

Comprehensive miRNA Analysis Using Serum From Patients With Noninfectious Uveitis

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PURPOSE. MicroRNAs (miRNAs) are noncoding RNAs and have attracted attention as a biomarker in a variety of diseases. However, extensive unbiased miRNAs analysis in patients with uveitis has not been completely explored. In the present study, we comprehensively analyzed the deregulated miRNAs in three major forms of uveitis (Behçet's disease [BD], sarcoidosis and Vogt-Koyanagi-Harada disease [VKH]) to search for potential biomarkers.

METHODS. This study included 10 patients with BD, 17 patients with sarcoidosis, and 13 patients with VKH. Eleven healthy subjects were used as controls. The miRNAs expression levels were studied by microarray using serum samples from patients with uveitis and healthy controls.

RESULTS. A total of 281 upregulated miRNAs and 137 downregulated miRNAs were detected in patients with BD, 35 upregulated miRNAs and 86 downregulated miRNAs in patients with sarcoidosis, and 153 upregulated miRNAs and 35 downregulated miRNAs in patients with VKH. Some deregulated miRNAs were involved in the mitogen-activated protein kinase signaling pathway and inflammatory cytokine pathways. Furthermore, we identified miR-4708-3p, miR-4323, and let-7g-3p as the best predictor miRNAs for BD, sarcoidosis, and VKH, respectively. Panels of miRNAs with diagnostic potential for the three diseases were generated using machine learning.

CONCLUSIONS. In this study, comprehensive miRNA analysis identified deregulated miRNAs in three major forms of noninfectious uveitis. This study provides new insights into molecular pathogenetic mechanisms and useful information toward developing novel diagnostic biomarkers and therapeutic targets for BD, sarcoidosis, and VKH.

Keywords: microRNA, uveitis, biomarker

Uveitis is defined as inflammation of the uveal tracts. This disease is a common cause of vision loss, accounting for 10% to 15% of legal blindness worldwide.¹ Behçet's disease (BD), sarcoidosis, and Vogt-Koyanagi-Harada disease (VKH) are the three most common noninfectious uveitis entities seen in Japan.^{2,3}

These three major forms of uveitis share many clinical features with other uveitis entities. Owing to the lack of diagnostic biomarkers, the diagnosis of uveitis is usually clinical and based on expert opinion. Especially for nonuveitis specialists or inexperienced ophthalmologists, misdiagnosis may occur, which would lead to a delay in diagnosis and treatment. Early diagnosis, close monitoring, and early and appropriate treatment are mandatory to decrease the risk of serious vision impairment, morbidity, and mortality associated with BD, sarcoidosis, and VKH. In this context, exploration of new diagnostic biomarkers that allow reliable and early diagnosis of these major uveitis is urgently needed. Efforts have been made to apply microRNAs (miRNAs) studies to solve these diagnostic difficulties in ophthalmology.

In recently published articles, miRNA analysis has been reported to be a potentially useful tool to help

ophthalmologists make medical decisions regarding diabetic retinopathy and age-related macular degeneration.^{4,5} MicroRNAs are small noncoding RNAs consisting of 20 or more bases that do not encode proteins in humans. More than 2600 miRNAs have been identified. Recently, numerous reports suggest that miRNAs are present in plasma at detectable levels, and that they are more stable than mRNAs in body fluids, resistant to degradation, and easily and rapidly measurable owing to their small size and stem-loop structure.⁶⁻⁸ Therefore, miRNA are expected to be useful biomarkers in many autoimmune diseases, without the need of conducting biopsy, surgery, or other invasive procedures.^{7,9,10}

Comprehensive miRNA analysis has been applied to delineate miRNA regulations and discover potential biomarker for uveitis. Recently, differential expression of miRNAs in BD, sarcoidosis, and VKH has been reported.¹¹⁻¹⁵ These studies suggest that miRNAs may provide clues to explain the different pathogenetic pathways leading to different forms of uveitis. However, these studies failed to identify disease-specific miRNAs, and the interpretation of signatures with respect to disease biology is unclear.

TABLE 1. Clinical and Laboratory Features of Cohorts of Patients With Noninfectious Uveitis and Controls

	BD	Sarcoidosis	VKH	Healthy Controls
<i>N</i>	10	17	13	11
Sex (male/female)	7/3	5/12	6/7	6/5
Age (years)	36.4 ± 17.1 (16–65)	60.4 ± 17.1 (26–85)	42.4 ± 12.5 (12–58)	54.1 ± 20.6 (27–89)
WBC (/μL)	6420.0 ± 1057.2	5176.5 ± 1703.5	6792.3 ± 2666.2	–
CRP (mg/dL)	0.18 ± 0.25	0.08 ± 0.07	0.06 ± 0.04	–
CH50 (U/mL)	66.6 ± 11.3	64.6 ± 12.7	58.6 ± 13.1	–
sIL-2R (U/mL)	399.2 ± 196.1	840.7 ± 403.8	–	–
ACE (IU/L)	11.8 ± 2.8	17.8 ± 5.7	12.1 ± 3.2	–
HLA-B51	3 (30)	–	–	–
HLA-A26	4 (40)	–	–	–
Anatomic type				
Anterior uveitis	1 (10)	6 (35.3)	0 (0)	–
Intermediate uveitis	0 (0)	2 (11.8)	0 (0)	–
Posterior uveitis	4 (40)	3 (17.6)	3 (23.1)	–
Panuveitis	5 (50)	6 (35.3)	10 (76.9)	–
Systemic activity	7 (70)	11 (64.7)	10 (76.9)	–

Values are mean ± SD (range) or number (%). ACE = angiotensin-converting enzyme; CH50 = 50% hemolytic unit of complement; CRP = C-reactive protein; sIL-2R = soluble IL-2 receptor; WBC = white blood cell.

Among the reported miRNA analyses of BD, sarcoidosis, and VKH, there was one comprehensive miRNA analysis using microarray in patients with BD (six patients, only two of whom had uveitis), whereas miRNA analyses in patients with sarcoidosis and VKH were limited to investigations of selected miRNAs (<40 miRNAs). There is no report of comprehensive miRNA analyses of BD, sarcoidosis, and VKH using serum samples. Serum biomarkers are highly desirable for investigations in ophthalmology clinics because of the minimally invasive nature of sample collection. Therefore, search for new biomarkers expressed in the serum of patients with the three common major forms of uveitis would greatly contribute to the diagnosis, especially for nonuveitis specialists. In this study, we performed an advanced high-throughput, untargeted, and unbiased comprehensive miRNA analysis using patients' serum samples to search for new biomarkers.

