The axial resolution of the Fourier-domain OCT (FD-OCT) is approximately 6 μm, which is significantly better than the 10 μm of standard OCTs. The external limiting membrane (ELM) (first highly reflective line), photoreceptor inner and outer segment junction (IS/OS) (second line), and the retinal pigment epithelium (RPE) (fourth line) can all be detected with an FD-OCT.[6–9] Disturbances of the IS/OS junction have been reported in some retinal diseases, eg, postoperative retinal detachment, central serous chorioretinopathy, and retinal dystrophy, which could not be detected ophthalmoscopically.[7,10–13] A disruption of the third highly reflective line of the FD-OCT images has been reported in cases of macular microhole.[13] Because the third line is considered to represent the outer segment of the photoreceptors because of its anatomical position, the disruption suggests an alteration of the photoreceptor outer segments. However, the origin of the third line has not been well investigated.

The adaptive optics (AO) fundus camera can obtain images with a transverse resolution of <2 μm, which makes it possible to resolve individual photoreceptors in living human eyes.[14–20] An increase in the cone spacing in retinas with cone dystrophy can be detected by AO imaging,[20,21] and the degree of the increased spacing is consistent with the decrease in visual function measured by mfERGs.[22] A disruption of the third bright line of the FD-OCT images is reported to cause a dark area in the AO fundus images.[14,15]

The purpose of this study was to determine whether the photoreceptor abnormalities in eyes with OMD can be detected tangentially in the FD-OCT images and en face in the AO fundus images.

Subjects and methods

Subjects

Eight eyes of four patients, who were diagnosed with OMD in the Department of Ophthalmology, Osaka University School of Medicine, were studied. The diagnosis of OMD was made by the following findings: normal fundus, normal fluorescein angiography, decreased visual acuity, normal full-field ERGs for both rod and cone components, and reduced amplitude of mfERGs in the central 5 degrees. All of the patients were classified as having sporadic OMD because none reported other family members with similar visual problems. Some of the characteristics of these OMD patients are summarized in Table 1.

The research protocol was approved by the Institutional Review Board of the Osaka University Medical School, and the procedures used conformed to the tenets of the Declaration of Helsinki. After the nature and possible consequences of the study were explained, a written informed consent was obtained from all patients.

The mfERGs were recorded with the Veris Clinic system (Mayo Co., Aichi, Japan) under standardized conditions. The stimulus array consisted of 103 hexagons, and the luminance of each hexagon was alternated between 200 cd/m^2 and 5 cd/m^2. A cross sectional image of the retina was obtained by a FD-OCT (RTVue-100; Optovue Inc., Fremont, CA). Horizontal and vertical scans were made through the fovea with a scan length of 6 mm. To improve the signal-to-noise ratio, consecutive images were averaged with the built-in software.

AO fundus images

The AO fundus images were taken through pupils dilated with topical tropicamide (0.5%) and phenylephrine (0.5%)

Table 1 Clinical characteristics of examined patients

Age	Sex	Eye	Visual acuity	Spherical equivalent (D)	Progression	Scotoma size	Fundus	Fullfield ergs	OCT findings	
									IS/OS	OS
48	M	OD	20/100	-0.25	3 years	None	Normal	Normal	Severely disrupted	-
		OS	20/66	0		central 10deg	Normal	Normal	Severely disrupted	-
38	M	OD	20/66	-6.25	Unknown	Perifoveal 2.5deg	Normal	Normal	Severely disrupted	-
		OS	20/66	-6		Central 3deg	Normal	Normal	Severely disrupted	-
43	M	OD	20/100	-1.75	18 years	Central 2.5deg	Normal	Normal	Severely disrupted	-
		OS	20/100	-1.75		Central 2.5deg	Normal	Normal	Disrupted	-
46	F	OD	10/100	-2.5	6 months	Central 2.5deg	Normal	Normal	Disrupted in fovea	+
		OS	20/20	-1.75		None	Normal	Normal	Almost normal	+

Abbreviations: erg, electroretinogram; F, female; IS, inner segment; M, male; OCT, optical coherence tomography; OS, outer segment.

and the ciliary muscle paralyzed. A detailed description of the custom-built AO fundus camera has been published,[23,24] and the principle of our flood illumination AO fundus camera is similar to that reported by Roorda and Williams.[15] Briefly, the main components of the camera were a nematic liquid crystal phase modulator (LCPM: X8267-12; Hamamatsu Photonics, Hamamatsu, Japan), a Hartmann-Shack wavefront sensor (HSWS: 28 × 28 lenslets; specially made by Topcon, Co., Tokyo, Japan), and a scientific CCD digital camera (C9100-02; Hamamatsu Photonics, Hamamatsu, Japan). The wavefront sensor measured the ocular wavefront up to the eighth Zernike order, and the phase modulator compensated for the measured wavefront aberrations. The system is also equipped with coaxial, 8-degree-wide viewing optics to identify the location and orientation of the highly magnified retinal images.

The retina was illuminated with a 2-ms flash (635-nm wavelength) from a laser diode, and a retinal image was obtained with a 6-mm diameter exit pupil. The patient was instructed to fixate a target in the center of the field. Frame-averaging was performed using custom software (Topcon) to improve the quality of the image. Overlapping images were merged using Photoshop (Adobe Systems Inc., San Jose, CA).

Results

The age of the patients ranged from 38 to 48 years. The best-corrected visual acuity (BCVA) at examination ranged from 20/200 to 20/20. None of the patients had an episode of sudden loss of visual acuity. The duration of decrease of vision ranged from 3 months to 3 years; six eyes out of eight had a central relative scotoma by Goldmann perimetry.

Multifocal ERGs

The amplitudes of the mfERGs in the central area were reduced in all eyes. The area of the decreased amplitude varied among eyes (Figure 1).

FD-OCT

All eyes showed a disruption of the IS/OS line except the left eye of patient 3 who had a visual acuity of 20/20; meanwhile the IS/OS line and the third line are easily identifiable in normal control. Both eyes of patients 1 and 2 had a severe disruption of IS/OS line in the center of the fovea, and both had a low-intensity space between the elevated external limiting membrane (ELM) and the retinal pigment epithelium (RPE) (Figures 2B–2E). In patient 3, the IS/OS line and the RPE line were disrupted, and the retina was thinner (Figures 2F and 2G). The outer segment layer between the IS/OS line and RPE line of the FD-OCT images was visible only in the center of the fovea in the left eye of patient 4 (Figure 2I).

AO fundus images

The AO images showed patchy dark areas in all eyes, which disrupted the mosaic of bright spots in the fovea (Figures 3B–3H), compared with normal control (Figure 3A). This suggested a degeneration of some of the photoreceptors in this area. Nonuniform bright spots with irregular shapes and higher brightness appeared around the dark areas. In patient 3, the normal cone mosaic was replaced by dark areas, and nonuniform bright spots appeared to be all that remained (Figures 3F and 3G). In the left eye of patient

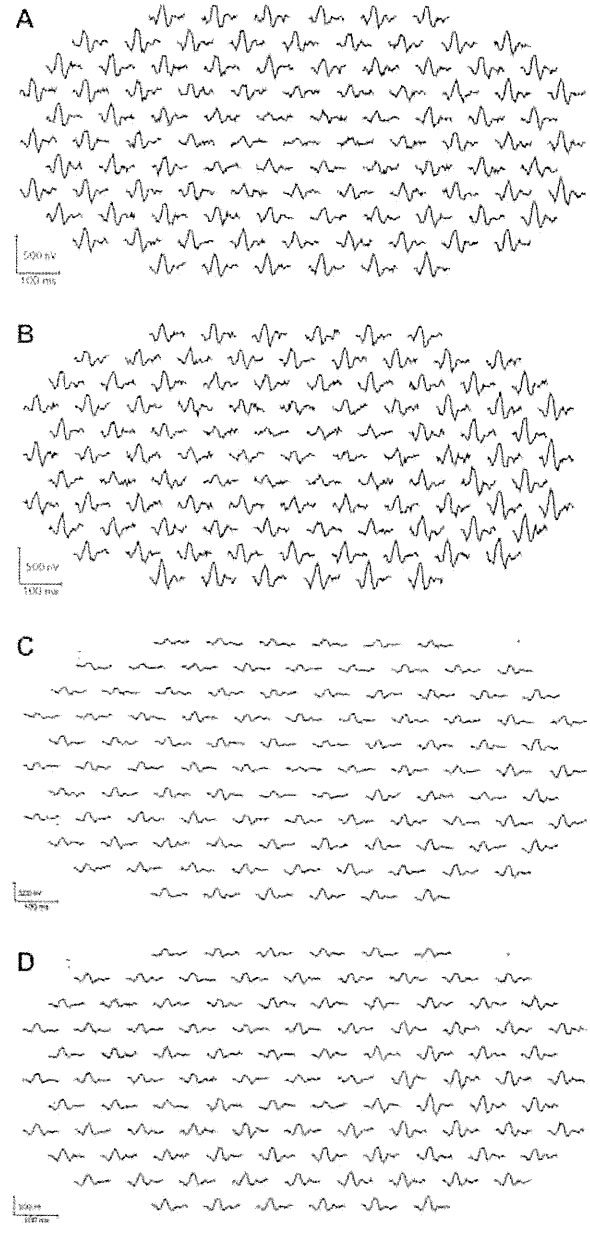


Figure 1 *(Continued)*

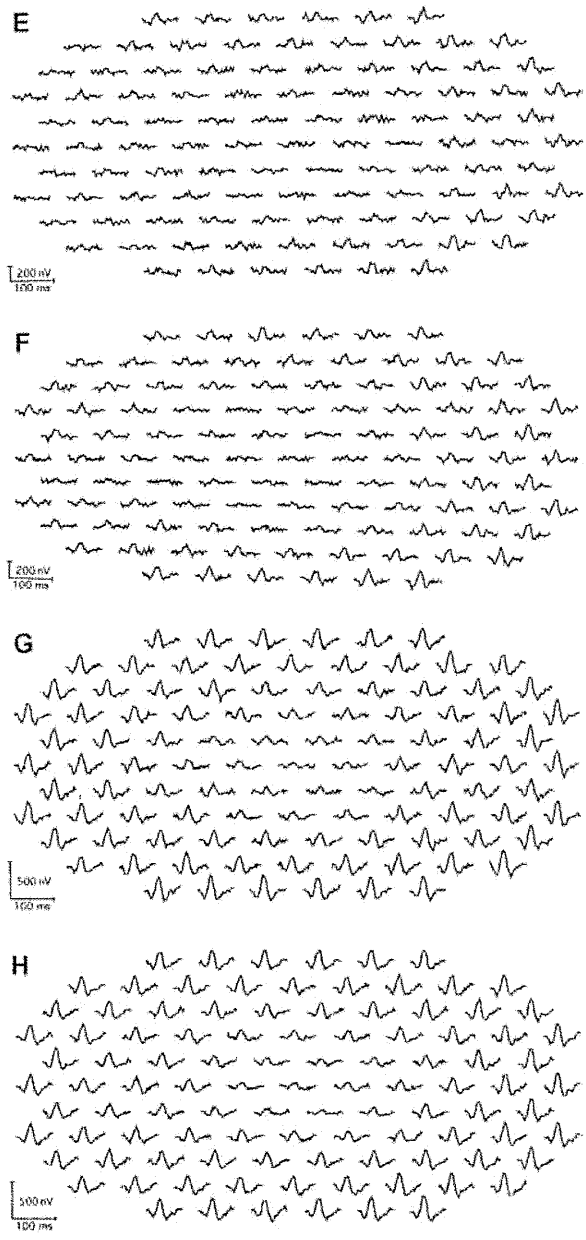


Figure 1 Multifocal electroretinogram (ERGs) of right and left eye of patient 1 (**A, B**), patient 2 (**C, D**), patient 3 (**E, F**), and patient 4 (**G, H**). Foveal amplitudes are decreased in all eyes. Especially in patient 3, amplitudes are attenuated widely including ring 5 and 6, although full-field ERG showed normal amplitude.

4, the mosaic of blight spots were in relatively good order with fewer dark areas in the center of the image (Figure 3I), although the mosaic was disrupted in the peripheral area.

