Pathway and Network Analysis in Primary Open Angle Glaucoma

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ABSTRACT

Glaucoma, a group of multifactor ocular diseases, is the second leading cause of blindness worldwide. Primary open angle (POA) is the most common type of glaucoma, characterized by progressive optic nerve degeneration. Numerous genes and proteins have been revealed to be associated with POAG, but the pathologic mechanisms of the disease are still poorly understood. Proteomics, the collective study of proteins in an organism at a given condition, has extensively been used for the high-throughput identification of proteins related to POAG. A significant obstacle in proteomics studies is the data variability which makes it hard to interpret the results. Pathway analysis and network topological information can help address the challenge and provide a greater appreciation of the disease mechanism and progression. The purpose of this paper is to determine POAG biological and network information to further understand the mechanisms associated with POAG. PANTHER classification system was used, including classification with gene ontology, protein class and pathway. 474 gene/protein IDs were extracted from previous proteomic studies. Among pathways found by PANTHER classification, apoptosis signaling pathway was the most significant pathway (with the p-value of 5.54E-12). Other PANTHER categories results demonstrated that developmental processes, receptor binding, extracellular region and extracellular matrix proteins were the most significant biological process, molecular function, cellular component and protein class respectively. Pathway analysis aids to find probable mechanisms involved in POAG. A network analysis on proteins was also performed using STRING database and cytoscape software. From network analysis, candidate biomarkers for the disease were introduced.

Keywords: Glaucoma; Pathway Analysis; Network; Proteomics.

INTRODUCTION

Glaucoma is an ocular disorder and neurodegenerative disease with multi-factorial etiology that causes damage to optic nerve [1, 2]. It is the second most common cause of irreversible blindness and approximately, 60 million people are affected by glaucoma worldwide [3, 4]. Glaucoma can be devided into two main categories: open-angle and closed-angle. Open-angle is the most common type of glaucoma and in this case, eyes have normal structure but the intraocular fluid has no flow through trabecular meshwork. Closed-angle glaucoma is less common and drainage may be poor because the angle between the iris and the cornea is too narrow. Primary open angle glaucoma (POAG) is characterized by the presence of glaucomatous optic neuropathy without an identifiable secondary cause [5]. Prevalence of POAG in Asian countries is between 1 to 4 % [6]. It is increasingly evident that besides intraocular pressure-generated stress and aging, glaucomatous neurodegeneration involves genetic predispositions and epigenetic risk factors. Although recent experimental studies have achieved many advances in understanding of glaucomatous neurodegeneration, key molecular mechanisms that can serve as treatment targets remain unclear [7].

Proteomics, the collective study of proteins in an organism at a given condition, has extensively been used for the high-throughput identification of proteins related to POAG. For instance, Tezel et al., identified a number of serum proteins by immunoproteomic study of human glaucoma [8]. They identified a number of proteins which were diseased tissue-related antigens and could serve as candidate biomarkers of glaucoma. Duan et al. analyzed

aqueous humor composition of POAG patients via two-dimensional gel electrophoresis and found seven increased proteins in aqueous humor of POAG patients including PGDS, caspase 14 precursor, transthyretin, cystain C, albumin precursor, and transferrin [9]. A primary hurdle that slows down the application of proteomics is the data's high variability which makes it difficult to interpret proteomics data analysis results biologically. Another challenge is how to extract functional and biological information from a long list of proteins discovered from high-throughput proteomics [10].

Pathways are intricate networks of molecular reactions in organisms [11]. Pathway analysis can help organize a long list of proteins onto a short list of pathway knowledge maps, making it easy to interpret molecular mechanisms underlying these altered proteins or their expressions. Network analysis is also used to gain systems-level biological meanings [10]. Understanding of the interactomes will provide a much greater appreciation of what triggers disease onset and progression and allow a disease profile to be constructed which can be validated and modified following future discoveries [12].

The purpose of this paper is to determine POAG biological and network information to further understand the mechanisms associated with POAG. Network analysis can introduce some candidate biomarkers for the disease.

