Bioinformatics analysis to identify the differentially expressed genes of glaucoma

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Abstract. The aim of the present study was to screen the differentially expressed genes (DEGs) associated with glaucoma and investigate the changing patterns of the expression of these genes. The GSE2378 gene microarray data of glaucoma was downloaded from the Gene Expression Omnibus database, which included seven normal samples and eight glaucoma astrocyte samples. Taking into account the corresponding associations between the probe ID and gene symbols, the DEGs were identified prior to and subsequent to the summation of probe level values using the Limma package in R language, followed by Gene Ontology (GO) and pathway enrichment analyses. Interaction networks of the DEGs were constructed using the Biomolecular Interaction Network Database, and cluster analysis of the genes in the networks was performed using ClusterONE. Subsequent to the summation of probe value, a total of 223 genes were identified as DEGs between the normal and glaucoma samples, including 74 downregulated and 149 upregulated genes. In addition, the DEGs were found to be associated with several functions, including response to wounding, extracellular region part and calcium ion binding. The most significantly enriched pathways were complement and coagulation cascades, arrhythmogenic right ventricular cardiomyopathy and extracellular matrix (ECM)-receptor interaction. Furthermore, interaction networks were constructed of the DEGs prior to and subsequent to the summation of probe values, and *HNF4A* and *CEBPD* were identified as hub genes. Additionally, 37 and 31 GO terms were identified to be enriched in the two DEGs of the networks prior to and subsequent to summation, respectively. The results indicated the identified genes associated with ECM as important, and the *CEBPD* gene was considered to be a critical gene in glaucoma. The findings of the present study offer a potential reference value in further investigations of glaucoma at the gene level.

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Introduction

Glaucoma is an ocular disorder, characterized by intraocular pressure-associated optic neuropathy, with open angle and closed angle glaucoma being the two predominant types. At present, glaucoma is the second leading cause of vision loss worldwide (1). The number of individuals with open angle glaucoma worldwide in 2000 was 44,700,000, and the number is projected to increase to 79,600,000 worldwide (1). In 2013, the population of patients aged between 40 and 80 years with glaucoma worldwide was estimated to be 64,300,000 (2).

The chronic increase in intraocular pressure, which results in eye pain, is considered a key risk factor for glaucoma (3). Dysfunction of the corneal endothelium results in bullous keratopathy, characterized by progressive optic nerve fiber loss and retinal ganglion cell death (4). In glaucoma, optic nerve fiber degeneration initially occurs at the lamina cribrosa (5), which is formed by extracellular matrix (ECM) and quiescent astrocytes (6,7), and functions as a fibroelastic structure, providing mechanical and biological support for optic nerve axons. Chronic elevated intraocular pressure results in ECM remodeling and activation of quiescent astrocytes (8). In turn, the reactive astrocytes express new ECM proteins, a number of which are considered to alter its composition or be neurotoxic to the retinal ganglion cells.

There is a genetic basis underlying a substantial fraction of glaucoma. It has been reported that ~5% of primary open angle glaucoma cases are currently attributed to single-gene or Mendelian forms of glaucoma (9). The vascular endothelial growth factor (*VEGF*) family and collagen gene family have been associated with glaucoma risk (10). It has been reported that the *VEGF* family consists of positive regulators of angiogenesis in the retina (11,12). In addition, VEGF has been demonstrated to be a key inducer of corneal neovascularization (13-15), which may contribute to the further understanding and treatment of glaucoma. Previous studies have implicated the involvement of collagen genes in the regulation of central corneal thickness, which is a risk factor of glaucoma and, thus, possibly associated with the pathogenesis of glaucoma (16). Variations in collagen genes, which lead to inter-individual differences in scleral and lamina cribrosa properties, have

been previously reported to result in different susceptibilities of individuals to elevated intraocular pressure (17). Therefore, it has been suggested that collagen mutations may cause glaucoma (18). Each of these investigations concerning glaucoma genetics have provided novel insights into gene therapy, which appears to be a promising approach in the treatment of glaucoma (19).

