• Basic Research •

Clinical utility of cytokine analysis in the diagnosis and efficacy monitoring of vitreoretinal lymphoma

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Abstract

● **AIM:** To investigate the value of cytokine analysis in aqueous humor (AH) for discriminating vitreoretinal lymphoma (VRL) from uveitis and for evaluating the efficacy of intravitreal methotrexate (MTX) injections.

● **METHODS:** This retrospective study was done on 28 VRL patients between 2013 and 2019. AH interleukin (IL)-10, IL-6, IL-8, vascular endothelial growth factor (VEGF), and vascular cell adhesion molecules (VCAM) were measured in 28 VRL patients and 38 uveitis patients. As to the respective examinations for distinguishing VRL from uveitis, the diagnostic accuracy was evaluated by receiver operating characteristic (ROC) curve analysis. The response to treatment was monitored by observing changes in best-corrected visual acuity (BCVA), ocular manifestation, and AH cytokine levels in 21 patients with VRL who had undergo multiple intravitreal injections of MTX.

● **RESULTS:** Compared with uveitis patients, VRL patients had higher IL-10 level (P<0.001) and IL-10/IL-6 ratio (P<0.001), whereas patients with uveitis had significantly higher IL-6 level than those with VRL (P=0.003). An ROC analysis was used to identify the diagnostic threshold values for VRL, and it was found that optimal sensitivity and specificity improved to 94.1% and 100%, respectively, for IL-10/IL-6>1.55 and 88.2% and 81.1%, respectively, for IL-10>76.7 pg/mL. In 21 patients who had undergo repeated injections, improvements in BCVA, clinical remission of VRL and continuous decrease in cytokine levels over time were observed. In those patients, the BCVA correlated with the aqueous levels of IL-10 and IL-6 during the course of disease treatment.

● **CONCLUSION:** The combination of the aqueous cytokine profiles can be instrumental for conventional diagnostic methods and for progression monitoring and treatment response.

● **KEYWORDS:** vitreoretinal lymphoma; intraocular lymphoma; primary central nervous system lymphoma; cytokine; methotrexate

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INTRODUCTION

Primary vitreoretinal lymphoma (PVRL) is a subgroup of primary central nervous system lymphoma (PCNSL) that mainly affects the vitreous and retina of the eye. PVRL is a relatively rare disease, but the most common form of intraocular lymphoma[1]. Vitreoretinal lymphoma (VRL) is a B-cell non-Hodgkin lymphoma in 95% of patients, mainly diffusing the large B-cell lymphoma (DLBCL) subtype. PVRL often masquerades as chronic uveitis, which is always manifest as vitritis and/or alone posterior uveitis, and therefore PVRL may be associated with diagnostic delay and misdiagnosis[2]. Early accurate diagnosis should be given not only for PVRL, but more importantly for the explicit goal of ameliorating the overall PCNSL-surviving rate. Some studies reported that 69%-90% of PVRL patients gradually fall victim to central nervous system (CNS) lymphoma in the following 29mo[3-4], with a considerable morbidity and a high mortality[5]. At present, PVRL remains a disease of poor prognosis. In general, owing to delays in diagnosis and lack of appropriate therapies, less than 25% of the patients will be alive after five years of progression[6].

The gold standard for PVRL diagnosis is cytological examination of vitreous. However, PVRL vitreous cytologic diagnosis is difficult because of the technical difficulties in preserving vitreous specimens, the effect of corticosteroids administered, the high vulnerability of lymphoma cells, and the high dependence on skilled cytopathologists, *etc*[7]. Therefore, several supplementary diagnostic methods, such as those based on cytokine level analysis, molecular analysis, flow cytometry, immunohistochemistry, and polymerase chain reaction (PCR), are currently used to assist the clinical diagnosis of VRL[8-10].

Among previous literatures that compared diagnostic value of cytology, PCR and cytokines, cytokine analysis has been regarded as the most sensitive method to diagnose VRL[11-13]. Secreted by malignant B lymphocytes, interleukin (IL)-10 further promotes the proliferation of these cells in intraocular lymphoma and CNS lymphoma[14]. IL-6 levels are associated with nonmalignant intraocular inflammation, and multiple studies have shown that the IL-10/IL-6 ratio greater than 1.0 can be used as a diagnostic tool for lymphoma[15]. In a word, cytokine analysis was superior to conventional procedures used in screening and diagnosing PVRL patients.

In this retrospective study, the clinical features and intraocular cytokine profiles of VRL in 41 eyes (28 patients) were investigated, with special attention paid to the diagnostic value of IL-10 levels and IL-10/IL-6 ratio in VRL patients. The therapeutic efficacy of intravitreal methotrexate (MTX) injection was also monitored by combining best-corrected visual acuity (BCVA), ocular manifestation and changes of intraocular cytokines. The results may be clinically helpful to give early and accurate diagnosis of PVRL, and to guide the further development of more precise and personalized therapeutic strategies.

