

Transcriptional profiling analysis predicts potential biomarkers for glaucoma: HGF, AKR1B10 and AKR1C3

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Abstract. Glaucoma results in damage to the optic nerve and vision loss. The aim of this study was to screen more accurate biomarkers and targets for glaucoma. The datasets E-GEOD-7144 and E-MEXP-3427 were screened for differently expressed genes (DEGs) by significance analysis of microarrays. Functional and pathway enrichment analysis were processed. Pathway relationship networks and gene co-expression networks were constructed. DEGs of disease and treatment with the same symbols were of interest. RT-qPCR was processed to verify the expression of key DEGs. A total of 1,019 DEGs of glaucoma were identified and 93 DEGs in transforming growth factor- β 1 (TGF- β 1) and TGF- β 1-2 treatment cases compared with the normal control group. Pathway relationship network of glaucoma was constructed with 25 nodes. The pathway relationship network of TGF- β 1 and -2 treatment groups was constructed with 11 nodes. Glaucoma-related DEGs in GO terms and pathways were inserted and 180 common DEGs were obtained. Then, gene co-expression network of glaucoma-related DEGs was constructed with 91 nodes. Furthermore, DEGs of TGF- β 1 and -2 treated glaucoma in GO terms and pathways were inserted, and 29 common DEGs were identified. Based on these DEGs, gene co-expression network was constructed with 12 nodes and 16 edges. Finally, a total of 6 important DEGs of disease and treatment were inserted and obtained. They were HGF, AKR1B10, AKR1C3, PPAP2B, INHBA and BCAT1. The expression of HGF, AKR1B10 and AKR1C3 was decreased in glaucoma samples and treatment samples. In conclusion, HGF, AKR1B10 and AKR1C3 may be key genes for glaucoma diagnosis and treatment.

Introduction

Glaucoma includes various types of open-angle, closed-angle and normal-tension glaucoma, and results in damage to the

optic nerve and vision loss (1). The risk factors of glaucoma include increased pressure in the eye and family history of high blood pressure, obesity and migraines (2). Once vision loss from glaucoma occurs, it will be permanent (3). Therefore, the exact pathogenesis requires research for prevention, diagnosis and treatment of glaucoma.

Several important genes and their molecular mechanisms have been found to be related with glaucoma. The research of Skarie and Link revealed that WDR36 has a negative correlation with p53 stress-response pathway in the primary open-angle glaucoma (4). In a large animal model, MMP-1 was confirmed to lower the intraocular pressure of glaucoma cases induced by steroid (5). Besides, LOXL1 indicates high prevalence of glaucoma, but with low specificity (6). Jelodari-Mamaghani *et al* (7) suggested that some sequence variations of latent transforming growth factor- β (TGF- β) binding protein 2 could induce primary open-angle glaucoma and other pseudoexfoliation syndromes. However, some genes are controversial because their underlying molecular mechanisms were uncovered. Kumar *et al* (8) showed that 3.59% of Indian primary open-angle glaucoma patients had mutations in MYOC, OPIT and CY1B1 genes. But in some primary congenital glaucoma patients, MYOC and FOXC1 mutations did not participate (9). Though various genes and related molecular mechanisms were investigated, targeted therapy for glaucoma has not achieved a breakthrough. Accurate targets for glaucoma are urgently needed.

Bioinformatics analysis was processed to screen more accurate biomarkers and therapy targets for glaucoma. Most importantly, two different datasets related to the disease and treatment were downloaded. Various networks including pathway-relationship network and gene co-expression networks were constructed. The final intersection was identified to achieve the goal and lay a theoretical foundation for further research.

Materials and methods

Microarray datasets. Interesting datasets for glaucoma were searched in ArrayExpress Archive (<http://www.ebi.ac.uk/arrayexpress/>). Two different datasets were obtained, including E-GEOD-7144 and E-MEXP-3427 (10). The platforms of the databases were [HG-U133A] Affymetrix Human Genome U133A Array and Affymetrix GeneChip Human Genome

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U133 Plus 2.0 [HG-U133_Plus_2], respectively. The expression profiling of E-GEOD-7144 contains 6 trabecular meshwork samples of glaucoma patients, including 2 glaucoma samples treated with ethyl alcohol as the normal control group and 4 glaucoma samples treated by TGF- β 1 and -2. The expression profile of E-MEXP-3427 contained 9 trabecular meshwork samples, including 2 glaucoma samples and 7 controls.

Data pretreatment and identification of DEGs. The downloaded raw data were corrected and normalized by Robust Multi-chip Average (RMA) algorithm (11), and then annotated based on Affymetrix (<http://www.affymetrix.com/support/technical/annotationfilesmain.affx>). Following, normalized unscaled standard errors (NUSE) controlling was used for quality control (12). Compared with the normal control group, the DEGs in glaucoma samples were screened by significance analysis of microarrays (SAM) (13). Similarly, the DEGs in TGF- β 1 and -2 treatment groups compared with the untreated control group were also identified by this method. The threshold of DEGs in the two groups was $P < 0.05$ and $|\log FCI| > 2$.

