The correlation between primary open-angle glaucoma (POAG) and gut microbiota: a potential towards predictive, preventive, and personalized medicine

Si Chen (chendd0522@163.com)
Xiangya Hospital, Central South University

Nan Wang
Xiangya Hospital Central South University

Siqi Xiong
Xiangya Hospital Central South University

Xiaobo Xia
Xiangya Hospital Central South University

Research Article

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Abstract

Background

Glaucoma is the leading cause of irreversible blindness worldwide. Emerged evidence has shown that glaucoma is considered an immune disorder. Gut is the largest immune organ in human body and gut microbiota (GM) plays irreversible role of maintaining immune homeostasis. But how does GM influences glaucoma remain unrevealed. This study aimed at investigating key molecules/pathways mediating GM and glaucoma and provide new biomarkers for future predictive, preventive, and personalized medicine.

Methods

Datasets from primary open-angle glaucoma (POAG) patients (GSE138125) and datasets for target genes of GM/GM metabolites were downloaded from public database. For GSE138125, the differentially expressed genes (DEGs) between healthy and POAG samples were identified. And the online Venn diagram tool was used to obtain the DEGs from POAG related to GM. After which GM-related DEGs were analyzed by correlation analysis, pathway enrichment analysis and protein-protein interaction (PPI) network analysis. Human trabecular meshwork cells were used for validation, and mRNA level of hub genes was verified by quantitative real-time polymerase chain reaction (RT-qPCR) in the in vitro glaucoma model.

Results

A total of 16 GM-related DEGs in POAG were identified from the above 2 datasets (9 up-regulated genes and 7 down-regulated genes). Pathway enrichment analysis indicated that these genes are mostly enriched in immune regulation especially macrophages related pathways. Then 6 hub genes were identified by PPI network analysis and construction of key modules. Finally, RT-qPCR confirmed that the expression of the hub genes in the in vitro glaucoma model was consistent with the results of bioinformatics analysis of mRNA chip.

Conclusion

This bioinformatic study elucidates NFKB1, IL18, KITLG, TLR9, FKBP2, and HDAC4 as hub genes for POAG and GM regulation. Immune response modulated by macrophages play an important role in POAG and may be potential targets for future predictive, preventive, and personalized diagnosis and treatment.

Introduction
Glaucoma is the leading cause of irreversible blindness worldwide \(^1\). It is predicted there will be over 1.18 hundred million glaucoma patients in 2040 \(^1\). This disease is characterized as progressive and irreversible loss of retinal ganglion cells (RGCs) mainly due to the increased intraocular pressure (IOP) \(^2\). The cause of glaucoma is a complex of multiple reasons, such as age, hypertension, thyroid disease, diabetes, genomic mutations, and so on \(^3\)–\(^5\). At present, the clinical methods for early diagnosis and treatment of glaucoma are very limited. Due to the selective retinal ganglion cells (RGCs) damage and characteristic visual field defects, a considerable number of patients with late-stage glaucoma can still maintain a distance vision of more than 5.0, which leads to subjective neglect and missed diagnosis. Meanwhile, in terms of treatment, the intraocular pressure of glaucoma patients is mainly reduced or controlled through 3 methods: medicine, laser and surgery, to achieve the purpose of protecting the patient's visual function \(^6\)–\(^8\). However, for some glaucoma patients, although the intraocular pressure has been controlled at normal level, visual field defects continue to develop, eventually leading to irreversible blindness. High intraocular pressure is the main factor of glaucoma, but it is not the only factor. The pathogenesis of glaucoma has not been thoroughly studied so far. Moreover, emerged evidence has suggested glaucoma could be considered an immune disorder \(^9\)–\(^11\). Glaucoma can be divided into multiple subtypes including primary open-angle glaucoma (POAG), primary angle closure glaucoma (PACG), secondary glaucoma etc \(^12\)\(^3\). Among these subtypes, POAG consist of 74% of glaucoma according to data from clinical field \(^13\). In POAG, both the anterior and posterior segments of the eye are affected, and serious damage may be caused upon the trabecular meshwork (TM) and optic nerve (ON) \(^14\). The trabecular meshwork, located within the iridocorneal angle, is the main pathway for drainage of aqueous humor (AH) out of the eye, and its dysfunction leads to IOP elevation \(^15\). Thus, trabecular meshwork (TM) is the key structure during the process of glaucoma including POAG.