METHODS

Subjects

Subjects were retrospectively identified from medical records at the Tokyo Medical University Hospital between 2016 and 2019. Patients with active uveitis who had not received anti-inflammatory therapies such as immunosuppressive agent, an anti-TNF- α agent, and systemic steroid therapy for 6 months were screened. Subjects were randomly selected from typical cases of each form of uveitis, and cases in which a uveitis specialists had difficulty in making a judgment of the diagnosis were excluded. Ten patients with BD, 17 patients with sarcoidosis, and 13 patients with VKH were studied. Eleven healthy subjects were included as controls. The demographic and clinical characteristics of the uveitis patients were assessed at the time of diagnosis and summarized in Table 1. Systemic activity in Table 1 was defined as oral aphthous ulcers, genital ulcers, arthralgia, or skin nodules in BD; headache or tinnitus in VKH; and active lesions in organs other than the eyes in sarcoidosis. BD was diagnosed according to the diagnostic criteria reported by the Designated Disease Study Group of the Ministry of Health, Labour, and Welfare in Japan.¹⁶ Sarcoidosis was diagnosed according the diagnostic criteria revised in

2019,¹⁷ and VKH according to the international diagnostic criteria.¹⁸ This study was approved by the Ethics Committee of Tokyo Medical University Hospital and written informed consent was obtained from all participants.

Blood Sample Collection

Serum samples were collected in BD vacutainer tubes using 21G needle. From each patient enrolled in this study, a sample of venous blood (approximately 5.0 mL) was collected in a tube, and then centrifuged (1000×g at room temperature for 15 minutes) to collect serum, which was stored at –80°C until study.

RNA Extraction and Microarrays

Gene tip miRNA was extracted from fresh frozen serum samples using the 3D-Gene RNA extraction reagent from a liquid sample kit (Toray Industries, Inc., Kanagawa, Japan) and concentrated. The extracted miRNA was fluorescently labeled using the 3D-Gene miRNA Labeling kit (Toray Industries, Inc.). The fluorescently labeled RNA was hybridized to a 3D-Gene Human miRNA Oligo Chip (Toray Industries, Inc.)¹⁹ designed to detect 2565 mature human miRNA sequences registered in miRBase Release 21 (<http://www.mirbase.org/>). The chip was scanned using a 3D-Gene Scanner. The miRNAs with signals higher than the background signal were first selected (positive call), and the background signal was subtracted from each positive call miRNA signal. MicroRNA signal values were standardized by global normalization as follows. For each sample, raw data were log-transformed, and the median was calculated. All data were shifted so that the median values were aligned, except when raw data = 2; in that case, the data were not shifted.

Bioinformatic Analysis and Statistical Analysis

A miRNA with fold change (FC) of 2.0 or greater or 0.5 or less ($|\log_2 \text{FC}| \geq 1$) and a *P* value of less than 0.05 in an unpaired *t* test was defined as miRNA that was deregulated in a disease. Unsupervised hierarchical clustering analysis was performed using an algorithm based

on Pearson correlation and the average-linkage method. Differentially expressed miRNAs between any two groups of samples were identified using the criteria, including *P* values and FC.

Targeted genes of significantly deregulated miRNAs were identified using the Database for Human MicroRNA Target Prediction (miRDB) (<http://mirdb.org/>). Pathway analysis using these miRNAs was performed using DNA Intelligent Analysis–miRPath v3.0 (<http://snf-515788.vm.okeanos.grnet.gr/>). Because more than 100 miRNAs cannot be input to the DNA Intelligent Analysis–miRPath, when there were more than 100 deregulated miRNAs, they were arranged in ascending order of FC, and the top 100 miRNAs were adopted. Pathway analysis using target genes were performed using the Database for Annotation Visualization and Integrated Discovery Bioinformatics Resources 6.8 (<https://david.ncifcrf.gov/>). Cytoscape 3.7.1 (<http://manual.cytoscape.org/en/stable/>) was used to create networks of relations between mitogen-activated protein kinase (MAPK) signaling pathway and miRNAs as well as between cytokines and miRNAs.

Statistical analyses were performed using R (3.6.2.) (<http://www.R-project.org>). Statistical analysis was performed by a two-tailed Student *t* test. Differences were considered significant at a *P* value of less than 0.05. Principal component analysis was used to discriminate the different biological samples based on the distances of a reduced set of new variables (principal components), using two principal components for depicting the results in two dimensions.

In addition, to perform predictions using multiple miRNAs, we decreased the number of miRNAs that had significant expression using Boruta selection (<https://notabug.org/mbq/Boruta/>), which output variable importance measure using random forest, which is a type of machine learning. And, we constructed receiver operator characteristic (ROC) curves using a panel of multiple miRNAs generated using R package random forest and compared with a single miRNA identified as the most important factor by random forest. ROC curve was plotted with Graph Pad Prism 6, which presented sensitivity and specificity metrics for each ROC curve at the optimal threshold to evaluate the predictive power of candidate miRNAs in serum for each form of uveitis.

RESULTS

Comparison With Healthy Controls

A total of 281 upregulated miRNAs and 137 downregulated miRNAs were detected in patients with BD, whereas 35 upregulated miRNAs and 86 downregulated miRNAs were found in patients with sarcoidosis, and 153 upregulated miRNAs and 35 downregulated miRNAs in patients with VKH (Supplementary Tables S1–S3). Among these miRNAs, 18 upregulated miRNAs and 27 downregulated miRNAs were common to the three diseases (Figs. 1a–1e, Table 2). For each uveitis, a hierarchical cluster analysis with an unsupervised approach was then performed to investigate the differences in expression of miRNAs between uveitis patients and healthy controls (Figs. 1f–1h). Obviously, for each of the uveitis, serum miRNA results of patients were clearly separated from those of healthy controls. These results thus suggest that patients with BD, sarcoidosis, or VKH had a significantly distinct serum miRNA profile.

TABLE 2. List of miRNAs Showing Altered Expression Levels in All Three Forms of Uveitis

Upregulated miRNA	Downregulated miRNA
hsa-miR-1226-3p	hsa-miR-1301-5p
hsa-miR-133b	hsa-miR-193a-5p
hsa-miR-193b-3p	hsa-miR-300
hsa-miR-204-5p	hsa-miR-302c-5p
hsa-miR-2277-5p	hsa-miR-3605-5p
hsa-miR-326	hsa-miR-3612
hsa-miR-422a	hsa-miR-379-5p
hsa-miR-4652-3p	hsa-miR-3936
hsa-miR-4698	hsa-miR-3978
hsa-miR-483-3p	hsa-miR-4461
hsa-miR-495-5p	hsa-miR-4633-3p
hsa-miR-519d-5p	hsa-miR-4681
hsa-miR-520f-5p	hsa-miR-4694-3p
hsa-miR-542-5p	hsa-miR-4724-5p
hsa-miR-548ab	hsa-miR-4755-3p
hsa-miR-6734-3p	hsa-miR-5190
hsa-miR-6744-3p	hsa-miR-5192
hsa-miR-6769b-3p	hsa-miR-6501-3p
	hsa-miR-6728-5p
	hsa-miR-6804-5p
	hsa-miR-6817-5p
	hsa-miR-6823-5p
	hsa-miR-6853-5p
	hsa-miR-7162-3p
	hsa-miR-758-5p
	hsa-miR-8060
	hsa-miR-920

Comparison Between the Three Forms of Uveitis

As a next step, we conducted pairwise comparisons between the three forms of uveitis. When BD and VKH were compared, expression of 104 miRNAs was enhanced in BD and expression of 151 miRNA was enhanced in VKH. When BD and sarcoidosis were compared, expression of 302 miRNA was enhanced in BD and expression of 72 miRNA was enhanced in sarcoidosis. When sarcoidosis and VKH were compared, none of the miRNAs showed enhanced expression in sarcoidosis, whereas the expressions of 55 miRNAs was enhanced in VKH (Figs. 2a–2c). When comparing each uveitis with another uveitis, the principal component analysis using miRNAs with $|\log_2 \text{FC}| \geq 1$ separated BD, sarcoidosis, and VKH into three groups by the first and second components, and the hierarchical cluster analysis using the average values of these miRNAs also distinctly classified the three major forms of uveitis (Figs. 2d–2e). These results indicate that BD, sarcoidosis, and VKH have different serum miRNA profiles that may contain biomarkers for each uveitis.

