BIOMARKERS, GENOMICS, PROTEOMICS, AND GENE REGULATION

Potential Biomarkers for Primary Open-Angle Glaucoma Identified by Long Noncoding RNA Profiling in the Aqueous Humor

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This study aimed to identify potential biomarkers for primary open-angle glaucoma (POAG) diagnosis. First, long noncoding RNA (lncRNA) and mRNA expression profiles in the aqueous humor (AH) from 10 POAG and 10 control patients were accessed by microarray analyses. Coding-noncoding gene coexpression networks were drawn to predict potential lncRNA functions. lncRNA T267384, ENST00000607393, and T342877 expression levels were further tested by real-time quantitative PCR in AH from 29 POAG and 30 cataract patients, in iris tissues from 16 POAG patients and 10 controls, and in plasma from 49 POAG patients and 55 healthy controls. Finally, ENST00000607393 function was characterized in an *in vitro* model of cell calcification. A total of 3627 lncRNAs and 2228 mRNAs in the AH of POAG patients were significantly up-regulated, and 1520 lncRNAs and 820 mRNAs were significantly down-regulated. Seven lncRNAs showed positive correlation with glaucoma-associated gene, bone morphogenetic protein 2. Moreover, real-time quantitative RT-PCR confirmed that T267384, ENST00000607393, and T342877 expression levels were significantly higher in the AH from a different cohort of POAG patients. ENST00000607393 was also significantly higher in the iris and plasma of POAG patients. Last, ENST00000607393 knockdown alleviated calcification of primary human trabecular meshwork cells *in vitro*. Therefore, lncRNAs T267384, ENST00000607393, and T342877 may be potential biomarkers for POAG diagnosis. ENST00000607393 might be a new therapeutic target for trabecular meshwork calcification. (*Am J Pathol* 2019; 189: 739–752; <https://doi.org/10.1016/j.ajpath.2018.12.011>)

Glaucoma is a group of heterogeneous diseases characterized by loss of retinal ganglion cells and their axons, resulting in gradual deficits of visual field.¹ Primary open-angle glaucoma (POAG) is the most common type of open-angle glaucoma, and the number of POAG patients is estimated to be 45 million around the world.² Although the main diagnosis basis of POAG is the thinning of the retinal nerve fiber layer, visual field loss, and pathologically high intraocular pressure (IOP),^{3,4} multiple factors, including genetic factors,^{5,6} environmental circumstances,⁷ and systemic diseases,⁸ can play an essential role in the pathogenesis of POAG.

It is generally appreciated that approximately 75% of the human genome can be transcribed into RNAs, >95% of

which are noncoding RNAs,⁹ including miRNAs, circular RNAs, and long noncoding RNAs (lncRNAs). lncRNAs are a class of non-protein-coding noncoding RNAs >200 nucleotides. Similar to miRNAs that are much shorter (containing approximately 22 nucleotides),¹⁰ lncRNAs can also regulate gene expression through interacting with mRNAs and other kinds of noncoding RNAs.¹¹ Indeed,

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Table 1 Clinical Characteristics of Samples Used in the Study

Characteristics	Aqueous humor		Iris		Plasma	
	Control (n = 50)	POAG (n = 48)	Control (n = 10)	POAG (n = 16)	Control (n = 55)	POAG (n = 49)
Age, mean ± SD, years	63.2 ± 3.5	61.0 ± 2.9	58.4 ± 1.4	60.2 ± 2.1	64.2 ± 1.2	61.9 ± 1.9
Sex, %						
Female	51.2	55.8	48.2	45.8	48.9	53.3
Male	48.8	44.2	51.8	54.2	51.1	46.7
IOP, mean ± SD, mmHg	18.8 ± 0.7	27.8 ± 2.1	NA	26.2 ± 1.8	NA	26.9 ± 2.3

IOP, intraocular pressure; NA, not applicable; POAG, primary open-angle glaucoma.

lncRNAs can participate in gene regulation at transcriptional, post-transcriptional, translational, and epigenetic levels during a variety of biological and pathologic processes.¹² In the past three decades, fundamental works have revealed the critical role of lncRNAs in the occurrence of neurodegenerative diseases, including glaucoma.^{13,14} For example, single-nucleotide polymorphisms in *CDKN2B-AS1*, an lncRNA in the antisense direction of the *INK* locus, are associated with the risk of developing POAG and optic nerve degeneration.^{14,15} Possible mechanisms of *CDKN2B-AS1* in glaucoma may involve effects on the transforming growth factor-β signaling pathway or regulation of gene expression of the neighboring genes.¹⁶

Herein, we sought to reveal disease-related extracellular lncRNA and mRNA profiles in the aqueous humor (AH) of POAG patients and to identify potential biomarkers for POAG diagnosis. Our results showed that lncRNAs T267384, ENST00000607393, and T342877 may be potential biomarkers for POAG diagnosis. Moreover, ENST00000607393 may be a new therapeutic target for trabecular meshwork calcification, a process involved in IOP elevation and glaucoma progression.

Materials and Methods

Study Participants

The study protocol was approved and monitored by the Chinese Clinical Trial Registry (registration number ChiCTR-ROn-16008492) and the Ethics Committee of The Second Xiangya Hospital (Changsha, China; project number 20160101). All processes in this study were in accordance with the tenets of the Declaration of Helsinki, and written informed consent was obtained from all subjects. All donated samples were obtained from The Second Xiangya Hospital.