SUBJECTS AND METHODS

Ethical Approval The Institutional Review Board approved this retrospective study (No.2018-4-3-3). The vitreous biopsy and its purpose were explained to all the patients and their informed consent was obtained. It was only after securing their informed consent and the Ethics Committee's approval that the aqueous humor (AH) samples of all the patients were obtained. All procedures were conducted in accordance with the principles of the Declaration of Helsinki.

Patients and Samples A total of 28 patients suffering from unilateral or bilateral VRL were recruited from the Ophthalmology Department of the Capital Medical University affiliated Beijing Chaoyang Hospital between April 2013 and August 2019. A retrospective study was performed consequently. Clinical data (including demographic data, medical history, course of disease from onset to diagnosis, ocular clinical manifestations, ocular complications, *etc*), imaging results, and laboratory examination results of each patient were reviewed in detail. The uveitis group consisted of 38 patients with diagnosed uveitis [16 acute retinal necrosis (ARN) patients[16], 16 Fuchs' uveitis syndrome patients[17], and 6 Vogt-Koyanagi-Harada (VKH) disease patients[18]]. There was no evidence of VRL or CNS lymphoma in those patients diagnosed with uveitis.

None of the VRL patients had immunodeficiency, human immunodeficiency virus-positive or T-cell lymphoma. During the follow-up, all patients with primary ocular lesions underwent cranial magnetic resonance imaging (MRI) and/or computed tomography (CT) scans to detect CNS involvement.

The diagnosis was determined by a comprehensive analysis of the patients' typical clinical manifestations, the results of the vitreal and AH samples, and the history of a PCNSL. VRL was not diagnosed until the clinical suspicion was confirmed according to vitreal or brain cytology. A combination of clinical and radiologic (MRI/CT) findings, which were confirmed by a brain biopsy, helped the neuro-oncology team achieve the diagnosis of PCNSL. The vitreous samples were collected by diagnostic vitrectomy. The vitreous samples were centrifuged and the concentrated cells were then submitted for histopathological examination. Parts of additional vitreous samples obtained by vitreous cutters were used for IgH gene rearrangement analysis.

All suspected VRL patients were diagnosed and treatment began within one month. All VRL patients were treated by intravitreal injection of MTX. After topical anaesthesia with opulvacaine hydrochloride drops, 400 mg/0.1 mL of MTX was injected at the level of the pars plana with a 30-gauge needle. At each follow-up after treatment, the ophthalmologic examination included measurements of BCVA and intraocular pressure (IOP), inflammatory responses in the anterior chamber and vitreous cavity, and malignant cellular infiltration in the retina and/or optic nerve head. BCVA was tested by using a Snellen chart, and transformed logarithm of the minimum angle of resolution (logMAR) values[19].

Treatment was decided by the comprehensive assessment of an experienced ophthalmologist individually according to the clinical symptoms, ocular manifestation, ophthalmologic imaging and intraocular cytokine levels. We monitored changes in BCVA, IOP, anterior chamber inflammatory response, and intraocular cytokine levels before and one week after each intravitreal MTX injection.

Aqueous Humor Cytokine Analysis All patients underwent anterior chamber paracentesis (ACP) during or before surgery and 100 μL of AH was extracted for cytokine measurement. All AH samples were immediately stored at 4°C and brought to the laboratory for cytokine and cell analysis. AH cytokines [IL-10, IL-6, IL-8, vascular endothelial growth factor (VEGF) and vascular cell adhesion molecules (VCAM)] were examined by using CBA with BD FACSCantoII flow cytometry.

Statistical Analysis The distribution of cytokine levels between VRL and uveitis were compared by using a non-parametric analysis (Mann-Whitney *U* tests). The sensitivity and the specificity of cytokines IL-10, IL-6, IL-8, VEGF, VCAM, and IL-10/IL-6 ratio in the diagnosis of VRL were illustrated by using the receiver operator characteristic (ROC) curves. Moreover, the area under the ROC curve (AUC) was calculated and the AUC of more than 0.7 units was considered to be positive. The Youden index defined as (sensitivity) + (specificity) -1, was used as a simple descriptive synthesis of

sensitivity and specificity. Statistical comparisons between before and after treatment were performed by the Wilcoxon signed rank sum test. The Spearman rank correlation coefficient was used for the analyses of the correlations between logMAR BCVA and cytokines in those patients who had undergone three or more injections.