Genome-wide analyses of glaucoma have been performed. Bettahi *et al* (20) selected the differentially expressed genes (DEGs) in healing corneal epithelial cells of normal, vs. diabetic corneas. Pieragostino *et al* (21) examined differential protein expression in the tears of patients with pseudoexfoliative and primary open angle glaucoma. Microarray data in leukocytes of patients with primary open angle glaucoma has also been analyzed to examine variations at a genetic level (22). The GSE2378 gene expression profile in the Gene Expression Omnibus (GEO) database is comprised of seven and eight astrocyte samples from donors with and without glaucoma, respectively, and has been previously downloaded to screen DEGs and cluster-associated functions (23-25). However, the interaction among DEGs, particularly the functional modules in the interaction network, remain to be elucidated.

In the present study, the GSE2378 gene expression array was used and, to eliminate the effects of mismatching between large quantities of probe IDs and gene symbols, the data were divided into two groups: Prior to and following the summation of probe values. The DEGs were screened, followed by Gene Ontology (GO) and pathway enrichment analysis and, to examine the potential mechanism of glaucoma, interactions between the DEGs were investigated and visualized and significant functional modules in the network were assessed.

Materials and methods

Derivation of genetic data. The GSE2378 gene expression profiles of optic nerve astrocytes (26,27) were downloaded from the public functional genomics data repository GEO database (http://www.ncbi.nlm.nih.gov/geo/) (28). In total, 15 specimens, including seven normal samples and eight glaucoma specimens, were available, based on the Human Genome U95 version 2 array from Affymetrix, Inc. (Santa Clara, CA, USA).

Normalization of data. The original GSE2378 data in the CEL files were converted into expression measures using the affy package in R language (29) (http://www.bioconductor.org/packages/3.0/bioc/), and background correction and quartile data normalization were performed using the robust multiarray average algorithm with default parameters in the R affy package (30,31).

Selection of DEGs. The Limma package in R (32) (http://www.bioconductor.org/packages/release/bioc/html/limma.html) was used to identify the DEGs at the probe level between the glaucoma samples and normal samples. P<0.01 and |log fold change (FC)|>0.5 were used as the cut-off criteria. The DEGs were determined pre- and post-summation of the probe value. In the treatment of post-summation of probe value, when multiple probe sets corresponded to the same gene, the expression values of the probes were added as the final value of gene expression for the differential expression screening.

Function and pathway enrichment of the DEGs. Functional enrichment analysis was conducted for DEGs, to identify changes in biological function or characteristics by calculating the whole significance of the gene expression (33). Gene-annotation enrichment analysis is a high-throughput strategy, which reduces the dimension of the data analysis and increases the likelihood of identifying the most relevant biological processes, making it a common approach in functional investigations of large-scale genomic or microarray data (34). Although a number of high-throughput enrichment tools can provide gene function enrichment analysis, the most widely used is Database for Annotation, Visualization and Integration Discovery (DAVID) (35) (http://david.abcc.Ncifcrf.gov/). In the present study, DAVID was applied to the enriched GO categories, based on a hypergeometric distribution with a count (gene number enriched in a specific GO term) >5 and the false discovery rate (FDR)<0.01. In addition, the over-represented Kyoto Encyclopedia of Genes and Genomes (KEGG) categories in the pathways (36) were identified.

Protein-protein interaction network and functional module analysis. The Biomolecular Interaction Network Database (BIND; http://bind.ca) (37) archives biomolecular interaction, complex and pathway information. Continued input from users has further improved the BIND data specification, which includes the ability to store detailed information about genetic interactions. Based on the available gene information of the DEGs in the above dataset, the interaction networks were analyzed using Cytoscape (http://www.cytoscape.org/) with a confidence threshold of 0.7. In addition, cluster analysis of genes in protein-protein interaction networks was performed to identify modules with the highest confidence levels using ClusterONE (http://www.paccanarolab.org.sci-hub.org/clusterone/) in the Cytoscape software. Subsequently, GO enrichment analysis of the clustered genes in the selected module was performed, using DAVID with parameters of count >5 and the FDR<0.01.

Results

Identification of DEGs. Based on the Limma package in R language, using P<0.01 and |logFC|>0.5 cut-offs, a total of 234 probes were identified to be differentially expressed in the glaucoma samples compared with the normal control samples, which included 79 downregulated probes, corresponding to 67 genes; and 155 upregulated probes, corresponding to 142 genes. A total of four probes matching the *MYH11* gene were significantly downregulated. Subsequent to statistical analysis, 2,000 genes were identified to match multiple probes. Accordingly, the expression profiles of the probes were added for the same gene to perform the differential analysis between the normal and glaucoma groups at the gene expression level, rather than at the probe level only. In total, 223 DEGs were identified post-summation of the probe value, including 74 downregulated and 149 upregulated genes. Compared with the results pre-summation, there were

Table I. Differentially expressed genes pre- and post-summation.