Samples for experimental groups were obtained from POAG patients who did not receive any glaucoma medication 4 months before surgery and had uncontrolled IOP. The control group for AH profiling consisted of age- and sex-matched age-related cataract patients who were candidates for cataract surgery (Table 1). Ninety-eight AH samples from 98 eyes of 98 participants (48 with POAG and 50 with age-related cataract), 26 iris samples from 26 eyes of

26 participants (16 with POAG and 10 donated human eyes without eye diseases), and 104 plasma samples from 104 participants (49 with POAG and 55 healthy controls) (Table 1) were also collected for either microarray or real-time quantitative PCR (qPCR) analysis. There was no statistical difference between glaucoma and control groups on age and sex. Individuals who experienced trauma or secondary glaucoma, those with other eye disorders, such as pigment dispersion syndrome, Posner-Schlossman syndrome, and Fuchs heterochromic iridocyclitis, and those who had systemic diseases, such as diabetes, were excluded. Individuals whose fixation loss, false-positive rate, or false-negative rate was >20% in computerized visual field examinations were also excluded.

All subjects underwent a complete ophthalmologic examination, including medical history, best-corrected visual acuity measurement, slit-lamp examination, fundus examination, Goldmann applanation tonometry, gonioscopy examination, central corneal thickness measurement, ultrasound biomicroscopy, optic disk ophthalmoscopy, optical coherence tomography, and computerized visual field (Humphrey 24-2) test.

Sample Collection

Aqueous humor (approximately 100 μL) was carefully collected from patients who underwent surgery by paracentesis of the anterior chamber, using a 27-gauge needle inserted through the peripheral cornea under a microscope. During paracentesis, the needle was kept away from touching the iris and lens. Irises were collected from glaucoma patients who underwent glaucoma filtering surgery and from donated human eyes. Irises from POAG patients were carefully dissected from the ciliary body at its radial edge (clockwise from the 12- to 2-o’clock position). Control irides from donated eyes were also dissected from the same location. The death-to-preservation time was <12 hours. Peripheral blood samples (approximately 5 mL) were drawn from forearm veins and collected into tubes containing anticoagulant (citrate). The blood was centrifuged at 3000 × g for 15 minutes at 4°C (Eppendorf Centrifuge 5810 R; Eppendorf, Hamburg, Germany). The upper layer of the plasma was collected into Eppendorf tubes. All samples

were immediately cooled at -80°C and protected from light in a dry place.

RNA Isolation and qPCR

Total RNA in aqueous humor, plasma, and iris tissues was extracted with TRIzol LS or TRIzol Reagent (Invitrogen, Carlsbad, CA) and purified with the RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. RNA was reverse transcribed into cDNA with the Transcriptor First Strand cDNA Synthesis Kit (Roche, Pleasanton, CA) using oligo-dT primers. Transcript levels were determined through qPCR using FastStart Universal SYBR Green Master (ROX) (Roche), according to the manufacturer's instructions. Primer pairs are listed in Table 2. Expression levels were normalized to β -actin and calculated as fold changes using the $2^{-\Delta\Delta\text{Ct}}$ method.¹⁷

Microarray Analysis

AH RNA samples from 10 control subjects and 10 POAG patients for lncRNA and mRNA microarray analyses were extracted, as described above. For microarray analysis, RNA integrity and genomic DNA contamination were tested by denaturing agarose gel electrophoresis. On average, 300 to 400 ng RNA was extracted per AH sample. RNA samples were amplified with the TargetAmpTM1-Round aRNA Amplification kit (Epicentre, Madison, Wisconsin) before loading, and 1 μg RNA was loaded per microarray. Gene expression profiling was performed according to the manufacturer's protocol using human lncRNA arrays with a total of 40,173 probes for lncRNAs and 20,730 probes for mRNAs (Arraystar, Shanghai, China). Data were extracted through the Agilent Feature Extraction software version 11.5 (Agilent, Santa Clara, CA). A train of data processing, including quantile normalization, was performed through the R software package version 3.5.2.¹⁸ All original data have been uploaded to Gene Expression Omnibus public database (<https://www.ncbi.nlm.nih.gov/geo/>; accession number GSE101727).

GO Analysis and KEGG

The Gene Ontology (GO) enrichment analysis was based on the comparison between differentially expressed genes and genetic backgrounds in the GO database (<http://www.geneontology.org>, last accessed August 25, 2016). The P value denotes the significance of GO term enrichment in differentially expressed genes. The lower the P value is, the more significant the GO term is.