MATERIALS AND METHODS

Gene/protein expression data

Gene/protein IDs were extracted from previous studies and 474 proteins were selected from the papers [2, 6, 8, 9, 13-21]. The proteins uniprot IDs were identified from uniprot database (http://uniprot.org).

Classification and pathway analysis methods

The 474 proteins were then functionally classified, using PANTHER (protein annotation through evolutionary relationship) classification system (http://pantherdb.org). PANTHER is a comprehensive system that combines gene function, ontology and pathways [22-24]. By running overrepresentation test, we compared our data list with a reference list of Homo sapience proteins in the PANTHER database. Over- and underrepresentation of ontology categories were determined statistically with Binomial distribution test.

Protein-protein interaction analysis methods

Network analysis on proteins was performed using STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) server (http://string-db.org) [25] and Cytoscape software [26]. STRING is a database of known and predicted protein interactions. The interactions include direct (physical) and indirect (functional) associations. A network from the gene list was constructed by STRING. Disconnected nodes were eliminated. This network was then analyzed using Cytoscape 3.0.2 software. Cytocluster application was used to cluster the network into subnetworks. The clustering was done using HC-PIN nonoverlapping algorithm and strong option with complex size threshold of 3.

RESULTS

Pathway analysis was performed using PANTHER classification system. Overrepresentation test was run which compares our data list to a reference list. Here, we used the default Homo sapiens reference list of the PANTHER database. Overrepresentation means we observe more genes than expected based on the reference list. Conversely, underrepresentation refers to less observed genes than expected from the reference list. Binomial distribution test is used for the comparison.

The gene ontology including biological process, molecular function and cellular component was performed on the list. Bonferroni correction is used on the results. Table 1 shows the biological processes related to POAG. According to the table, developmental process, response to stimulus and cellular process were the most significant with least p-values. Table2 shows the molecular functions. Receptor binding, extracellular matrix structural constituent and protein binding are the most important molecular functions related to POAG. Table 3 shows the cellular components found from the protein list. Extracellular region and extracellular matrix have the least p-values in this category.

Protein classes were also identified by PANTHER (table 4). Extracellular matrix proteins, signaling molecules and extracellular matrix structural proteins were the most important ones. Pathway analysis was done both by overrepresentation test and functional classification.

Table 1. Top 10 biological processes related to POAG.

GO Biological Process	Homo sapiens (REF) #	#	Expected	+/_	▲ P-value
Unclassified	9422	68	143.46	-	**4.00E-16**
developmental process	2846	103	43.33	+	**2.46E-15**
response to stimulus	1671	73	25.44	+	**4.57E-14**
cellular process	5952	156	90.63	+	**2.94E-12**
immune system process	1733	70	26.39	+	**9.96E-12**
cell death	697	39	10.61	+	**8.26E-10**
apoptotic process	697	39	10.61	+	**8.26E-10**
Death	699	39	10.64	+	**8.99E-10**
macrophage activation	289	21	4.40	+	**1.14E-06**
cell communication	3221	89	49.04	+	**1.73E-06**
cell adhesion	890	38	13.55	+	**2.68E-06**

+/- shows over- and under representations. Second and third column contains the number of genes in reference list and our list respectively. P-value threshold is considered 0.05

Table 2. Top 10 molecular functions related to POAG

Molecular Function	Homo sapiens (REF) #	#	Expected	+/_	▲ P-value
Unclassified	10605	93	161.48	_	**2.27E-12**
receptor binding	1017	40	15.49	+	**8.83E-06**
extracellular matrix structural constituent	91	11	1.39	+	**3.56E-05**
protein binding	2855	77	43.47	+	**5.81E-05**
cytokine activity	188	14	2.86	+	**2.65E-04**
peptidase activity	747	30	11.37	+	**2.98E-04**
structural molecule activity	1261	42	19.20	+	**3.15E-04**
hydrolase activity	2332	64	35.51	+	**4.13E-04**
cytokine receptor binding	64	8	0.97	+	**1.26E-03**
receptor activity	1576	47	24.00	+	**1.40E-03**
peptidase inhibitor activity	193	12	2.94	+	**8.29E-03**