POAG and immune response are not traditionally perceived to be related, but recently, mounting evidence suggest the pathogenesis in POAG is related to immune system disorders \(^16\). Gut is considered the largest immunological organ in the human body with an irreplaceable role in regulating immune homeostasis \(^17\). Researches have shown that there is over $10^{14}$ microbes in human digest system: from stomach to distal colon, and intestinal microbes are highly varied from individuals \(^18\). Gut microbiota (GM) and its metabolites have adverse health effects on the human body, and GM dysbiosis can result in a variety of chronic diseases, including metabolic diseases, gastrointestinal diseases, cardiovascular diseases (the gut-heart-axis), and neurodegenerative diseases (the gut-brain-axis) \(^19\)–\(^23\).

Recently, the concept of gut-eye-axis have been raised \(^24\), as in human body, biological signals released by the intestinal microbes could leads to immune response from the eye. Researchers have shown series of ocular diseases such as uveitis, macular degeneration \(^25\), diabetic retinopathy \(^26\), corneal disease \(^27\) is relevant with GM dysbiosis. Studies also discovered the possible link between gut microbiota and glaucoma, for example: irritable bowel disease patients have higher risk of glaucoma \(^28\), and there is distinct difference in gut microbiota composition and serum metabolic phenotype between POAG patients and healthy individuals \(^29\)\(^,\)\(^30\). Despite the above evidence, the knowledge of how gut microbiota...
corelated and influenced the process of POAG targeting predictive, prevention, and personalization medicine (PPPM) is still very limited.

**Working hypothesis**

Thus, understanding the specific mechanism and mode of action of immune response and immune regulation in POAG will help to obtain novel approach for the early diagnosis of POAG, to expand effective neuroprotective strategies, and to develop personalized treatment methods, which in line with the principle of PPPM. Recently, several studies have revealed how non-ocular factors contribute to diagnosis of ocular diseases, for example: red blood cell distribution width (RDW) could be a potential laboratory parameter for disease prediction of primary angle-closure glaucoma (PACG)\[^{31}\]. Moreover, systemic inflammatory biomarkers and metabolomics have been proved to allow early identification and timely implementation of replacement therapies for ocular diseases\[^{32, 33}\].

In this study, we analyzed RNA-seq data from primary open-angle glaucoma (POAG) patients and co-analyzed them with the genetic data from the gut microbiota database. We found several genes/pathways differentially expressed in POAG patients that are gut microbiota related, among which are critical genes/pathways who played an important role in regulating macrophages differentiation and polarization. Our findings could be regarded as biomarkers for early identification of POAG and could be developed into personalized treatment therapy from the prospect of gut microbiota regulation. Our research provided a new perspective to expand the understanding of the gut-eye-axis, the identification of hub genes and pathways could represent potential therapeutic strategies for glaucoma, and point towards future predictive, preventive, and personalized medicine (PPPM / 3PM).

**Methods & Materials**

Study design and data collection

Datasets of POAG patients were downloaded from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). The keywords “glaucoma” was used to search related gene expression datasets and non-human tested specimens were excluded. The GSE138125\[^{34}\] data set is consisted of the gene expression profiles of 4 POAG patients and 4 normal controls. Datasets of gut microbiota were obtained from gutMGene (http://bio-annotation.cn/gutmgene/home.dhtml), which is a database for target genes of gut microbes and microbial metabolites released by Harbin Medical University.

Differentially Expressed Gene Analysis

For GSE138125, the differentially expressed genes (DEGs) between healthy and POAG samples were screened by the “limma” package in R software. The genes with P value < 0.05 and logFC > 1 were to be DEGs. In addition, the online Venn diagram tool was used to obtain the gut microbiota related DEGs (http://bioinformatics.psb.ugent.be/webtools/Venn/). Then, the “heatmap” and “ggplot2” packages in R
software are used to draw heatmap and volcano plot and box plot respectively to visualize the gut microbiota-related DEGs\textsuperscript{[35]} The co-DEGs obtained from the two datasets were visualized using the R packages “complex heatmap” and “ggplot2” to generate the heat maps and volcano maps, respectively. These co-DEGs were retained for subsequent analysis. After screening out the gut microbiota-related genes, the "complex heatmap" and "ggplot2" R packages were used for visualization, generating heatmaps and volcano maps, respectively.