Gene Ontology (GO) functional enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. GO database was built by Gene Ontology Consortium, which described gene and their protein functions (14). Based on this database, screened DEGs in the two groups were functional annotated, and then significant GO terms were selected by Fisher exact test and multiple hypothesis test with the threshold of false discovery rate (FDR) < 0.05 . Benjamini-Hochberg (BH) method was utilized for correcting P-value to FDR (15).

Based on KEGG database, the screened DEGs in the two groups were gathered in eight categories, including total network, metabolic process, genetic information transfer, environmental information transfer, intracellular biological process, biological systems, human disease and drug development (16). The enriched pathways were calculated by Fisher's exact test with the criteria of FDR < 0.05 .

Construction of pathway relationship network. KEGG database provides the relationship of genes and pathways. In this study, the pathway relationship network was constructed based on this database. According to the information of signal transduction in the network, upstream and downstream signal pathways were obtained.

Intersection of DEGs in GO terms and pathways. DEGs in GO terms and pathways with the same symbols were of interest, and common DEGs of the two groups were obtained.

Gene co-expression network construction. Gene co-expression network reflected the regulatory relationship among DEGs, and showed the hub nodes with higher degrees. This network was constructed with the threshold of correlation coefficient > 1 .

Intersection of DEGs of disease and treatment. DEGs of disease and treatment with the same symbols were of interest, and the important DEGs were obtained.

Trabecular meshwork cell culture and treatment. As described in previous studies, primary culture of HTM-2 cells was prepared from glaucoma and normal donors. The experiment included 3 groups: the normal control group, the disease group and the treatment group. All cells were maintained at 37°C in 5% CO₂ in low glucose Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum. All reagents were purchased from Invitrogen; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). After 24 h treatment, HTM cells from glaucoma in the treatment group were subcultured in free culture medium for 48 h, and then treated by TGF- β 1 and -2 (1 ng/ml of free culture medium) for 1 h. The cells in the normal control and disease groups were also processed by the above steps but treated by normal saline. The experiment design with three replications was used.

Determination of expression levels of important DEGs by RT-qPCR. Total RNA of cells in the three groups were reverse transcribed by oligo(dT) primer and Superscript II Reverse Transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.). The primers of DEGs were designed as follows: HGF (upstream) 5'-ACAGCTTTTTCCTTCGAGCTATCGGGGTAAAGACCTACAGG-5'; (downstream) 5'-CATCAAAGCCCTTATCGGGGATA-3'; AKR1B10 (upstream) 5'-GGACCTGTTCATCGTCAGCAA-3'; (downstream) 5'-CCCCAGACTTGAATCCCTGTG-3'; AKR1B10 (upstream) 5'-GTAAAGCTTTGGAGGTCAC-3'; (downstream) 5'-CACCCATCGTTTGTCTCGT-3'; PPAP2B2B (upstream) 5'-CCTCTTCTGCCTCTTCATGG-3'; (downstream) 5'-GCCACATACGGGTTCTGAGT-3'; GAPDH (upstream) 5'-AATGCATCCTGCACCACCAA-3'; (downstream) 5'-GTAGCCATATTCATTGTCATA-3'.

All amplifications were performed on the Rotor-Gene 3000 real-time cycler (Corbett Research, Sydney, Australia) instrument. The reaction conditions were 1 cycle of 95°C for 10 min, 42 cycles of 95°C for 20 sec, and 60°C for 20 sec and 72°C for 20 sec. SYBR Premix Ex Taq™ (Takara, Otsu, Japan) was used in the following PCR procedure. The experiment was repeated three times.

This study has been approved by the Ethics Committee of The Second People's Hospital of Jinan (Jinan, China) and all patients provided informed consent.

Results

Identification of DEGs. Compared with the normal control group, a total of 1019 DEGs of glaucoma were identified, including 471 up- and 548 downregulated genes. Similarly, total 93 DEGs in TGF- β 1 and -2 treatment cases compared with the untreated control group were obtained, including 71 upregulated and 22 downregulated DEGs.

Functional and pathway enrichment analysis. As shown in Table I, the screened glaucoma-related DEGs were enriched in various GO terms, including negative regulation of cell proliferation (FDR=9.56E-11), endoplasmic reticulum unfolded protein response (FDR=9.56E-11), activation of signaling protein activity involved in unfolded protein response (FDR=7.04E-08) and cellular protein metabolic process (FDR=4.22E-07). Simultaneously, these DEGs involved in pathways, such as protein processing in endoplasmic

Table I. The top 5 GO and KEGG pathways enriched by glaucoma-related DEGs.

