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Alterations in anterior lens capsule structure and LTBP-2 expression in primary angle-closure glaucoma

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ABSTRACT

Objective This study investigated the role of latent-transforming growth factor β-binding protein 2 (LTBP-2) in primary angle-closure glaucoma (PACG) by analysing its expression and the ultrastructure of the anterior lens capsule in PACG patients with age-related cataract (ARC).

Methods Tissue samples of the anterior lens capsule were collected from patients undergoing cataract phacoemulsification surgery. Patients in the experimental group were diagnosed with primary angle-closure (PAC) combined with ARC (PAC+ARC) and PACG combined with ARC (PACG+ARC). The control group consisted of patients with only ARC. The techniques used included scanning electron microscopy, real-time fluorescence quantitative polymerase chain reaction (RT-qPCR), western blotting and immunofluorescence.

Results Ultrastructural analysis revealed disordered connections in PAC+ARC, loose connections in PACG+ARC and well-ordered connections in ARC. RT-qPCR and western blotting showed significantly lower LTBP-2 mRNA and protein expression in PAC+ARC and PACG+ARC than in ARC, with PAC+ARC having the lowest levels. Immunofluorescence confirmed these findings, showing varying LTBP-2 fluorescence intensities across groups.

Conclusion The study identified ultrastructural changes in the anterior lens capsules in PACG accompanied by reduced LTBP-2 expression, especially in PAC+ARC patients. This suggests a potential role for LTBP-2 in PACG development, warranting further investigation.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Primary angle-closure glaucoma (PACG) is a major cause of blindness, particularly in Asian populations. It is known that mutations in genes related to extracellular matrix components, such as latent-transforming growth factor β-binding protein 2 (LTBP-2), may contribute to the development of PACG. However, the specific role and expression patterns of LTBP-2 in the anterior lens capsule of PACG patients have not been thoroughly investigated.

WHAT THIS STUDY ADDS

⇒ This study reveals significant ultrastructural changes and decreased expression of LTBP-2 in the anterior lens capsule of PACG patients. Specifically, primary angle-closure+age-related cataract (ARC) patients showed the most pronounced reduction in LTBP-2 levels. These findings suggest that LTBP-2 plays a crucial role in maintaining the structural integrity of the lens capsule and that its dysregulation may contribute to the pathogenesis of PACG.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ The insights from this study highlight the importance of LTBP-2 in the structural maintenance of the lens capsule, suggesting potential therapeutic targets for PACG. Future research could explore interventions aimed at restoring or compensating for LTBP-2 function. Clinically, these findings may prompt the development of new diagnostic markers and treatment strategies to prevent or mitigate the progression of PACG.

INTRODUCTION

Glaucoma, which will affect approximately 111.8 million people worldwide in 2040, is the most prevalent neurodegenerative disease and the second leading cause of blindness.[1 2] Primary angle-closure glaucoma (PACG), responsible for 25% of all glaucoma cases globally, is more visually damaging than the more common variant, primary open-angle glaucoma.[3] A substantial majority of PACG patients reside in Asia, with China alone accounting for nearly 1.5 million cases of unilateral blindness and an additional 1.5 million cases of bilateral blindness due to PACG. Additionally, China has over 28 million people classified as primary angle-closure suspects (PACS), with an estimated 9 million individuals having primary angle-closure (PAC) and 4.5 million diagnosed with PACG.[4 5]

PACS is diagnosed using gonioscopy if ≥180° iridotrabecular contact (ITC) is present. To diagnose PAC, the presence of ≥180° ITC must be accompanied by an elevated intraocular pressure (IOP) or the existence of peripheral anterior synechiae without any known ocular disease or other underlying cause. If glaucomatous optic neuropathy is present, PAC is then classified as PACG. For classification purposes, glaucomatous nerve damage is defined as an abnormality in the

optic disc or retinal nerve fibre layer or a reliably reproducible abnormality in the visual field.[6 7]

Multiple susceptibility gene loci have been identified in PACG, including *PLEKHA7*, *COL11A1*, *PCMTD1-ST18*, *EPDR1*, *GLIS3*, *DPM2-FAM102A*, *CHAT* and *FERMT2*.[8 9] However, these findings are not definitive, as independent studies have not consistently replicated them. Recent studies have suggested that mutations in *LTBP-2* may be associated with PACG.[10] Latent-transforming growth factor β-binding protein 2 (LTBP-2), a member of the fibrillin and latent-transforming growth factor-β (TGF-β) binding protein (LTBP) superfamily, plays a role in extracellular matrix (ECM) synthesis and is highly expressed in the lens capsule.[11 12] LTBP-2 forms a latent complex with TGF-β, facilitating its storage and release.[13]

