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Distinct microRNA-155 expression in the vitreous of patients with primary vitreoretinal lymphoma and uveitis

Jingsheng Tuo¹, Defen Shen¹, Howard Hua Yang², and Chi-Chao Chan^{1,†}

¹Laboratory of Immunology, National Eye Institute, National Institutes of Health, Bethesda, MD

²Office of the Director, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Rockville, MD

Abstract

Purpose—To use micro- ribonucleic acid (microRNA) profiles in the vitreous for differential diagnosis of primary vitreoretinal lymphoma and uveitis.

Design—A prospective cross-sectional study design

Methods—This prospective cross-section study included 17 diffuse large B-cell primary vitreoretinal lymphoma and 12 uveitic patients. The supernatant of ocular fluid was subjected to total RNA extraction, followed by complementary deoxyribonucleic acid (cDNA) synthesis. Selected samples (primary vitreoretinal lymphoma n=3, uveitis n=3) were arrayed by a real time polymerase chain reaction (RT-PCR)-based microRNA panel that detects 168 human mature microRNAs. The markers promising in distinct levels between uveitis and lymphoma were further tested for all other 23 samples by individual RT-PCR analysis.

Results—Of 168 microRNAs in the array, 66.5% were detectable with consistent higher microRNA-484, microRNA-197, and microRNA-132 in the primary vitreoretinal lymphoma vitreous and higher microRNA-155, microRNA-200c, and microRNA-22* in the uveitic ocular fluids. The results were normalized by different combinations of 7 control microRNAs (microRNA-103, microRNA-191, microRNA-42-5p, microRNA-16, microRNA-425, microRNA-93 and microRNA-451). After optimization, normalization against microRNA-16 was as equally reliable as the average of the 7 control microRNAs. Individual assays of all samples supported the pattern yielded from the array analysis. But only microRNA-155 was significantly higher in the uveitic vitreous compared to that with lymphoma.

Conclusions—Mature microRNAs are detectable in ocular fluid samples. Primary vitreoretinal B-cell lymphoma and uveitis might be characterized by distinct microRNA signatures. Quantification of ocular microRNA-155 might be helpful in the differential diagnosis of these two diseases.

[†]To whom correspondence may be addressed. 10 Center Dr., Bldg. 10, Rm. 10N103, NIH/NEI, Bethesda, MD 20892-1857, Phone: (301) 435-4577, Fax: (301) 402-8664, chanc@nei.nih.gov.

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Introduction

Primary vitreoretinal lymphoma, also known as primary intraocular lymphoma, is a subset of primary central nervous system lymphoma and is mostly classified as a diffuse large B-cell lymphoma. Primary vitreoretinal lymphoma affects the retina, vitreous, and optic nerve head, with the most common symptoms being blurred or decreased vision due to tumor cells in the vitreous and retina.^{1, 2} In general, primary vitreoretinal lymphoma cells first emerge between the retinal pigment epithelial cell (RPE) and Brush's membrane, followed by infiltration in the neuroretina, optic nerve head, and vitreous. Primary vitreoretinal lymphoma is a fatal ocular malignancy due to its frequent involvement with the brain; thus, it is important to have the diagnosis early and treat it promptly. However, the clinical appearances of primary vitreoretinal lymphoma can be quite similar to uveitis, leading to a misdiagnosis of a uveitic entity and initial treatment with anti-inflammatory agents such as corticosteroids, which can further confound the diagnosis. The ratio of interleukin-10 (IL-10) to interleukin-6 (IL-6) or interferon (IFN)-gamma in the vitreous has been used for assisting differential diagnosis because B-cell primary vitreoretinal lymphoma secretes high levels of IL-10 while uveitis leads to high IL-6 and IFN-gamma levels.³⁻⁵