GO ID	GO name	Diff gene counts in GO	Enrichment score	P-value	FDR
GO:0008285	Negative regulation of cell proliferation	32	5.306158859	5.10×10^{-14}	9.56×10^{-11}
GO:0030968	Endoplasmic reticulum unfolded protein response	17	12.15861552	8.56×10^{-14}	9.56×10^{-11}
GO:0006987	Activation of signaling protein activity involved in unfolded protein response	13	12.05803873	9.46×10^{-11}	7.04×10^{-8}
GO:0044267	Cellular protein metabolic process	32	3.688553148	7.55×10^{-10}	4.22×10^{-7}
GO:0007050	Cell cycle arrest	15	6.956560809	9.88×10^{-9}	4.22×10^{-6}

Pathway ID	Pathway name	Diff gene counts in pathway	Enrichment score	P-value	FDR
4141	Protein processing in endoplasmic reticulum	21	7.464764652	2.17×10^{-12}	4.74×10^{-10}
4151	PI3K-Akt signaling pathway	25	4.276848144	3.28×10^{-9}	3.51×10^{-7}
5200	Pathways in cancer	24	4.356891907	4.81×10^{-9}	3.51×10^{-7}
1100	Metabolic pathways	45	2.246694155	1.70×10^{-6}	9.32×10^{-5}
5410	Hypertrophic cardiomyopathy (HCM)	9	6.285457295	2.74×10^{-5}	1.06×10^{-3}

DEGs, differently expressed genes; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; FDR, false discovery rate.

Table II. The top 5 GO and KEGG pathways enriched by TGF- β 1 and -2 treatment-related DEGs.

GO ID	GO name	Diff gene counts in GO	Enrichment score	P-value	FDR
GO:0044597	Daunorubicin metabolic process	4	327.380597	6.92×10^{-10}	1.77×10^{-7}
GO:0044598	Doxorubicin metabolic process	4	327.380597	6.92×10^{-10}	1.77×10^{-7}
GO:0071395	Cellular response to jasmonic acid stimulus	3	491.0708955	2.72×10^{-8}	4.63×10^{-6}
GO:0030198	Extracellular matrix organization	7	21.82537313	7.10×10^{-8}	9.06×10^{-6}
GO:0051897	Positive regulation of protein kinase B signaling cascade	5	48.14420544	1.38×10^{-7}	1.41×10^{-5}

Pathway ID	Pathway name	Diff gene counts in pathway	Enrichment score	P-value	FDR
1100	Metabolic pathways	12	6.608186988	4.52×10^{-7}	2.35×10^{-5}
4512	ECM-receptor interaction	5	37.62995368	4.79×10^{-7}	2.35×10^{-5}
5146	Amoebiasis	5	30.03491716	1.47×10^{-6}	3.84×10^{-5}
4510	Focal adhesion	6	19.07071439	1.57×10^{-6}	3.84×10^{-5}
4974	Protein digestion and absorption	4	29.76187246	2.10×10^{-5}	4.12×10^{-4}

DEGs, differently expressed genes; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; FDR, false discovery rate.

reticulum (FDR=4.74E-10), PI3K-Akt signaling pathway (FDR=3.51E-07), pathways in cancer (FDR=3.51E-07) and metabolic pathways (FDR=9.32E-05).

Similarly, the DEGs of TGF- β 1 and -2 treatment patients participated in different GO terms, such as daunorubicin metabolic process (FDR=1.76E-07), doxorubicin metabolic process (FDR=1.76E-07), cellular response to jasmonic acid stimulus (FDR=4.62E-06). These DEGs were also

enriched in various pathways including metabolic pathways (FDR=2.34E-05), ECM-receptor interaction (FDR=2.34E-05) and amoebiasis (FDR=3.84E-05) (Table II).

Construction of pathway relationship network. Pathway relationship network of glaucoma was constructed with 25 nodes and 87 edges (Fig. 1). The hub nodes were MAPK signaling pathway (degree 18), apoptosis (degree 18) and cell

