Expression of the Primary Angle Closure Glaucoma (PACG) Susceptibility Gene PLEKHA7 in Endothelial and Epithelial Cell Junctions in the Eye

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PURPOSE. The role of the recently identified primary angle closure glaucoma (PACG) susceptibility gene, pleckstrin homology domain containing, family A member 7 (PLEKHA7), in PACG is unknown. PLEKHA7 associates with apical junctional complexes (AJCs) and is thus implicated in paracellular fluid regulation. We aimed to determine PLEKHA7’s localization in the eye and its association with AJCs to elucidate its potential role in PACG.

METHODS. Total RNA from ocular tissues was isolated and analyzed by real-time PCR. Frozen and paraffin-embedded human globes were sectioned and used for immunohistochemistry and immunofluorescence analysis.

RESULTS. Specific PLEKHA7 expression was found in the muscles, vascular endothelium, and epithelium of the iris, ciliary body and ciliary processes, trabecular meshwork (TM), and choroid. PLEKHA7 expression in musculature and vascular endothelium was confirmed with smooth muscle marker, SMA, and endothelium marker, PECAM-1, respectively. At the above sites, PLEKHA7 colocalization was seen with adherens junction markers (E-cadherin and β-catenin) and tight junction markers (ZO-1).

CONCLUSIONS. Specific localization of PLEKHA7 was found within PACG-related structures (iris, ciliary body, and choroid) and blood–aqueous barrier (BAB) structures (posterior iris epithelium, nonpigmented ciliary epithelium, iris and ciliary body microvasculature). The association of PLEKHA7 with AJCs in the eye suggests a potential role for PLEKHA7 in PACG via fluidic regulation. Novel expression of PLEKHA7 was also seen in the ocular smooth muscles and vascular endothelia.

Keywords: glaucoma, gene expression, blood–ocular barrier

Primary angle closure glaucoma (PACG) is a major cause of glaucoma in Asia and carries a 3-fold increase in risk of severe bilateral visual impairment compared to primary open angle glaucoma.1 Angle closure disease covers a spectrum that starts with primary angle closure suspects (PACS) and culminates in PACG. In PACG, the elevation in intraocular pressure (IOP) occurs from iris–trabecular meshwork contact in the narrowed angles of PACS eyes that prevents aqueous outflow. The iridocorneal apposition, a key feature of PACG, is known to occur due to the interplay of both anatomical and dynamic factors in angle closure eyes.2 However, the cellular and biochemical mechanisms that give rise to PACG, or how they interact during the progression of the disease, is not known.

In order to better understand the biological pathways and mechanisms for PACG pathogenesis, we recently conducted a two-stage genome-wide association study (GWAS) to identify PACG susceptibility genes.1 This led to the identification of three loci, one of which was an intragenic variant within the PLEKHA7 gene on chromosome 11. This gene encodes the protein PLEKHA7, which belongs to the pleckstrin homology domain containing protein family. Studies have suggested that PLEKHA7 protein is capable of stabilizing apical junctional adhesion complexes (AJCs).3

Apical junctional adhesion complexes are composed of adherens junctions (AJs) and tight junctions (TJs) and are involved in cell-to-cell adhesion and the regulation of paracellular permeability, respectively, and thus the maintenance of tissue homeostasis. Adherens junctions regulate the epithelial and endothelial permeability via the maintenance of TJ integrity. Tight junctions also help to maintain the apical–basal polarity in addition to regulating the paracellular transport of ions, water, and solutes. PLEKHA7 protein has been found to...
stabilize these AJCs by bridging transmembrane cadherins, such as E-cadherin, to the intracellular microtubule network of proteins. Recent studies have also indirectly linked PLEKHA7 protein to the TJ and the actin cytoskeleton through the interaction with the paracoding-ZO-1 complex, a major scaffold of TJs.3–5

