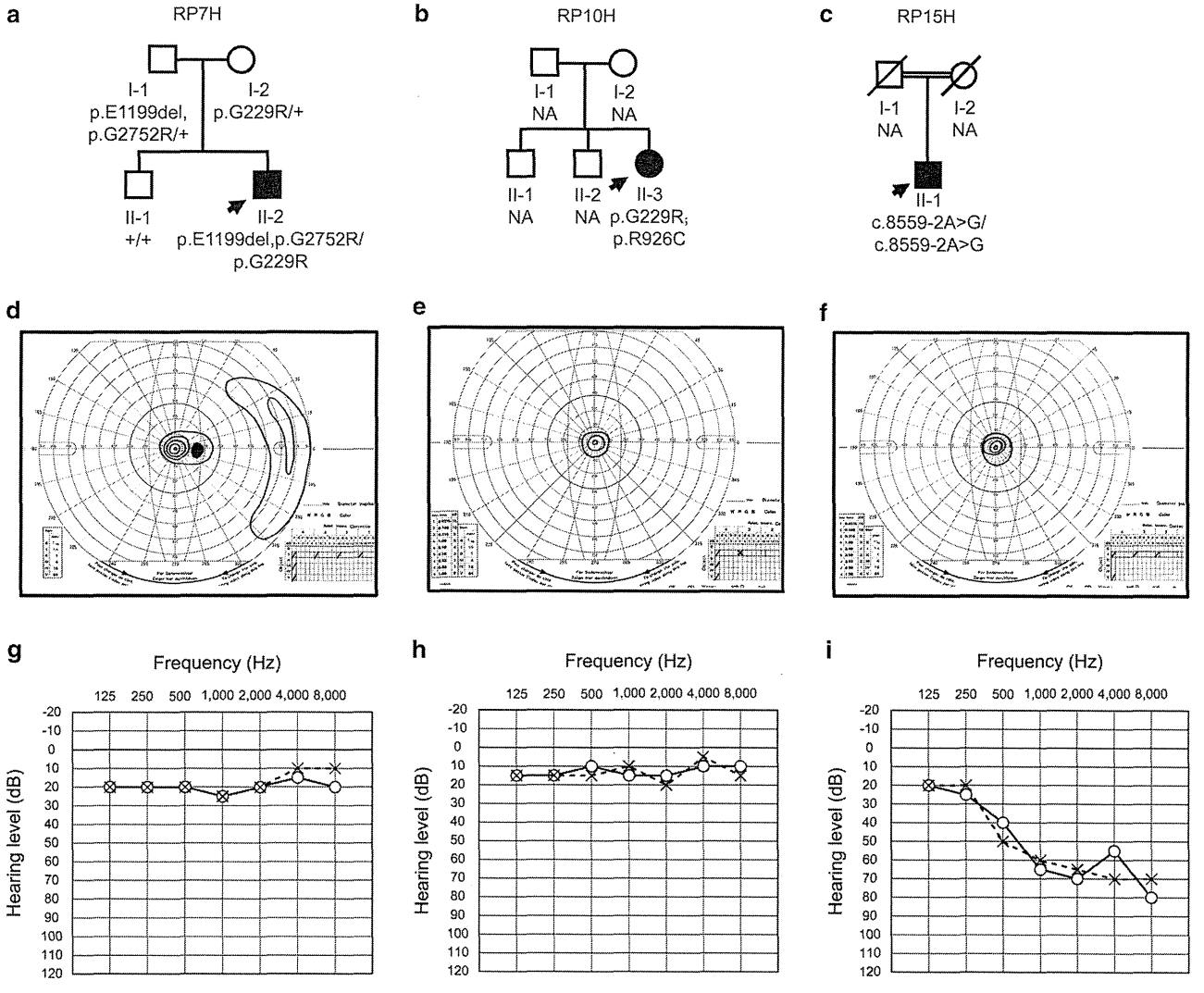

Figure 1 Pedigree and clinical examination data of patients with mutations in the *USH2A* gene. (**a–c**) Pedigrees of patients RP7H (**a**), RP10H (**b**) and RP15H (**c**). The genotypes are presented for p.E1199del, p.G229R, p.G2752R, p.R926C and c.8559-2A>G. The genotype of each evaluated individual is indicated below the symbol: square boxes and circles denote male and female individuals, respectively; black symbols indicate affected individuals; and slashed symbols indicate deceased individuals. The probands are indicated with arrows. NA, unavailable DNA samples. For example, c.8559-2A>G/c.8559-2A>G, homozygous mutation carriers; p.G229R/+, heterozygous carriers; +/+, individuals carrying two wild-type alleles; p.E1199del/p.G229R, individuals who were compound heterozygous for both mutations. p.G229R;p.R926C could not be segregated (See Table 1 footnote). (**d–f**) Right visual fields of patients RP7H (**d**), RP10H (**e**) and RP15H (**f**). The constriction of visual fields was found to be symmetric. The concentric constriction started in their twenties or thirties, and no effective residual visual field was observed after their fifties. (**g–i**) Audiograms of patients RP7H (**g**), RP10H (**h**) and RP15H (**i**). Circles and crosses indicate hearing thresholds of the right and left ears, respectively.