Discussion

We had hypothesized that the main structures affected in eyes with OMD were the photoreceptors as in other types of retinal dystrophy, and the morphological changes of the photoreceptor could be detected by high-resolution retinal imaging techniques. This is important in eyes with OMD because histopathological sections of eyes with OMD have not

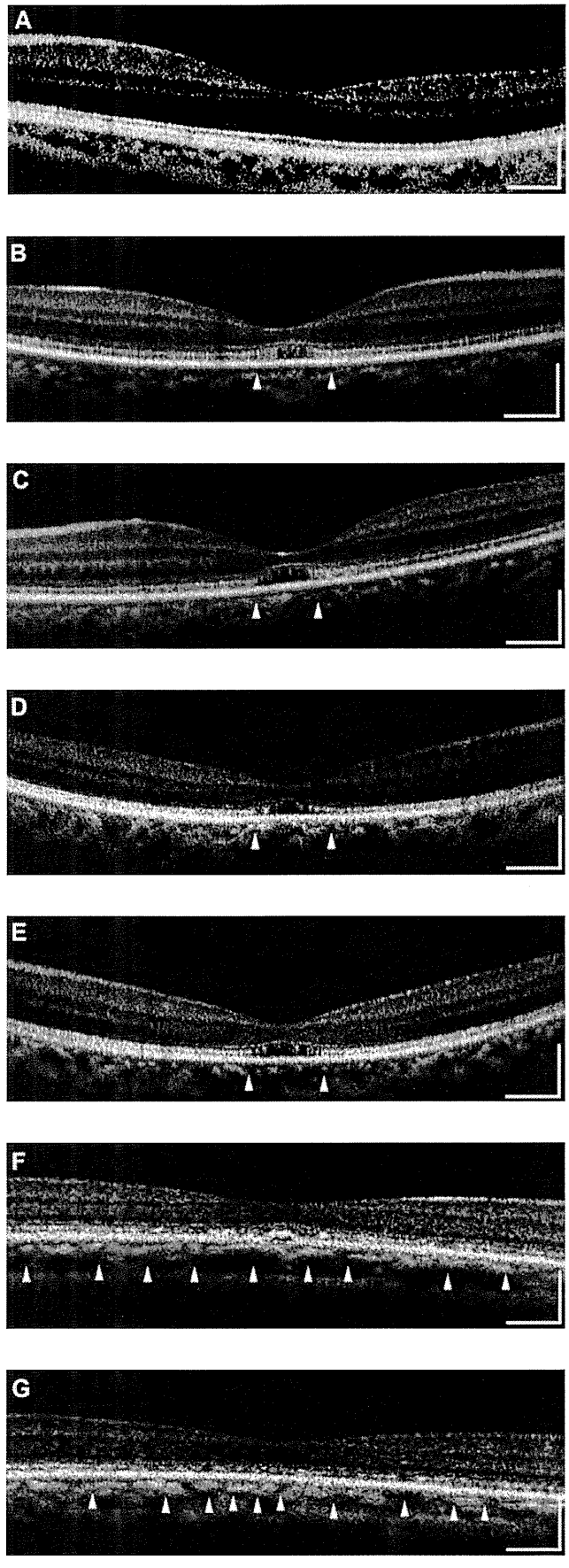


Figure 2 *(Continued)*

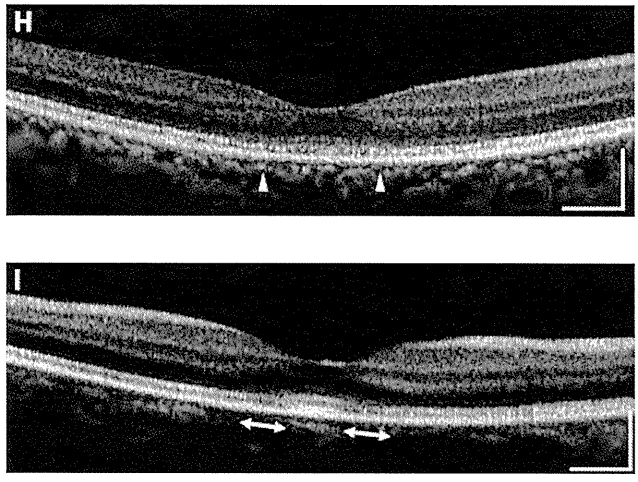


Figure 2 Fourier-domain optical coherence tomography (FD-OCT) images (horizontal scan) of normal eye (**A**), and right/left eyes of patient 1 (**B, C**), patient 2 (**D, E**), patient 3 (**F, G**), and patient 4 (**H, I**). Horizontal bars represent 500 μm, and vertical bars represent 200 μm. The eyes in patients 1–4 had a bilateral symmetric decline in visual acuity, whereas those in patient 5 had an assymmetric decline (20/200 right eye, 20/20 left eye). FD-OCT in normal eye provided clear images of the retinal layers. The external limiting membrane (ELM), photoreceptors inner and outer segment (IS/OS) junction, third line, and retinal pigment epithelium (RPE) are distinguishable. Meanwhile, the retinal photoreceptor layer is not clear in the eyes of the patients. Although ELM was visualized in all eyes, IS/OS is elevated and disrupted in fovea (**B, C, D, E, H**), widely disrupted and not clear (**F, G**), and clearly visualized in one eye (**I**). The third line was visualized only in one eye (**I**), just in the fovea.

been published. In cone-rod dystrophy, a loss of cones in the perifoveal area has been reported, and the number of cones is reduced in the extrafoveal and peripheral areas.[25,26] In addition, the length of photoreceptor outer segments has been reported to be shortened,[25,26] and an accumulation of lipofuscin granules in the RPE has also been reported in these eyes.[26]

The FD-OCT images showed a disruption of the IS/OS line and a loss of the third highly reflective line in the center of the fovea in all eyes except for the left eye of patient 4 whose BCVA was good. These findings are consistent with recent reports that there was a significant correlation between the disturbance of the IS/OS junction and the BCVA.[7,10,11]

The origin of the third bright line in the FD-OCT images has not been determined. It cannot be detected in highly myopic eyes even if the patient has good visual acuity. This suggests that the third line cannot be resolved if the length of photoreceptor outer segments is not long enough. In our patient, the third line was not detected even though they were not highly myopic. We suggest that the shortening of the photoreceptor outer segments is the reason why the third bright line cannot be seen in the FD-OCT images. However, the third line was seen in the center of the foveal area of the left eye of patient 4. In this case, we suggest that the photoreceptor outer segments were long enough in this area, and the visual acuity had not yet decreased. The AO images

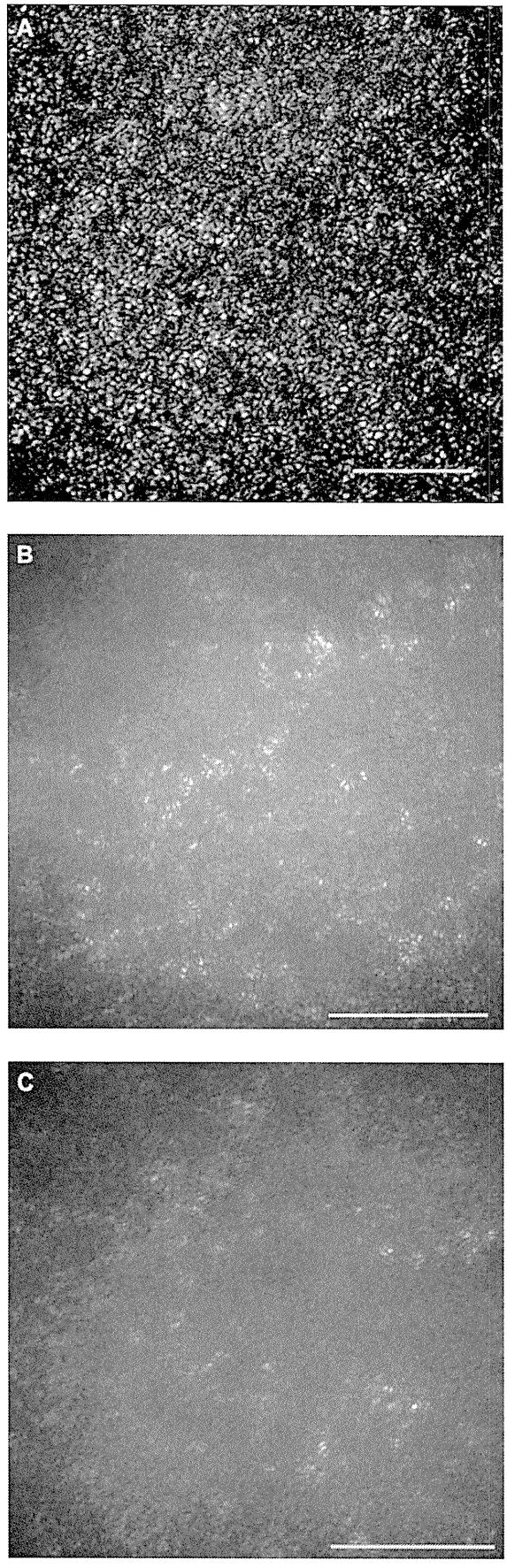


Figure 3 (*Continued*)

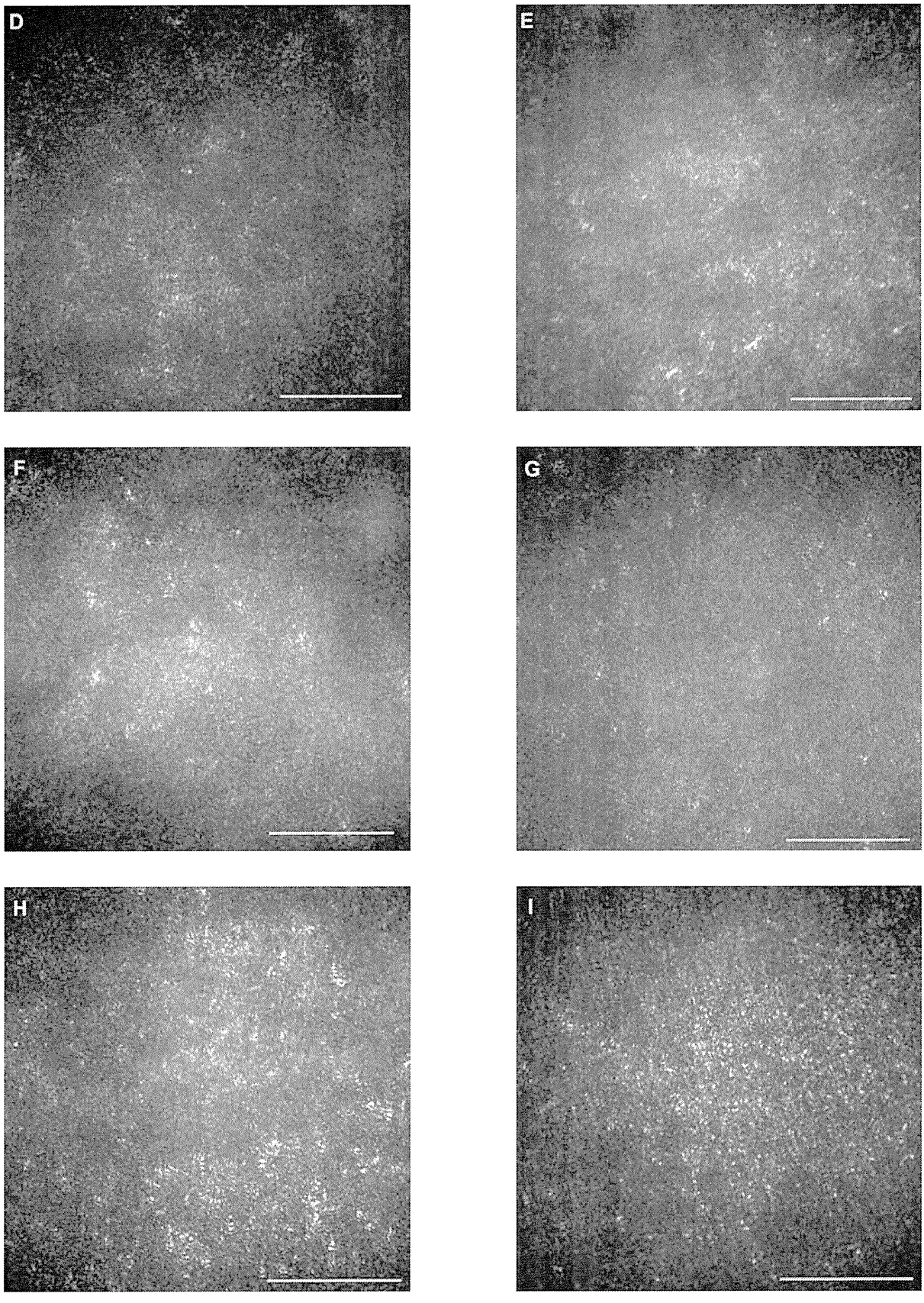


Figure 3 Adaptive optics (AO) images of the fovea of a normal eye (**A**), and right/left eyes of patient 1 (**B, C**), patient 2 (**D, E**), patient 3 (**F, G**), and patient 4 (**H, I**). Bars represent 100 μm. In the eyes of the patients, signals from the cone mosaic were attenuated, and the bright spots were distorted (**B–H**). One eye which had normal visual acuity had an almost normal appearance in the foveal center, with some dark areas around the fovea.

showed the lateral extent of the photoreceptor changes with patchy dark areas and irregular bright spots around the foveal center. There are reports of the AO findings in patients with cone-rod dystrophy (CRD) with increased cone spacing.

