ORIGINAL ARTICLE

Gene expression profiling of primary vitreoretinal lymphoma

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Abstract

The characteristics of tumor cells of primary vitreoretinal lymphoma (PVRL) have not been defined, although researches have shown that most cases are of diffuse large B-cell lymphoma (DLBCL). To determine the subtype and biological characteristics of tumor cells of PVRL, we performed a gene expression profiling analysis. RNA was extracted from the vitreous fluid of 7 PVRL patients and from nodal samples of 10 DLBCL patients: 6 of germinal center B-cell (GCB) type and 4 of activated B-cell (ABC) type determined by Hans' criteria. Six PVRL samples showed gene expression profiles that were similar to each other. The patterns were different from those of the ABC-type nodular DLBCL but relatively close to those of the GCB-type nodular DLBCL. Interestingly, all of the 6 examined PVRL samples had either $MYD88^{L265P}$ or mutation in the immunoreceptor tyrosine-based activation motif (ITAM) region of $CD79B$. Five PVRL patients with similar gene expression profiles were treated with a standardized regimen: intravitreal administration of methotrexate (MTX) followed by six courses of systemic high doses of MTX. As a result, 2 patients had $CD79B$ mutations and showed early central nervous system (CNS) progression. Patients without CNS progression did not have this mutation. In conclusion, PVRL had unique genetic features: an expression pattern different from ABC-type and relatively close to GCB-type DLBCL. $CD79B$ mutations showed potential to serve as prognostic markers for CNS progression.

KEYWORDS
CD79B, diffuse large B-cell lymphoma, GCB-type DLBCL, gene expression profiling, primary vitreoretinal lymphoma

1 | INTRODUCTION

Primary vitreoretinal lymphoma (PVRL) is a rare form of non–Hodgkin lymphoma, with primary lesions limited to intraocular tissues: the vitreous and the retina. Although morphological and genetic findings show that most cases are diffuse large B-cell lymphoma (DLBCL),[1,2] the characteristics of tumor cells of PVRL have not been defined to date. DLBCL is subdivided into two groups based on the genes expressed, the differentiation step or the biological response of the B cells: activated B-cell (ABC) type and germinal center B-cell (GCB) type.[3] To determine the subtype and biological characteristics of tumor cells of PVRL, we performed gene expression profiling analysis using RNA extracted from vitreous samples upon diagnosis.

2 | MATERIALS AND METHODS

2.1 | Patients

The patients were diagnosed and treated at Tokyo Medical and Dental University (TMDU) hospital from 2011 to 2018. Samples from the patients whose vitreous RNA could be obtained were analyzed. The study was approved by the ethics board of TMDU and written informed consent was obtained from the patients.

2.2 | Diagnosis of primary vitreoretinal lymphoma

Primary vitreoretinal lymphoma was diagnosed using the following criteria according to the previous report[2]: (a) typical eye involvement: a cloudy vitreous body and/or subretinal proliferative lesions; (b) presence of lymphoma cells in the vitreous fluid; and (c) clonality of the infiltrating lymphoma cells in the vitreous fluid using either PCR analysis of IgH gene rearrangements or flow cytometry analysis. Patients who had (a) accompanied by either (b) or (c) were diagnosed with VRL. VRL confined to the eyes at diagnosis was defined as PVRL.

2.3 | Flow cytometry

The infiltrating cells were isolated from the vitreous fluid and used for flow cytometry. We examined the surface expression of B-cell markers (CD19 and CD20), T-cell markers (CD3, CD4, CD5 and CD8), κ light chains and λ light chains. We defined a monoclonal κ population as one in which the κ/λ ratio was 3:1 or greater, and monoclonal λ population as one that had a λ/κ ratio in excess of 2:1, according to the criteria suggested by Levy et al.[3]

2.4 | Samples and microarray

RNA was extracted from the vitreous fluid of PVRL patients with an RNeasy Mini Kit (QIAGEN, Hilden, Germany). Lymph nodes of patients with nodal DLBCL were used as controls. RNA from the lymph nodes of nodal DLBCL was isolated using ISOGEN II (Nippon Gene, Tokyo, Japan). Their pathological subtypes were determined by Hans criteria.[4]

Five nanograms of total RNA were used for amplification with an Ovation RNA Amplification System V2 (NuGEN Technologies). One microgram of purified, amplified cDNA was used as the input in the Genomic DNA Enzymatic Labeling Kit (Agilent Technologies). The amplification and labeling reactions were carried out according to the manufacturer's instructions. Labeled cDNA was hybridized to a SurePrint G3 Human GE Microarray 8x60K v2 (Agilent Technologies) according to the manufacturer's instructions. Relative hybridization intensities and background hybridization values were calculated using Agilent Feature Extraction Software (9.5.1.1).

