation primary response gene 88	Encodes a cytosolic adapter protein plays a central role in the innate and adaptive immune response and enables interleukin-1 and Toll-like receptor pathways
PDHX	Pyruvate dehydrogenase complex component X	Encodes a non-catalytic subunit, E3 binding protein, of the pyruvate dehydrogenase complex that converts pyruvate to acetyl coenzyme A
SDHA	Succinate dehydrogenase complex flavoprotein subunit A	Encodes a major catalytic subunit of succinate-ubiquinone oxidoreductase that is part of the mitochondrial respiratory chain
TMEM214	Transmembrane protein 214	Encodes a protein that is possibly involved in apoptosis
TNFAIP3	Tumor necrosis factor alpha induced protein 3	Encodes a protein that is a zinc-finger protein and a ubiquitin-editing enzyme, involved in NF-kB activation and TNF-mediated apoptosis

VRL = vitreoretinal lymphoma, mRNA = messenger ribonucleic acid, NF-kB = nuclear factor kappa B, TNF = tumor necrosis factor

Table 4.

Visual acuity at baseline and follow-up visits in patients with suspected VRL

LogMar Va, median (IQR)	Overall	Confirmed VRL	Unconfirmed VRL
Baseline visit	*N=13* 0.30 (1.82)	*N= 6* 0.24 (1.40)	*N=7* 0.30 (0.39)
Follow-up at 4–7 months	*N=7* 0.30 (0.92)	*N=4* 0.30 (0.40)	*N=3* 0.54 (0.95)
Follow-up at 10–14 months	*N= 8* 0.30 (0.38)	*N=4* 0.10 (0.05)	*N=4* 0.48 (0.63)

VRL = vitreoretinal lymphoma, logMAR = logarithmic minimum angle of resolution, Va = visual acuity, IQR = interquartile range, N = number of patients