Until now, ophthalmologists could detect photoreceptor degeneration only by conventional ophthalmoscopy and electroretinography. In OMD patients, the photoreceptor damage is mild, and it cannot be detected by conventional ophthalmoscopy. The mfERGs are useful for detecting reduced cone function, although the result of mfERGs may be unreliable in subjects with fixation problems, such as young children and patients with eccentric fixation.[27] FD-OCT and AO are noninvasive and effective methods to observe photoreceptor damage and confirm a diagnosis.

The future applications of AO fundus examinations include monitoring disease progression and measuring the effect of treatment. Further investigations are needed to interpret and quantify the features of these images.

In conclusion, the morphological changes of OMD patients can be seen tangentially by FD-OCT and en-face by AO.

This study was supported by Health Sciences Research Grants (H19-sensory-001) from the Ministry of Health, Labor and Welfare, and by Grants (19209053) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Disclosure

The authors report no conflicts of interest in this work.

References

1. Miyake Y, Horiguchi M, Tomita N, et al. Occult macular dystrophy. *Am J Ophthalmol*. 1996;122(5):644–653.
2. Piao CH, Kondo M, Tanikawa A, et al. Multifocal electroretinogram in occult macular dystrophy. *Invest Ophthalmol Vis Sci*. 2000;41(2): 513–517.
3. Fujii S, Escaño MF, Ishibashi K, et al. Multifocal electroretinography in patients with occult macular dystrophy. *Br J Ophthalmol*. 1999;83(7): 879–880.
4. Kondo M, Ito Y, Ueno S, Piao CH, et al. Foveal thickness in occult macular dystrophy. *Am J Ophthalmol*. 2003;135(5):725–728.
5. Brockhurst RJ, Sandberg MA. Optical coherence tomography findings in occult macular dystrophy. *Am J Ophthalmol*. 2007;143(3):516–518.
6. Drexler W, Sattmann H, Hermann B, et al. Enhanced visualization of macular pathology with the use of ultrahigh-resolution optical coherence tomography. *Arch Ophthalmol*. 2003;121(5):695–706.
7. Schocket LS, Witkin AJ, Fujimoro JG, et al. Ultrahigh-resolution optical coherence tomography in patients with decreased visual acuity after retinal detachment repair. *Ophthalmology*. 2006;113(4):666–672.
8. Alam S, Zawadzki R, Choi S, et al. Clinical application of rapid serial Fourier-domain optical coherence tomography for macular imaging. *Opthalmology*. 2006;113(8):1425–1431.
9. Ergun E, Hermann B, Wirtitsch M, et al. Assessment of central visual function in Stargardt disease/fundus flavimaculatus with ultrahigh-resolution optical coherence tomography. *Invest Ophthalmol Vis Sci*. 2005;46(1):310–316.
10. Ojima Y, Hangai M, Sasahara M, et al. Three-demensional imaging of the foveal photoreceptor layer in central serous chorioretinopathy using high-speed optical coherence tomography. *Ophthalmology*. 2007;114(12):2197–2207.
11. Piccolino FC, de la Longrais RR, Ravera G, et al. The foveal photoreceptor layer and visual acuity loss in central serous chorioretinopathy. *Am J Ophthalmol*. 2005;139(1):87–99.
12. Iida T, Hagimura N, Sato T, et al. Evaluation of central serous chorioretinopathy with optical coherence tomography. *Am J Ophthalmol*. 2000;129(1):16–20.
13. Kitaguchi Y, Fujikado T, Bessho K, et al. Adaptive optics fundus camera to examine localized changes in the photoreceptor layer of the fovea. *Ophthalmology*. 2008;115(10):1771–1777.
14. Liang J, Williams DR, Miller DT. Supernormal vision and high resolution retinal imaging through adaptive optics. *J Opt Soc Am A Opt Image Sci Vis*. 1997;14(11):2884–2892.
15. Kitaguchi Y, Fujikado T, Kusaka S, et al. Imaging of titanium:sapphire laser retinal injury by adaptive optics fundus imaging and Fourier-domain optical coherence tomography. *Am J Ophthalmol*. 2009;148(1):97–104.e2.
16. Roorda A, Williams DR. The arrangement of three cone classes in the living human eye. *Nature*. 1999;397(6719):520–522.
17. Roorda A, Williams DR. Optical fiber properties of individual human cones. *J Vis*. 2002;2(5):404–412.
18. Roorda A, Romero BF, Donnelly WJ, et al. Adaptive optics scanning laser ophthalmoscopy. *Opt Express*. 2002;10(9):405–412.
19. Pallilkaris A, Williams DR, Hofer H, et al. The reflectance of single cones in the living human eye. *Invest Ophthalmol Vis Sci*. 2003;44(10): 4580–4592.
20. Wolfing JI, Chung M, Carroll J, et al. High-resolution retinal imaging of cone-rod dystrophy. *Ophthalmology*. 2006;113(6):1019.e1.
21. Duncan JL, Zhang Y, Gandhi J, et al. High-resolution imaging with adaptive optics in patients with inherited retinal degeneration. *Invest Ophthalmol Vis Sci*. 2007;48(7):3283–3291.
22. Choi SS, Double N, Hardy JL, et al. In vivo imaging of the photoreceptor mosaic in retinal dystrophies and correlations with visual function. *Invest Ophthalmol Vis Sci*. 2006;47(5):2080–2092.
23. Kitaguchi Y, Bessho K, Yamaguchi T, et al. In vivo measurements of cone photoreceptor spacing in myopic eyes from images obtained by adaptive optics fundus camera. *Jpn J Ophthalmol*. 2007;51(6):456–461.
24. Yamaguchi T, Nakazawa N, Bessho K, et al. Adaptive optics fundus camera using a liquid crystal phase modulator. *Optical Review*. 2008; 15(3):173–180.
25. Rabb MF, Tso MO, Fishman GA. Cone-rod dystrophy. A clinical and histopathologic report. *Ophthalmology*. 1986;93(11):1443–1451.
26. Demirci FY, Gupta N, Radak AL, et al. Histopathologic study of X-linked cone-rod dystrophy (CORDX1) caused by a mutation in the RPGR exon ORF15. *Am J Ophthalmol*. 2005;139(2):386–388.
27. Kondo M, Miyake Y, Horiguchi M, et al. Clinical evaluation of multifocal electroretinogram. *Invest Ophthalmol Vis Sci*. 1995;36(10):2146–2150.

Mutations in the *TSPAN12* Gene in Japanese Patients with Familial Exudative Vitreoretinopathy

HIROYUKI KONDO, SHUNJI KUSAKA, AKI YOSHINAGA, EIICHI UCHIO, AKIHIKO TAWARA, KENSHI HAYASHI, AND TOMOKO TAHIRA

• PURPOSE: To search for mutations in the *TSPAN12* gene in 90 Japanese probands with familial exudative vitreoretinopathy (FEVR) and their family members and to determine the types and frequencies of the mutations.

• DESIGN: Laboratory investigation and clinical case analyses.

• METHODS: Direct sequencing after polymerase chain reaction of the coding exons of *TSPAN12* was performed for 90 probands with FEVR and some of their family members. The clinical signs and symptoms that were characteristic of individuals with *TSPAN12* mutations were determined.

• RESULTS: Three families were found to carry 2 mutations in *TSPAN12*. One of these mutations was a new missense change, L245P, and the other was an already reported nonsense mutation, L140X, in 2 families. Mutations in *TSPAN12* accounted for 3% of Japanese FEVR patients and 8% of the FEVR families who did not have mutations in any of the known FEVR genes, *FZD4*, *LRP5*, and *NDP*. The clinical signs and symptoms varied among the patients, but the retinal findings with *TSPAN12* mutations were not different from those with mutations in the known FEVR-causing genes.

• CONCLUSIONS: Mutant *TSPAN12* is responsible for approximately 3% of FEVR patients in Japan. The results provide further evidence that mutations in *TSPAN12* are FEVR causing and that the gene products most likely play a role in the development of retinal vessels. (Am J Ophthalmol 2011;151:1095–1100. © 2011 by Elsevier Inc. All rights reserved.)

FAMILIAL EXUDATIVE VITREORETINOPATHY (FEVR) IS a hereditary disorder that is characterized by defects in the development of retinal vessels and is manifested by different retinal pathologic features, including retinal folds and retinal detachments.[1,2] The expressivity of the disease differs widely between and within families. Most individuals remain asymptomatic, and the consistent signs of FEVR are abnormal retinal vessels and avascularization of the peripheral retina.[2]

FEVR is genetically heterogeneous, and 3 genes are known to be responsible for FEVR. Mutations in the genes coding for the Wnt receptor pair, frizzled-4 (*FZD4*), and low-density lipoprotein receptor-like protein 5 (*LRP5*), are known to cause FEVR.[3,4] Mutations in genes coding for the ligand of the receptor pair, norrin (*NDP*), also cause FEVR and Norrie disease (ND).[5] The ligand–receptor complex activates canonical Wnt signaling and controls vascular development in the retina.[6] Mutations in *FZD4* cause autosomal dominant FEVR, mutations in *LRP5* cause autosomal dominant or recessive FEVR, and mutations in *NDP* cause X-linked recessive FEVR.[3–7]

Recently, a transmembrane protein, TSPAN12, was found to be expressed in the retinal vascular endothelial cells and to enhance Wnt signaling through FZD4 and LRP5.[8] This study was followed by 2 studies that demonstrated 9 mutations of this gene in autosomal dominant FEVR patients.[9,10] Because of our interest in the genetic basis of FEVR, we have examined our Japanese patients with FEVR to determine whether *TSPAN12* mutations were present in them. We show that mutations in the *TSPAN12* gene were found in only approximately 3% of the Japanese FEVR patients.

METHODS

• PARTICIPANTS AND CLINICAL EXAMINATIONS: Ninety probands, 39 familial and 51 simplex, with FEVR and 7 cases with ND were studied. All patients were Japanese and were born at term of normal weight. The diagnosis of FEVR was based on the presence of peripheral retinal avascularization with abnormal retinal vascular changes as well as the other typical clinical signs: severe retinal exudates, retinal neovascularization, peripheral fibrovascular mass, ectopic macula, retinal folds, and retinal detachment. The diagnosis of ND was made for boys who had bilateral retinal detachment or retinal folds with retrolental fibrous tissue and blindness within the first year of life. Ocular examinations included refraction, visual acuity, intraocular pressure, slit lamp, fundus, and ultrasonog-

Accepted for publication Nov 19, 2010.