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis is a functional analysis mapping genes to KEGG pathways (<http://www.genome.jp/kegg>, last accessed July 16, 2018). The P value (EASE score, Fisher P value, or hypergeometric P value) denotes

Table 2 Primer Sequences

Primer name	Primer sequence
β -Actin	F: 5'-GTGGCCGAGGACTTTGATTG-3' R: 5'-CCTGTAACAACGCATCTCATATT-3'
ENST00000506335	F: 5'-AGAGGGAAACCATCGGAAAT-3' R: 5'-CCGACGCTAAACTGTCTGAATA-3'
ENST00000508241	F: 5'-TTTGGGTTTGGCTGTACTCA-3' R: 5'-GACCACATTTCCCAAGAACT-3'
ENST00000607393	F: 5'-GGCGTCTGTTTGTATTGGC-3' R: 5'-TCACTTTTGTCTGGGGAGATT-3'
ENST00000564363	F: 5'-ACAAAAACATAAAGGCCGGG-3' R: 5'-AGAGCACCCCCAAGCCCT-3'
TCONS_00025577	F: 5'-TCCCTGAACTACGACTTCCTCA-3' R: 5'-GACCACATTTCCCAAGAACT-3'
T034489	F: 5'-TGCCACAGCCTTTACGACA-3' R: 5'-TACCAACCAGGAGGGGAGAT-3'
T267384	F: 5'-TCTGGGCATTGAAGAGTGAAG-3' R: 5'-AGAGGGATGGGTACAAATAAGC-3'
T273036	F: 5'-TGGCTTGAAGATGAAGGACTCC-3' R: 5'-GGGCCTCTCTCAGCTGCTAA-3'
T342877	F: 5'-TCTGCGGGACATTCATACCT-3' R: 5'-ATTTCCTTCTCTCTCCTGGTCTC-3'
NR_026887	F: 5'-GCTTCGTTTTCGGTCCAGA-3' R: 5'-TTTACTCCCTCCCGTCCAA-3'

F, forward; R, reverse.

the significance of the pathway correlated to the conditions. Lower P values indicate higher significance. The recommended P -value cutoff is 0.05.

CNC Gene Expression Network

The coding-noncoding (CNC) gene expression network was constructed according to the correlation coefficient between the normalized lncRNA and mRNA expression values (Pearson's correlation coefficient ≥ 0.9 , false discovery rate ≤ 0.05 , and $P \leq 0.05$). The CNC gene expression network was generated using Cytoscape2.8.3.¹⁹

Cell Isolation, Culture, and Treatment

The primary human trabecular meshwork (HTM) cells were isolated from dissected trabecular meshwork tissues of donated human eyes (without known history of ocular diseases or eye surgeries), as previously described.²⁰ Donors were obtained from The Second Xiangya Hospital. Trabecular meshwork (TM) cells from four different donors (mean $\pm$ SD age = 54.3 ± 5.3 years; female = 50%, male = 50%) were isolated and used in the experiments. Cells were used at passages 4 to 6. The primary HTM cells were grown in Dulbecco's modified Eagle's medium (4.5 g/L glucose; Gibco by Life Technologies, Grand Island, NY) containing 10% fetal bovine serum (Gibco by Life Technologies), 1% penicillin/streptomycin (Gibco by Life Technologies), and 1% L-glutamine (Gibco by Life Technologies) at 37°C in a humidified atmosphere of 5% CO_2 .

To induce cell calcification, the primary HTM cells were treated with 0, 500, 600, 700, 800, and 900 $\mu\text{mol/L}$ H_2O_2 (Sigma-Aldrich, St. Louis, MO) for 48 hours.

FISH Probe Hybridization

Three fluorescence *in situ* hybridization (FISH) probes targeting ENST00000607393 were designed and synthesized by GenePharma Inc. (Shanghai, China), and their sequences were 5'-AGAAGGCTCGGCGTAGGGA-3', 5'-TGA-TAATGAGAAGGCTCGGCGTA-3', and 5'-GAGCCC-GAGTTCGCTGGAAT-3'. A negative control FISH probe, based on human sequence, was designed and synthesized by GenePharma Inc., and the sequence was 5'-GTGTAA-CACGTCTATACGCCCA-3'. FISH was performed according to the manufacturer's protocol. The subcellular localization of ENST00000607393 in HTM cells was determined with a fluorescence microscope (Olympus, Tokyo, Japan).

siRNA Treatment

ENST00000607393 knockdown in HTM cells was performed with siRNA designed and synthesized by GenePharma Inc. The human ENST00000607393 siRNA target sequence was 5'-GCAGGCGUGUGCAUUCUUTT-3', and the negative control siRNA sequence was 5'-UUCUCCGAACGUGU-CACGUTT-3'. For siRNA transfection, 2500 ng of siRNA oligonucleotide dimers and 3.75 μL of Lipofectamine 3000 (Invitrogen) were mixed together in 250 μL serum-free Opti-MEM medium (Gibco by Life Technologies), according to the manufacturer's protocol. HTM cells were incubated with the siRNA-Lipofectamine complexes for 10 hours in serum- and antibiotic-free Dulbecco's modified Eagle's medium (Gibco by Life Technologies) at 37°C. After incubation, the growth medium was replaced with complete medium (Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% L-glutamine). After 24 hours, HTM cells were harvested for RT-qPCR or follow-up experiments.

Alizarin Red Staining and ALP Determination

Hydrogen peroxide-treated primary HTM cells were washed with phosphate-buffered saline three times and fixed with 4% paraformaldehyde for 15 minutes. Cells were then exposed to fresh 1.5% alizarin red (pH 4.2; Sigma-Aldrich) for 5 minutes, washed with distilled water, and imaged with an optical microscope (Olympus).

Alkaline phosphatase (ALP) activity was measured with the Alkaline Phosphatase Assay Kit (Colorimetric) (ab83369 and ab134678; Abcam, Burlingame, CA), according to the manufacturer's protocol, and the absorbance was determined at OD 405 nm using an EnSpire multimode reader (PerkinElmer, Waltham, MA).

