FULL LENGTH ARTICLE

Identification of differently expressed mRNAs by peripheral blood mononuclear cells in Vogt-Koyanagi-Harada disease

Yujing Li a, Guannan Su a, Fanfan Huang a, Ying Zhu a, Xiang Luo a, Aize Kijlstra b, Peizeng Yang a,*

a The First Affiliated Hospital of Chongqing Medical University, Chongqing Key Lab of Ophthalmology, Chongqing Eye Institute, Chongqing 400016, PR China
b University Eye Clinic Maastricht, Maastricht 6211, the Netherlands

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Abstract Vogt-Koyanagi-Harada disease (VKH) is a rare autoimmune disease characterized by diffuse and bilateral uveitis, alopecia, tinnitus, hearing loss, vitiligo and headache. The transcriptional expression pattern of peripheral blood mononuclear cells (PBMC) in VKH remains largely unknown. In this study, mRNA sequencing was conducted in PBMC from VKH patients with active uveitis before treatment (n = 7), the same patients after prednisone combined with cyclosporine treatment (n = 7) and healthy control subjects strictly matched with gender and age (n = 7). We found 118 differentially expressed genes (DEGs) between VKH patients and healthy control subjects, and 21 DEGs between VKH patients before and after treatment. TRIB1 was selected as a potential biomarker to monitor the development of VKH according to the mRNA sequencing. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were performed to predict the possible biological functions and signaling pathways of DEGs. Neutrophil degranulation, peptidase regulator activity, secretory granule membrane, cellular response to peptide, growth factor binding and cell projection membrane were enriched as GO annotations of DEGs. Arachidonic acid metabolism and mitogen-activated protein kinase (MAPK) signaling pathway were potential signaling pathways involved in pathogenesis and drug response of VKH. A protein–protein interaction (PPI) network was constructed by STRING, and colony stimulating factor 1 receptor (CSF1R) was identified as the hubgene of all DEGs by Cytoscape. The cell type presumed to contribute to the aberrant expression of DEGs was analyzed with the use of publicly available single-cell sequencing data of PBMC from a healthy donor and single-cell sequencing dataset of monocytes from VKH patients. Our findings may help to decipher the underlying cellular and molecular pathogenesis of VKH and may lead novel therapeutic applications.

* Corresponding author.
E-mail address: peizengycmu@126.com (P. Yang).

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Introduction

VKH is a rare autoimmune disease,¹ and various melanin-containing organs are affected by this systemic disorder.² A rapidly decreasing visual acuity with blurred vision accompanied with headache, neck and back stiffness, hearing loss, tinnitus, vitiligo and alopecia are the major symptoms of VKH.³ Cataract, glaucoma, choroidal neovascular membranes, subretinal fibrosis may occur as complications in half of the patients with VKH.⁴ A definite etiology of VKH is not clear, whereby HLA-DRB1*0405 carriers,⁵ single nucleotide polymorphism loci of different genotypes,⁶ vaccine injection,⁷ viral infections⁸,⁹ and recognition of melanin-related antigens² have all been implicated in the etiology of VKH. Diagnosis of VKH mainly depends on clinical manifestations supplemented with auxiliary examinations like fluorescein angiography, angiography with indocyanine green, and optical coherence tomography.¹⁰ To date, no biomarkers are available for the diagnosis of VKH, although elevated carbonic anhydrase 2 (CA2) and ras-related protein Rap-1b (RAP1B) in exosomes isolated from plasma have been proposed as biomarkers in early stage VKH.¹¹ The therapy of VKH often requires long-term oral corticosteroids¹² combined with cyclosporine.¹³ The long-term use of corticosteroids and cyclosporine may lead to adverse side effects.¹⁴,¹⁵ In addition, some VKH patients respond poorly to the corticosteroid-cyclosporine therapy, which necessitates the search for novel drugs.¹⁶ Untimely or inappropriate treatment can lead to blindness in VKH. Therefore, elucidating the detailed cellular and molecular mechanisms underlying the pathogenesis and finding potential therapeutic targets for this chronic and relapsing disease is urgently needed.

