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Exploring the potential mechanism and screening small molecule drugs for glaucoma by using bioinformatics approach

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Abstract. – BACKGROUND: Glaucoma is a neurodegenerative disease which is the second most common cause of blindness worldwide.

AIM: To investigate the mechanism of glaucoma and identify small molecule drugs.

MATERIALS AND METHODS: Gene expression profiles of GSE2378 were downloaded from Gene Expression Omnibus (GEO) database which included 15 astrocytes from 8 and 7 donors with and without glaucoma, respectively. Then the raw data were normalized by Robust Multichip Averaging and the differentially expressed genes (DEGs) were identified with limma package in R. Moreover, the Gene Ontology and pathway enrichment analyses were performed by GOEAST and Gene Set Analysis Toolkit V2, respectively. In addition, the potential target sites of transcription factors were detected using MSigDB. Finally, small molecule drugs were screened for glaucoma treatment by Connectivity Map.

RESULTS: A total of 961 DEGs between glaucoma and normal cells were identified. These DEGs were discovered mainly involved in cell surface, molecule binding, changes in protein activity and signal transduction. Additionally, the most significant pathway was pathway in cancer (FDR = 0.0051). Some DEGs shared target sites of the transcription factor, such as NFκB. (FDR = 0.0132) and PBX1 (FDR = 0.0158). Luteolin (enrichment = 0.87) can simulate the state of normal cells, while vancomycin (enrichment = -0.883) and Prestwick-1082 (enrichment = -0.882) might be potential pathogenic substances.

CONCLUSIONS: We hypothesize that glaucoma cells may be not only caused by the optic nerve cells themselves, but also caused by infections due to resistance decline. All these results may facilitate glaucoma treatment with a new breakthrough.

Key Words:
Glaucoma, Differentially expressed gene, Gene Ontology, Pathway enrichment analysis, Small molecules.

Introduction

Glaucoma is a dangerous eye disease that can cause blindness by optic nerve damage[1]. Some glaucoma cases have rapid onset and great harm [2]. Glaucoma has become the second leading cause of blindness globally according to statistics gathered by World Health Organization in 2002 [3]. People over 40 years old are more likely to suffer from glaucoma, and it is popular in female patients[4,5]. Therefore, the research and treatment of glaucoma are of great significance to human health.

With the development of bioinformatics, a number of researchers have utilized this approach to explore the potential molecular mechanism of glaucoma[6]. Johnson et al[7] have discovered that during the pathogenesis of glaucoma the most significant up-regulated gene may affect cell cycle, cytoskeleton, and immune system process, and the down-regulated genes change glucose and lipid metabolism. Miao et al[8] have predicted that the potential mechanism of glaucoma is the changes of signal transduction, response to stress, ECM genes, migration and cell adhesion functions. Paylakhi et al[9] have found many of glaucoma related genes are enriched in biologic pathways, including focal adhesion and extracellular matrix. Gene set enrichment analysis of altered transcriptional pathways in glaucoma shows that the changes in apoptotic, pro-sur-

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vival and pro-death may be associated with the pathogenesis of glaucoma[10]. Despite there are many studies about glaucoma, the mechanism of it are still not fully understood.

Many diseases are caused by hereditary mutations and an increasing number of the identified disease-related mutations occur in gene regulatory sequences. Laurila and Lähdesmäki[11] have investigated the effect of mutations on transcription factor binding affinity computationally. For example, the mutation in ALOX changes its binding status with transcription factor SPI1, which results in inflammatory effects[12]. Mutation of HBD also affect its binding with transcription factor GATA1, which finally leads to δ-thalassemia[13]. Erb C. has discovered some risk factors can induce the transcription factor NF□B to stimulate differential gene expression[14]. Therefore, the target sites of transcription factor play important roles in the pathogenesis of glaucoma.