RESULTS

Demographic Data and Clinical Characteristics A total of 28 patients (41 eyes) diagnosed with VRL were included in the study. The demographic data and clinical characteristics of the 28 patients were summarized in Table 1. All the 28 PVRL patients had VRL of the B cell type. The average age at disease onset of VRL was 54.6y (range 34-81y) and this was similar in the male and female patients (P=0.306). The majority of the patients (20/28, 71.4%) were female, and the ratio of male to female is 1:2.5. During the observation period of the study, 13 of the 28 patients (46.4%) had VRL in one eye and 15 patients (53.6%) had bilateral eyes involved. Of the 28 patients, 21 (75.0%) manifested primary intraocular lymphoma first, whereas 7 patients (25.0%) developed PCNSL before onset of the ocular lesion. During the observation period, 18 patients (64.3%) suffered two-eye and CNS lesions, and 3 patients (10.7%) suffered damages of both eyes, the CNS, and other organs. The organs involved in these patients were the neck lymph nodes in 1 case, the mammary lymph nodes in 1 case, and the testicles in 1 case. The most common initial ocular symptoms were, in descending order, blurred vision or reduced vision (26/28, 92.9%), floater symptom (10/28, 35.7%), flashing lights (3/28, 10.7%), and red/sore eye (3/28, 10.7%). Among the 11 samples in which IgH gene rearrangements were performed, 8 patients (72.7%) had positive IgH results. Eleven eyes (26.8%) showed prominences of retinal pigment epithelium (RPE) and hyper-reflective deposits under RPE on optical coherence tomography (OCT) images.

All the patients were examined for visual function during their first presentation and the follow-up period (Table 2). The mean logMAR BCVA was 1.06±0.88 (range: 0-2.7) at the first visit of the 41 eyes involved. Most VRL patients had significant visual impairment at the first presentation, with 17 eyes (41.5%) showing severe visual impairment (BCVA≤0.1), 15 eyes (36.6%) had moderate visual impairment (BCVA: 0.1-0.4), and 9 eyes (21.9%) had mild visual impairment (BCVA≥0.5). The occurrence of keratic precipitates (KP) was recorded in 20 eyes (48.8%). The mean aqueous flare was 19.4±28.0 (range 3.2-150.2) in VRL patients, and anterior chamber cells were observed in 17 (41.4%) eyes. Vitreous opacification was the most common initial ocular manifestations, found in all of the patients (100.0%); this was followed by inflammatory cells in vitreous in 35 eyes (85.4%), subretinal infiltration in 15 eyes (36.6%), retinal vasculitis in 9 eyes (21.9%), retinal detachment in 5 eyes (12.2%), optic neuropathy in 4 cases (9.8%), and other vitreous retinopathy in 3 cases (7.3%).

Table 1 Clinical characteristics of patients with VRL *n* (%)

Items	Data
Number of patients (eyes involved)	28 patients (41 eyes)
Age (y, mean±SD)	54.6±11.9
Gender	
Male	8 (28.6)
Female	20 (71.4)
One-eye or two-eye cases	
One-eye cases	15 (53.6)
Two-eye cases	13 (46.4)
Onset patterns	
Primary intraocular lymphoma	21 (75.0)
Primary CNS lymphoma	7 (25.0)
Organs involved	
Eye and CNS	18 (64.3)
Eye, CNS and other organ systems	3 (10.7)
Symptoms	
Blurred or reduced vision	26 (92.9)
Floater symptom	10 (35.7)
Flashing lights	3 (10.7)
Red and sore eyes	3 (10.7)

VRL: Vitreoretinal lymphoma; CNS: Central nervous system; SD: Standard deviation.

Table 2 Ocular manifestations of VRL *n* (%)

Items	Data
Number of patients (eyes involved)	28 patients (41 eyes)
BCVA at initial manifestation	
≤0.1	17 (41.5)
0.1-0.4	15 (36.6)
≥0.5	9 (21.9)
logMAR initial BCVA, mean±SD	1.06±0.88
IOP (mm Hg), mean±SD	16.7±4.6
Keratic precipitates	20 (48.8)
Aqueous flare, mean±SD	19.4±28.0
Anterior chamber cells	17 (41.4)
Vitreous retinopathy	
Vitreous opacification	41 (100.0)
Inflammatory cells in vitreous	35 (85.4)
Subretinal infiltration	15 (36.6)
Retinal vasculitis	9 (21.9)
Retinal detachment	5 (12.2)
Optic neuropathy	4 (9.8)
Others	3 (7.3)

VRL: Vitreoretinal lymphoma; BCVA: Best-corrected visual acuity; IOP: Intraocular pressure; SD: Standard deviation.