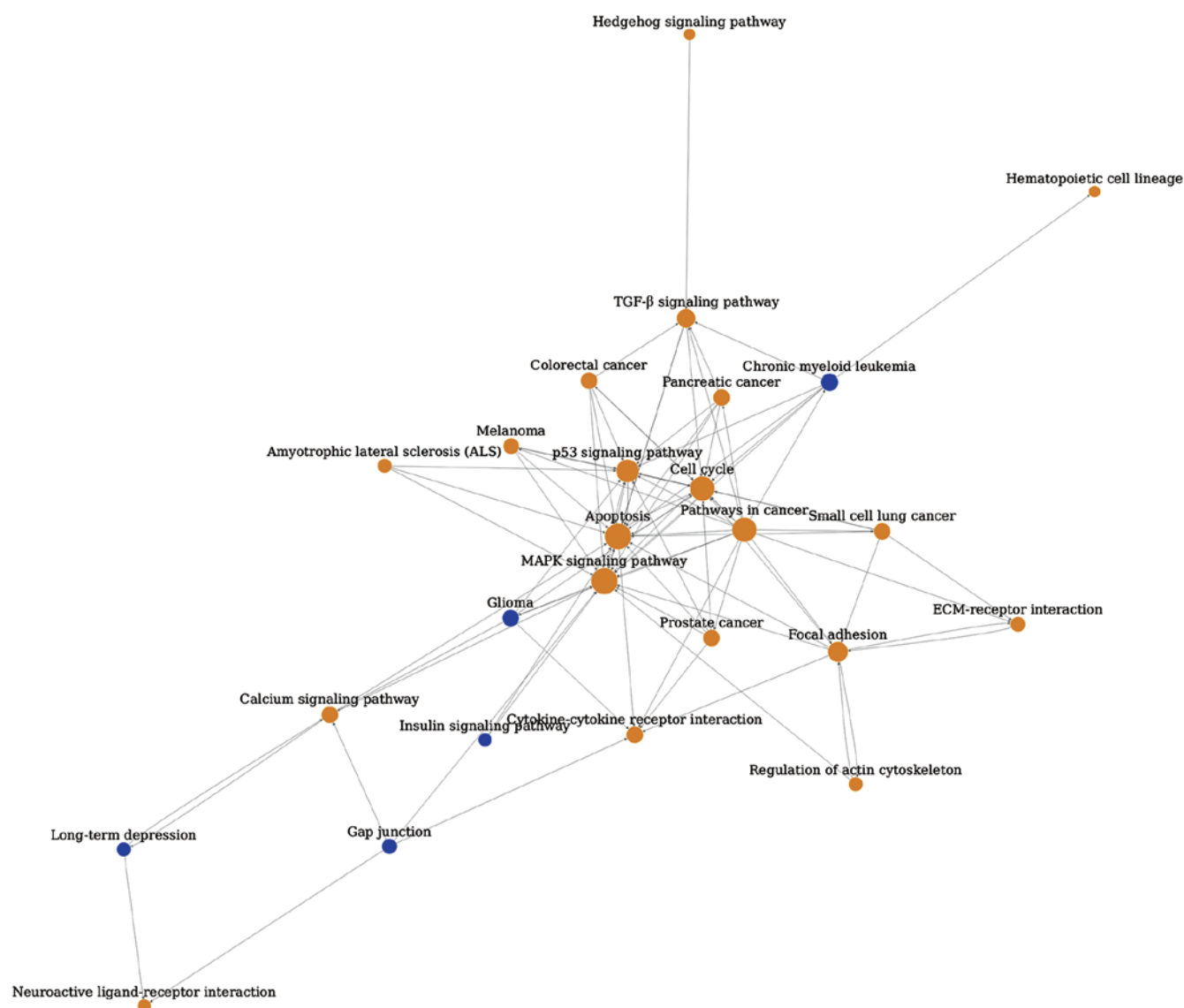


Figure 1. Pathway relationship network of glaucoma-related DEGs. The blue nodes represent pathways involving downregulated DEGs, and the yellow nodes represent pathways involving both up- and downregulated DEGs. The arrows represent regulatory direction. DEG, differently expressed gene.

cycle (degree 15). In addition, several upstream pathways were identified, including pathways in cancer and gap junction. Cytokine-cytokine receptor interaction was shown as a downstream pathway.

As shown in Fig. 2, pathway relationship network of TGF- β 1 and -2 treatment groups was constructed with 11 nodes and 20 edges. Focal adhesion, pathways in cancer and Wnt signaling pathway were the hub nodes with degree of 7.7 and 6, respectively.

Gene co-expression network construction of common DEGs. Based on gene symbol, glaucoma-related DEGs in GO terms and pathways were inserted and 180 common DEGs were obtained. Then, gene co-expression network of glaucoma-related DEGs was constructed, including 91 nodes and 166 edges (Fig. 3). In addition, the hub nodes in this network were protein disulfide isomerase family A, member 4 (PDIA4, degree 13), osteosarcoma amplified 9, endoplasmic reticulum lectin (OS9, degree 12), derlin 3 (DERL3, degree 10) and sel-1 suppressor of lin-12-like (SEL1L, degree 9).

Furthermore, DEGs of TGF- β 1 and -2 treated glaucoma in GO terms and pathways were inserted, and 29 common DEGs were identified. Based on the DEGs, gene co-expression network was constructed with 12 nodes and 16 edges (Fig. 4). The hub node of this network was HGF (degree 4).