This association of PLEKHA7 with AJCs and its putative function in cell-cell adhesion and paracellular permeability led to our hypothesis that PLEKHA7 may play a role in PACG. In the pathogenesis of PACG, there is increasing evidence that in addition to the above-mentioned anatomical factors, dynamic (nonpupil block) mechanisms such as iris volume changes during pupillary dilation and choroidal effusion may be involved.6 Some authors have attributed the increase in iris volume (or lesser reduction in volume) during dilation to the movement of extracellular fluid between the iris stroma and anterior chamber, suggesting that there is aberrant fluid movement within the iris microvasculature and posterior pigmented iris epithelium.7 Similarly, a proposed mechanism for choroidal effusion in PACG is increased vascular permeability to proteins and thus an increase in the extravascular space.8 Other studies have shown that in both acute and chronic angle closure eyes,9 breakdown of the blood-aqueous barrier (BAB) leads to inflammation in the anterior chamber.9 Although it is yet unclear if the breakdown occurs as a primary or secondary event, it has been suggested that this leakage of inflammatory proteins and cells may further contribute to pressure elevation in angle closure development.9 Furthermore, AJs and TJs have also been implicated in the maintenance of the BAB.10

Based on these previously reported findings, the aberrant intercellular fluidics and the breakdown of the BAB are possible mechanisms that underlie the pathogenesis of PACG. The association of PLEKHA7 at a cellular level with AJCs may reflect its potential function in regulating paracellular fluid movement in PACG-related structures.

The primary aim in this study was therefore to determine if PLEKHA7 protein’s distribution within the eye includes the iridocorneal angle outflow pathway involved in PACG. In addition, we aimed to confirm the association of PLEKHA7 with AJCs by colocalization with TJ- and AJ-specific markers. Together, these findings would support our hypothesis that PLEKHA7 is present in PACG-related structures of the eye, that its function is mediated by its association with AJCs, and that it may contribute to alter intercellular fluid dynamics that have recently been suggested to be involved in PACG.

Materials and Methods

Antibodies

Primary antibodies utilized included Sigma-Aldrich Corp., (St. Louis, MO, USA) polyclonal rabbit anti-PLEKHA7 (1:50); Santa Cruz Biotecnology (Dallas, TX, USA) monoclonal mouse anti-Myc (1:50); Cell Signaling Technology (Boston, MA, USA) monoclonal mouse anti-β-catenin (1:50); Molecular Probes (Carlsbad, CA, USA) monoclonal mouse anti-ZO-1 (1:50); Sigma-Aldrich Corp. monoclonal rat anti-DECMa for detection of E-cadherin (1:50); Zuelig Pharma (Hong Kong) monoclonal rat anti-PECAM-1 (1:50); Santa Cruz Biotechnology monoclonal mouse anti-Occludin (1:50); and Dako (Glostrup, Denmark) monoclonal mouse anti-SMA (1:50). Secondary fluorescently labeled antibodies used included anti-mouse or anti-rabbit FITC (1:300) and Cy3 (1:300) (Jackson Laboratories, Westgrove, PA, USA).

Immunofluorescence Confocal Microscopy

Enucleated human eyes were purchased from Lions Eye Institute for Transplant and Research (Tampa, FL, USA) or Singapore General Hospital. Eyes were fixed in 4% paraformaldehyde (PFA) for 16 hours and embedded in optimal cutting temperature (OCT) embedding medium (Tissue-Tek OCT Compound; Sakura Finetek USA, Torrance, CA, USA). The eyes were cryosectioned (10 μm) and transferred to glass slides.

Frozen cryostat sections were left to dry for 1 hour at room temperature (RT); slides were rehydrated with 0.1% PBS-Tween for 10 to 15 minutes, and the PBS-Tween was removed. Sections were blocked for 1 hour at RT with the antibodies dilution buffer. Primary and secondary antibodies were diluted in 10% FBS, 0.1% PBS-Tween; 1× pen/strep were incubated overnight at 4°C and 1 h at RT, respectively. Vectashield with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA) was applied to the cryosections, which were then overlaid with coverslips (Fisher Scientific, Pittsburgh, PA, USA) and stored in the dark at 4°C until viewing with confocal microscopy. Confocal microscopy was performed with an Olympus Fluvoview 1000 confocal microscope (Center Valley, PA, USA).

Plasmid Construction

Complementary DNA clone RC209861 for PLEKHA7 was purchased from Origene Technologies (Rockville, MD, USA). Full-length and truncated forms of PLEKHA7 were amplified by standard PCR methods and subcloned into pCI-puro vector. pCI-puro was constructed from pCI-neo (Clontech Laboratories, Mountain View, CA, USA) backbones.

Short Hairpin RNA (shRNA) Plasmids

Bacterial clones of pLKO.1-nontarget control with GCGGCCGCATAGCCTAAATTTTCTCAGAAAATATTAGCGCTATCGCGCTTTTTTT and pLKO.1-M53 with shRNA sequence of CCGGGACCTTCTCAAGGATCGAAGTCTCGAGACTTCGCTATCGCGCTTTTTT were purchased from Sigma-Aldrich Corp.