Pathway Enrichment Analysis of miRNAs Deregulated in Uveitis

In the third part of our analysis, we sought to identify all the molecular pathways that were targeted by the selected miRNAs by performing a pathway enrichment analysis based on annotated gene targets in DNA Intelligent Analysis–miRPath. For detailed analysis of the most relevant pathways of each uveitis, the Kyoto Encyclopedia of Genes

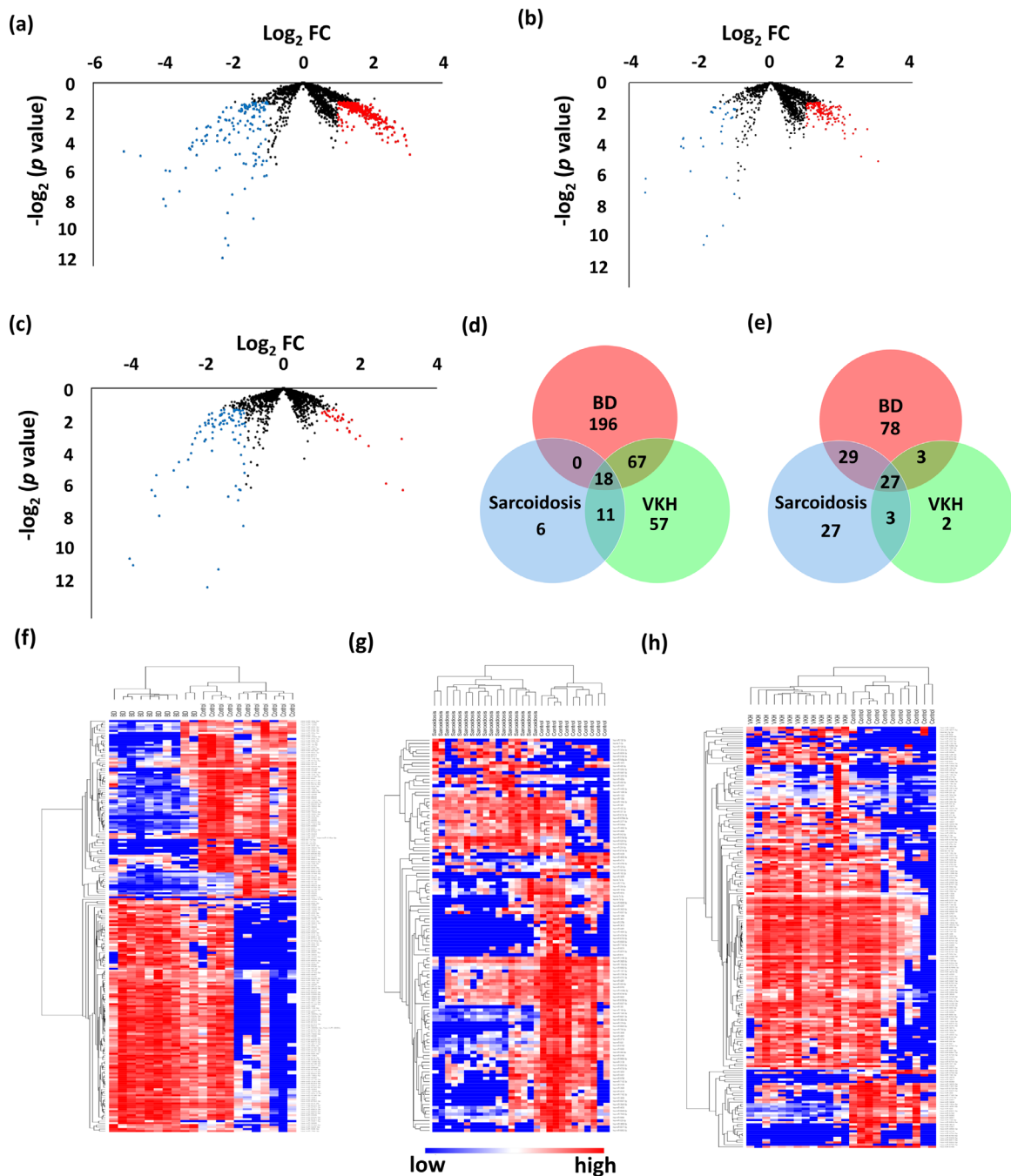


FIGURE 1. Differentially expressed miRNAs compared with healthy controls. (a–c) Volcano plots of serum miRNAs in (a) BD, (b) sarcoidosis, and (c) VKH. Blue dots indicate downregulated and red dots indicate upregulated miRNAs. Only miRNAs with $P < 0.05$ and $|\log_2 \text{ FC}| \geq 1$ are included in blue or red dots. Horizontal axis: $\log_2 \text{ FC}$, vertical axis: P value. (d and e) Venn diagrams showing the numbers of (d) upregulated miRNAs and (e) downregulated miRNAs in each uveitis. The diagram depicts the number of deregulated miRNAs specific for each disease and the number of miRNAs overlapping with other diseases. (f–h) Unsupervised hierarchical clustering analysis with a heatmap using serum miRNAs in (f) BD, (g) sarcoidosis, and (h) VKH. The cluster analysis shows a good separation between each uveitis and healthy controls based on markedly different miRNAs. The red to blue colors corresponds with high to low values, respectively.

and Genomes database was used to search for potential compound identities and relevant pathways. The software allowed us to evaluate the miRNA regulatory effect and to identify regulated pathways based on predicted and validated miRNA–target interactions. Pathway analysis using miRNAs that were downregulated in all three diseases compared with healthy controls suggested that a total of

33 pathways, including the TGF- β signaling pathway, AMPK signaling pathway, and MAPK signaling pathway, were associated with each uveitis by gene union analysis (Fig. 3). These results suggest that the serum miRNA profiles of BD, sarcoidosis, and VKH may provide clues to explain the different pathogenetic pathways leading to different forms of uveitis.

