Gene Expression Profile in Human Trabecular Meshwork From Patients With Primary Open-Angle Glaucoma

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PURPOSE. To identify the specific genes in human trabecular meshwork (TM) related to POAG.

METHODS. Primary open-angle glaucoma TM specimens were obtained from routine trabeculectomy surgery. Nonglaucomatous control TM specimens were dissected from donor eyes using the same approach as a standard trabeculectomy. All cases were screened for myocilin (*MYOC*) mutations. Total RNA was extracted, labeled, and hybridized to Illumina HumanWG-6 BeadChips. Expression data were normalized and analyzed using the R package *limma* in Bioconductor. Pathway analyses were performed using DAVID Bioinformatics Resources.

RESULTS. Our study included surgical TM specimens from 15 cases and 13 controls. One case was identified with a heterozygous *Q368X MYOC* mutation. If TMs were available from both eyes in an individual, the expression data were combined for analysis. The following three comparisons were performed for differential analyses: (1) *MYOC* POAG case versus 14 non-*MYOC* POAG cases, (2) *MYOC* POAG case versus 13 controls, and (3) 14 non-*MYOC* POAG cases versus 13 controls. Limited by one *MYOC* case in comparisons 1 and 2, expression changes were reported comparing the fold changes but without *P* values. Comparison 3 identified 483 genes, including 36 components of TM exosomes. Gene ontology analysis identified several enriched functional clusters, including cell adhesion, extracellular matrix, and secretion.

CONCLUSIONS. This is the largest TM expression study of POAG cases and controls performed to date and represents the first report of TM expression in a patient having POAG with a *Q368X MYOC* mutation. Our data suggest the potential role of endocytic and exosome pathways in the pathogenesis of POAG.

Keywords: gene expression, myocilin, exosome, trabecular meshwork, *PAX6*

Glaucoma is the second leading cause of blindness worldwide, affecting more than 60 million people.^{1,2} Primary open-angle glaucoma (Online Mendelian Inheritance in Man 137760) is the most common form of glaucoma.^{1,3} It is defined as progressive loss of retinal ganglion cells and visual field without any identifiable cause. Recognized risk factors for POAG include elevated IOP, positive family history of glaucoma, refractive error, and African or Hispanic ancestry.^{4,5} Primary open-angle glaucoma is considered a complex genetic disorder with a strong hereditary component because of the increased 7-fold to 10-fold higher risk in first-degree relatives of affected individuals.^{6–8}

A number of genomic regions have been linked to POAG in family-based linkage analysis.^{4,5,9–11} Although it remains a major challenge to identify mutations in most of the reported POAG loci,^{5,8,12} mutations in myocilin (*MYOC*),¹³ optineurin,¹⁴ and the *WDR36* gene¹⁵ have been associated with glaucoma. It appears that *MYOC* mutations do not cause glaucoma via haploinsufficiency or overexpression.^{4,16} Animal models in which *MYOC* expression is either reduced or overexpressed do not seem to produce glaucoma. It has recently been reported

that *MYOC* release into the aqueous humor is associated with the shedding of small vesicles called exosomes.^{17–19} Exosomes contain ligands and RNAs involved in autocrine and paracrine signaling. These ligands may have a role in the homeostasis of the trabecular meshwork (TM). More recently, genome-wide association studies have successfully identified genomic regions associated with POAG risk, including the *CAV1/CAV2* gene,²⁰ *TMCO1* gene,²¹ *CDKN2B-AS1* gene,^{21,22} *SIX1/SIX6* gene,²² and a chromosome 8q22 locus.²² In addition, DNA copy number variants containing the *TBK1* gene^{23,24} and the *GALC* gene²⁵ have been associated with POAG. The role that these genes have in human POAG remains to be established. It is important to characterize these identified genes using additional information such as gene expression in human ocular tissues.