	Pre-summation			Post-summation		
Probe ID	Gene symbol	logFC	P-value	Gene symbol	logFC	P-value
32582_at	MYH11	-3.00	6.37E-04	MYH11	-8.96	1.44E-03
34235_at	GPR116	-2.36	0.007138566	ITGA6	-3.06	5.35E-04
37407_s_at	MYH11	-2.29	0.002362727	STAT1	-2.54	2.13E-03
767_at	MYH11	-1.97	0.003229233	GPR116	-2.36	7.83E-03
40488_at	DMD	-1.49	9.75E-04	RBPMS	-2.14	4.54E-03
39710_at	NREP	-1.48	0.003015513	CSPG4	-1.90	1.30E-03
37279_at	GEM	-1.40	1.35E-03	SLC1A1	-1.85	8.53E-03
38004_at	CSPG4	-1.38	0.000514187	TEK	-1.82	2.11E-03
40899_at	KRT19	-1.27	0.007598562	ITGA3	-1.63	2.60E-03
774_g_at	MYH11	-1.23	0.005252688	PDLIM5	-1.57	7.72E-03
41215_s_at	41215_s_at	2.05	0.007785332	SEPP1	2.43	9.40E-05
36686_at	ALDH1A3	2.09	5.06E-03	ADH1B	2.48	1.51E-06
38379_at	GPNMB	2.18	0.002595973	CLU	2.49	1.24E-03
1380_at	FGF7	2.25	4.40E-03	PDE1A	2.77	2.28E-03
34363_at	SEPP1	2.43	7.77E-05	AKR1C3	2.88	8.90E-05
35730_at	ADH1B	2.49	1.14E-06	32805_at	3.25	5.81E-04
36780_at	CLU	2.49	0.00108886	ID1	3.45	1.26E-03
36311_at	PDE1A	2.78	0.00202481	CTSK	3.56	6.69E-05
37399_at	AKR1C3	2.89	7.35E-05	PTGDS	4.63	1.37E-04
32805_at	32805_at	3.25	5.00E-04	FGF7	6.20	2.18E-03

Top 10 differentially expressed genes were determined based on a logFC values >0.5. FC, fold change.

189 DEGs in common, with the most significant gene listed in Table I.

The *MYH1*, *CSPG41* and *GPR116* genes were identified to be the most significantly downregulated DEGs prior and subsequent to probe value summation. Similarly, among the upregulated genes, *FGF7*, *ADH1B*, *CLU*, *ARR1C3*, *SEPP1* and *PDE1A* were in the top 10 significant DEGs. Scatter diagrams of pre- and post-summation data demonstrated that no significant difference existed in the number of DEGs (Fig. 1A). Excluding the repeated genes, the common DEGs pre- and post-summation of probe value were revealed using Venn analysis (Fig. 1B). A total of 128 common upregulated genes and 61 downregulated genes were identified. No genes contradicted each other in the four categories.

Enrichment analysis of the DEGs. To determine the functions of DEGs in glaucoma, the 189 common DEGs were mapped to the GO database. GO terms in biological process (BP), including response to wounding, regulation of cell proliferation and vasculature development; terms in cellular component (CC), including extracellular region part, extracellular region and cytoplasmic membrane-bounded vesicle lumen; and terms in molecular function (MF), including calcium ion binding, carbohydrate binding and calmodulin binding, were enriched (Table II).

In order to further investigate changes to the biological pathways in glaucoma cells, the significant pathways associated with the DEGs were identified. The five pathways identified with significant P-values are listed in Table III. The most significant enrichment pathways were complement and coagulation cascades, arrhythmogenic right ventricular cardiomyopathy and ECM-receptor interaction.