Statistical Analysis

Categorical variables were compared by χ^2 test. Numeric variables were compared using *t*-test or *U*-test. Results were expressed as means $\pm$ SD or median $\pm$ interquartile range, depending on the results of the Shapiro-Wilk normality test. The correlation between lncRNA and mRNA expression was evaluated by Pearson correlative analysis. lncRNA expression levels were assessed for correlation with glaucoma types and clinical features using Spearman rank correlation analysis and Pearson correlative analysis, respectively.

Experimental groups related to HTM cells were compared by one-way analysis of variance analysis, followed by post-hoc tests to compare difference between two groups. Receiver operating characteristic (ROC) curves were obtained through ROC curve analyses. The ROC curve is constructed by plotting the true-positive rate (alias sensitivity) against the false-positive rate (calculated as 1 – specificity) at various threshold settings. The best possible prediction method would generate a point in the upper left corner (0, 1), which means 100% sensitivity and 100% specificity. A random classifier's ROC curve point tends around the diagonal (green) line. Therefore, an area under the curve value of >0.5 is generally considered as significantly meaningful. The data set used herein was the lncRNA expression data by RT-qPCR in both control and POAG groups. All statistical analyses were performed using SPSS software version 19.0 (IBM, Armonk, NY). $P < 0.05$ was considered statistically significant.

Results

Microarray Expression Profiling of Extracellular lncRNAs and mRNAs in AH from Individual Subjects

lncRNAs were shown to take part in the development and progression of glaucoma.^{21,22} To identify potential biomarkers for POAG diagnosis, expression analyses were performed and candidate biomarkers were prioritized using a few bioinformatic tools. The experimental design is shown in Figure 1. First, to detect and identify differentially expressed extracellular lncRNAs and mRNAs in the AH of POAG patients, AH samples from 10 POAG patients and 10 control subjects (age- and sex-matched cataract patients) were collected and quantified by microarray assays containing 40,173 lncRNA and 20,730 mRNA probes (Figure 2). A total of 3627 lncRNAs and 2228 mRNAs were significantly up-regulated (fold change ≥ 2 , false discovery rate ≤ 0.05 , $P \leq 0.05$), and 1520 lncRNAs and 820 mRNAs were significantly down-regulated (fold change ≤ 0.5 , false discovery rate ≤ 0.05 , $P \leq 0.05$), in POAG patients compared with control subjects (Figure 2, A–D). To confirm the results, 10 lncRNAs that had highly significant P values ($P < 0.01$) and false discovery rate (<0.025) and large fold changes (>4.5) and showed positive correlation

Figure 1 Graphical summary of the experimental design. First, long noncoding RNA (lncRNA) and mRNA expression profiles in the aqueous humor (AH) from 10 primary open-angle glaucoma (POAG) and 10 control patients were accessed by microarray analyses. Moreover, coding-noncoding (CNC) gene coexpression networks were drawn to predict potential lncRNA functions. On the basis of CNC networks and Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses, 10 lncRNAs were chosen and their expression was assessed by individual real-time quantitative RT-PCR (RT-qPCR) assays. Consistently, 7 lncRNAs (highlighted in **red**) are significantly up-regulated in the POAG group and 3 lncRNAs (highlighted in **blue**) have no significant differences. lncRNA T267384, ENST00000607393, and T342877 (highlighted in **red**) expression levels were further tested by real-time quantitative PCR in AH, iris tissues, and plasma. Finally, the function of ENST00000607393 (highlighted in **red**) was characterized in an *in vitro* model of cell calcification. Scale bar = 50 μ m. TM, trabecular meshwork.

with POAG-relevant mRNA were chosen, and their expression was assessed by individual RT-qPCR assays using the same samples for the initial microarray analysis. Consistent with the microarray results, T267384 (located on chromosome 4), ENST00000607393 (located on chromosome 8), T342877 (located on chromosome 8), ENST00000564363 (located on chromosome 12), NR_026887 (located on chromosome 12), TCONS_00025577 (located on chromosome 17), and ENST00000508241 (located on chromosome 4) were significantly up-regulated in the POAG group (Figure 2E). However, there were no significant differences in expression levels of T034489 (located on chromosome 1), ENST00000506335 (located on chromosome 5), and T273036 (located on chromosome 4) in AH samples between cataract and POAG groups (Figure 2E). Most genes

tested by qPCR (7/10) were consistent with the direction of changes obtained by microarray analysis, confirming the validity of the microarray data.

GO and KEGG Pathway Enrichment Analysis

To explore potential functions of differentially expressed genes and correlated pathways, GO (<http://www.geneontology.org>) and KEGG (<http://www.genome.jp/kegg>) pathway enrichment analyses were performed (Figure 3). A GO enrichment analysis showed that biological processes of up-regulated mRNAs include protein targeting to endoplasmic reticulum, ribosome biogenesis, viral transcription, and so forth (Figure 3A), whereas biological processes of down-regulated mRNAs include glomerulus vasculature development, response to

protozoan and endocrine process, and so forth (Figure 3B). Top enriched cellular components and molecular function are also shown in Figure 3, A and B.