Quantitative measurement of mRNA, a high throughput technology, for the detection of genes with good sensitivity and reproducibility,¹⁷ may contribute to the diagnosis, drug discovery¹⁸,¹⁹ and surveillance of disease.²⁰ Aberrant mRNA expression profiles of PBMC have been described in several autoimmune diseases such as rheumatoid arthritis, inflammatory bowel disease,²¹ psoriasis²² and multiple sclerosis.²³ Abnormal expression of suppressor of cytokine signaling 1 (SOCS1),²⁴ interleukin 23 (IL-23),²⁵ nucleotide-binding oligomerization domain-containing protein 1 (NOD1), NOD2²⁶ in PBMC have been reported in VKH.

To date, a comprehensive mRNA sequencing of PBMC in VKH has not yet been reported and was therefore the subject of the study represented here. In this study we compared the mRNA profile of PBMC in healthy control subjects and in VKH patients before and after treatment. We found several DEGs, which are potentially involved in the pathogenesis of VKH and which might be engaged in drug responses. GO annotation, KEGG enrichment, PPI network and analysis of single-cell sequencing data of PBMC from a healthy donor and single-cell sequencing data of monocytes from VKH patients were performed to deepen our understanding of these DEGs.

Materials and methods

Subjects

Diagnosis of VKH was based on the revised diagnostic criteria for VKH²⁷ in combination with those reported earlier by our group.²⁸ VKH patients with cells and flare in the anterior chamber and vitreous, keratic precipitates and diffuse choroiditis²⁹ and who did not receive any treatment for more than 4 months before this attack were defined as VKH patient with active uveitis before treatment.

This study was approved by the Institutional Ethical Committee of The First Affiliated Hospital of Chongqing Medical University (#2018-048). Informed consents were obtained before the collection of samples. In this project, 7 VKH patients with active uveitis before treatment were recruited, their blood samples were also collected after the therapy with cyclosporine and prednisone for 8 weeks. Exclusions to the enrollment included diabetes mellitus, hypertension, dyslipidaemia, history of other autoimmune disease, immunodeficiency and other disorders. Healthy controls (n = 7) were strictly age- and gender-matched with the VKH patients.

Preparation of PBMC

Venous blood was collected in tubes containing ethylene diamine tetraacetic acid (EDTA), then kept on ice, and PBMC were isolated by gradient centrifugation within 1 h after collection. PBMC were isolated by Ficoll (TBD Science, Tianjin, China) density-gradient centrifugation (600 g for 30 min at 18 °C) according to the instruction manual, cells were suspended in ice-cold phosphate buffered solution and centrifuged at 300 g for 5 min at 4 °C twice. Purified PBMC were stored in liquid nitrogen until use. VKH PBMC samples used for RT-qPCR were collected previously by our lab.

mRNA sequencing and identification of DEGs

RNA extraction, library construction and mRNA sequencing were performed by Novogene (Beijing, China). Briefly, total RNAs were extracted by TRIzol, mRNAs were purified by poly-T oligo-attached magnetic beads. Sequencing libraries were generated by NEBNext Ultra RNA Library Prep Kit (NEB, USA) for Illumina, then sequenced on an Illumina Novaseq platform and 150 bp paired-end reads were generated. Differently expressed genes (DEGs) between two groups were analyzed by DESeq2 R package. ³⁰ P-values were adjusted by Benjamini and Hochberg’s approach for
controlling the false discovery rate. Genes with an adjusted P-value (padj) < 0.05 identified by DESeq2 were considered as DEGs. Hierarchical clustering analyses were performed on Fragments Per Kilobase per Million (FPKM) values and Z-score was used for row normalization. The color of each grid represents the normalized value of gene expression.