Although many drugs for the treatment of glaucoma have been found in the past decades, there is an urgent need to discover some molecular drugs with more efficient and selective. Based on the genes related with disease phenotype, researchers screen drugs to repress these genes. Drug versus Disease (DvD) provides a pipeline for the comparison of drug and disease gene expression profiles from public microarray repositories[15]. Yeh et al[16] have screened trifluoperazine as a potential anticancer stem cell agent that could overcome EGFR-tyrosine kinase inhibitor and chemotherapy resistance. Chen et al[17] have compared the expression pattern of CCA-related genes and genes perturbed by small molecules in connectivity map, and then NVP-AUY922 is regarded as an effective treatment option for patients with CCA. In addition, using microarray technology, Claerhout et al[18] have discovered vorinostat as a candidate therapy for gastric cancer. Therefore, the bioinformatics approach has been widely used in the molecule drugs identification.

In this study, to explore the molecular mechanism of glaucoma, the differentially expressed genes (DEGs) between normal and glaucoma samples were identified and conducted functional analyses. In addition, the potential target sites of transcription factors were also detected, helping to regulate expression of DEGs. Moreover, small molecules for glaucoma treatment were screened out. Our findings set new insight into the pathogenesis of glaucoma and provide several molecular drugs which have the potential to combat the disease.

Materials and Methods

Data Source

The microarray data were downloaded from Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/, accession number GSE2378 [19]) based on the GPL8300 [HG_U95Av2] platform. Total 15 samples were obtained which included 8 and 7 astrocytes from Caucasian American donors with and without glaucoma, respectively. Meanwhile, the original CEL files and the platform probe annotation information of HG_U95Av2 were downloaded for the present study.

Screen of Differentially Expressed Genes

The raw chip data were divided into two sets, including glaucoma cells and normal cells. We used R language to analyse chip data (version v.2.13.0)[20]. First, RMA(Robust Multi-chip Averaging) method[21] was used for chip normalization. Then the limma package in R[22] was utilized to screen the DEGs and the multiple testing correction were conducted to adjust the raw p values to false discovery rate (FDR) by Benjamini and Hochberg (BH)[23]. Finally, the genes with FDR < 0.05 were identified as the DEGs.

Detecting Significant Gene Ontology (GO) Terms of DEGs

To explore changes of DEGs occurring at the cellular level and detect the functions of DEGs, we elaborated gene function and cellular location based on Gene Ontology (GO)[24]. GO enrichment analysis was performed using Gene Ontology Enrichment Analysis Software Toolkit (GOEAST)[25] based on hypergeometric algorithm. Finally, the significant cellular component, molecular function and biological process, in which the DEGs were involved, were identified with FDR less than 0.1, 0.1 and 0.05, respectively.

If DEGs contains valid IDs of k genes from a microarray with a total of t genes, for a given GO term, there are q genes within k and m genes within t, then the possibility that whether genes associated with this GO term is enriched by hypergeometric algorithm,

$$p\,(X = x > q) = \sum_{x\text{-}q}^{m} \frac{\binom{m}{x}\binom{t\text{-}m}{k\text{-}x}}{\binom{t}{k}}$$

Detecting Significant Pathways

After elaborating gene function and cellular location based on GO, we further explored how these DEGs interacted with other genes in a pathway. All the metabolic and non-metabolic pathways were obtained from KEGG (Kyoto encyclopedia of genes and genomes) Pathway database[26] and the enriched pathways were identified using Gene Set Analysis Toolkit V2 [27, 28] based on hypergeometric algorithm. If DEGs contains valid IDs of k genes from a microarray with a total of t genes, for a given pathway, there are q genes within k and m genes within t, the possibility is calculated as following,

$$p\,(X = x > q) = \sum_{x\text{-}q}^{m} \frac{\binom{m}{x}\binom{\text{t-m}}{k\text{-}x}}{\binom{\text{t}}{\text{k}}}$$

Then BH method [23] was used to adjust the raw p-values into false discovery rate (FDR). Finally, the significant pathways were identified with FDR < 0.05 and count > 2.

Potential Transcription Factor Target Sites

The gene enrichment analysis was performed based on gene annotation data in MSigDB (molecular signature database, http://www.broadinstitute.org/gsea/msigdb/index.jsp) using hypergeometric algorithm, and then BH adjustment was utilized for multiple test correction. Finally, the potential target sites of the transcription factors were identified with FDR < 0.05.