Patient RP7H was born in the Hamamatsu area and was considered as an isolated case (Figure 1a). In RP7H, the proband (II-2) was compound heterozygous for the novel missense mutation c.685G>C (p.G229R) and the novel deletion mutation c.3595_3597delGAA (p.E1199del) (Figure 1a). p.G229R was also identified in patient RP10H, who was unrelated to RP7H (Tables 1 and 2). The amino-acid residue at G229 of human USH2A was compared with those encoded by the orthologous genes of some vertebrates (bovine, dog, rat, mouse, chicken and zebrafish) and was found to be highly conserved across species (Table 2). p.G229R was predicted to be pathogenic by four different computational prediction programs (SIFT, Polyphen2, SNAP and Align-GVGD). On the other hand, p.E1199del is a 3-base pair (bp) in-frame deletion that results in the loss of the amino-acid residue E1199 in the second fibronectin type 3 domain (Figure 2). E1199 was also compared with the equivalent residue in other species' orthologous genes and was highly conserved among mammals and chicken (Table 2). p.E1199del was analyzed by the PSIPRED program to determine its effect on the secondary structure of usherin. The predicted effect was the shortening of the beta-sheet stretch from seven contiguous amino acids (QPCVSYE-1199) to five (QPCVS-1197), which suggests that the mutation affected the normal protein structure and was pathogenic. Interestingly, we also found another missense mutation c.8254G>A (p.G2752R), which we previously found in USH2 patient C212 as one of thee candidates for probable pathogenic mutations, but we could not determine the pathogenicity because of difficulty in the

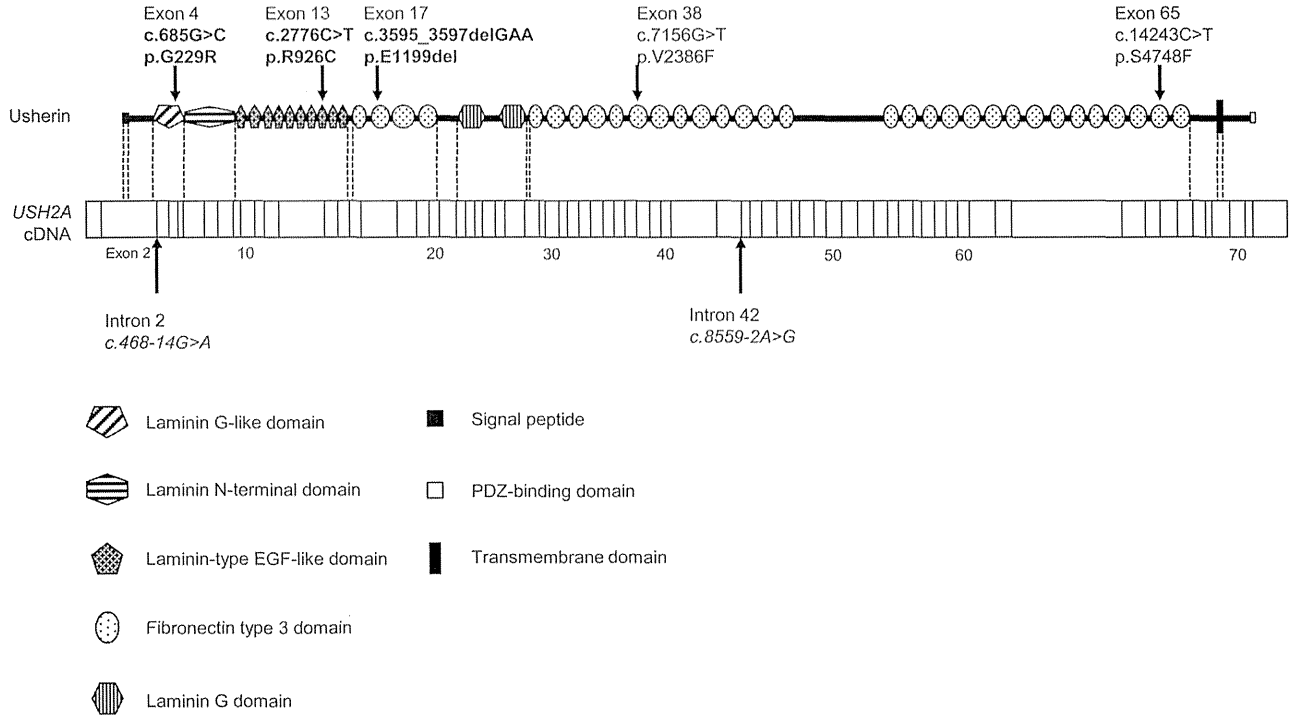

Figure 2 Schematic distribution of the *USH2A* mutations identified in this study. Upper, usherin domains encoded by *USH2A*; lower, *USH2A* cDNA with exon boundaries. Novel very likely pathogenic mutations, novel possible pathogenic mutations and previously described mutations are shown in bold, normal and italic fonts, respectively. Identified mutations were widely distributed throughout almost the entire *USH2A* gene without any clear hot spot.

segregation analysis.[13] However, in this study, p.G2752R was assigned to the group of possible non-pathogenic sequence alterations (Supplementary Table S2), because it has been described in the dbSNP database (rs201863550) and the 1000 Genomes database. As shown in Figure 1a, the mutations were found to co-segregate with the disease phenotype as follows. The unaffected father (I-1) and mother (I-2) were heterozygous for p.E1199del and p.G229R, respectively, whereas the unaffected brother (II-1) carried the wild-type alleles. In addition, p.G2752R was identified in *cis* to p.E1199del in the unaffected father, indicating that these two mutations were genetically linked in this family.

Patient RP10H was born in the Hamamatsu area and was considered to be an isolated case (Figure 1b). RP10H was heterozygous for two missense mutations, c.685G>C (p.G229R) and c.2776C>T (p.R926C). p.G229R, also found in RP7H, was classified as very likely pathogenic as described above. Similarly, the novel missense mutation p.R926C was predicted to be pathogenic by four different computational prediction programs (SIFT, Polyphen2, SNAP and Align-GVGD) (Table 2). Like G229, the R926 residue was also found to be highly conserved across species (Table 2). No segregation analysis could be performed in this patient owing to difficulties in collecting samples from the patient's family. Although both these two missense mutations were considered pathogenic, we were not able to confirm whether they were located on different chromosomes (Figure 1b, Table 1).