Gene expression, especially differential expression data obtained from diseased and control tissues, can provide key information that can help determine which genes may be involved in pathologic processes.^{26,27} A number of studies^{28–41} have examined gene expression profiles in TM tissue or cells and provided critical information about POAG. However, it remains unclear how the expression profile is affected in TM

TABLE 1. Clinical Characteristics of Patients Having POAG With Surgical TM Samples

Case No.	Sex	Ethnicity	Age at Diagnosis, y	MYOC Mutation	Eyes Assayed, <i>n</i>
1	M	C	72	No	1
2	M	AA	67	No	1
3	M	C	55	No	1
4	F	C	86	No	1
5	F	C	73	No	1
6	M	C	65	No	1
7	M	AA	61	No	1
8	M	AA	40	No	1
9	F	AA	82	No	1
10	F	AA	69	No	1
11	F	C	60	No	1
12	F	C	61	No	1
13	F	C	79	No	1
14	F	C	54	No	1
15 (MYOC case)	F	C	71	Q368X	2

AA, African American; C, Caucasian.

tissue from patients with POAG compared with that from healthy control subjects. This study was conducted to compare gene expression in TMs from patients with POAG versus that in TMs from nonglaucomatous controls to identify genes that have a role in glaucoma pathogenesis, as well as to provide additional information that can be used to prioritize candidate genes for studies in human genetics.

METHODS

Procurement of Tissue and RNA Extraction

This research conforms to all tenets of the Declaration of Helsinki. The study was approved by the Institutional Review Board at Duke University Medical Center. Written informed consent was obtained from all participating individuals.

Trabecular meshwork from patients with POAG was obtained during routine trabeculectomy surgery performed by one surgeon (RRA) for uncontrolled glaucoma. Primary open-angle glaucoma case inclusion criteria were the following: (1) age at POAG diagnosis older than 35 years, (2) glaucomatous optic nerve damage with associated visual field damage in the eye from which TM tissue was obtained, and (3) no evidence of secondary glaucoma (i.e., history of ocular trauma, exfoliation syndrome, or other condition associated with glaucoma). All control TM tissue was obtained from donor eyes without a clinical history of glaucoma or glaucoma-associated condition, elevated IOP, or the use of glaucoma medications or steroids. The method used to obtain TM specimens from donor eyes was the same as that used for trabeculectomy and was performed by the surgeon (RRA) who obtained the surgical specimens.⁴² All POAG cases were screened for mutations in myocilin using the standard PCR-based Sanger sequencing method.^{43,44}

All POAG TM specimens obtained in the operating room were immediately placed in RNeasy Lysis Buffer (Qiagen, Crawfordsville, IN). Surgical TM samples were incubated for 24 hours at room temperature to facilitate RNeasy Lysis Buffer (Qiagen, Crawfordsville, IN) permeation and then frozen at -80°C . Similarly, on receipt by the North Carolina Eye Bank (within 7 hours postmortem), donor eyes were cut open several clock hours 4 mm posterior to the limbus and immersed in RNeasy Lysis Buffer (Qiagen, Crawfordsville, IN) to preserve the RNA in the donor eyes. The TM specimens were excised

from donor eyes within 72 hours of receipt by the Center for Human Genetics at Duke University Medical Center and then stored at -80°C until RNA extraction. Total RNA from TM tissue was extracted using the RiboPure Kit from Ambion, Inc., following the manufacturer's protocol. The quantity of RNA yield was determined via NanoDrop (Thermo Fisher Scientific Inc., Wilmington, DE), and the quality was assessed using the RNA 6000 Pico Kit (Agilent Technologies, Inc., Santa Clara, CA).

Gene Expression Analysis

The extracted total RNA from TMs was amplified using the MessageAmp II aRNA Amplification Kit (Ambion, Inc.). The amplified RNA was quantitated, and 100 ng of all samples was labeled using the TotalPrep RNA Amplification Kit (Illumina, San Diego, CA). The resultant labeled RNA was quantitated, and 1.5 μg was hybridized to Illumina HumanWG-6 BeadChips, following the manufacturer's protocol. The chips were scanned using the Illumina iScan system. Probe and intensity data were exported from Illumina's proprietary software BeadStudio. The data included a detection *P* value, which was a measure of the probability of seeing a specific probe intensity level by chance. Probes with detection $P \geq 0.05$ were excluded from analysis. Data from probes expressed in fewer than half of POAG case or control TM samples were excluded from further analysis. The RNA expression data were imported into the R package *limma* in Bioconductor (<http://www.bioconductor.org> in the public domain).⁴⁵ All raw data were log2 transformed, and the quantile normalization function in *limma* was used to normalize the intensity data between samples.