Comparative Cytokine Profiles of Vitreoretinal Lymphoma and uveitis Additionally, cytokine concentration was analyzed to see if a difference in the distribution of IL levels exists

between eyes with VRL and uveitis (Table 3). In the AH of the VRL patients, IL-10 were significantly elevated compared with those of the uveitis patients: the mean IL-10 concentration in the VRL samples was 2378.8 pg/mL (range: 16.2-22329.9) and in the uveitis samples, 47.9 pg/mL (range: 0-302.0; P<0.001). Conversely, the mean IL-6 concentration was lower for the VRL (135.1 pg/mL) than for the uveitis (12094.6 pg/mL) samples (P=0.003). Accordingly, the ratio of IL-10/IL-6 was much higher for the VRL (mean±SD: 51.1±112.2) than for the uveitis (mean±SD: 0.05±0.06) samples (P<0.001). In addition, The VCAM concentration in the patients with uveitis was higher than in the VRL patients (P=0.047). There were no significant differences between the two groups as to AH concentration of IL-8 and VEGF.

Diagnostic Value of Cytokines for Vitreoretinal Lymphoma

In this study, the cytokine profiles concerned were analyzed, including IL-10, IL-6, IL-10/IL-6 ratio, IL-8, VEGF, and VCAM, in AH samples from patients with VRL, and other uveitis patients. ROC curves for IL-10, IL-6, IL-10/IL-6 ratio, IL-8, VEGF and VCAM to distinguish VRL from other uveitis are shown in Figure 1.

IL-10/IL-6 ratio showed the highest diagnostic accuracy with AUC=1.0. The IL-10, IL-6, IL-8, VEGF, and VCAM had diagnostic accuracies of AUC=0.919 (95%CI, 0.849-0.989, P<0.001), 0.679 (95%CI, 0.539-0.819, P=0.035), 0.676 (95%CI, 0.531-0.821, P=0.040), 0.596 (95%CI, 0.444-0.749, P=0.259) and 0.669 (95%CI, 0.528-0.81, P=0.047), respectively (Figure 1). The sensitivity and specificity of IL-10 and IL-10/IL-6 ratio using the optimal cutoff value are shown in Table 4. The sensitivity and specificity at an IL-10 cutoff level of 76.7 pg/mL were 88.2% and 81.1%, respectively (P<0.001). IL-10/IL-6 ratio had the highest sensitivity of 94.1% and specificity of 100.0% with the cutoff value of 1.55 (P<0.001).

Response to Repeated Intravitreal Methotrexate Injections

Of the 28 patients, 21 patients (29 eyes) underwent repeated intravitreal MTX injections. The follow-up time after MTX injection was 2-27mo (median, 11mo). Seventeen patients (81.0%) manifested PVRL first, whereas 4 patients (19.0%) developed primary CNS lymphoma before onset of the intraocular lymphoma. In 6 PVRL patients, during the follow-up period, the ocular involvement either preceded the CNS or was a presenting CNS symptom. The average interval between the onset of ocular and CNS involvement was 8mo (range 3-12mo). The average interval of ocular involvement in these 4 PCNSL patients was 5mo (range 2-10mo). All the CNS lymphoma patients had already completed systemic therapy when intravitreal MTX injections were started; therefore, there is no confounding effect as a result of systemic therapy.

The 21 patients underwent repeated intravitreal MTX injections, with an average of 2.6 times (range 1-13 times). Most patients (89.3%) underwent less than or equal to 3 injections, 2 patients underwent 4 injections, 1 patient underwent 7 injections, and 1 patient underwent 13 injections. Changes of BCVA, IOP, aqueous flare and intraocular cytokines levels before and after the first 3 injections were observed and analyzed (Table 5). BCVA improved in 28 eyes (96.6%) after treatment and remained unchanged in only 1 eye; all affected eyes with improvements of bothersome floaters and blurred

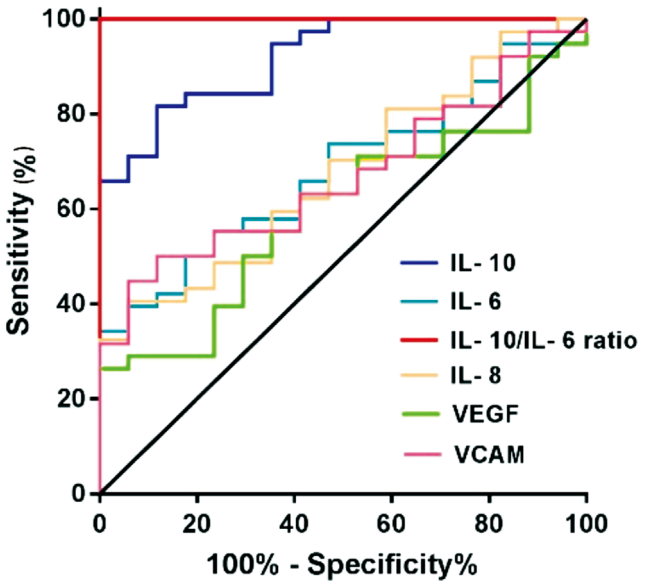

Figure 1 ROC curves for IL-10, IL-6, IL-10/IL-6 ratio, IL-8, VEGF and VCAM for differentiation of VRL from uveitis IL: Interleukin; VEGF: Vascular endothelial growth factor; VCAM: Vascular cell adhesion molecules; ROC: Receiver operating characteristic; VRL: Vitreoretinal lymphoma.