Patient RP15H carried the homozygous c.8559-2A>G mutation; RP15H was an isolated case and his parents were second cousins from the Hamamatsu area; that is, he was the product of a consanguineous marriage (Figure 1c). The proband (II-1) was homozygous for the splicing mutation c.8559-2A>G. No segregation analysis was performed, because both parents were deceased. Although the mutation has been previously reported as disease causing in four Japanese and one Chinese USH2 patients,[13,15,32] all of these five patients were heterozygous for c.8559-2A>G. To the best of our knowledge, this study is the first to report of a patient homozygous for the c.8559-2A>G mutation.

Patient RP66K was born in Kobe and was considered as an isolated case. In RP66K, we found the splicing mutation, c468-14G>A, which has been previously reported as disease causing in a French USH2 patient and shown to create a new AG (acceptor consensus) sequence, resulting in abnormal splicing.[33]

None of these five very likely pathogenic mutations was found among the Japanese controls or in a public SNP database (Table 2).

Families with novel possible pathogenic mutations

Here we report two novel missense mutations in two different patients (RP82K and RP85N), none of which was identified in 400 Japanese control alleles or a public SNP database. Patients RP82K and RP85N were born in the Kansai and Hiroshima areas, respectively, and were considered as isolated cases. RP82K and RP85N each had one novel missense mutation (p.V2386F and p.S4748F, respectively; Tables 1 and 2). The amino-acid residues of USH2A affected by the two novel missense mutations (V2386 and S4748) are not evolutionally conserved compared with those encoded by the orthologous genes of some vertebrate species (Table 2). For pathogenicity, the *in silico* analysis with at least two of the five different computational programs described these two mutations as pathogenic but did not exclude the possibility that these mutations were non-pathogenic (Table 2). Nevertheless, these two mutations were assigned to the group of possible

pathogenic mutations, and further analyses are necessary to determine the precise nature of these mutations.

Summary of the possible non-pathogenic sequence alterations in the *USH2A* gene identified in this study

Overall, 78 possible sequence alterations were identified among 82 patients, and 7 of them have never been reported (Supplementary Table S2). These alterations did not fulfill the assessment of pathogenicity in this study (See Materials and methods); therefore, they were assigned to the group of possible non-pathogenic sequence alterations (Supplementary Table S2).

Clinical findings

The age of the four patients with one or two deleterious mutations ranged from 19 to 52 years at the time of diagnosis and from 37 to 60 years at the time of initial examination for this study. In addition, all the four patients had night blindness. The constriction of visual fields was found to be symmetric. The concentric constriction started in their twenties or thirties, and no effective residual visual field was observed after their fifties (Figures 1d–f). In all cases, the fundus displayed changes typical of RP, including attenuated retinal vessels and bone spicule deposits over 360° of the fundus, all of which were increased in density with age. Spectral-domain OCT images also showed a marked reduction in retinal thickness resulting from the loss of photoreceptor layers. The photoreceptor inner segment/outer segment junction was either completely absent or was only detectable at the fovea of four subjects. The electroretinographic responses were consistent with severe generalized rod-cone dysfunction. On the other hand, none of these four patients had difficulties in daily conversation. Although the hearing tests for RP7H and RP10H yielded normal results (Figures 1g and h), the test for RP15H showed moderate hearing loss, suggesting USH2 (Figure 1i).

The age of the two patients with one possible mutation (RP82K and RP85N) was 40–46 years at the time of diagnosis and 50–66 years at the time of the initial examination for this study. Both patients had night blindness. The ocular findings including visual field, fundus, OCT and electroretinogram were compatible with the ocular findings in four patients with one or two deleterious mutations. Audiological examination, including pure-tone audiometry, was not performed, because the patients did not consent to the study.

DISCUSSION

This study is the first to analyze mutations in the *USH2A* gene among Japanese arRP patients with no systemic manifestations. In total, we detected 85 *USH2A* sequence alterations, of which 12 were novel. Among these 85 sequence alterations, 5 were classified as very likely pathogenic mutations (1 deletion, 2 splicing and 2 missense mutations), 2 were possibly pathogenic mutations (2 missense mutations) and 78 were possible non-pathogenic sequence alterations (Tables 1 and 2, and Supplementary Table S2). Among the 7 very likely and possible pathogenic mutations, a deletion and 4 missense mutations were novel, whereas the other 2 splicing mutations have been reported as disease causing in USH2 patients.[13,15,32,33] Similar to our previous study of Japanese USH2 patients, our current study did not detect the most prevalent mutations, p.E767fs and p.C759F, which account for approximately 23–39% and 1–14% of mutated alleles, respectively, in Caucasian individuals.[7–11] These results indicate that the profile of *USH2A* gene mutations differs largely between Japanese patients and previously reported Caucasian populations.[4–12,23,24,26–29]

We previously screened all *EYS* gene exons in 100 unrelated Japanese arRP patients with no systemic manifestations, with the exclusion of families showing obvious autosomal dominant inheritance, and, as a result, detected *EYS* gene mutations in 18–26% of the patients.[30] Excluding 18 RP patients with very likely pathogenic *EYS* gene mutations, 82 of these 100 patients were employed in this study. Among them, we found at least one very likely pathogenic or possible pathogenic *USH2A* gene mutation in six cases, of which three had two mutations and three had one mutation (Tables 1 and 2). Previous studies reported that 23 out of the 96 USH2 patients carried heterozygous *USH2A* gene mutations,[5] implying that this finding could be due to relatively large heterozygous deletions or deep intronic mutations.[34] In this study, because the direct sequences of PCR-amplified samples were used, we were not able to detect large deletions, insertions or rearrangements. In addition, because audiograms were not obtained from all patients with *USH2A* gene mutations, some of them may be USH2 patients without documented hearing loss. Therefore our results can only provide an estimate of the prevalence of *USH2A* mutations among Japanese arRP patients without documented systemic manifestations, including hearing loss. Considering only one or two deleterious mutations, the minimum observed prevalence of distinct *USH2A* gene mutations is 4% (4/100). If the patients with one heterogeneous possible pathogenic mutation are included in the estimation, the prevalence increases to 6% (6/100). A few previous studies on *USH2A* mutations employed large sets of non-syndromic RP patients, which accounted for 7–23% of arRP patients.[23,26–29] A possible reason for why the estimated prevalence in our study was lower than that of previous reports may be the fact that the Japanese population does not carry the p.E767fs or p.C759F mutation.

