·Experimental Research·

Specific expression of transmembrane protein, TMEM26, in retinas and its association with primary open-angle glaucoma

Yin Yi, Mao Yao, Yang Zhenglin, Huang Lulin

School of Medicine, University of Electronic Science and Technology of China, Department of Medical Genetics, Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital, Sichuan Provincial Key Laboratory for Human Disease Gene Study, Chengdu 610072, China

Corresponding author: Huang Lulin, Email: huangluling@yeah.net

[Abstract] Objective To predict the transmembrane structure of transmembrane protein 26 (TMEM26), we investigated its expressions in human and mouse retinas, and we investigated the relationship between *TMEM26* and primary open-angle glaucoma (POAG).

Methods The transmembrane structures of TMEM26 protein in humans and mice were obtained by inputting the amino acid sequences of TMEM26 into the transmembrane protein structure prediction software, MemBrain. The expression and location of TMEM26 in human and mouse retinas were observed using frozen sections of human and mouse retinas stained with anti-TMEM26 antibody. The sections were from a human donor and five SPF-grade C57BL/6 mice. The possible function of the *TMEM26* gene and its influence on eyes were implied on the basis of its specific expression in the retina. The association of single nucleotide polymorphisms (SNPs) in the *TMEM26* region with POAG was searched in a published database. The use and care of animals complied with Regulations on the Management of Experimental Animals. This research protocol was approved by an Ethics Committee of Sichuan Provincial People's Hospital (No. 2019-36).

Results Both human and mouse TMEM26 involved eight transmembrane proteins with eight similar hydrophobic transmembrane domains, four hydrophilic cytoplasmic domains, and five hydrophilic extracellular membrane domains. Small differences in the number of amino acid residues in the domains of TMEM26 were found between humans and mice. In both human and mouse retinas, TMEM26 was specifically expressed only in the outer (OPL) and inner (IPL) plexiform layers. TMEM26 was weakly associated with POAG in published data.

Conclusions TMEM26 is a multi-pass transmembrane protein, mainly expressed in the IPL and OPL of the retina, and the *TMEM26* gene is weakly associated with POAG.

[Key words] Transmembrane structure of transmembrane protein 26 protein, human; Tmem26 protein, mouse; Protein transmembrane structure; Retina; Inner plexiform layer; Outer plexiform layer; Primary open-angle glaucoma

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As functional proteins, membrane proteins are important in living organisms, and are involved in cell proliferation and differentiation, growth and metabolism, material exchange, and energy transformation[1]. Membrane proteins can be classified into membrane-integrated, membrane-anchored, and extrinsic membrane proteins. Transmembrane proteins, the membrane-integrated proteins, are responsible for signal transduction and transmembrane transport of small molecule substances or ions[2]. Because there is at least one segment of transmembrane structure in transmembrane proteins, the transmembrane protein structure includes the transmembrane, extracellular, and cytoplasmic domains, which are important for the function of these proteins. Located on chromosome 10, transmembrane protein 26 (TMEM26) is a 6-exon gene expressed in both human and mouse, which encodes multi-pass transmembrane proteins with a relative molecular mass of about 41,600[3]. Currently, it has been verified that TMEM26 is expressed in mouse embryos and is regulated by time of development and space[3]; it is expressed in breast cancer cells, and its protein structure affects the response to drug therapy[4]. It is also expressed in airway epithelium and lung parenchymal cells, and is a candidate gene related to decreased lung function and the pathogenesis of chronic obstructive pulmonary disease[5]. Moreover, TMEM26 has been identified as a surface marker for beige adipocytes[6-7]. Although TMEM26 is expressed in various tissues and cells, its specific functions, especially in the eye, remain unclear. Glaucoma is a major cause of blindness, and commonly includes chronic angle-closure glaucoma, primary open-angle glaucoma (POAG), and exfoliation glaucoma. The loss of retinal ganglion cells (RGCs) is a key pathogenesis of glaucoma[8-9]. The inner plexiform layer (IPL) is composed of a fibrous reticular layer formed by RGC dendrites, bipolar cell axons, and amacrine cells. Its thickness is a potential biomarker for POAG[10]. This study therefore aimed to characterize the expression of TMEM26 in the retina and its association between variations of *TMEM26* gene POAG, based on the reported data of this study.