We performed the following three grouped comparisons to identify patterns of differential gene expression: (1) MYOC POAG case versus 14 non-MYOC POAG cases, (2) MYOC POAG case versus 13 controls, and (3) 14 non-MYOC POAG cases versus 13 controls. If TMs were available from both eyes in an individual, the RNA expression data were averaged. Additional analysis was conducted to determine whether postmortem delay before preservation in RNeasy Lysis Buffer (Qiagen, Crawfordsville, IN) affected RNA extraction. *P* values from these comparisons were corrected for multiple testing using the false discovery rate method.⁴⁶ Genes with corrected $P < 0.05$ were considered to be differentially expressed. Because the annotations for the Illumina gene expression chip are outdated, all differentially expressed probes were reannotated using BLAT (BLAST-like Alignment Tool; available in the public domain at <http://genome.ucsc.edu/cgi-bin/hgBlat?>) to map the probe sequences to human genome build 37. Because of the limited MYOC POAG case sample size (two eyes in one patient) in comparisons 1 and 2, we report the results by directly comparing the expression using the fold changes instead of *P* values.

For comparison 3, based on the differentially expressed genes between cases and controls, we used the Database for Annotation, Visualization, and Integrated Discovery (DAVID) Bioinformatics Resources (<http://www.david.niaid.nih.gov> in the public domain) to search for overrepresented or under-represented gene ontology terms, protein families, and biological pathways in the Kyoto Encyclopedia of Genes and Genomes (KEGG; <http://www.genome.ad.jp/> in the public domain).^{47,48} The locations of differentially expressed genes were mapped to the genome, and their locations were compared with published genomic regions linked to POAG. The expression data for both cases and controls have been deposited into the National Center for Biotechnology Information Gene Expression Omnibus (<http://www.ncbi.nlm.nih>

TABLE 2. Clinical Characteristics of Nonglaucomatous Control Subjects

Control No.	Sex	Ethnicity	Age at Death, y	Postmortem Delay, h	Eyes Assayed, <i>n</i>	Cause of Death
1	F	C	67	3:43	1	Sepsis, small cell lung cancer
2	M	C	64	5:38	1	Sepsis, metastatic lung cancer
3	F	AA	55	2:25	1	Breast cancer
4	F	C	94	3:17	1	Alzheimer's disease
5	M	C	64	3:24	1	Pneumonia, pulmonary fibrosis
6	M	AA	49	4:07	1	Renal cancer
7	F	C	72	4:53	1	Pancreatic cancer
8	F	C	48	5:24	2	Cerebral vascular accident, leukemia
9	M	C	67	5:42	2	Colon cancer
10	F	C	53	4:18	2	Sepsis, cerebral vascular accident
11	M	C	59	5:04	2	Pulmonary edema, cardiomyopathy
12	F	C	59	4:50	2	Chronic obstructive pulmonary disease
13	F	AA	61	6:24	2	Cerebral vascular accident, intracerebral hemorrhage

AA, African American; C, Caucasian.

gov/geo/ in the public domain) database with the accession number GSE27276.

RESULTS

Clinical Features of Glaucoma Cases and Nonglaucomatous Controls

Our study included 35 human TMs from 15 patients with POAG and 13 healthy controls (as summarized in Table 1 for POAG cases and Table 2 for controls), including TMs in both left and right eyes from six controls and one POAG case. The clinical features of these 15 patients with POAG (six men and nine women) are listed in Table 1. There were five African American POAG cases and 10 Caucasian POAG cases. Their ages at diagnosis ranged from 40 to 86 years. All patients with POAG had bilateral disease. Trabecular meshwork was obtained in all eyes when possible. Only one patient had trabeculectomy with TM collection in both eyes; this patient was later determined to have *Q368X MYOC*-related POAG (designated hereafter as the *MYOC* POAG case). Both TM specimens were included for analysis in this case. The RNA expression values were averaged for the expression analysis. Trabecular meshworks were obtained from 13 nonglaucomatous donors (five men and eight women) (Table 2). There were three African American and 10 Caucasian donors. Postmortem delays between death and tissue storage in RNALater (Ambion, Inc.) ranged from 2 hours to approximately 7 hours (average time, 4 hours and 33 minutes). This relatively short interval from death to RNA preservation enabled us to obtain high-quality total RNA with minimal RNA degradation. Our analysis

on postmortem delay and gene expression indicated that postmortem delay did not affect the gene expression significantly. The age at death for eye donors ranged from 48 to 94 years. The cause of death varied among individuals, but cancer was most common (in seven of 13 individuals).