The KEGG pathway enrichment analyses showed that 10 pathways were significantly enriched among up-regulated genes, including phosphatidylinositol signaling system, ribosome, mRNA surveillance pathway, proximal tubule bicarbonate reclamation, inositol phosphate metabolism, melanogenesis, long-term potentiation, biosynthesis for amino acids, protein digestion and absorption, and oxidative phosphorylation (Figure 4A). Of interest, mRNAs of Wnt, Frizzled, and Go, three important upstream components of the Wnt signaling pathway, were significantly up-regulated in AH of POAG patients. Ten pathways were enriched in down-regulated genes, including mitogen-activated protein kinase signaling pathway, homologous recombination, fanconi anemia pathway, pertussis, prostate cancer, base excision repair, taste transduction, prion diseases, pathway in cancer, and calcium signaling pathway (Figure 4B).

lncRNA–mRNA Coexpression Network

To explore potential functions of differentially expressed lncRNAs, the CNC gene coexpression network analysis, based on the strength of correlation between differentially

expressed mRNAs and lncRNAs, was used.^{23,24} To this end, 15 mRNAs that are associated with POAG or trabecular meshwork^{25–32} and were differentially expressed in the AH samples (fold change ≥ 2 , false discovery rate ≤ 0.05 , $P \leq 0.05$) were selected to construct a coexpression network according to the correlation coefficient (Pearson’s correlation coefficient ≥ 0.9 , false discovery rate ≤ 0.05 , and $P \leq 0.05$). Among them, four mRNAs, bone morphogenetic protein 2 (*BMP2*), forkhead box E3 (*FOX E3*), growth hormone secretagogue receptor (*GHSR*), and optineurin (*OPTN*), were correlated with 1270, 76, 25, and 24 lncRNAs, respectively (Supplemental Figure S1). No expression correlation was found for the rest of selected mRNAs, including integrin subunit β 5 (*ITGB5*), transforming growth factor- β 1 (*TGFB1*), forkhead box C1 (*FOX C1*), transmembrane and coiled-coil domains 1 (*TMC O1*), NF- κ B subunit 1 (*NFKB1*), FasL (*FASLG*), ataxin 2 (*ATXN2*), caveolin 1 (*CAV1*), ghrelin and obestatin prepropeptide (*GHRL*), syndecan 4 (*SDC4*), and chloride voltage-gated channel 2 (*CLCN2*).

To select possible glaucoma biomarkers, seven differentially expressed lncRNAs from the aforementioned qPCR analysis were studied, and their expression correlation with the four differentially regulated, POAG-relevant mRNAs was assessed. All of the seven lncRNAs, T267384,

Figure 3 Gene Ontology (GO) enrichment analyses. **A** and **B**: GO enrichment analysis showing top 10 biological processes, cellular components, and molecular functions enriched in up-regulated (**A**) and down-regulated (**B**) mRNAs. ER, endoplasmic reticulum; NA, nicotinamide adenine dinucleotide phosphate; R, RNA.

ENST00000607393, T342877, ENST00000564363, TCONS_00025577, ENST00000508241, and NR_026887, showed positive correlation with *BMP2*. The correlation between expression of these lncRNAs and POAG-relevant gene may indicate their importance in the occurrence and development of the disease.

The Diagnostic Values of T267384, ENST00000607393, and T342877 in AH, Iris, and Plasma

To identify robust biomarkers for glaucoma diagnosis, three lncRNAs, T267384, ENST00000607393, and T342877, that had the smallest *P* values in the RT-qPCR analysis were

studied. They showed positive correlation with POAG-relevant gene, *BMP2*, based on the CNC analysis. Moreover, because that iris is a component of the ocular anterior segment that contacts AH with large areas and affects its physiology and pathology,³³ and plasma-based diagnosis might offer an alternative noninvasive strategy to improve sampling flexibility,³⁴ the expression profiles and diagnostic values of those three lncRNAs were compared in AH, iris, and plasma. First, their expression level was examined in the AH, iris, and plasma from three independent cohorts of POAG patients and their corresponding controls (Figure 5). The expression levels of T267384, ENST00000607393, and T342877 were significantly higher in the AH of POAG

Figure 4 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses. **A** and **B**: KEGG pathway enrichment analyses showing 10 pathways enriched among up-regulated genes (**A**) and 10 pathways enriched among down-regulated genes (**B**). DE, differentially expressed; MAPK, mitogen-activated protein kinase.

Figure 5 T267384, ENST00000607393, and T342877 expression in the aqueous humor, iris, and plasma. **A–C:** Scatter plots of expression levels of T267384 (**A**), ENST00000607393 (**B**), and T342877 (**C**) in the aqueous humor. **D–F:** Scatter plots of expression levels of T267384 (**D**), ENST00000607393 (**E**), and T342877 (**F**) in the iris. **G–I:** Scatter plots of expression levels of T267384 (**G**), ENST00000607393 (**H**), and T342877 (**I**) in plasma. Data are expressed as means $\pm$ SD (**A–F**) or median (**G–I**). $n = 30$ (**A–C**, control); $n = 29$ [**A–C**, primary open-angle glaucoma (POAG)]; $n = 10$ (**D–F**, control); $n = 16$ (**D–F**, POAG); $n = 55$ (**G–I**, control); $n = 49$ (**G–I**, POAG). $**P < 0.01$, $***P < 0.001$. lncRNA, long noncoding RNA.