**Extraction of RNA and reverse-transcription quantitative polymerase chain reaction (RT-qPCR)**

Total RNA was extracted using the TRizol reagent (Invitrogen, USA). The concentration of total RNA was measured with a NanoPhotometer (IMPLEN, German). Reverse transcription was performed using Go-Taq polymerase (Promega, USA). A SYBR Green qPCR Master Mix (MCE, USA) for RT-qPCR was used with Applied Biosystems 7500 (Applied Biosystems, USA). \( \beta \)-Actin was used as internal control. Relative expression of target gene was determined by 2\(^{-\Delta\Delta C_t} \). The forward primer of \( \beta \)-actin was CATGGTCGTTGCTGCGAATG and reverse primer of \( \beta \)-actin was CCTTCTTAATGTCACGCG. The forward primer of SGK1 was GAACACAACGACACGACT and reverse primer of SGK1 was CCATACGGATCTCAGCAG. The forward primer of TRIB1 was CTCCTCGGTGGAGAGGATAC and reverse primer of TRIB1 was CAAGAGGCTGCGAATGAG.

**GO and KEGG enrichment analysis**

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of DEGs were performed with the clusterProfiler R package. Terms or pathways with a P-value less than 0.05 were identified as statistically enriched.

**Protein—protein interaction**

The interactions between differentially expressed genes were analyzed using the STRING database. The following settings were used: full network, evidence, all active interaction sources, medium confidence (0.400) and hide disconnected nodes in the network. The hub genes were calculated and visualized by cytoHubba in Cytoscape and ranked by degree.

**Single-cell RNA sequencing**

The data of the PBMC single-cell sequencing data set from a healthy donor was downloaded from https://support.10xgenomics.com/single-cell-gene-expression/datasets, and then analyzed by the Seurat R package (3.2.0). Genes expressed in less than 3 cells were filtered out. Cells with 200–4000 detected genes and expressing <20% mitochondrial genes were retained for subsequent analysis. Data were normalized using the NormalizeData function, PCA dimensional reduction was performed by the RunPCA function. Twelve clusters were identified with the use of the FindNeighbors and FindClusters function at a resolution of 0.4. Using this approach we extracted the five clusters we focused on with canonical markers. Data of the single-cell sequencing of monocytes from healthy controls and VKH patients were downloaded from GSE148020, the processing workflow, parameters and marker genes were the same as they published. We extracted the monocytes, integrated the monocytes of one group and identified the different expressed genes in monocytes between VKH patients and healthy volunteers by the FindMarkers function with threshold settings of Fold Change >1.5 and padj <0.05.

**Statistical analysis**

The statistical significance of DEGs from the bulk sample was determined with the DESeq2 R package, and the statistical significance of DEGs from the single-cell sample was determined with the FindMarkers function, whereby a padj <0.05 was considered as statistically significant. The statistical analyses, as well as the diagrams, were performed with SPSS software and GraphPad Prism 7 software. The Shapiro–Wilk test was applied to test normality. Nonparametric test was used to examine the statistical relationship between two non-normally distributed data, and parametric test was used to examine the statistical relationship between two normal distributed data, P value less than 0.05 was considered as statistically significant. Detailed statistical methods were described in figure legends.

**Results**

**Identification of DEGs**

The basic information of the included VKH patients is shown in Table 1. The discovery of DEGs between two groups was identified by the R-package DESeq2 with threshold settings of Fold Change (FC) > 1.5 and padj < 0.05. DEGs were graphed as volcano plots. In total, 118 altered genes (107 up-regulated and 11 down-regulated) were detected when comparing healthy control subjects and VKH patients with active uveitis before treatment (Fig. 1A). Another 21 differentially expressed genes (3 up-regulated and 18 down-regulated) were found between VKH patients before and after therapy with cyclosporine and prednisone (Fig. 1B). DEGs with a similar expression pattern within a group were visualized by hierachical clustering heatmap analysis (Fig. 1C, D).