Differential Expression Analysis

The differential expression analysis was performed using three separate comparisons. The included the following: (1) *MYOC* POAG case versus 14 non-*MYOC* POAG cases, (2) *MYOC* POAG case versus 13 controls, and (3) 14 non-*MYOC* POAG cases versus 13 controls.

Comparison 1 was designed to identify expression differences specific to myocilin mutations. Because of the limited sample size of one POAG case with myocilin mutation, we only give the top nine genes in the fold changes with the largest effect (at least 3-fold upregulation or less than −3 fold downregulation) in Table 3. Fifty-five genes were found to have expression changes of more than 2-fold, either upregulated or downregulated. Although myocilin, optineurin, and *CYP11B1* were highly expressed in all individuals, their expression levels were not significantly different between the *MYOC* POAG case and the non-*MYOC* POAG cases. Other known glaucoma genes (*LOXLI*, *WDR36*, *CAV1/CAV2*, *SIX6/SIX1*, *TBK1*, and *CDKN2B*) were expressed at relatively low levels.

Comparison 2 examined the expression differences between the single *MYOC* POAG case and the 13 control TMs. Because of the limitation of only one *MYOC* POAG case, we also reported expression changes with the fold changes only

TABLE 3. Genes That Are Differentially Expressed in the *MYOC* POAG Case Compared With 14 Non-*MYOC* POAG Cases

Gene Symbol	Description	Probe Identification	Fold Change
<i>RPS4Y</i>	Ribosomal protein S4, Ylinked	GI_17981706	−4.7
<i>ALDH3A1</i>	Aldehyde dehydrogenase 3 family, member A1	GI_22907048	−4.9
<i>HLA-DRB5</i>	Major histocompatibility complex, class II, DR beta 5	GI_26665892	3.0
<i>MARCH6</i>	Membrane-associated ring finger (C3HC4) 6, E3 ubiquitin protein ligase	GI_27479011	−3.3
<i>BGN</i>	Biglycan	GI_34304351	4.2
<i>PENK</i>	Proenkephalin	GI_40254835	4.5
<i>CHI3L1</i>	Chitinase 3-like 1 (cartilage glycoprotein-39)	GI_4557017	3.1
<i>FCER1G</i>	Fc fragment of IgE, high affinity I, receptor for gamma polypeptide	GI_4758343	3.0
<i>MIA</i>	Melanoma inhibitory activity	GI_5729924	3.1

Because of the limited sample size of the *MYOC* case (two eyes in one patient), we only reported the relative fold changes with large effect (at least 3-fold upregulation or downregulation).

TABLE 4. Genes That Are Differentially Expressed in the MYOC POAG Case Compared With 13 Control Subjects