A previous report employing Japanese USH2 patients detected the c.8559-2A>G mutation in 4 out of the 19 cases, suggesting a possible frequent *USH2A* gene mutation among the Japanese population.[13,15] Here we identified that RP15H was homozygous for the c.8559-2A>G mutation, supporting the possibility of a frequent *USH2A* gene mutation among the Japanese population. To the best of our knowledge, this study is the first to report a patient homozygous for the c.8559-2A>G mutation. Although RP15H did not have documented hearing loss or communication problems, the hearing test demonstrated that the patient had moderate sensorineural hearing loss. A detailed medical interview revealed that the patient, a 61-year-old male, noticed a slight difficulty in hearing but considered it as age-appropriate. Elderly subjects, especially those aged >60 years, are affected by age-related hearing deterioration that makes it difficult to distinguish hearing loss from age-appropriate hearing. Therefore, auditory examination, including pure-tone audiometry, may be recommended for accurate evaluation of auditory function in elderly subjects. However, in our opinion, we could determine the presence or absence of hearing loss and select RP patients without auditory examination, because most of the subjects included in this study were middle-aged or younger.

In RP85N, we also found another missense mutation, c.2802T>G (p.C934W), which was previously reported as disease causing in a Chinese RP patient without hearing loss, although it has also been identified in two Chinese individuals among 100 normal Chinese controls.[25] p.C934W was also listed in the dbSNP database (rs201527662) and the 1000 Genomes database and was detected in 1 of the 400 control alleles in this study. Therefore we evaluated p.C934W as a possible non-pathogenic sequence alteration in this study (Supplementary Table S2).

In our previous report, we were unable to assign three sequence alterations (p.C691T, p.G2752R and p.T3747R) identified in patient C212 or determine which one of them was pathogenic.[13] In this study, we found that RP7H was heterozygous for p.G2752R, which was absent from 400 control alleles. However, because p.G2752R was described in the public SNP database (rs201863550) and was found to be genetically linked to the 3-bp in-frame deletion mutation p.E1199del in the patient's family (Figure 1a), it was assigned to the group of possible non-pathogenic sequence alterations. We speculate that patient C212 may be compound heterozygous for p.C691T and p.T3747R.

Studies have reported the phenotype of non-syndromic RP caused by *USH2A* gene mutations among the Caucasian.[35] The patients in this present study shared a relatively uniform phenotype, characterized by a symptom-free interval in the first and second decades of life, followed by a rapid decline in visual functions due to concentric constriction. The four patients with one or two deleterious mutations did not have any documented hearing loss or pronunciation problems. Although the hearing test results for RP7H and RP10H were normal (Figures 1g and h), the results for RP15H showed moderate hearing loss, indicating USH2 (Figure 1i). These findings suggest that RP without documented hearing loss occasionally includes moderate type of USH2.

In conclusion, the profile of *USH2A* gene mutations in arRP patients with no systemic manifestations differs largely between Japanese and Caucasian. Considering only one or two deleterious mutations, the observed prevalence of distinct *USH2A* gene mutations among Japanese arRP patients with no systemic manifestations was 4% (4/100). Based on these data, if both *EYS* and *USH2A* genes are analyzed among Japanese arRP patients with no systemic manifestations, gene defects could be detected in 22–32% of the patients in total (18–26% and 4–6%, respectively). We believe that screening for these two genes is effective for genetic testing and counseling of RP patients in Japan.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Fluorescein angiographic observations of peripheral retinal vessel growth in infants after intravitreal injection of bevacizumab as sole therapy for zone I and posterior zone II retinopathy of prematurity

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ABSTRACT

Aim To evaluate vascularisation of the peripheral retina using fluorescein angiography (FA) digital recordings of infants who had been treated with intravitreal bevacizumab (IVB) as sole therapy for zone I and posterior zone II retinopathy of prematurity (ROP).

Methods A retrospective evaluation was performed of medical records, RetCam fundus images and RetCam fluorescein angiogram videos of 10 neonates (20 eyes) who received intravitreal bevacizumab injections as the only treatment for zone I and posterior zone II ROP between August 2007 and November 2012.

Results All eyes had initial resolution of posterior disease after IVB injection as documented by RetCam colour fundus photographs. Using a distance of 2 disc diameters from the ora serrata to vascular termini as the upper limit of allowable avascular retina in children, the FA of these infants demonstrated that 11 of 20 eyes had not achieved normal retinal vascularisation.

Conclusions Although bevacizumab appears effective in bringing resolution of zone I and posterior zone II ROP and allowing growth of peripheral retinal vessels, in our series of 20 eyes, complete normal peripheral retinal vascularisation was not achieved in half of the patients.