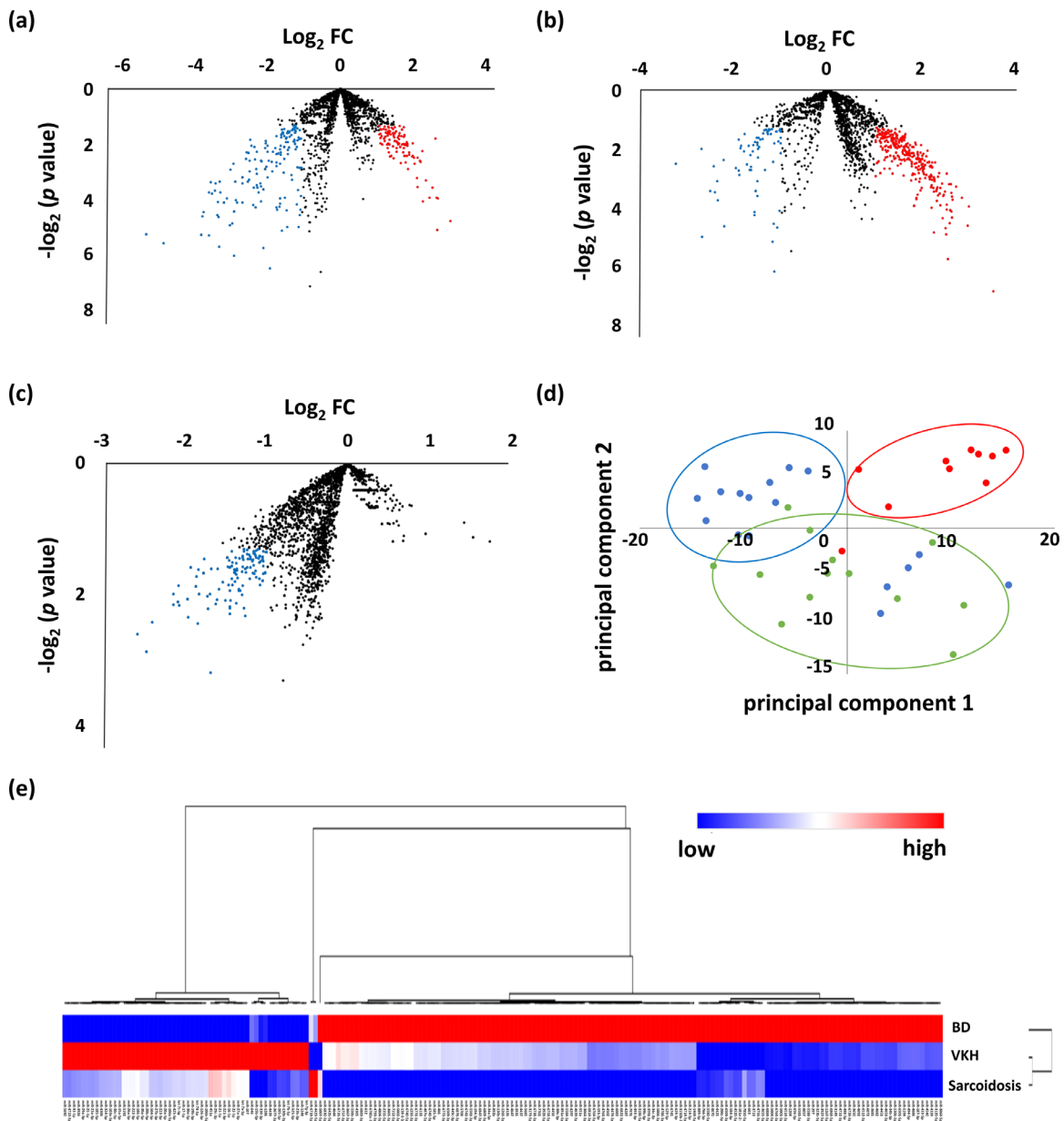


FIGURE 2. Differentially expressed miRNAs in pairwise comparisons of the three forms of uveitis. (a) Volcano plot of BD versus sarcoidosis. Blue dots indicate miRNAs upregulated in sarcoidosis and red dots miRNAs upregulated in BD. (b) Volcano plot of BD versus VKH. Blue dots indicate miRNA upregulated in VKH and red dots miRNAs upregulated in BD. (c) Volcano plot of VKH versus sarcoidosis. Blue dots indicate miRNA upregulated in VKH. Only miRNAs having $P < 0.05$ and $|\log_2 \text{FC}| \geq 1$ are included in blue or red dots. (d) Principal component analysis plot (using miRNAs with $|\log_2 \text{FC}| \geq 1$) separates BD (red dots), sarcoidosis (blue dots), and VKH (green dots) into three groups. (e) Cluster analysis using average values of miRNAs in each uveitis shows a good separation between the three groups of uveitis. The red to blue colors correspond with high to low values, respectively.

Comparative Analysis of Selected miRNA Gene Targets and Differentially Expressed Genes in Uveitis

To better define the roles played by miRNAs in the pathogenesis of uveitis, we searched for miRNAs that target deregulated genes in uveitis. Therefore, we used the more sophisticated integrative database for human miRNA target prediction (miRDB: <http://www.mirdb.org/>) to obtain lists of genes targeted by the downregulated miRNAs with very high prediction scores (≥ 95) common to the three forms of uveitis compared with healthy controls. A Database for

Annotation Visualization and Integrated Discovery analysis using these target genes suggested a pathway relationship, as shown in Table 3. This analysis also suggested that the MAPK signaling pathway was a relevant pathway for the three major forms of uveitis (Fig. 4). Thus, we were able to identify miRNAs that may control gene modulation involved in disease pathogenesis. Table 4 shows the targeted genes (target prediction score of ≥ 95) and their corresponding targeting miRNAs. MicroRNA target many genes, but only genes with prediction scores of 95 and higher were used in this analysis, which limited the numbers of genes analyzed. Nevertheless, pathway analysis strongly

TABLE 3. Pathway Analysis of Genes Targeted by Downregulated miRNAs Found in All Three Forms of Uveitis

Category	Pathway	P Value
KEGG_PATHWAY	MAPK signaling pathway	2.10E-03
KEGG_PATHWAY	PI3K-Akt signaling pathway	1.30E-02
KEGG_PATHWAY	Circadian rhythm	3.40E-02
KEGG_PATHWAY	Signaling pathways regulating pluripotency of stem cells	4.30E-02
KEGG_PATHWAY	Focal adhesion	4.90E-02

KEGG = Kyoto Encyclopedia of Genes and Genomes.