Table 3 Cytokines levels in the AH of VRL and uveitis patients

mean±SD (pg/mL)

Parameters	VRL	Uveitis	P^a
IL-10	2378.8±4790.6	47.9±80.9	<0.001
IL-6	135.1±181.1	12094.6±27015.1	0.003
IL-10/IL-6 ratio	51.1±112.2	0.05±0.06	<0.001
IL-8	135.5±179.5	1829.9±4049.4	0.141
VEGF	37.7±30.5	2800.8±15900.86	0.105
VCAM	2587.8±2498.2	13665.8±21232.8	0.047

AH: Aqueous humor; VRL: Vitreoretinal lymphoma; IL: Interleukin; VEGF: Vascular endothelial cell growth factor; VCAM: Vascular cell adhesion molecules; SD: Standard deviation. [a]Mann-Whitney U test.

Table 4 Sensitivity, specificity and optimal cutoff values of parameters for differentiation of VRL from uveitis

Parameters	AUC	P	Cutoff value	Sensitivity	Specificity
IL-10	0.917	<0.001	76.7 pg/mL	88.2%	81.1%
IL-10/IL-6 ratio	1.000	<0.001	1.55	94.1%	100.0%

VRL: Vitreoretinal lymphoma; AUC: Area under the receiver operating characteristic curve; IL: Interleukin.

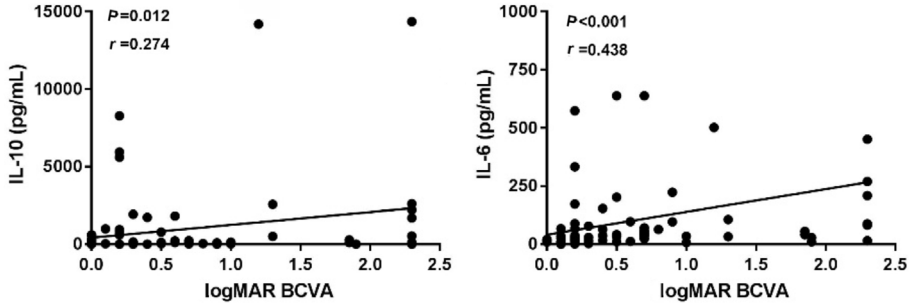

Figure 2 Correlation between the logMAR BCVA and the aqueous cytokines in VRL patients who were received repeated intravitreal MTX injections BCVA: Best-corrected visual acuity; IL: Interleukin; VRL: Vitreoretinal lymphoma; MTX: Methotrexate.

Table 5 Changes of the BCVA, ocular manifestation and aqueous cytokines in VRL patients receiving repeated intravitreal MTX injections

mean±SD

Parameters	1st injection			2nd injection			3rd injection		
	Before	After	P^a	Before	After	P^a	Before	After	P^a
logMAR BCVA	1.08±0.81	0.60±0.66	<0.001	0.68±0.67	0.39±0.53	0.002	0.76±0.88	0.50±0.64	0.008
IOP (mm Hg)	16.8±5.1	15.0±4.1	0.280	15.2±4.2	14.9±2.6	0.867	15.4±4.2	15.8±3.1	0.219
Aqueous flare	20.6±31.6	16.0±24.1	0.484	17.4±17.1	14.9±9.7	0.760	21.0±24.2	15.1±11.9	0.008
Cytokines in AH, pg/mL									
IL-10	2194.8±4759.3	643.3±2128.2	<0.001	765.7±1926.1	363.0±1317.6	0.001	1889.4±3938.9	208.6±510.8	0.013
IL-6	161.6±202.2	190.4±288.5	0.909	205.1±327.2	74.2±102.8	0.064	155.7±339.4	28.6±37.5	0.035
IL-10/IL-6	45.3±116.5	6.97±13.1	<0.001	12.5±23.0	4.8±12.9	0.007	20.2±43.7	4.6±10.8	0.003

BCVA: Best-corrected visual acuity; IOP: Intraocular pressure; AH: Aqueous humor; IL: Interleukin; SD: Standard deviation. [a]Wilcoxon signed rank sum test.

vision. Clinical remission was observed in all affected eyes with absence of cells from vitreous and complete or partial resolution of retinal infiltration. Patients who had undergone repeated intravitreal MTX improved their BCVA from the initial mean BCVA logMAR value of 1.08 to 0.60 after the first injection, from the mean BCVA logMAR value of 0.68 to 0.39 after the second injection, and from the mean BCVA logMAR value of 0.76 to 0.50 after the third injection. There was no significant change in the IOP before and after the three treatments, and the IOP was within the normal range. The aqueous flare decreased after each injection, although no statistically significant differences were found. IL-10, IL-6, and IL-10/IL-6 ratio response to treatment with intravitreal MTX was also summarized in Table 5. Each intravitreal injection was associated with rapid and steady reduction in aqueous IL-10 levels and IL-10/IL-6 ratio. We also found that IL-10 levels rose again after the second injection, suggesting early MTX retreatment. In our observation, IL-6 levels began to decline after the second injection, but no statistical significance was found.