Interactive network analysis. The DEGs were mapped to the BIND database and significant interactions were screened with a confidence coefficient >0.7. By integrating these associations, interaction networks of the DEGs were constructed. In the networks of DEGs prior to summation (Fig. 2Aa), *HNF4A* was connected with multiple modules. The protein in the network serves as a node, and the degree of a node denotes the number of proteins interacting with the specific node, which is indicated by the lines between them. The 'hub nodes' were defined as the nodes which had high degrees within the network. The *IGF1R*, *RUNX1T1* and *STAT1* DEGs were identified as hub nodes. Following cluster analysis using ClusterONE, a module containing *FOS* and *CEBPD* DEGs, and non-DEGs belonging to the *HNF4A* and *CEBP* families, were obtained (Fig. 2Ab). The module contained a total of 18 nodes, with a module density of 0.542, quality of 0.874 and P-value of 2.222E-7.

The networks of the post-summation DEGs are shown in Fig. 2Ba and b. *HNF4A* was connected with multiple modules, and the *HDAC1* and *EGFR* DEGs were identified as the hub nodes. Following cluster analysis, a module of 12 nodes, with a density of 0.758, quality of 0.847 and P-value of 1.765E-5 was obtained, including one DEG (*CEBPD)* (Fig. 2Bb).

Table II. Top five significantly enriched GO terms of the differentially expressed genes.

Category	Term	Count	P-value	Fold enrichment	FDR
BP	GO:0009611~response to wounding	27	5.08E-09	3.893572	8.59E-06
BP	GO:0042127~regulation of cell proliferation	33	7.11E-09	3.20479	1.20E-05
BP	GO:0001944~vasculature development	16	9.78E-07	4.871992	1.65E-03
BP	GO:0006954~inflammatory response	18	1.16E-06	4.233012	1.96E-03
BP	GO:0007167~enzyme linked receptor protein signaling pathway	17	1.01E-05	3.799121	1.70E-02
CC	GO:0044421~extracellular region part	31	2.19E-06	2.579701	2.84E-03
CC	GO:0005576~extracellular region	44	1.71E-04	1.748781	2.22E-01
CC	GO:0060205~cytoplasmic membrane-bounded vesicle lumen	6	2.05E-04	10.89375	2.66E-01
CC	GO:0031983~vesicle lumen	6	2.54E-04	10.42011	3.29E-01
CC	GO:0005615~extracellular space	21	3.24E-04	2.449106	4.20E-01
MF	GO:0005509~calcium ion binding	24	1.58E-03	2.018187	2.207535
MF	GO:0030246~carbohydrate binding	13	2.09E-03	2.837957	2.902738
MF	GO:0005516~calmodulin binding	8	2.21E-03	4.415986	3.064494
MF	GO:0005539~glycosaminoglycan binding	8	2.21E-03	4.415986	3.064494
MF	GO:0003779~actin binding	12	3.29E-03	2.844654	4.545023

BP, biological process; CC, cellular component; MF, molecular function; GO, Gene Ontology; FDR, false discovery rate.