1 Materials and Methods

1.1 Materials

1.1.1 Material sources. Five healthy SPF C57BL/6 mice (21 weeks old, 25−31 g) were provided by Jiangsu GemPharmatech, Nanjing, China), and their feeding and use conformed to the *Regulations on the Management of Experimental Animals*. One human eyeball was from a cadaver donor. This study was approved by the Ethics Committee of Sichuan Provincial People's Hospital [Approval No.: LS (Y) 2019 No. 36], and the families of the donors provided written consent for the use of human specimens.

1.1.2 Main reagents and instruments. The following reagents and instruments were used: 4% paraformaldehyde universal tissue fixative solution (Biosharp, Beijing, China); 30% sucrose solution (Beijing Solarbio Science & Technology, Beijing, China); optimal cutting temperature (OCT) cryo embedding medium (Sakura Finetek, Torrance, CA, USA); rabbit anti-*TMEM26* polyclonal antibody (TA330777; Origene, San Diego, CA, USA); Isolectin B4 594 (MKbio, Yamaguchi, Japan); 0.25% Triton X-100 (Beijing Solarbio Science & Technology); 5% goat serum, Alexa Fluor 488-labeled goat anti-rabbit fluorescent secondary antibody, 4',6-diamidino-2-phenylindole (DAPI) and phosphate-buffered saline (PBS) (Shanghai Beyotime Biotechnology, Shanghai, China); a dissecting microscope (SZX10; Olympus, Tokyo, Japan); microdissecting instruments (Shenzhen Biotechnology, Shenzhen, China); a freezing microtome (BK-2318; Biobase Biodustry (Shandong) Co., Ltd, Jinan, China); a constant temperature oven (DHP-9052; Shanghai Hecheng Instrument Manufacturing, Shanghai, China); and a laser scanning confocal microscope (LSM800; Carl Zeiss, Jena, Germany).

1.2 Methods

1.2.1 Prediction of transmembrane protein structures. The amino acid sequences of human and mouse *TMEM26* were

obtained from the UCSC website (http://genome.ucsc.edu/), and then inputted into MemBrain 3.0 (http://www.csbio.sjtu.edu.cn/bioinf/MemBrain/).

1.2.2 Observation of expression and localization of *TMEM26* in human and mouse retinas by immunofluorescence staining. The cornea was removed using a dissecting microscope, and the eyeball was fully immersed in 4% paraformaldehyde. After 2 h of fixation, residual paraformaldehyde was removed using phosphate-buffered saline (PBS) solution. The lens was then gently removed using tweezers and a dissecting microscope. The eyeball without the lens was dehydrated with 30% sucrose for 2 h, embedded with OCT embedding medium, and stored in a refrigerator at -80°C, followed by frozen sectioning. The sections were then baked in an oven at 37°C for 1 h to completely fix them on the slides. The position of the sections was circled with an immunohistochemical pen, and the sections were blocked with PBS containing 5% goat serum and 0.25% Triton X-100 for 1 h, then incubated with rabbit anti-*TMEM26* polyclonal antibody (1:200) in the dark at 4°C overnight with Alexa Fluor 488-labeled goat anti-rabbit secondary antibody (1:500), and then with DAPI in the dark for 2 h. After washing with PBS, the sections were observed and photographed using a laser scanning confocal microscope.

1.2.3 Co-localization of *TMEM26* and vessels by double immunofluorescence staining. *TMEM26* immunofluorescence staining was performed in accordance with Section 1.2.2, and retinal vascular endothelial cells were marked by Isolectin B4 staining. The cells were stained with Isolectin B4 (1:200) in the dark at 4°C overnight, washed with PBS for 10 min, mounted, and observed, and photographed using a laser scanning confocal microscope. DAPI, *TMEM26,* and retinal vessels were labeled blue, green, and red, respectively.

1.2.4 Single nucleotide polymorphism (SNP) analysis of *TMEM26*. Based on previous data of our group [11], the SNP variation of *TMEM26* was analyzed in 1,007 POAG patients and 1,009 normal controls. The data were then summarized to identify significant differences ($p < 0.01$).

