

FIGURE 5. Graphs showing the percentage changes in the retinal and choroidal thicknesses during the follow-up period. (Top) Graph showing the percentage changes in the retinal and choroidal thicknesses after photodynamic therapy (PDT) compared with baseline. The open triangles with the connecting dashed line indicate the retinal thickness; the open circles with the connecting solid line indicate the changes in the choroidal thickness. Both the retinal and choroidal thicknesses increased at 1 and 2 days after treatment and decreased at 1 week and 1 month. The retinal thickness at 3 and 6 months increased slightly from that at 1 month. Baseline = before photodynamic therapy (PDT); 1 D = 1 day after PDT; 2 D = 2 days after PDT; 1 W = 1 week after PDT; 1 M = 1 month after PDT; 3 M = 3 months after PDT; 6 M = 6 months after PDT. (Bottom) Graph showing changes in the ranibizumab plus PDT group. Percentage changes in the retinal and choroidal thickness after intravitreal ranibizumab injections and PDT are compared with baseline. The open triangles with the connecting dashed line indicate the retinal thickness; the open circles with the connecting solid line indicate the changes in the choroidal thickness. Both the retinal and choroidal thicknesses were the same at 0 day, increased at 1 and 2 days, and decreased at 1, 3, and 6 months. The choroidal thickness at 1 week returned to baseline, and the retinal thickness at 1 week already decreased below baseline. The retinal thickness at 6 months remained lower than at baseline. Baseline = before intravitreal ranibizumab injection; 0 D = before PDT; 1 D = 1 day after PDT; 2 D = 2 days after PDT; 1 W = 1 week after PDT; 1 M = 1 month after PDT; 3 M = 3 months after PDT; 6 M = 6 months after PDT.

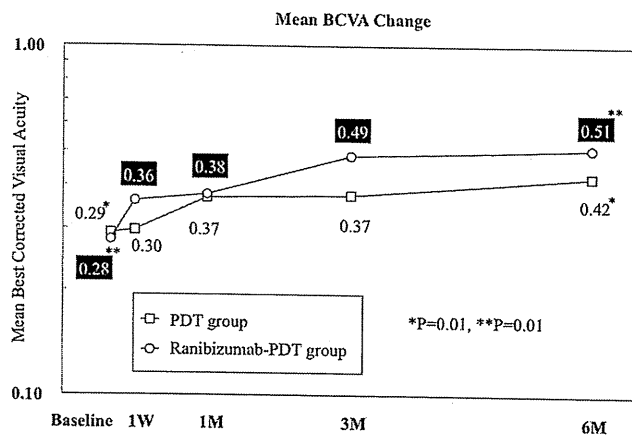


FIGURE 6. The best-corrected visual acuity (BCVA) changes in the photodynamic therapy (PDT) group and the ranibizumab plus PDT group. The open circles indicate the ranibizumab plus PDT group; the open squares indicate the PDT group. The mean BCVA between baseline and 6 months improved significantly more in the ranibizumab plus PDT group ( $P = .01$ ) than in the PDT group ( $P = .01$ ). Baseline = before intravitreal ranibizumab injection in the ranibizumab PDT group and before PDT in the PDT group; 1 W = 1 week after PDT; 1 M = 1 month after PDT; 3 M = 3 months after PDT; 6 M = 6 months after PDT.

from permeating the retinal pigment epithelium from the choroid intraretinally or subretinally. Although a study with more patients and longer follow-up is needed, combination therapy may help to resolve the medical and economic problems.

This retrospective study had several weaknesses. It was a pilot study with few patients and a short-term follow-up period for a new technique. Another study should evaluate the interactions between the choroidal thickness and age and refractive error. However, we believe the current findings suggest the importance of measuring the choroidal thickness during follow-up and understanding the treatment effectiveness.

In conclusion, both PDT monotherapy and combination therapy caused regression of the polypoidal lesions and decreased the retinal and choroidal thicknesses because of reduced choroidal vascular hyperpermeability. Combination therapy could reduce the transient exudation immediately after PDT, could maintain the retinal thinning with monthly intravitreal ranibizumab injections, and could improve the vision better than PDT monotherapy. EDI OCT noninvasively monitored the proposed site for the pathophysiologic changes in the retina and choroid and provided information not available by other means.

THE AUTHORS INDICATE NO FINANCIAL SUPPORT OR FINANCIAL CONFLICT OF INTEREST. INVOLVED IN DESIGN AND CONDUCT OF STUDY (I.M., T.I., M.S.); Collection of data (I.M., Y.S.); Management, analysis, and interpretation of data (I.M., T.I., T.S.); Preparation and review of manuscript (I.M., T.I.); and Approval of the manuscript (I.M., T.I., Y.S., M.S., T.S.). This study followed the tenets of the Declaration of Helsinki. The Institutional Review Board at Fukushima Medical University School of Medicine approved optical coherence tomography observation for

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## Fluorescein Staining of the Vitreous During Vitrectomy for Retinopathy of Prematurity

Vitreous staining using triamcinolone acetonide<sup>1</sup> or fluorescein<sup>2</sup> generally has been used even in children<sup>3</sup> to visualize the preretinal membrane and vitreous during vitrectomy.

During the surgery for retinopathy of prematurity (ROP), careful segmentation and extensive vitreous cutting are required around the base of tractional retinal detachments, vitreous base, and fibrovascular tissue.<sup>4</sup> Removal of the formed vitreous around the fibrovascular tissue and the vitreous base is a key factor for a successful surgery.<sup>4</sup> To remove the vitreous safely, good intraoperative visualization of the vitreous is essential.

In the current study, we included cases of aggressive posterior ROP in which wide-field vitrectomies are necessary to evaluate the staining of the entire vitreous. We describe a technique for staining the vitreous with fluorescein and compared it with staining using triamcinolone in these patients with ROP.

### Patients and Methods

This study included 45 consecutive eyes with Stage 4A aggressive posterior ROP, which required vitrectomies with lensectomies to be performed in 30 babies (mean age, 24 weeks; range, 22–30 weeks); no infant had undergone a previous surgery. The same surgeon (N.A.) performed all the surgeries in our hospital between June 2005 and May 2008. All aspects of this study were approved by the institutional ethics committee, and the parents of the patients provided informed consent before the infants were enrolled in the study. The mean follow-up duration in the triamcinolone group was 32 months (range, 15–38 months), and in the fluorescein group, the mean follow-up duration was 28 months (range, 12–32 months).

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The demographics of the study group are shown in Table 1. There was no difference in the severity of ROP between the two groups. All patients underwent a 3-port 25-gauge vitrectomy using a small contact lens designed for premature eyes. The other instruments, including infusion pipes, cannulas, and light pipes, were used as in the conventional 25-gauge system. We previously described the procedures of early vitreous surgery for ROP.<sup>4,5</sup> In 27 eyes of 18 patients, sodium intravenous fluorescein (0.1 mL/kg) was injected preoperatively for fluorescein angiography and to visualize the vitreous gel intraoperatively. The intervals between fluorescein angiography and vitrectomy ranged from 10 minutes to 20 minutes. When both eyes required surgery, the mean time to surgery in the other eye was approximately 50 minutes. In the other study group, 0.2 mL of triamcinolone was injected repeatedly into the vitreous cavity in 18 eyes of 12 patients to visualize the residual vitreous after lensectomy and core vitrectomy. The authors evaluated the staining pattern of the vitreous, the postoperative results, and any complications. Analyses were performed, and categorical differences were compared using Fisher's test. All *P* values were 2-sided, and *P* < 0.05 was considered statistically significant. Analyses were conducted using GraphPad Prism5.0 statistical software (GraphPad Prism Software Institute, La Jolla, CA).

### Results

Injections of triamcinolone were required three to five times during the surgery because we could not achieve full visualization of the distance between the residual vitreous and the retinal surface; triamcinolone was only on the cut surface of the vitreous, and the vitreous gel beneath remained transparent. In contrast, fluorescein dye produced homogenous and full-thickness staining (Figure 1), especially around the fibrovascular tissue and the vitreous base (Figure 2), which was sufficiently stained green but remained transparent.

When fluorescein was used, an iatrogenic break occurred in 1 eye (3.7%) as a result of an unstable 25-gauge infusion cannula. The break was repaired by

Table 1. Demographic Characteristics of Eyes Undergoing Vitrectomy for Stage 4A ROP

Characteristic	Fluorescein Injection	Triamcinolone Injection
Number of eyes/patients	27/18	18/12
Gestational age, mean (range), weeks	24 (22–29)	25 (22–30)
Birth weight, mean (range), g	782 (366–1585)	834 (466–1,676)
Age at surgery, mean (range), weeks	15	14
Bilateral eyes	18	12
Zone of disease, eyes		
Zone	7	6
Posterior zone	16	8
Anterior zone	4	4
Extent of fibrous tissue, mean (range), cumulative clock hours	7.9 (1–12)	7.6 (2–12)

endophotocoagulation and fluid–gas exchange. When triamcinolone was used, 3 breaks (16.7%) occurred during cutting of the vitreous on the detached retina because of insufficient visualization.

Table 2 shows the postoperative reattachment rates at the final examination in each group. In the fluorescein group, the rate of complete retinal reattachment was 85%; in the triamcinolone group, the retinal reattachment rate was 67%. The retinas were partially reattached in 15% of the eyes in the fluorescein group and 26% of the eyes in the triamcinolone group. The average follow-up duration was 26 months (range, 15–37 months).

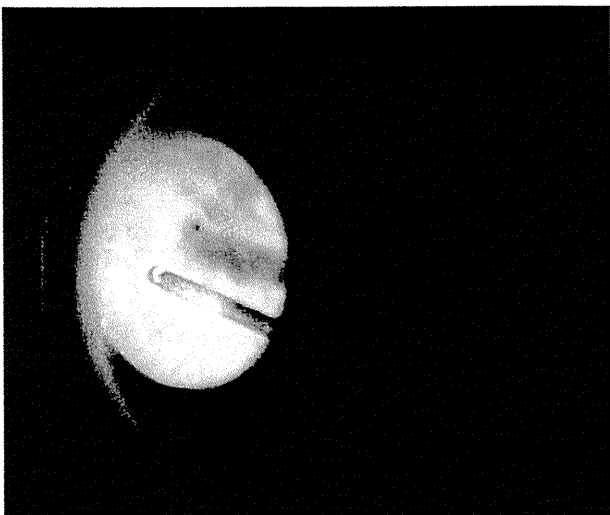


Fig. 1. Injection of fluorescein before vitrectomy to stain the vitreous in patients with Stage 4A ROP. The fluorescein is well distributed over the entire vitreous gel. The posterior vitreous cortex is clearly seen but remains transparent.