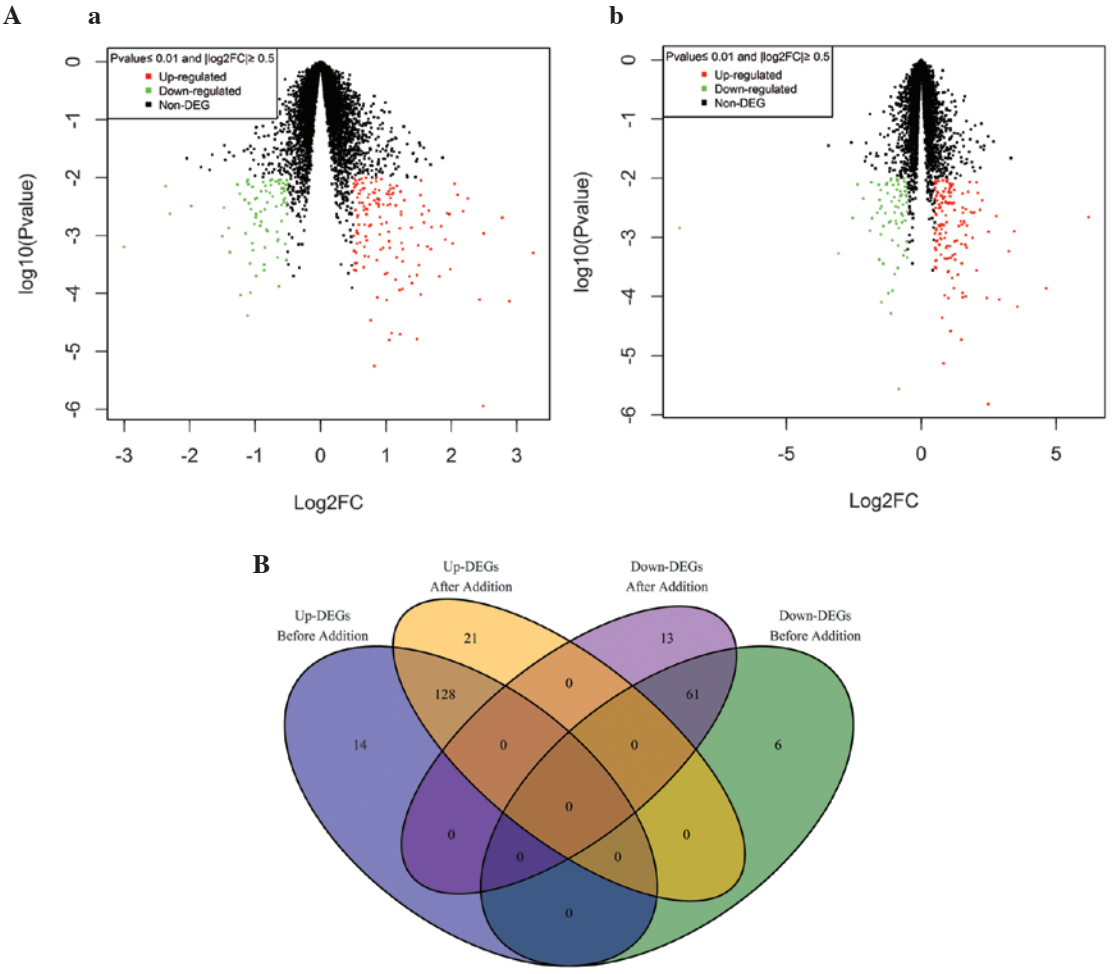

Figure 1. Gene expression analysis pre- and post-summation. (A) Scatter diagram of the gene expressing profiles (Aa) pre- and (Ab) post-summation. The log2FC value is on the x-axis and log10 (P-value) is on the y-axis. (B) Comparison between the number of genes pre- and post-summation. DEGs, differentially expressed genes; FC, fold-change; Up-DEGs, upregulated DEGs; Down-DEGs, downregulated DEGs.

Table III. Top five significantly enriched KEGG pathways of differentially expressed genes.

KEGG term	Count	P-value	Fold enrichment	FDR
hsa04610:Complement and coagulation cascades	10	2.96E-06	8.010397	3.38E-03
hsa05412:Arrhythmogenic right ventricular cardiomyopathy	8	3.73E-04	5.818078	4.26E-01
hsa04512:Extracellular matrix-receptor interaction	8	6.89E-04	5.263975	7.83E-01
hsa05200:Pathways in cancer	15	1.86E-03	2.527671	2.101618
hsa04510:Focal adhesion	11	2.92E-03	3.024822	3.283705

KEGG, Kyoto Encyclopedia of Genes and Genomes; FDR, false discovery rate.