1.3 Data processing

TMEM26 gene and protein information was obtained from NCBI (https://www.ncbi.nlm.nih.gov/) and the UCSC website. The transmembrane protein structure was predicted using MemBrain 3.0. DNAMAN (https://www.lynnon.com/) was used for amino acid sequence alignment, and transmembrane protein images were edited by AI.

2 Results

2.1 The transmembrane structure of TMEM26

According to the UCSC website, there were 368 amino acid residues in human TMEM26 and 366 amino acid residues in mouse TMEM26. The amino acid sequences of TMEM26 from the two sources were compared, showing a homology of 69.46%. The predicted transmembrane structure of human and mouse TMEM26 is shown in Figure 1, and the amino acid sequences are shown in Table 1. Human TMEM26 contained eight hydrophobic transmembrane domains, four hydrophilic cytoplasmic domains, and five hydrophilic extracellular domains, and had a predicted hydrophilic cytoplasmic structure connected by two helical transmembrane structures, with only two amino acid residues, which was a small cytoplasmic domain. Similar to the predicted transmembrane structure of human TMEM26 (Figure 1 and Table 1), mouse TMEM26 also contained eight predicted transmembrane domains, five hydrophilic extracellular domains, and four hydrophilic cytoplasmic domains. Both human and mouse TMEM26 had polypeptide chain N-termini and C-termini outside the cell membrane, and their transmembrane structures were similar, with similar transmembrane, extracellular, and cytoplasmic domains. However, the numbers of amino acid residues of the domains were slightly different. The predicted two "back-to-back" helical transmembrane structures in human TMEM26 consisted of 29 amino acid residues of E2-V30 and 25 amino acid residues of E33-K57, while those of mouse TMEM26 consisted of 30 amino acid residues of E2-K31 and 24 amino acid residues of H34-K57. In addition, the number of amino acid residues showed the greatest difference in the extracellular domain at the terminal of the polypeptide, i.e., there were two more amino acid residues in human TMEM26 than mouse TMEM26.

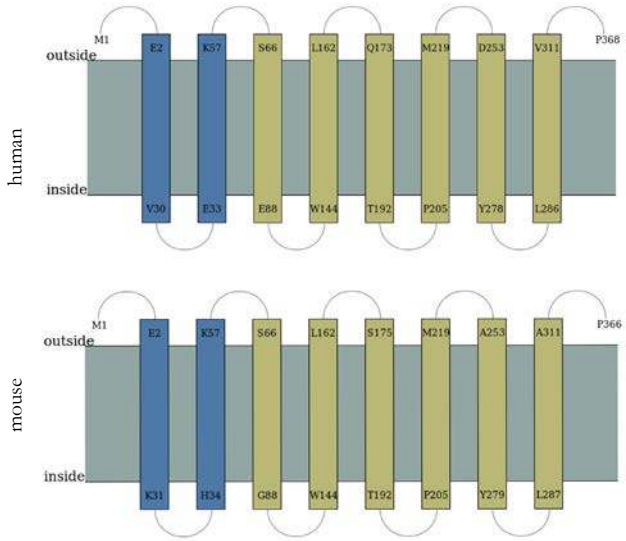

Figure 1. Transmembrane structure of the TMEM26 protein in humans and mice. The blue denotes back-to-back transmembrane helices, and the yellow denotes non-back-to-back transmembrane helices.

Table 1 Specific amino acid sequences of the transmembrane structure of TMEM26 protein [12-16]

	Amino acid residue sequence	Position		Amino acid residue sequence	Position
Human	1 - 1	Extracellular domain	Mouse	1 - 1	Extracellular domain
	2-30	Transmembrane domain		2-31	Transmembrane domain
	31-32	Cytoplasmic domain		32-33	Cytoplasmic domain
	33-57	Transmembrane domain		34-57	Transmembrane domain
	58-65	Extracellular domain		58-65	Extracellular domain
	66- 88	Transmembrane domain		66- 88	Transmembrane domain
	89-143	Cytoplasmic domain		89-143	Cytoplasmic domain
	144-162	Transmembrane domain		144-162	Transmembrane domain
	163-172	Extracellular domain		163-174	Extracellular domain
	173-192	Transmembrane domain		175-192	Transmembrane domain
	193-204	Cytoplasmic domain		193-204	Cytoplasmic domain
	205-219	Transmembrane domain		205-219	Transmembrane domain
	220-252	Extracellular domain		220-252	Extracellular domain
	253-278	Transmembrane domain		253-279	Transmembrane domain
	279-285	Cytoplasmic domain		280-286	Cytoplasmic domain
	286-311	Transmembrane domain		287-311	Transmembrane domain
	312-368	Extracellular domain		312-366	Extracellular domain