Cell Culture and Transfection

MDCK cell line was cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS. Cells were transfected using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA), according to the manufacturer’s protocol. Transfected cells were harvested 1 day post transfection for confocal analysis.

Immunohistochemistry

Enucleated eyes were fixed in 10% formalin. Paraffin sections were cut at 4 μm and placed onto coated slides. Immunohistochemistry was performed using Leica Bond Polymer Refine detection kit DS9800 (Leica Microsystems GmbH, Wetzlar, Germany). Slides were heated for 20 minutes at 60°C and then loaded onto the Leica Bond III autostainer for immunohistochemical staining. Staining on the autostainer consisted of dewaxing, antigen retrieval using Leica Bond ER2 solution for 20 minutes at 100°C, and antibody incubation for 20 minutes at RT followed by staining using the Bond polymer refine kit, after which the slides were dehydrated and coverslipped.

Immunofluorescence Confocal Microscopy of Cells

Cells grown on coverslips (Fisher Scientific) were washed two times with PBS. The cells were then fixed with a 1:1 ratio of
Gene Expression Analysis

**PLEKHA7** gene expression was assessed by real-time RT-PCR, using primers **PLEKHA7-F** 5′-TAAAAGCACGCCGAAGAAGG-3′ and **PLEKHA7-R** 5′-TGTCGGCAGTAAGTAGTGA-3′, with product size 206 bp. **ACTB** was used as an amplification and normalization control for real-time RT-PCR, using primers **ACTB-F** 5′-CAACCGGAGAAGAGTGA-3′ and **ACTB-R** 5′-CCAGAGGGTGACGAGGG-3′, with product size 97 bp.

Total RNA was extracted from a variety of ocular tissues (sclera, lens, iris, ciliary body, trabecular meshwork [TM], cornea epithelium, cornea endothelium, cornea stroma, retina, choroid, optic nerve and optic nerve head) with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer’s protocol. First-strand cDNA synthesis was performed with the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) using random primers. Real-time quantitative RT-PCR was performed with Applied Biosystems SYBR Fast qPCR kit and analyzed with ABI 7500 real-time PCR system (Applied Biosystems, Carlsbad, CA, USA) following manufacturer’s instructions. β-actin was selected as endogenous control for normalization. All PCR reactions were done in duplicate for the target gene, **PLEKHA7**, and endogenous control, β-actin (**ACTB**).

Statistical Analyses

**PLEKHA7** gene expression levels were calculated using the 2^-ΔΔCt^ comparative threshold cycle method. All relative values of different parts of the eye were normalized using β-actin as endogenous control. Experiments were carried out three times (n = 3), while mean and standard deviations were calculated and presented as averages ± SD (error bars). Statistical data were analyzed by unpaired homoscedastic t-test; two-tailed P values less than 0.05 were considered statistically significant, and values less than 0.01 were considered more statistically significant. Accordingly, one asterisk corresponds to P values less than 0.05; two asterisks indicates P values less than 0.01.

## Results

**PLEKHA7 Antibody Specificity**

To verify the specificity of PLEKHA7 antibody, confocal immunofluorescence microscopy was performed on canine MDCK cells transiently transfected with PLEKHA7-Myc. Exogenously expressed PLEKHA7 in MDCK cells (Figs. 1B-E) was present at cell–cell junctions, where it partially colocalized with the AJ marker E-cadherin (Figs. 1F-I). The PLEKHA7 antibody, raised against a peptide sequence downstream of the PH domain of human PLEKHA7 (Fig. 1A), did not detect the endogenous canine PLEKHA7 protein in MDCK cells (data not shown). Cells depleted of overexpressed PLEKHA7 via a PLEKHA7-specific shRNA showed a significant reduction in PLEKHA7 immunofluorescence staining along the cell–cell junctions (Figs. 1J–M), without affecting the junctional staining of E-cadherin.

Western blot analysis of PLEKHA7 protein levels confirmed a dose-dependent downregulation of PLEKHA7 protein levels in PLEKHA7 shRNA-treated cells, consistent with the reduced staining (Fig. 1N, lanes 4, 5), whereas PLEKHA7 protein levels remained unaffected in cells transfected with the nontarget control (Fig. 1N, lane 3). These data thus confirm the specificity of the PLEKHA7 antibody.