INTRODUCTION

The incidence of retinopathy of prematurity (ROP) has increased globally due to advances in the care of very-low-weight premature infants. In a recent review on the incidence of ROP,[1] the incidence of all ROP was found to be approximately 60% for infants less than 1500 g in high-income countries. Most cases of ROP regress spontaneously; however, more severe cases need treatment to prevent blindness. In middle-income countries greater numbers of premature infants are being saved; however, screening and treatment of severe ROP is often lacking, which in turn is leading to an increase in blindness due to ROP. Six different studies in India have reported the incidence of severe ROP, ranging from 6.3% to 44.9%.[1] Aggressive posterior ROP (AP-ROP) is a severe form of ROP located in zone I or posterior zone II of the retina, and is characterised by rapid progression to advanced stages of disease.[2 3] Even with early laser treatment as suggested in the 'Early Treatment for ROP' (ETROP) study,[4] poor outcomes are still frequently seen in AP-ROP.[5 6] Recently, there have been several encouraging reports of the use of intravitreal

Table 1 Patient characteristics

Patient no.	GA in weeks PMA	Weight (g)	Diagnosis at IVB	PMA at IVB	PMA at last FA (weeks)	Weeks IVB to Last FA	Eye	Avascular peripheral retina, Y/N	Leakage, Y/N
1	28	1200	AP-ROP	37	64	27	Right	N	N
							Left	N	N
2	35	1700	AP-ROP	37	64	27	Right	Y	Y
							Left	Y	Y
3	28	1700	POST-ROP	35	46	42	Right	N	N
							Left	N	N
4	31	1700	AP-ROP	38	84	46	Right	N	N
							Left	N	N
5	28	1200	AP-ROP	32	78	46	Right	N	N
							Left	N	N
6	30	1700	AP-ROP	34	92	58	Right	Y	Y
							Left	Y	Y
7	30	1200	POST-ROP	37	123	86	Right	Y	Y
							Left	N	N
8	30	1150	POST-ROP	34	123	89	Right	Y	Y
							Left	Y	Y
9	29	1200	AP-ROP	36	202	166	Right	Y	N
							Left	Y	N
10	31	1182	AP-ROP	35	259	224	Right	Y	Y
							Left	Y	Y

AP-ROP, aggressive posterior retinopathy of prematurity; FA, fluorescein angiogram; GA, gestational age; IVB, intravitreal bevacizumab injection; PMA, postmenstrual age; POST-ROP, posterior retinopathy of prematurity.

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bevacizumab as an off-label first line of treatment in neonates with severe ROP.[7–13]

One of the reported benefits of intravitreal bevacizumab as treatment for zone I and posterior zone II ROP is that the development of peripheral retinal vessels continues after treatment, whereas conventional laser therapy leads to permanent destruction of the peripheral retina.[14] In the present work, we report on the results of fluorescein angiography (FA) performed on 10 neonates (20 eyes), which we had treated up to 5 years previously with intravitreal bevacizumab as sole therapy for zone I and posterior zone II ROP. We have evaluated the extent of peripheral retinal vessel growth and remaining avascular retina after a single injection of intravitreal bevacizumab.

All cases were treated and examined at Klinik Mata Nusantara (KMN), an eye hospital in Jakarta, Indonesia. This retrospective study was approved by the Medical Committee of KMN.

Patients

In this retrospective study, we reviewed the records of 17 neonates who had FA after IVB for zone I and posterior zone II ROP. For the purposes of this study, we included 10 neonates who had achieved regression of posterior disease in both eyes with a single injection of bevacizumab and had a minimal follow-up period of 24 weeks after IVB. We excluded six neonates who did not achieve resolution of posterior disease or needed additional treatment before resolution of ROP: one neonate with AP-ROP had resolution of zone I ROP in one eye but developed stage 5 ROP in the other eye; another neonate with AP-ROP needed a second IVB injection to achieve resolution of zone I disease in both eyes; two neonates had not achieved resolution of posterior zone II disease at the last follow-up, and another two neonates needed vitrectomy. One neonate had to be excluded because the child was lost to follow-up after 10 weeks.

At time of IVB, 7 of these 10 cases had been diagnosed as having AP-ROP and 3 cases as having posterior zone II ROP without plus disease. When FA was performed more than once, we evaluated the last FA. Fluorescein angiograms of 10 neonates (20 eyes) were thus evaluated. These neonates had been treated with IVB as a first-line therapy between August 2007 and November 2012. In all cases, regression of posterior disease was documented by RetCam fundus photographs. Gestational age at birth ranged from 28–35 weeks post menstrual age (PMA) (mean=30 weeks), birth weight ranged from 1150–1700 g (mean=1393.2 g), PMA at time of IVB ranged from 32–38