Micro ribonucleic acid (RNA), also known as microRNA, are small non-coding RNA molecules that play key regulatory role in many biological processes.⁶⁻⁸ MicroRNAs belong to a highly conserved class of 17-25 nucleotide RNA molecules, which have multiple roles in negative regulation of gene expression including transcript degradation, transcript sequestering, and translational suppression, as well as possible involvement in positive regulation of gene expression via transcriptional and translational activation. The microRNA expression is deregulated in cancer through multiple mechanisms, such as gene amplification, deletion, mutation and epigenetic silencing. There is now ample evidence that microRNAs are involved in the initiation and progression of cancer. MicroRNAs are stably present within microvesicles (exosomes) in many biofluids, including serum, plasma, urine, cerebrospinal fluid, aqueous humor and vitreous.^{9, 10} The extracellular microRNAs can be isolated even after long-term storage. Recently, studies have reported the high relative stability of microRNAs in biofluids and the correlation of microRNA expression profiles with diseases and disease states.¹¹⁻¹³ A technique breakthrough for detecting short microRNAs by stem-loop quantitative real time polymerase chain reaction (RT-PCR)¹⁴ has sparked tremendous interest in using microRNA from biofluids as biomarkers for many diseases. In this study, we used quantitative RT-PCR to determine the microRNA profiles in the vitreous samples from primary vitreoretinal lymphoma and uveitis patients.

Methods

Study subjects

This prospective cross-sectional study followed the tenets of the Declaration of Helsinki and was approved by the IRB of National Eye Institute (NEI), National Institutes of Health (NIH). All participants signed the informed consent forms. Demographic and clinical features of study participants are listed in Table 1. The study included 17 primary vitreoretinal B-cell lymphoma and 12 uveitic patients. The diagnosis of primary vitreoretinal lymphoma was based on the definitive identification of malignant lymphoid cells in the eye.^{2, 5, 15} The uveitic patients were mostly non-infectious (only one case with *Propionibacterium acnes* (*P. acnes*) endophthalmitis), posterior or pan-uveitis. Vitreous specimens from all lymphoma patients and 9 uveitic patients were obtained through a standard three-port pars plana vitrectomy and centrifuged (200g, 10 minutes, room temperature) to collect supernatant.¹⁶ Three aqueous specimens were obtained from aseptic aqueous aspiration. The supernatant was aliquoted for quantification of cytokines, including

IL-10 and IL-6 by enzyme-linked immunosorbent assay (ELISA), and for ribonucleic acid (RNA) extraction.

Micro Ribonucleic Acid array

Total ribonucleic acid including microRNAs in vitreous supernatant was extracted using a commercial column-based system (Qiagen miRNeasy® Mini Kit). Reverse transcription of RNA was performed by using mature microRNA as templates and microRNACURY LNA™ Universal complementary deoxyribonucleic acid (cDNA) Synthesis Kit according to manufacturer's instructions (Exiqon A/S, Vedbaek, Denmark). Six randomly selected samples (primary vitreoretinal lymphoma n=3, uveitis n=3) were arrayed by a real-time polymerase chain reaction (RT-PCR)-based microRNA panel that detects 168 human mature microRNAs (<http://www.exiqon.com/microrna-microarray-analysis-microrna-array>) (Exiqon microRNACURY LNA™ Serum/Plasma Focus microRNA PCR Panel, Part# 203826, Vedbaek, Denmark). RT-PCR was carried out on a StepOnePlus RT-PCR System (Life technologies, Applied Biosystems). Cycling conditions were as follows: 95°C for 10 minutes, 40 cycles of 15 seconds at 95°C, and 60 seconds at 60°C. Fluorescent data were converted into cycle threshold (Ct). The results were normalized by different combinations of 7 control microRNAs (microRNA-103, microRNA-191, microRNA-42-5p, microRNA-16, microRNA-425, microRNA-93 and microRNA-451) as recommended by the manufacturer. The microRNA levels were determined with DataAssist™ v2.0 Software (Applied Biosystems, CA). The promising microRNAs with distinct levels between uveitis and lymphoma in the array were further quantified by individual analysis.