**PLEKHA7 mRNA Expression Profiling in Ocular Tissues**

Quantitative real-time PCR (qRT-PCR) performed using β-actin as the endogenous control showed that **PLEKHA7** expression was most abundant at sites within the anterior chamber that are relevant to PACG, such as the iris, ciliary body (nonpigmented epithelium of the ciliary processes and ciliary body muscles), TM, and optic nerve (including the optic nerve head). As the expression of **PLEKHA7** in retina had been established previously, mRNA expression levels of **PLEKHA7** in other ocular tissues were compared to that of retina. Retinal expression of **PLEKHA7** was also used to stratify the expression levels of **PLEKHA7** into high (>1.5-fold elevation in comparison to retinal expression of **PLEKHA7**), moderate (similar to retinal expression), and low (lower than retinal expression). High levels of **PLEKHA7** expression were detected in TM, iris, ciliary body, and optic nerve, which expressed 1.5- to 2.7-fold more **PLEKHA7** transcripts compared to retina. The corneal epithelium showed **PLEKHA7** expression levels similar to those in the retina, whereas lower expression was seen in the corneal stroma and endothelium, lens, and choroid (Fig. 2A). **PLEKHA7** expression in sclera was not detected by qRT-PCR.

**PLEKHA7 Protein Distribution in Ocular Tissues**

Immunohistochemistry localization of anti-PLEKHA7 antibodies demonstrated high PLEKHA7 protein expression within the anterior segment of the eye (Fig. 2C). These regions included Schlemm’s canal, TM, ciliary muscle, the bilayered epithelium of the ciliary processes, the anterior border of the iris, and the iris dilator muscle, as well as blood vessels in the various parts of the eye. We also found PLEKHA7 in the retinal pigment epithelium (Fig. 2B) as previously shown. Mirroring the results of the qRT-PCR, PLEKHA7 protein expression was also absent in the sclera.

**Localization of PLEKHA7 With Smooth Muscle Actin and PECAM-1**

Localization of PLEKHA7 With Smooth Muscle Actin (SMA). The positive staining of PLEKHA7 in the smooth muscles of the ciliary body and iris corresponded to smooth muscle and the myofibroblastic lining of the larger blood vessel walls, stained with smooth muscle actin (alpha-SMA, a smooth muscle– and myofibroblast-specific marker; Fig. 2C) by immunohistochemistry.

Localization of PLEKHA7 With PECAM-1. PLEKHA7 was also detected within the microvasculature of the ciliary body, iris (Fig. 2D), and choroid (Fig. 2B). Distinct positive staining for PLEKHA7 corresponded to the endothelial cells lining the blood vessels, stained with the endothelial cell–specific marker PECAM-1.
Subcellular Localization of PLEKHA7 With TJ and AJ Markers

Colocalization of PLEKHA7 With ZO-1. Colocalization of PLEKHA7 with the TJ marker ZO-1 was detected in anterior segment structures relevant to PACG such as the iris, TM, epithelium of the ciliary processes, and ciliary body musculature as well as the endothelium of vessels.

ZO-1 was present in the iris dilator muscle (IDM) and the polarized bilayer epithelia of the ciliary processes, and ciliary body musculature as well as the endothelium of vessels.

ZO-1 was present in the iris dilator muscle (IDM) and the polarized bilayer epithelia of the ciliary processes (Fig. 3A). ZO-1 also colocalized with PLEKHA7 at the anterior border of the posterior iris epithelium located just posterior to the IDM (Fig. 3B), while little staining for ZO-1 or PLEKHA7 was observed at the basal side of the posterior iris epithelium (Fig. 3B).

Using confocal fluorescent microscopy, PLEKHA7 within the PECAM-1-positive endothelial vasculature at a subcellular level was found to colocalize with ZO-1 in the intercellular clefts of the endothelium of the fenestrated capillaries of the ciliary body (Fig. 3C, upper). At the apical pole of the PECAM-1-positive iris vascular endothelial cells, ZO-1 also appeared as a punctate junctional staining that partially colocalized with PLEKHA7 (Fig. 3C, lower).

ZO-1 staining at the bilayer ciliary epithelium showed partial colocalization with PLEKHA7 in the region where the apical domains of nonpigmented ciliary epithelium and pigmented ciliary epithelium are juxtaposed (Fig. 3D).