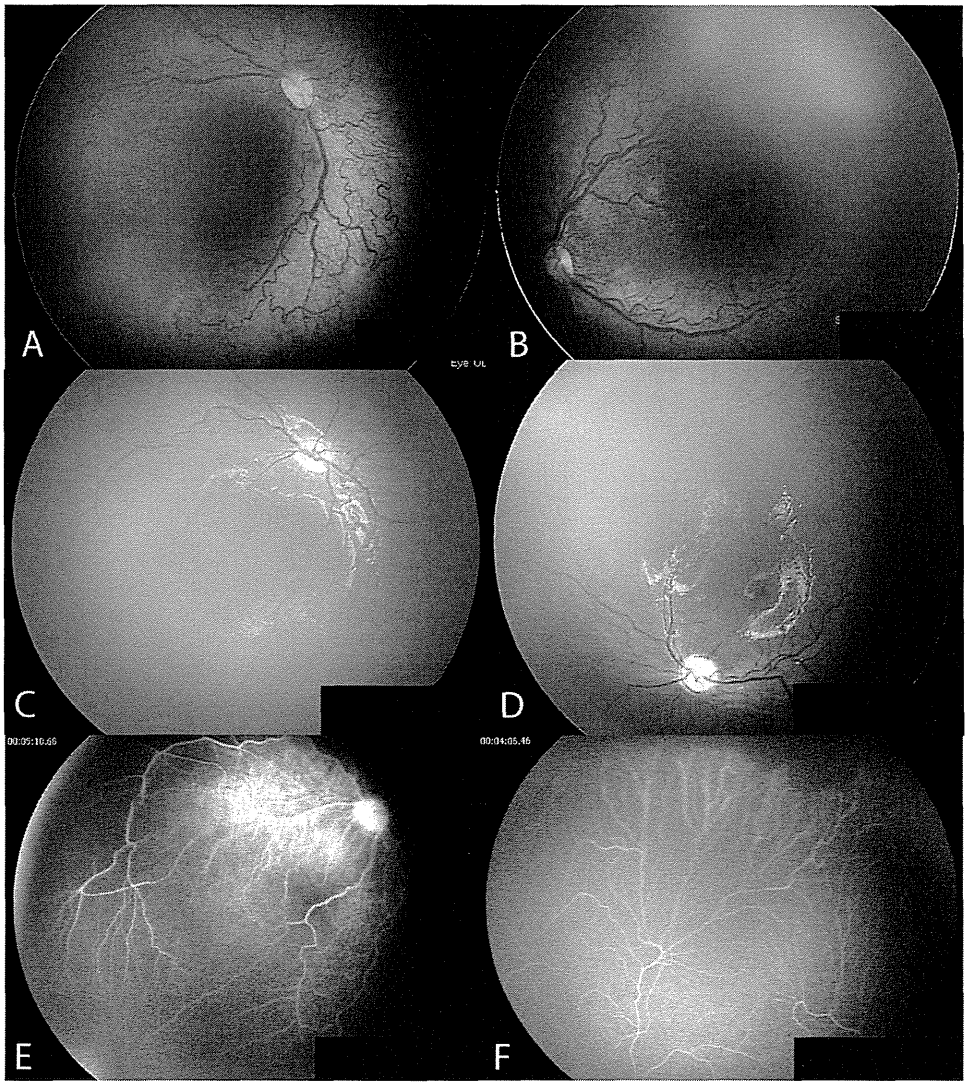

Figure 1 Case no. 5: aggressive posterior retinopathy of prematurity. Posterior fundus before intravitreal bevacizumab injection (IVB) (A,B); after injection of IVB (C,D); fluorescein angiography 46 weeks after IVB, demonstrates less than 2 disc diameters (DD) of avascular retina (E,F).

weeks (mean=35.5 weeks). The interval between treatment with IVB and FA ranged from 27–224 weeks.

RetCam FA was performed under general anaesthesia in an operating room at KMN. A 10% solution of fluorescein was injected intravenously at a dose of 0.1 mL/kg followed by an isotonic saline flush. None of the patients experienced systemic complications related to FA.

METHODS

A retrospective analysis of the medical records of all infants that had been treated with IVB at KMN was performed. We extracted medical records of infants who had demonstrated resolution of zone I and posterior zone II ROP with IVB as sole treatment as documented by RetCam colour fundus photographs. Although we have been treating zone I and posterior zone II ROP with IVB since 2006, RetCam FA only became available to us in the latter part of 2011. The medical records of 10 neonates (20 eyes) who had RetCam FA after IVB were used to document resolution of posterior disease. We reviewed the fluorescein digital videos of these 20 eyes to evaluate the extent of remaining avascular retina.

An estimate of the peripheral retinal non-perfusion in the infants was compared to previously published descriptions of FA in children.[15] Blair *et al*[15] concluded that avascular retina extending more than 2 disc diameters (DD) from the ora serrata should be considered abnormal.

RESULTS

General patterns

Digital video recordings of RetCam FA allowed us to distinctly visualise the anterior border of retinal vessel growth and the vascular–avascular junction of 10 infants who had achieved RetCam documented resolution of posterior disease after treatment with IVB for zone I and posterior zone II ROP. Of 20 eyes examined with FA, 11 had incomplete peripheral retina vascularisation (table 1). Of these 11 eyes, 9 had fluorescein leakage at the vascular–avascular junction. The IVB-FA interval of these eyes with incomplete vascularisation ranged from 27 to 224 weeks (median 87.5 weeks). At the time of IVB, the diagnosis in these children with incomplete retinal vascularisation was AP-ROP in seven cases and posterior zone II ROP without plus disease in three cases. The birth weight of these infants ranged