In clinical practice, during continuous curvilinear capsulorhexis in cataract surgery with phacoemulsification for PACG patients, signs such as decreased elasticity of the lens capsule, relaxation of capsule folds, instability of the capsular bag and deviation of the capsulorhexis opening are often observed. Some patients also have relaxation or even rupture of the zonular. Multiple studies have confirmed that zonular lesions are involved in the pathogenesis of PACG.[14] Whether structural and functional abnormalities exist in the lens capsule of PACG is still unclear. It is also unknown if relaxation of the anterior lens capsule is involved in the development of PACG. Furthermore, no reports have investigated whether the distribution and changes of LTBP-2 in the anterior lens capsule differ between PAC and PACG. Therefore, further research is necessary to explore the varying expression of LTBP-2 across different stages of PACG and its potential influence on lens capsule relaxation.

METHODS

Subjects

From December 2019 to January 2022, at the Tianjin Ophthalmic Hospital, 101 cases (101 eyes) diagnosed in the cataract department with age-related cataract (ARC) were used as the control group. A total of 100 cases (100 eyes) diagnosed in our hospital's glaucoma department with PAC and ARC (PAC+ARC) and 105 cases (105 eyes) diagnosed with PACG and ARC (PACG+ARC) were included in the experimental group. Both the experimental and control groups excluded patients with other ocular diseases, such as pseudoexfoliation syndrome, uveitis, vitreous haemorrhage, retinal diseases and optic pathway disorders, or a history of ocular trauma. It also excluded patients with systemic diseases, such as hyperthyroidism, hypothyroidism, connective tissue diseases and diseases requiring long-term use of glucocorticoids, anticonvulsants, chemotherapy drugs and other medications that have significant systemic effects. During the operation, continuous circular capsulorhexis of 5.5 mm was performed, and the anterior capsule of the human lens was removed. Subsequently, the specimens were collected.

Scanning electron microscopy (SEM)

Samples of the anterior lens capsules were obtained from five patients each in the PAC+ARC, PACG+ARC and ARC groups during surgery. After 5.5 mm continuous circular capsulorhexis, the specimens were washed in saline solution to remove residual red blood cells. The specimens in each group were fixed in Eppendorf tubes containing 2.5% glutaraldehyde at 4°C for 24 hours. Following fixation, the specimens were washed two times with Phosphate-buffered saline (PBS) and fixed with 1% osmium tetroxide for 1 hour at 4°C and then washed two additional times with PBS. Dehydration was performed using a mixture of acetone and isoamyl acetate, followed by pure isoamyl acetate. Critical point drying was performed by replacing ethanol with liquid carbon dioxide, gradually increasing the temperature and pressure to reach the critical point and slowly releasing the carbon dioxide. The dried specimens were subjected to gold coating using a rotating evaporation source. Finally, the samples were observed using SEM (Hitachi-SU8200).

Quantitative real-time PCR (RT-qPCR)

Samples were obtained from 28, 30 and 30 patients in the PAC+ARC, PACG+ARC and ARC groups, respectively. After performing 5.5 mm continuous circular capsulorhexis during surgery, the anterior lens capsule samples were gently washed to remove residual red blood cells. The samples were stored in Eppendorf tubes containing RNA stabilisation reagent (RNAwait) at −80°C. RNA was extracted using RNeasy Micro Kit columns (Qiagen, Valencia, California, USA) according to the manufacturer's instructions. The RNA concentration was measured using a NanoDrop 2000 system (Thermo, Boston, Massachusetts, USA) and 500 µg of RNA was used to synthesise cDNA using HiScript II Reverse Transcriptase (Vazyme, Nanjing, China). The expression levels of LTBP2 were measured using the SYBR Green system (Roche, Pleasanton, California, USA). The cycle threshold for each mRNA was normalised to that of glyceraldehyde-3-phosphate dehydrogenase and averaged. Each experiment was independently repeated three times. The primer sequences are listed in online supplemental table 1.