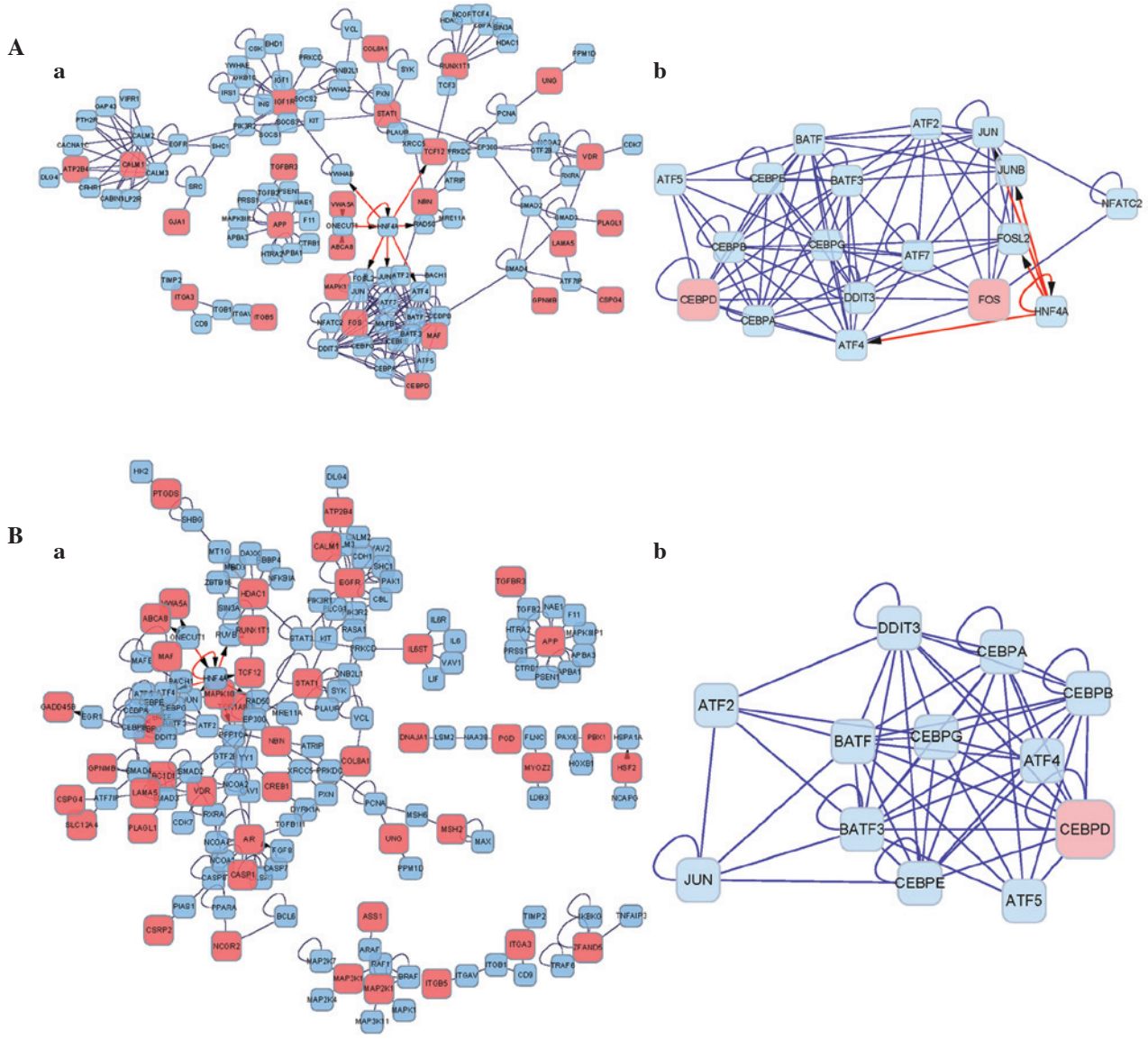

Figure 2. Interaction networks of the DEGs. (A) Pre-summation network; (Aa) whole network; (Ab) most significant subnetwork. (B) post-summation network; (Ba) whole network; (Bb) most significant subnetwork. Pink nodes represent DEGs; blue nodes represent their interaction genes. Red arrows indicate the interaction between protein and DNA, blue lines indicate interaction between proteins. DEGs, differentially expressed genes.

Functional annotation analysis of modules. Functional annotation analysis of the modules available in ClusterONE was performed. A total of 37 and 31 GO terms were enriched in the two modules of the pre- and post-summation networks, respectively. The top three BP, CC and MF enriched functions are listed in Table IV. The enriched genes were

Table IV. Top three function enrichment terms of genes in the interaction network of differentially expressed genes.