Note: The sequence of amino acid residues was N-terminal to C-terminal, and the position was relative to the cell membrane.

2.2 Expression of TMEM26 in retinas

In human retinas, the TMEM26 protein was mainly specifically expressed in the outer plexiform layer (OPL) and inner plexiform layer (IPL) of the retina, rather than in the nucleus (Figure 2). In a similar manner, in the mouse retinas, TMEM26 was expressed primarily in the OPL and IPL of the retina, rather than in the nucleus (Figure 3). In addition, isolectin B4 staining showed that TMEM26 was not expressed in vascular cells (Figure 4).

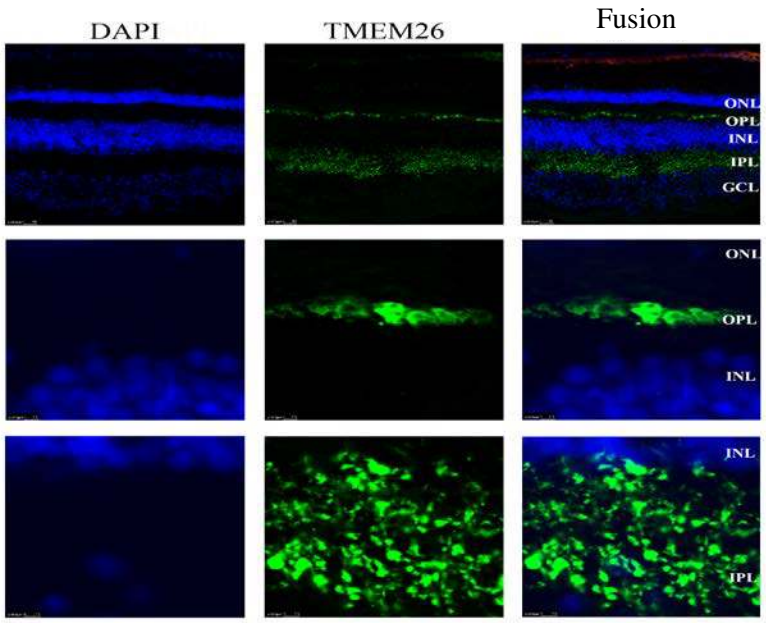

Figure 2. Expression of TMEM26 in human retina. TMEM26-positive cells are denoted by green fluorescence (Alexa Fluor 488), and nuclei are denoted by blue fluorescence (DAPI). A: Distribution of TMEM26 in human retina. TMEM26 was mainly expressed in the OPL and IPL (400×, bar = 50 μm). B: Expression of TMEM26 in the OPL (630×, bar = 7.5 μm) C: Expression of TMEM26 in the IPL (630×, bar = 7.5 μm). DAPI: 4,6-diamidino-2-phenylindole; TMEM26: transmembrane protein 26; OPL: outer plexiform layer; IPL: inner plexiform layer.