2.5 | Raw data analysis and filter criteria

Raw signal intensities and flags for each probe were calculated from hybridization intensities (gProcessedSignal) and spot information (e.g. gIsSaturated) according to the procedures recommended by Agilent Technologies. The raw signal intensities of all the samples were log2-transformed and normalized by quantile algorithm with the "preprocessCore" library package[5] on Bioconductor software.[6] We selected the probes that call the "P" flag in at least one sample to identify differentially expressed genes. Then, we applied the Linear Models for Microarray Analysis (limma) package in the Bioconductor software.[7] The criteria for differentially expressed genes were a limma P-value < 0.01 and ratio ≥ 10 (upregulated genes) or a P-value < 0.01 and ratio ≤ 0.1 (downregulated genes).

2.6 | Data analysis

Data were extracted, and agglomerative hierarchical clustering was performed on the basis of genes discriminating the GCB and ABC signatures, which were initially published by Alizadeh et al.[3] A clustering diagram of gene trees and heat maps was generated using MeV software (http://mev.tm4.org).

A pathway analysis of the PVRL gene expression dataset was performed using gene set enrichment analysis with the KEGG/BioCarta pathway gene sets. A gene ontology (GO) analysis was performed using DAVID (https://david.ncifcrf.gov/).

Microarray data have been deposited at the Gene Expression Omnibus (GEO), which is hosted by the National Center for Biotechnology Information, under accession number GSE127761. Further information about the GEO can be found at https://www.ncbi.nlm.nih.gov/geo/info/linking.html.

2.7 | Mutation analysis

To detect *IgH* gene rearrangements, a PCR assay was performed as described previously. Direct sequencing analysis for the section of *CD79B* encoding the immunoreceptor tyrosine-based activation motif region (ITAM) was performed on the basis of methods described previously.[8] Direct sequencing analysis for $MYD88^{L265P}$ was performed on the basis of methods described previously.[9]

3 | RESULTS

We performed gene expression profiling analysis on 7 samples from PVRL patients. The clinical findings of the patients are shown in Table 1. We also examined 10 samples from nodal DLBCL patients, who served as controls; 4 samples were the ABC-type, and 6 samples were the GCB-type (Table S1). They had no CNS lesions.

First, we performed hierarchical clustering analysis of the selected genes discriminating the GCB and ABC signatures to clarify the

subtypes of PVRL. As shown in Figure 1, 6 of the 7 PVRL showed independent patterns from those of the nodular samples but were relatively close to 3 of the 6 GCB-type nodular DLBCL. The gene expression pattern of PVRL was different from ABC-type nodular DLBCL.

Next, the expression signatures of PVRL were compared with those of nodal DLBCL. The top 100 genes that were significantly upregulated and downregulated in PVRL compared with nodal DLBCL were selected using the limma shown in Tables S2 and S3,

TABLE 1 Patient characteristics

Cases	Age	Gender	Involved eye	Cytology	FCM	PCR	IL10/ IL6	Mutation	Treatment	CNS progression	Outcomes	Follow-up period
PVRL-1	72	M	R	+	NE	NA	>1	MYD88^{L265P}	v MTX + sMTX	–	Alive in CR	67 Mo
			L	+	+	+	>1	MYD88^{L265P}				
PVRL-2	78	F	R	NE	+	+	>1	CD79B$^{D193E, Y196N}$	v MTX + sMTX	+ (19 Mo)	Lost to follow-up after CNS progression	21 Mo
			L	+	+	NA	>1	NA				
PVRL-3	78	F	L	NA	NA	+	>1	MYD88^{L265P}	v MTX	+ (24 Mo)	Lost to follow-up after CNS progression	25 Mo
PVRL-4	78	F	R	+	+	+	>1	CD79B^{Y196N}	v MTX + sMTX	+ (11 Mo)	Alive in 2nd CR (CNS)	50 Mo
			L	NA	NA	NA	NA	NA				
PVRL-5	78	F	R	+	+	+	>1	NE	v MTX	–	Alive in CR	23 Mo
PVRL-6	76	F	R	+	+	NA	>1	MYD88^{L265P}	v MTX + sMTX	–	Alive in CR	38 Mo
PVRL-7	76	F	R	+	–	+	>1	MYD88^{L265P}	v MTX + sMTX	–	Alive in CR	36 Mo