The logMAR BCVA and the AH levels of IL-10, IL-6, and IL-10/IL-6 ratio in VRL patients who had undergone repeated intravitreal MTX injections were collected and the potential correlations between those data were also analyzed. Correlation analyses showed that the logMAR BCVA of VRL patients correlated positively with the aqueous IL-10 concentration in AH (P=0.012 r=0.274; Figure 2). Furthermore, it was found that the logMAR BCVA of VRL patients also positively correlated well with the aqueous IL-6 level in AH (P<0.001 r=0.438). No significant correlation was found between the logMAR BCVA of VRL patients and the ratio of IL-10/IL-6 (P=0.318).

Treatment with intravitreal MTX injections resulted in few ocular complications. All of the patients developed conjunctival hyperaemia that resolved within a few days. Nineteen patients developed local subconjunctival hemorrhage. Two patients developed keratopathy, which appeared after the 6th injection. Acceleration of existing cataract appeared in 1 patient, which required surgical intervention. No serious complications, such as neovascular glaucoma (NVG), toxic anterior segment syndrome (TASS), and endophthalmitis, were observed in this study.

DISCUSSION

In this study, the clinical characteristics of VRL patients were described in detail, and the aqueous cytokine profiles between VRL and uveitis patients were compared. Optimal diagnostic sensitivities and specificities were also identified for AH cytokines IL-10, IL-6, and IL-10/IL-6 ratio, and the therapeutic response to treatment with intravitreal injections of MTX was monitored.

VRL is a vision and potentially life-threatening ophthalmic disease. VRL most often occurs in patients over 50y of age, with the ratio of male to female reported as 1:1.2-1.6[2,20]. In this study, the average age of VRL onset was 54.6y, and there were more female patients (20 cases) than males (8 cases). In this study, the incidence of bilateral VRL was 53.6%, which was closer to the previously reported range (60%-90%)[4,20]. The percentage of patients who had onset of intraocular lymphoma was 67.9%, which is consistent with the percentage (66.7%) reported by Keisuke *et al*[21] in their study. The study also found that a majority of VRL patients (64.3%) had CNS involvement, consistent with the previously reported high rate (60%-90%)[22-23].

In our observation, blurred vision, reduced vision, and floater symptom are the most common ocular symptoms in VRL patients. Vitreous opacification, vitreous inflammatory cells and subretinal infiltration are the most common ocular manifestations. In this study, vitreous opacity in VRL patients was characterized by aggregation of large cells, strand formation, and a similar appearance of auroras confirmed by kinetic observation. However, it is difficult for ophthalmologists inexperienced in VRL diagnosis to make a correct diagnosis based only on the appearance of vitreous opacity.

PVRL is marked by insidious onset, diverse and atypical symptoms, nonspecific ocular manifestations, and transient improvement after being treated with corticosteroids[1]. For these reasons, many VRL patients are misdiagnosed as refractory uveitis, or have undergone a protracted workup before a correct diagnosis can be made. Nevertheless, it must be borne in mind that a rapid definitive diagnosis is necessary for appropriate treatment to forestall the substantially worse prognosis of the CNS associated with the progression of the disease. For PVRL patients, it is beneficial for their final visual acuity to initiate treatment earlier when possible. Knowledge of VRL clinical characteristics, together with an understanding of the pathologic developments and prognosis of VRL will help ophthalmologists identify suspicious cases in time and conduct correct examinations to diagnose VRL clearly.