**Validation of some important DEGs**

The mRNA-sequence analysis showed that the so called serum glucocorticoid regulated kinase 1 (SGK1) was upregulated in VKH patients with active uveitis before treatment when compared with healthy control subjects with the minimum padj value. The expression of TRIB1 was increased in VKH patients with active uveitis before treatment and was downregulated in VKH patients after treatment according to the mRNA sequencing (Fig. 2A), which indicates that TRIB1 is a potential biomarker to monitor the development of VKH. Uregulated mRNA expression of SGK1 and TRIB1 were further confirmed by RT-qPCR in another cohort of VKH patients with active uveitis before treatment and healthy control subjects (Fig. 2B, C).
Functional analysis of DEGs

To ascertain the potential biological functions of the identified DEGs, we annotated them by Gene Ontology (GO) analysis. GO analysis includes three items: Cellular Component (CC), Molecular Function (MF) and Biological Process (BP).

The DEGs identified by comparing healthy controls with VKH patients with active uveitis before treatment revealed that neutrophil degranulation, neutrophil activation involved in immune response, response to molecule of bacterial origin, regulation of innate immune response and leukocyte migration were enriched in the BP category (Fig. 3A). Peptidase regulator activity, peptide binding, enzyme inhibitor activity and nucleotide receptor activity were enriched in the MF category (Fig. 3B). Secretory granule membrane, secretory granule lumen and cytoplasmic vesicle lumen were enriched in the CC category (Fig. 3C).

The DEGs found by comparing the same group of VKH patients before and after therapy with cyclosporine and prednisone included enriched growth factor binding, kinase regulator activity and mitogen-activated protein kinase (Fig. 3D). Cellular response to peptide, regulation of cell—cell adhesion, regulation of leukocyte cell—cell adhesion and positive regulation of cytokine production were enriched in the BP category (Fig. 3E). Cell projection membrane, anchored component of membrane, endocytic vesicle, and secretory granule membrane were enriched in the MF category (Fig. 3F).

Pathway enrichment analysis of DEGs by KEGG

To explore the potential signaling pathway involved in the pathogenesis and treatment of VKH, we analyze the KEGG pathway enrichment. Arachidonic acid metabolism, phagosome, malaria, cholesterol metabolism, and glutathione metabolism pathways were enriched when comparing healthy control subjects and VKH patients with active uveitis before treatment (Fig. 4A). The MAPK signaling pathway, Ras signaling pathway, C-type lectin receptor signaling pathway and regulation of actin cytoskeleton pathways were enriched when comparing the same group of VKH patients before and after treatment (Fig. 4B).

Combined analysis of DEGs with single-cell sequencing

Since PBMC mainly consists of T cells, B cells, NK cells and monocytes, we extracted CD4\(^+\) T cell, CD8\(^+\) T cell, NK cell, B cell and monocyte from the single-cell sequencing dataset of PBMC (Fig. 6A). Then, we examined the distribution of our DEGs in these clusters. We found that there were mainly 5 types of distribution features in these DEGs. Type 1 included genes with almost no obvious expression in all clusters (Fig. 6B). Type 2 included genes that were sporadically expressed in very few cells in all clusters (Fig. 6C). Type 3 included genes expressed in part of the cells with a preference to certain clusters (Fig. 6D). Type 4 included genes that were mainly expressed in only one certain cluster (Fig. 6E). Type 5 included genes that were expressed in most cells among all clusters (Fig. 6F).

Interestingly, we found that approximately 60% DEGs (healthy controls vs. VKH patients with active uveitis) were expressed only in monocytes according to the single-cell sequencing of the PBMC from a healthy donor (i.e., type 4). We wondered whether the expression changes of these DEGs could be attributed to monocytes. Therefore, we identified the DEGs of monocytes from healthy individuals and VKH patients with the use of single-cell sequencing of monocytes published earlier by others.36 However, the DEGs of PBMC shown in our study and DEGs of monocytes exhibited no intersection.