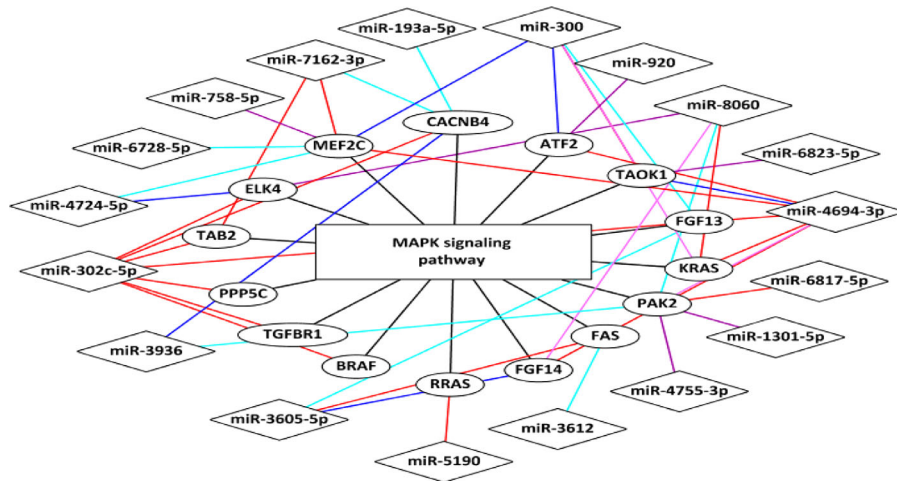


FIGURE 4. Predicted genes in the MAPK signaling pathway targeted by downregulated serum miRNAs (miRNAs) in BD, sarcoidosis, and VKH. Selected inflammatory genes targeted by each group of miRNAs are depicted in a network diagram. Differentially expressed miRNAs that do not target any of the selected genes are not shown. The lines connecting the miRNAs and the genes are color coded for prediction score (*red*, score ≥ 90 ; *pink*, $90 > \text{score} \geq 80$; *purple*, $80 > \text{score} \geq 70$; *blue*, $70 > \text{score} \geq 60$; *light blue*, $60 > \text{score} \geq 50$). ATF2 = Activating transcription factor 2; BRAF = B-Raf proto-oncogene, serine/threonine kinase; CACNB4 = calcium voltage-gated channel auxiliary subunit beta 4; ELK4 = ETS transcription factor ELK4; FAS = Fas cell surface death receptor; FGF13 = fibroblast growth factor 13; FGF14 = fibroblast growth factor 14; KRAS = KRAS proto-oncogene, GTPase; MEF2C = myocyte enhancer factor 2C; PAK2 = p21 (RAC1) activated kinase 2; PPP5C = protein phosphatase 5 catalytic subunit; RRAS = RAS related; TAB2 = TGF-beta activated kinase 1 (MAP3K7) binding protein 2; TAOK2 = TAO kinase 1; TGFBR1 = transforming growth factor beta receptor 1.

sarcoidosis,^{21,22} and VKH,^{23,24} we examined the relations of these cytokines with the deregulated miRNAs in these diseases. First, we searched miRDB to find miRNAs targeting these cytokines. The miRNAs were classified into seven modules according to the form of uveitis with altered expression. Figure 5 presents the network generated using Cytoscape showing the relations of these cytokines with miRNAs that were deregulated in all three forms of uveitis. These results indicate that multiple miRNAs are involved and may activate or suppress these cytokines as a mechanism of pathophysiology. These results suggest that cytokines involved in inflammation are regulated by miRNAs and that the serum miRNA profile reflects changes in cytokines.

Machine Learning and miRNA Expression Validation in Uveitis

Machine learning using random forest was performed using Boruta (<https://notabug.org/mbq/Boruta/>) for miRNAs with a *P* value of less than 0.05 when compared with other uveitis. Multivariate analysis identified 52, 30, and 24 miRNAs in BD, sarcoidosis and VKH, respectively (Table 5). Among these miRNAs, random forest analysis revealed that miR-4708-3p, miR-4323, and let-7g-3p were the best predictors for BD, sarcoidosis and VKH, respectively. The areas under the ROC curves (AUCs) for BD, sarcoidosis, and VKH were 0.963,

0.847, and 0.821 using miR-4708-3p, miR-4323, and let-7g-3p, respectively (95% confidence intervals, 0.91–1.02, 0.70–0.99, and 0.67–0.97; $P < 0.0001$, $P < 0.0002$, and $P < 0.0012$).

We subsequently evaluated whether a combination of all the miRNAs extracted by the machine learning method using Boruta discriminate the three major forms of uveitis. The AUCs using the panels of miRNAs extracted by Boruta were 0.963, 0.900, and 0.940 for BD, sarcoidosis, and VKH, respectively (95% confidence intervals, 0.91–1.02, 0.81–0.99, and 0.87–1.01; $P < 0.0001$ for all), which were the same (BD) or higher (sarcoidosis and VKH) than the AUCs using single most predictive miRNAs (Fig. 6). The expression levels of the miRNAs did not correlate with age or sex. Consequently, correction for age and sex did not influence the results of analyses.

DISCUSSION

In this study, we sought to identify novel miRNAs that alter in expression level specifically in the three major forms of uveitis by performing a comprehensive search with the aim to examine (1) whether BD, sarcoidosis, and VKH possess distinct miRNA expression profiles when compared with healthy controls and (2) whether the miRNA signatures identified for the three major forms of uveitis allow discrimination between them when compared with each other. Among

TABLE 4. MicroRNAs Altered in All Three Forms of Uveitis and Their Respective Targeted Genes (for Genes With Prediction Scores of ≥ 95)