Western blotting

Samples of anterior lens capsules were collected during surgery from 61 patients with PAC and ARC, 64 patients with PACG and ARC and 60 patients with ARC alone. The collected samples were gently cleaned and preserved at −80°C for subsequent analysis. Proteins in these samples were extracted using radioimmunoprecipitation assay (RIPA) lysis buffer (P0013K; Beyotime, Shanghai, China), and the supernatant containing the proteins was isolated by centrifugation. The protein concentration was determined using a bicinchoninic acid (BCA) assay. The samples were mixed with sample buffer and stored at −80°C. Subsequently, western blotting was performed by initially preparing an 8% gel and conducting

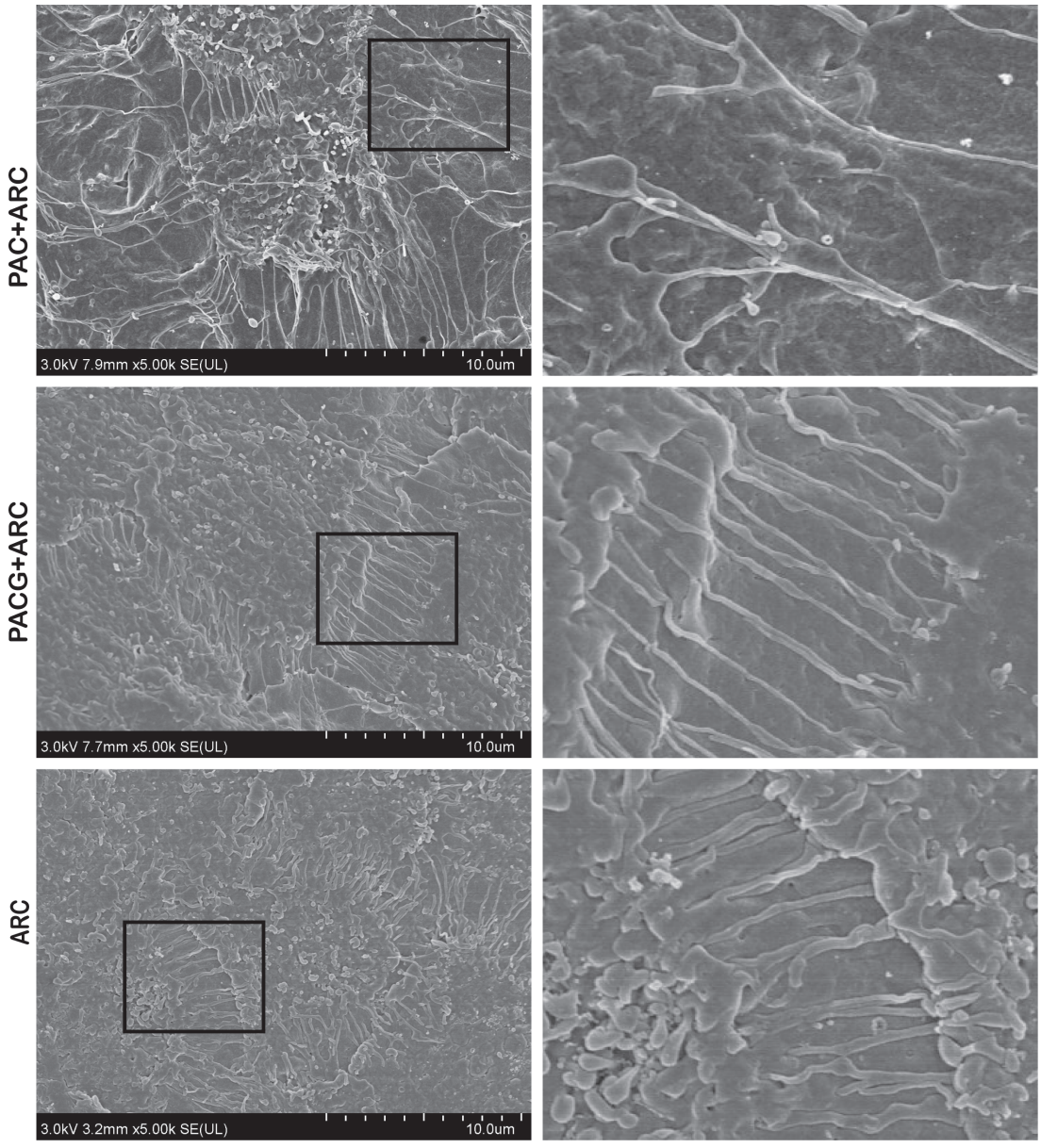

Figure 1 Scanning electron microscopy at ×5000 magnification. The image on the left shows an enlarged view of the area highlighted by the black box on the right. ARC, age-related cataract; PAC, primary angle-closure; PACG, primary angle-closure glaucoma.