Category	Term	Count	P-value	Fold-enrichment	FDR
Pre-summation					
BP	GO:0006355~regulation of transcription, DNA-dependent	18	9.29E-16	7.630006	1.24E-12
BP	GO:0051252~regulation of RNA metabolic process	18	1.36E-15	7.461666	1.87E-12
BP	GO:0045449~regulation of transcription	18	6.43E-13	5.201077	8.98E-10
CC	GO:0031981~nuclear lumen	11	1.83E-08	7.458992	1.46E-05
CC	GO:0070013~intracellular organelle lumen	11	1.35E-07	6.079561	1.08E-04
CC	GO:0043233~organelle lumen	11	1.68E-07	5.942604	1.35E-04
MF	GO:0043565~sequence-specific DNA binding	18	1.96E-23	21.3888	1.89E-20
MF	GO:0046983~protein dimerization activity	17	1.12E-21	22.6231	1.08E-18
MF	GO:0003700~transcription factor activity	18	6.75E-20	13.3159	6.50E-17
Post-summation					
BP	GO:0006355~regulation of transcription, DNA-dependent	12	1.91E-10	7.630006	2.53E-07
BP	GO:0051252~regulation of RNA metabolic process	12	2.44E-10	7.461666	3.23E-07
BP	GO:0006350~transcription	12	1.24E-09	6.438839	1.64E-06
CC	GO:0031981~nuclear lumen	7	1.33E-05	7.713276	1.06E-02
CC	GO:0070013~intracellular organelle lumen	7	4.45E-05	6.286818	3.53E-02
CC	GO:0043233~organelle lumen	7	5.09E-05	6.145192	4.03E-02
MF	GO:0046983~protein dimerization activity	12	6.09E-16	23.95387	5.00E-13
MF	GO:0043565~sequence-specific DNA binding	12	2.14E-15	21.3888	1.88E-12
MF	GO:0003700~transcription factor activity	12	4.07E-13	13.3159	3.63E-10

Top three BP, CC and MF terms, were determined based on the lowest P-values. BP, biological process; CC, cellular component; MF, molecular function; FDR, false discovery rate.

predominantly involved in the progress of gene transcription and expression.

Discussion

Among the selected DEGs, *MYH11* was significantly downregulated pre- and post-summation. Notably, the four probes of *MYH11* were all among the 10 most significantly downregulated genes. Accordingly, the different transcripts of *MHY11* may be involved in the development of glaucoma. In addition, *FGF7*, *ADH1B*, *CLU*, ARR1C3, *SEPP1* and *PDE1A* were all significantly upregulated DEGs pre- and post-summation. Although a number of these have been reported to be involved in Alzheimer's disease or different types of cancer (38-43), there is little information regarding the systematic mechanism underlying the effect of these genes in glaucoma (44). Therefore, the functions of these genes require further investigation. The minimal difference between the pre- and post-summation DEGs, and the absence of contradiction between the upregulated and downregulated genes indicated the analysis used in the present study was reliable.

In the present study, GO functional annotation of the DEGs assisted in identifying associated genes involved in different biological progresses. In the BP term, functions associated with cell division and structure were enriched; in the CC term, functions associated with plasma lumen and vesicles were enriched; and in the MF term, the functions were predominantly involved in calcium signal transduction. These results reflected that the structures of the cell vesicles and microtubules were markedly altered in glaucoma, which was in accordance with the results of a previous study (4). DEGs were found to be enriched in the hsa04512: ECM-receptor interaction KEGG pathway, the genes of which have been reported to be closely associated with glaucoma (45).

In the interaction network analysis of the DEGs, *HNF4A* was associated with multiple modules, indicating that this gene was important in regulating the expression of numerous genes and connecting various pathways. *HNF4A* has been reported to be associated with the pancreas and liver (46). A mutation in the *HNF-4*A gene has been reported to result in monogenic diabetes, of which glaucoma is a common complication (47). Therefore, further analysis of the association between *HNF4A* and glaucoma is required. In addition, the roles of *CEBPD*, a member of *CEBP* family, in the network confirmed the reliability of GO enrichment

analysis, as GO:0042127: regulation of cell proliferation was significantly altered. It has been reported that the binding of the *CCAAT* enhancer to the *CEBPD* transcription factor regulates the cell cycle (48) and its expression may inhibit the proliferation of tumor cells (49). In addition, cell proliferation, rather than astrocyte hypertrophy, characterizes early pressure-induced optic nerve head injury, leading to glaucoma (50). These findings suggested the possibility of identifying how the *CEBPD* transcription factor assists in the inhibition of cell proliferation in glaucoma.

In conclusion, the present study identified DEGs using bioinformatics analysis and observed that CEBP family genes, in particular, *CEBPD*, may be important in the progression of glaucoma. Genes associated with the ECM were also suggested to be important. However, further experiments are required to confirm the results of the present study. Due to the increasing public availability of genomic data, similar approaches are likely to become more popular as a basis for future investigations.

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