Pathway Enrichment Analysis and Functional Annotation

The above differentially expressed gut microbiota-related genes in POAG were submitted to GO function enrichment analysis, which consisted of biological process (BP), cellular component (CC), and molecular function (MF), and the Kyoto Encyclopedia of Genes and Genomes (KEGG) signaling pathway enrichment analysis using an R package “clusterProfiler”. The enriched GO terms and KEGG pathways with an adjusted p value < 0.05 was selected.

PPI Network Construction and hub Genes Identification

The protein–protein interaction (PPI) network was analyzed using the Search Tool for the Retrieval of Interacting Genes (STRING; http://string-db.org). An interaction with a combined score > 0.4 was selected and used to construct a PPI network with Cytoscape software (version 3.9.0). Hub genes were identified for further analysis by using the cytoHubba plugin.

Cell Culture and Cell Grouping

Human trabecular meshwork cell was purchased from the IMMOCELL company and cultured in DMEM/F12 medium containing 15% fetal bovine serum (FBS) (Gibco, USA) and 1% Antibiotic-Antimycotic (Gibco, USA) at 37°C with 5% carbon dioxide. When the cell density reached 80%, it was washed with PBS (Gibco, USA) and treated with 0.05% trypsin (Gibco, USA) for passage at the proportion of 1:3.

The logarithmic growth phase cells with good growth condition were inserted into 6-well plates with 1.2×10\textsuperscript{6} cells per well. The cells were divided into two groups: the H2O2 treated group to mimic glaucoma in vitro, and the control. The H2O2 treated group was cultured in the medium containing 200 nM H2O2, and the normal group was cultured in the SG medium. For H2O2 treated group, 200uM H2O2 was added to each well and cultured at 37°C for 24 hours to create the in vitro glaucoma model.

RNA Extraction and RT-qPCR

RNA was extracted from human trabecular meshwork stem cells using Trizol reagent (Fisher Scientific, #15-596-018) and cDNA generated using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). For each treatment group, 3 biological replicates were collected. RT-qPCR was performed on a CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories) using the 2X SYBR Green Pro TaqHS Premix (Accurate Biotechnology Co.). Primer sequences are provided in Table S3. Gene expression fold change
was calculated using the comparative CT method ($2^{-\Delta \Delta CT}$) and expression in untreated human trabecular meshwork cells was normalized to 1 as the control[36]. GAPDH was used as the housekeeping gene in these experiments.

Statistics

RT-qPCR data were analyzed utilizing Prism 9 (version 9.5.0) software, the gene expression fold change was analyzed using one-way ANOVA and the difference between groups were statistically significant when P value < 0.05.

Results

In this study, we analyzed RNA-seq data of human trabecular meshwork tissue collected from 4 POAG patients and 4 healthy controls. We further co-analyzed 1606 differential expressed genes(DEGs) screened out from POAG dataset and co-analyzed those DEGs with the gut microbiota database gutMGene v1.0, which contains target genes of gut microbiota and gut microbiota metabolites. The results are presented as follows.

Identification of GM related differentially expressed genes in POAG and correlation analysis