Colocalization of PLEKHA7 with TJs was further confirmed with occludin (another TJ marker) using confocal fluorescent microscopy, which showed a colocalization profile similar to that of ZO-1 and PLEKHA7 (Fig. 3E).

Colocalization of PLEKHA7 with TJs was further confirmed with occludin (another TJ marker) using confocal fluorescent microscopy, which showed a colocalization profile similar to that of ZO-1 and PLEKHA7 (Fig. 3E). In addition, the staining profile of occludin in the anterior chamber structures was similar to that reported by Sonsino et al., who analyzed localization of occludin and ZO-1 within the human BAB.

**Figure 1.** Characterization of PLEKHA7 antibody. (A) Schematic representation of location of the epitope recognized by the commercial antibody. The pleckstrin homology domain (PH), WW protein interaction domain, SbcC ATPase domain (SbcC), and DUF domain (DUF) of PLEKHA7 are shown (not drawn to scale). (B–E) MDCK cells transfected with C-terminally tagged pCI-puro-PLEKHA7-Myc construct stained with anti-Myc (red) and anti-PLEKHA7 (green) antibodies and DAPI (DNA; blue) (Scale bars: 5 μm). (F–M) MDCK shRNA knockdown control cells transfected with PLEKHA7-Myc cDNA and either pLKO-nontarget control (F–I) or pLKO-MS3 shRNA to mediate knockdown of exogenously expressed PLEKHA7 (J–M). Cells were stained with anti-E-cadherin (red) and anti-PLEKHA7 (green) antibodies and DAPI (DNA; blue) (Scale bars: 2 μm). (N) Efficiency of shRNA-mediated knockdown of PLEKHA7-Myc was assessed by Western blotting of cell lysates with anti-Myc antibody to detect PLEKHA7-Myc within MDCK cells transfected with either pLKO-nontarget (pLKO-NT) control or PLEKHA7-specific shRNA containing plasmid pLKO-MS3. Lane 1: mock; Lane 2: PLEKHA7-Myc only; Lane 3: PLEKHA7-Myc with 5 μg pLKO-NT; Lane 4: PLEKHA7-Myc with 2.5 μg pLKO-MS3; Lane 5: PLEKHA7-Myc with 5 μg pLKO-MS3.

**PLEKHA7 Expression**

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**Figure 2.** PLEKHA7 distribution within the anterior chamber of the eye. (A) Quantitative analysis of PLEKHA7 mRNA expression in the sclera, retina, trabecular meshwork (TM), iris, ciliary body (CB), optic nerve head (ONH), optic nerve (ON), cornea epithelium (C-Epi), cornea endothelium (C-Endo), cornea stroma (C-stroma), lens, and choroid assessed by real-time PCR. Relative values were normalized using β-actin as endogenous control. \(N = 3;\) error bars: SD of the mean. \(^* P < 0.05; \) \(^** P < 0.01.\) (B) Immunofluorescence labeling of PLEKHA7 in the retinal pigment epithelium (RPE) and choroid (Scale bar: 150 μm). (C) PLEKHA7 staining at Schlemm’s canal (SC), ciliary muscle (CM), ciliary processes (CP), and iris dilator muscle (IDM) (inset, black arrow) but not at the sclera (S). (PLEKHA, red chromogen; magnification: ×10.) SMA staining at the SC, IDM, and CM (SMA, diaminobenzidine; magnification: ×40). (D) PLEKHA7 and SMA cytoplasmic staining at the CM and IDM (magnification: ×40); PLEKHA7 also stains the CD31-positive endothelial cell lining of the ciliary body vessel (CBV) and iris vessel (IV) (magnification: ×40).
**FIGURE 3.** PLEKHA7 expression colocalization with ZO-1 (TJ marker). (A) Colocalization of the TJ marker ZO-1 (green) and PLEKHA7 (red) at the anterior and posterior chambers of the adult eye. Extensive colocalization is observed at regions such as the trabecular meshwork (TM), ciliary processes (CP), and ciliary muscle (CM). ZO-1 staining is not observed at the iris dilator muscle (IDM) within the iris (I) but is observed at the sclera (S). (B) Colocalization of ZO-1 (green) with PLEKHA7 (red) along the lateral membranes of anterior border of the posterior iris epithelium (arrowhead) (Scale bars: 40 μm). (C) High magnification showed colocalization of ZO-1 (green) with PLEKHA7 (red) at intercellular clefts within ciliary body vessels (CBV), and partial colocalization was also detected at the apical lumen-facing surface of the iris vessel endothelium (IV).
Colocalization of PLEKHA7 With E-Cadherin and β-Catenin. The AJ marker E-cadherin in the IDM and epithelia of the ciliary processes colocalized with the PLEKHA7 staining profile (Fig. 4A).