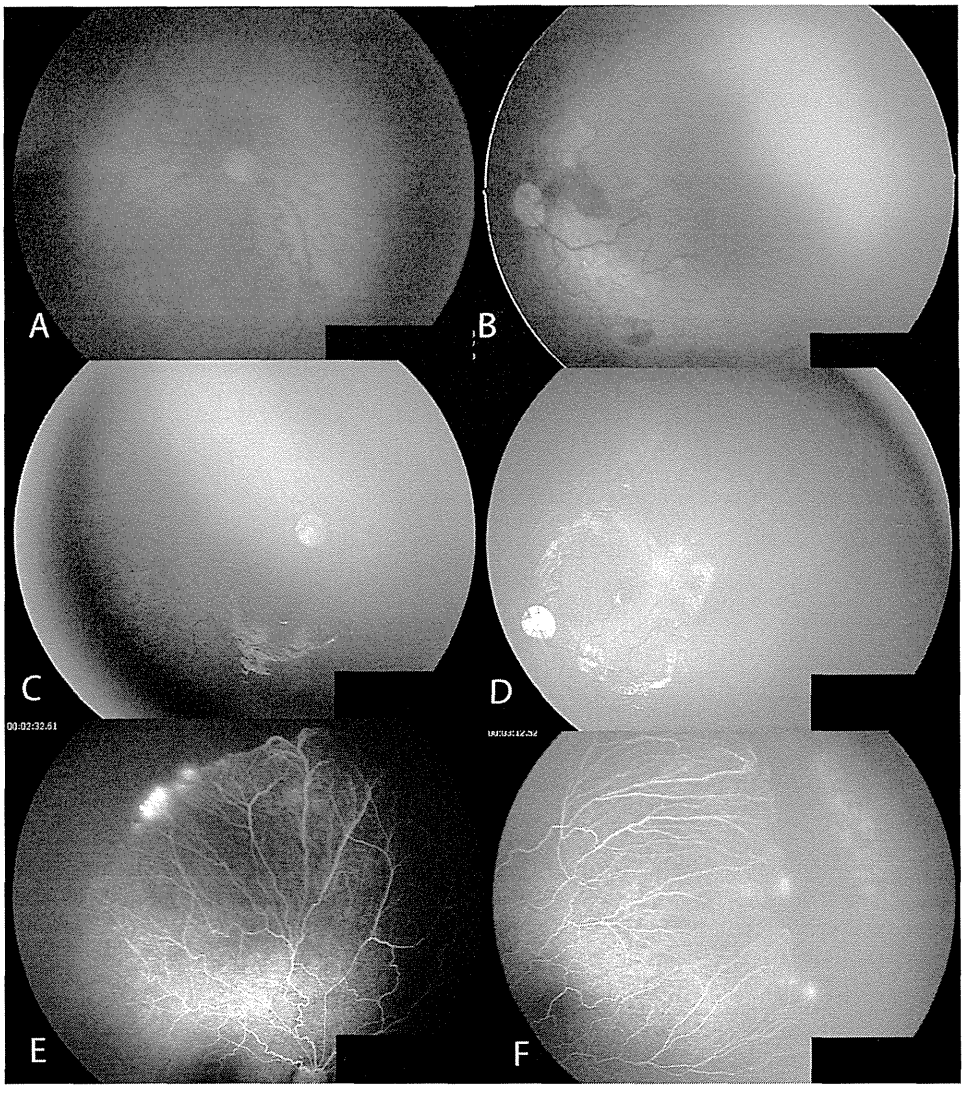

Figure 2 Case no. 10: aggressive posterior retinopathy of prematurity. Posterior fundus before intravitreal bevacizumab injection (IVB) (A,B); resolution of posterior disease after IVB (C,D). The peripheral retina remains avascular with fluorescein leakage more than 4 years after IVB (E,F).

from 1150–1700 g with a mean of 1393.2 g. The gestational age ranged from 28–35 weeks with a mean of 30 weeks PMA.

Case reports

Case no. 5 was a case of AP-ROP (figure 1A,B), which resolved after a single injection of IVB (figure 1C,D). FA performed at 46 weeks after IVB (figure 1E,F) shows less than 2 DD of avascular peripheral retina and no vascular leakage.

Case no. 10 was a case of AP-ROP (figure 2A,B), where there was resolution of posterior disease after IVB (figure 2C,D) but the peripheral retina remained avascular with fluorescein leakage at the vascular–avascular junction more than 4 years after IVB treatment (figure 2E,F).

Cases no. 8 and no. 7 are twin neonates who presented with posterior ROP (figures 3A,B and 4A,B, respectively). Case no. 8 had avascular peripheral retinas without fluorescein leakage 21 weeks after IVB treatment (figure 3C,D). FA at 89 weeks after IVB demonstrated that the retina in both eyes remained avascular with fluorescein leakage at the vascular–avascular junction (figure 3E,F).

Case no. 7 had avascular peripheral retinas without leakage 18 weeks after IVB treatment (figure 4C,D). FA performed at 86 weeks after IVB demonstrated that the left peripheral retina was vascularised while the right peripheral retina remained unvascularised and had fluorescein leakage at the vascular–avascular junction (figure 4E,F).

DISCUSSION

Although numerous authors have reported their experience using bevacizumab in the management of ROP,[9 10 13] at the time of writing there has been only one controlled trial comparing intravitreal bevacizumab to conventional treatment of ROP, the BEAT ROP trial.[10] In that study, the authors concluded that development of peripheral retinal vessels continued after treatment with IVB. In our study, we aimed to evaluate the extent of peripheral retinal growth in eyes with zone I and posterior zone II ROP that were treated with a single injection of intravitreal bevacizumab. Fluorescein angiographic imaging was chosen, as it allowed us to accurately visualise the extent of peripheral retinal vessel growth in these eyes.

In our series of 20 eyes from 10 patients we found that, despite resolution of zone I and posterior zone II ROP after a single injection of IVB, the peripheral retina remained incompletely vascularised in 11 (55%) of the eyes. In addition, we