Individual micro Ribonucleic Acid assay

The cDNA was synthesized by miScript II RT Kit (Qiagen). The individual microRNA assay was performed with miScript SYBR Green PCR Kit and miScript Primer Assays according to manufacturer's instructions (Qiagen). A master mix containing 2x QuantiTect SYBR Green PCR Master Mix, 10x miScript Universal Primer, 10x miScript Primer Assay, and RNase-free water was prepared for the Primer Assay specific to the microRNA of interest. The assays for microRNA-484, microRNA-197, microRNA-132, microRNA-155, microRNA-200c, microRNA-22* and the reference microRNA-16 were performed in the same plate in triplicate using StepOnePlus real-time PCR system (Life Technologies, ABI). Cycling conditions were as follows: 95°C for 10 minutes, 40 cycles of 15 seconds at 95°C, and 60 seconds at 60°C followed by a melting curve analysis. To determine the Ct values, the threshold level of fluorescence was set manually in the early phase of PCR amplification. ABI SDS 1.3.1 software and the $2^{-\Delta\Delta C_t}$ method was used to determine the relative microRNA level that was normalized first by the level of microRNA-16 from the same cDNA sample and again normalized to the microRNA level in the lower expression group.

Quantitation of vitreous IL-10 and IL-6 and IgH gene rearrangement

The IL-10 and IL-6 levels in vitreous supernatant were measured by ELISA (Quantikine; R&D Systems, Abingdon, UK). Immunoglobulin heavy chain (IgH) gene rearrangement was detected by PCR of extracted DNA from cells collected from ocular fluids using primer pairs of complementarity determining region (CDR)3, framework region (FR)3A and/or FR2A.^{3, 17} All three primer sets are included the CDR3 region of the IgH gene, and all can detect rearrangement(s) in CDR3.

Statistical analysis

The means for microRNA level (fold changes that are in normal distribution) between primary vitreoretinal lymphoma and uveitis groups were compared using the Student's t-

test. Differences were considered significant when $P < 0.05$. The interaction of microRNA and transcriptional factors were predicted using web-based transMicroRNA database.¹⁸

Results

Six vitreous samples (primary vitreoretinal lymphoma $n=3$, uveitis $n=3$) out of 29 samples were selected for array analysis of 168 human mature microRNAs (Table 1). The array detected 110 microRNAs (66.5%) in these samples out of 168 microRNAs printed in the array when the cut-off was set as $Ct < 36$. For balancing the sample input, the results were normalized by different combinations of 7 control microRNAs (microRNA-103, microRNA-191, microRNA-42-5p, microRNA-16, microRNA-425, microRNA-93 and microRNA-451) as recommended by the manufacturer. After optimization, the normalization against microRNA-16 showed as equally reliable as the average of the 7 control microRNAs. MicroRNA-484, microRNA-197, and microRNA-132 were consistently higher in the primary vitreoretinal lymphoma vitreous compared to that of uveitis, while microRNA-155, microRNA-200c, and microRNA-22* were consistently higher in the uveitis vitreous compared to that of lymphoma. The average microRNA levels of 3 primary vitreoretinal lymphoma and 3 uveitis samples normalized against microRNA-16 are plotted in Fig. 1.

The markers with distinct levels between uveitis and lymphoma were further quantified in the other 23 samples. No difference of microRNA155 between the specimens from aqueous and vitreous in uveitis was measured. After calibration against microRNA-16, groupwise comparisons indicated lower microRNA-484, microRNA-197, and microRNA-132, but higher microRNA-155, microRNA-200c, and microRNA-22* in the primary vitreoretinal lymphoma than those in uveitic fluids. The microRNA patterns from the individual assays were consistent with that from the array analysis. However, the comparison only shows that microRNA155 in B-cell Primary vitreoretinal lymphoma is significantly lower than that in uveitis (Fig. 2). Heavy overlap of microRNA155 levels between B-cell primary vitreoretinal lymphoma and uveitis samples was noted.