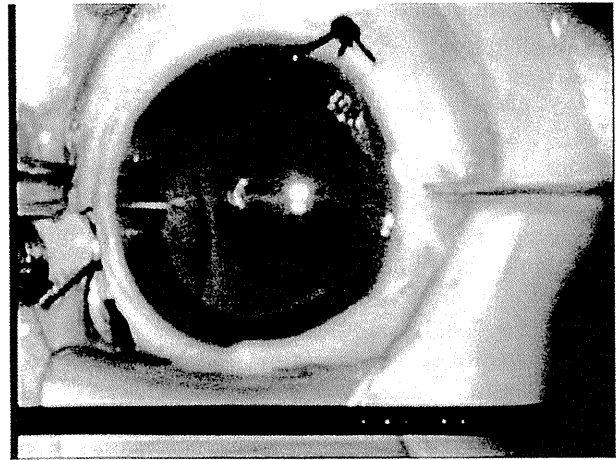


Fig. 2. The vitreous base is stained sufficiently, facilitating good visualization of residual vitreous.

There were no statistical differences between the two groups in the iatrogenic breaks ( $P = 0.64 > 0.05$ ) and the reattachment rates at 18 months postoperatively ( $P = 0.17 > 0.05$ ). No infants had systemic abnormalities intraoperatively or postoperatively. The degrees of postoperative inflammation were similar between the groups. No steroid-induced glaucoma developed postoperatively in the triamcinolone group.

## Discussion

The current study suggested that fluorescein may be more useful than triamcinolone because fluorescein facilitated clear visualization around the vitreous base and the fibrovascular tissue. The base of the tractional retinal detachment was clearly visible and distinguishable from the retina, the fibrous tissue, and the stained peripheral vitreous, making cutting of the vitreous safer.

In the current study, there was no statistical significance between the groups possibly because of the small number of patients. However, an iatrogenic break in one eye stained with fluorescein resulted from the instability of the infusion cannula, and we assume that this break might have occurred during staining

Table 2. Postreattachment at Final Examination

	Triamcinolone Injection (n = 18 eyes)	Fluorescein Injection (n = 27 eyes)
Completely reattached, n (%)	12 eyes (67)	23 eyes (85)
Partially reattached, n (%)	4 eyes (22)	4 eyes (15)
Detached, n (%)	2 eyes (11)	0 eyes (0)
Follow-up (range), months	32 (15–38)	28 (12–32)

with either fluorescein or triamcinolone, which may not be related to the vitreous staining.

Three breaks occurred during cutting of the vitreous stained with triamcinolone, which seemed high; however, we had anticipated that cutting the vitreous without staining during the surgery for Stage 4A ROP would have resulted in more breaks than when using triamcinolone. In addition, the absence of staining would not have facilitated cutting of sufficient vitreous to achieve retinal reattachment in Stage 4A ROP because the solid and transparent vitreous adheres firmly to fibrovascular tissue, and it could not be separated from the retina especially at the vitreous base.

The use of triamcinolone in pediatric cases is controversial and may carry the risk of development of endophthalmitis and glaucoma, and the most common method of staining with triamcinolone in adults is direct injection into the vitreous.<sup>1</sup> However, fluorescein is an ophthalmic angiographic agent that leaks from fibrous tissue and then stains vitreous, and it is used in routine evaluations of patients with ROP.<sup>5</sup> Therefore, in the current study, we evaluated which agent was more suitable for this surgery.

No allergic reactions developed in any cases in which fluorescein was used, although such reactions in adults have been reported.<sup>6</sup> Therefore, these surgeries for ROP should be performed with the infants under carefully controlled general anesthesia. Compared with the use of triamcinolone, which required several vitreous injections into each eye, one intravenous injection of fluorescein eliminated complicated surgical procedures and reduced the risk of the development of endophthalmitis and glaucoma.

In addition, angiography confirmed the status and extent of the ROP preoperatively. Obtaining this

information and a clear full-thickness view using fluorescein may reduce the incidence of iatrogenic dialysis and ensure successful surgeries.

**Key words:** retinopathy of prematurity, early vitreous surgery, fluorescein, staining, vitreous, triamcinolone acetonide.

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# Clinical Features of Congenital Retinal Folds

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• **PURPOSE:** To investigate the clinical features and prognosis of congenital retinal folds without systemic associations.

• **DESIGN:** Retrospective observational case series.

• **METHODS:** The characteristics, clinical course, ocular complications, and best-corrected visual acuity (BCVA) of eyes with congenital retinal folds were studied during the follow-up periods. The affected and fellow eyes were examined by slit-lamp biomicroscopy, binocular indirect ophthalmoscopy, and fundus fluorescein angiography. The parents and siblings of each patient also underwent ophthalmoscopic examinations. The BCVA was measured using a Landolt ring VA chart.

• **RESULTS:** One hundred forty-seven eyes of 121 patients with congenital retinal folds were examined. Fifty-five patients (45.5%) were female. The fold was unilateral in 95 patients (78.5%), and 69 of those patients (72.6%) had retinal abnormalities in the fellow eye. The meridional distribution of folds was temporal in 136 eyes (92.5%). The family history was positive in 32 patients (26.4%). Secondary fundus complications, including fibrovascular proliferation and tractional, rhegmatogenous, and exudative retinal detachments, developed in 44 eyes (29.9%). The BCVAs could be measured in 119 eyes and ranged from 20/100 to 20/20 in 5 eyes (4.2%), 2/100 to 20/200 in 45 eyes (37.8%), and 2/200 or worse in 69 eyes (58.0%). The follow-up periods ranged from 4 to 243 months (mean,  $79.7 \pm 58.9$  months).

• **CONCLUSIONS:** These clinical features suggested that most congenital retinal folds may result from insufficient retinal vascular development, as in familial exudative vitreoretinopathy, rather than persistent fetal vasculature. Adequate management of active retinopathy and late-onset complications, especially retinal detachment, is required. (Am J Ophthalmol 2011;xx:xxx. © 2011 by Elsevier Inc. All rights reserved.)

**A** CONGENITAL RETINAL FOLD (ABLATIO FALCIFORMIS congenital), extending radially from the optic disc toward the peripheral fundus, was first described in 1935 as a rare congenital anomaly.<sup>1,2</sup> The pathogenesis was investigated histologically, and the

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anomaly was hypothesized to be attributable to persistent hyaloid vessels leading to a pulled dysplastic retina. In 1955, Reese reported the clinical and pathologic features of persistent hyperplastic primary vitreous (PHPV).<sup>3</sup> In 1965, Michaelson<sup>4</sup> introduced the term “posterior PHPV,”<sup>4</sup> and in 1970 Pruett and Schepens<sup>5</sup> described a new clinical entity called “posterior hyperplastic primary vitreous,” the posterior form of PHPV, characterized by vitreous membranes extending from the disc toward the peripheral fundus. Those investigators used the term posterior PHPV as a synonym for falciform retinal folds and the term anterior PHPV as a synonym for the PHPV described by Reese.<sup>3</sup> Thus, congenital retinal folds often were diagnosed as posterior PHPV afterward. The term PHPV now has evolved to persistent fetal vasculature (PFV), which usually occurs as a nonheritable set of vascular malformations affecting 1 eye of an otherwise normal infant.<sup>6</sup> However, based on the fundus drawings of Pruett and Schepens,<sup>5</sup> vitreous membranes and retinal folds were not clearly distinguished. Those authors reported that the vitreous band and retinal folds extended toward the fundus periphery in various meridians but were most commonly nasal.<sup>5</sup> They also described the pleomorphism of posterior PHPV and complications such as microcornea, retinal detachment, vitreous hemorrhage, cataract, and glaucoma.<sup>7</sup> In most cases, posterior PHPV is unilateral and rarely familial.

In 1969, familial (dominant) exudative vitreoretinopathy (FEVR), a developmental disorder of the retinal vasculature, was described and suggested to be the possible origin of congenital retinal folds.<sup>8-10</sup> Recently, congenital retinal folds were thought to occur even after birth and were caused by various infantile diseases such as FEVR, retinopathy of prematurity (ROP), Norrie disease, incontinentia pigmenti, and congenital toxoplasmosis. However, clinically distinguishing retinal folds without systemic associations is often difficult, and their pathogenesis remains controversial.

We conducted the current study to clarify the clinical features of congenital retinal folds without systemic associations.

## METHODS

ONE HUNDRED FORTY-SEVEN EYES OF 121 PATIENTS WITH unilateral or bilateral congenital retinal folds, diagnosed at the National Center for Child Health and Development,