From the Department of Ophthalmology, University of Occupational and Environmental Health Japan, Kitakyushu, Japan (H.K., A.T.); the Department of Ophthalmology, Fukuoka University, Fukuoka, Japan (H.K., E.U.); the Department of Ophthalmology, Sakai Hospital Kinki University Faculty of Medicine, Sakai, Japan (S.K.); the Division of Genome Analysis, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan (A.Y., T.T.); and the Faculty of Agriculture, Kyushu University, Fukuoka, Japan (K.H.).

Inquiries to Hiroyuki Kondo, 1-1, Iseigaoka, Yahatanishiku, Kitakyushu 807-8555, Japan; e-mail: kondohi@med.uoeh-u.ac.jp

0002-9394/$36.00
doi:10.1016/j.ajo.2010.11.026

TABLE 1. Sequences of Polymerase Chain Reaction Primers Used to Amplify *TSPAN12* Coding Exons

Exon	Primer		PCR Product Size (bp)
	Forward (5′→3′)	Reverse (5′→3′)	
2	attGGTGAGATGTCCCGTGTTCT	gtTAATGCTTAGCCATGCCCTT	270
3	aTTTCAAGATGCAGCAAATGG	GTTGCTATGGGCAGGAAAAA	333
4	atTGCTATGTCTTGGGTGCATT	gttAAACGAAAGCGTCCCTTCTT	331
5	aTTTCCCCATCTGCTTCTGAG	gttAAAAGGCTGAACTGTTGTTTTAGA	267
6	attGAGCTACAGCTGTTGATATTTTGC	gttAAACATCTGGTTTGAAGGTGC	210
7	atTGATGACAGATATAGCTCTGGGT	gttGGAAAATTTCATTGGCATATTG	346
8	attGCTTTCCCTGAGAACCACTG	gtTGCTTAGGTGTTATTTTATGGCAA	574

PCR = polymerase chain reaction.

The 5′-end of each primer was designed to have an ATT or GTT for postlabeling purposes.[12] When necessary, extra nucleotides (lowercase) were attached.

raphy. Fluorescein angiography was performed on 20 probands.

• LABORATORY STUDIES: Deoxyribonucleic acid samples were extracted from peripheral blood using a deoxyribonucleic acid extraction kit (QiaAmp; Qiagen, Chatsworth, California, USA). To identify mutations in the coding exons (exons 2 to 8) of the *TSPAN12* gene, oligonucleotide primers on the flanking intron and untranslated region sequences were designed (Table 1). Polymerase chain reaction and sequencing were conducted as described.[11] The annealing temperature for polymerase chain reaction was 60 C for all exons. After sequence changes were detected in the probands, samples from other family members were analyzed by direct sequencing as well as denaturing high-performance liquid chromatography. Before this study, mutations in 3 genes, *FZD4*, *LRP5*, and *NDP*, known to cause FEVR had been analyzed in these patients.[11,12]

RESULTS

TWO NEW NONSYNONYMOUS SEQUENCE CHANGES IN THE coding sequence of the *TSPAN12* gene were found in 2 probands from Families 1 and 2 with autosomal dominant FEVR (Figure 1): c.734T→C (L245P) and c.154G→C (E52Q). L245 is located at the C-terminal cytoplasmic tail region and could provide specific functional links to cytoskeletal or signaling proteins.[13] E52 is located in the short extracellular loop. Both residues and the surrounding regions were conserved in humans and other vertebrates (Figure 1).

None of the sequence changes were found in 380 chromosomes from 190 healthy volunteers. Direct sequencing as well as denaturing high-performance liquid chromatography analysis revealed that both changes were transmitted heterozygously and were cosegregated with the disease except for a sister of the proband in Family 2 (Figure 1). This patient was diagnosed with FEVR because of abnormal retinal vessels with vitreous degeneration, but did not have the E52Q change. Therefore, we could not conclude that E52Q is responsible for FEVR.

One recurrent mutation c.419T→A (L140X) also was found in a sporadic patient (Family 3) and in a proband with autosomal dominant FEVR (Family 4). The mutation in Family 4 was reported previously.[10] Subsequent analysis of family members revealed a total of 6 mutations when the E52Q change was excluded (Figure 1). The clinical symptoms varied among the patients carrying the *TSPAN12* mutations from mild vascular changes with retinal degeneration to severe bilateral retinal folds (Table 2 and Figure 2). The clinical signs and symptoms of patients with the *TSPAN12* mutation were not different from those with mutations in known FEVR-causing genes.

Thirty-three FEVR patients who carried mutations either in *FZD4* or *LRP5* had no mutations in *TSPAN12*. Seven ND patients who had been shown to carry mutations in *NDP* had no mutations in *TSPAN12*. Thus, *TSPAN12* mutations may not be responsible for typical ND.

In addition, we found 3 known polymorphisms, 2 new nucleotide changes in introns, and 1 new synonymous nucleotide change in *TSPAN12*: IVS2-23G→A, c.91A→G, IVS6-80T→A, IVS7-81A→G (rs17142959), c.765G→T (rs41623), and c.*39C→T (rs41622). These were not considered to be responsible for FEVR.

DISCUSSION

WE EXAMINED 90 JAPANESE PATIENTS WITH FEVR FOR MUtations in the *TSPAN12* gene. Two patients with familial FEVR and 1 with sporadic FEVR were found to carry heterozygous mutations in *TSPAN12*. Our data indicated

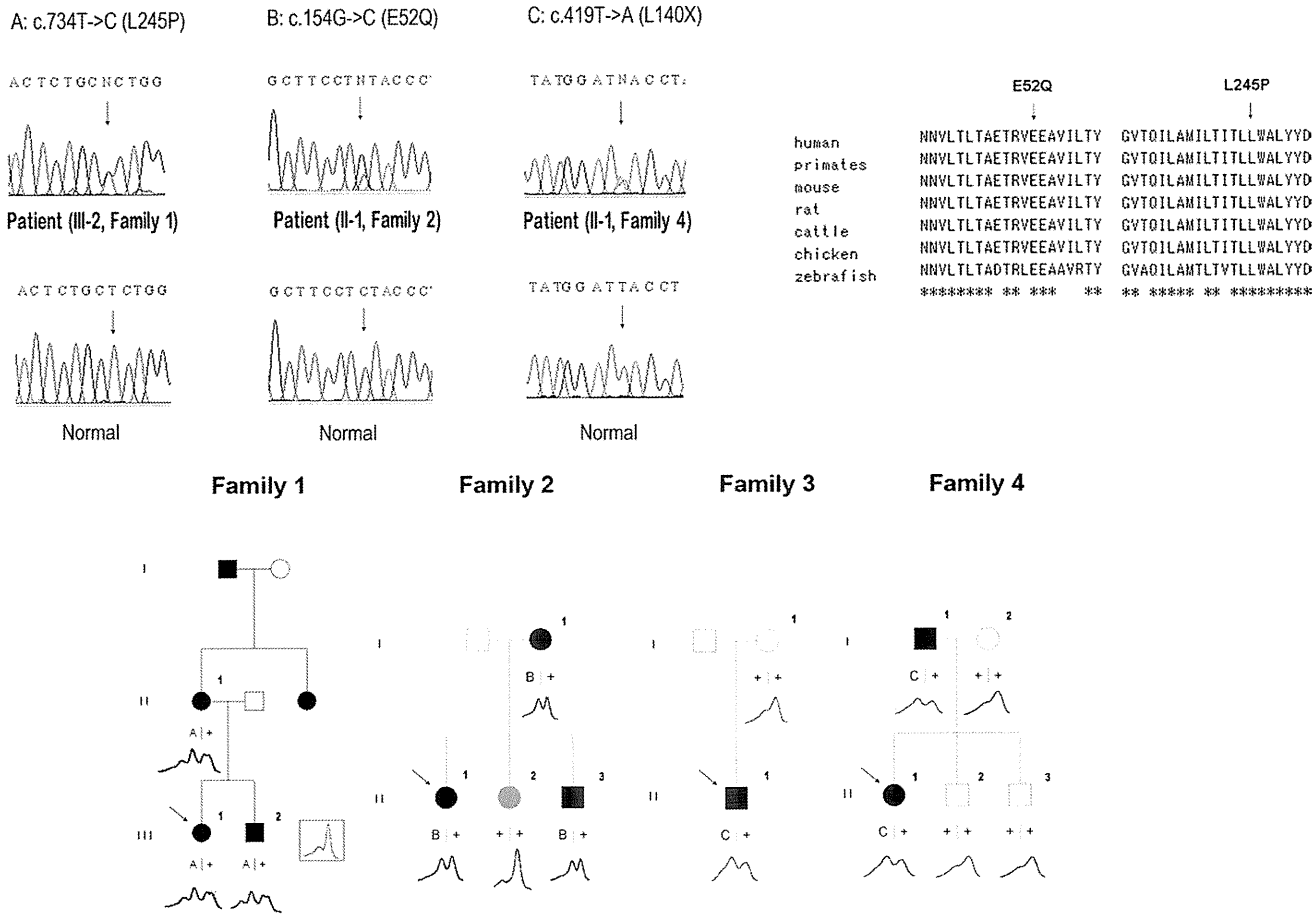


FIGURE 1. Chromatograms and pedigrees of 4 families with familial exudative vitreoretinopathy. (Top left) Mutations and nonsynonymous changes in the *TSPAN12* genes in patients with familial exudative vitreoretinopathy. Arrows indicate the positions of the altered nucleotides. E52Q is shown in the antisense direction. (Top right) Protein sequence alignment of TSPAN12 with homologues from human and other vertebrates with arrows indicating the amino acid changes. Sequence data were derived from GenBank or SwissProt based on a previous study (Poulter and associates[10]). Asterisks (*) indicate highly conserved amino acids. (Bottom) Pedigrees of 4 families illustrating the cosegregation of the *TSPAN12* mutations with familial exudative vitreoretinopathy in Families 1 through 4. Solid symbols indicate individuals with a diagnosis of familial exudative vitreoretinopathy. Arrows indicate probands. Individuals from whom sequence data were obtained are numbered. A, B, and C indicate the sequence changes L245P, E52Q, and L140X, respectively, which also are indicated above the trace data at the top. Plus (+) indicates a wild-type sequence. Results of denaturing high-performance liquid chromatography (DHPLC) are shown below each genotype. For Family 1, a wild-type pattern of DHPLC is shown in the Inset because deoxyribonucleic acid for an unaffected individual is unavailable. Note that a sister of the proband of Family 2 (filled with gray) was diagnosed with mild familial exudative vitreoretinopathy, but did not carry the mutation in *TSPAN12*. The mutation in Family 4 has been reported previously.[10]

that mutations in *TSPAN12* accounted for 3% of Japanese families with FEVR and 8% of the families in which no mutations were found in any of the genes known to be responsible for FEVR.

Our findings confirm 2 recent studies that reported that *TSPAN12* causes FEVR. Both reports showed that mutations in *TSPAN12* accounted for approximately 10% of mainly white patients in whom mutations have not been identified in the known genes.[9,10] Thus, the frequencies of mutations in this gene are similar in the 2 populations.

TSPAN12 is one of the members of tetraspanin superfamily. These proteins share 2 highly conserved features; the 4 transmembrane domains contain well-conserved residues, and the second extracellular loop has a Cys-Cys-Gly sequence and additional cysteines (Figure 3). Tetraspanins are known to participate in a spectrum of membrane-associated activities involving cell adhesion, cell proliferation, and activation of signaling pathway.[14] These proteins not only build homomultimer but also bind specifically and directly to other proteins.[15] *TSPAN12* interacts specifically with Norrin or *LRP5* and enhances the mutlimerization of the norrin/FZD4/LRP5 complex in the retina.[8] Defective *TSPAN12* possibly causes a reduction in norrin/FZD4/LRP5 signaling, which controls the angiogenic program.