IL-10/IL-6 ratio differentiated primary vitreoretinal B-cell lymphoma from uveitis, with the ratios >1 in all the primary vitreoretinal lymphoma cases, and <1 in all the uveitis cases (Table 2). There was reverse correlation between Log IL-10 and microRNA155 level in vitreous samples (Fig. 3). The IgH gene rearrangement was detected using three different primers. These molecular data confirmed the diagnosis of B-cell lymphoma. Since no correlation between the primer set and phenotype has been found in our previous large study of 57 intraocular lymphoma cases,¹⁶ further analysis of each specific primer set was not performed among these smaller number cases.

Discussion

MicroRNA (microRNA) are transcribed from a DNA sequence but are not translated into a protein. The microRNAs are abundantly present in all cells and are able to regulate gene expression mainly by inhibiting protein translation.⁶⁻⁸ Deregulation of microRNAs has been previously associated with cancer, and many microRNAs are expressed in a tissue-specific manner and exhibit different expression profiles in the normal tissue compared to distinct biological or pathological conditions.¹⁹ This study reported the distinct microRNA profiles in human vitreous samples from B-cell primary vitreoretinal lymphoma and uveitic patients. Most primary vitreoretinal lymphomas are diffuse large B-cell lymphomas characterized by aggressive growth and are often fatal.^{3, 5, 20} It is known that the specific cellular factors secreted from tumor cells can serve as a specific biomarker.²¹⁻²³ Recently, exosomes carrying RNAs (messenger RNA [mRNA], microRNA, and other small RNAs) from the

donor cells were successfully used to predict disease onset, progression, and prognosis.^{11-13, 24} Because of the significant programmed cell death of tumor cells in clinical conditions but stable level of exosomes in the biofluids, the characteristics of a well-preserved biological signature from donor cells such as exosome-packed microRNA profiling has an advantage over traditional methods of tumor diagnosis that depend on the identification of tumor cells. In this study, we have detected 110 microRNAs out of 168 human mature microRNAs from patients' various samples, indicating the feasibility using various diagnostic materials, such as vitreous and aqueous humor, in microRNA profiling for ocular diseases.

Studies have reported an increase of serum microRNA-155 in lymphoma patients.^{25, 26} The expression levels of microRNA-155 were 5.25 fold higher in serum samples from diffuse large B-cell lymphoma patients than from those of healthy controls.²⁵ Interestingly, in a study of patients with classical Hodgkin lymphoma, microRNA-155 levels were elevated in plasma samples but slightly lower in the primary lymph node.²⁷ The discrepancy of microRNA-155 expression in plasma and the tumor site may explain our finding of relatively low microRNA-155 in the vitreous of primary vitreoretinal lymphoma. The vitreal microRNA-155 should closely relate to its level in ocular lymphoma. However, plasma microRNA-155 was not available and microRNA-155 in normal vitreous was not measured in this study.

An *in vitro* study detected an approximately 8-fold increase in microRNA-155 expression and an 11-fold increase in the primary transcript of the B-cell integration cluster for microRNA-155 (BIC/microRNA-155, which contains the microRNA-155 precursor) in RPE after individual treatment with IFN- γ , TNF- α or IL-1 β .²⁸ This implies that the microRNA-155 expression response to inflammatory stimulation could be very robust. Moreover, some specific individual treatments or specific general situations (e.g., additional infections) leading to an increased or to an overexpressed release of such inflammatory cytokines, may negatively influence the possible value of the microRNA-155 expression. Therefore, this influence could help (for the diagnosis of uveitis) or decisive marker in the differential diagnosis of primary vitreoretinal B-cell lymphoma and uveitis.