Expression Profiles of cell Lines Perturbed by Small Molecules

Connectivity Map[29] (CMap, http://www.broad.mit.edu/Connectivity Map/) consists of more than 7,000 gene expression profiles treated with 1,309 small molecules. These expression profiles represent about 6,000 instances, each of which comprises a treatment and vehicle pair. By comparing the expression pattern of DEGs and the genes perturbed by molecules in CMap, a list of significant molecules related to the disease were identified with FDR < 0.05. Finally, a correlation score for each perturbagen was calculated, ranging from -1 to 1.

KS scores for both up () and down (KS_{down}) were calculated as follows:

$$a = \max_{j=l}^{t}\left[\frac{j}{t} - \frac{V(j)}{N}\right] \quad b = \max\left[\frac{V(j)}{N} - \frac{j\text{-}l}{t}\right]$$

$$KS_{up/down} = \begin{cases} a, (a > b) \\ -b, (b > a) \end{cases}$$

Where, t is the number of genes in either the up- or down-regulated gene group, N is the total number genes in array, j denotes the jth gene in the rank ordered up- or down-regulated groups according to the extent of differential expression, $V(j)$ denotes the position of the jth gene in the rank ordered whole gene list (also ranked according to the extent of differential expression). The connectivity S scores set to zero where KS_{up} and KS_{down} have the same sign, otherwise, to be KS_{up}-KS_{down}.

Results

DEGs Between Glaucoma Cells and Normal Cells

We identified DEGs between glaucoma cells and normal cells using linear models with FDR < 0.05. Finally, 1110 probes were discovered differential expression, which involved 961 DEGs.

Enriched GO terms of DEGs

DEGs were enriched into GO terms using GOEAST from three aspects, including cellular component, molecular function and biological process.

From the aspect of cellular component (Figure 1), differential genes of glaucoma mainly involved in cell surface and extracellular matrix, including cell membrane and extracellular matrix composition. In addition, DEGs were found mainly affected the molecular functions included molecule binding and changes in protein activity (Figure 2). Finally, multiple biological processes in which the DEGs anticipated, including signal transduction, MAPK Cascade pathway, JAK-STAK pathways, cell response were identified (Figure 3).

Pathways Disturbed in Glaucoma Cells

After the pathway enrichment analysis, total 12 pathways with FDR < 0.05 and count > 2 were identified (Table I). Among thses pathways, the most significant one was pathway in cancer (FDR = 0.0051), and the other pathways