β-catenin, which interacts with E-cadherin,11 also colocalized with PLEKHA7 (Fig. 4B) in tissue sites associated with PACG and the iris muscles. In the TM region, however, the labeling pattern for PLEKHA7 and β-catenin showed only partial colocalization.

PLEKHA7 immunostaining of the endothelium of the greater arterial circle of the iris also showed a diffuse β-catenin staining (Fig. 4B). PLEKHA7 colocalized with β-catenin at the interendothelial clefs within the endothelium of fenestrated ciliary body capillaries (Fig. 4C, upper) and at focal sites within the nonfenestrated iris capillaries (Fig. 4C, lower).

At higher resolution, the immunofluorescence labeling of AJ β-catenin (Fig. 4D) differed from TJ ZO-1 (Fig. 3D), as β-catenin signals were detected at cell borders of both nonpigmented ciliary epithelium and pigmented ciliary epithelium cells (Fig. 4D) while ZO-1 signals were restricted to the region where apical domains of nonpigmented ciliary epithelium and pigmented ciliary epithelium are juxtaposed.

**DISCUSSION**

In the eye, PLEKHA7 has been described only in the outer limiting membrane of the retina and at the basal layer of the corneal epithelium.3 In our study, examination of PLEKHA7 mRNA in an expanded repertoire of ocular tissue confirmed its expression in structures that are particularly relevant to PACG, such as the iris, ciliary body, TM, and also optic nerve (including the optic nerve head) (Fig. 2A). Further confirmation on histological analysis, using a PLEKHA7 antibody validated for specificity, showed strong PLEKHA7 immunoreactivity that corresponded to the gene expression profile (Figs. 2C, 2D). We also confirmed, using histology and immunohistochemistry, that PLEKHA7 is distributed to the (1) epithelial cells of the posterior iris epithelium and nonpigmented epithelium of the ciliary processes (Fig. 2C); (2) smooth muscles of the IDM, ciliary body muscles, and TM (Fig. 2D, right); and (3) endothelial cells within the iris and ciliary body microvasculature (Fig. 2D, left) as well as in the choroidal vasculature.

The specific localization of PLEKHA7 to these anterior segment structures involved in the pathogenesis of PACG support the hypothesis that PLEKHA7 is a plausible candidate gene for PACG susceptibility and that changes in the protein could be relevant to the pathogenesis of PACG within the eye. In addition, the localization of PLEKHA7 protein in the vascular endothelium particularly within the microvasculature of the iris, ciliary body (Fig. 2D), and choroid (data not shown) is of interest, as this is the first time that PLEKHA7 has been described in vascular endothelial cells in addition to epithelia.

PLEKHA7 has been shown to stabilize AJCs in other studies;3-7 however, in the eye, its subcellular role and functions are unknown. As the secondary aim in this study, we used confocal microscopy to determine the colocalization of PLEKHA7 with AJ (E-cadherin and β-catenin)- and TJ (ZO-1)-specific markers. We show that PLEKHA7 colocalizes with AJs (e.g., E-cadherin or β-catenin) in the IDM, iris, and ciliary body microvasculature and epithelia of the ciliary processes (Figs. 4A–C). PLEKHA7 also colocalizes with ZO-1-associated TJs in the anterior border of the posterior iris epithelium (Fig. 3B), which were recently found to also play a role in the BAB.1,2,13 In addition to the other sites of the BAB such as the nonpigmented ciliary epithelium and the iris and ciliary body microvasculature (Figs. 3A, 3C). As ZO-1 expression in the eye is not exclusive to TJs,14,15 a second TJ marker, occludin, was used to confirm the PLEKHA7 colocalization with the TJ (Supplementary Fig. S1). These findings support PLEKHA7 presence in AJCs of the eye, suggesting that PLEKHA7 is involved in stabilizing AJC through its association with AJs and TJs.5,15 within cells of the ocular tissue associated with dynamic factors for PACG (such as the iris, ciliary process, anterior segment microvasculature, and choroidal vasculature).