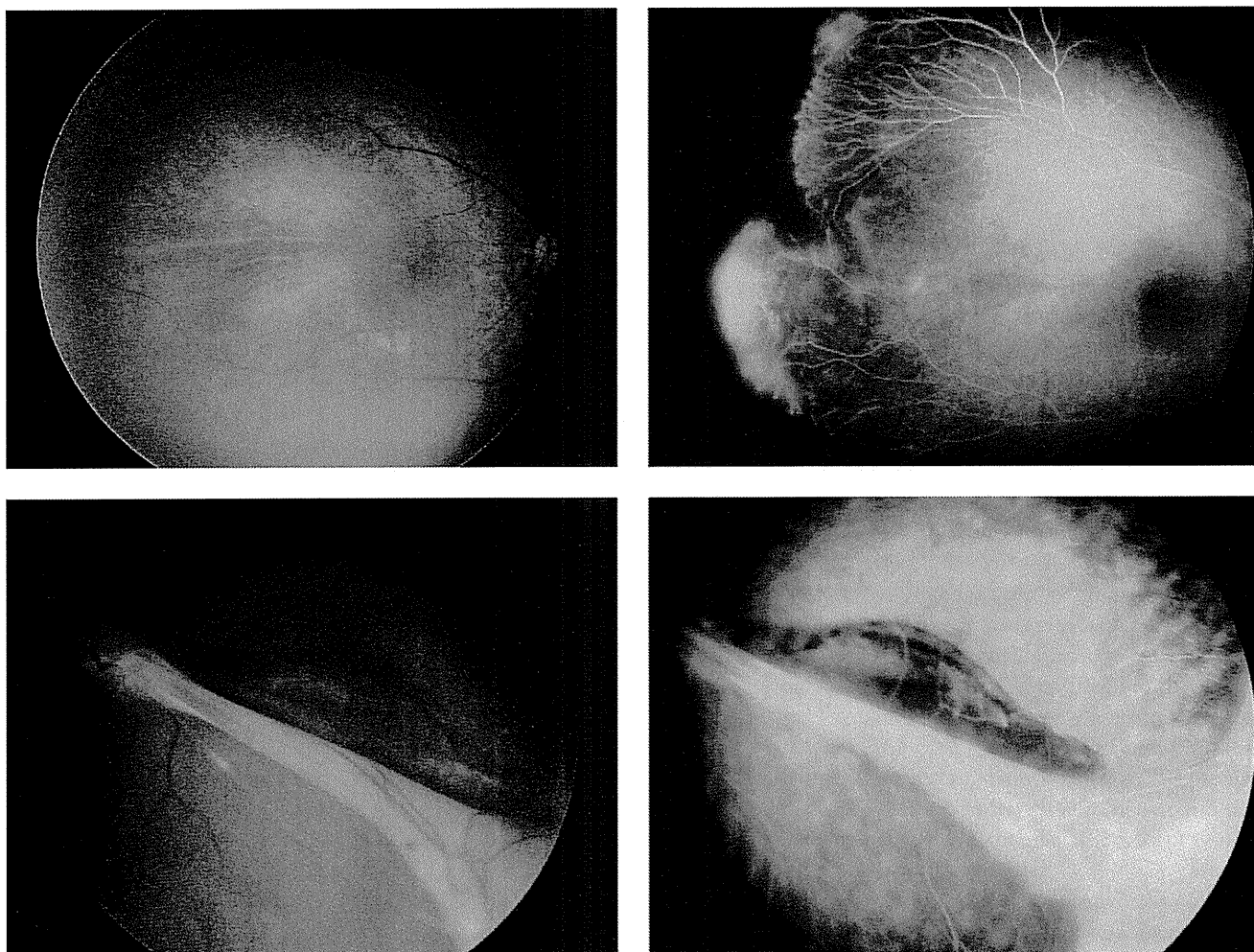


FIGURE 1. Unilateral congenital fold with retinal vascular abnormalities in the fellow eye. Fundus photographs and fluorescein angiography (FA) of a unilateral retinal fold in the left eye (Bottom left and right) and the fellow right eye (Top left and right) in a 4-month-old boy. (Top left) Retinal vascular abnormalities in the peripheral fundus are seen in the fellow right eye. (Top right) FA shows a peripheral avascular zone, supernumerous vascular branchings, arteriovenous shunt formation, a V-shaped area of degeneration, and neovascularization with dye leakage in the fellow right eye. Laser photocoagulation was applied to the peripheral avascular retina. (Bottom left) The retinal vessels within the fold are bundled and pulled toward the peripheral fibrous tissue and decreased in number in the stretched retina. (Bottom right) FA shows hyperfluorescence from folds in which the vessels are bundled and dye leakage from the fibrovascular tissue. Scleral buckling with laser photocoagulation was applied.

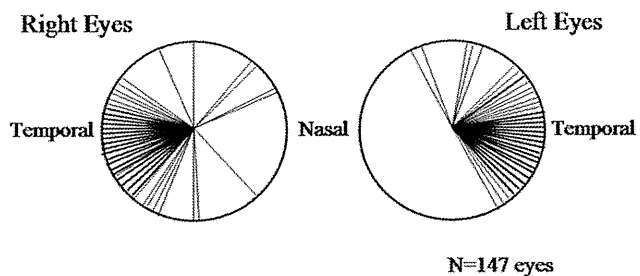


FIGURE 2. Meridional distribution of congenital retinal folds. The meridional distribution of the folds was temporal, superotemporal, or inferotemporal in 92.5% (136/147) eyes.

Tokyo, Japan, between June 1986 and February 2009, and examined between March 2002 and April 2009, were studied retrospectively. Patients with a history of premature birth, oxygen therapy, systemic associations, or positive laboratory examinations for infectious diseases were excluded. Eyes with anterior segment dysgenesis also were excluded.

The characteristics of retinal folds in affected eyes and findings in fellow eyes were examined by slit-lamp biomicroscopy and binocular indirect ophthalmoscopy. Thirty-six patients (29.8%) underwent fundus fluorescein angiography (FA) under general anesthesia. In patients with a unilateral retinal fold, the fundus periphery of the fellow eye also was examined and the retinal vascular development was evaluated. The criteria used to diagnose



**TABLE.** Features of Secondary Ocular Complications in the Fundus in Eyes With Congenital Retinal Folds (N = 44 Eyes)

	Tractional Retinal Detachment N = 19 Eyes	Rhegmatogenous Retinal Detachment N = 12 Eyes	Fibrovascular Proliferation N = 11 Eyes	Exudative Retinal Detachment N = 2 Eyes
Age at onset (months)	1–88 (mean, 25.8 ± 27.2)	33–195 (mean, 87.0 ± 56.5)	2–121 (mean, 19.4 ± 35.8)	31, 167
Origin of complications	Excessive fibrovascular proliferation, 15 (79%) Regrowth of fibrovascular tissue, 4 (21%)	Ocular trauma, 5 (42%) Unknown, 7 (58%)	NV, 10 (91%) Recurrence of NV, 1 (9%)	Unknown 2 (100%)
Treatment	V + L, 6 (32%) B + PC, 4 (21%) None, 9 (47%)	V + L, 5 (42%) V + L + B, 4 (33%) B, 2 (17%) None, 1 (8%)	V + L, 5 (46%) PC, 4 (36%) B + PC, 1 (9%) None, 1 (9%)	None, 2 (100%) <sup>a</sup>
Surgical outcomes	Retinal reattachment, 7/10 (70%)	Retinal reattachment, 3/11 (27%)	NV stabilization, 8/10 (80%)	

B = scleral buckling; B + PC = scleral buckling with laser photocoagulation; NV = neovascularization; PC = laser photocoagulation; V + L = vitrectomy with lensectomy; V + L + B = vitrectomy with lensectomy and scleral buckling.

<sup>a</sup>Untreated retinas reattached spontaneously.

retinal vascular abnormalities were the presence of a peripheral avascular zone, vitreoretinal adhesions, arteriovenous shunt formation, supernumerous vascular branchings, a V-shaped area of retinal degeneration, neovascularization, and cystoid degeneration.<sup>11–13</sup> Ophthalmoscopic examinations of the parents and siblings of each patient were performed when possible. A family history was judged to be present if retinal vascular abnormalities were found in any family members. The clinical course and the secondary ocular complications were investigated during the follow-up periods. The best-corrected visual acuities (BCVAs) were measured with a standard Japanese VA chart using Landolt rings at 5 meters and converted to Snellen VA. The follow-up periods ranged from 4 to 243 months (mean, 79.7 ± 58.9 months).

## RESULTS

• **CHARACTERISTICS OF EYES AND PATIENTS:** Sixty-six of the 121 patients (54.5%) were male and 55 (45.5%) were female. The ages of the patients at the first examination at our hospital ranged from 4 weeks to 9 years 1 month (mean, 17.9 ± 21.6 months). However, the families or pediatricians had observed the clinical manifestations, that is, poor fixation behavior, nystagmus, or strabismus, by 12 months of age in 105 patients (86.8%), and 91 patients (75.2%) had been examined by other ophthalmologists within the first year. A unilateral retinal fold in 16 patients (13.2%) identified after 13 months of age was confirmed not to have any acquired pathogenesis and diagnosed as a congenital retinal fold.

The retinal vessels within the fold were bundled and pulled toward the peripheral fibrous tissue and decreased in number in the stretched retina in 144 of 147 eyes (98.0%) (Figure 1, Bottom left). A peripheral avascular zone was seen more than 3 disc diameters' width in all eyes. Other ophthalmoscopic findings in affected eyes were intravitreal neovascularization in 13 eyes (8.8%), retinal hemorrhages in 8 eyes (5.4%), disc anomalies in 4 eyes (2.7%), retinal exudates in 3 eyes (2.0%), and coloboma and medullated nerve fiber in 1 eye (0.7%) each. Fundus FA, performed on 46 eyes of 36 patients, showed hyperfluorescence from bundling of the retinal vessels in the folds and fibrovascular tissue at the periphery of the folds in all eyes (100%). Dye leakage from an arteriovenous shunt and intravitreal neovascularization within the fibrovascular tissue was detected in 13 eyes (28.3%) (Figure 1, Bottom right).

• **MOST CASES OF CONGENITAL RETINAL FOLD WERE UNILATERAL AND ORIGINATED IN THE TEMPORAL QUADRANTS:** The fold was unilateral in 95 of 121 patients (78.5%) and bilateral in 26 patients (21.5%). The meridional distribution of the folds was temporal, superotemporal, or inferotemporal in 136 of 147 eyes (92.5%) (Figure 2). All folds in the other 11 eyes were unilateral, extending nasally, superonasally, inferonasally, superiorly, or inferiorly.

• **MOST CASES OF UNILATERAL RETINAL FOLD HAD IDENTIFIABLE ABNORMALITIES IN THE FELLOW EYE:** Only 26 cases (27.4%) of the 95 unilateral retinal folds identified demonstrated no pathology in the fellow eye. The remaining 72.6% had identifiable abnormalities as

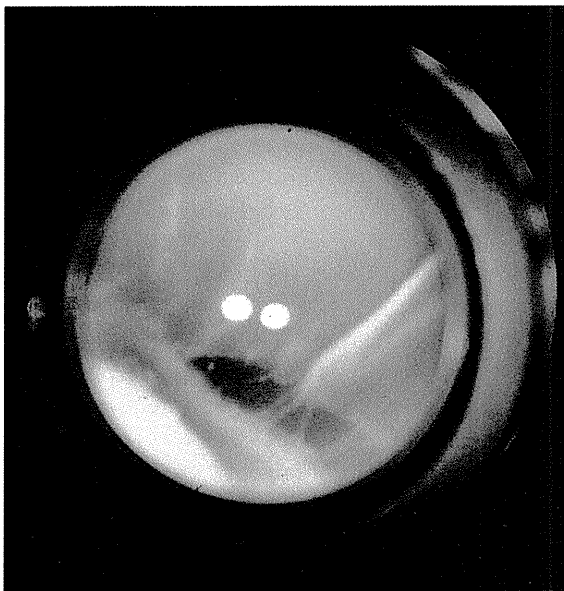
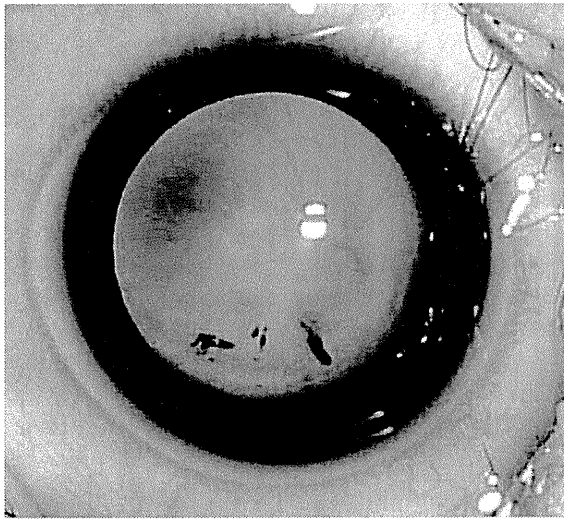
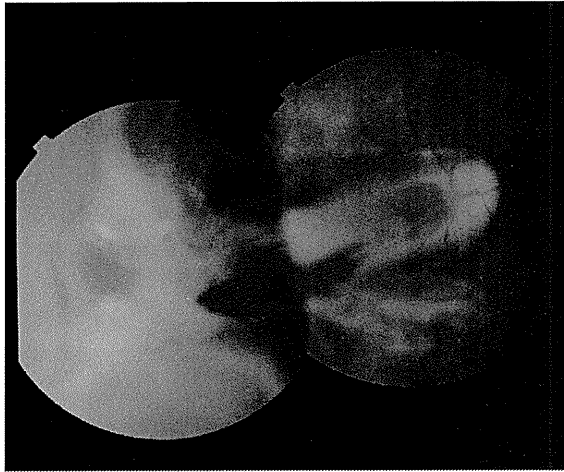


FIGURE 3. Secondary complications of congenital retinal folds. Photographs of secondary complications in the fundus of a 4-month-old girl (Top and Middle) and a 37-month-old boy (Bottom). (Top) Prominent fibrovascular proliferation

follows. Retinal vascular abnormalities in the peripheral fundus were identified in 33 of 95 cases (34.7%): an avascular zone in all eyes (100%), supernumerous vascular branchings in 15 eyes (45.5%), cystoid degeneration in 12 eyes (36.4%), a V-shaped area of retinal degeneration in 9 eyes (27.3%), vitreoretinal adhesions and fibrous membrane in 7 eyes (21.2%) each, arteriovenous shunt formation in 5 eyes (15.2%), and neovascularization in 4 eyes (12.1%) (Figure 1, Top left and right). A total retinal detachment and leukokoria, a dragged retina, and coloboma were found in 18 (18.9%), 17 (17.9%), and 1 (1.1%) of 95 cases, respectively.