Table 3. Most significant cellular components related to POAG

Cellular Component	Homo sapiens (REF) #	#	Expected	+/_	▲ P value
extracellular region	533	32	8.12	+	**4.00E-09**
extracellular matrix	376	25	5.73	+	**6.91E-08**
cell part	1592	40	24.24	+	**6.94E-02**
Organelle	1051	28	16.00	+	**1.67E-01**
Intracellular	1434	35	21.83	+	**2.18E-01**
Cytoskeleton	786	22	11.97	+	**2.57E-01**
intermediate filament cytoskeleton	73	5	1.11	+	**2.84E-01**

Table 4. Top 10 protein classes related to POAG

PANTHER protein class	Homo sapiens (REF) #	#	expected	+/_	▲ P-value
extracellular matrix protein	466	28	7.10	+	**2.24E-07**
signaling molecule	1048	40	15.96	+	**2.20E-05**
extracellular matrix structural protein	93	11	1.42	+	**5.01E-05**
defense/immunity protein	572	25	8.71	+	**6.03E-04**
Cytokine	178	13	2.71	+	**8.71E-04**
complement component	81	9	1.23	+	**1.00E-03**
Surfactant	44	7	0.67	+	**1.15E-03**
Hydrolase	1654	48	25.18	+	**2.55E-03**
interleukin superfamily	36	6	0.55	+	**4.09E-03**
Receptor	1597	46	24.32	+	**4.73E-03**

Table 5. Most significant pathways derived from overrepresentation test

Pathways	Homo sapiens (REF) #	#	expected	+/_	P-value
apoptosis signaling pathway	113	19	1.72	+	**5.54E-12**
huntington disease	142	18	2.16	+	**2.66E-09**
interleukin signaling pathway	95	10	1.45	+	**4.79E-04**
integrin signaling pathway	175	13	2.66	+	**7.15E-04**
FAS signaling pathway	31	4	0.47	+	**2.48E-01**
insulin/IGF pathway-mitogen activated protein kinase/MAP kinase cascade	32	4	0.49	+	**2.79E-01**
parkinson disease	88	6	1.34	+	**4.46E-01**
Angiogenesis	152	8	2.31	+	**4.56E-01**

Table 5 shows the results from over-representation test. It is shown in table5 that the top 5 significant pathways are apoptosis signaling pathway, Huntington disease, interleukin signaling pathway, integrin signaling pathway and Fas signaling pathway. In tables 1 to 5, second and third columns contain the number of genes in reference list and our list respectively. Plus and minus signs in the fourth column show over- and under-representations. P-value threshold is considered 0.05. Pathways from functional classification by PANTHER are shown in figure 1.

The pie chart shows the pathways related to our protein list. According to the chart, apoptosis signaling pathway, Huntington disease, integrin signaling pathway and gonadotropin releasing hormone receptor pathway are the most involved pathways in POAG patients. Figure 2 shows the overrepresentation diagram of the POAG pathways.

Figure 1. Pie chart of the pathways from functional classification analysis by PANTHER. Most important pathways are shown

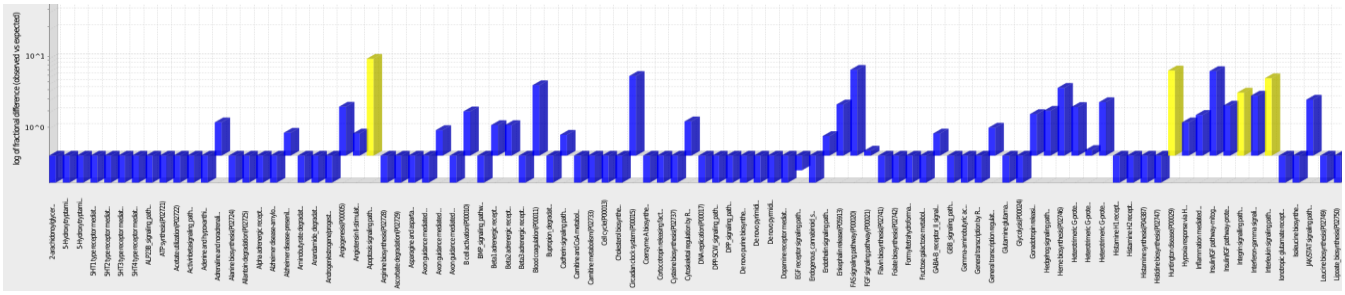


Figure 2. Pathway over representation diagram. Yellow bars show apoptosis signaling pathway, Huntington disease pathway, integrin signaling pathway and interleukin signaling pathway. Y axis is the log of fractional difference (observed vs expected)