1606 DEGs were screened between POAG patients and healthy controls using the ‘limma’ package. Compared with datasets obtained from gutMGene v1.0, a total of 16 differentially expressed GM related genes in POAG patients were identified after taking the intersection of the Venn, including 9 common upregulated genes and 7 common downregulated genes. These genes were identified as GM related DEGs in POAG(Fig. 2, Table 1).
Table 1
The 16 differentially expressed gut microbiota (GM)-related genes in POAG samples compared to healthy samples.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>logFC</th>
<th>Changes</th>
<th>P-value</th>
<th>Adj. P-value</th>
<th>probe_id</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR9</td>
<td>2.4128</td>
<td>up</td>
<td>5.08E-05</td>
<td>0.0036</td>
<td>ASHV40034404</td>
</tr>
<tr>
<td>FKBP2</td>
<td>1.1549</td>
<td>up</td>
<td>0.00016</td>
<td>0.0074</td>
<td>ASHV40008587</td>
</tr>
<tr>
<td>NFKB1</td>
<td>1.4539</td>
<td>up</td>
<td>0.0035</td>
<td>0.0564</td>
<td>ASHV40039215</td>
</tr>
<tr>
<td>HDAC4</td>
<td>1.1963</td>
<td>up</td>
<td>0.0053</td>
<td>0.0755</td>
<td>ASHV40028293</td>
</tr>
<tr>
<td>MUC2</td>
<td>1.2995</td>
<td>up</td>
<td>0.0094</td>
<td>0.1129</td>
<td>ASHV40007933</td>
</tr>
<tr>
<td>KITLG</td>
<td>3.7384</td>
<td>up</td>
<td>0.0158</td>
<td>0.1629</td>
<td>ASHV40010359</td>
</tr>
<tr>
<td>GLP1R</td>
<td>1.8892</td>
<td>up</td>
<td>0.0186</td>
<td>0.1821</td>
<td>ASHV40045007</td>
</tr>
<tr>
<td>SLC22A6</td>
<td>1.8091</td>
<td>up</td>
<td>0.0333</td>
<td>0.2150</td>
<td>ASHV40008556</td>
</tr>
<tr>
<td>MGAT3</td>
<td>1.2228</td>
<td>up</td>
<td>0.0395</td>
<td>0.2150</td>
<td>ASHV40033182</td>
</tr>
<tr>
<td>SLC9A3</td>
<td>-6.63</td>
<td>down</td>
<td>5.62E-08</td>
<td>8.17E-05</td>
<td>ASHV40001585</td>
</tr>
<tr>
<td>IL18</td>
<td>-1.12</td>
<td>down</td>
<td>2.69E-05</td>
<td>0.0024</td>
<td>ASHV40007630</td>
</tr>
<tr>
<td>CTSD</td>
<td>-1.15</td>
<td>down</td>
<td>0.00017</td>
<td>0.0078</td>
<td>ASHV40006502</td>
</tr>
<tr>
<td>DAPK3</td>
<td>-1.04</td>
<td>down</td>
<td>0.0045</td>
<td>0.0668</td>
<td>ASHV40025025</td>
</tr>
<tr>
<td>CCL16</td>
<td>-1.07</td>
<td>down</td>
<td>0.0099</td>
<td>0.1173</td>
<td>ASHV40020203</td>
</tr>
<tr>
<td>GPX3</td>
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<td>0.0200</td>
<td>0.1909</td>
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<tr>
<td>SSTR3</td>
<td>-1.27</td>
<td>down</td>
<td>0.0265</td>
<td>0.2150</td>
<td>ASHV40056858</td>
</tr>
</tbody>
</table>

To explore the expression correlation of these 16 GM related DEGs in POAG, the correlation analysis has been performed by bioinformatics methods. The results showed that there was a high correlation between up-regulated genes and down-regulated genes, respectively (Fig. 3).

Pathway enrichment analysis

To further explore the underlying biological processes of these GM related DEGs in POAG, gene-ontology (GO) enrichment analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed. The results showed that in terms of GO analysis, the genes were mainly enriched in signaling pathways related to regulation of macrophage activity such as: positive regulation of granulocyte macrophage colony stimulating factor production, granulocyte macrophage colony stimulating factor production, positive regulation of macrophage derived foam cell differentiation.

Meanwhile, KEGG analysis showed that DEGs related to GM were mainly enriched in PD-L1 expression and PD-L1 checkpoint pathway in cancer, and series of parasite diseases including Chagas disease and...
Amoebiasis (Fig. 4, Table 2).
Table 2
Functional and pathway enrichment analyses for differentially expressed gut microbiota (GM)-related genes in POAG.