patients ($4.05\times$, $2.41\times$, and $2.87\times$, respectively; $P < 0.001$; $n = 30$ and $n = 29$ for control and POAG, respectively) (Figure 5, A–C). The expression level of ENST00000607393 was also significantly higher in iris tissues of POAG patients ($2.74\times$; $P = 0.007$; $n = 10$ and $n = 16$ for control and POAG, respectively) (Figure 5E) and in the plasma of POAG patients ($1.96\times$; $P < 0.001$; $n = 55$ and $n = 49$ for control and POAG, respectively) compared with controls (Figure 5H). However, no statistical differences were found in expression of T267384 and T342877 in the iris and plasma between POAG and control groups ($P = 0.661$, $P = 0.740$, $P = 0.904$, and $P = 0.068$, respectively) (Figure 5, D, F, G, and I).

Next, ROC curve analyses were performed to assess the diagnostic sensitivity and specificity of T267384, ENST00000607393, and T342877 in the AH, iris, and plasma using their expression data in corresponding tissues. The areas under the curves (represent diagnosis ability) for T267384, ENST00000607393, and T342877 in AH and ENST00000607393 in the iris and plasma were 0.966,

0.966, 0.931, 0.6883, and 0.612, respectively (Figure 6). Furthermore, when the cutoff values were 1.588, 1.173, 2.326, 0.245, and 1.005, the diagnostic sensitivities/specificities of T267384, ENST00000607393, and T342877 in AH and ENST00000607393 in iris and plasma were 0.966/1.000, 0.966/1.000, 0.931/0.967, 0.688/0.700, and 0.612/0.673, respectively (Table 3).

Because these three lncRNAs showed much higher sensitivity and specificity in AH samples than in the iris and plasma, the expression of additional lncRNAs in the AH was examined to identify additional molecules that may also serve as good diagnostic biomarkers in the AH. Four lncRNAs (ENST00000564363, NR_026887, TCONS_00025577, and ENST00000508241) that had significantly higher expression in AH samples of POAG patients than in controls were identified. As the sensitivities and specificities of T267384, ENST00000607393, and T342877 in AH were much higher than when in iris and plasma, the diagnostic values of those additional lncRNAs (ENST00000564363, NR_026887, TCONS_00025577, and

Figure 6 Receiver operating characteristic (ROC) curves of T267384, ENST00000607393, and T342877 for glaucoma diagnosis. **A–C:** ROC curves of T267384, ENST00000607393, and T342877 in the aqueous humor for glaucoma diagnosis. The areas under the curves were 0.998, 0.998, and 0.984, respectively. **D:** ROC curve of ENST00000607393 in iris for glaucoma diagnosis. The area under the curve was 0.788. **E:** ROC curve of ENST00000607393 in plasma for glaucoma diagnosis. The area under the curve was 0.720. The **blue lines** were generated by plotting the sensitivity in the y axis versus the 1 – specificity on the x axis at various threshold settings. The **diagonal green lines** indicate where assay sensitivity = 1 – specificity. Usually, a good assay with high diagnosis specificity and sensitivity has an area under the curve value > 0.5, which is indicated by having a ROC curve within the top left corner divided by the **green line**.

ENST00000508241) in AH were further explored (Supplemental Figure S2). The expression levels of these lncRNAs were significantly higher in the AH of POAG patients than in controls ($P = 0.004$, $P = 0.001$, $P = 0.002$, and $P = 0.004$, respectively; $n = 19$). Moreover, the areas under the curves for ENST00000564363, NR_026887, TCONS_00025577, and ENST00000508241 in AH were 1.000, 0.906, 0.933, and 0.867, respectively (Supplemental Figure S3).

To further investigate the possible correlation between the expression of T267384, ENST00000607393, and T342877 and a range of glaucoma-related features, including mean deviation and pattern SD obtained from the computerized visual field test, retinal nerve fiber layer (RNFL) average thickness obtained from optical coherence tomography, central corneal thickness, and IOP obtained from Goldmann applanation tonometry test, association analyses were conducted (Table 4). First, no significant association was observed for the expression of T267384 with any specific glaucoma feature. However, ENST00000607393 expression level was associated with pattern SD and RNFL average thickness ($n = 15$; Pearson's correlation coefficients = 0.689 and 0.551, respectively; $P = 0.005$ and $P = 0.033$, respectively). Similarly, T342877 expression level was associated with pattern SD and RNFL average thickness in POAG patients ($n = 15$; Pearson's correlation coefficients = 0.649 and < 0.818, respectively; $P = 0.009$ and $P < 0.001$, respectively). Moreover, the strongest result was the

association between T342877 expression and RNFL average thickness ($n = 15$; Pearson's correlation coefficient = 0.818; $P < 0.001$).

ENST00000607393 Knockdown Alleviates Calcification of Primary Human Trabecular Meshwork Cells *in Vitro*

ENST00000607393 expression levels were significantly higher in AH, iris, and plasma of glaucoma patients, and they showed positive correlation with *BMP2*, which is involved in TM calcification,³⁵ a process important for intraocular pressure elevation. Therefore, it was tested if ENST00000607393 could also influence TM calcification (Figure 7). To this end, the subcellular localization of ENST00000607393 was first examined in TM cells by FISH as the subcellular localization of lncRNAs may indicate their biological function.³⁶ ENST00000607393

Table 3 Diagnostic Values of T267384, ENST00000607393, and T342877 in AH, Iris, and Plasma

Cutoff value	Index	Sensitivity	Specificity
1.588	T267384 in AH	0.966	1.000
1.173	ENST00000607393 in AH	0.966	1.000
2.326	T342877 in AH	0.931	0.967
0.245	ENST00000607393 in iris	0.688	0.700
1.005	ENST00000607393 in plasma	0.612	0.673

AH, aqueous humor.