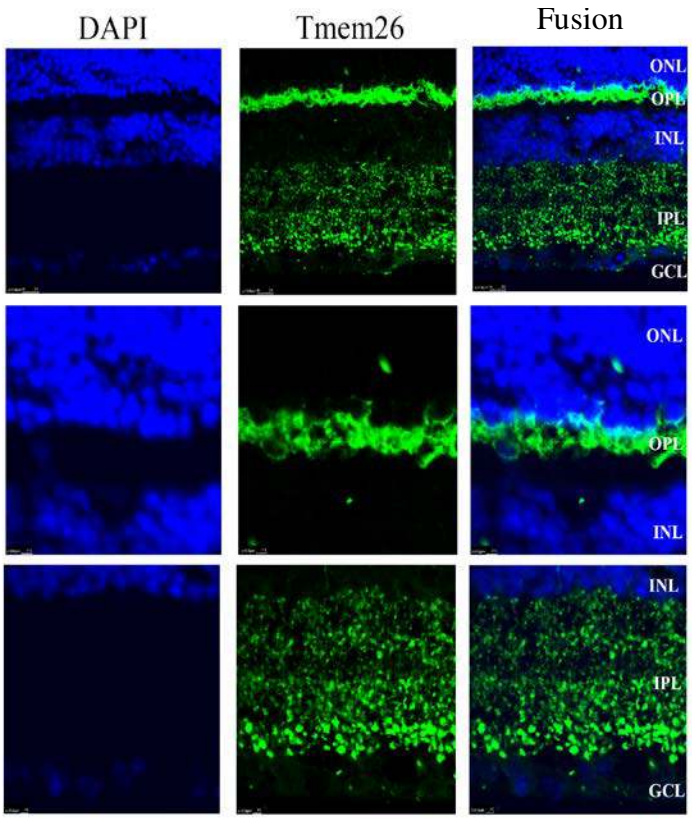

Figure 3. Expression of TMEM26 in mouse retina. TMEM26-positive cells are denoted by green fluorescence (Alexa Fluor 488), and nuclei are denoted by blue fluorescence (DAPI). A: Distribution of TMEM26 in mouse retina TMEM26 was mainly expressed in the OPL and IPL (400×, bar = 25 μm) B: Expression of TMEM26 in the OPL (630×, bar = 7.5 μm). C: Expression of TMEM26 in the IPL (630×, bar = 10 μm) DAPI: 4′,6-diamidino-2-phenylindole; TMEM26: transmembrane protein 26; ONL: outer nuclear layer; OPL: outer plexiform layer.

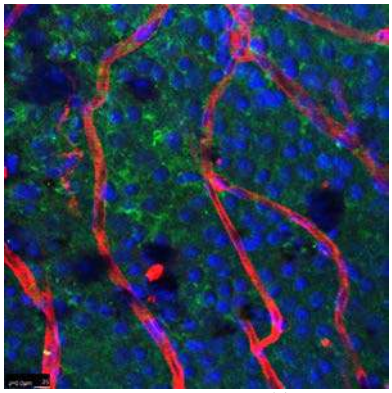

Figure 4. The co-location of TMEM26 protein and vessels in mouse retina. TMEM26 was not expressed in vessel cells (200×, bar = 25 μm). TMEM26-positive cells are denoted by green florescence (Alexa Fluor 488), mouse retinal vessels are denoted with red fluorescence (GS-IB4), and nuclei presented as blue fluorescence (DAPI).). DAPI: 4,6´-diamidino-2-phenylindole; TMEM26: transmembrane protein 26.

2.3 Association between TMEM26 SNP and POAG

TMEM26 was mainly expressed in the IPL and OPL of the retina, showing a specific expression pattern. To determine whether the presence of *TMEM26* was associated with eye disease, the association between *TMEM26* and POAG was determined, based on the published data of the Genome-wide Association Study (GWAS) [11]. The results showed that among 1,007 POAG patients with high intraocular pressure and 1,009 controls, 26 *TMEM26* SNP sites were weakly correlated with POAG (p = E^{-3}), with the 10:63203054 site having the strongest signal (p = 0.0017, odds ratio = 0.76), suggesting that it may have a weak protective effect on POAG (Table 2).

Table 2 Variation of the *TMEM26* gene in chromosome 10 among 1,007 POAG patients and 1,009 normal controls