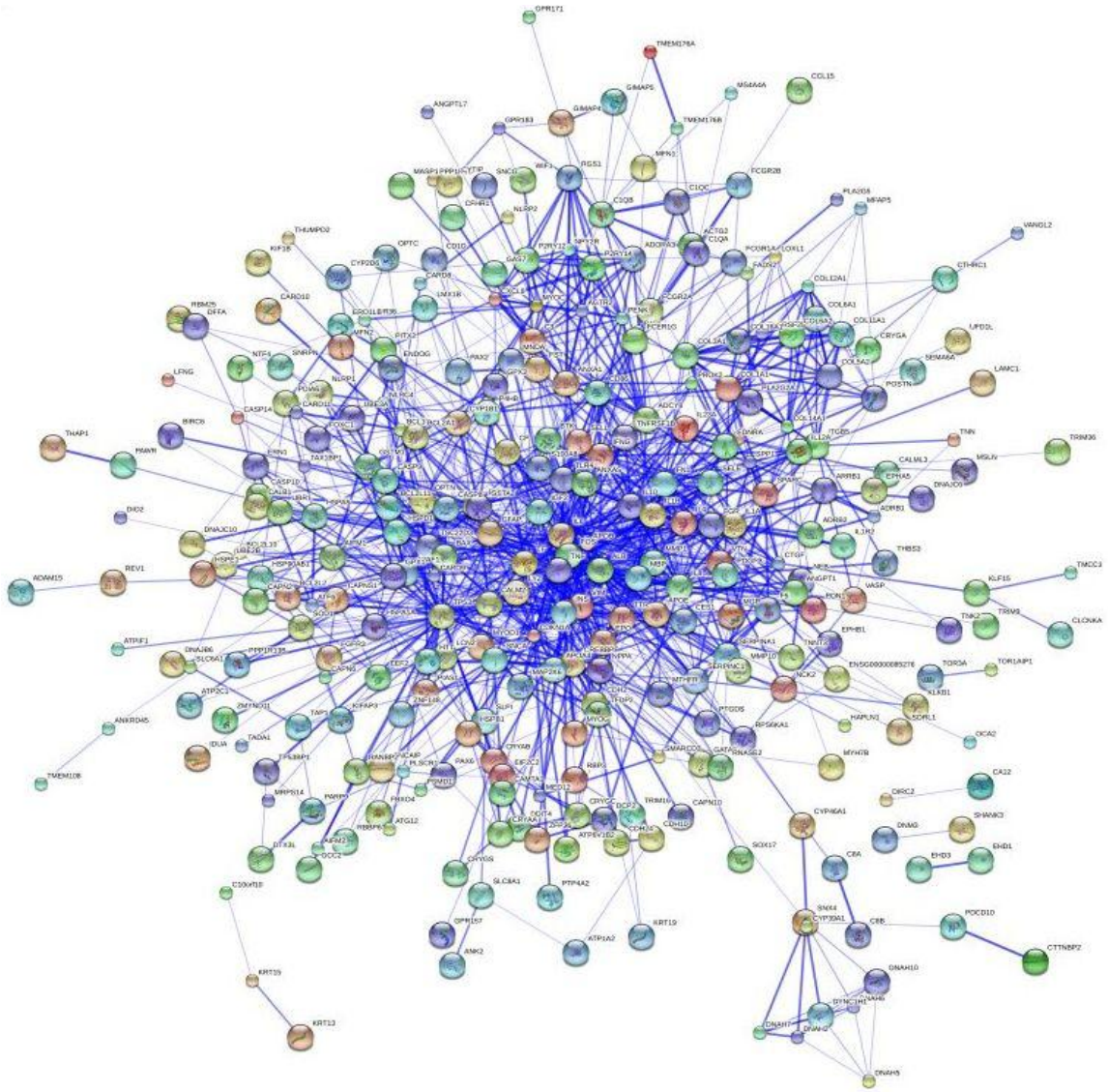
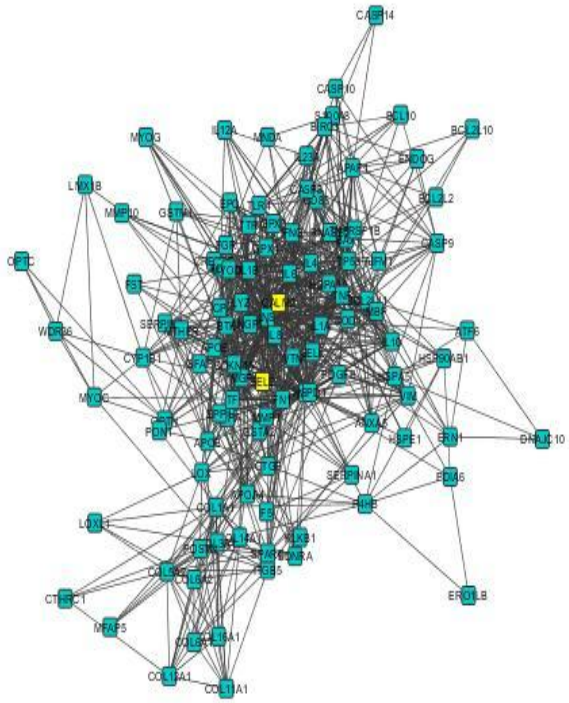