TABLE 2. Mutations in *TSPAN12* Gene and the Associated Clinical Findings in Patients with Familial Exudative Vitreoretinopathy

Family	ID[a]/Age (yo)/Sex	Sequence Change	Visual Acuity (Refraction)	Peripheral Avascular Retina	Retinal Vessels Abnormality	Vitreous Degeneration	Ectopic Macula	Fibrous Tissue	Falciform Retinal Fold	Comments
1	II-1/37/F	L245P	1.2 (nc) BE	BE	BE	BE	No	No	No	
	III:1/15/F (proband)	L245P	0.06 (−2.5 D) RE; 0.6 (−6.0 D) LE	BE	BE	BE	No	RE	RE	PHC LE
	III:2/13/M	L245P	1.2 (nc) BE	BE	BE	BE	No	No	No	
3	II-1/11/M (proband)	L140X	NLP RE; 0.1 (+13.0 D) LE	LE[b]	LE[b]	NA	No	BE	BE	VxLx BE at 1 yo, phthisical RE, aphakic BE
4	I-1/42/M	L140X	1.2 (−4.0 D) BE	BE	BE	No	No	No	No	
	II-1/12/F (proband)	L140X	0.07 (+18.0 D); 0.1 (+18.0 D)	BE	BE	NA	BE	BE	No	VxLx BE at 0 yo, aphakic BE

BE = both eyes; D = diopters; Esx = esotropia operation; F = female; LE = left eye; Lx = lensectomy; M = male; NA = not analyzed; nc = not correctable; PHC = photocoagulation; RE = right eye; Vx = vitrectomy; yo = year(s) old.

[a]Identifications are referable to Figure 1, Bottom.

[b]Findings on the right eye were not available because of phthisis.

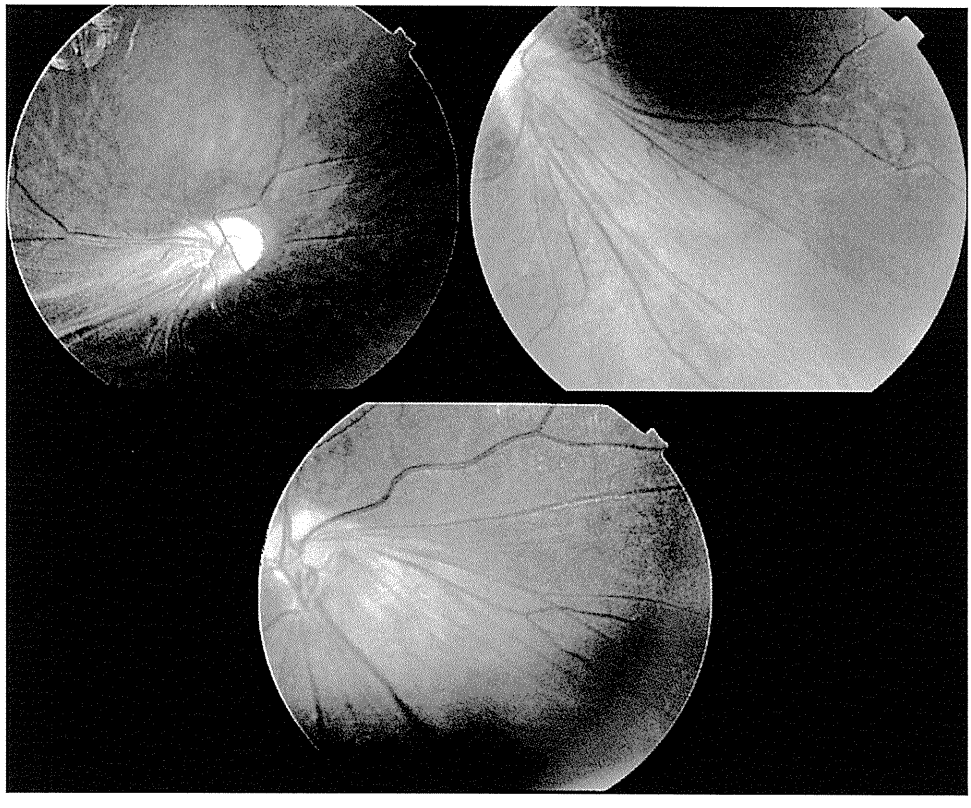


FIGURE 2. Fundus photographs of patients with familial exudative vitreoretinopathy carrying mutations in *TSPAN12*. (Top left) Fundus photograph of the right eye of the proband of Family 1 showing a retinal fold resulting from retrolental fibrous tissues. (Top right and Bottom) Fundus photographs of the left eyes of the probands of Families 3 and 4 showing a dragged macula.

So far, 9 mutations in *TSPAN12* have been identified (Figure 3). Of these mutations, at least 5 (insertion, deletion, nonsense, and splicing) are predicted to result in truncated proteins that may not be synthesized because of nonsense-mediated decay of the messenger ribonucleic acid.[10] One missense mutation, A237P, was suggested to be subjected to proteolytic degradation.[9] Based on these data, haploinsufficiency of *TSPAN12* was proposed as the cause of FEVR.

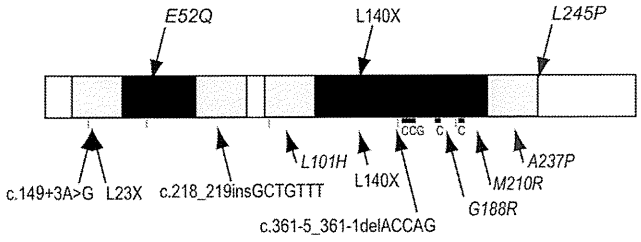


FIGURE 3. Schematic diagram of the structure of TSPAN12 and locations of mutations and nonsynonymous change identified in the *TSPAN12* gene in familial exudative vitreoretinopathy patients. TSPAN12 contains 4 transmembrane domain (shaded boxes), and the first and second extracellular loop domains (filled boxes) are highly conserved. White boxes indicate intracellular regions. Vertical bars indicate exon–intron boundaries. Horizontal bars indicate a conserved Cys-Cys-Gly sequence (CCG) and the partner cysteins (C) that form disulfide bridges. One nonsense mutation and 2 nonsynonymous sequence changes identified in this study are at the top. Note that E52Q did not cosegregate with disease and may not be responsible for familial exudative vitreoretinopathy (asterisk). Mutations previously reported by Nikopoulos and associates and Poulter and associates are at the bottom.[9,10] Missense mutations are shown in italics.

The expression of the clinical features of the patients with *TSPAN12* mutations differed widely, as shown in Table 2 and Figure 2. The probands showed relatively severe retinopathy, for example, retinal folds, whereas the other family members often were asymptomatic, as has been reported in individuals who carry mutations in other FEVR-causing genes. The retinal findings in patients with *TSPAN12* mutations were not different from those with mutations in *FDZ4* and *LRP5*,[10] although retinal exudates were not found in our patients. Mutations in *LRP5* are known to cause reduced bone density,[11] but we did not examine the systemic changes in the patients with *TSPAN12* mutations in detail.

For Family 2, a change in E52Q was found in 3 individuals with FEVR, whereas the same change was not found in a sister of the proband who also had mild FEVR. The lack of cosegregation suggests that E52Q is a nonpathogenic polymorphism. However, the genetic background of FEVR is likely to be more complex than that of Mendelian pedigree patterns.[11,16] A possibility remains that this family has an unknown genetic background that may make them susceptible to the disease depending on the E52Q change. Functional analysis is required to assess the effect of this change.

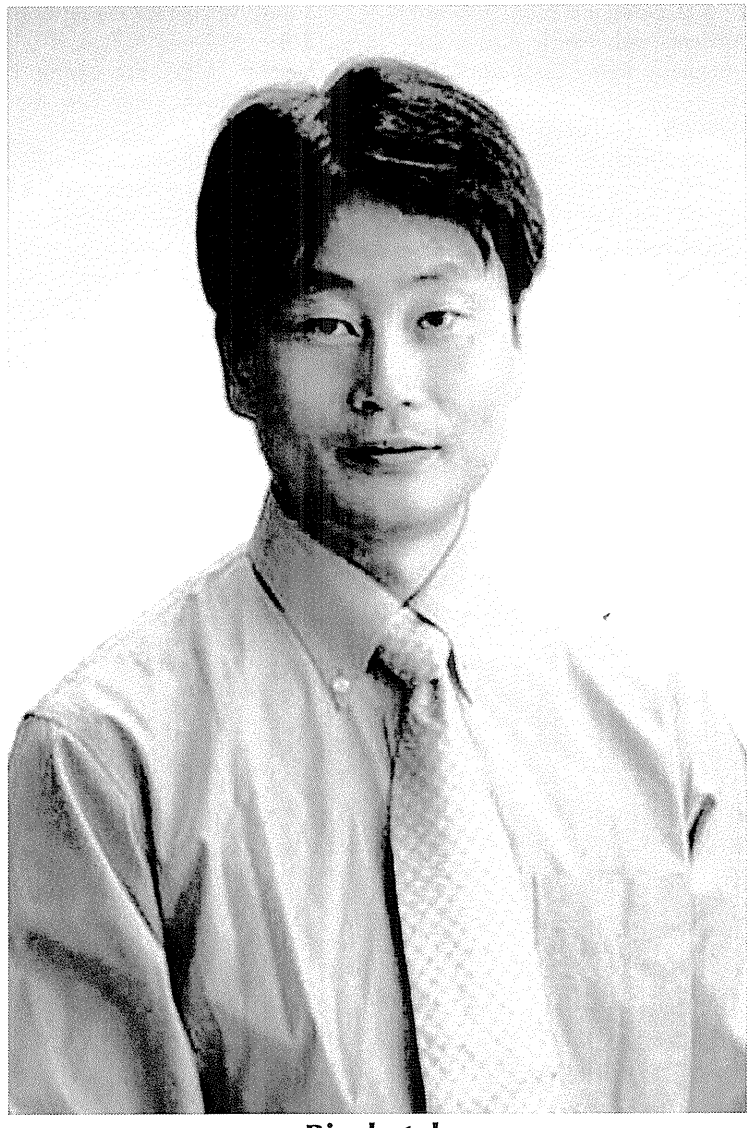
In conclusion, we examined 90 patients with FEVR for mutations in the *TSPAN12* gene, and 3 families were found to carry heterozygous mutations in *TSPAN12*. These findings indicate that mutant *TSPAN12* is responsible for approximately 3% of FEVR in Japan. The results provide additional evidence that mutations in *TSPAN12* are FEVR causing that and *TSPAN12* is crucial for the development of the retinal vessels.

PUBLICATION OF THIS ARTICLE WAS SUPPORTED BY THE JAPAN SOCIETY FOR THE PROMOTION OF SCIENCE, TOKYO, JAPAN (Grants-in-Aid for Scientific Research 22591956), and the Ministry of Health, Labour and Welfare of Japan (Grants for Research on Intractable Diseases 22141101). The authors indicate no financial conflict of interest. Involved in Design of study (H.K., T.T.); Conduct of study (H.K., S.K., K.H.); Data collection (H.K., A.Y.); Analysis and interpretation of data (H.K., T.T., K.H.); Writing and preparation of the manuscript (H.K.); Review and approval of the manuscript (E.U., A.T.). The procedures used conformed to the tenets of the Declaration of Helsinki. Informed consent was obtained from all patients, and the experimental protocol was approved by the Ethics Review Board of Fukuoka University. The authors thank the patients and their families and Duco Hamasaki, Professor Emeritus, Bascom Palmer Eye Institute, University of Miami, Miami, Florida, for his critical comments and valuable assistance.