The “true” unilateral congenital fold was seen in 26 patients out of all 121 patients (21.5%). Among these 26 patients, the meridional distribution of the folds was temporal in 17 eyes (65.4%) and nasally, superiorly, or inferiorly in 9 eyes (34.6%).

Fundus FA was performed on 24 fellow eyes in patients with a unilateral retinal fold and clearly showed various retinal vascular abnormalities in 18 eyes (75.0%). Hyperfluorescence of the vascular abnormalities in the periphery was seen in 8 of the 24 eyes (33.3%), in which dye leakage from the neovascularization was detected in 4 eyes (4/24; 16.7%) (Figure 1, Top right).

Among the 50 fellow eyes with retinal vascular abnormalities in the periphery or dragged retina, laser photocoagulation was applied to the peripheral avascular retina in 7 eyes (14.0%) and the neovascularization stabilized in all eyes (100%). Scleral buckling was performed in 3 fellow eyes (6.0%) for a late-onset tractional or rhegmatogenous retinal detachment, and retinal reattachment was achieved in all eyes (100%).

• **MOST CASES OF CONGENITAL RETINAL FOLD SUGGEST FAMILIAL INHERITANCE:** Family members were examined in 50 cases (41.3%), and a positive family history was identified in 32 cases (64.0%), with ophthalmoscopic findings of retinal vascular abnormalities in the periphery (81.2%), retinal folds (9.4%), dragged retina (6.3%), and leukokoria (3.1%). A negative family history was suspected by ocular examination of the parents in 18 cases (36.0%). In all cases with a positive family history, the trait originated in 1 of the family lines. In positive cases, the fold was bilateral in 12 cases (37.5%) and unilateral with abnormal retinal vascular changes in the fellow eye in the other 20 cases (62.5%). In negative cases,

progresses with the retinal hemorrhage in the right eye. Laser photocoagulation was applied to the peripheral avascular retina. (Middle) Two months later, a tractional retinal detachment has progressed rapidly. Vitrectomy with lensectomy was performed. (Bottom) Multiple retinal breaks in the periphery at the edge of the retinal fold have induced a rhegmatogenous retinal detachment in the left eye. Vitrectomy with lensectomy and scleral buckling were performed.

the fold was bilateral in 5 cases (27.8%), unilateral with abnormal retinal vascular change in the fellow eye in 9 cases (50.0%), and unilateral with normal fellow eye in 4 cases (22.2%).

• **SECONDARY COMPLICATIONS OF CONGENITAL RETINAL FOLD ARE COMMON AND VISUALLY DEVASTATING:** During the follow-up periods, secondary ocular complications developed in the fundus in 44 of 147 eyes (29.9%) with congenital retinal folds; progression of a tractional retinal detachment in 19 eyes (12.9%), rhegmatogenous retinal detachment in 12 eyes (8.2%), fibrovascular proliferation from the neovascularization in 11 eyes (7.5%), and exudative retinal detachment in 2 eyes (1.4%). The secondary complications in the fundus of 44 eyes are summarized in the Table.

Among the 26 patients with “true” unilateral congenital fold, secondary ocular complications also developed in the fundus in 6 eyes (23.1%); progression of a tractional retinal detachment in 2 eyes (7.7%), rhegmatogenous retinal detachment in 1 eye (3.8%), fibrovascular proliferation from the neovascularization in 2 eyes (7.7%), and exudative retinal detachment in 2 eyes (3.8%).

• **TRACTIONAL RETINAL DETACHMENTS:** Progression of tractional retinal detachment occurred in patients ranging in age from 1 to 88 months (mean,  $25.8 \pm 27.2$  months). Among the 19 eyes the tractional retinal detachment originated from excessive fibrovascular proliferation and contraction in 15 eyes (79%) (Figure 3, Top and Middle) and regrowth of fibrovascular tissue in 4 eyes (21%). Ten eyes (53%) were treated: vitrectomy with lensectomy was performed in 6 eyes (32%) and scleral buckling with laser photocoagulation in 4 eyes (21%). Retinal reattachment was achieved in 7 of 10 treated eyes (70%).

• **RHEGMATOGENOUS RETINAL DETACHMENTS:** A rhegmatogenous retinal detachment developed in patients ranging in age from 33 to 195 months (mean,  $87.0 \pm 56.5$  months). Among the 12 eyes, ocular trauma including the digito-ocular sign was involved in 5 eyes (42%). Multiple or expanded retinal breaks were seen in the periphery within the stretched and fragile retina at the edge of the retinal folds in 8 eyes (67%) (Figure 3 Bottom), dialysis developed in 1 eye (8.3%), and no breaks were seen in 3 eyes. Nine eyes (75%) with a rhegmatogenous retinal detachment had a total retinal detachment with proliferative vitreoretinopathy (PVR). Treatment was performed in 11 eyes (92%): vitrectomy with lensectomy in 5 eyes (42%), vitrectomy with lensectomy and scleral buckling in 4 eyes (33%), and scleral buckling in 2 eyes (17%); however, retinal reattachment occurred in 3 of 11 treated eyes (27%).

• **FIBROVASCULAR PROLIFERATION:** Fibrovascular proliferation progressed in patients ranging in age from 2 to 121 months (mean,  $19.4 \pm 35.8$  months), within the first year in 9 of 11 eyes (82%). Growth of neovascularization was identified in 11 eyes (100%). Treatment was performed in 10 eyes (91%): vitrectomy with lensectomy in 5 eyes (46%), laser photocoagulation applied to the peripheral avascular retina in 4 eyes (36%), and scleral buckling with laser photocoagulation in 1 eye (9%); treatment stabilized the neovascularization and prevented a retinal detachment in 8 of 10 treated eyes (80%).

• **ANTERIOR SEGMENT COMPLICATIONS:** Secondary complications in the anterior segments developed in 16 of 147 eyes (10.9%) with congenital retinal folds; glaucoma in 9 eyes (6.1%), cataract in 8 eyes (5.4%), and band keratopathy and keratoconus in 1 eye (0.7%) each. Two glaucoma eyes developed cataracts and 1 cataract eye developed glaucoma after cataract surgery. Glaucoma developed in patients ranging in age from 2 to 137 months (mean,  $60.1 \pm 46.8$  months). The main cause was fibrovascular proliferation and contraction that resulted in anterior lens displacement and angle-closure glaucoma. Neovascular glaucoma was identified in 1 eye. Treatment was performed in 6 eyes (67%): medical treatment in 4 eyes and lensectomy and peripheral iridectomy in 1 eye each. Cataract developed in patients ranging in age from 11 to 113 months (mean,  $59.1 \pm 40.2$  months). Lensectomy was performed in 4 eyes (50%).

Among the 26 patients with “true” unilateral congenital fold, secondary complications in the anterior segments developed in 4 eyes (15.4%): glaucoma in 1 eye (3.8%) and cataract in 3 eyes (11.5%).

• **VISUAL OUTCOMES WERE GENERALLY POOR:** The VA could be measured in 119 eyes. Of these, the final BCVA ranged from 20/100 to 20/20 in 5 eyes (4.2%) with macular formation, 2/100 to 20/200 in 45 eyes (37.8%), 2/200 to light perception in 46 eyes (38.7%), and no light perception in 23 eyes (19.3%). Among the total of 147 eyes, an ocular prosthesis was used in 8 eyes (5.4%) with phthisis bulbi or microphthalmos to facilitate orbital growth.

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## DISCUSSION

IN THE CURRENT SERIES, THE RETINAL VESSELS WITHIN THE folds were bundled and pulled toward the temporal periphery in most cases. The retinal vessels may appear not to enter the fold but to have developed before the retina became folded. The folds mostly were composed of stretched retina rather than vitreous membranes, described by Michaelson<sup>4</sup> and by Pruett and Schepens,<sup>5</sup> extending from the disc toward the peripheral fundus. Pruett and Schepens reported that the meridional distribution of

vitreous bands and retinal folds was commonly on the nasal side,<sup>5</sup> but in the current study, the folds extended temporally in 92.5% (136/147) eyes, although in the “true” unilateral congenital fold group of 26 patients (26 eyes), the folds extended nasally, superiorly, or inferiorly in higher rate of 34.6% (9/26) eyes compared to 7.5% (11/147) eyes.

The affected eye also had a peripheral avascular zone and retinal vascular abnormalities including neovascularization, hemorrhage, and exudates that indicated active retinopathy. The retinal folds were unilateral in 78.5% of eyes; however, 71.5% of patients with a unilateral fold had abnormal retinal vascular changes, a dragged retina, or total retinal detachment and leukokoria in the fellow eye. Insufficient retinal vascular development and abnormal vascular changes were seen frequently in the temporal periphery of the fellow eyes (34.7%). Since the growth of retinal vessels is more likely to be delayed temporally than nasally, these features seemed to indicate that most retinal folds in the current series may have resulted from bilateral incomplete and abnormal vascular retinal development, similar to that of ROP. Most congenital retinal folds may be caused by insufficient retinal vascular development, as in FEVR, rather than by PFV. It is interesting that features in each eye of the same patient are often quite different in this series. It is distinctly unusual in ROP for patients to develop severe retinopathy in 1 eye but not develop a similar degree of pathology in the other eye. Insufficiency of vascular development of this series may originate from gene mutations that related to morphogenesis of the retinal vessels. The molecular mechanism needs further elucidation.

The family history was positive in 64.0% of cases in which family members were examined. All positive cases had bilateral manifestations of incomplete and abnormal vascular development that confirmed the diagnosis of FEVR. Most positive cases were transmitted by autosomal dominant inheritance, while none was transmitted by autosomal recessive or X-linked recessive inheritance. Sporadic cases may exist within 77.8% of negative cases with bilateral manifestations. Gene studies to detect mutations in *FZD4*, *LRP5*, and *NDP* are under way to clarify the genetic characteristics of Japanese patients.