miRNA	Target Genes									
miR-1301-5p	GPD2	C5orf30	SLC6A1	KCNMB2						
miR-193a-5p	BLID									
miR-300	USP1	CNOT6L	EMP2	PTBP3	ACTR3	TMEM132B	GPR37	MED14	HOXA9	LRP6
	KLF4	DGKH	ZFPM2	MRS2	KIF11	GPR34	BARD1	NECTIN1	GPR65	RICTOR
	B3GNT2	SCAI	RIMKLB	CDK19	KIAA0408	SNAPC1	ZFY	FYTTD1	ADGRG2	CEMIP2
	PAN3	WIP1	GRM8	RABGGTB	CEPT1	PSD3	MARK1	KPNA3	CEP76	ANO4
	LEF1	ANKRD31	PNISR	CCNA2	COL4A1	DCUN1D4	ITGAV	NCOA2		
miR-302c-5p	SBF2	ADAM23	EIF4A2	DPY19L1	GBE1	IBTK	AMMECR1	NRARP	ZNF195	ELK4
	NUDT12	CHD6	SLC7A11	TMEM243	ZFPM2	HNRNPF	EPHA7	PPP5C	KHDRBS3	SLC38A4
	ATP6V0A2	ASF1A	FXR1	B3GNT2	STC1	CREBZF	CERS3	PHKB	MYT1	APBB2
	EIF5A2	HTR2C	AP1AR	HNRNPR	SYNCRIP	NAA50	KCTD8	CD200R1	RNF145	DNA2
	MBNL3	UBR3	STRN	CBL1	MBNL1	BMP2K	PRDM11	FGF13	SPRY1	BRAF
	TGFBR1	CDK8	CHL1	LYSMD3	CACNB4	HNRNPD1	ZNF275	CGGBP1	SLC2A2	HNRNPH1
	RASSF3	LRP1B	ERBB4	MIER3						
miR-3605-5p	KRTAP9-3	HMG3	UTY	FAS	CHRM1	KIAA0408	FAM169B			
miR-3612	KLF6	CHST2	THEM5	NONO	SYDE1	PIK3C2B				
miR-379-5p	TXLNG	MTMR2								
miR-3936	FBXW4	CFHR5								
miR-3978	PP15K2	AP3S2	CHIC2	PRICKLE2	AP1S2	SNAP25	ZW10	LRRTM2	TRANK1	KDM7A
miR-4461	-									
miR-4633-3p	-									
miR-4681	-									
miR-4694-3p	NUP153	ZIC3	RGS8	YIPF6	ANXA5	HECW2	CCDC179	PTPN4	RORA	ELL2
	FGF14	PRKAA1	RYR2	CWC27	FGF13	ARSJ	KCNIP4	AUTS2	TNRC6B	PCNP
	SEZ6L	ZNF441	GOLGA1	MEF2C	CNOT6L	MBL2	EXOC5	PSMA7	KHSRP	TET3
	ZC3H12C									
miR-4724-5p	PKD2L2	DR1	ZDHHC17	CDYL						
miR-4755-3p	NEURL1B	CDH22	DIO3	ANKRD52	ACVRL1	LZTS1	FOXO3			
miR-5190	LYSMD4	TAPT1	AGBL3	LGALS1	TTBK1	RRAS				
miR-5192	KDM3B	ABRAXAS2	GORASP2							
miR-6501-3p	N4BP2	DCAF7	DIRAS2	GALE	SULT2A1	TMEM168	GIPC3	ZFR	TRIO	SORT1
miR-6728-5p	ZCRB1	BCL6	DCAF8	NUTM1						
miR-6804-5p	JMJD8									
miR-6817-5p	DAB1	CSTF3	GGACT	PRIM1	GRM5	PAK2	RADIL	ATRX		
miR-6823-5p	SENP8									
miR-6853-5p	-									
miR-7162-3p	PPARGC1B	GALNT2	RBFOX1	PDSS2	CEP164	ACVR2B	FIGNL2	MFHAS1	SPECC1	IKZF3
	DLC1	GALNT4	POC1B-GALNT4	IRGQ						
miR-758-5p	-									
miR-8060	LRIT2	FBXO21	SEMA5A	ZC3H12B	MAPK6	JARID2	SYF2	CPEB3	NPAS2	
miR-920	TNRC6B									

the three major forms of uveitis, biomarkers, such as sIL-2R and angiotensin-converting enzyme, have been identified in sarcoidosis,²⁵ but not in the other two diseases. To date, the diagnosis of BD and VKH is based on clinical symptoms because no sensitive and specific biomarkers are available. In this context, it is significant that our study achieved complete discrimination of these three major forms of uveitis from each other and from a healthy condition, and that the miRNAs we identified may contain novel biomarkers for these diseases.

The elucidation of serum biomarkers is highly desirable owing to the minimal invasive nature of sample collection. Our results show that local intraocular inflammation leaves a molecular footprint in peripheral blood, although the sources of serum miRNAs remain unknown. Nevertheless, given that serum miRNAs are derived not only from the eye affected by the disease but also from systemic immune responses, serum miRNAs may carry more comprehensive local and systemic information about the diseases. MicroRNAs function by negatively regulating gene expres-

sion at mRNA and protein levels. To understand the functions of miRNAs expressed differentially in the major forms of uveitis, we need to focus on the expression of their respective target genes. However, it is not feasible to explore all the targets of differentially expressed miRNAs in a single study. Therefore, we selected 27 downregulated miRNAs common to the three diseases to study their gene regulatory function. An analysis of pathways that were enriched by the selected target genes confirmed the essential roles of these transcripts and their corresponding targeting miRNA in the pathogenesis of BD, sarcoidosis, and VKH. Although the etiology and pathogenesis of these three major forms of uveitis remain unclear, the evidence obtained in the present study suggests that 45 miRNAs (18 upregulated miRNAs and 27 downregulated miRNAs) showing deregulated expression levels in all the three diseases may also play crucial roles in the pathogenesis of other forms of uveitis. Among the 45 miRNAs distinguishing uveitis from healthy condition, 18 miRNAs (miR-2277-5p, miR-519d-5p, miR-520f-5p, miR-548ab, miR-6744-3p, miR-6769-3p, miR-1301-5p, miR-3936,

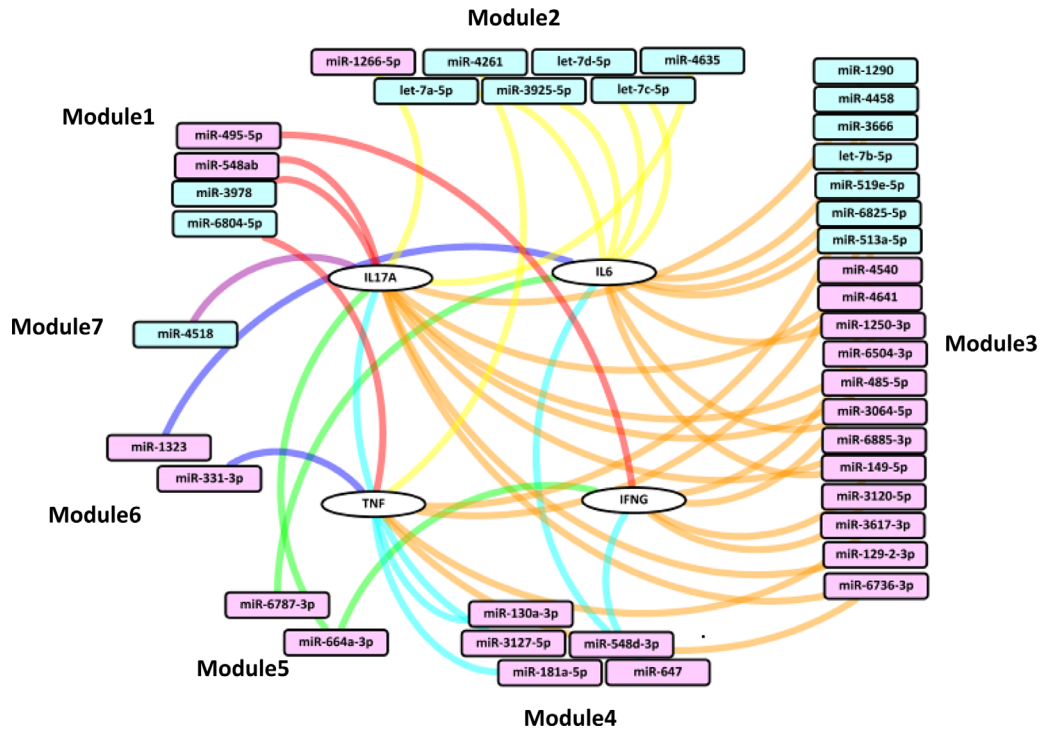


FIGURE 5. miRNAs–cytokine gene interaction networks in BD, sarcoidosis, and VKH. Twenty-four upregulated miRNAs (*pink boxes*) and 16 downregulated miRNAs (*blue boxes*) in the serum of patients with uveitis have interactions with cytokine genes related to uveitis. The lines connecting the miRNAs and genes are color-coded for each module. Module 1 = altered in all uveitis; Module 2 = Altered in BD and sarcoidosis; Module 3 = altered in BD; Module 4 = altered in VKH; Module 5 = altered in BD and VKH; Module 6 = altered in sarcoidosis and VKH; Module 7 = altered in sarcoidosis.

miR-4633-3p, miR-4681, miR-4694-3p, miR-5192, miR-6501-3p, miR-6728-5p, miR-6804-5p, miR-6823-5, miR-7162-3p, and miR-8060) have not been reported previously. Further studies are needed to analyze the functions of these miRNAs.