REFERENCES

1. Criswick VG, Schepens CL. Familial exudative vitreoretinopathy. Am J Ophthalmol 1969;68(4):578–594.
2. Warden SM, Andreoli CM, Mukai S. The Wnt signaling pathway in familial exudative vitreoretinopathy and Norrie disease. Semin Ophthalmol 2007;22(4):211–217.
3. Robitaille J, MacDonald ML, Kaykas A, et al. Mutant frizzled-4 disrupts retinal angiogenesis in familial exudative vitreoretinopathy. Nat Genet 2002;32(2):326–330.
4. Toomes C, Bottomley HM, Jackson RM, et al. Mutations in LRP5 or FZD4 underlie the common familial exudative vitreoretinopathy locus on chromosome 11q. Am J Hum Genet 2004;74(4):721–730.
5. Chen ZY, Battinelli EM, Fielder A, et al. A mutation in the Norrie disease gene (NDP) associated with X-linked familial exudative vitreoretinopathy. Nat Genet 1993;5(2):180–183.
6. Xu Q, Wang Y, Dabdoub A, et al. Vascular development in the retina and inner ear: control by Norrin and Frizzled-4, a high-affinity ligand-receptor pair. Cell 2004;116(6):883–895.
7. Jiao X, Ventruto V, Trese MT, Shastry BS, Hejtmancik JF. Autosomal recessive familial exudative vitreoretinopathy is associated with mutations in LRP5. Am J Hum Genet 2004;75(5):878–884.
8. Junge HJ, Yang S, Burton JB, et al. TSPAN12 regulates retinal vascular development by promoting Norrin- but not Wnt-induced FZD4/beta-catenin signaling. Cell 2009;139(2):299–311.
9. Nikopoulos K, Gilissen C, Hoischen A, et al. Next-generation sequencing of a 40 Mb linkage interval reveals TSPAN12 mutations in patients with familial exudative vitreoretinopathy. Am J Hum Genet 2010;86(2):240–247.
10. Poulter JA, Ali M, Gilmour DF, et al. Mutations in TSPAN12 cause autosomal-dominant familial exudative vitreoretinopathy. Am J Hum Genet 2010;86(2):248–253.
11. Qin M, Hayashi H, Oshima K, Tahira T, Hayashi K, Kondo H. Complexity of the genotype-phenotype correlation in familial exudative vitreoretinopathy with mutations in the LRP5 and/or FZD4 genes. Hum Mutat 2005;26(2):104–112.

12. Kondo H, Qin M, Kusaka S, et al. Novel mutations in Norrie disease gene in Japanese patients with Norrie disease and familial exudative vitreoretinopathy. Invest Ophthalmol Vis Sci 2007;48(3):1276–1282.
13. Stipp CS, Kolesnikova TV, Hemler ME. Functional domains in tetraspanin proteins. Trends Biochem Sci 2003;28(2): 106–112.
14. Garcia-Espana A, Chung PJ, Sarkar IN, Stiner E, Sun TT, Desalle R. Appearance of new tetraspanin genes during vertebrate evolution. Genomics 2008;91(4):326–334.
15. Hemler ME. Tetraspanin functions and associated microdomains. Nat Rev Mol Cell Biol 2005;6(6):801–811.
16. Kondo H, Qin M, Tahira T, et al. Severe form of familial exudative vitreoretinopathy caused by homozygous R417Q mutation in frizzled-4 gene. Ophthalmic Genet 2007;28(4): 220–223.



Biosketch

Hiroyuki Kondo, is an Associate Professor at the Department Ophthalmology, University of Occupational and Environmental Health, Japan. His interests include ophthalmic genetics and vitreoretinal surgery, with specialization in pediatric vitreous surgery.

Vitreous Levels of Angiopoietin-1 and Angiopoietin-2 in Eyes With Retinopathy of Prematurity

TATSUHIKO SATO, CHIHARU SHIMA, AND SHUNJI KUSAKA

• PURPOSE: To determine the vitreous levels of angiopoietin (Ang)-1 and Ang-2 in eyes with retinopathy of prematurity (ROP), and to determine the correlation between the 2 levels.
• DESIGN: Retrospective case-control study.
• METHODS: Forty-eight eyes with stage 4 ROP were studied. Six eyes with congenital cataract were used as controls. The ROP eyes were classified by the vascular activity into highly (n = 22), moderately (n = 15), and mildly (n = 11) vascular-active ROP. Eyes with highly vascular-active ROP initially received 0.5 mg of intravitreal bevacizumab (IVB) and underwent vitrectomy within 1 week. The others underwent vitrectomy without IVB. Vitreous samples were collected at the beginning of vitrectomy, and the vitreous levels of Angs were measured by enzyme-linked immunosorbent assay.
• RESULTS: The mean concentrations of Ang-1 and Ang-2 were 201.9 and 7832.1 pg/mL in highly vascular-active ROP eyes, 216.1 and 7731.2 pg/mL in moderately vascular-active ROP eyes, 533.8 and 1685.9 pg/mL in mildly vascular-active ROP eyes, and 0 and 41.5 pg/mL in control eyes. The vitreous Ang-1 level was significantly higher ($P < .05$) in highly, moderately, and mildly vascular-active ROP eyes than in control eyes. The vitreous Ang-2 level was significantly higher ($P < .05$) in highly and moderately vascular-active ROP eyes than in control eyes. There was a significant negative correlation ($r = -0.406$; $P = .040$) between the Ang-1 and Ang-2 levels in moderately and mildly vascular-active ROP eyes.
• CONCLUSIONS: The balance of Ang-1 and Ang-2 in the vitreous may be important in the pathogenesis of ROP. (Am J Ophthalmol 2011;151:353–357. © 2011 by Elsevier Inc. All rights reserved.)

RETINOPATHY OF PREMATURITY (ROP), FIRST REported as retrolental fibroplasia by Terry in 1942,[1] is a retinal vascular disorder that develops in eyes with incomplete blood vessel development at birth. The sprouting of new blood vessels, pathologic neovascularization, induced by hypoxia is the crucial event in the pathology of ROP. In severe cases, the pathologic neovascularization leads to fibrovascular proliferation, vitreoretinal traction, and tractional retinal detachment, which then leads to severe loss of vision. Various factors may be involved in regulating the pathologic neovascularization, and among them vascular endothelial growth factor (VEGF) has been reported to be the dominant factor.[2–7]

The vitreous level of VEGF has been shown to be higher in stage 4 ROP eyes than in control (congenital cataract) eyes, and the VEGF level was correlated with the vascular activity.[3–5] In addition, anti-VEGF therapy, for example, intravitreal injection of bevacizumab, a humanized monoclonal antibody against VEGF, can reduce the angiogenic activity in ROP eyes.[6,7]

The angiopoietins (Angs) are growth factors that modulate the processes of not only physiological angiogenesis but also pathologic neovascularization, particularly associated with VEGF.[8] Ang-1, Ang-2, Ang-3, and Ang-4 are members of the Ang family.[8] Among them, Ang-1 and Ang-2 are ligands of tyrosine kinase receptor Tie2 and have similar binding affinities for Tie2.[8,9] Ang-1 is expressed by pericytes in vitro and in vivo.[10] It induces autophosphorylation of Tie2 and promotes remodeling, maturation, and stabilization of blood vessels by recruiting surrounding support cells and extracellular matrix.[11–14] Ang-2, on the other hand, is expressed by endothelial cells in vivo and is an antagonist for the Tie2 receptor by inhibiting its autophosphorylation.[9,15,16] Ang-2 induces endothelial destabilization and promotes angiogenesis in the presence of VEGF, and destabilization by Ang-2 in the absence of VEGF leads to the regression of fragile vessels.[14,17] The production of Ang-2 is upregulated by hypoxia[18] and VEGF.[18,19]

The purpose of this study was to measure the vitreous levels of Ang-1 and Ang-2 in ROP eyes and to compare the levels to that in eyes with congenital cataracts. We also determined the correlation between the Ang-1 and Ang-2 levels in the vitreous.

METHODS

FORTY-EIGHT EYES OF 36 INFANTS (17 FEMALE AND 19 MALE infants) with stage 4 ROP (4A, 36 eyes; 4B, 12 eyes) were studied. The mean gestational age of the infants was 24.2 weeks (range, 22–26 weeks), and the mean birth weight was 640 grams (range, 332–977 grams). All of the infants

Accepted for publication Aug 25, 2010.
From the Departments of Applied Visual Science (T.S., S.K.) and Ophthalmology (C.S.), Osaka University, Graduate School of Medicine, Suita, Japan.
Inquiries to Shunji Kusaka, Department of Ophthalmology, Sakai Hospital Kinki University Faculty of Medicine, 2-7-1 Harayamadai, Minami-ku, Sakai, 590-0132 Osaka, Japan; e-mail: kusaka-ns@umin.net

0002-9394/$36.00
doi:10.1016/j.ajo.2010.08.037

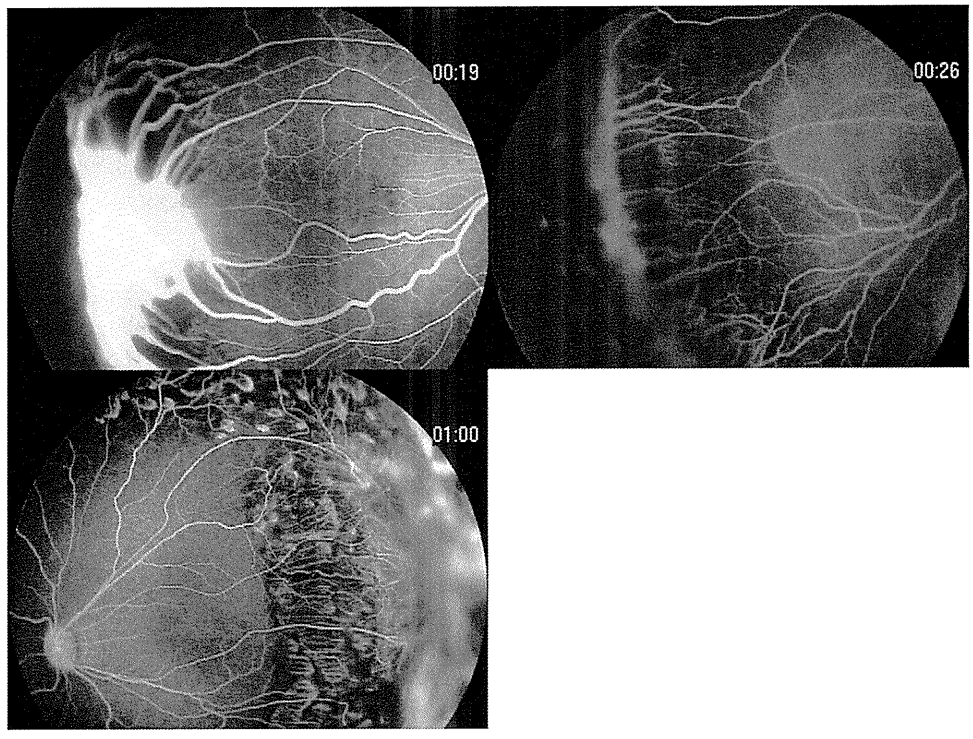


FIGURE 1. Fluorescein angiography in eyes with stage 4 retinopathy of prematurity (ROP). (Top left) Representative fluorescein angiogram in an eye with highly vascular-active ROP. The angiogram shows dilation and tortuosity of the posterior retinal vessels and marked fluorescein leakage from the neovascularization. (Top right) Representative fluorescein angiogram in an eye with moderately vascular-active ROP. The angiogram shows dilation and tortuosity of the posterior retinal vessels and weak fluorescein leakage from neovascularization. (Bottom left) Representative fluorescein angiogram in an eye with mildly vascular-active ROP. The angiogram shows that the posterior retinal vessels are not dilated and tortuous and shows the mild fluorescein leakage from the neovascularization.

underwent primary vitreous surgery at the Osaka University Hospital, Osaka, Japan from July 5, 2007 through December 24, 2009. All of the eyes underwent indirect photocoagulation of the avascular peripheral retina before the vitrectomy.

Fundus examinations with a slit lamp and contact lens (Volk Quad Pediatric Lens; Volk Optical Inc, Mentor, Ohio, USA) were performed under general anesthesia. During the examinations, fundus photographs and fluorescein angiograms were taken with a RetCam 120 digital fundus camera (Massie Research Laboratories, Inc, Pleasanton, California, USA). The stage of the ROP was based on the International Classification of Retinopathy of Prematurity.[20] In addition, the stage 4 ROP eyes were classified into 3 groups: highly vascular-active ROP, moderately vascular-active ROP, and mildly vascular-active ROP, as described (Figure 1).[4,5] Eyes with highly vascular-active ROP initially received 0.5 mg of intravitreal bevacizumab (IVB) and underwent vitrectomy 1 to 7 days after the injection.[7] Vitrectomy was performed on moderately and mildly vascular-active ROP eyes without IVB.

Six eyes of 5 infants with congenital cataract (3 female and 2 male infants), whose ages ranged from 1 month to 4 years, were studied as controls. All of these infants were full-term babies and did not have any other ocular or systemic complications.