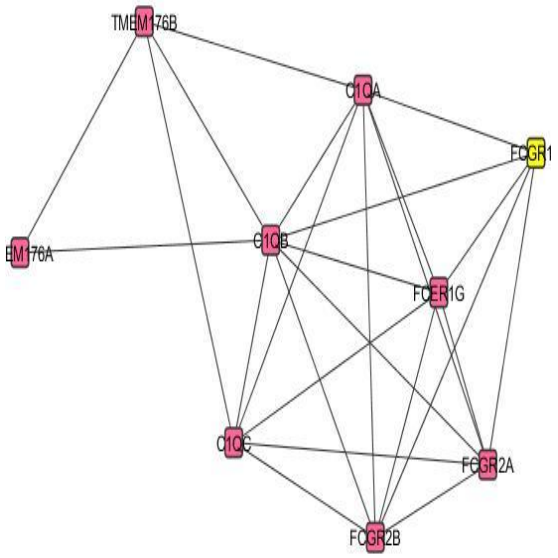
Figure 3. POAG network created by STRING server. Disconnected nodes are eliminated.

The network analysis was done using STRING server. The created network contained 312 nodes and 1348 edges after removing the disconnected nodes. Figure 3, shows the network created by string server. The resulting network was then analyzed by CytoScape software. We used CytoCluster plugin to cluster our network into smaller subnetworks. Nine subnetworks were resulted. We used these clusters to find candidate biomarkers for POAG disease. Subnetworks were ranked according to their size. In each cluster, seed nodes were identified. Seed nodes are the highest weighted nodes in a graph. In the human protein-protein network, each node (protein) with corresponding gene expression value is regarded as "seed node." For a seed node i, this node and its neighbors j within the shortest distance k, form a connected subnetwork with n nodes [27].