Regarding secondary fundus complications, a high rate of fibrovascular proliferation and rapid progression of tractional retinal detachments indicate the characteristics of active FEVR. Van Nouhuys<sup>9</sup> and Nishimura and associates<sup>10</sup> reported similar features of retinal folds. Various retinal involvements in FEVR have been studied and reported since 1982 in Japan.<sup>10–12</sup> There may be differences among races, but FEVR is supposed to be a rather common origin of congenital retinal folds without systemic associations.

The “true” unilateral congenital fold, the small group of patients that most closely resemble “congenital retinal folds” as previously described, seems to have different

pathology. PFV may play a role in the pathogenesis of congenital retinal folds in unilateral cases, especially those associated with coloboma in the affected or fellow eye,<sup>14</sup> in which a tent-shaped retinal detachment (fold) extends inferiorly along with the fetal fissure. In those cases, the tractional fetal tissue pulled on the retina and caused a tent-like configuration.<sup>7</sup> The term anterior-peripheral PFV and not posterior PHPV should be used for the origin of congenital retinal folds pulled by the fetal fibrous tissue in the periphery. However, it is rare that PFV results in peripheral fibrous proliferation, because PFV usually proliferates along the hyaloid artery.

Few reports have been published on the long-term prognosis of retinal folds. Van Nouhuys reported that 3 different factors play an etiologic role in the pathogenesis of retinal detachments in eyes with FEVR: traction from vitreous membranes, atrophy of the peripheral retina, and subretinal exudation.<sup>15</sup> In that study, the most frequent late complication was a retinal detachment, which developed in 20% of 180 eyes with FEVR, and traction was the most important cause of the retinal detachment. Recently, surgery, including peripheral laser ablation and vitrectomy, has been advocated in FEVR including retinal folds. Previous reports of vitrectomy to treat FEVR mainly involved cases of tractional retinal detachment.<sup>16,17</sup>

In the current study, nearly 30% of affected eyes with congenital retinal folds developed secondary fundus complications including fibrovascular proliferation, tractional retinal detachment, rhegmatogenous retinal detachment, and exudative retinal detachment. Even in the group of patients with “true” unilateral congenital fold, secondary fundus complications developed in 23.1%. The complication rate with the “true” unilateral fold seems to be also high in fundus and rather higher in the anterior segments.

Fibrovascular proliferation developed from neovascularization of active retinopathy in 7.5% of eyes, mostly within the first year of life. Tractional retinal detachments developed from excessive fibrovascular proliferation and regrowth in 12.9% in infants and younger children under 4 years of age. However, it is noteworthy that fibrovascular proliferation and tractional retinal detachments may develop from regrowth in older children aged 7 to 10 years. Meanwhile, rhegmatogenous retinal detachments and exudative retinal detachments developed in 8.2% and 1.4%, respectively, in older children from 2 to 16 years old. Ocular trauma was highly involved in the development of rhegmatogenous retinal detachments. Retinal breaks mostly occurred in the periphery within the stretched retinal folds, resulting in intractable PVR. Laser photocoagulation, scleral buckling, and vitrectomy with lensectomy were performed in the affected eyes with useful vision; however, the success rates for eyes complicated with fibrovascular proliferation, a tractional retinal detachment, and a rhegmatogenous retinal detachment were 80%, 70%, and 27%, respectively.

These results indicated that very early diagnosis within the first months of life, frequent examinations at a young age, and early intervention with laser and vitreoretinal surgery are essential to prevent serious complications and preserve useful vision. Fundus FA is recommended in cases suspected to arise from neovascularization of active retinopathy. The current findings also confirmed the need for a thorough ophthalmoscopic examination of the fellow eye in patients with unilateral retinal folds and for examinations of siblings at an early age. Early detection of a retinal detachment was extremely hard in eyes with a unilateral fold or in worse eyes with bilateral folds. We also recommend that older children undergo follow-up every 3 months, avoid sports associated with a high risk of ocular trauma, and wear protective glasses. Secondary complications in the anterior segment also developed in nearly 11% with congenital retinal folds in the current series. Glaucoma

and cataract developed in 6.1% and 5.4%, respectively, in patients around 5 years of age; however, those diseases may develop in infants to older children older than 9 years of age. Longer follow-up may increase the morbidity of the anterior and posterior complications. Thus, life-long observation is needed to preserve vision in eyes with a retinal fold.

The final BCVAs were 20/100 to 20/20 in 5 eyes (4.2%), 2/100 to 20/200 in 45 eyes (37.8%), and 2/200 or worse in 69 eyes (58.0%), because the temporal retina including the macula was folded in most eyes. In 5 eyes with VA of 20/100 or better, the folds were pulled nasally, superiorly, or inferiorly to the periphery and the normal macular morphology was preserved. It is suggested that even in eyes with congenital retinal folds, if the macula is rotated, appropriate treatment for amblyopia should be performed to facilitate development of good vision and binocular function.<sup>18</sup>

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# Two Novel Mutations in the *EYS* Gene Are Possible Major Causes of Autosomal Recessive Retinitis Pigmentosa in the Japanese Population

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## Abstract

Retinitis pigmentosa (RP) is a highly heterogeneous genetic disease including autosomal recessive (ar), autosomal dominant (ad), and X-linked inheritance. Recently, arRP has been associated with mutations in *EYS* (Eyes shut homolog), which is a major causative gene for this disease. This study was conducted to determine the spectrum and frequency of *EYS* mutations in 100 Japanese arRP patients. To determine the prevalence of *EYS* mutations, all *EYS* exons were screened for mutations by polymerase chain reaction amplification, and sequence analysis was performed. We detected 67 sequence alterations in *EYS*, of which 21 were novel. Of these, 7 were very likely pathogenic mutations, 6 were possible pathogenic mutations, and 54 were predicted non-pathogenic sequence alterations. The minimum observed prevalence of distinct *EYS* mutations in our study was 18% (18/100, comprising 9 patients with 2 very likely pathogenic mutations and the remaining 9 with only one such mutation). Among these mutations, 2 novel truncating mutations, c.4957\_4958insA (p.S1653KfsX2) and c.8868C>A (p.Y2956X), were identified in 16 patients and accounted for 57.1% (20/35 alleles) of the mutated alleles. Although these 2 truncating mutations were not detected in Japanese patients with adRP or Leber's congenital amaurosis, we detected them in Korean arRP patients. Similar to Japanese arRP results, the c.4957\_4958insA mutation was more frequently detected than the c.8868C>A mutation. The 18% estimated prevalence of very likely pathogenic mutations in our study suggests a major involvement of *EYS* in the pathogenesis of arRP in the Japanese population. Mutation spectrum of *EYS* in 100 Japanese patients, including 13 distinct very likely and possible pathogenic mutations, was largely different from the previously reported spectrum in patients from non-Asian populations. Screening for c.4957\_4958insA and c.8868C>A mutations in the *EYS* gene may therefore be very effective for the genetic testing and counseling of RP patients in Japan.

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## Introduction

Retinitis pigmentosa (RP [MIM 268000]) is a highly heterogeneous genetic disease characterized by night blindness and visual field constriction leading to severe visual impairment. The disease appears with different modes of inheritance including autosomal recessive (ar), autosomal dominant (ad), and X-linked, and currently over half of cases are isolated in Japan.

To date, 53 causative genes and 7 loci of RP have been identified (<http://www.sph.uth.tmc.edu/Retnet/>), including the eyes shut homolog (*EYS*) gene encoding an ortholog of *Drosophila*

spacemaker (spam), a protein essential for photoreceptor morphology. *EYS* spans over 2 Mb, making it one of the largest known genes expressed in the human eye [1,2]. *EYS* gene mutations, primarily truncating and some missense mutations, have been detected in arRP families of different ancestral origin and have reported to account for 5–16% of arRP [3–6]. Most gene mutations (e.g., *RHO*, *PRPH2*, *PRPF31*, *RPI*, and *IMPDI1*) have been found in Japanese patients with adRP, with few reports describing mutations in arRP [7,8]. Therefore, the genes causing arRP in most Japanese families have yet to be identified.

In this study, we screened all *ETS* gene exons in 100 unrelated Japanese RP patients. We found 2 novel truncating *ETS* gene mutations that were surprisingly related to 16% of Japanese arRP patients, but were not detected in Japanese patients with either adRP or Leber's congenital amaurosis (LCA [MIM204000], the earliest onset and most severe form of hereditary retinal dystrophy with several clinical features overlapping with those of RP). Additionally, these mutations were also detected in 9% of Korean arRP patients.

## Methods

### Patients and clinical evaluation

We screened all *ETS* gene exons in 100 unrelated Japanese RP patients with no systemic manifestations, excluding families with obvious autosomal dominant inheritance. Some pedigrees showed a pattern compatible with the recessive mode of inheritance; the other patients were considered isolated cases. In addition, 200 unrelated and non-RP Japanese individuals were screened as controls to evaluate the frequency of the mutations found in the patient samples. We also screened a part of *ETS* gene exons 26 and 44 in 19 unrelated Japanese adRP patients, 28 unrelated Japanese LCA patients, and 32 unrelated Korean arRP patients. The 19 Japanese adRP patients had already been screened for some principal adRP-causing genes, but the pathogenic mutations have not yet been detected. Examples of the screening list for adRP-causing genes and targeted exons include exon 3 and 4 in *RPI*; exon 1, 2, 3, 4, and 5 in *RHO*; exon 1, 2, and 3 in *PRPH2*; exon 2, 3, and 4 in *CRX*; exon 11 in *PRPF3*; exon 10, 11, and 12 in *IMPDH1*; exon 2 in *NRL*; exon 43 in *PRPF8*; exon 1 and 2 in *ROM1*; exon 5 and 6 in *RP9*; exon 2, 3, 5, 6, 7, 8, 11, and 12 in *PRPF31*; exon 11 and 15 in *SEMA4A*; exon 1 in *CA4*; exon 3 in *GUCA1B*; exon 3 in *SP4*; and exon 3 in *TOPORS*.

Japanese RP patients were examined either at the Department of Ophthalmology, Hamamatsu University Hospital in Hamamatsu (by YH), Department of Ophthalmology, Kobe City Medical Center General Hospital in Kobe (by MT), or Department of Ophthalmology, Nagoya University Hospital in Nagoya (by MK). Patients' origin varied widely, from the Tokyo to Osaka areas in Japan. Japanese LCA patients were examined at the Department of Ophthalmology and Laboratory of Cell Biology, National Center for Child Health and Development in Tokyo (by NA). LCA patients' origin varied widely, from all over Japan except the Okinawa islands. Meanwhile, Korean RP patients were examined at the Department of Ophthalmology, Kyungpook National University Hospital in Daegu (by ITK). The Korean patients' origin varied widely, from Daegu to Yeongju and Pohang areas in Gyeongsangbuk-do, Korea. A full ophthalmic examination was performed. Clinical diagnosis for RP was based on visual field, fundus examination, and electroretinogram findings, and clinical diagnosis for LCA was based on fundus examination and electroretinogram findings.