electrophoresis to separate the proteins. The proteins were then transferred onto 0.45 µm polyvinylidene fluoride membranes. After transfer, the membranes were washed three times with tris-buffered saline with tween 20 (TBST) for 10 min each, blocked with 5% non-fat milk at room temperature for 1 hour, washed three times with TBST for 10 min each and incubated overnight at 4°C with primary antibodies against LTBP-2 (1:500; cat. no. sc-166199; Santa Cruz Biotechnology, Dallas, Texas, USA) and β-actin (1:5000; cat. no. 66009-1-ig; Proteintech). The primary antibodies were diluted using QuickBlock primary antibody dilution buffer for western blot (Beyotime: P0256). The membranes were then washed and treated with a goat anti-mouse Horseradish Peroxidase (HRP) -conjugated IgG (H+L) cross-adsorbed secondary antibody (G-21040; BioLegend, San Diego, California, USA). Finally, the protein bands were visualised using a chemiluminescent substrate, and their expression was analysed using ImageJ software.

Immunofluorescence staining

Samples of the anterior lens capsules were obtained from six patients in the PAC+ARC group, six patients in the PACG+ARC group and six patients in the ARC group during surgery. The samples were gently cleaned and fixed with 4% paraformaldehyde for 10 min and then washed three times with PBS for 5 min each wash. Subsequently, the samples were placed on adhesive glass slides and air dried for 30 min. Permeabilisation was achieved using phosphate-buffered saline with Triton X-100 (PBST), and blocking was performed using a 10% goat serum solution. The samples were incubated overnight

at 4°C with primary antibodies targeting LTBP-2 (1:50; cat. no. sc-166199; Santa Cruz Biotechnology, Dallas, Texas, USA). After washing, the samples were incubated with (1:200; proteintech, China) for 1 hour at room temperature in the dark. One drop of HelixGen antifade fluorescence mounting medium containing 4′,6-diamidino-2-phenylindole was added to each sample, and the samples were photographed using a laser scanning microscope (Leica SP8 confocal microscope, Germany). Image analysis was performed using ImageJ software.

Statistical analysis

Descriptive statistics were calculated for the PAC+ARC, PACG+ARC and ARC groups. One-way analysis of variance (ANOVA) was used to test for differences in continuous variables across groups, while the χ^2 test was employed to compare categorical variables, such as sex distribution, between groups. These analyses were conducted using GraphPad Prism software (V.8.0.1; GraphPad Software, San Diego, California, USA). Statistical significance was set at p<0.05.

RESULTS

Summary of demographic comparisons between study groups

Online supplemental table 2 provides a comparison of the sex distribution, mean age and number of subjects across the three groups: PAC+ARC, PACG+ARC and ARC. Sex was distributed evenly among the groups, with males constituting between 41.9% and 42.6%, and females comprising between 57.4% and 58.1% of patients. The mean age across the groups was comparable, ranging from 62.9 to 63.3 years. Each group had 100–105 participants. Statistical analyses revealed no significant differences in age (p=0.926) or sex distribution (p=0.960) across the groups. Overall, the descriptive statistics and p values in the table demonstrate that key demographic variables, such as age and sex, were evenly matched among the PAC+ARC, PACG+ARC and ARC patient groups.

Ultrastructural organisation of the anterior lens capsule

Under a scanning electron microscope at ×5000 magnification, the ultrastructure of the anterior lens capsule was observed in the three patient groups. In the PAC+ARC group, the connections between lens epithelial cells were disorganised and sparse. In the PACG+ARC group, connections between the lens epithelial cells were visible, but the intercellular connections were relatively loose. In the ARC group, the cellular connections were dense and neatly arranged (figure 1).

Expression status of LTBP-2 mRNA in the anterior lens capsule

The expression of LTBP-2 mRNA in the anterior lens capsule of the human eye was evaluated using RT-qPCR. As shown in figure 2, the relative expression levels of LTBP-2 mRNA were 0.11±0.03 in the PAC+ARC group and 0.32±0.08 in the PACG+ARC group. Both groups exhibited significantly lower relative expression levels of LTBP-2 mRNA than the control group (ARC group), which had a relative expression level of 1.00±0.00 (p<0.0001). The PAC+ARC group demonstrated a significantly lower relative expression level of LTBP-2 mRNA than the PACG+ARC group (p<0.01).