Intersection of DEGs of disease and treatment. Finally, a total of 6 important DEGs of disease and treatment were inserted and obtained. They were hepatocyte growth factor (HGF), aldo-ketoreductase family 1, member B10 (AKR1B10), aldo-ketoreductase family 1, member C3 (AKR1C3), phosphatidic acid phosphatase type 2B (PPAP2B), inhibin β A (INHBA) and branched chain amino-acid transaminase 1, cytosolic (BCAT1). These genes may be biomarkers and targets for glaucoma diagnosis and treatment.

DEG expression level by RT-qPCR. The expression level of HGF, AKR1B10, AKR1C3 and PPAP2B were determined by RT-qPCR. As shown in Fig. 5, the expression of HGF, AKR1B10 and AKR1C3 was significantly decreased in

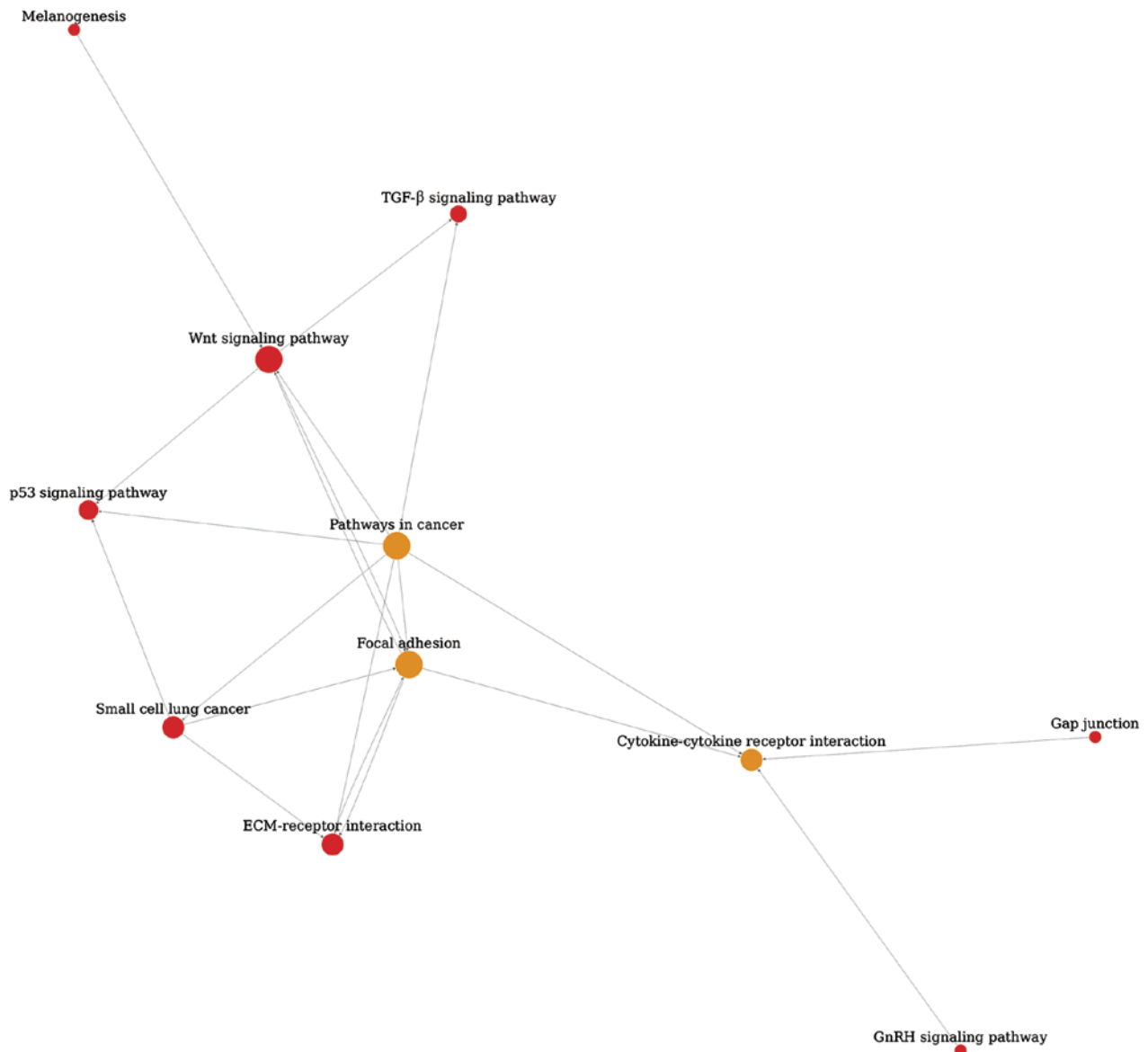


Figure 2. Pathway relationship network of TGF- β 1 and -2 treatment-related DEGs. The red nodes represent pathways involving upregulated DEGs, and the yellow nodes represent pathways involving both up- and downregulated DEGs. The arrows represent regulatory direction. DEG, differently expressed gene; TGF- β 1, transforming growth factor- β 1.

glaucoma and treatment samples. However, no significant difference of AKR1C3 was found in the three groups.