Gene Symbol	Description	Probe Identification	Fold Change
<i>PENK</i>	Proenkephalin	GI_40254835	7.8
<i>ECRG4</i>	Esophageal cancer related gene 4 protein	GI_14165275	5.5
<i>MGP</i> *	Matrix Gla protein	GI_4505178	8.5
<i>HLA-DPA1</i>	Major histocompatibility complex, class II, DP alpha 1	GI_24797073	5.7
<i>TAGLN</i> *	Sapiens transgelin	GI_12621918	5.3
<i>KRT19</i> *	Keratin 19	GI_40217850	−28.3
<i>FCGBP</i> *	Fc fragment of IgG binding protein	GI_4503680	5.8
<i>BGN</i> *	Biglycan	GI_34304351	6.7
<i>BCAS1</i>	Breast carcinoma amplified sequence 1	GI_4502372	−6.8
<i>TNNT3</i>	Troponin T3, skeletal, fast	GI_5803202	−7.0
<i>SLPI</i> *	Secretory leukocyte protease inhibitor (antileukoprotease)	GI_15834622	−7.1
<i>CALML3</i> *	Calmodulin-like 3 (CALML3)	GI_36031099	−14.5
<i>GUCY1A3</i>	Guanylate cyclase 1, soluble, alpha 3	GI_4504212	5.1
<i>ALDH3A1</i>	Aldehyde dehydrogenase 3 family, member A1	GI_22907048	−10.7
<i>PAX6</i>	Paired box gene 6 (aniridia, keratitis)	GI_4505614	−7.4
<i>KRT13</i> *	Keratin 13	GI_24234693	−7.6
<i>LCN2</i> *	Lipocalin 2 (oncogene 24p3)	GI_38455401	−7.0
<i>S100A9</i> *	S100 calcium binding protein A9 (calgranulin B)	GI_9845520	−8.2
<i>TGM1</i> *	Transglutaminase 1 (K polypeptide epidermal type I, protein-glutamine-gamma-glutamyltransferase)	GI_4507474	−5.0
<i>IMUP</i>	Immortalization-upregulated protein	GI_27485203	−8.2
<i>KRT15</i> *	Keratin 15	GI_24430189	−11.0
<i>IMUP</i>	Immortalization-upregulated protein	GI_41281602	−5.6
<i>S100A8</i> *	S100 calcium binding protein A8 (calgranulin A)	GI_21614543	−8.4
<i>KRT14</i> *	Keratin 14 (epidermolysis bullosa simplex, Dowling-Meara, Koebner)	GI_15431309	−5.4

Because of the limited sample size of the MYOC case (two eyes in one patient), we only reported the top genes with the largest relative fold changes (at least 5-fold upregulation or less than −5 fold downregulation) without *P* values.

* These genes have been reported as exosome components according to the database from ExoCarta (www.exocarta.org in the public domain).

instead of *P* values. There are approximately 102 genes with expression changes (upregulation or downregulation) of at least 3-fold and approximately 400 genes with expression changes of at least 2-fold. We list the top 24 genes with the largest effect of at least 5-fold (upregulated or downregulated) in Table 4. Of these 24 genes, 14 have been identified as exosome components, which are listed with an asterisk in Table 4. As we observed in comparison 1, the expression of relatively highly expressed POAG genes (myocilin, optineurin, and *CYP11B1*) was not significantly different between the MYOC POAG case and the nonglaucomatous controls. The lack of myocilin expression change between the MYOC POAG case and the controls is consistent with earlier findings that myocilin mutations affect protein function but not RNA expression.¹⁶

Comparison 3 analyzed the difference in expression between non-MYOC POAG cases and nonglaucomatous controls. This analysis identified 483 genes that were differentially expressed, including 387 upregulated and 96 downregulated genes. Table 5 lists the 30 genes with the most significant differential expression. Notably, *PAX6* (paired box gene 6), which has been implicated in developmental forms of glaucoma, is significantly downregulated (>6-fold) in non-MYOC POAG cases. A TM marker, *MGP* (matrix GLA protein), is significantly upregulated in the TM of patients with POAG. A complete list of differentially expressed genes in comparison 3 is included in Supplementary Table S1. Thirty-six differentially expressed genes are reported as constituents of exosomes derived from TM cells¹⁹ (Supplementary Table S2). Gene ontology analysis using DAVID Bioinformatics Resources identified a number of enriched functional clusters, including signal peptide (adjusted *P* value, 1.0E-13), secreted (adjusted *P* value, 1.4E-11), glycoprotein (adjusted *P* value, 1.9E-11), extracellular region (adjusted *P* value, 1.4E-8), cell adhesion (adjusted *P* value, 1.1E-5), extracellular matrix (adjusted *P*

value, 2.7E-8), phosphate transport (adjusted *P* value, 1.2E-4), and calcium ion binding (adjusted *P* value, 1.1E-5). Several of these enriched clusters overlap those identified between the MYOC POAG case and controls.

In comparisons 2 and 3, a number of genes overlapped such as *CALML3*, *MGP*, *LCN2*, *PAX6*, *TNNT3*, *KRT13*, and *KRT19*. These genes shared similar differential expression (i.e., the direction of expression change) between the MYOC POAG case and the non-MYOC POAG cases compared with controls. For example, *MGP* expression is upregulated in the TMs of both MYOC and non-MYOC POAG cases, while *PAX6* is downregulated in the TMs from both groups. This similar differential expression pattern may suggest a similarity between myocilin-associated POAG and nonmyocilin POAG.