### Ethics statements

This study was approved by the Institutional Review Board for Human Genetic and Genome Research at the 6 participating institutions (Hamamatsu University School of Medicine, RIKEN Center for Developmental Biology, Nagoya University Graduate School of Medicine, National Center for Child Health and Development, Chiba University Graduate School of Medicine, and Kyungpook National University Hospital), and its procedures conformed to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all participants before molecular genetic studies.

### Mutation analysis

Genomic DNA in Japanese samples was extracted from the peripheral lymphocytes using standard procedures. In Korean samples, whole blood samples were collected on FTA cards (GE Healthcare). Blood samples were spotted onto the cards and air-dried for 1 h at room temperature. For polymerase chain reaction (PCR) amplification, a 1.2-mm disk was punched from a dried blood spot using a Harris micro-punch tool (GE Healthcare) and processed according to the manufacturer's instructions. PCR was performed using the KOD -Plus- ver. 2 PCR kit (Toyobo) with the primer sets described in Table S1 for 35 cycles of 98°C for 10 s, 60°C for 30 s, and 68°C for 1 min in an automated thermal cycler (GeneAmp PCR System 9700; Applied Biosystems). PCR products were purified with Wizard SV Gel and PCR Clean-up System (Promega) or treated with Exonuclease I and Antarctic Phosphatase (New England Biolabs). Direct sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI3100 autosequencer (Applied Biosystems). For Japanese arRP patients, all 44 exons, including 3 non-coding exons (exons 1–3) that cover the 5' untranslated region and 41 coding exons (exons 4–44), were analyzed in both sense and antisense directions. For Japanese adRP and LCA patients, and Korean arRP patients, parts of exons 26 and 44 were analyzed (Table S1).

### Assessment of pathogenicity

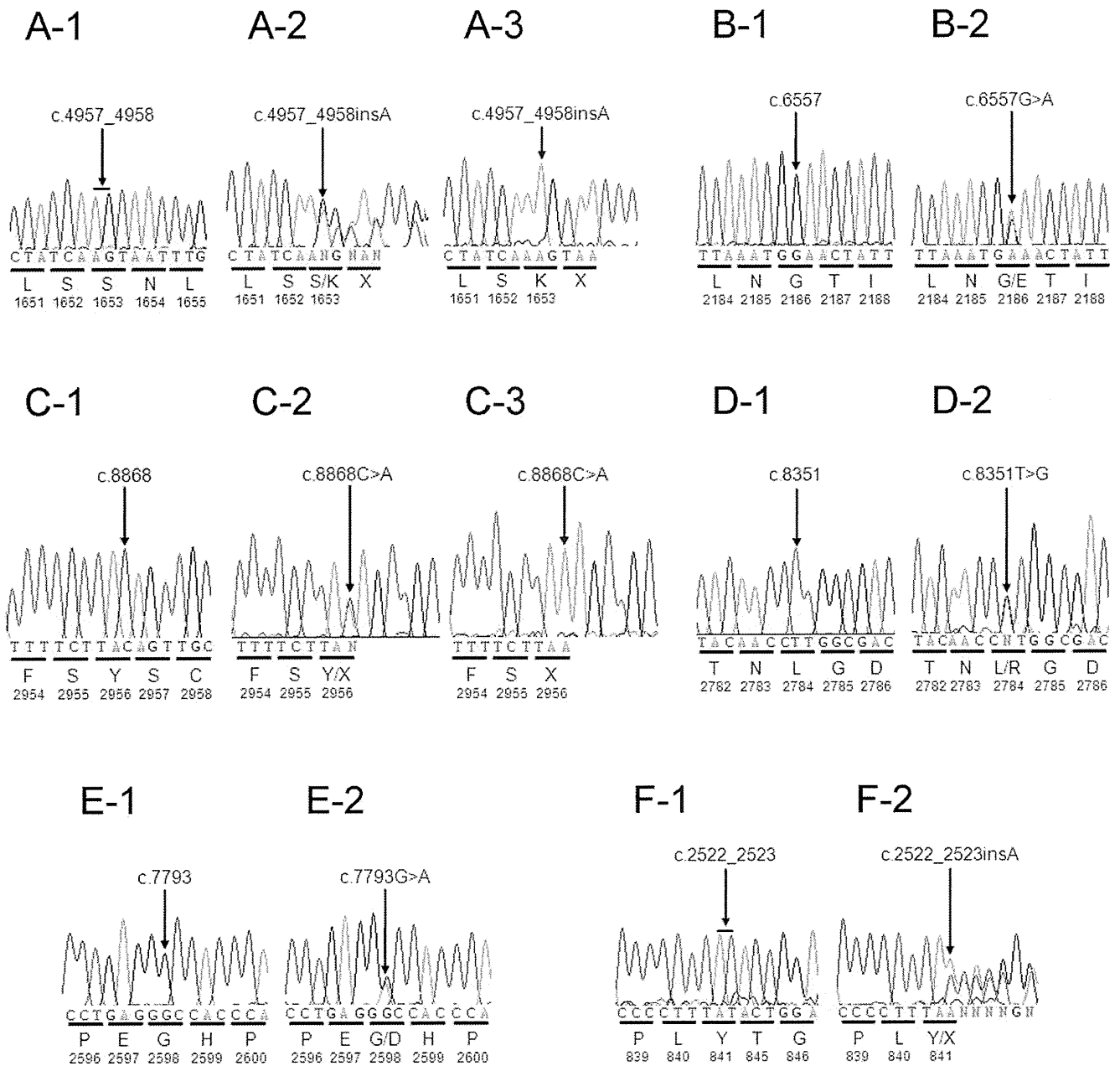
A sequence variant was considered pathogenic if it represented a truncating mutation (nonsense or frameshift), large-scale deletion mutation, or missense mutation affecting a conserved amino acid residue and did not appear in control samples (number of alleles studied  $\leq 400$ ) and/or in a public SNP database (<http://www.ncbi.nlm.nih.gov/projects/SNP/>). Particularly, missense mutations were considered pathogenic if found together with a second variant, especially if it was truncating. As reference data, we employed 4 computational algorithms to evaluate the pathogenicity of missense mutations: SIFT ([http://sift.jcvi.org/www/SIFT\\_seq\\_submit2.html](http://sift.jcvi.org/www/SIFT_seq_submit2.html)), PolyPhen2 (<http://genetics.bwh.harvard.edu/pph2/>), PMut (<http://mmb.pcb.ub.es/PMut/>), and SNAP (<http://rostlab.org/services/snap/>).

## Results

### Mutation analysis

Mutation analysis of *ETS* in 100 unrelated Japanese patients revealed 7 very likely pathogenic mutations in 18 patients (18%). Of these 18 patients, a second mutant allele could not be detected in 9 patients. The very likely pathogenic mutations consisted of 3 truncating mutations, 1 deletion mutation, 2 missense mutations, and 1 previously described mutation (Fig. 1, Table 1, and Table 2). In addition, we also identified 6 possible pathogenic mutations in 8 separate patients (Table 1 and Table 2).

A novel truncating insertion, c.4957\_4958insA, was detected in 12 patients and accounted for 15 of the 35 mutated alleles detected (42.9%) (Table 1 and Table 2). Three patients were homozygous for the c.4957\_4958insA mutation, and the other 9 patients were heterozygous. Of the latter, 3 patients showed the second mutation while 6 did not. This insertion creates a frameshift mutation that predicts a premature stop at codon 1654 (p.S1653KfsX2). A novel truncating nonsense mutation c.8868C>A (p.Y2956X) was identified in 4 patients and accounted for 5 of the 35 mutated alleles detected (14.3%). Thus, these 2 novel truncating mutations were identified in 16 separate patients, resulting in a very high frequency of the 2 mutations in Japanese arRP patients.



**Figure 1. Electropherograms of the 6 likely pathogenic *EYS* mutations.** Partial sequence of the *EYS* gene showing the normal control sequences (A-1 through F-1), heterozygous mutation sequences (A-2 through F-2), and homozygous mutation sequences (A-3 and C-3). Deduced amino acids are indicated under the sequence trace. The mutation location is indicated either by an arrow (for a nucleotide change) or a horizontal line (to show 2 nucleotides between which the insertion occurred). (A) c.4957\_4958insA; p.S1653KfsX2 (Exon 26), (B) c.6557G>A; p.G2186E (Exon 32), (C) c.8868C>A; p.Y2956X (Exon 44), (D) c.8351T>G; p.L2784R (Exon 44), (E) c.7793G>A; p.G2598D (Exon 40), (F) c.2522\_2523insA; p.Y841X (Exon 16). doi:10.1371/journal.pone.0031036.g001

#### Families with very likely pathogenic mutations and both alleles affected

Nine of the 18 patients bearing very likely pathogenic mutations appeared to have both alleles affected, suggesting that they received one mutated allele from each unaffected parent (Table 1 and Table 2). In 4 patients (RP3H, RP48K, RP56K, and RP81K), segregation analysis was performed, and the 2 pathogenic alleles were considered to be on different chromosomes (Fig. 2).

1. In RP3H, proband (II-6) was homozygous for c.4957\_4958insA. The mutation co-segregated with the

phenotype: the unaffected brother (II-4) demonstrated wild-type alleles, while the affected brother (II-5) was homozygous for the mutation.

2. In RP48K, proband (II-1) was homozygous for c.4957\_4958insA. The unaffected brother (II-2) was heterozygous for the mutation.

3. In RP56K, proband (II-1) was compound heterozygous for c.4957\_4958insA and missense mutation c.8351T>G (p.L2784R). The mutation co-segregated with the phenotype: the affected brother (II-2) also showed both mutations, while the unaffected brother (II-3) was heterozygous for c.4957\_4958insA.