SNP	BP	A1	F_A	F_U	A2	CHISQ	*P*	OR	SE	L95	U95
10:63177991	63177991	T	0.1979	0.2316	C	6.752	0.009363	0.8185	0.07713	0.7037	0.9521
10:63192407	63192407	A	0.2776	0.3186	AT	8.062	0.00452	0.8217	0.0692	0.7175	0.9411
10:63192414	63192414	T	0.2826	0.3221	A	7.412	0.00648	0.829	0.0689	0.7243	0.9489
10:63203054	63203054	C	0.1308	0.166	A	9.845	0.001703	0.7558	0.08943	0.6343	0.9006
10:63205791	63205791	C	0.1994	0.2331	T	6.714	0.009568	0.8194	0.07693	0.7047	0.9528
10:63206161	63206161	G	0.1994	0.2331	C	6.714	0.009568	0.8194	0.07693	0.7047	0.9528
10:63206361	63206361	A	0.1994	0.2331	G	6.714	0.009568	0.8194	0.07693	0.7047	0.9528
10:63216093	63216093	T	0.1979	0.2331	A	7.344	0.006728	0.8117	0.07705	0.6979	0.944
10:63221214	63221214	T	0.0732	0.0974	C	7.562	0.005963	0.7312	0.1142	0.5846	0.9146
10:63221768	63221768	T	0.1944	0.2301	C	7.649	0.005681	0.8073	0.07749	0.6935	0.9397
10:63230738	63230738	A	0.1969	0.2316	T	7.172	0.007406	0.8134	0.07721	0.6991	0.9462
10:63232038	63232038	T	0.1969	0.2321	C	7.372	0.006624	0.8111	0.07718	0.6972	0.9436
10:63233671	63233671	C	0.2595	0.2992	G	7.837	0.005118	0.8209	0.07055	0.7149	0.9426
10:63236941	63236941	T	0.1969	0.2316	G	7.172	0.007406	0.8134	0.07721	0.6991	0.9462
10:63240804	63240804	G	0.1969	0.2311	GA	6.974	0.008271	0.8156	0.07724	0.7011	0.9489
10:63248358	63248358	A	0.1969	0.2311	G	6.974	0.008271	0.8156	0.07724	0.7011	0.9489
10:63257002	63257002	C	0.2099	0.247	T	7.821	0.005163	0.8099	0.07545	0.6986	0.939
10:63259495	63259495	T	0.2099	0.247	C	7.821	0.005163	0.8099	0.07545	0.6986	0.939
10:63264872	63264872	C	0.1959	0.2336	T	8.446	0.003658	0.7993	0.07718	0.6871	0.9298
10:63267630	63267630	T	0.2074	0.2445	TA	7.891	0.004969	0.8085	0.07575	0.6969	0.9379
10:63270969	63270969	A	0.2074	0.244	C	7.687	0.005563	0.8107	0.07577	0.6988	0.9405
10:63271946	63271946	AT	0.2069	0.244	A	7.905	0.004931	0.8082	0.07581	0.6966	0.9377
10:63280046	63280046	C	0.2059	0.243	G	7.933	0.004854	0.8076	0.07593	0.6959	0.9372
10:63281627	63281627	A	0.2004	0.2386	G	8.518	0.003516	0.7999	0.07657	0.6884	0.9294
10:63286848	63286848	C	0.1949	0.2326	A	8.478	0.003594	0.7986	0.07731	0.6863	0.9293
10:63286859	63286859	A	0.1964	0.2331	C	8.005	0.004665	0.804	0.07717	0.6912	0.9353

Note: POAG: primary open-angle glaucoma; SNP: single nucleotide polymorphism; BP: base pairs (Hg19); A1: base of major allele; F_A: allele of affected patients; F_U: allele of healthy controls; A2: base of minor allele; CHISQ: χ^2 test; *P*: *P* value of fixed effect meta-analysis; OR: assessed value of the fixed effects; SE: standard error of coefficient; L95: lower confidence interval for the odds ratio in CMH; U95: upper confidence interval for the odds ratio in the Cochran–Mantel–Haenszel test.