A comparison of the genomic location of differentially expressed genes and published linkage regions for POAG was made. Thirty-three differentially expressed genes are located within POAG linkage regions (Supplementary Table S3). No genes linked or associated with POAG or POAG-related traits were differentially expressed in TM, including: myocilin, optineurin, *WDR36*, *CYP11B1*, *CAV1/CAV2*, *CDKN2B*, *TMC01*, *SIX1/SIX6*, *ATOH7*, *TBK1*, and *GALC*.

DISCUSSION

This is the largest comparison of gene expression in the TM of patients with POAG and controls reported to date. In addition, it is the first report of gene expression in tissue obtained from a patient having POAG with the most commonly reported MYOC mutation (*Q368X*). In the comparison of the MYOC TM sample and non-MYOC tissue, we identified a small number of genes with large effect on expression. In the comparison of the MYOC POAG case versus controls, a number of genes with relative large expression changes shared similar differential

TABLE 5. Top 30 Genes Among More Than 480 Genes Differentially Expressed Between 14 Non-MYOC POAG Cases and 13 Control Subjects With at Least 1.5-Fold Change in Gene Expression

Gene Symbol	Description	Probe Identification	Fold Change	Adjusted P Value*
<i>ARRB1</i>	Arrestin, beta 1	GI_20482945-S	2.79	5.94E-06
<i>CALML3</i>	Calmodulin-like 3	GI_36031099-S	-12.00	7.69E-05
<i>KRT19</i>	Keratin 19	GI_40217850-S	-15.11	1.08E-04
<i>PRSS8</i>	Protease, serine, 8 (prostasin)	GI_21536453-S	-4.04	1.54E-04
<i>CA12</i>	Carbonic anhydrase XII	GI_9951924-S	3.55	2.06E-04
<i>LCN2</i>	Lipocalin 2 (oncogene 24p3)	GI_38455401-S	-6.49	3.22E-04
<i>TNNT3</i>	Troponin T3, skeletal, fast	GI_5803202-S	-4.54	6.59E-04
<i>TNFRSF25</i>	Tumor necrosis factor receptor superfamily, member 25, transcript variant 7	GI_23200030-A	2.57	6.59E-04
<i>MYH14</i>	Myosin, heavy polypeptide 14	GI_33563339-S	-2.24	7.58E-04
<i>KRT13</i>	Keratin 13, transcript variant 2	GI_24234693-A	-8.34	8.39E-04
<i>MASPI</i>	Mannan-binding lectin serine protease 1 (C4/C2 activating component of Ra-reactive factor), transcript variant 2	GI_21264358-I	-2.44	8.39E-04
<i>ANKH</i>	Ankylosis, progressive homolog (mouse)	GI_34452701-S	2.69	8.39E-04
<i>PAX6</i>	Paired box gene 6 (aniridia, keratitis)	GI_4505614-A	-6.37	8.49E-04
<i>FCGBP</i>	Fc fragment of IgG binding protein	GI_4503680-S	5.35	8.49E-04
<i>KLKB1</i>	Kallikrein B, plasma (Fletcher factor) 1	GI_9257226-S	1.82	8.87E-04
<i>TMEM130</i>	Transmembrane protein 130	GI_23097273-S	2.24	8.87E-04
<i>C2orf40</i>	Chromosome 2 open reading frame 40	GI_14165275-S	5.06	8.87E-04
<i>MGP</i>	Matrix Gla protein	GI_4505178-S	6.08	8.87E-04
<i>MSLN</i>	Mesothelin, transcript variant 1	GI_7108357-A	-2.84	9.90E-04
<i>C19orf33</i>	Chromosome 19 open reading frame 33	GI_41281602-S	-5.34	1.21E-03
<i>MNDA</i>	Myeloid cell nuclear differentiation antigen	GI_4505226-S	1.89	1.26E-03
<i>SLPI</i>	Secretory leukocyte protease inhibitor (antileukoproteinase)	GI_15834622-S	-6.03	1.45E-03
<i>C15ORF52</i>	Chromosome 15 open reading frame 52	GI_37540822-S	1.67	1.45E-03
<i>CDH2</i>	Cadherin 2, type 1, N-cadherin (neuronal)	GI_14589888-S	3.33	1.48E-03
<i>KRT15</i>	Keratin 15	GI_24430189-S	-9.43	1.58E-03
<i>COL5A2</i>	Collagen, type V, alpha 2	GI_16554580-S	2.44	1.58E-03
<i>HLA-DPA1</i>	Major histocompatibility complex, class II, DP alpha 1	GI_24797073-S	4.29	1.74E-03
<i>MUC21</i>	Mucin 21, cell surface associated	GI_42657617-S	-2.92	1.81E-03
<i>C10orf10</i>	Chromosome 10 open reading frame 10	GI_5901937-S	-2.69	1.81E-03
<i>FADS2</i>	Fatty acid desaturase 2	GI_14141180-S	2.59	1.81E-03