**Table 1.** Mutation spectrum of the *EYS* gene in Japanese families.

Family ID	Nucleotide change	Predicted effect	Domain <sup>a</sup>	Location in gene	Type of change	Reference
Families with very likely pathogenic mutations and both alleles affected						
RP3H <sup>b</sup>	c.4957_4958insA/ c.4957_4958insA	p.S1653KfsX2/ p.S1653KfsX2	Close to coiled-coil/ Close to coiled-coil	Exon 26/Exon 26	Homozygous	This study
RP48K <sup>b</sup>	c.4957_4958insA/ c.4957_4958insA	p.S1653KfsX2/ p.S1653KfsX2	Close to coiled-coil/ Close to coiled-coil	Exon 26/Exon 26	Homozygous	This study
RP54K	c.4957_4958insA/ c.4957_4958insA	p.S1653KfsX2/ p.S1653KfsX2	Close to coiled-coil/ Close to coiled-coil	Exon 26/Exon 26	Homozygous	This study
RP44K	c.4957_4958insA/ c.6557G>A	p.S1653KfsX2/ p.G2186E	Close to coiled-coil/ Laminin G	Exon 26/Exon 32	Heterozygous/ Heterozygous	This study/Abd El-Aziz et al., 2010; Littink et al., 2010; This study
RP56K <sup>b</sup>	c.4957_4958insA/ c.8351T>G	p.S1653KfsX2/ p.L2784R	Close to coiled-coil/ Laminin G	Exon 26/Exon 44	Compound Heterozygous	This study
RP87N	c.4957_4958insA/ c.7793G>A	p.S1653KfsX2/ p.G2598D	Close to coiled-coil/ Close to Laminin G	Exon 26/Exon 40	Heterozygous/ Heterozygous	This study
RP81K <sup>b</sup>	c.2522_2523insA/ c.6557G>A	p.Y841X/p.G2186E	EGF/Laminin G	Exon 16/Exon 32	Compound Heterozygous	This study/Abd El-Aziz et al., 2010; Littink et al., 2010; This study
RP21H	deletion exon32/ deletion exon32	p.D2142_S2191delinsG/ p.D2142_S2191delinsG	Laminin G/Laminin G	Exon 32/Exon 32	Homozygous	This study
RP35K	c.8868C>A/c.8868C>A	p.Y2956X/p.Y2956X	EGF/EGF	Exon 44/Exon 44	Homozygous	This study
Families with single very likely pathogenic mutations						
RP1H	c.4957_4958insA	p.S1653KfsX2	Close to coiled-coil	Exon 26	Heterozygous	This study
RP6H	c.4957_4958insA	p.S1653KfsX2	Close to coiled-coil	Exon 26	Heterozygous	This study
RP12H	c.4957_4958insA	p.S1653KfsX2	Close to coiled-coil	Exon 26	Heterozygous	This study
RP51K	c.4957_4958insA	p.S1653KfsX2	Close to coiled-coil	Exon 26	Heterozygous	This study
RP96H	c.4957_4958insA	p.S1653KfsX2	Close to coiled-coil	Exon 26	Heterozygous	This study
RP100N	c.4957_4958insA	p.S1653KfsX2	Close to coiled-coil	Exon 26	Heterozygous	This study
RP8H	c.8868C>A	p.Y2956X	EGF	Exon 44	Heterozygous	This study
RP25H	c.8868C>A	p.Y2956X	EGF	Exon 44	Heterozygous	This study
RP80K <sup>b</sup>	c.8868C>A	p.Y2956X	EGF	Exon 44	Heterozygous	This study
Families with single possible pathogenic mutations						
RP4H	c.9272T>C	p.I3091T	Laminin G	Exon 44	Heterozygous	This study
RP9H	c.8875C>A	p.L2959M	EGF	Exon 44	Heterozygous	This study
RP49K	c.9272T>C	p.I3091T	Laminin G	Exon 44	Heterozygous	This study
RP53K	c.5884A>G	p.T1962A	Laminin G	Exon 28	Heterozygous	This study
RP55K	c.9272T>C	p.I3091T	Laminin G	Exon 44	Heterozygous	This study
RP74K	c.5404C>T	p.L1802F	Close to Laminin G	Exon 26	Heterozygous	This study
RP79K	c.77G>A	p.R26Q	Close to signal peptide cleavage site	Exon 4	Heterozygous	This study
RP83K	c.2923T>C	p.C975R	EGF	Exon 19	Heterozygous	This study

Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence FM209056, according to the nomenclature recommended by the Human Genome Variation Society ([www.hgvs.org/mutnomen](http://www.hgvs.org/mutnomen)). The initiation codon is codon 1. None of these 13 mutations were found in the Japanese controls.

<sup>a</sup>EYS has a signal peptide, a putative coiled-coil, 29 EGF, and 5 Laminin G domains. See Fig. 3.

<sup>b</sup>Segregation analysis has been performed. See Fig. 2.

In RP56K and RP81K, 2 pathogenic alleles were considered to be on different chromosomes (compound heterozygous). See Fig. 2.  
doi:10.1371/journal.pone.0031036.t001

4. In RP81K, proband (II-5) was compound heterozygous for truncating insertion c.2522\_2523insA (p.Y841X) and missense mutation c.6557G>A (p.G2186E). This insertion results in premature termination of the encoded protein at codon 841 (p.Y841X). Missense mutation c.6557G>A has been previously reported as disease causing in one Korean/American and one Chinese patient [3,6]. The unaffected mother (I-2) was heterozygous for c.2522\_2523insA, while the unaffected sister (II-6) was heterozygous for c.6557G>A.

For the other patients, segregation analysis could not be performed due to difficulties in collecting samples from the families of patients (Table 1). RP54K and RP35K were homozygous for truncating mutation c.4957\_4958insA and c.8868C>A, respectively. RP21H was homozygous for deletion in exon 32, an in-frame deletion that results in the replacement of amino acids from D2142 to S2191 with G2142 (p.D2142\_S2191delinsG) and disrupts the second laminin G domain (Fig. 3). RP44K and RP87N were heterozygous for truncating and missense mutations, c.4957\_4958insA/c.6657G>A (p.G2186E) and

**Table 2.** Summary of the very likely and possible pathogenic mutations identified in 100 Japanese arRP patients.

		Nucleotide change	Predicted effect	Location in gene	Domain <sup>a</sup>	Conservation in hu/o/m/ho/d/op/p/c/z/dr <sup>b</sup>	Allele frequency			Reference	Species	Computational prediction <sup>c</sup>		
							Control	Patient	Family ID			SIFT	PolyPhen2 (HumDiv)	PMut
<b>Very likely pathogenic mutations</b>	<b>Insertion</b>	c.2522_2523insA	p.Y841X	Exon 16	EGF	not applicable	0/400	1/200	RP81K	This study	Japanese			
		c.4957_4958insA	p.S1653KfsX2	Exon 26	Close to coiled-coil	not applicable	0/400	15/200	RP1H, RP3H, RP6H, RP12H, RP48K, RP51K, RP54K, RP44K, RP56K, RP87N, RP96H, RP100N	This study	Japanese			
	<b>Nonsense</b>	c.8868C>A	p.Y2956X	Exon 44	EGF	not applicable	0/400	5/200	RP8H, RP25H, RP35K, RP80K	This study	Japanese			
	<b>Deletion</b>	Deletion exon 32	p.D2142_S2191delinsG	Exon 32	Laminin G	not applicable	0/200 <sup>d</sup>	2/200	RP21H	This study	Japanese			
	<b>Missense</b>	c.6557G>A	p.G2186E	Exon 32	Laminin G	G/G/G/G/G/-/-/-/-	0/400	2/200	RP44K, RP81K	Abd El-Aziz et al., 2010; Littink et al., 2010; This study	Chinese, South Korean/American, Japanese	Probably damaging	Pathological	Non-neutral
		c.7793G>A	p.G2598D	Exon 40	Close to Laminin G	G/G/G/-/-/-/-/G/I/T	0/400	1/200	RP87N	This study	Japanese	Probably damaging		Non-neutral
	c.8351T>G	p.L2784R	Exon 44	Laminin G	L/L/L/L/L/L/L/L/L/G	0/400	1/200	RP56K	This study	Japanese	Probably damaging		Non-neutral	
<b>Possible pathogenic mutations</b>	<b>Missense</b>	c.77G>A	p.R26Q	Exon 4	Close to signal peptide cleavage site	R/R/R/K/K/-/-/-/-	0/400	1/200	RP79K	This study	Japanese	Affected protein function		Pathological
		c.2923T>C	p.C975R	Exon 19	EGF	C/C/C/-/-/-/-/-/-	0/400	1/200	RP83K	This study	Japanese	Possibly damaging	Pathological	Non-neutral
		c.5404C>T	p.L1802F	Exon 26	Close to Laminin G	L/L/L/-/-/-/-/-/-	0/400	1/200	RP74K	This study	Japanese	Possibly damaging		
		c.5884A>G	p.T1962A	Exon 28	Laminin G	T/T/T/T/-/-/-/-	0/400	1/200	RP53K	This study	Japanese	Possibly damaging		
		c.8875C>A	p.L2959M	Exon 44	EGF	L/L/L/L/L/L/A/N/-/S	0/400	1/200	RP9H	This study	Japanese	Possibly damaging		
		c.9272T>C	p.I3091T	Exon 44	Laminin G	I/I/I/I/I/I/I/I/I/L	0/400	3/200	RP4H, RP49K, RP55K	This study	Japanese	Affected protein function	Probably damaging	

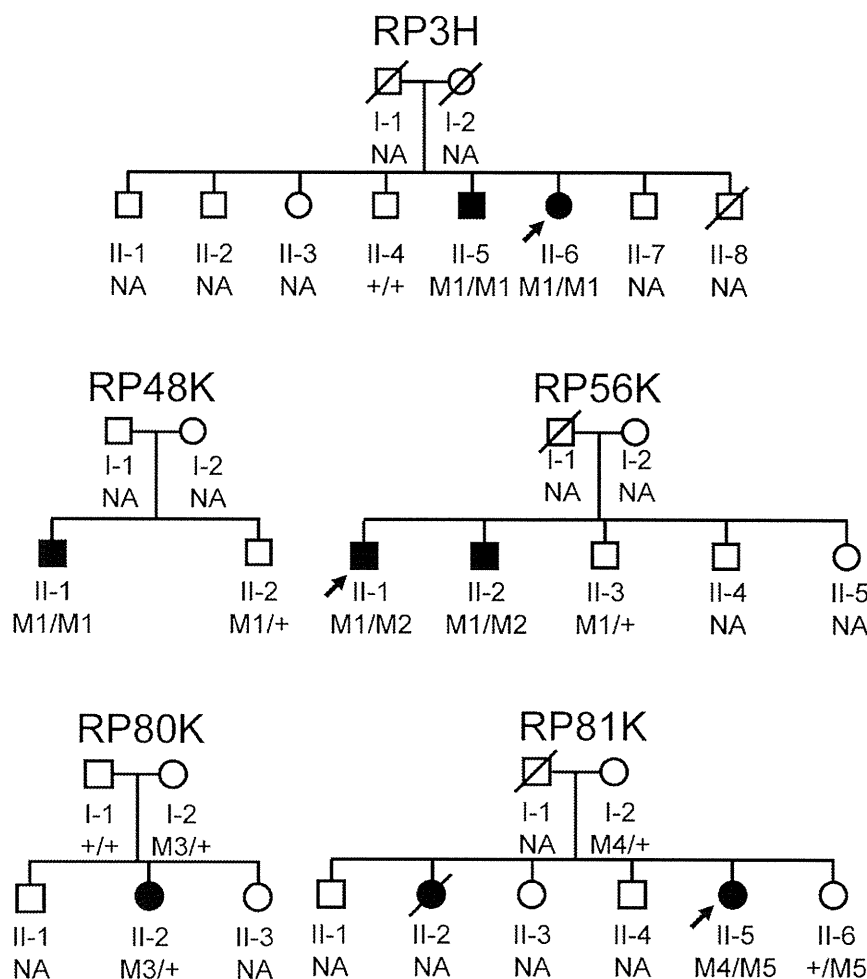
<sup>a</sup>EYS contains a signal peptide, a putative coiled-coil, 29 EGF, and 5 laminin G domains. See Fig. 3.