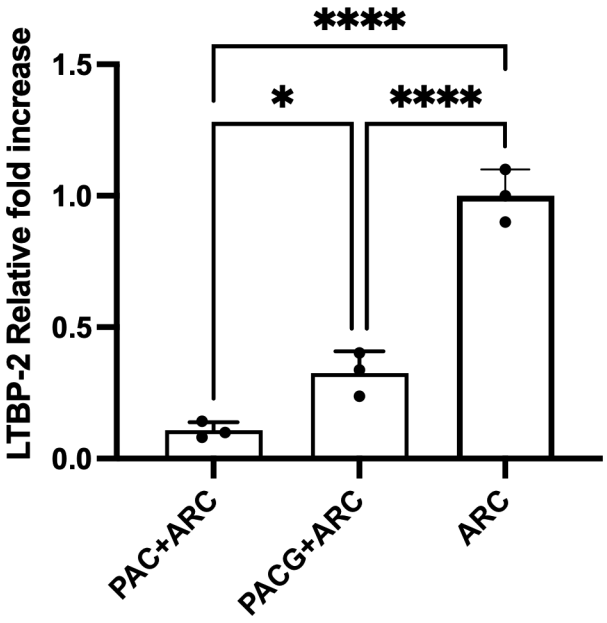

Figure 2 Relative mRNA expression levels of LTBP-2 in the anterior lens capsule in each group (*p<0.01, ****p<0.0001). Data are presented as mean±SD. ARC, age-related cataract; LTBP-2, latent-transforming growth factor β-binding protein 2; PAC, primary angle-closure; PACG, primary angle-closure glaucoma.

Expression status of LTBP-2 protein in the anterior lens capsule

Western blotting was performed to semiquantitatively analyse the protein expression of LTBP-2 in the anterior lens capsule of the human eye (figure 3). The results showed that the relative protein expression levels of LTBP-2 (normalised to the reference protein β-actin) in the different groups were as follows: PAC+ARC: 0.27±0.03, PACG+ARC: 0.75±0.09, ARC: 0.99±0.12. The protein expression levels of LTBP-2 in both the PAC+ARC and PACG+ARC groups were significantly lower than that in the ARC group (p<0. 05). Furthermore, the protein expression level of LTBP-2 in the PAC+ARC group was significantly lower than that in the PACG+ARC group (p<0. 05). These findings are consistent with the mRNA expression levels of LTBP-2.

Expression and distribution of LTBP-2 in the anterior lens capsule

LTBP-2 is an ECM protein that interacts with fibronectin and contains microfibrils. Immunofluorescence staining revealed that LTBP-2 in the anterior lens capsule was

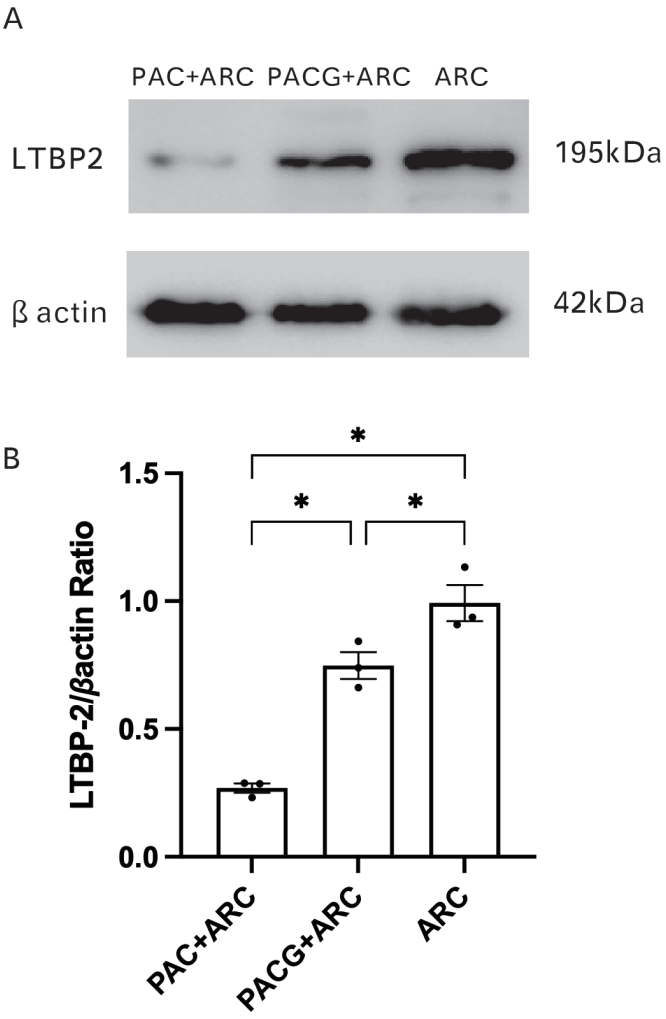

Figure 3 Analysis of LTBP-2 protein expression in the human anterior lens capsule ((A) depiction of LTBP-2 and β-actin protein bands; (B) quantitative representation of LTBP-2 protein levels across different groups. '*' denotes a significance level of p<0. 05.). ARC, age-related cataract; LTBP-2, latent-transforming growth factor β-binding protein 2; PAC, primary angle-closure; PACG, primary angle-closure glaucoma.