* P values from these comparisons were corrected for multiple testing using the false discovery rate method.

expression with those identified in the comparison of non-MYOC POAG cases versus controls. Although the data are limited to a single MYOC-associated case, they suggest that there are many similarities between the expression profiles of MYOC and non-MYOC POAG cases and indicate that there may be shared molecular mechanisms between these genetically different forms of POAG.

When MYOC-associated TM was compared with non-glaucomatous control samples, MYOC was not differentially expressed in either MYOC or non-MYOC POAG cases, confirming the previous finding that myocilin mutations do not seem to alter MYOC transcription.^{4,8,12,16} This is consistent with the location of the Q368X myocilin mutation; because it is in the third and last exon, the mutated myocilin transcript will not be subject to nonsense-mediated decay,^{49,50} which normally leads to the degradation of the mutated transcripts and decreased expression.

When non-MYOC cases were compared with controls, we identified a number of coding genes with differential expression. Among these, *PAX6* was significantly downregulated. *PAX6*, as a transcription factor, regulates the development of the eye and has been implicated in the pathogenesis of developmental glaucoma and anterior segmental dysgenesis, including aniridia, Peters' anomaly, Axenfeld's anomaly, optic nerve malformations, and foveal hypoplasia.^{5,51-53} We observed decreased expression (>6-fold) of *PAX6* in the TMs from non-MYOC POAG cases, which is consistent with the loss

of *PAX6* expression in aniridia and other ASD disorders.⁵¹ A recent study by Xie et al.⁵⁴ identified a large number of *PAX6* direct and indirect target genes using chromatin immunoprecipitation and expression data. We observed that 11% (53 of 483) of differentially expressed genes in non-MYOC POAG cases are potential *PAX6* target genes in the lens and that 23% (109 of 483) of these genes are potential *PAX6* targets in at least one of the three tissues (lens, forebrain, and β -cells from the islets of pancreas). Downregulation of *PAX6* observed in the TM of POAG cases raises the possibility that this protein may have a role in normal TM physiology.

We also identified a number of noncoding RNAs that are differentially expressed in the TM obtained from patients with POAG. These include, among others, two microRNAs (*miR-205* and *Let-7b*), antisense RNAs (*ZFH4-AS1* and *PPP2R3B-AS1*), and lincRNAs (*linc00341*, *FLJ33630*, *SHISA4*, *LYRM2*, *LOC202781*, and *RUNX1-IT*). A recent study by Li and colleagues⁵⁵ identified 25 differentially expressed miRNAs in stress-induced premature senescence in primary cultures of human TM cells. *Let-7b* has been reported to activate Toll-like receptor 7 (*TLR7*) and through *TLR7* to cause neurodegeneration in mouse neurons.⁵⁶ Furthermore, *miR-205* has been reported to have an important role in the cornea and lens; it also regulates the function of *INPPL1* and *ITGB4* by competing with *miR-184*.⁵⁷ In addition, *miR-205* modulates cellular invasion and migration by targeting *ZEB2* (zinc finger E-box binding homeobox 2) in cancer cells.⁵⁸ Because several

thousand target genes have been predicted for either *Let-7b* (>5500 possible targets) or *miR-205* (>7800 possible targets) according to the database microRNA.org (in the public domain),^{59,60} it is not possible to specify which role or roles are critical in POAG. However, these data suggest that there is a potential role of these and possibly other noncoding RNAs in POAG. Notably, variants in *CDKN2B-AS1* have been associated with POAG and glaucoma-related ocular phenotypes; however, these antisense RNAs are not included in the gene expression array used in this study.^{21,22,61,62}