<sup>b</sup>hu/o/m/ho/d/op/p/c/z/dr denotes Human/Orangutan/Marmoset/Horse/Dog/Opossum/Platypus/Chicken/Zebrafish/Drosophila EYS orthologs, respectively. The hyphen (-) indicates that genomic sequence of corresponding region in the species was reported to be unknown [5].

<sup>c</sup>SIFT, PolyPhen2 (only the HumDiv data are shown), PMut, and SNAP were used as reference data to evaluate the pathogenicity of the missense mutations. c.77G>A, c.2923T>C, c.7793G>A, c.8351T>G, and c.9272T>C were predicted to be pathogenic by a number of different computational prediction programs. In addition, the c.6557G>A mutation, which had been previously reported as disease causing, was classified as pathogenic by the PolyPhen2, PMut, and SNAP programs.

<sup>d</sup>Homozygous exon 32 deletion mutation was not detected in 200 controls.

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**Figure 2. Pedigrees of the families that was available for mutation analysis.** Below the individuals, genotypes are presented for either p.S1653KfsX2 (M1), p.L2784R (M2), p.Y2956K (M3), p.Y841X (M4), or p.G2186E (M5) detected to segregate with RP. M1/M1 represents homozygous mutation. M1/+ indicates heterozygous carriers, +/+ indicates individuals carrying 2 wild-type alleles, whereas M1/M2 represents individuals presenting both mutations as compound heterozygous. Square boxes indicate men, circles denote women, and affected individuals are pointed out by a black symbol. Slashed symbols indicate deceased individuals. The probands are indicated with an arrow. NA denotes unavailable DNA samples. doi:10.1371/journal.pone.0031036.g002

c.4957\_4958insA/c.7793T>G (p.G2598D), respectively. None of these 7 very likely pathogenic mutations were found in the Japanese controls.

#### Families with single novel very likely pathogenic mutations

The rest of the patients comprising the group with very likely pathogenic mutations presented only single truncating mutations (Table 1 and Table 2). RP1H, RP6H, RP12H, RP51H, RP96H, and RP100N were heterozygous for c.4957\_4958insA. RP8H, RP25H, and RP80K were heterozygous for c.8868C>A. Segregation analysis was performed in patient RP80K. The unaffected father (I-1) demonstrated wild-type alleles, and the unaffected mother (I-2) was heterozygous for the mutation (Fig. 2). In RP96H, we found very likely pathogenic missense mutation c.8923T>C (p.F2975L), which was not detected in any of the 400 control alleles. However, as c.8923T>C has been described as rs79036642 in the dbSNP database, it was assigned to the group of possible non-pathogenic sequence alterations (Table 3).

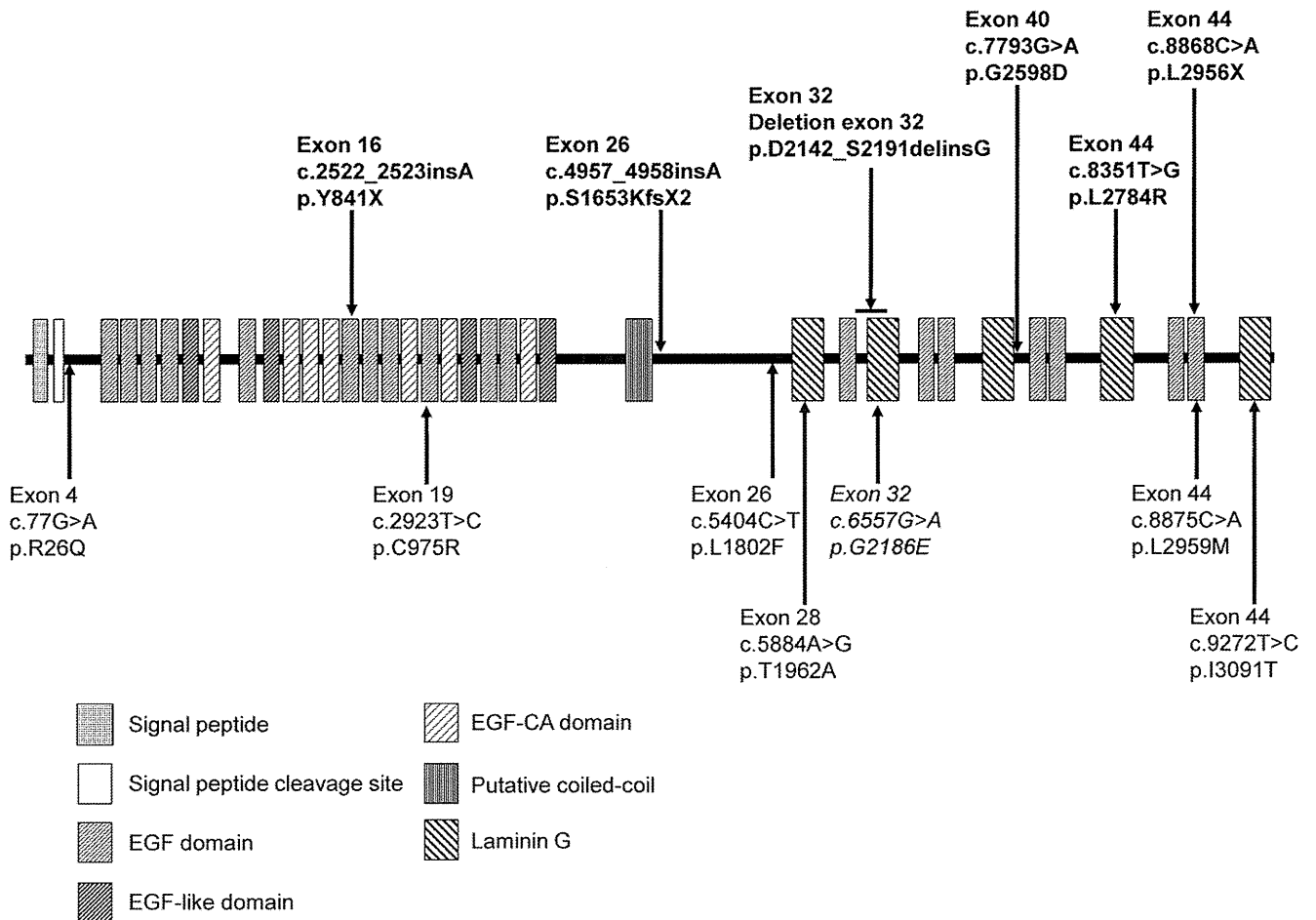
#### Families with single novel possible pathogenic mutations

A group of patients with possible pathogenic mutations had only single missense mutations (Table 1 and Table 2). We report 6

novel missense mutations in 8 different patients (Table 1 and Table 2), none of which were identified in the 400 Japanese control alleles. All amino acid residues affected by these mutations were compared with those encoded by orthologous genes of various vertebrates (orangutan, marmoset, horse, dog, opossum, platypus, chicken, and zebrafish) and *Drosophila* and found to be highly conserved across species (Table 2). The novel missense mutation c.2923T>C (p.C975R) was predicted to be pathogenic by 3 different computational prediction programs (PolyPhen2, PMut, and SNAP) (Table 2). RP4H, RP49K, and RP55K were heterozygous for the same missense mutation c.9272T>C (p.I3091T), which was predicted to be pathogenic by SIFT and PolyPhen2 programs (Table 2). In addition, 54 possible non-pathogenic sequence alterations were found, of which 9 were previously unreported (Table 3).

#### Screening of the 2 truncating mutations

We focused on 2 truncating mutations, c.4957\_4958insA in exon 26 and c.8868C>A in exon 44, which were identified in 16 separate Japanese arRP patients in this study. The frequency of the 2 mutations was very high in this Japanese arRP cohort. However, we did not detect the 2 mutations in 19 Japanese arRP



**Figure 3. Predicted domain structure and distribution of identified *EYS* mutations.** SMART (<http://smart.embl-heidelberg.de/>) and Pfam (<http://pfam.sanger.ac.uk/>) were used to search protein functional domains. A coiled-coil domain identified by Barragán et al. (2010) between the EGF-like domain and laminin G domain was also indicated. Novel very likely pathogenic mutations, novel possible pathogenic mutations, and a previously described mutation are shown in bold, normal, and italic type, respectively. Six out of 9 missense mutations were found in the EGF or laminin G domains. Furthermore, 7 were located in the latter half of the protein between the putative coiled-coil region and C-terminus. doi:10.1371/journal.pone.0031036.g003

patients and 28 LCA patients who were recruited and screened to evaluate the frequency of the mutations. We also recruited 32 unrelated Korean arRP patients and screened for the 2 *EYS* gene mutations. The c.4957\_4958insA mutation was detected in 2 patients and accounted for 3 of 64 Korean patient alleles (4.7%). One patient was homozygous and the other was heterozygous. The c.8868C>A mutation was identified in 1 patient and accounted for 1 of the 64 Korean patient alleles (1.6%).

### Clinical findings

Nine Japanese patients with very likely pathogenic *EYS* gene mutations in both alleles, 9 Japanese patients with single very likely pathogenic changes, and a Korean patient with homozygous c.4957\_4958insA mutation demonstrated classic RP with mostly night blindness as the initial symptom, followed by gradual constriction of the visual field. The fundus displayed bone spicules increasing in density with age and attenuated retinal vessels. Electroretinogram responses were not detectable, consistent with severe generalized rod-cone dysfunction. The remaining visual field determined using Goldmann kinetic perimetry with V-4 target ranged from approximately 10° to 60° of the central and inferior visual fields, respectively, in a 74-year-old woman (RP100N) to complete blindness in a 54-year-old man (RP21H).

No remarkable clinical difference was observed between 9 patients with very likely pathogenic *EYS* gene mutations in both alleles and 9 patients with single very likely pathogenic changes.

### Discussion

This study is the first to analyze mutations in the *EYS* gene among Japanese arRP patients. We detected 67 sequence alterations in the *EYS* gene, of which 21 were novel. Of these, 7 were very likely pathogenic mutations, 6 were possibly pathogenic mutations, and 54 were possible non-pathogenic sequence alterations (Table 1, Table 2, and Table 3).

Considering only the very likely pathogenic mutations, the minimum observed prevalence of distinct *EYS* gene mutations in our study is 18% (18/100, 9 patients with 2 very likely pathogenic mutations and 9 with only one such mutations). Additionally, if the possible pathogenic mutations are included in the prevalence estimation, prevalence increases to 26% (26/100, with 17 of 26 patients presenting single mutations). The estimated prevalence in our study may be extremely high compared with those in the previous studies [3–6]. Until recently, mutations in 34 genes have been associated with arRP (<http://www.sph.uth.tmc.edu/Retnet/>). The most frequently mutated gene is *USH2A*, accounting for