predominantly diffusely distributed in the ECM. In figure 4A, grey fluorescence represents LTBP-2, whereas blue fluorescence represents cell nuclei. In the PAC+ARC group, LTBP-2 exhibited weak fluorescence; in the PACG+ARC group, LTBP-2 showed moderate fluorescence; and in the ARC group, LTBP-2 displayed strong fluorescence.

Semiquantitative analysis of LTBP-2 protein expression was performed for each group (figure 4B). The total fluorescence intensity values were as follows: PAC+ARC group: 245.2±112.2; PACG+ARC group: 725.8±252.7; and ARC group: 1316.0±146.5. The total fluorescence intensity in the PAC+ARC group was significantly lower than that in the ARC group (p<0.05). Similarly, the total fluorescence intensity in the PACG+ARC group was significantly lower than that in the ARC group (p<0.001). Additionally, the total fluorescence intensity in the PAC+ARC group was significantly lower than that in the PACG+ARC group (p<0.05).

DISCUSSION

Glaucoma is considered a leading cause of irreversible blindness worldwide, with approximately half of all glaucoma patients residing in Asia. The prevalence of PACG is higher in Asia than in Europe and Africa.[3 15] PACG can be further classified into three categories: PACS, PAC and PACG. During the continuous circular capsulorhexis step in cataract surgery, we observed that the anterior lens capsule was more relaxed in patients with coexisting PACG and PAC compared with those with ARC alone, characterised by reduced elasticity, increased deformability and the presence of folds. Within the PACG group, the anterior lens capsule was more relaxed in patients with PAC than in those with PACG.

Healthy eyes exhibit strong lens zonules connecting the ciliary body and lens capsule. Instability of these structures may cause capsular bag relaxation during capsulorhexis, suggesting potential weaknesses. This is mirrored in patients with pseudoexfoliation syndrome, where pseudoexfoliative material from the ciliary and lens epithelium accumulates, becomes altered and eventually detaches lens zonules.[16 17] These changes can cause intraoperative symptoms, including capsular bag instability characterised by anterior capsule wrinkling, whole-bag movement, posterior capsule laxity, ciliary sulcus collapse and vitreous prolapse around the capsular bag.[18] These findings suggest that there may be issues with the lens capsule, ciliary body or zonules in patients with PACG. To further explore these issues, we conducted SEM of the anterior lens capsule in three groups of patients. The PAC+ARC group exhibited disrupted and sparse intercellular connections, whereas the PACG+ARC group showed intercellular connections that were relatively loose. In contrast, the ARC group displayed dense and orderly arranged intercellular connections (figure 1). These microstructural changes suggest that alterations in the structure of the anterior lens capsule in angle-closure glaucoma may be related to decreased elasticity of the lens capsule. Decreased elasticity and relaxation of the lens capsule could potentially cause anterior displacement and convexity of the lens, further resulting in shallowing of the anterior chamber and induction of angle closure. The specific mechanisms require further research.

LTBP-2, encoded by 36 exons, is a matrix protein and a member of the superfamily comprising fibrillin and LTBP.[19] TGF-β, a multifunctional pleiotropic growth factor, primarily inhibits cell proliferation and regulates ECM production. Secreted as a latent complex composed of mature dimeric growth factors, latent-associated peptides and LTBP, these complexes are stored in the fibrillin-rich ECM, where they await activation by appropriate signals.[20 21] LTBP-2 is expressed in elastic tissues and interacts with fibrillin-1 and fibrillin-5 via their carboxyl and amino terminals, respectively, but not with fibrillin-2. LTBP-2 promotes fibrillin-5 fibrous deposition