MicroRNA-155 has been implicated in several immunological diseases including multiple sclerosis, arthritis, systemic lupus erythematosus, and experimental autoimmune encephalomyelitis.²⁹⁻³³ Mechanistic studies have revealed that the upregulation of microRNA-155 might be related to IL-17, macrophage, and NF-kappaB induction.^{29, 30, 32, 34} Lower levels of IFN- γ are reported in the vitreous of patients with primary vitreoretinal lymphoma.⁴ While this study did not include vitreous sample from healthy donors, we nevertheless found significantly higher microRNA-155 levels in uveitic fluids than in primary vitreoretinal B-cell lymphoma samples. There are plenty of pro-inflammatory cytokines secreted by various infiltrating inflammatory cells in the eyes with uveitis. While the vitreous microRNAs might be derived from lymphoma cells or from infiltration of inflammation cells, it is equally possible that these microRNAs were secreted from local ocular tissue and resident cells as a response to tumors or to inflammatory stimulation.

A negative correlation between microRNA-155 and IL-10 was noted in our data. The current knowledge about the interaction between microRNA-155 and IL-10 is that IL-10 may inhibit the expression of microRNA-155 in response to lipopolysaccharide stimulation of macrophages in a STAT3-dependent manner.³⁵ An *in vivo* study echoed this finding and reported that IL-10 deficient mice had enhanced expression of 10 microRNAs including microRNA-155 in their colonic tissues and peripheral blood lymphocytes.³⁶

In our study cohort, the differentiation of primary vitreoretinal lymphoma from uveitis by vitreous microRNA-155 level did not offer advantage to the ratio of vitreous IL-6 and IL-10 levels. Moreover, the group comparison using the same platform simultaneously might not be feasible in the evaluation of individual cases. The small sample size also limited the significance of the study. However, characteristics of the specific microRNA profiling could lead to better understanding of the mechanisms of the two diseases. In human tissues, microRNA-155 regulates numerous transcriptional factors such as FOXP3, NFKB1, TGFB1, and AKT1 (web available TransMicroRNA, <http://202.38.126.151/hmdd/microRNA/tf/>). The interaction of microRNA-155 and those transcriptional factors plays an important role in innate immune responses and tumorigenesis. In general, microRNAs have strong regulatory function by targeting complementary regions of the mRNA in the 3'-UTR of genes, thus disrupting gene expression by either mRNA degradation or translational inhibition.³⁷ Because a single microRNA may influence the expression of a cluster of genes and possibly an entire pathway, moderate changes in microRNA profiling may have great impact on pathogenesis. As such, microRNAs have a strong potential to become targeting elements for therapy development.

We conclude that mature microRNAs in ocular fluid could be used as novel markers for clinical diagnosis. Quantification of ocular microRNA-155 might be helpful in the differential diagnosis of primary vitreoretinal B-cell lymphoma and uveitis.

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Figure 1.

The Pair Plot of 110 Detectable micro Ribonucleic Acids in Vitreous Samples from Primary Vitreoretinal Lymphoma and Uveitis. The data are the average of 3 biological samples from each condition and normalized against microRNA-16. The level of the majority of microRNAs does not differ substantially between the pair, as shown in the distance from the bisecting line. However, the microRNA-484, microRNA-197, and microRNA-132 were higher in the primary vitreoretinal lymphoma vitreous while microRNA-155, microRNA-200c, and microRNA-22* higher in the uveitis vitreous. The three pairs of the array showed the same pattern. miR: microRNA, PVRL: primary vitreoretinal lymphoma

Figure 2.

Quantitative RT-PCR of Individual Assay of 6 micro Ribonucleic Acids. Significantly higher expression of microRNA-155 was found in uveitis vitreous compared to the primary vitreoretinal lymphoma vitreous. *: $p < 0.05$, miR: microRNA, PVRL: primary vitreoretinal lymphoma

Figure 3. Correlation of micro Ribonucleic Acid-155 and Interleukin-10 Levels in Vitreous from Primary Vitreoretinal Lymphoma and Uveitis Vitreous. There is a reverse correlation between vitreal microRNA-155 and IL-10 levels from the two diseases. miR: microRNA, IL: interleukin