Recent data suggest that four genes associated with POAG are involved in the endocytic pathway. These genes include *WDR36*,⁶³ optineurin,⁶⁴ tank binding protein kinase-1,⁶⁵ and myocilin.⁶⁶ In addition to the endocytic pathway, these genes may have a role in exosome release from multivesicular bodies. Abnormalities in the exosome pathway have been proposed as a potential pathologic mechanism in POAG.^{17,19,66–68} We compared the recently published protein profile of TM exosomes¹⁹ with the expression profiles from normal and glaucoma TM samples and found that approximately 25% of total exosome proteins identified from TM demonstrated differential expression in POAG TM specimens. Of note, two proteins typically enriched in exosomes, a lipid raft protein (flotilin-2) and a tetraspanin (tetraspanin-6), were differentially expressed in TM from POAG cases.¹⁹ In addition, protein products of a few characteristic TM genes, including the matrix GLA protein, *SPARC*, and *TIMP2* (which were identified in exosomes), were differentially expressed in TMs from non-MYOC POAG cases.

A number of studies have contributed to the expression profiling of the TM with either sequencing-based or microarray-based approaches. Sequencing-based expression was mainly done via serial analysis of gene expression, which involves direct measurement of mRNA transcripts and generates a nonbiased expression profile without a reference sample.^{69,70} We and others have applied this approach to several ocular tissues, including human TMs.^{42,71,72} Microarray analysis is based on the use of predesigned probes for selected genes or genome annotation and measures the level of gene expression relative to a reference sample (e.g., tissue of a different type or from a different individual).^{73,74} This approach has been extensively applied with human TMs and/or cultured TM cells in studies.^{29,31–34,36–41,55,75–80} In comparison, our study includes more POAG cases and controls, and our Illumina array contains probes for more than 47,000 genes and transcripts. Our study used TM samples collected from POAG cases during trabeculectomy, rather than postmortem tissue, as in other studies. We have confirmed the differential expression of glycogenes (*SULF1*, *LUM*, and *OGN*) from a previous study.³² We confirmed the differential expression of several genes in response to elevated IOP, including *COCH*, *MGP*, *OGN*, and *SPARC*.⁸⁰ As expected, we did not find shared differential expression between our study and previous studies^{31,33,76,81,82} of steroid-treated TM. We did not replicate the increased expression of serum amyloid- β in POAG TMs,⁴⁰ which may be explained by differences in experimental design; this study used TMs from postmortem donors and pooled RNAs from cases and controls for their analysis. No significant overlap was found between differentially expressed genes in our study and a previous study by Liton et al.³⁷ This could be related to the larger sample size in our study and the use of different array platforms.

There are limitations to this study. First, because of the small TM samples obtained from the surgical specimens, only limited amounts of RNA could be extracted per POAG specimen, eliminating the opportunity to perform validation of differential expression using real-time PCR. Regardless of this limitation, additional studies are necessary to replicate the

findings from this study. Second, all patients with POAG from whom TM was obtained were receiving medical treatment. Medical or surgical treatments likely affect RNA expression and thus the results and interpretation of TM expression studies. Third, we had only one patient having POAG with the *Q368X MYOC* mutation, limiting the power of statistical analysis for differential expression involving that case. Clearly, additional *MYOC*-related POAG TM specimens need to be studied. However, the extraordinary rarity of these samples, as made clear from the paucity of similar studies in the literature, nonetheless makes reporting these data valuable in our view.

In summary, we have performed the first comparison to date of RNA expression profiles obtained from TM of POAG cases, including one secondary to a myocilin mutation, with that of nonglaucomatous controls. These data provide support for the role of a number of specific cellular pathways in POAG, including the pathway associated with exosomes. Our preliminary data support the notion that *MYOC*-associated POAG may involve mechanisms that are similar to those of non-*MYOC* forms of POAG. Finally, these data help clarify the processes that ultimately cause POAG and in so doing improve the prospects of a better understanding of this disease process, along with more rational approaches for the development of new therapies.

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