Undiluted vitreous samples were collected from eyes with ROP during 3-port closed vitrectomy with a 23-gauge system before the infusion valve was opened. In the eyes with congenital cataract, the 2-port limbal approach was used. After lens aspiration, the aqueous humor in anterior chamber was replaced by viscoelastic material. A posterior continuous curvilinear capsulorrhexis was performed,[21] and undiluted vitreous samples were collected during the anterior vitrectomy.

The vitreous samples were collected in sterile tubes, which were then placed on dry ice and stored at −80°C until the assay. For the protein assay, the vitreous samples were thawed and centrifuged at 15 000 rpm for 10 minutes at 4°C. The supernatants were used to determine the vitreous levels of Ang-1 and Ang-2 by enzyme-linked immunosorbent assay with kits for human anti-Ang-1 and anti-Ang-2 (R & D Systems, Minneapolis, Minnesota, USA). The minimum detectable levels of the tests were 3.45 pg/mL for Ang-1 and 8.29 pg/mL for Ang-2. If the raw data were less than the minimum detectable levels, they were set to 0 for the statistical analyses. Each assay used 30 μL for Ang-1 and 25 μL for Ang-2 of the vitreous

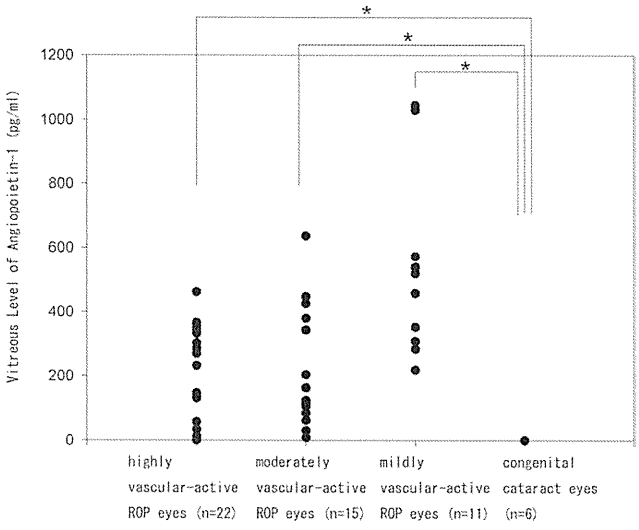


FIGURE 2. Vitreous level of angiopoietin-1 in eyes with retinopathy of prematurity and congenital cataract. The horizontal axis represents the sample type and the vertical axis represents the vitreous level of angiopoietin-1. Statistical analyses were performed by Kruskal-Wallis 1-way analysis of variance, followed by Dunn's method (*$P < .05$). ROP = retinopathy of prematurity.

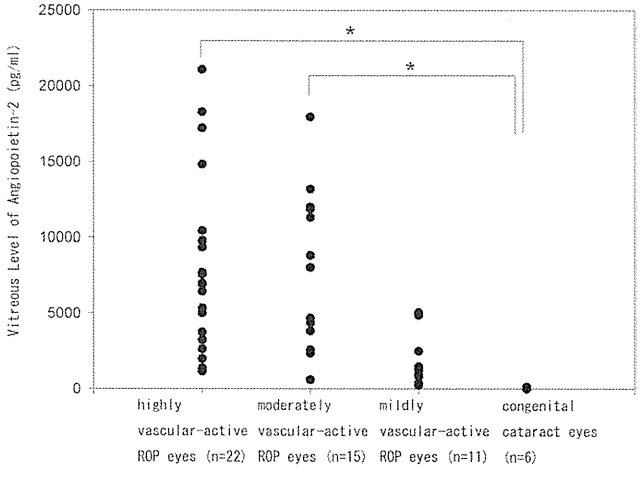


FIGURE 3. Vitreous level of angiopoietin-2 in eyes with retinopathy of prematurity and congenital cataract. The horizontal axis represents the sample type and the vertical axis represents the vitreous level of angiopoietin-2. Statistical analyses were performed by Kruskal-Wallis 1-way analysis of variance, followed by Dunn's method (*$P < .05$). ROP = retinopathy of prematurity.

sample/well, and the assay was performed twice. The optical density was determined at 450 nm with an absorption spectrophotometer ($ARVO_{MX}$; PerkinElmer Japan, Kanagawa, Japan) with the correction wavelength set at 540 nm.

Statistical analyses were performed using SPSS software (Sigma Stat; Systat Software, Inc, San Jose, California, USA). Data are presented as the means and ranges.

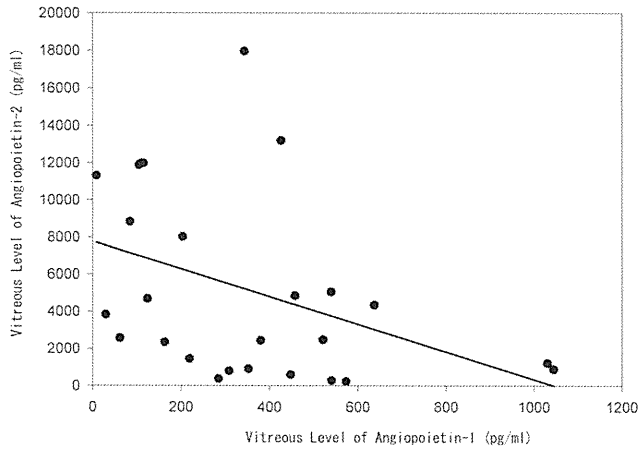


FIGURE 4. Correlation between angiopoietin-1 and -2 levels in the vitreous of eyes with retinopathy of prematurity (ROP). The horizontal and vertical axes represent the vitreous levels of angiopoietin-1 and -2 in moderately and mildly vascular-active ROP eyes, respectively. Statistical analyses were performed by Pearson product moment correlation ($r = -0.406$, $P = .040$).

Kruskal-Wallis 1-way analysis of variance (ANOVA) on ranks was used to compare the vitreous concentrations of Ang-1and Ang-2 among the groups, followed by Dunn's method to detect significant difference between 2 groups. The correlation between the Ang-1 and Ang-2 levels was determined by the Pearson product moment correlation. A P value less than .05 was considered to be statistically significant.

RESULTS

AMONG THE EYES WITH STAGE 4 ROP, 22 EYES WERE CLASsified as highly vascular-active ROP, 15 eyes as moderately vascular-active ROP, and 11 eyes as mildly vascular-active ROP. The mean vitreous level of Ang-1 was 201.9 pg/mL (range, 0 to 461.7 pg/mL) in the highly vascular-active ROP eyes, 216.1 pg/mL (range, 8.5 to 637.0 pg/mL) in the moderately vascular-active ROP eyes, 533.8 pg/mL (range, 218.9 to 1044.9 pg/mL) in the mildly vascular-active ROP eyes, and 0 pg/mL (range, 0 to 0 pg/mL) in the control eyes. The vitreous levels of Ang-1 were significantly different ($P < .001$) among the 4 groups. The levels of Ang-1 in the highly, moderately, and mildly vascular-active ROP eyes were significantly ($P < .05$) higher than that in the control eyes (Figure 2).

The vitreous levels of Ang-2 were significantly different ($P < .001$) among the 4 groups. The mean vitreous level of Ang-2 in the highly vascular-active ROP eyes that had received IVB was 7832.1 pg/mL (range, 1136.6 to 21 078.1 pg/mL). The mean vitreous level of Ang-2 was 7731.2 pg/mL (range, 599.5 to 17 956.0 pg/mL) in the moderately vascular-active ROP eyes, 1685.9 pg/mL (range, 231.0 to 5055.1 pg/mL) in the mildly vascular-active ROP eyes, and

41.5 pg/mL (range, 0 to 125.5 pg/mL) in the control eyes. The levels of Ang-2 in the highly and moderately vascular-active ROP eyes were significantly ($P < .05$) higher than that in control eyes (Figure 3).

Because the vitreous level of Ang-2 in highly vascular-active ROP eyes could be affected by the IVB,[18,19] the correlation of the vitreous levels between Ang-1 and Ang-2 was analyzed only in the moderately and mildly vascular-active ROP eyes. The analysis showed a significant negative correlation ($r = -0.406$; $P = .040$) between the Ang-1 and Ang-2 levels in the vitreous of these ROP eyes (Figure 4).

DISCUSSION

THE MAJOR FINDINGS IN THIS STUDY ARE: FIRST, THE VITreous level of Ang-1 was significantly higher in the highly, moderately, and mildly vascular-active ROP eyes than in control eyes. Second, the vitreous Ang-2 level was significantly higher in the highly and moderately vascular-active ROP eyes than in control eyes. And third, there was a significant negative correlation between the Ang-1 and Ang-2 levels in the vitreous fluid of the moderately and mildly vascular-active ROP eyes.

Ang-1 was first described in 1996 to be a ligand for the Tie2 receptor.[12] In vivo experiments on transgenic mice that overexpressed Ang-1[22] or of recombinant adenoviruses expressing Ang-1[23] showed that Ang-1 induced the development of blood vessels that were not leaky and vessels that did not leak when exposed to inflammatory agents. Mice lacking Ang-1 had angiogenic deficits similar to those seen in mice lacking Tie2. These findings indicated that Ang-1 was a primary physiologic ligand for Tie2, and that it played a critical role in in vivo angiogenic activity.[13] Ang-1 has also been demonstrated in diabetic rats where it prevents and reverses diabetic retinal vascular changes, such as blood-retinal barrier breakdown, in both new and established diabetes.[24] We found that the vitreous level of Ang-1 was significantly higher in the highly, moderately, and mildly vascular-active ROP eyes than that in the control eyes. Taking these results together, Ang-1 may contribute to the antipermeability and stabilization of new blood vessels in ROP eyes with any increased degree of vascular activity.

Ang-2 was first described in 1997 as a naturally occurring antagonist against Ang-1 and Tie2.[9] Transgenic mice overexpressing Ang-2 had disruptions in the formation of embryonic blood vessels.[9] Ang-2 also caused pericyte dropout in normal retinas.[25] The inhibition of Ang-2 by an intravitreal injection of sTie2-Fc reduced retinal neovascularization in murine oxygen-induced retinopathy (OIR), a commonly used mouse model of ROP.[16] The vitreous level of Ang-2 has been shown to be higher in eyes with proliferative diabetic retinopathy than that in eyes without proliferative diabetic retinopathy, and the level increased as the vascular activity increased.[26] We found that the level of Ang-2 was significantly higher in the highly and moderately vascular-active ROP eyes than that of control eyes. Taken together, Ang-2 may contribute to vascular plasticity and angiogenesis in ROP eyes.

We also found that the vitreous levels of Ang-1 and Ang-2 were significantly negatively correlated in the moderately and mildly vascular-active ROP eyes. These results indicated that the balance of positive and negative regulation by both Ang-1 and Ang-2 may be crucial to angiogenic events in ROP.

We did not determine the vitreous level of VEGF. However, we have measured the vitreous level of VEGF in stage 4 ROP eyes[4,5] and found that the level in highly vascular-active ROP eyes that had received 0.5 mg of IVB was not significantly different from that of control (congenital cataract) eyes.[4] This suggested that a dose of 0.5 mg IVB may be enough to inhibit the activity of VEGF in ROP eyes. On the other hand, although the IVB led to reduced neovascular activity in most cases of ROP, we have never had a complete resolution of leakage after IVB.[7] The reason for this discrepancy between the VEGF and neovascular activity has not been determined. One possibility is that a molecule (eg, Ang-2) other than VEGF may play a significant role in the vascular activity in ROP eyes.

The origins of vitreous Ang-1 and Ang-2 were also not determined. Because the serum concentrations of the Angs were not measured, we cannot eliminate the possibility of leakage of the Angs from blood into the vitreous cavity. However, the vitreous Angs are more likely derived from the eye. One of the reasons for this is that the production of Ang-2 has been demonstrated to be upregulated by hypoxia and VEGF,[18,19] which is similar to the condition in ROP eyes. In fact, we have investigated the comprehensive gene expression changes in murine OIR, and demonstrated that the mRNA level of Ang-2 is upregulated at the time when the extraretinal neovascularization is the most prominent.[27] These results suggested that the Angs may be produced in ROP eyes in response to the clinical conditions. Further studies are needed to determine the origin of Angs in the vitreous.