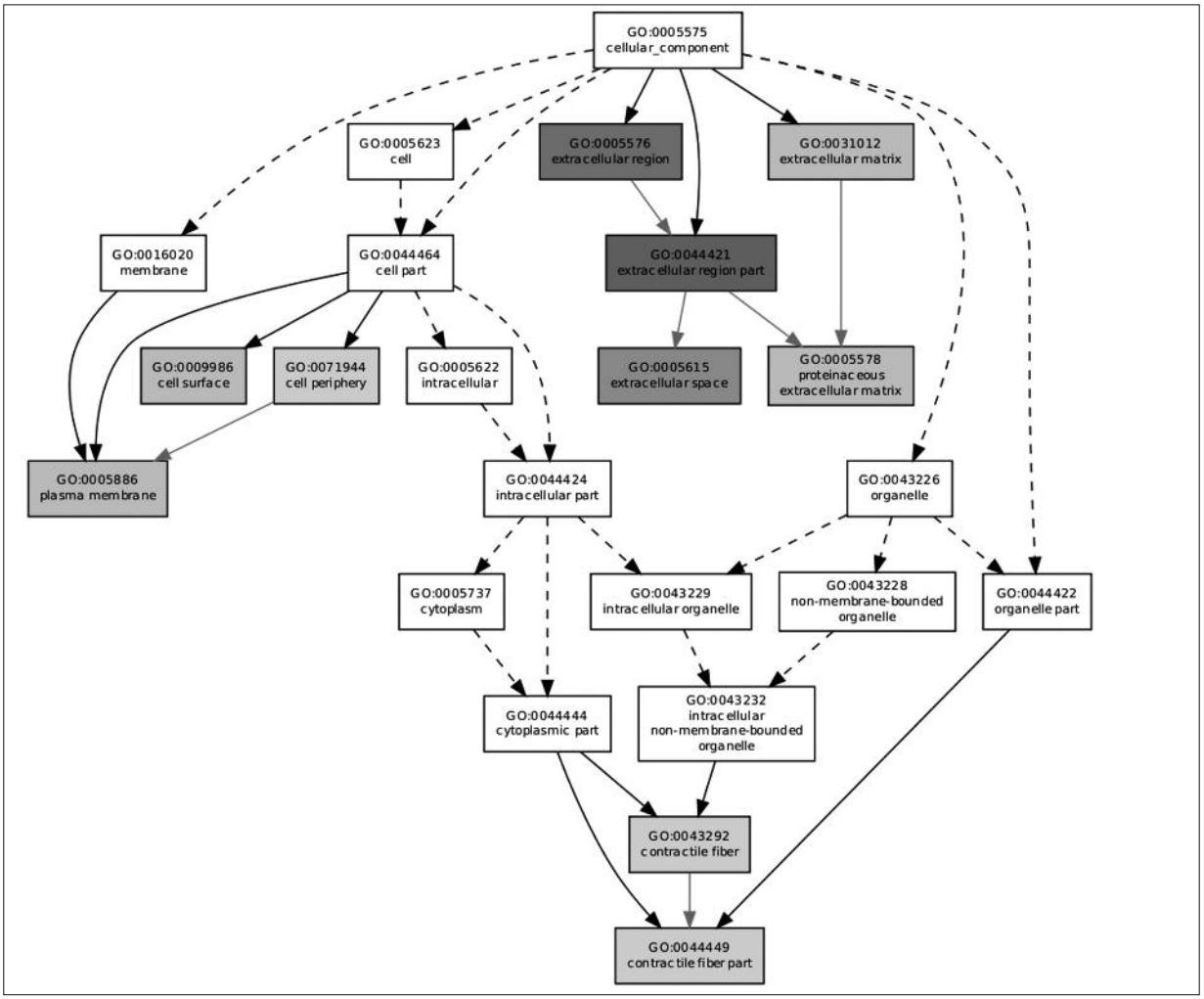

Figure 1. Enriched cellular components of DEGs. Significantly enriched terms are colored gray (FDR < 0.1) and the deeper color represents the more significance.

mainly included MAPK signaling pathway (FDR = 0.0219) and Jak-STAT signaling pathway (0.0306).

Potential Transcription Factor Target Sites

The potential target sites of the transcription factors were identified based on MSigDB with FDR < 0.05 (Table II). The potential target sites of 30 transcription factors were detected, including NF□B (FDR = 0.0132) and PBX1 (FDR = 0.0158).

Glaucoma Related Small Molecules

In order to screen out small molecule drugs, we performed computational bioinformatics analysis of DEGs using the CMap. Total 20 related small molecules with a highly significant correlation were identified, including 13 negative molecules and 7 positive ones. The most significant molecules included Luteolin (enrichment = 0.87), vancomycin (enrichment = -0.883) and Prestwick-1082 (enrichment = -0.882) (Table III).