Table 1

Age, Gender, and Diagnosis of Patients with Primary Vitreoretinal Lymphoma and Uveitis

ID	Age	Gender	Disease	Comments
1*	80	F	B-cell Primary vitreoretinal lymphoma	IgH gene arrangement identified
2	67	M	B-cell Primary vitreoretinal lymphoma	IgH gene arrangement identified
3	73	F	B-cell Primary vitreoretinal lymphoma	IgH gene arrangement identified
4*	76	M	B-cell Primary vitreoretinal lymphoma	IgH gene arrangement identified
5	74	F	B-cell Primary vitreoretinal lymphoma	IgH gene arrangement identified
6	64	F	B-cell Primary vitreoretinal lymphoma	IgH gene arrangement identified
7	69	M	B-cell Primary vitreoretinal lymphoma	IgH gene arrangement identified
8	55	M	B-cell Primary vitreoretinal lymphoma	IgH gene arrangement identified
9	66	F	B-cell Primary vitreoretinal lymphoma	IgH gene arrangement identified
10	63	F	B-cell Primary vitreoretinal lymphoma	IgH gene arrangement identified
11	58	M	B-cell Primary vitreoretinal lymphoma	IgH gene arrangement identified
12	74	F	B-cell Primary vitreoretinal lymphoma	IgH gene arrangement identified
13	70	M	B-cell Primary vitreoretinal lymphoma	IgH gene arrangement identified
14	45	F	B-cell Primary vitreoretinal lymphoma	IgH gene arrangement identified
15	50	F	B-cell Primary vitreoretinal lymphoma	IgH gene arrangement identified
16	80	F	B-cell Primary vitreoretinal lymphoma	IgH gene arrangement identified
17*	68	M	B-cell Primary vitreoretinal lymphoma	IgH gene arrangement identified
18*	35	F	Uveitis	Vitreous inflammation granulomatous
19	72	F	Uveitis	Vitreous subacute inflammation
20	72	F	Uveitis	Vitreous chronic inflammation
21	60	F	Uveitis	Aqueous inflammation
22	45	F	Uveitis	Aqueous chronic inflammation
23	52	F	Uveitis	Vitreous infection (<i>P. acnes</i>)
24	69	F	Uveitis	Vitreous choroid chronic inflammation
25	79	F	Uveitis	Vitreous inflammation granulomatous
26	24	M	Uveitis	Vitreous inflammation granulomatous
27*	76	F	Uveitis	Vitreous chronic inflammation
28	52	F	Uveitis	Aqueous chronic inflammation
29*	62	F	Uveitis	Vitreous chronic inflammation

* : sample used for array assay

P. acnes: *Propionibacterium acnes*

Table 2
Interleukin-6, Interleukin-10, and Their Ratio in Ocular Fluids of Patients with Primary Vitreoretinal Lymphoma and Uveitis

Primary Vitreoretinal B-cell Lymphoma				Uveitis			
sample	IL-6 (pg/ml)	IL-10 (pg/ml)	IL-10/IL-6	sample	IL-6 (pg/ml)	IL-10 (pg/ml)	IL-10/IL-6
1*	265	1805	6.8	18*	162	23	0.1
2	15	106	7	19	9512	135	0.01
3	180	1092	6.6	20	114	23	0.2
4*	116	18154	156.5	21	581	46	0.1
5	98	969	9.8	22	115	47	0.4
6	15	785	52.3	23	2171	NA	
7	17	479	28.1	24	28	23	0.8
8	60	97	1.6	25	641	152	0.2
9	242	3313	13.7	26	16	NA	
10	16	215	13.4	27*	32	23	0.7
11	15	37	2.4	28	732	67	0.1
12	60	118	1.9	29*	16	NA	
13	29	1887	65				
14	96	929	9.6				
15	16	88	5.5				
16	62	246	3.9				
17*	250	5912	23.6				

* sample used for array assay

IL: interleukin