Discussion

Glaucoma is treated by daily eye drop drugs, but with unsatisfactory results (17). Gene therapy has successfully progressed for other eye diseases, and may be an effective method for the treatment of glaucoma in the future (17). In this study, various DEGs were screened and their association with glaucoma was examined. We also examined DEGs related to treatment, including HGF, AKR1B10, AKR1C3 and PPAP2B.

HGF encodes a protein which activates a tyrosine kinase signaling cascade and further regulated cell growth, motility and morphogenesis (18). In the patients with eyes suffering from primary open-angle glaucoma, the concentration of HGF was significantly elevated (19). In addition, in 2010, four SNPs were found closely related with primary angle closure

glaucoma, including rs12536657, rs17427817, rs5745718 and rs12540393 (20). Moreover, the level of HGF was confirmed to stimulate the expression of MMP-1, and further affected the migration of human corneal epithelial cells (21). In this study, the gene was involved in various functions and pathways, including the negative regulation of the apoptotic process, mitosis, PI3K-Akt signaling pathway and pathways in cancer. In addition, the expression of HGF was significantly lower in the glaucoma and treatment groups than in the normal control group. In a previous study, the aqueous humor factors significantly affected mitosis in the molecular pathogenesis of primary open-angle glaucoma (22). In various diseases, HGF has been confirmed to be an effective target of TGF- β 1 and -2 treatments. For example, tumor-derived TGF- β 1 promoted HGF-dependent invasion of squamous carcinoma cells (23). Furthermore, TGF- β was involved in HGF-c-Met pathway, and then induced oligodendrocyte precursor cell chemotaxis (24). Besides, Tripathi *et al* (25) found that the level of TGF- β 2 was