Discussion

Glaucoma is a dangerous eye disease that can cause blindness by optic nerve damage. Some glaucoma cases have rapid onset and great harm. In this study, we identified 961 DEGs based on gene expression profile of glaucoma cells and normal cells. Then the DEGs were discovered involved in several intracellular signal and metabolic processes during the GO and KEGG pathway analyses. Additionally, the potential target

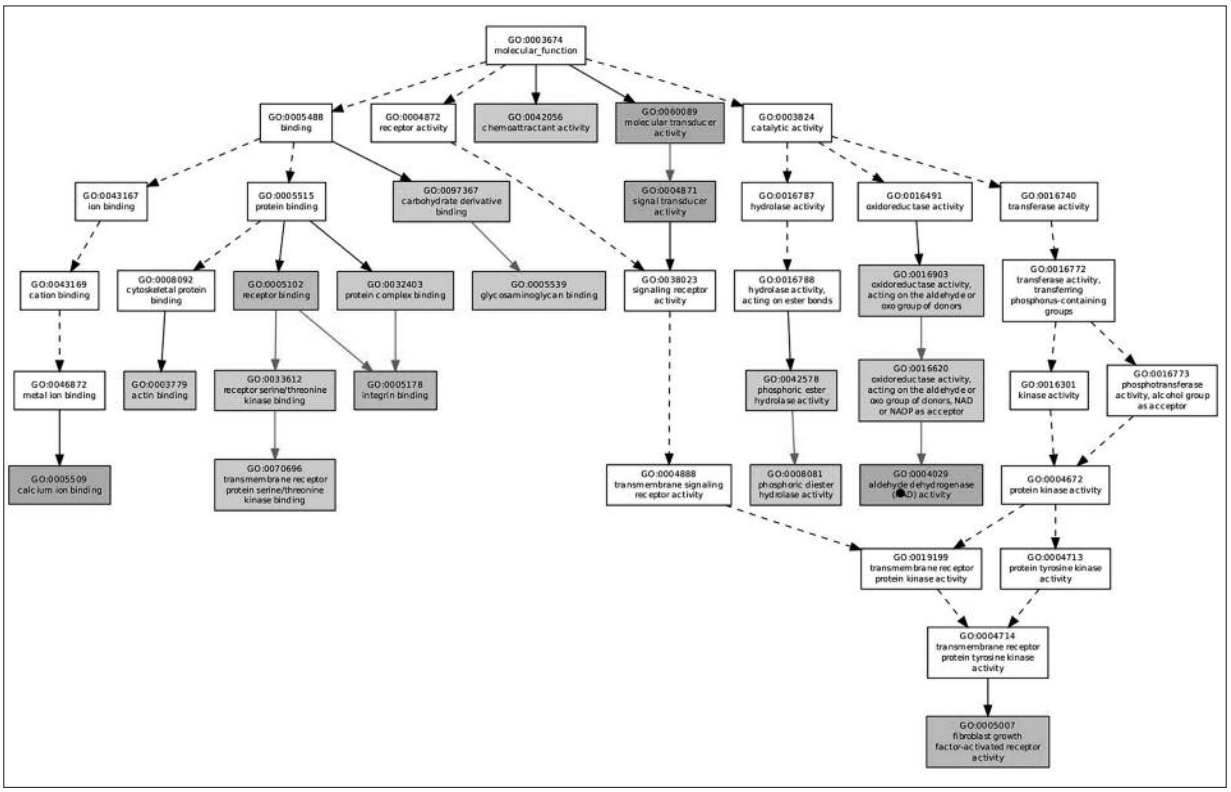

Figure 2. Enriched molecular function of DEGs. Significantly enriched terms are colored gray (FDR < 0.1) and the deeper color represents the more significance.

sites of the transcription factor were dected. Finally, small molecule drugs were identified for glaucoma treatment.

From GO and KEGG pathway enrichment analyses, a lot of changes occur in glaucoma cells compared to normal cells. Besides intracellular changes in signal, metabolic processes are also dramatically changed. DEGs involve in many biological aspects, including cell surface, extracellular matrix and intracellular shrinkage fiber, which is consistent with the previous studies[30,31]. Changes in terms, such as the calcium ion binding, actin binding, kinase binding, integrin protein binding, and the activity of the signal transduction indicates that glaucoma cells may change in signal transduction[32,33]. Metabolic related pathways are also related with glaucoma, such as glycolysis/gluconeogenesis, valine, leucine and isoleucine degradation[34-37]. These abnormal metabolites also help early diagnosis and treatment of glaucoma. Cell morphology related pathways are also included such as regulation of actin cytoskeleton[38]. Signaling transduction related pathways involves MAPK signaling pathway, Jak-STAT signaling pathway and focal adhesion[7,39,40]. DEGs of glaucoma enrich in multiple pathways, some of which are also shared by other diseases, such as pathway in cancer. Therefore, further study on these same pathways not only provides a theoretical basis for glaucoma treatment, but also facilitates the treatment of other diseases. Besides, immune related pathway is also included such as complement and coagulation cascades[2,41].