Anti-TNF- α drugs such as adalimumab are used to treat refractory noninfectious uveitis.²⁶ The MAPK signaling pathway is a well-known inflammatory cytokine pathway associated with TNF- α . A pathway analysis revealed that several downregulated miRNAs found in the three uveitis entities were involved in the MAPK signaling pathway, possibly through post-transcriptional regulation by miRNAs. The fact that the MAPK signaling pathway ranked the top pathway in the analysis using target genes provides additional evidence that this pathway is activated in various forms of uveitis, which may be the reason for the therapeutic effect of anti-TNF- α drugs.

In recent years, genome-wide association studies have reported genetic polymorphisms of *IL-10* and *IL-23R/IL-12RB2a* as disease susceptibility genes in BD.^{27,28} The products of BD-susceptible genes were also found in our study to be related to serum miRNAs such as miR-4261 (targeting *IL10* and *IL23R*), and miR-6788-3p (targeting *IL10* and *IL12RB2*).

One report showed that miR-146a was related to VKH but not BD,¹¹ and another report found that miR-146a was related to BD.¹² In our study, miR-146a expression was upregulated in VKH, but did not change in BD, and these results are consistent with those of Hou et al.¹¹ In addition, miR-155 that regulates helper T cell 17 cell differentiation^{12,29} and miR-196a³⁰ have been reported in BD. In sarcoidosis, alterations of miR-34a,³¹ miR-150-5p, miR-202-

3p, miR-204-5p, miR-222-3p,³² miR-16-5p, miR-425-5p, miR-93-5p, miR-21-5p, and miR-340-5p¹³ expression have been reported, but the studies were conducted on pulmonary sarcoidosis. The miRNA profile may differ depending on the affected organ. Our search of the literature found no reports of miRNAs in ocular sarcoidosis. We report for the first time a study to delineate the serum miRNA profile of ocular sarcoidosis by comparing with BD and VKH. Finally, miR-301a, miR-23a,¹¹ and miR-20a-5p³³ have been reported to be related to VKH. We also found altered expression of miR-23a in patients with VKH in this study. Our results for BD agree with those of Puccetti et al.¹⁴ for miRNAs with relatively large FCs such as miR-27b-3p and miR-126-3p, and miR-29b-1-5p showed different expression when compared with sarcoidosis and VKH. Other miRNAs that we identified, such as miR-185 and miR-196a in BD, as well as miR-301a and 20a-5p in VKH, did not match those reported previously. Note that many previous studies analyzed peripheral blood mononuclear cells and CD4-positive T cells, whereas we used serum samples in this study. Differences in miRNAs present in various tissues may have led to discrepancies in the results. In the search of biomarkers, samples that can be collected with minimal invasiveness and processed by simple procedures are desirable. In that respect, our approach of using serum sample that does not require separation of peripheral blood mononuclear cells seems to be suitable.

Among the deregulated miRNAs, we focused on miR-4708-3p. In microarray analysis, miR-4708-3p was downregulated in the serum of patients with BD compared with other uveitis and healthy controls. Furthermore, machine

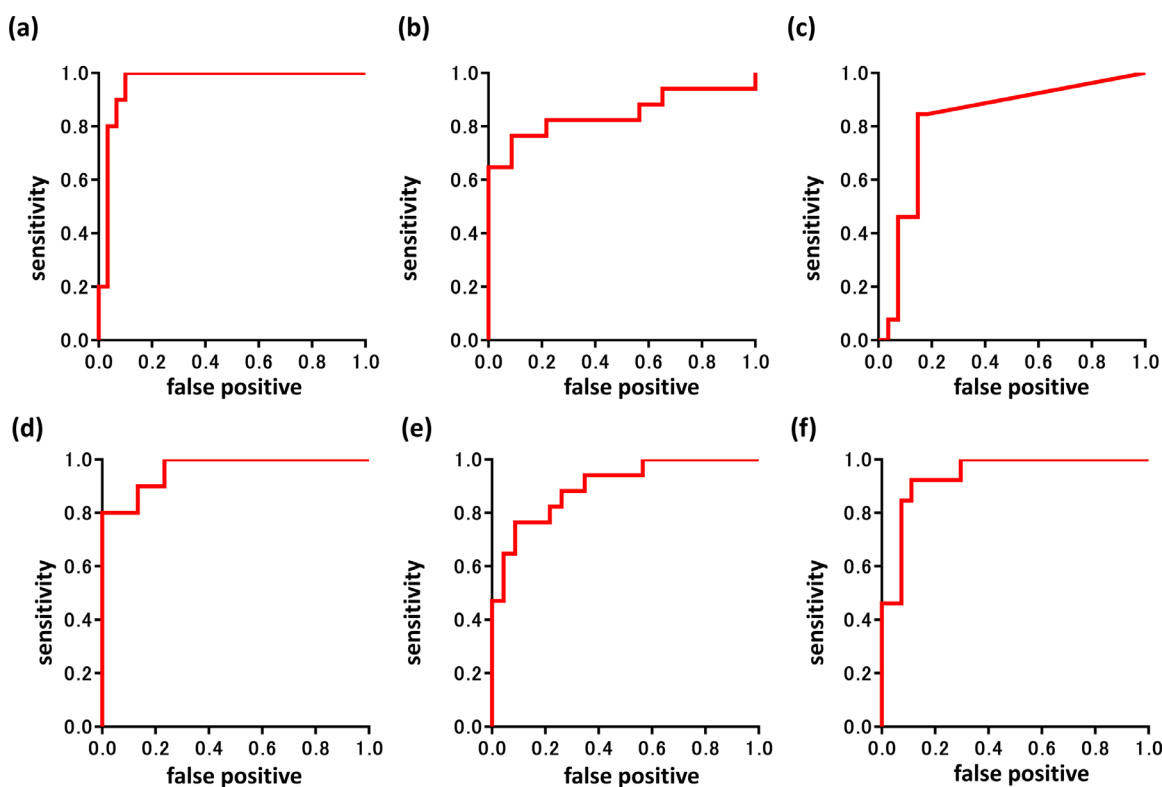


FIGURE 6. ROC curves using the miRNAs with the best prediction and using panels of combined miRNAs. (a) ROC curve of BD versus others using miR-4708-3p. (b) ROC curve of sarcoidosis versus others using miR-4323. (c) ROC curve of VKH versus others using let-7g-3p. (d) ROC curve of BD versus others using 52 miRNAs combined. (e) The ROC curve of sarcoidosis versus others using 30 miRNAs combined. (f) The ROC curve of VKH versus others using 24 miRNAs combined. The area under the ROC using a panel of miRNAs is the same (BD) or higher (sarcoidosis or VKH) than that using single miRNA.