3 Discussion

TMEM26, a member of the transmembrane protein family, is located on the cell membrane. Based on immunohistochemical staining, TMEM26 is expressed on the cytoplasmic side rather than the nucleus. Its expression is thought to occur in synapses, which secrete neurotransmitters. Membrane proteins, especially transmembrane proteins, are important for material transport and receptor recognition. The transmembrane structure of multi-pass transmembrane proteins can form material transport channels, while the hydrophilic domains of transmembrane proteins exert regulatory effects in channel switching and protein recruitment. Studying the transmembrane protein structure is therefore of great significance for understanding and identifying the function of transmembrane proteins. Comparisons of the transmembrane structures of human and mouse TMEM26 can provide the basis for animal experiments, and can provide possible etiologies of human eye diseases. Slight difference in the structures of human and mouse TMEM26 may suggest differences in the regulatory effects and material transport, but its specific significance remains to be determined. There have been few studies on the structure and function of TMEM26. As a protein-coding gene, the 6-exon mRNA of about 6 kb in *TMEM26* is the most common transcript. In addition, there are other transcripts. For example, an 82 bp exon 2a is present between exon 2 and exon 3 of *TMEM26* in mice, and the exon 2a insert leads to a frameshift mutation. Finally, early stop codons have been introduced into exon 3, resulting in early termination of translation and abnormal *TMEM26* gene expression [3].

The presence or absence of N-terminal signal peptides and the number of C-terminal domains will affect protein functioning[17]. It has been shown that TMEM26 is an

N-glycosylated protein in Jurkat T cells and breast cancer cells [7], and N-glycosylated TMEM26 can be retained on the plasma membrane for a longer time than non-glycosylated TMEM26, suggesting the important function of N-glycosylation of TMEM26 [4]. Hansel *et al.* [5] detected several isoproteins of TMEM26 in human breast cancer cells using western blotting, including the non-N-glycosylated protein $p40^{TMEM26}$ containing 368 amino acids, and two isoproteins of $p44^{TMEM26}$ and $p53^{TMEM26}$ formed by N-glycosylation modification [4]. In addition, errors may exist in the predicted eight spanning transmembrane regions due to the complex protein structural configuration. For example, Town *et al.* [3] and Yuan *et al.* [18] predicted that TMEM26 may have a seven-span transmembrane structure, however, the specific transmembrane structure of TMEM26 still needs further verification. TMEM26 protein is a surface marker for beige adipocytes. The browning and differentiation of white adipose tissues into beige adipose tissues is highly effective in the treatment of obesity. TMEM26 improves glucolipid metabolism in obese patients and ameliorates cardiac metabolism in HIV-infected patients, and can be used to detect the incremental effect of browning promoters on beige cells [19-22]. The GWAS has shown that *TMEM26* was associated with the depth of sleep, treatment of lung cancer with taxane, treatment of schizophrenia with antipsychotic clozapine, food allergies in European and American children, pathogenesis of diisocyanate asthma, and blood pressure elevation [23-28]. However, studies of the function of TMEM26 in the retina are rare.

There are two plexiform layers (OPL and IPL) between RGCs. Photoreceptor cells in the OPL are connected with longitudinal bipolar cells, and horizontal cells in the IPL are connected with RGCs. RGCs form synapses through interneurons in the OPL and IPL, which are connected with photoreceptors [29]. Photoreceptor cells are responsible for receiving light stimuli and transmitting light signals to nerve cells, and thus to the central nervous system via the optic nerve, resulting in the generation of visual images. In the present study, the results showed that TMEM26 was specifically expressed in the OPL and IPL of human and mouse retinas. Further studies of the function of TMEM26, especially in the retina and in vision, are of great significance.

Based on the specific expression patterns of TMEM26 in the OPL and IPL, its expression is thought to be located in the synapse, which can secrete neurotransmitters, and these neurotransmitters may affect RGCs or optic nerves. It has been reported that expression of TMEM26 was the highest in the IPL, and the IPL thickness was significantly correlated with POAG [10]; therefore, it has been speculated that *TMEM26* gene expression may be associated with POAG. According to data from our group, *TMEM26* gene expression is weakly correlated with POAG.

In conclusion, TMEM26 was expressed in both the OPL and IPL of the retina, and its expression was the highest in the IPL. SNP analysis confirmed that *TMEM26* gene expression was weakly correlated with POAG, but its specific function remains to be determined. In the future, *TMEM26*-knockout mouse models should be established to study the possible function of this gene during various human diseases.

Conflicts of interest: All authors declare no conflict of interest

Author Contribution Statement: Yin Yi: topics and experimental design, experimental studies, data analyses, statistical analysis, writing, and revision; Mao Yao: experimental studies and data analyses; Yang Zhenglin: work support and experimental guidance; and Huang Lulin: topics and experimental design, experimental guidance, revision, and funding.

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