<table>
<thead>
<tr>
<th>Term</th>
<th>Description</th>
<th>Count</th>
<th>P-value</th>
<th>Adj. P-value</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biological processes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GO:0032725</td>
<td>positive regulation of granulocyte macrophage colony-stimulating factor production</td>
<td>2</td>
<td>7.14E-05</td>
<td>0.022724817</td>
<td>TLR9, IL18</td>
</tr>
<tr>
<td>GO:0032604</td>
<td>granulocyte macrophage colony-stimulating factor production</td>
<td>2</td>
<td>9.24E-05</td>
<td>0.022724817</td>
<td>TLR9, IL18</td>
</tr>
<tr>
<td>GO:0032645</td>
<td>regulation of granulocyte macrophage colony-stimulating factor production</td>
<td>2</td>
<td>9.24E-05</td>
<td>0.022724817</td>
<td>TLR9, IL18</td>
</tr>
<tr>
<td>GO:0010744</td>
<td>positive regulation of macrophage derived foam cell differentiation</td>
<td>2</td>
<td>0.000116091</td>
<td>0.022724817</td>
<td>NFKB1, IL18</td>
</tr>
<tr>
<td>GO:0070555</td>
<td>response to interleukin-1</td>
<td>3</td>
<td>0.000227134</td>
<td>0.028746332</td>
<td>NFKB1, HDAC4, CCL16</td>
</tr>
<tr>
<td>GO:0070665</td>
<td>positive regulation of leukocyte proliferation</td>
<td>3</td>
<td>0.000261452</td>
<td>0.028746332</td>
<td>TLR9, KITLG, IL18</td>
</tr>
<tr>
<td>Cellular component</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GO:0035580</td>
<td>specific granule lumen</td>
<td>2</td>
<td>0.001153971</td>
<td>0.044166957</td>
<td>NFKB1, CTSD</td>
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<tr>
<td>GO:0045121</td>
<td>membrane raft</td>
<td>3</td>
<td>0.002366087</td>
<td>0.044166957</td>
<td>SLC22A6, CTSD, DAPK3</td>
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<tr>
<td>GO:0098857</td>
<td>membrane microdomain</td>
<td>3</td>
<td>0.002366087</td>
<td>0.044166957</td>
<td>SLC22A6, CTSD, DAPK3</td>
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<td>Molecular functions</td>
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<tr>
<td>GO:0005126</td>
<td>cytokine receptor binding</td>
<td>4</td>
<td>7.33E-05</td>
<td>0.007407743</td>
<td>TLR9, KITLG, IL18, CCL16</td>
</tr>
<tr>
<td>KEGG pathway</td>
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<td></td>
<td></td>
<td></td>
</tr>
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</table>
Table 3

<table>
<thead>
<tr>
<th>Term</th>
<th>Description</th>
<th>Count</th>
<th>P-value</th>
<th>Adj. P-value</th>
<th>Genes</th>
</tr>
</thead>
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<tr>
<td>hsa04623</td>
<td>Cytosolic DNA-sensing pathway</td>
<td>2</td>
<td>0.005025394</td>
<td>0.092589797</td>
<td>NFKB1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IL18</td>
</tr>
<tr>
<td>hsa05321</td>
<td>Inflammatory bowel disease</td>
<td>2</td>
<td>0.005341719</td>
<td>0.092589797</td>
<td>NFKB1</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IL18</td>
</tr>
<tr>
<td>hsa05235</td>
<td>PD-L1 expression and PD-1 checkpoint pathway in cancer</td>
<td>2</td>
<td>0.009823429</td>
<td>0.114747773</td>
<td>TLR9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NFKB1</td>
</tr>
<tr>
<td>hsa04061</td>
<td>Viral protein interaction with cytokine and cytokine receptor</td>
<td>2</td>
<td>0.012284424</td>
<td>0.114747773</td>
<td>IL18</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CCL16</td>
</tr>
<tr>
<td>hsa04620</td>
<td>Toll-like receptor signaling pathway</td>
<td>2</td>
<td>0.013240128</td>
<td>0.114747773</td>
<td>TLR9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NFKB1</td>
</tr>
</tbody>
</table>

Relationship between enriched pathways

The relationship between the above enriched pathways was shown (Fig. 4). There were 7 common GM related genes in POAG patients in the 10 most prominent pathways, which are CCL16, nuclear factor kappa B subunit 1 (NFKB1), interleukin 18 (IL18), KITLG, TLR9, MUC2, and HDAC4. The 10 most prominent pathways include positive regulation of granulocyte macrophage colony-stimulating factor production, maintenance of gastrointestinal epithelium, regulation of macrophage derived foam cell differentiation, response to interleukin 1 and positive regulation of leukocyte proliferation. Besides, we analyzed the expression of differential genes in the significantly enriched pathway and showed the results in the Heatmap-like functional classification map (Fig. 5).
PPI Network Construction of GM related DEGs and Identification of Hub Genes

To discover the potential relationships between proteins encoded by GM related DEGs in glaucoma and to identify hub genes, the PPI network of the DEGs was screened by Search Tool for the Retrieval of Interacting Genes (STRING), including 16 nodes and 6 edges, with a PPI enrichment p value < 0.0153 (Fig. 6A). Then, the obtained results were imported into Cytoscape software for visual analysis. The interaction number of each gene was also shown. The PPI network was analyzed by Cytoscape plug-in CytoHubba to identify hub genes (Fig. 6B). The top 6 genes identified as potential hub genes are: nuclear factor kappa B subunit 1 (NFKB1), interleukin 18 (IL18), KITLG, TLR9, FKBP2, and HDAC4.