Clinical examination is always not sensitive enough to distinguish vitreal cells from malignant cells, benign lymphocytes and resident hyalocytes. Cytological examination of vitreous biopsy specimens is considered to be a necessary condition for the definitive diagnosis of VRL, but the cytological evaluation for the diagnosis of VRL is associated with relatively low sensitivity and a significant false-negative rate[13]. Possible reasons for this low positive rate include the influence of corticosteroids, cell damage by the vitreous cutter, inappropriate sample processing, the time interval between onset of ocular symptoms and histological diagnosis, and the effects of systemic treatment performed before vitrectomy. Another possible reason for the low positive rate of cytological diagnosis is that lymphoma cells degenerate easily and become necrotic rapidly. Moreover, experienced technicians and cytopathologists play an important role in the cytological diagnosis of VRL[7,24]. Thus, much effort has been made to identify multiplex technologies to facilitate lymphoma diagnosis. Many research centers have proposed to provide adjunctive diagnostic evidence for VRL cytology by detecting cytokine levels within intraocular fluid (AH and the vitreous)[25-26]. Kimura *et al*[21] found that the IL-10/IL-6 ratio is more sensitive than PCR or cytological examination in the diagnosis of VRL. Frenkel *et al*[13] conducted a study involving 150 patients with a presumed diagnosis of VRL, and they concluded that cytokine analysis had the highest sensitivity and accuracy in diagnosis of VRL when compared with cytology and PCR. In our analysis, both the AH concentration of IL-10 and the IL-10/IL-6 ratio were significantly elevated in VRL compared with uveitis. Inversely, IL-6 and VCAM levels were higher in eyes with uveitis. With optimal threshold values available, good sensitivity (>90%) and high specificity (100%) can be achieved to differentiate VRL from uveitis. In this study, the sensitivity and specificity at an IL-10/IL-6 ratio cutoff level of 1.55 were 94.1% and 100%, respectively, and thus the sensitivity was higher than that found in previous studies[11,27]. A high level of IL-10 was highly indicative of lymphoma, and adding the IL-10/IL-6 ratio increased the accuracy of lymphoma diagnosis. The combination of the intraocular concentration of IL-10 and IL-10/IL-6 ratios was highly informative in distinguishing between VRL and uveitis.

Intraocular injection of MTX is an effective method to treat VRL. All the 29 treated eyes showed partial or complete remission of VRL, manifesting in the form of improved BCVA, reduced vitreous opacity, absence of the vitreous cells, and resolution of retinal infiltration. At the same time, the aqueous IL-10, IL-6, and IL-10/IL-6 ratio markedly decreased and continued to decrease over the span of the treatment. IL-10 became undetectable within 3 intravitreal MTX injections in 4 patients. Previous studies confirmed that aqueous levels of IL-10 correlated well with the severity of vitreous cells and disease activity[28]. Along with those reports, the present study also found that the aqueous levels of IL-10 and IL-6 correlated well with the visual function of VRL patients during the follow-up period after treatment. Our study further confirms that monitoring intraocular cytokine levels is useful for observing therapeutic outcomes in VRL patients. It is difficult to diagnose and monitor lymphoma by using clinical parameters only. The current technique of determining the number of vitreous cells cannot be quantified. The need for precise and quantitative indicators to monitor therapeutic

effectiveness makes very important the adjunctive use of cytokine levels in following treatment effects. Elevated levels of IL-10 in intraocular fluid, even in a clinically quiescent eye, may indicate an impending recurrence of the disease; the severity of clinical disease should be monitored closely. Whenever the cytokine levels disagree with the clinical severity of the disease, the clinician should take all of the available data into account before making an overall clinical response to therapy. This study provides further considerable support for the argument that the serial measurements of cytokines are expected to be a quantitative tool for monitoring disease status and therapeutic efficacy in VRL patients[29].

Increased AH IL-10 level of ≥50 pg/mL has an 89% sensitivity and 93% specificity at the diagnosis of primary intraocular lymphoma, and has been recommended as a diagnostic screening value[30]. In another study, the threshold values IL-10>100 pg/mL and IL-10/IL-6>1.0 successfully identified 18 of the 22 patients (82%) with lymphoma[31]. Moreover, in vitreous specimens, the IL-10/IL-6 ratio>1.0 was considered the diagnostic criterion for the disease, based on a sensitivity of 75%-90%[14,27]. The difference in sensitivity in different studies may be due to the use of different cutoff values. Also, the exact cutoff value for the IL-10 level or IL-10/IL-6 ratio may vary between laboratories. The main reasons may lie in the different methods and sample collection and storage conditions, techniques, and manufacturers' equipment and supplies, dilution of vitreous and AH samples, and the laboratory's experience. Anyway, a large number of studies are needed to identify the optimal cutoff for IL-10 level and IL-10/IL-6 ratio to make a more accurate diagnosis.

Due to the lack of large-scale comparative clinical studies, no specific consensus has been reached on the treatment of PVRL without signs of CNS involvement. Previous literature has reported that the treatment strategy for isolated PVRL depends on the preference of each clinical center[32-33]. Various drugs are currently used in intraocular chemotherapy, and MTX is now used widely across the world[34]. Treatment of isolated VRL by intravitreal injections of MTX is now used widely with encouraging results, including successful visual outcome and acceptable tolerance. Most ophthalmologists inject MTX in the vitreous at a dose of 400 μg (in 0.1 mL). However, the frequency of injections and the follow-up scheme vary quite widely. In 2002, Smith *et al*[35] reported the routine use of intravitreally injected MTX in 3 phases: induction (*i.e.*, twice a week for 1mo), consolidation (*i.e.*, once a week for 1-2mo) and maintenance (*i.e.*, once a month for 9-12mo). Under this therapeutic regimen, patients must receive a total of 15-22 injections for up to 11-15mo.