Abbreviations: CNS, central nervous system; CR, complete response; F, female; FCM, flow cytometry; L, left; M, male; Mo, months; NA, not available; NE, not examined; R, right; sMTX, systemic methotrexate; vMTX: intravitreal methotrexate; WT, wild type.
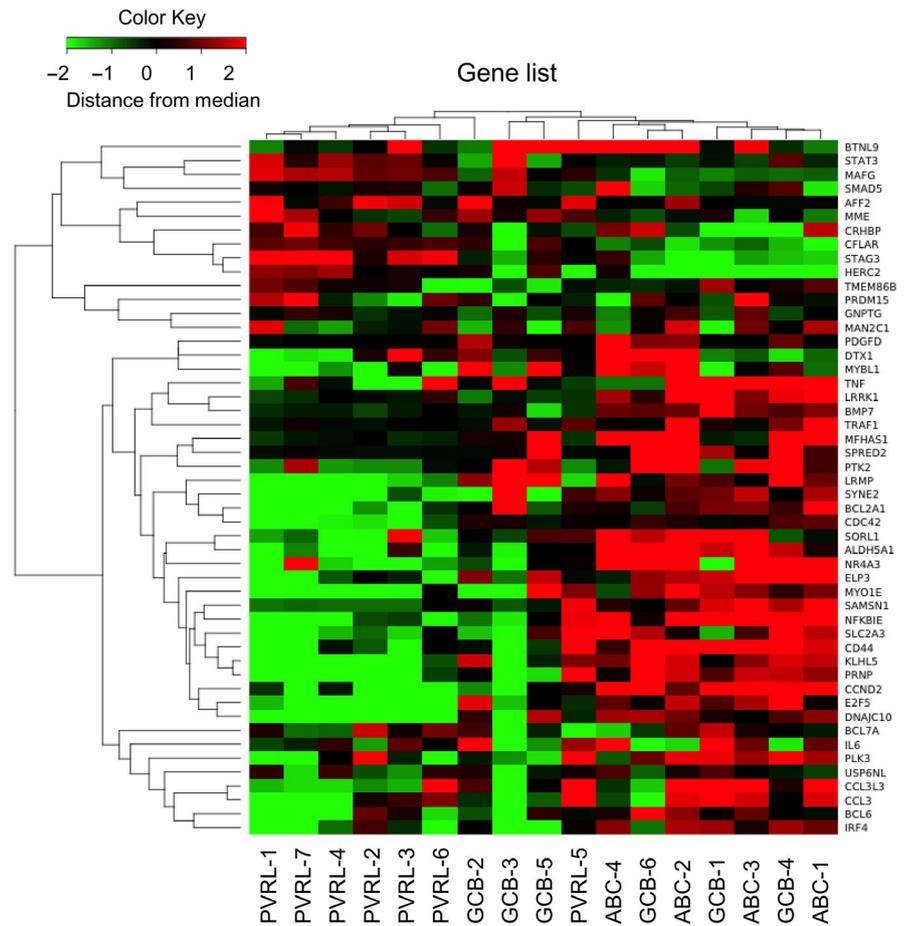

FIGURE 1 Hierarchical clustering of genes discriminating the germinal center B-cell (GCB) and activated B-cell (ABC) signatures previously published by Alizadeh et al. The RNA samples from 7 patients with primary vitreoretinal lymphoma (PVRL) and 10 patients with nodal diffuse large B-cell lymphoma (DLBCL) were examined

respectively. For genes with significant fluctuations in expression (*P*-value < .01), we performed a GO analysis using the DAVID bioinformatics resource. The analysis identified several gene clusters with altered expression (Table S4). The genes with high enrichment scores were linked to antigen processing and presentation, T-cell receptor signaling pathway, viral process, cell division and cell proliferation. A pathway analysis showed that ribosome, antigen processing and presentation, and neurological disease-associated genes fluctuated (Table S5).