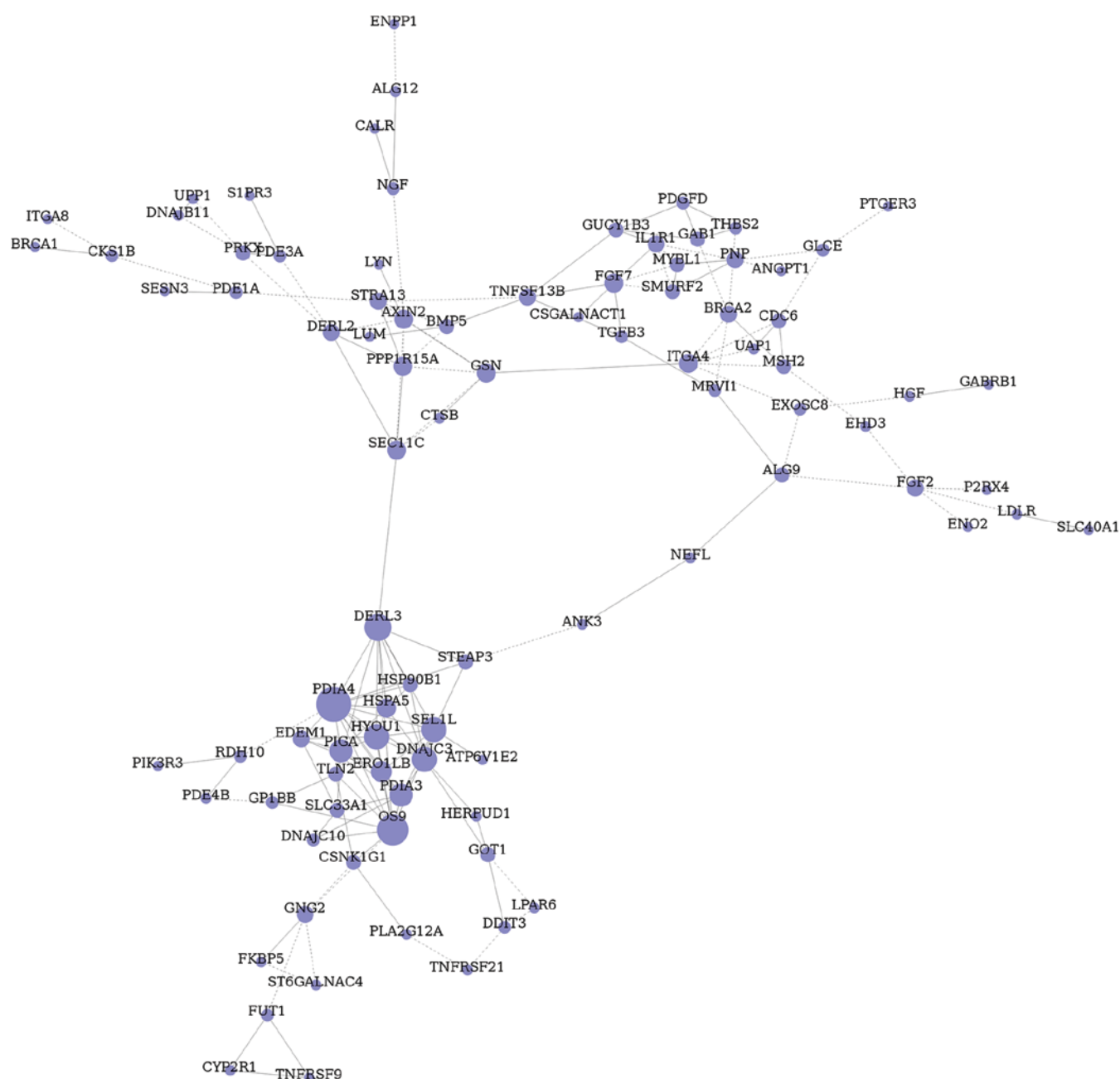


Figure 3. Gene co-expression network of important glaucoma-related DEGs. The solid lines represent DEGs with positive correlation, and the dotted lines represent DEGs with negative correlation. DEG, differently expressed gene.

significantly increased in aqueous humor in glaucomatous eyes. Overall, HGF is an important biomarker for glaucoma and also a key target for glaucoma treatment.

Another key DEG, AKR1B10, was also identified in both glaucoma and TGF- β 1 and -2 treatment-related gene co-expression network. This gene encoded a member of aldo/ketoreductase superfamily, which could effectively reduce aliphatic and aromatic aldehydes. As known, NADPH-dependent aldo-ketoreductase is an important rate limiting enzyme of polyol pathway, which accelerates glucose metabolism and also affects the diabetic cataract and retinopathy (26). In this study, AKR1B10 was found enriched in the daunorubicin metabolic process, doxorubicin metabolic process, and farnesol catabolic process. As shown

in previous results, intraoperative daunorubicin could decrease the intraocular pressure safely and effectively in high-risk surgical cases of glaucoma (27). Moreover, doxorubicin was also confirmed to be an effective adjunct to glaucoma surgery (28). Thereby, we inferred that AKR1B10 participated in the pathogenesis of glaucoma by being involved in the daunorubicin and doxorubicin metabolic process. Interestingly, AKR1C3 and AKR1B10 had positive relationships in the gene co-expression network of glaucoma. AKR1C3 is also a member of the aldo-ketoreductase family 1. Besides the daunorubicin and doxorubicin metabolic process, AKR1C3 is enriched in immune response, G-protein coupled receptor signaling pathway and positive regulation of protein kinase B signaling cascade. Among these functions