Validation of the hub gene expression in the in vitro glaucoma model

In this study, we incubated human trabecular meshwork cells with H2O2 (200uM) to simulate a glaucoma model in vitro. We performed RT-qPCR to validate the expression change of the hub genes IL18, HDAC4, TLR9 and NFKB1. NFKB1, TLR9 and HDAC4 were predicted to be upregulated in glaucoma and their mRNA level increased according to the RT-qPCR data (Fig. 7), meanwhile, IL18 was predicted to be downregulated in glaucoma and its mRNA level was also increased according to RT-qPCR.

Discussion

Glaucoma has a huge impact on public health worldwide and primary open-angle glaucoma (POAG) is responsible for over 70% of glaucoma \[8\]. Current consensus on glaucoma is not solely based on intraocular pressure (IOP), especially in cases of normal-tension glaucoma (NTG). Although high IOP is a well-established risk factor for glaucoma, there is increasing evidence that other factors, such as vascular and neurodegenerative mechanisms \[20,21\], also play a role in the development and progression of the disease. Lowering IOP is currently the only intervention available \[22\]. However, clinical evidence indicates that lowering IOP does not prevent progression in all POAG patients \[23\]. Thus, non-IOP factors are involved in POAG. Considering these findings, the treatment of glaucoma has shifted from a sole focus on lowering IOP to a more individualized approach that considers other factors that may be contributing to the disease. Previous studies have revealed inflammation and immune response mediate the process of POAG from several different aspects \[9\], yet much remains to be understood with regards to cellular processes regulating the maintenance of the TM and its relevance to IOP.

Recent advances have identified the significant associations between gut microbiota and several disorders in multiple organs besides the digest trac\[37,38\]. Emerging evidence has suggested gut microbiota could be the regulator of several central nervous system disorder, including the ocular system disorders\[39\]. Although data from clinical filed showing the possible link between gut microbiota and the occurrence of POAG\[29,40−42\], the mechanism underlying its connections and the key molecules remained unknown.
In this study, we aimed at discovering the key genes and pathways in POAG that are gut microbiota related. After analysis, NFKB1, IL18, TLR9, FKBP2, KITLG, and HDAC4 were recognized as hub genes in POAG patients and in the database of gut microbiota regulation. They showed the most remarkable correlation with regulation and stimulation of leukocytes such as macrophages. Thus, we conclude these 6 hub genes might play important roles via affecting the activity of macrophages during the progression of POAG and in regulating gut microbiota. We further verified the expression level of 4 hub genes (NFKB1, TLR9, HDAC4 and IL18) through RT-qPCR. The above 4 hub genes were shown to be regulating immune response from different aspects. Based on our RT-qPCR results, the mRNA level of all 4 genes were increased in H$_2$O$_2$ treated group compared to control.

NFKB1 has been reported to be playing a critical role in suppressing inflammation, aging and cancer$^{[43]}$. NFKB1$^{-/-}$ mice have reduced numbers of conventional and plasmacytoid dendritic cells and in response to TLR9 stimulation$^{[44]}$. Moreover, NFKB1$^{-/-}$ bone marrow-derived macrophages show an increased expression of interferon-inducible genes, including IFN-β, in response to TLR-4 and TLR-9 stimulation$^{[45]}$. Toll like receptor 9 (TLR9) is an intracellular TLR, expressed in different immunological and non-immunological cells$^{[46]}$. Gut microbiota are a source of TLR ligands, including TLR9. Though its role in glaucoma via regulating gut microbiota remained mystical, previous research have indicated that TLR9 deficiency induces osteoclastic bone loss via gut microbiota-associated systemic chronic inflammation$^{[47]}$, thus, it is possible that TLR9 mediate the occurrence of glaucoma through chronic inflammation process. Targeting immune responses mediated by TLR9 is a potential therapeutic strategy for preventing disease-associated inflammation, and autoimmune diseases$^{[48]}$. Several previous studies have also described a wide range of oxidative stress-related makers which are found in glaucomatous patients, including the activation of the NFKB pathway and the up-regulation of pro-inflammatory cytokines$^{[49]}$.