As mentioned earlier, the expression of PLEKHA7 protein in the vascular endothelium has not been described previously, and its colocalization with the AJ marker β-catenin raises the possibility of PLEKHA7 playing an important role in the regulation of vascular permeability and thus intercellular fluidics within the eye.

The presence of PLEKHA7 within the AJCs of PACG-related sites, such as the iris stromal vessels and posterior iris epithelium, provides a possible explanation linking dysregulated paracellular fluid movement that may contribute to the variations in iris volume during dilation. PLEKHA7 in the choroidal vasculature may also play a role in the increased permeability of the choroid vessels and contribute to choroidal effusion that has recently been described in PACG.

The BAB incorporates several morphological components—(1) the endothelium of capillaries within the ciliary body and iris, (2) iris epithelium, (3) nonpigmented ciliary epithelium, and (4) the unidirectional valve created by the iris resting on the lens—that help to ensure that plasma-derived proteins are prevented from entering the posterior chamber of the eye.1,16 The principal subcellular components of the BAB are the TJs between nonpigmented ciliary epithelial cells and the TJs between endothelial cells of the iris microvasculature as well as the iris posterior epithelium (a continuation of the nonpigmented ciliary epithelium).15 Hence, the location of PLEKHA7 at AJs and TJs at sites representing the BAB also suggests that PLEKHA7 may play role in maintenance of the BAB and its breakdown as described in PACG.9

Finally, we also found expression of PLEKHA7 within the SMA-positive smooth muscles cells of the ciliary body and TM (Figs. 2C, 2D). Previous studies have shown that the TM contains smooth muscle-like properties and expresses SMA.17 Together with the smooth muscles of the ciliary body, these play a role in the regulation of the outflow through the contractility of the TM and ciliary body.18 Our finding of PLEKHA7 in the TM and ciliary body muscles (Figs. 2B, 2C) raises the possibility that PLEKHA7 may also play a role in the regulation of aqueous outflow that may contribute to the development of PACG.

As a next step, our current histological analysis will require supportive functional data. Unfortunately, incorporation of expression data from ocular tissues of patients with PACG carrying the risk variant was not possible due to the lack of availability of relevant ocular tissue at the present time. Indeed, in order to conclusively prove that PLEKHA7 is the PACG gene behind the association signal at this genetic locus, the causal variant needs to be identified and characterized in relevant cells. Based on the protein localization data presented here and the analysis of PLEKHA7 in other cell types, we have suggested several physiological functions for PLEKHA7 at multiple sites in...
FIGURE 4. PLEKHA7 expression colocalization with AJ markers. (A) Immunofluorescence localization of the AJ marker E-cadherin (green) and PLEKHA7 (red). Extensive colocalization is observed at iris dilator muscle (IDM) within the iris (I), ciliary muscle (CM), and nonpigmented ciliary epithelium (NPCE) located at the ciliary processes (CP). No PLEKHA7 is observed at the sclera (S). (B) PLEKHA7 (red) and the AJ marker β-catenin (green) colocalize at trabecular meshwork (TM) and greater arterial circle of iris (GAC). (C) Colocalization of β-catenin (green) with PLEKHA7 (red) at intercellular clefts within ciliary body vessels (CBV) and partial colocalization at the iris vessel endothelium (IV) with focal β-catenin staining (Scale bars: 10 μm). (D) Staining of β-catenin (green) occurs at the border between NPCE and PCE with β-catenin labeling the lateral membranes of both NPCE and PCE epithelial layers (Scale bars: 10 μm).
the eye. These putative functions will need to be further investigated in transgenic animal models or in relevant ocular cell lines where the gene is depleted or overexpressed so that PLEKHA7’s role in maintaining the barrier properties of these cells and/or contractility can be evaluated. Currently, based on its localization in the eye and potential physiological role, which are in good agreement with clinically observed features of PACG, PLEKHA7 is an attractive candidate gene worthy of further investigation. In summary, this study describes the expression profile and localization of PLEKHA7 protein within the ocular structures of the eye that are related to PACG. Our findings show that the subcellular role of PLEKHA7 in these PACG-relevant sites and vascular endothelium may be related to AJ and TJs, which are important in the maintenance of cell-to-cell adhesion and paracellular fluid transport and may thus contribute to the altered regulation of fluid dynamics that have recently been linked to the pathogenesis of PACG. Future functional studies will aim to validate the role of PLEKHA7 in PACG pathogenesis.

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