In vitro and *in vivo* in a monkey model of experimental glaucoma (ExpG), NF-kB mRNA expression and nuclear NF-kB protein was higher in glaucomatous optic nerve heads (ONH) astrocytes than in normal ONH astrocytes[42]. The transcriptional regulatory activity of FOXC1, whose mutations results in an increased susceptibility to glaucoma, is impaired by PBX1 in a filamin A-mediated manner[43]. Combining the previous and the present results, we predict that the target sites of transcription factor play important roles in the pathogenesis of glaucoma.

A number of small molecules are obtained by comparing the expression pattern of DEGs and that of genes perturbed by small molecules. Small molecules vancomycin can simulate the

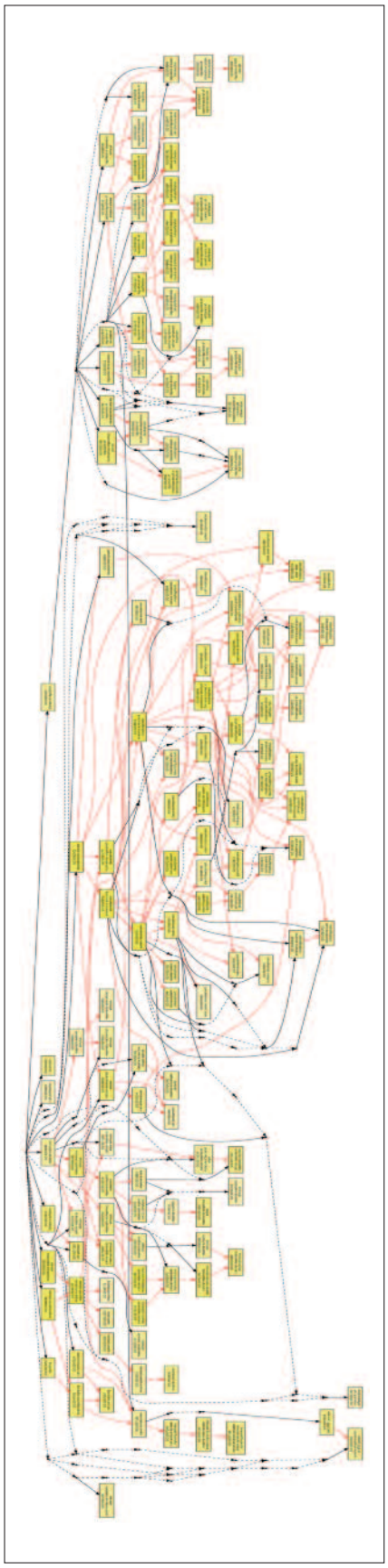

Figure 3. Enriched biological processes of DEGs. Significantly enriched terms are colored gray (FDR < 0.05) and the deeper color represents the more significance.

Table I. The significant pathways in glaucoma cells with FDR < 0.05.

KEGG pathway	FDR
Pathways in cancer	0.0051
Focal adhesion	0.0051
Ascorbate and aldarate metabolism	0.0204
Regulation of actin cytoskeleton	0.0219
Complement and coagulation cascades	0.0219
MAPK signaling pathway	0.0219
Glycolysis/Gluconeogenesis	0.0219
Limonene and pinene degradation	0.0230
Jak-STAT signaling pathway	0.0306
Valine, leucine and isoleucine degradation	0.0306
beta-Alanine metabolism	0.0334
Bladder cancer	0.0446

Note: KEGG is Kyoto encyclopedia of genes and genomes and the KEGG PATHWAY database contains pahway maps for molecular systems in both normal and perturbed states.