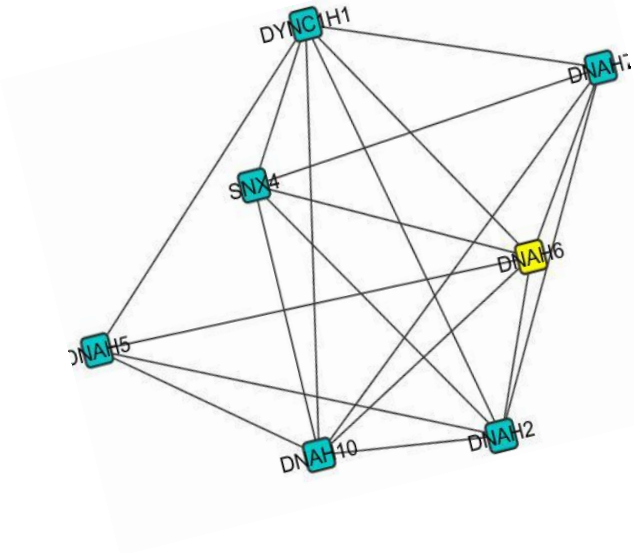
Here, we had seed nodes in clusters 1, 3, 4 and 6 as can be seen in figure 4.



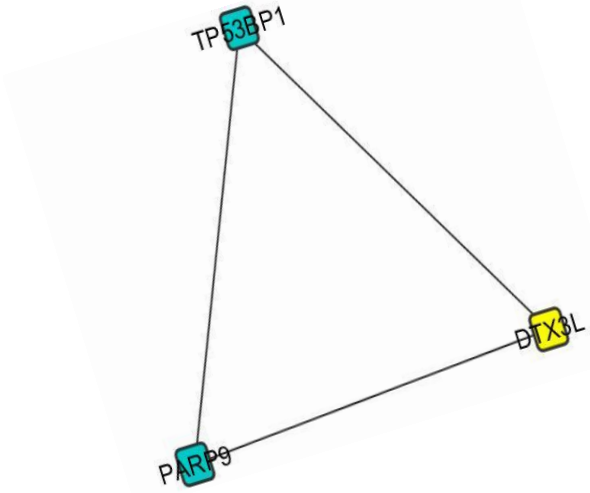
(a)



(b)



(c)



(d)

Figure 4. Clusters 1 (a), 3 (b), 4 (c) and 6 (d) are shown which had seed nodes. Seed nodes are colored yellow

DISCUSSION

As mentioned, PANTHER classification system was used to classify the protein list of primary open angle glaucoma. Pathway analysis was done by overrepresentation test. As shown in table 5, the most significant pathway in our classification related to POAG is apoptosis signaling pathway. Apoptosis is critical for the normal development and function of multicellular organisms. Abnormalities in cell death control can contribute to a variety of diseases [28]. Evidence indicates that apoptosis may be the final common pathway for retinal ganglion cell (RGC) death in glaucoma. Apoptotic RGC death has been demonstrated in animal models of glaucoma [29, 30] . Markers of apoptosis have also been observed in the human glaucomatous retina [31-33].

The second most significant pathway in POAG was the Huntington disease. Anholt and carbone (2013) proposed that the accumulation of overexpressed or misfolded proteins such as myocilin associated with age-dependent onset of glaucoma, leads to ER stress when the unfolded protein response (UPR) pathway becomes unable of removing these proteins via the proteasome. The UPR is a conserved cellular pathway among all eukaryotes that protects cells against the accumulation and aggregation of misfolded proteins during protein synthesis. When the extent of aggregation of misfolded or damaged proteins exceeds the capacity of the UPR, it is no longer able to protect the ER from stress and this results in activation of apoptosis. This is the same process as in other neurodegenerative diseases such as Alzheimer, Parkinson and Huntington. Thus POAG can be placed in the same context as other adult late-onset neurodegenerative diseases [34].

The third most significant pathway in our classification was the interleukin signaling pathway. Several studies show the involvement of interleukins in the pathogenesis of glaucoma [35-37]. Johnson et al., showed that interleukin-6-type cytokine signaling is implicated by gene expression responses in early optic nerve head injury in rat glaucoma [35].

Fini et al., studied the role of interleukin-1-regulated signaling pathways in normal and glaucomatous trabecular meshwork cells and showed that glaucomatous TM cells are unable to adapt to changes in extracellular levels of interleukin-1 [37].