Discussion

In this study, where we used mRNA sequencing of PBMC, we identified 118 DEGs when comparing healthy control subjects...
Figure 1  Visualization of DEGs. (A, B) Volcano plot of DEGs. Green and red dots represent downregulated and upregulated genes respectively with the criterion of FC > 1.5 and padj < 0.05. Blue dots are genes not meeting the inclusion criterion of DEG. (C, D) Hierarchical clustering heatmap of DEGs. The color of each grid represents the normalized value of gene expression, the redder the color, the higher the expression level; the bluer the color, the lower the expression level. HC (n = 7), Va (n = 7), Vt (n = 7). HC, healthy control. Va, VKH patients with active uveitis before treatment. Vt, VKH patients after the therapy of cyclosporine and prednisone. The same number after Va and Vt represents the same patient.
with VKH patients, and 21 DEGs between patients before and after therapy with prednisone and cyclosporine. Bioinformatics analysis was performed to predict the biological role of DEGs, and two datasets of public available single-cell sequencing were used to infer the cell-specific origin of these DEGs. Our data suggest that peripheral immune cells may also participate in the pathogenesis of VKH as well as in the response to treatment.

When comparing DEGs from healthy controls with those obtained in VKH patients with active uveitis, several genes showed an evident expression change in VKH. SGK1 was upregulated in VKH, which is in agreement with earlier studies showing that SGK1 is also increased in PBMC from ulcerative colitis patients.\(^{37}\) SGK1, a serine/threonine kinase, is a sensor of multiple environmental signals.\(^{38}\) It has been reported that cytosolic Ca\(^{2+}\), reactive oxygen species,
Figure 4  KEgg pathway enrichment analysis of DEGs. The enriched KEgg pathways of DEGs were revealed by bar graph. (A) The enriched KEgg pathways enriched for DEGs between healthy control and VKH patients with active uveitis before treatment. (B) The enriched KEgg pathways enriched for DEGs between VKH patients before and after treatment.

Figure 5  Construction of PPI network among all DEGs. Each circle represents a differently expressed gene. The rank of the connection was sorted by color from deep red to light yellow.
phosphatidylinositide-3-kinase, cyclic AMP, stress-activated protein kinase-5, stress-activated protein kinase-2, protein kinase C signaling could regulate the transcription of SGK1. Glucocorticoid receptor, nuclear factor kappa-B, reticuloendotheliosis viral oncogene homolog, activating transcription factor 6 have been shown to bind the promoter of SGK1. It is evident that the regulation of SGK1 is quite complex and which mechanisms are exactly operational in VKH is unclear and deserves further study.

The arachidonic acid metabolism pathway is the most obvious enriched KEGG pathway of the DEGs when comparing healthy control subjects and VKH patients with active uveitis before treatment. Arachidonic acid can be metabolized by enzymes to a spectrum of bioactive mediators that includes prostanoids, leukotrienes, epoxyeicosatrienoic acids, dihydroxyeicosatetraenoic acid, eicosatetraenoic acids, and lipoxins, which are strongly correlated with inflammation. Arachidonic acid not only plays an important role in cardiovascular biology and carcinogenesis, but also in autoimmune inflammatory diseases, such as asthma and arthritis. This enrichment indicates that activation of arachidonic acid metabolism pathway closely associates with the inflammation of VKH with active uveitis. Pathway analysis showed that MAPK, Ras and C-type lectin receptor signaling pathways were enriched when studying VKH patients longitudinally before and after treatment, suggesting that these pathways are involved in the process of disease remission. Suppressors of cytokine signaling 1 (SOCS1) was upregulated in treated VKH patients as compared to its expression before treatment. SOCS1 has been reported to exert an anti-inflammatory effect through the regulation of reactive oxygen species (ROS). Downregulated SOCS1 is associated with an induction of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6. We could not detect a difference when comparing SOCS1 in active uveitis stage VKH versus controls either by mRNA sequencing or RT-qPCR, which is not in agreement with earlier studies showing that SOCS1 was expressed at a 4.92-fold higher level in PBMC of VKH patient with active uveitis when compared with normal controls. Interestingly, the latter cohort of VKH patients with active uveitis had a history of corticosteroids use. Based on these results, it seems that upregulated SOCS1 emerges after the use of corticosteroids.