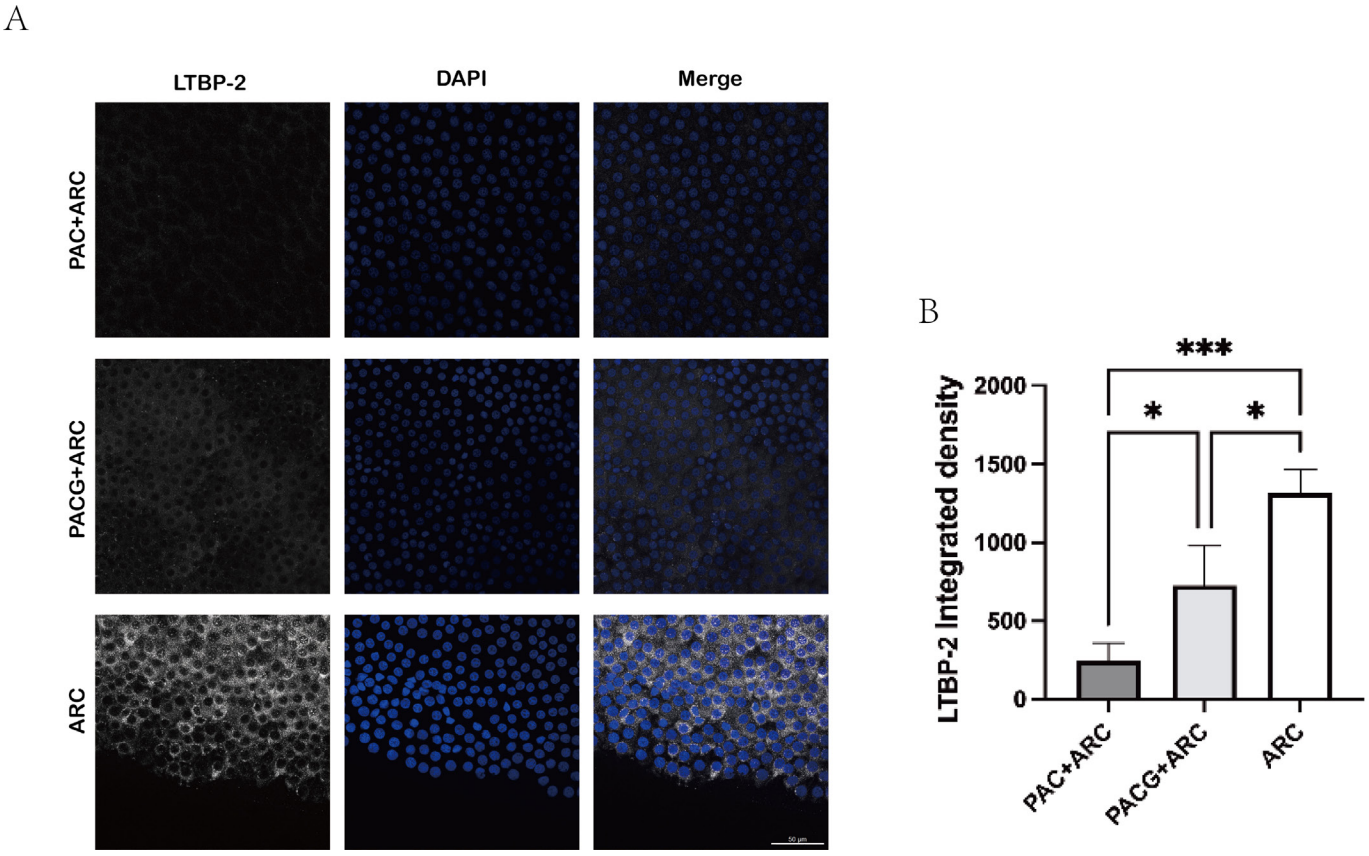

Figure 4 Immunofluorescence staining and quantitative analysis of LTBP-2 in the human anterior lens capsule. (A) Immunofluorescence staining for LTBP-2 (grey) and DAPI nuclear staining (blue) in human anterior lens capsule sections at ×400 magnification. () Quantitative analysis of LTBP-2 fluorescence intensity. Data are presented as mean±SD. *P<0.05, **p<0.01, ***p<0.001. ARC, age-related cataract; DAPI, 4′,6-diamidino-2-phenylindole; LTBP-2, latent-transforming growth factor β-binding protein 2; PAC, primary angle-closure; PACG, primary angle-closure glaucoma.

