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.
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 specialist 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 to 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 fluorescent labeled using the 3D-Gene miRNA Labeling kit (Toray Industries, Inc.). The fluorescent labeled RNA was hybridized to a 3D-Gene Human miRNA Oligo Chip (Toray Industries, Inc.) 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 (|log₂ FC| ≥ 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://smf-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**

<table>
<thead>
<tr>
<th>Upregulated miRNA</th>
<th>Downregulated miRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-1226-3p</td>
<td>hsa-miR-1301-5p</td>
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<tr>
<td>hsa-miR-133b</td>
<td>hsa-miR-1934-5p</td>
</tr>
<tr>
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<td>hsa-miR-300</td>
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<tr>
<td>hsa-miR-204-5p</td>
<td>hsa-miR-302-5p</td>
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<td>hsa-miR-2277-5p</td>
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<td>hsa-miR-3612</td>
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<td>hsa-miR-6224</td>
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<td>hsa-miR-4633-3p</td>
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<td>hsa-miR-4681</td>
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<td>hsa-miR-4694-3p</td>
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<tr>
<td>hsa-miR-542-5p</td>
<td>hsa-miR-4724-5p</td>
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<tr>
<td>hsa-miR-58ab</td>
<td>hsa-miR-4755-5p</td>
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<td>hsa-miR-6734-3p</td>
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<td>hsa-miR-6769b-3p</td>
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<td>hsa-miR-6728-5p</td>
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<td>hsa-miR-6817-5p</td>
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<tr>
<td>hsa-miR-758-5p</td>
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</tr>
<tr>
<td>hsa-miR-8060</td>
<td>hsa-miR-920</td>
</tr>
</tbody>
</table>

**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 $|\log_{2} FC| \geq 1$ 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 correspond 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-\( \beta \) 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.
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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

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 |log₂ FC| ≥ 1 are included in blue or red dots. (d) Principal component analysis plot (using miRNAs with |log₂ FC| ≥ 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.
suggests the involvement of the MAPK signaling pathway, and analysis using the target genes further substantiated the involvement of the MAPK pathway in the pathologic conditions of all three forms of uveitis.

**Inflammatory Cytokines and miRNAs**

Because the functional roles of cytokines, including IL-6, IL-17, IFN-γ, and TNF-α, have been examined in BD.\(^ {20} \)}
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TABLE 3. Pathway Analysis of Genes Targeted by Downregulated miRNAs Found in All Three Forms of Uveitis

<table>
<thead>
<tr>
<th>Category</th>
<th>Pathway</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>KEGG_PATHWAY</td>
<td>MAPK signaling pathway</td>
<td>2.10E-03</td>
</tr>
<tr>
<td>KEGG_PATHWAY</td>
<td>PI3K-Akt signaling pathway</td>
<td>1.30E-02</td>
</tr>
<tr>
<td>KEGG_PATHWAY</td>
<td>Circadian rhythm</td>
<td>3.40E-02</td>
</tr>
<tr>
<td>KEGG_PATHWAY</td>
<td>Signaling pathways regulating pluripotency of stem cells</td>
<td>4.30E-02</td>
</tr>
<tr>
<td>KEGG_PATHWAY</td>
<td>Focal adhesion</td>
<td>4.90E-02</td>
</tr>
</tbody>
</table>

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 groups 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 > score ≥ 80; purple, 80 > score ≥ 70; blue, 70 > score ≥ 60; light blue, 60 > score ≥ 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.

FIGURE 5. Network showing the relations of these cytokines with downregulated miRNAs. The miRNAs were classified into seven modules according to the form of uveitis with altered expression. 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.
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 completediscrimination 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 expression 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-1301-5p, miR-3936, miR-3605-5p, and others) were found to be significantly altered in all three forms of uveitis.

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

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Target Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-1301-5p</td>
<td>GPD2, C5orf30, SLC6A1, KCNMB2</td>
</tr>
<tr>
<td>miR-1303a-5p</td>
<td>BLID, USP1, KLF4, B3GNT2, PAN3, Lef1, SFB2, NUDT12, ATP6V0A2, EIF5A2, MBNL3, TGFBR1, RASSF3, KRTAP9-3, miR-3605-5p, miR-3612, miR-379-5p, miR-3978, miR-4661, miR-4633-3p, miR-4654-3p, miR-4694-3p, miR-4724-5p, miR-4755-3p, miR-5190, miR-5192, miR-6501-3p, miR-6728-5p, miR-6804-5p, miR-6823-5p, miR-6853-5p, miR-7162-3p, miR-8060, miR-920, miR-2277-5p, miR-519d-5p, miR-520f-5p, miR-548ab, miR-6744-3p, miR-1301-5p, miR-3936,</td>
</tr>
</tbody>
</table>

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**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-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. 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. 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. 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. In addition, miR-155 that regulates helper T cell 17 cell differentiation and miR-196a have been reported in BD. In sarcoidosis, alterations of miR-34a, miR-150-5p, miR-202-3p, miR-204-5p, miR-222-3p, miR-16-5p, miR-125-5p, miR-93-5p, miR-21-5p, and miR-340-5p 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, and miR-20a-5p 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. 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 of 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
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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 IL18 binding protein (IL18BP), IL22 (IL22), and IL23 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. Blood IL-18 mRNA expression in patients with BD is significantly higher than in healthy subjects. 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. IL23R encodes IL-23 receptor, and blood IL23R mRNA expression in patients with BD is upregulated. IL23R is a BD susceptibility gene, 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) and uric acid (AUC = 0.821) for BD, as well as angiotensin-converting enzyme (AUC = 0.727), the erythrocyte sedimentation rate, and C-reactive protein (AUC = 0.795 and 0.644) 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

**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.
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 VHK, 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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