Little is known of what the function of HDAC4 in glaucoma is, but emerging evidence have shown HDAC4 has its importance in regulating neurogenesis$^{[50]}$. In vivo selective inhibition of HDAC4 via MS-275 (entinostat) or LMK-235 could prevent ongoing RGC degeneration$^{[51]}$. Reduced HDAC4 expression is associated with blood-brain barrier (BBB) breakdown contributing to ischemia/reperfusion injury-induced infarct in ischemic stroke model rats, while increased HDAC4 expression ameliorates BBB injury, contributing to the reduced infarct volume$^{[52]}$.

IL18 is a pro-inflammatory cytokine which has varies function in different cell types$^{[53]}$. As an important regulator for both innate and acquired immune response, IL18 plays an important role in inflammatory/autoimmunity diseases. Research have shown the intestinal epithelial cell secretion of IL18 is necessary for gut microbiota homeostasis(dysbiosis)$^{[54]}$. Meanwhile, increased IL18 expression was observed in glaucomatous trabecular meshwork$^{[55]}$. Our bioinformatic prediction showed downregulation of IL18 while the RT-qPCR result showed significantly increased mRNA level of IL18, and RT-qPCR result is consistent with most previous research. In that case, we hypothesized the reason for the downregulation of IL18 showed in bulk RNA-seq data is due to the small sample number.
Pathways enriched in both GO and KEGG database are mostly related to the activity of leukocytes especially macrophages. Substantial amount of research concerning the role of the immune system in glaucoma has been performed in the recent years. Researchers have provided evidence of macrophages participate in the process of glaucomatous pathogenesis [56], studies analyzing the trabecular meshwork of patients with POAG found macrophages in this tissue by scanning electron microscopy [57], and the presence of macrophage inhibitory factor were observed in healthy human doners’ TM. Meanwhile, it is shown using macrophage activator drug Zymosan could induce neurodegenerative effect in glaucoma model[58]. Moreover, TM has been shown to display characters of several cell types including express behavior pattern of macrophages when operate to maintain homeostasis of IOP [59], which further support the accuracy of our bioinformatic analysis. It seems like macrophages played the role of the ‘double sided sword’ in glaucoma. Still, deeper discovery of the protective factors of the macrophages is needed to bring forward a therapeutic approach.

In this study, we utilized a hydrogen peroxide (H$_2$O$_2$) model to mimic glaucomatous ocular hypertension in vitro. H$_2$O$_2$ induces acute oxidative stress to TM which leads to the pathogenesis of POAG [60], this model was proved to be a useful platform which resulted in TM cellular dysfunction without destruction of TM structure [61], which made this model suitable for the hypothesis that gut microbiota dysbiosis regulates TM cellularity through innate immune response.

Gut microbiota and its metabolites contributes to a variety of diseases with inflammation components via regulation of macrophages, for example, intracranial aneurysm [62], IBD (inflammatory bowel disease) [63], Alzheimer’s disease [64], and more. Gut microbiota derived metabolite SCFAs (short-chain fatty acids) promotes anti-inflammatory functions of macrophages [65], as another gut microbiota metabolite TMAO (trimethylamine-N-oxide) induces M1 macrophage polarization [66]. Currently, it is still unclear how gut microbiota effects glaucoma via regulation of macrophages, it will be crucial to testify this mechanism in the real world. The gut-eye-axis is a relatively new concept that has gained attention in the scientific community in recent years.

Utilizing non-ocular laboratory examinations to help with early identification of ocular diseases has been proved to have a great potential [67, 68], for example: key molecules and pathways affected by glaucoma pathology can be developed into predictive diagnosis strategy [69]. In our case, we identified hub genes and pathways linked POAG with gut microbiota, which could be further used as biomarkers indicating POAG at an early stage from common non-invasive examination including fecal examinations.

While in this study, we discovered hub genes linked POAG with gut microbiota, those hub genes are also common inflammation related genes that could be altered during either POAG, or microbiota alone. Thus, the gut-eye-axis hypothesis is still an emerging field of research, and further studies are needed to fully understand the mechanisms behind this relationship and to establish a causal link towards future predictive and preventive medicine.
In the end, our study has its limitations. We showed gut microbiota could be influencing the occurrence of glaucoma via affecting macrophages, but lack of evidence from patients with glaucoma and gut microbiota dysbiosis at the same time. Moreover, sample number of POAG patients is relatively small (n = 4). As a matter of fact, human trabecular meshwork tissue is very rare, and incomparable with other animal or in vitro cell models. Considering our results are statistically significant, we believe that the four groups of samples can still reflect the situation of POAG. In a word, as a pilot study, our observations provide expanding insight of the gut-eye-axis and possible therapeutic target for future predictive, preventive and personalized treatment of glaucoma.