learning indicated that miR-4708-3p was the best predictor for BD, and the AUC for this miRNA was the same as that for the panel of multiple miRNAs for BD. This miRNA has not been reported to be related to diseases including cancer or inflammatory diseases. According to miRDB, miR-4708-3p targets 430 genes, including IL 18 binding protein (*IL18BP*), IL 22 (*IL22*), and IL 23 receptor (*IL23R*) genes. *IL18BP* encodes IL-18-binding protein, which binds to IL-18 to inhibit the binding of IL-18 to its receptor, consequently inhibiting IFN- γ production.^{34,35} Blood IL-18 mRNA expression in patients with BD is significantly higher than in healthy subjects.³⁶ It is possible that downregulated miR-4708-3p may enhance the expression of *IL18BP*, resulting in suppressed IL-18 production. In contrast, helper T cell 22 cells derived from patients with BD produce a larger amount of IL-22 than cells from healthy subjects.³⁷ *IL23R* encodes IL-23 receptor, and blood *IL23R* mRNA expression in patients with BD is upregulated.³⁸ *IL23R* is a BD susceptibility gene,^{27,28} and may be involved in disease susceptibility at miRNA level. Downregulation of miR-4708-3p is consistent with increased expression of IL-22 protein expression and *IL23R* mRNA expression. It remains unknown whether the function of this miRNA is anti-inflammatory. Further research is needed to elucidate the functions of miR-4708-3p.

In the ROC analysis of this study, the AUC using the novel miRNA panel, including the most predictive miRNA biomarker, was equivalent or superior to the AUC using the single most predictive miRNA biomarker for BD, sarcoidosis, and VKH. Hence, it was possible to increase the AUC

to nearly 90% by combining multiple miRNAs. A combination of biomarkers representing different biologic pathways may improve diagnostic accuracy. Previous studies have proposed various biomarkers such as hemoglobin with neutrophil-to-lymphocyte ratio (AUC = 0.897)³⁹ and uric acid (AUC = 0.821)⁴⁰ for BD, as well as angiotensin-converting enzyme (AUC = 0.727),⁴¹ the erythrocyte sedimentation rate, and C-reactive protein (AUC = 0.795 and 0.644)⁴² for sarcoidosis. To our knowledge, no reliable diagnostic biomarker with AUC data is available for VKH. The results of our study using a panel of miRNAs yielded higher AUC than previous reports. Because miRNAs bind to multiple mRNAs in a complementary manner, they may show complex changes in expression level. In this respect, it seems better to design a panel that combines multiple miRNAs as a biomarker to enhance predictive accuracy rather than using one type of miRNA. To our knowledge, this is the first published report of a serum-based miRNA panel with a very high accuracy for detecting the three major forms of uveitis. These miRNA panels robustly identify (with an accuracy of >90%) each of the three diseases. We believe that the statistical data indicate that the panels have excellent diagnostic power and provide definitive and reliable discrimination between the three major uveitis entities. However, these miRNA panels require external validation before further development for clinical use.

Although this study has revealed several interesting findings, there are possible biases and limitations, including its retrospective nature and the fact that the data were collected from a single institution. Although this is the most

TABLE 5. List of miRNAs in Each Uveitis Selected by Multivariate Analysis Using Boruta: With the Best Predictor Underlined

BD	Sarcoidosis	VKH
miR-1204	miR-1236-3p	<u>let-7g-3p</u>
miR-124-3p	miR-1304-3p	miR-1224-5p
miR-1246	miR-1469	miR-126-3p
miR-1343-3p	miR-1909-3p	miR-1343-3p
miR-145-5p	miR-1910-5p	miR-20b-5p
miR-187-3p	miR-1913	miR-2110
miR-191-5p	miR-296-5p	miR-22-3p
miR-194-3p	miR-3192-3p	miR-22-5p
miR-22-3p	miR-3194-5p	miR-3135b
miR-2467-3p	miR-326	miR-3622a-5p
miR-29c-3p	miR-3663-5p	miR-3677-3p
miR-30b-3p	miR-3944-5p	miR-4299
miR-3153	miR-4268	miR-4433a-5p
miR-3160-5p	<u>miR-4323</u>	miR-451a
miR-342-5p	miR-4731-3p	miR-4635
miR-3591-3p	miR-4758-5p	miR-4640-3p
miR-3605-3p	miR-508-5p	miR-525-5p
miR-3934-5p	miR-583	miR-6073
miR-423-3p	miR-664a-3p	miR-646
miR-4278	miR-6716-5p	miR-6822-5p
miR-4286	miR-6759-5p	miR-7108-3p
miR-433-5p	miR-6846-3p	miR-920
miR-4428	miR-6859-3p	miR-92a-3p
miR-4448	miR-6877-3p	miR-92b-3p
miR-4454	miR-711	
miR-4456	miR-7113-3p	
miR-451a	miR-7113-5p	
miR-4540	miR-764	
<u>miR-4708-3p</u>	miR-874-5p	
miR-4727-3p	miR-887-3p	
miR-4753-3p		
miR-4787-3p		
miR-5001-3p		
miR-508-5p		
miR-551b-5p		
miR-5698		
miR-6072		
miR-6073		
miR-6131		
miR-614		
miR-650		
miR-6512-3p		
miR-6514-3p		
miR-6515-5p		
miR-668-5p		
miR-6717-5p		
miR-6745		
miR-6748-5p		
miR-6883-5p		
miR-7975		
miR-8057		
miR-8059		

comprehensive miRNA study to date to explore new diagnostic biomarkers, the study had a small sample size; particularly, the number of patients with BD was small owing to the rareness of treatment-naïve BD. Serum is easier to obtain and costs less to prepare, making it more useful for screening in large-scale clinical trials and for future clinical use. Before further development for clinical use, the biomarker panels require external validation with similar rigorous clinical classification. Therefore, a further prospective study with

more patients from multiple centers is necessary, and such additional validation should be conducted in a more diverse demographic group than the initial cohort.

In conclusion, to our knowledge, this report is the first to be published of a comprehensive miRNA analysis that identifies distinct serum miRNA profiles in BD, sarcoidosis, and VKH, providing new insight into the pathophysiology and diagnosis of these three major uveitis entities. The lack of established biomarkers is the primary reason for the difficulty in making a diagnosis of these three diseases. The identification of new diagnostic biomarkers may improve the clinical outcome of uveitis. Some of the miRNAs identified in this study have not been hitherto reported, and these miRNAs may contain novel biomarkers and potential therapeutic targets. Serum samples from untreated patients were used in this study, and further research is needed to investigate whether therapeutic treatment impacts the miRNA profile. In the future, the functions of these miRNAs will be analyzed using animal models such as experimental autoimmune uveoretinitis model.

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