Table 4 P Values (Pearson’s Correlation Coefficients) for Correlations between lncRNA Expression and Glaucoma-Related Features in POAG Patients

Glaucoma-Related Features in POAG Patients	lncRNAs		
	T267384	ENST00000607393	T342877
MD	0.447 (−0.212)	0.541 (0.172)	0.957 (0.015)
PSD	0.434 (−0.219)	0.005 (0.689)	0.009 (0.649)
RNFL average thickness	0.849 (0.054)	0.033 (0.551)	<0.001 (0.818)
CCT	0.588 (−0.152)	0.111 (−0.429)	0.135 (−0.404)
IOP	0.430 (0.143)	0.235 (0.209)	0.523 (0.192)

Bold text is used to emphasize significant data.
CCT, central corneal thickness; IOP, intraocular pressure; lncRNA, long noncoding RNA; MD, mean deviation; POAG, primary open-angle glaucoma; PSD, pattern SD; RNFL, retinal nerve fiber layer.

localized in both the nucleus and cytoplasm of primary human TM cells (Figure 7A). An *in vitro* model was developed to induce TM calcification in culture. Hydrogen peroxide is well known to be a crucial element inducing calcification,^{37–39} and ALP activity is regarded as a marker of calcification precursor.⁴⁰ Therefore, primary human TM cells were treated with different concentrations of hydrogen peroxide. The ALP activity in TM cells was significantly

up-regulated after being treated with H₂O₂ (500, 600, 700, and 800 μmol/L) (Figure 7B). In addition, hydrogen peroxide-treated TM cells were stained with another well-established calcification marker, alizarin red, and nodules of red-orange were detected, indicative of positive staining in cells treated with 500 μmol/L H₂O₂ but not in nontreated controls (Figure 7C). Thus, hydrogen peroxide treatment could successfully induce TM calcification.

Figure 7 ENST00000607393 knockdown alleviates primary human trabecular meshwork cell calcification *in vitro*. **A**: ENST00000607393 localization in primary human trabecular meshwork (TM) cells is detected through fluorescence *in situ* hybridization. **B**: The alkaline phosphatase (ALP) activities of human primary TM cells increase after H₂O₂ treatment (500, 600, 700, and 800 μmol/L). **C**: After the treatment of 500 μmol/L H₂O₂, red-orange nodules are observed through alizarin red staining. **D**: Real-time quantitative RT-PCR analysis shows that ENST00000607393 expression levels increase after the treatment of different concentrations of hydrogen peroxide. **E**: ENST00000607393 expression levels in human primary TM cells are down-regulated through siRNA. **F**: The ALP activity of the ENST00000607393 targeted siRNA-treated cells is significantly decreased from that of the negative control (NC) siRNA-treated cells. Data are expressed as means ± SD (**B** and **D–F**). *P < 0.05, **P < 0.01, and ***P < 0.001. Scale bars: 50 μm (**A**); 200 μm (**C**). lncRNA, long noncoding RNA.

ENST00000607393 expression was quantified in primary human TM cells treated with hydrogen peroxide, and it was significantly increased compared with untreated cells when cells were treated with 500 and 600 $\mu\text{mol/L}$ of H_2O_2 (Figure 7D). To determine the function of ENST00000607393 on TM calcification, ENST00000607393 was down-regulated in TM cells by siRNA (Figure 7E). The ALP activity of siRNA-treated cells was significantly decreased compared with that of the negative control siRNA-treated cells (Figure 7F), indicating that ENST00000607393 knockdown alleviates calcification of primary human TM cells.

Discussion

lncRNAs play a crucial role in diverse cellular activities, and dysregulation of their expression plays a key role in the pathogenesis of neurodegenerative diseases.^{41,42} However, detailed analyses elaborating on expression profiling of lncRNAs in POAG patients have yet to be reported. Herein, lncRNA expression in AH from eyes with POAG and controls (subjects with cataract only) was systematically profiled and compared. In addition, potential functions of differentially expressed mRNAs and lncRNAs were explored through GO/KEGG pathway enrichment analyses and CNC networks were constructed. Furthermore, the expression of three lncRNAs, T267384, ENST00000607393, and T342877, was analyzed in AH, iris, and plasma of POAG patients and corresponding controls and showed that lncRNAs T267384, ENST00000607393, and T342877 may serve as potential biomarkers for POAG diagnosis. Moreover, ENST00000607393 was identified to be a new therapeutic target for the prevention of TM calcification that is relevant to glaucoma disease progression.