Table II. The enriched potential target sites of the transcription factor with FDR < 0.05

Target	FDR
hsa_RTAAACA_V$FREAC2_01	0.0003
hsa_AACTTT_UNKNOWN	0.0003
hsa_V$PAX4_04	0.0008
hsa_CTTTGT_V$LEF1_Q2	0.002
hsa_TTGTTT_V$FOXO4_01	0.0029
hsa_V$HFH3_01	0.0099
hsa_V$FOX_Q2	0.0132
hsa_V$NFKB_Q6	0.0132
hsa_V$HSF2_01	0.0132
hsa_V$CHX10_01	0.0137
hsa_AAAYWAACM_V$HFH4_01	0.0137
hsa_V$HFH4_01	0.0137
hsa_CAGCTG_V$AP4_Q5	0.0137
hsa_V$MYOD_Q6	0.0158
hsa_V$PBX1_02	0.0158
hsa_AAANWWTGC_UNKNOWN	0.0186
hsa_V$HNF4_Q6	0.0231
hsa_V$AREB6_04	0.0231
hsa_WWTAAGGC_UNKNOWN	0.025
hsa_V$SRF_Q4	0.0311
hsa_V$MEF2_03	0.0311
hsa_WGGAATGY_V$TEF1_Q6	0.0351
hsa_V$SRF_Q5_01	0.0387
hsa_CATTGTYY_V$SOX9_B1	0.0428
hsa_V$SRF_Q6	0.0428
hsa_V$AFP1_Q6	0.0457
hsa_V$MYOGNF1_01	0.0495
hsa_V$NFKB_C	0.0495
hsa_V$SRF_C	0.0495
hsa_V$FOXJ2_02	0.0495

Table III. List of glaucoma related small molecules.

CMap name	FDR	Enrichment
Genistein	0.0001	-0.506
Luteolin	0.0004	0.87
Thiamphenicol	0.00042	-0.825
Vancomycin	0.00044	-0.883
Eucatropine	0.0005	0.755
Cinchonine	0.00054	-0.875
Rimexolone	0.00058	0.855
Canadine	0.00068	-0.861
Metamizole sodium	0.00072	0.745
Naringenin	0.00092	-0.853
Monensin	0.00097	-0.72
Thiocolchicoside	0.00101	0.841
Ambroxol	0.00105	-0.844
Colchicine	0.00153	0.714
Felbinac	0.00173	-0.826
Eticlopride	0.00292	-0.803
Finasteride	0.003	-0.678
Zimeldine	0.00308	0.733
Adiphenine	0.0031	-0.729
Prestwick-1082	0.0032	-0.882

Note: CMap represents the Connectivity Map which can connect small molecules, genes and disease using gene-expression signatures.

cell state of glaucoma[44]. Nine percent of the 68 glaucoma patients are treated with vancomycin[45]. Twenty-one percent of glaucoma patients choose a combination of fortified aminoglycoside and vancomycin[46]. All gram-positive patients are sensitive to vancomycin[47]. Prestwick-1082 (enrichment = -0.882) was associated with high negative scores, which suggest that this small molecules is capable to cause opposite effect with glaucoma, that is, they can be used to improve therapeutic effect for ovarian cancer. Prestwick-1082 has recently garnered some attention for its possible therapeutic efficacy on ovarian cancer[48]. Such small molecules provide new possibility for glaucoma treatment. It is also found that many DEGs have the same target sites of the transcription factor. These sites play vital roles in the regulation of gene expression.

Conclusions

Through the functional analysis of DEGs, we hypothesize that glaucoma may not merely caused by the optic nerve cells themselves, it might also be caused by infections due to immunity decline. The target sites of transcription factors play vital roles in the regulation of gene expression. In addition, although it may be premature to suggest that these screened small molecules might be ready for glaucoma clinical trials, it is clearly a direction that warrants additional consideration. Furthermore, we plan to test the treatment results of these molecules on glaucoma in our future research. All these results may facilitate glaucoma treatment with a new breakthrough.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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