The treatment appeared to be effective at inducing ocular remission of the VRL, and was also used to re-induce remission in recurrent tumor cases. However, some problems are unavoidable and cannot be ignored in frequent and repeated MTX injections. First, complications and adverse events may be associated with frequent intraocular MTX injections. Possible complications occurring in injected eyes during the treatment period include keratopathy, acceleration of existing cataract, maculopathy, vitreous hemorrhage, NVG, TASS, and endophthalmitis[34,36]. In one study, different forms of keratopathy were observed in all the patients treated with repeated intravitreal MTX, and the symptoms improved as the frequency of the injections decreased. Cataract formation or acceleration is another common complication due to MTX toxicity and frequent intraocular injections. Anterior chamber angle neovascularization (NVI), with subsequent NVG, was noted at the 17th and 18th injection of MTX in two patients. Then intravitreal MTX injections were discontinued in the two patients. Although the two patients did not complete the treatment regimen, no recurrence of lymphoma was found during the follow-up. The most serious complication—endophthalmitis—was observed in patients who had undergone more than 8 repeated intravitreal MTX injections[32]. Another patient developed severe anterior segment sterile inflammation resembling TASS after his 20th injection. These complications are closely related to frequent and repeated injections. Frenkel *et al*[24] conducted a 10-year follow-up study and they reported that remission was reached after 6.4 injections. Additionally, other studies reported protocols involving less intensive injection schedules[37]. Therefore, we need to consider carefully whether all VRL patients need conventional frequent and repeated intraocular injections. Second, the patients underwent 22 injections over the whole course of 11 to 15mo in these previous studies. During this long course of treatment, many patients received systemic treatment with PCNSL at the same time, making it difficult to analyze the specific therapeutic effect of MTX on the vitreous. Although these studies reported remission of the disease, it was not possible to accurately analyze the effect of local treatment due to concurrent systemic chemotherapy. Third, MTX resistance during intraocular MTX is rare, but has been reported. The routine MTX injections initially were highly effective, but later the disease began to become resistant to the cytotoxic effect of the drug. This study suggested that after multiple injections, the patient's tumor had acquired the ability to reduce accumulation and metabolism of the drug[38].

In order to overcome the side effects of frequent intraocular injections, reduce the frequency of intravitreal injections of MTX, and effectively deal with VRL, we proposed a tailor-made therapeutic protocol for each VRL patient according to the ocular symptoms, signs, and response to treatment. Remission was achieved in all 29 eyes of 21 patients after a mean of 2.6 injections and maximum of 13 injections per eye.

Personalized treatment plans may fend off potentially serious complications of more extensive anti-lymphoma therapies. This is an important consideration because most VRL patients are elderly. PCNSL/PVRL is a heterogeneous disease, with different patients carrying different risks for CNS progression and VRL recurrence. The development of treatment protocols of PCNSL/PVRL requires a multidisciplinary collaboration, and therefore future research is needed to determine the optimal MTX-based intravitreal chemotherapy regimens in initial disease presentation and recurrent VRL.

Above all, in this study, the high specificity with good sensitivity suggested that analysis of aqueous cytokine is an effective diagnostic tool in patients of suspected VRL. IL-10 and IL-6 were also found to parallel well with visual functions, and this demonstrated the utility of aqueous IL levels as an adjunct. Furthermore, cytokine levels analysis does not require special samples or an experienced cytopathologist for reading the results. And the feature of cytokine analysis also improves the operability of VRL diagnosis. Last but not least, although the absolute levels of cytokines in the vitreous cavity and anterior chamber are different, some studies reported that the ratio of cytokines was constant in the vitreous and AH of patients[39-40]. This is especially important because AH is easier to tap and can be harvested several times during the course of the disease. Collecting an AH sample was considered to be a safe and repeatable procedure compared with collecting a vitreous sample. Based on the results of this study, intraocular cytokine analysis may be valuable not only for diagnosis but also for monitoring disease progression and therapeutic response of patients with VRL.

This study also has its limitations. First, the number of the VRL patients enrolled in the study was insufficient, and the retrospective nature of the study limits the ability to draw definitive conclusions. Second, we only detected the aqueous level of the cytokines. It would have been much better if we had detected the aqueous and vitreous levels simultaneously, which could contribute to probing intraocular cytokines profiles of VRL patients. Third, we did not observe the CNS progression or relapse of the patients with intravitreal MTX injections, and these patients were not followed for long enough. Further long-term prospective multicenter studies are needed to investigate clinical characteristics, disease progression and therapeutic effect of VRL patients to better customize treatment for individual patients and improve disease prognosis.

In conclusion, the combination of the intraocular concentration of IL-10 and IL-10/IL-6 ratios could be very helpful as an initial screening to diagnose and differentially diagnose VRL and monitor indicators to assess therapeutic effects during treatment for VRL.

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