The integrin signaling pathway is the other most important pathway in the classification. Integrins are crucially important because they are the main receptor proteins that cells use to both bind to and respond to the extracellular matrix. They are heterodimers and function as transmembrane linkers between the extracellular matrix and actin cytoskeleton. A cell can regulate the adhesive activity of its integrins from within. Integrins also function as signal transducers, activating various intracellular signaling pathways when activated by matrix binding. Integrins and conventional signaling receptors often cooperate to promote cell growth, cell survival, and cell proliferation [38].

Research efforts have been put in the last decade to elucidate cells response and ECM remodeling processes in the trabecular meshwork and the optic nerve head in glaucoma and integrins have been identified as very important participants in this process. In vitro and in vivo data strongly indicate that integrin-mediated signaling events can modulate the organization of actin cytoskeleton in TM cells and are associated with astrocytes migration and microglia activation in the optic nerve head of glaucoma patients. As a result, increase in resistance in the TM outflow pathways and ECM remodeling of the optic nerve head occur. While increase in outflow resistance causes an increase in IOP, and the remodeling of the optic nerve head accompanies the optic nerve axons damage. Increase in IOP further adds mechanical stress and strain to optic nerve axons and accelerates axon damages. Integrins appear to be ideal candidates for translating physical stress and strain into cellular responses known to occur in glaucomatous optic neuropathy [39].

Another significant signaling pathway related to POAG was the Fas signaling pathway. The Fas receptor (CD95) mediates apoptotic signaling by Fas-ligand expressed on the surface of other cells. The Fas-FasL interaction plays an important role in the immune system and lack of this system leads to autoimmunity, indicating that Fas-mediated apoptosis removes self-reactive lymphocytes. Fas signaling is also involved in immune surveillance to remove transformed cells and virus infected cells. Binding of Fas to FasL on another cell activates apoptotic signaling through a cytoplasmic domain termed the death domain that interacts with signaling adaptors including FAF, FADD

and DAX to activate the caspase proteolytic cascade. Fas signaling pathway may involve in glaucoma mechanism. Agrawal et al. (1999), showed that human TM cells express Fas receptor and undergo apoptosis after activation of the receptor by monoclonal IgM. Razeghinejad and Kamali (2007), investigated aqueous humor levels of soluble Fas in glaucomatous patients and hypothesized that lower levels of S-Fas may provide proper microenvironment for increased apoptosis of trabecular meshwork cells in primary open angle glaucoma [40].

The network analysis by CytoScape software developed 9 clusters of proteins which are highly condensed in the POAG network. In each cluster, seed nodes were determined presented in figure 4. The seed nodes which existed in our data set included SELE, CALM2 and DNAH6. These seed nodes can serve as candidate biomarkers for POAG disease. SELE is found in cytokine-stimulated endothelial cells and is thought to be responsible for the accumulation of blood leukocytes at sites of inflammation by mediating the adhesion of cells to the vascular lining. It is a part of the selectin family of cell adhesion molecules. Diseases associated with SELE include vasculitis, and leukostasis. GO annotations related to this gene include transmembrane signaling receptor activity and sialic acid binding. CALM2 (calmodulin 2 (phosphorylase kinase, delta)) mediates the control of a large number of enzymes, ion channels, aquaporins and other proteins by Ca(2+). Among the enzymes to be simuldated by the calmodulin-Ca(2+) complex are a number of protein kinases and phosphatase. DNAH6 (dynein, axonemal, heavy chain 6) is the other seed protein. GO annotations related to this protein include microtubule motor activity and ATPase activity. Dyneins are microtubule-associated motor protein complexes composed of several heavy, light, and intermediate chains. Two major classes of dyneins, axonemal and cytoplasmic, have been identified. DNAH6 is an axonemal dynein heavy chain (DHC).

CONCLUSION

In this study, using network and pathway analysis, GO biological process, protein class and cellular component, relationship between genes in POAG was identified that can be useful in identifying biomarker candidates and drug targets and to investigate possible disease mechanisms. Network analysis of POAG proteins, can help diagnose and predict treatments for the disease. From network analysis, 9 clusters were identified and 3 seed nodes were determined to be in our protein list which included SELE, CALM2 and DNAH6 which can serve as candidate biomarkers for POAG disease but for detailed evaluation of these proteins, more investigations are needed.

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