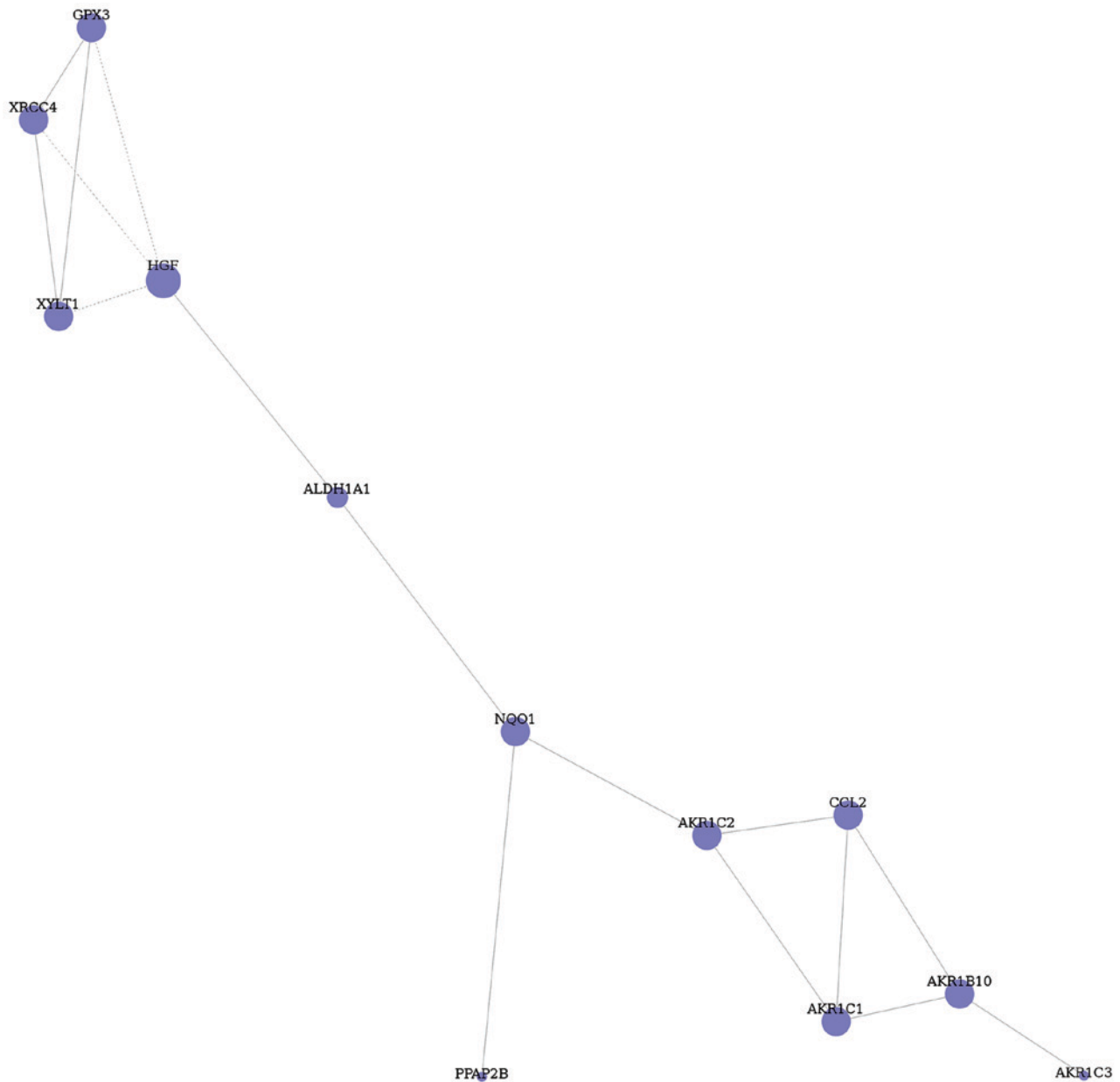


Figure 4. Important gene co-expression network TGF- β 1 and -2 treatment-related DEGs. The solid lines represent DEGs with positive correlation, and the dotted lines represent DEGs with negative correlation. DEG, differentially expressed gene; TGF- β 1, transforming growth factor- β 1.

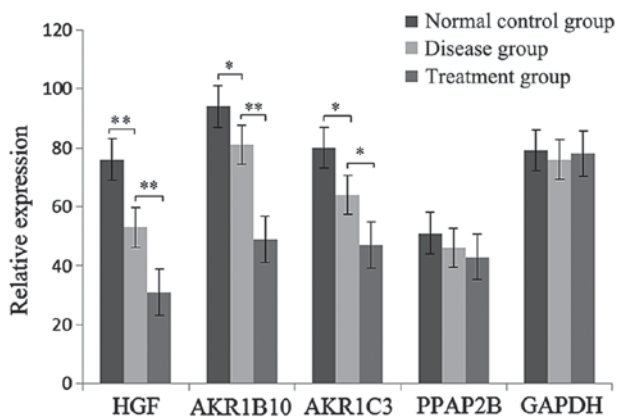


Figure 5. The expression level of HGF, AKR1B10, AKR1C3 and PPAP2B. HGF, hepatocyte growth factor; AKR1B10, aldo-ketoreductase family 1, member B10; AKR1C3, aldo-ketoreductase family 1, member C3; PPAP2B, phosphatidic acid phosphatase type 2B. * $p < 0.05$, ** $p < 0.01$.

and pathways, immune response, especially oxidative stress, played a key role in keeping a physiological balance by participating in the pathogenic cellular processes of glaucoma (29). In addition, G-protein activation mediated by shear stress was confirmed to be a possible step in molecular mechanism of glaucoma formation (30). Most importantly, the expression of AKR1B10 and AKR1C3 was significantly lower in the glaucoma and treatment groups than in the normal control group. According to the above information, AKR1C3 was inferred to be a key gene for prevention and treatment of glaucoma.

Simultaneously, PPAP2B was screened with a higher degree in this study and also involved in different functions and pathways, including small molecule metabolic process, negative regulation of protein phosphorylation and Fc γ R-mediated phagocytosis. PPAP2B encoded a member of the

phosphatidic acid phosphatase family which participate in the formation of diacylglycerol and glycerolipids (31). Moreover, Chiasseu *et al* (32) showed that the signature pathological features of glaucoma included altered phosphorylation of tauopathies. Besides, various metabolic abnormalities such as carbohydrate and uric acid metabolic abnormalities were confirmed to play an important role in pathogenesis of glaucoma damage (33). Thereby, PPAP2B may be a key gene in the pathogenesis of glaucoma through its involvement in the small molecule metabolic process. However, no significant difference of PPAP2B was found in the three groups.

In conclusion, HGF, AKR1B10 and AKR1C3 may be key genes for glaucoma diagnosis and treatment.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QN constructed pathway relationship network. XZ helped with gene co-expression network construction. QN and XZ were also involved in the conception and design of the study. Both authors have read and approved the final manuscript.

Ethics approval and consent to participate

This study has been approved by the Ethics Committee of The Second People's Hospital of Jinan (Jinan, China). All patients in this study provided informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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