on fibrillin-1 microfibrils, serving as a structural and regulatory protein, and impacting microfibril function in elastic fibre assembly.[11 22] These results suggest that the function of LTBP-2 is related to that of microfibrils and elastic fibres. Defects in microfibrils may cause glaucoma by altering tissue biomechanical properties and/or influencing TGF-β signalling.[23] Homozygous LTBP-2 mutations reportedly cause human glaucoma, and mutations also affect other ocular diseases, such as megalocornea, spherophakia and Weill-Marchesani syndrome.[24–27] Symptoms in many LTBP-2 mutation patients include laxity or dislocation of the lens zonules, and LTBP-2 deficiency weakens the strength and durability of zonular fibres.[28] Currently, there is no consensus regarding how LTBP-2 causes glaucoma. Possible explanations include anterior displacement of the lens that pushes the iris forward and causes angle closure,[29 30] or trabecular meshwork ECM changes that cause increased IOP.[31 32]

Elevated IOP is a significant risk factor for glaucoma, primarily due to damage to the trabecular meshwork and Schlemm's canal aqueous humour drainage pathways.[33] The trabecular meshwork consists of irregular networks of connective tissue bundles, through which the aqueous humour enters Schlemm's canal and eventually flows into the venous system. While only LTBP-2 among the various LTBP proteins does not covalently interact with TGF-β, non-covalent interactions between LTBP-2 and TGF-β cannot be ruled out.[34] The ECM of the trabecular meshwork is a critical component of IOP regulation.[31] Some studies have suggested that the impact of oxidative stress and LTBP-2 gene expression downregulation on the ECM in glaucoma may be mediated by activation of the TGF-β and BMP signalling pathways.[35] Oxidative stress can disrupt ECM morphology and cell-to-cell interactions. In cultured human scleral cells, oxidative stress induces the production of TGF-β1, thereby increasing the expression of ECM protein-encoding genes.[36] Oxidative stress promotes the secretion of TGF-β2 in reactive astrocytes in the human optic nerve head. Hydrogen peroxide and TGF-β2 both increase Hsp27 expression by activating p38MAP kinase and reduce oxidative stress, and TGF-β2 may help reduce the characteristic changes in reactive astrocytes in the optic nerve head in glaucoma patients.[37] Various subtypes of TGF-β and BMP are cytokines that belong to the TGF-β protein superfamily. Members of this family affect various cells and physiological functions throughout the body and influence developmental processes, and TGF-β2 and BMP can promote and reduce the expression of ECM proteins in lenses and TM cells.[38]

Increased TGF-β2 expression in the aqueous humour and trabecular meshwork in primary open-angle glaucoma leads to trabecular ECM deposition, causing elevated IOP, during which BMP may regulate the production of ECM induced by TGF-β2.[39]

In our study, we examined the expression of LTBP-2, which is known to be most highly expressed in the lens capsule among the various ocular tissues. LTBP-2 is expressed in the transition region between the trabecular meshwork, non-pigmented ciliary epithelium, corneal stroma and sclera, but is minimally expressed in the cornea, iris and sclera.[40] Thus, we selected the anterior lens capsules of the enrolled patients as the study targets. Through RT-qPCR, immunoblotting and immunofluorescence staining analysis, we found that the expression of LTBP-2 mRNA and protein was lower in both the PAC+ARC and PACG+ARC groups than in the ARC group, with the PACG+ARC group showing lower LTBP-2 expression than the PAC+ARC group. We hypothesise that changes in the expression and distribution of LTBP-2 in the anterior lens capsule of angle-closure glaucoma lead to alterations in zonular microfibrils and the ECM. This results in decreased elasticity of the lens capsule and may be an important component in the pathogenesis of PACG.

This study had several limitations. First, the inherent constraints of SEM may have led to artefacts during sample preparation. Second, the surgical instruments may have damaged the lens capsule during the procedure. Finally, while our study noted a decline in the nucleic acid and protein expression of LTBP-2 in the anterior capsule in the PAC+ARC and PACG+ARC groups compared with the control group, our understanding of LTBP-2's role in PACG is speculative and based on previous research. Further animal experiments are needed to clarify the role of LTBP-2 and develop new therapeutic strategies for PACG.

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Contributors The research design was orchestrated by XT and XY, while LY conducted the meticulous data collection and analysis. The initial manuscript was collaboratively crafted by LY and XT, with subsequent writing and comprehensive revisions jointly undertaken by XT and XY. XT is the guarantor.

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Competing interests None declared.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants. The research followed the tenets of the Declaration of Helsinki. This research plan was approved by the ethics committee of Tianjin Ophthalmic Hospital (2020051). Participants gave informed consent to participate in the study before taking part.

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