Another hitherto not yet described factor in VKH, included the enzyme tribble pseudokinase 1 (TRIB1). TRIB1 was upregulated in our VKH patients with active uveitis when compared to healthy controls and decreased again after treatment. It has been reported that TRIB1 plays a role in inflammatory signaling pathways. TRIB1 controls the polarization of macrophages and the mRNA level of TRIB1 in PBMC is correlated with chronic antibody-mediated allograft failure. GO analysis showed that TRIB1 is associated with the response to molecule of bacterial origin, and enzyme inhibitory activity. Our data suggest that TRIB1 may act as an immune regulator in VKH and
that it might serve as a potential biomarker to monitor disease activity and response to treatment.

Aberrant infiltrations of mononuclear immune cells in VKH lesions have been observed. Infiltration of T cells, B cells and macrophages in the choroid,34 3:1 ratio of CD4+ T cells and CD8+ T cells in vitiligo skin,45 abnormal frequency of lymphocytes in aqueous humor46 and cerebrospinal fluid47 have been reported in VKH. Based on these limited studies, it can be concluded that multiple types of mononuclear immune cell are involved in the pathological progress of VKH. However, the disease-specific cell type in VKH needs to be identified. Single-cell sequencing is a novel method for the disease researches and exploration of immune cell heterogeneity, which permits the detection of sequence at a single-cell resolution, it facilitates the identification of disease-associated cell type and the identification of categories in heterogeneous samples.48–50

To our knowledge, single-cell sequencing of PBMC has not yet been reported in VKH. In this study, the single-cell sequencing of PBMC from a healthy donor and single-cell sequencing in monocytes of VKH patients with corresponding normal control were used to further investigate the detail of DEGs identified by our bulk sample sequencing. We found that over half of our DEGs (VKH patients with active uveitis vs. healthy control subjects) were constitutively expressed in monocytes according to the single-cell sequencing of PBMC from a healthy donor. The expression of gene and the regulation of gene expression exhibit cell-type-specific properties,51,52 from which we hypothesized that monocytes significantly contribute to our identified DEGs. In addition, the most connected hubgene of our PPI is CSF1R, which is a known macrophage marker.53 However, we found no intersection between DEGs of monocytes and DEGs of PBMC. We speculate that this may be due to the following reasons. Firstly, there are differences in sequencing method, sequencing processes, the assembly of sequencing data and data processing between bulk sample sequencing and single cell sequencing, and therefore, comparing sequencing data obtained from different methods may produce biases. Secondly, the aberrant expression changes seen in PBMC were elicited by other non-monocyte clusters in VKH. Thirdly, we cannot exclude the possibility that there is a change in the frequency of monocytes in PBMC. Interestingly, CD14, a marker of monocyte,54 is upregulated in VKH patients with active uveitis before treatment when compared with healthy subjects. Fourthly, VKH is a rare disease, and the collection of sufficient samples from different untreated active patients is problematic. Our patient sample size is therefore relatively small. There were no females in the sequencing cohort, since most female VKH patients objected to donate blood samples for research purposes. However, men and women are equally affected by VKH in the Chinese Han population,55 and the same expression pattern of DEGs are observed in females by RT-qPCR as shown in Figure 2. Despite these limitations, we believe that elucidating which cell type contributes to the aberrant expression alterations detected in our study will help the discovery of disease-associated cell type.

Conclusions

This study is the first to our knowledge, which uses mRNA sequencing in PBMC from healthy control subjects and VKH patients with active uveitis before and after treatment. The differences in the mRNA expression profile among the tested groups provide evidence that peripheral immune cells are involved in the pathogenesis and treatment response of VKH. The data on mRNA expression of TRIB1 suggests that this gene may function as a biomarker in the management of VKH. Our data suggest that arachidonic acid metabolism may play an important role during the inflammatory events occurring during the development of VKH, and that treatment with prednisone combined with cyclosporine may target the MAPK signaling pathway. We speculate that monocyte, an ignored cell type in most previous VKH studies, plays an active role during VKH. We hope that our studies will help to clarify the underlying pathogenesis of VKH and lead to the development of novel drugs for the treatment of this rare disease.

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Conflict of interests

No conflict of interest declared.

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