In summary, the results of this study showed that the vitreous levels of Ang-1 and Ang-2 in stage 4 ROP eyes were significantly higher than those of control eyes. The levels of Ang-1 and Ang-2 showed a significant negative correlation. These results indicate that the balance of positive and negative regulation by both Ang-1 and Ang-2 may be important to the pathology of ROP.

PUBLICATION OF THIS ARTICLE WAS SUPPORTED BY GRANT-IN-AID 17591832 AND 20592041 FROM THE MINISTRY OF Education, Culture, Sports, Science and Technology, Tokyo, Japan. The authors indicate no financial conflict of interest. Involved in conception and design (T.S., S.K.); analysis and interpretation (T.S., S.K.); writing the article (T.S., S.K.); critical revision (T.S., S.K.); final approval (T.S., C.S., S.K.);

data collection (T.S., C.S., S.K.); provision of patients and resources (T.S., C.S., S.K.); statistical expertise (T.S., S.K.); and literature search (T.S., S.K.). The procedures used in this study conformed to the tenets of the Declaration of Helsinki and were approved by the Institutional Review Board of Osaka University Hospital. The parents of all of the patients provided written informed consent after an explanation of the nature and possible consequences of this study.

REFERENCES

1. Terry TL. Fibroblastic overgrowth of persistent tunica vasculosa lentis in infants born prematurely: II. Report of cases-clinical aspects. Trans Am Ophthalmol Soc 1942;40: 262–284.
2. Mechoulam H, Pierce EA. Retinopathy of prematurity: molecular pathology and therapeutic strategies. Am J Pharmacogenomics 2003;3(4):261–277.
3. Sonmez K, Drenser KA, Capone A Jr, Trese MT. vitreous levels of stromal cell-derived factor 1 and vascular endothelial growth factor in patients with retinopathy of prematurity. Ophthalmology 2008;115(6):1065–1070.
4. Sato T, Kusaka S, Shimojo H, Fujikado T. Vitreous levels of erythropoietin and vascular endothelial growth factor in eyes with retinopathy of prematurity. Ophthalmology 2009;116(9): 1599–1603.
5. Sato T, Kusaka S, Shimojo H, Fujikado T. Simultaneous analyses of vitreous levels of 27 cytokines in eyes with retinopathy of prematurity. Ophthalmology 2009;116(11):2165–2169.
6. Mintz-Hittner HA, Kuffel RR Jr. Intravitreal injection of bevacizumab (avastin) for treatment of stage 3 retinopathy of prematurity in zone I or posterior zone II. Retina 2008;28(6):831–838.
7. Kusaka S, Shima C, Wada K, et al. Efficacy of intravitreal injection of bevacizumab for severe retinopathy of prematurity: a pilot study. Br J Ophthalmol 2008;92(11):1450–1455.
8. Koh GY, Kim I, Kwak HJ, Yun MJ, Leem JC. Biomedical significance of endothelial cell specific growth factor, angiopoietin. Exp Mol Med 2002;34(1):1–11.
9. Maisonpierre PC, Suri C, Jones PF, et al. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science 1997;277(5322):55–60.
10. Sundberg C, Kowanetz M, Brown LF, Detmar M, Dvorak HF. Stable expression of angiopoietin-1 and other markers by cultured pericytes: phenotypic similarities to a subpopulation of cells in maturing vessels during later stages of angiogenesis in vivo. Lab Invest 2002;82(4):387–401.
11. Sato TN, Tozawa Y, Deutsch U, et al. Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. Nature 1995;376(6535):70–74.
12. Davis S, Aldrich TH, Jones PF, et al. Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. Cell 1996;87(7):1161–1169.
13. Suri C, Jones PF, Patan S, et al. Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. Cell 1996;87(7):1171–1180.
14. Holash J, Wiegand SJ, Yancopoulos GD. New model of tumor angiogenesis: dynamic balance between vessel regression and growth mediated by angiopoietins and VEGF. Oncogene 1999;18(38):5356–5362.
15. Chi JT, Chang HY, Haraldsen G, et al. Endothelial cell diversity revealed by global expression profiling. Proc Natl Acad Sci U S A 2003;100(19):10623–10628.
16. Takagi H, Koyama S, Seike H, et al. Potential role of the angiopoietin/tie2 system in ischemia-induced retinal neovascularization. Invest Ophthalmol Vis Sci 2003;44(1):393–402.
17. Asahara T, Chen D, Takahashi T, et al. Tie2 receptor ligands, angiopoietin-1 and angiopoietin-2, modulated VEGF-induced postnatal neovascularization. Circ Res 1998; 83(3):233–240.
18. Oh H, Takagi H, Suzuma K, Otani A, Matsumura M, Honda Y. Hypoxia and vascular endothelial growth factor selectively up-regulated angiopoietin-2 in bovine microvascular endothelial cells. J Biol Chem 1999;274(22):15732–15739.
19. Mandriota SJ, Pepper MS. Regulation of angiopoietin-2 mRNA levels in bovine microvascular endothelial cells by cytokines and hypoxia. Circ Res 1998;83(8):852–859.
20. International committee for the classification of retinopathy of prematurity. The international classification of retinopathy of prematurity revisited. Arch Ophthalmol 2005;123(7):991–999.
21. Gimbel HV, DeBroff BM. Posterior capsulorhexis with optic capture: maintaining a clear visual axis after pediatric cataract surgery. J Cataract Refract Surg 1994;20(6):658–664.
22. Thurston G, Suri C, Smith K, et al. Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1. Science 1999;286(5449):2511–2514.
23. Thurston G, Rudge JS, Ioffe E, et al. Angiopoietin-1 protects the adult vasculature against plasma leakage. Nat Med 2000;6(4):460–463.
24. Joussen AM, Poulaki V, Tsujikawa A, et al. Suppression of diabetic retinopathy with angiopoietin-1. Am J Pathol 2002; 160(5):1683–1693.
25. Hammes HP, Lin J, Wagner P, et al. Angiopoietin-2 causes pericyte dropout in the normal retina: evidence for involvement in diabetic retinopathy. Diabetes 2004;53(4):1104–1110.
26. Watanabe D, Suzuma K, Suzuma I, et al. Vitreous levels of angiopoietin 2 and vascular endothelial growth factor in patients with proliferative diabetic retinopathy. Am J Ophthalmol 2005;139(3):476–481.
27. Sato T, Kusaka S, Hashida N, Saishin Y, Fujikado T, Tano Y. Comprehensive gene expression profile in murine oxygen-induced retinopathy. Br J Ophthalmol 2009;93(1):96–103.

Biosketch

Tatsuhiko Sato, MD, received his medical degree from Osaka University Graduate School of Medicine, Suita, Japan, in 2001. He completed residency at Osaka University Hospital. Now he is an attending staff in Ophthalmology at Osaka Rosai Hospital. His field of interest includes surgical treatment of vitreoretinal disease such as diabetic retinopathy, retinal detachment, and retinopathy of prematurity.

Serum Concentrations of Bevacizumab (Avastin) and Vascular Endothelial Growth Factor in Infants With Retinopathy of Prematurity

TATSUHIKO SATO, KAZUKO WADA, HITOMI ARAHORI, NORIYUKI KUNO, KENJI IMOTO, CHIHARU IWAHASHI-SHIMA, AND SHUNJI KUSAKA

• PURPOSE: To determine the serum concentrations of bevacizumab and vascular endothelial growth factor (VEGF) in infants with retinopathy of prematurity (ROP) who received intravitreal bevacizumab; and to determine whether the changes in the serum concentration of bevacizumab were significantly correlated with the serum concentration of VEGF after intravitreal bevacizumab.

• DESIGN: Case series.

• METHODS: Eleven infants (4 girls and 7 boys) with ROP were studied. They received 0.25 mg or 0.5 mg of intravitreal bevacizumab to either 1 eye (unilateral cases) or both eyes (bilateral cases) with vascularly active ROP. Serum samples were collected before and 1 day, 1 week, and 2 weeks after the intravitreal bevacizumab. The serum concentrations of bevacizumab and VEGF were measured by enzyme-linked immunosorbent assay, and the correlation in the serum levels between the 2 was determined.

• RESULTS: The serum concentration of bevacizumab before and 1 day, 1week, and 2 weeks after a total of 0.5 mg of intravitreal bevacizumab was 0 ng/mL, 195 ± 324 ng/mL, 946 ± 680 ng/mL, and 1214 ± 351 ng/mL, respectively. The serum bevacizumab level before and 1 day and 1 week after a total 1.0 mg of intravitreal bevacizumab was 0 ng/mL, 248 ± 174 ng/mL, and 548 ± 89 ng/mL, respectively. The serum concentration of VEGF before and 1 day, 1 week, and 2 weeks after a total of 0.5 mg intravitreal bevacizumab was 1628 ± 929 pg/mL, 427 ± 140 pg/mL, 246 ± 110 pg/mL, and 269 ± 157 pg/mL, respectively. There was a significant negative correlation ($r = -0.575$, $P = .0125$) between the serum concentration of bevacizumab and VEGF when a total of 0.25 mg or 0.5 mg of bevacizumab was injected.

• CONCLUSIONS: These results indicate that bevacizumab can escape from the eye into the systemic circulation and reduce the serum level of VEGF in infants with ROP. Continued extensive evaluations of infants are warranted for possible effects after intravitreal bevacizumab in ROP patients. (Am J Ophthalmol 2011; xx:xxx. © 2011 by Elsevier Inc. All rights reserved.)

Accepted for publication Jul 22, 2011.

From the Departments of Applied Visual Science (T.S., S.K.), Pediatrics (K.W., H.A.), and Ophthalmology (C.I.S.), Osaka University Graduate School of Medicine, Suita, Japan; Research and Development Center, Santen Pharmaceutical Co, Ltd (N.K., K.I.), Nara, Japan; and Department of Ophthalmology (S.K.), Sakai Hospital, Kinki University Faculty of Medicine, Osaka, Japan.

Inquiries to Shunji Kusaka, Department of Ophthalmology, Sakai Hospital, Kinki University Faculty of Medicine, 2-7-1 Harayamadai, Minami-ku, Sakai, 590-0132 Osaka, Japan; e-mail: kusaka-ns@umin.net

RETINOPATHY OF PREMATURITY (ROP) IS THE LEADING cause of infant blindness, especially in developed countries. Retinal photocoagulation of the peripheral avascular retina is commonly used to treat eyes with ROP without retinal detachment, and scleral buckling or vitrectomy is used in ROP eyes with retinal detachment. Recently, early vitrectomy has been used to treat eyes with ROP to obtain favorable functional and structural outcomes.[1] However, some of the ROP eyes have high vascular activity, and vitrectomy in these eyes usually results in poor surgical outcomes.[2]

For such cases with high vascular activity, we have performed vitrectomy combined with a preoperative intravitreal injection of an antibody against vascular endothelial growth factor (VEGF).[3] VEGF is the main growth factor responsible for angiogenesis and is considered to be the primary angiogenic factor that mediates retinal neovascularization in eyes with ROP.[4] Studies of patients with stage 4 ROP showed that the vitreous concentration of VEGF in eyes with vascularly active ROP was significantly higher than in eyes with vascularly inactive ROP,[5–7] and anti-VEGF therapy has been shown to be effective in reducing the angiogenic activity in eyes with ROP.[3,8,9]

Bevacizumab (Avastin; Genentech Inc, South San Francisco, California, USA) is a recombinant humanized monoclonal antibody that is directed against all isoforms of VEGF. Many studies have reported on the effectiveness of intravitreal bevacizumab on neovascular disorders, for example, age-related macular degeneration,[10] proliferative diabetic retinopathy,[11] neovascular glaucoma,[12] and ROP.[3,8,9] In addition, the results of a randomized clinical trial that compared intravitreal bevacizumab as monotherapy with laser therapy in the treatment of ROP have been published.[13] As intravitreal bevacizumab was shown to be of significant benefit compared to laser therapy in zone I stage 3+ ROP, the use of intravitreal bevacizumab in the treatment of ROP is likely to be more common in the near future. However, there are also studies that have reported that intravitreal bevacizumab had adverse sys-

0002-9394/$36.00
doi:10.1016/j.ajo.2011.07.005