Finally, we focused on dysregulated pathways of PVRL with CNS progression using the results of the expression analysis. Five GCB-like PVRL (PVRL-1, 2, 4, 6 and 7) were treated with the same regimen: intravitreal administration of methotrexate (MTX) followed by six courses of systemic high doses of MTX according to the protocol reported previously.[10] Among them, PVRL-2 and PVRL-4 showed early progression in the CNS within 2 years after treatment, whereas the others did not. Recently, it was reported that *MYD88*[L265P] and mutations in the ITAM region of *CD79B* around Y196 were frequently detected in PVRL at rates of approximately 80%[11,12] and 35%,[13] respectively. As shown in Table 1, our PVRL cases had at least one of these mutations. PVRL-1, 6 and 7 had an *MYD88*[L265P] mutation (Table 1). Interestingly, PVRL-2 and PVRL-4 with CNS progression had a *CD79B* mutation. Patients without CNS progression did not have this mutation. We examined genes with significant fluctuations in expression (*P*-value <0 .01) in CNS progression. The top 100 genes that were significantly upregulated and downregulated in PVRL with CNS progression compared with those without are shown in Tables S6 and S7, respectively. Using the genes, we performed functional annotation analysis. Enriched functional annotation terms associated with significantly fluctuated genes in PVRL with CNS progression are shown in Table S8. As shown in Table S9, the most enriched pathway in patients with PVRL with CNS progression (PVRL-2 and PVRL-4) was linked to oxidative phosphorylation. The genes related to Toll-like receptor signaling pathway were also enriched.

4 | DISCUSSION

The DLBCL subtype of PVRL has been controversial. Wallace et al detected t(14;18) in more than 70% of patients with PVRL.[14] The high frequency of translocation in PVRL in this report suggests that lymphoma cells originate from GCB with high BCL2 expression. By contrast, another group reported that the immunophenotype of PVRL tumor cells was MUM1/IRF4+;BCL-6+/−;CD10−, suggesting ABC-type DLBCL.[15] Our results indicated that the majority of PVRL samples exhibited a characteristic gene expression profile: different from that of ABC-type nodal DLBCL and relatively close to that of GCB-type nodal DLBCL. Interestingly, 6 of 7 patients with vitreous DNA available for analysis had either *MYD88*[L265P] or mutations in the ITAM region of *CD79B*. It was reported that *MYD88*[L265P] and the mutations in *CD79B* were frequently detected in PVRL.[11-13] However, these mutations were reported to be infrequent in GCB-type DLBCL.[16,17] The present findings suggest that PVRL is not identical

to GCB-type nodal DLBCL and is independent of the reported subtypes of DLBCL.

The genes that were significantly fluctuated in PVRL in comparison with nodal DLBCL were those related to antigen processing and presentation, T-cell receptor signaling pathways and viral processes. The pathways associated with neurological diseases were also enriched. The genes and the pathways indicated characteristics unique to PVLR, although they may reflect the infiltration of reactive cells in PVRL. Two patients, PVRL-2 and PVRL-4, showed CNS progression after a standardized regimen including systemic administration of high dose MTX. They had a mutation in *CD79B*. *CD79B* codes the Igβ protein of the B-cell receptor (BCR), and the mutations in *CD79B* coding of the ITAM region cause persistently active BCR signaling, which leads to cell activation and survival. Reports have shown that *CD79B*[Y196] mutations are not related to the prognosis of DLBCL.[18-20] In immune-privileged sites, however, the mutation can give lymphoma cells survival benefits without antigen stimulation. Interestingly, the genes associated with Toll-like receptor signaling pathway located downstream of *CD79B* were significantly fluctuated in PVRL with CNS disease. Toll-like receptor signaling pathway mediates survival-promoting molecules NF-κB and may have roles for development of PVRL. Further study is necessary to investigate whether the mutation can be used as a PVRL prognostic marker.

The present study has some limitations. First, the amount of RNA obtained from the vitreous fluid was small and RNA was amplified prior to gene expression profiling analysis. Some bias might have occurred during this step. In addition, the number of the samples was small. Additional studies with a large number of samples are required to confirm the results.

In summary, PVRL had unique genetic features. *CD79B* mutations showed a potential to serve as prognostic markers for CNS progression.

CONFLICTS OF INTEREST

All authors declare no competing financial interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.