Conclusion and Expert recommendations

This study identified IL18, TLR9, NFKB1, HDAC4, FKBP2 and KITLG as hub genes in glaucoma that are gut microbiota related as well as showed that regulation of immune response by gut microbiota is associated with glaucoma. Those key molecules and pathway could contribute to early prediction of POAG, thus, reduce the negative effect such as economic and clinical burden associated with unnecessary medical treatments. Our findings offered strong evidence to prove the existence of the gut-eye-axis and support the potential of systemic immune therapy for targeting preventive and personalized treatment of POAG, especially for those with gut microbiota dysbiosis \[70\], which could further provide expanding insight in line with the paradigm shift from reactive treatment to 3PM.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

Datasets of POAG patients (GSE138125) were downloaded from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). Datasets of gut microbiota were obtained from gutMGene (http://bio-annotation.cn/gutmgene/home.dhtml).

Author Contributions

Conceptualization, Si Chen; Funding acquisition, Siqi Xiong and Xiaobo Xia; Investigation, Siqi Xiong and Xiaobo Xia; Methodology, Si Chen; Project administration, Xiaobo Xia; Software, Nan Wang; Supervision, Siqi Xiong and Xiaobo Xia; Validation, Si Chen and Nan Wang; Writing – original draft, Si Chen and Nan Wang; Writing – review & editing, Siqi Xiong and Xiaobo Xia. All authors will be informed about each step of manuscript processing including submission, revision, revision reminder, etc. via emails from our
system or assigned Assistant Editor. All authors have read and agreed to the published version of the manuscript. # These authors contributed equally to this work.

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**Conflicts of Interest**

The authors declare no conflict of interest.

**References**


Figures
Figure 1

The conception atlas of the gut-eye-axis
Figure 2

Differentially expressed gut microbiota-related genes in POAG patients and normal control. (A) The heatmap of 16 differentially expressed GM related genes in POAG patients. Red represents up-regulated genes and blue represents downregulated genes. GM, gut microbiota; (B) Volcano plot of 16 differentially expressed GM related genes in POAG patients. The red dots in the picture represent significantly up-regulated genes, blue dots represent significantly down-regulated genes, black dots represent genes that
are not differentially expressed, and the five genes that are most significantly up-regulated or down-regulated are marked; (C) A total of 201 differentially expressed GM related genes shown in Venn diagram; (D) The boxplot of 16 differentially expressed GM related genes in POAG and normal control, including 9 up-regulated genes and 7 down-regulated genes.

**Figure 3**

Correlation analysis of 16 differentially expressed GM-related genes in POAG patients and normal control. (A, B) Correlation heatmap.
Figure 4

Pathway enrichment analysis of 16 differentially expressed GM-related genes in POAG, including BPs, CCs and MFs. (A) Bar plot of enriched GO terms; (B) Bar plot of enriched KEGG terms; (C) Chordal graph of enriched GO terms. It shows the correlation between the up-regulated differentially expressed GM related genes in POAG and the first 10 enriched GO pathways; (D) Eight Diagrams of enriched GO terms. GO, Gene Ontology; BPs, biological processes; CCs, cellular components; MFs, molecular functions.
Figure 5

Correlations between enriched pathways (A) Relationships between enriched GO pathways; (B) Common genes in the top enriched GO pathways; (C) Heatmap-like functional classification. Genes that are most significantly up-regulated or down-regulated are marked.
Figure 6

PPI network and identification of hub genes. (A) PPI network of the differentially expressed GM related genes in POAG patients, constructed by STRING. STRING, Search Tool for the Retrieval of Interacting Genes; (B) Significant gene module and enrichment analysis of the modular genes.
Figure 7

The mRNA level of 4 hub genes. The mRNA level of NFKB1, IL18, TLR9 and HDAC4 were measured in human TM cells by RT-qPCR. P-values were calculated using one way ANOVA. *P < 0.05; ***P < 0.001; ns, non-significant. RT-qPCR, quantitative real-time polymerase chain reaction.