Because of tissue accessibility,^{43,44} AH is an attractive source of novel biomarkers of eye diseases. Moreover, the used sample preparation approach and global PCR-amplification method allow sensitive detection of lncRNAs within individual eyes, thus allowing better preservation of information than using pooled AH samples.⁴⁵ The origin of lncRNAs in AH remains obscure. AH is primarily produced by active transfer of water and solutes across the blood-eye barrier around the iris root.⁴⁶ Therefore, it is expected that lncRNAs in AH may also exist in plasma and iris. In support of this assumption, three differentially expressed lncRNAs in AH, T267384, ENST00000607393, and T342877, were also detectable in iris and plasma. Although abnormal lncRNA expression levels in AH, iris, and plasma are all applicable for glaucoma diagnosis, the expression in AH is more effective, with higher specificity and sensitivity than in the other two tissues. The possible explanation for this finding is that AH is a type of circulating liquid that is constantly being produced by ciliary processes and flows through the pupil to enter the anterior chamber and then exits the eye. Therefore,

AH could effectively reflect the pathophysiological status of the eye. Moreover, because of the existence of blood-retinal and blood-aqueous barriers, RNA and DNA derived from other organs have relative difficulty entering into the AH,^{47,48} although RNA, DNA, and protein levels in AH might possibly increase without the breakdown of the blood-aqueous barrier.⁴⁹ Therefore, compared with the expression in plasma, lncRNAs in AH might be better indicators of ocular physiology and pathology.⁵⁰

Finding lncRNA expression levels correlated to glaucoma-related features is of great importance, because this pattern of association may contribute toward monitoring glaucoma progression. According to these results, ENST00000607393 expression was associated with two glaucoma-related features, pattern SD and RNFL average thickness. Moreover, ENST00000607393 knockdown may alleviate primary human trabecular meshwork cell calcification, which would significantly alter outflow facility and biomechanical scleral stress and promote the elevation of IOP in glaucoma.⁵¹ Thus, it seems plausible that ENST00000607393 expression in AH could reflect glaucoma-related ocular damage through regulating the outflow pathway of AH. Similarly, the genotypes of *CDKN2B-AS1* were associated with risk of developing POAG.⁵² Single-nucleotide polymorphisms of *CDKN2B-AS1* were also associated with more specific features of glaucoma, such as greater cup/disc ratio and reduced IOP.¹⁴ Therefore, correlations between certain lncRNAs and specific glaucoma-related features need to be further investigated.

Among the selected 15 POAG-related mRNAs, *BMP2* expression was correlated with the greatest number of differentially expressed lncRNAs. As previously reported, as one of the most potent calcification inducer genes,⁵³ *BMP2* encodes a preproprotein that contributes to the generation of a subunit of a disulfide-linked homodimer, which is essential for bone and cartilage development.^{54,55} Moreover, *BMP2* is highly expressed in the TM and the peripapillary scleral region and is involved in regulating TM softness and sclera stiffness.^{56,57} Overexpressing *BMP2* by gene transfer in rats can induce TM calcification and elevated IOP,^{25,35,58} suggesting its importance in glaucoma pathogenesis. Of interest, ENST00000607393, whose expression levels were significantly up-regulated in AH, iris, and plasma of glaucoma patients, showed positive correlation with *BMP2*. In addition, ENST00000607393 knockdown alleviated primary human TM cell calcification induced by hydrogen peroxide *in vitro*. Although further work is needed to explore the role of ENST00000607393 in TM calcification and glaucoma *in vivo*, these results suggest ENST00000607393 to be a potential target for prevention of TM calcification.

KEGG pathway enrichment analyses revealed possible molecular pathways that might be altered in glaucoma patients.⁵⁹ However, none of these pathways is clearly known to be involved in glaucoma, and the mechanism for

alleviated TM cell calcification in the ENST00000607393 knockdown cell model is unclear. Of interest, however, the mRNA expression levels of upstream components of the Wnt signaling pathway, Wnt, Frizzled, and Go/Gq, were significantly up-regulated in AH of glaucoma patients. Wnt and BMP signaling pathways can regulate similar biological processes and signal cross talks between the two may exist depending on the biological context.^{60,61} For instance, both canonical WNT/ β -catenin and BMP pathways play important roles in bone formation and cell calcification.^{62–64} On BMP2 stimulation, calcification and skeletal development processes could be accelerated through activation of BMP and Wnt pathways.⁶⁵ Moreover, BMP2 deficiency could block the ability of the WNT signaling pathway to promote osteogenesis.⁶⁶ Thus, although it is still necessary to investigate mechanistic links of ENST00000607393 expression and glaucoma, it is possible that ENST00000607393 knockdown might exert its action through the Wnt signaling pathway.

The potential limitation of this study is, because of the ethical and sampling restriction, age-related cataract patients, rather than the general population, were chosen as the control group. However, similar samples were also used as proper controls in other molecular studies using human AH.^{67,68} Moreover, the sample size in this study was relatively small, and the samples only came from the Chinese population. Therefore, it is difficult to analyze the correlation between lncRNA expression and glaucoma-related features in greater detail. Nevertheless, this study represents one of the first few reports that assessed lncRNA expression profiles in individual AH samples of POAG patients and evaluated the possibility of using lncRNAs from AH as POAG diagnostic biomarkers. These results may serve as a basis for further research in this area.

Conclusion

lncRNAs T267384, ENST00000607393, and T342877 may be potential biomarkers for POAG diagnosis. Moreover, ENST00000607393 might be a new therapeutic target for trabecular meshwork calcification that is involved in IOP misregulation and glaucoma.

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Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2018.12.011>.

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