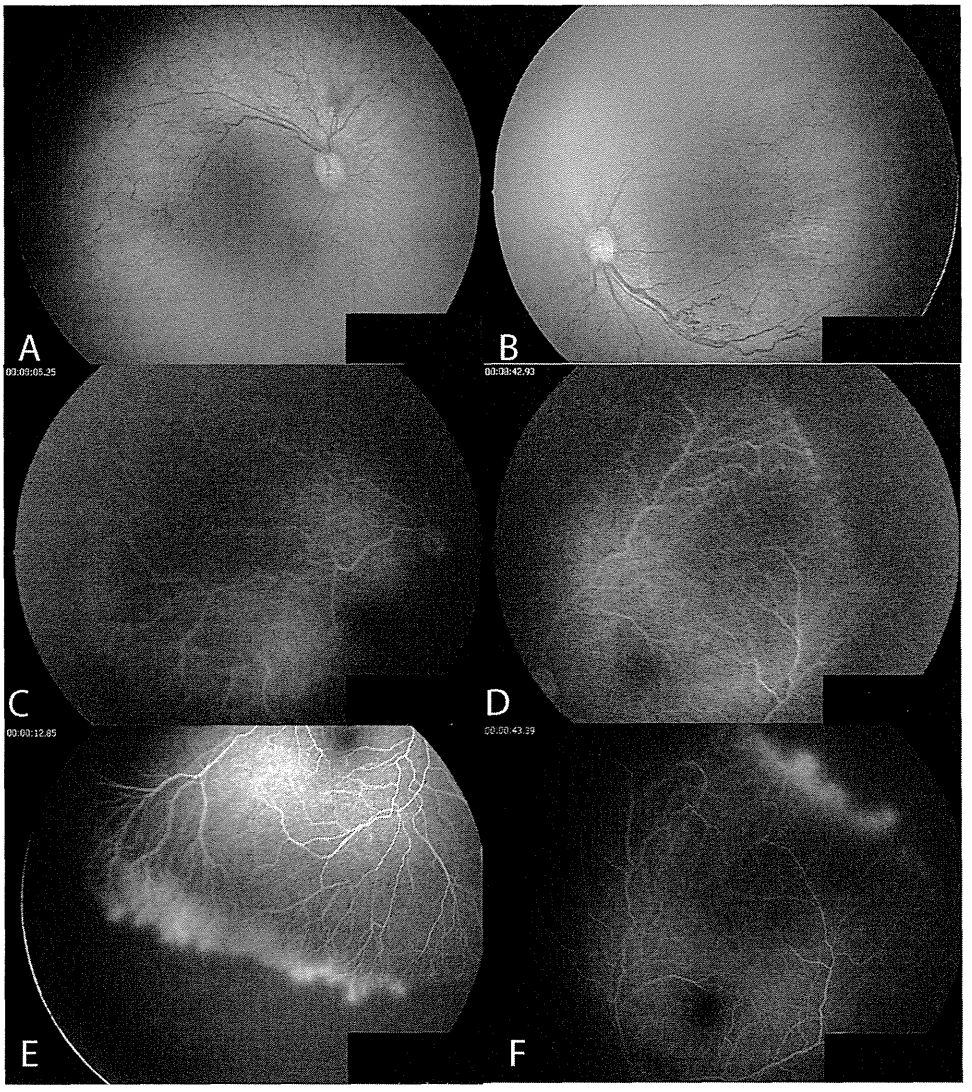

Figure 3 Case no. 8: posterior retinopathy of prematurity. Posterior fundus before intravitreal bevacizumab injection (IVB) (A,B); avascular peripheral retina without leakage 21 weeks after IVB (C,D); avascular peripheral retina with fluorescein leakage in both eyes 89 weeks after IVB (E,F).

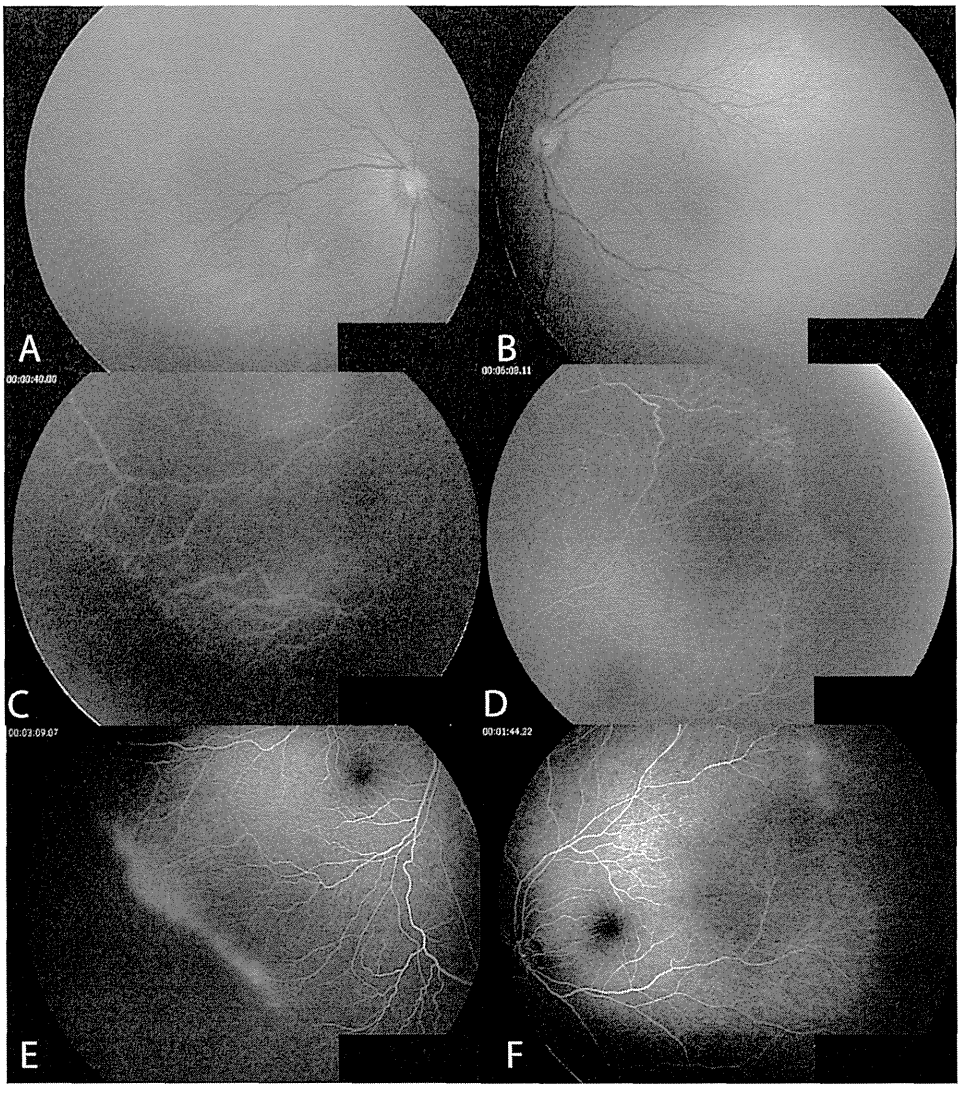

Figure 4 Case no. 7: posterior retinopathy of prematurity, the twin sister of case no. 8. Posterior fundus before intravitreal bevacizumab injection (IVB) (A,B); avascular peripheral retina without fluorescein leakage in both eyes 18 weeks after IVB (C,D); fully vascularised peripheral retina in the left eye, and avascular retina with fluorescein leakage in the right eye 86 weeks after IVB (E,F).

observed fluorescein leakage at the vascular–avascular junction in 9 of these 11 eyes with avascular peripheral retina (82% of total).

The safety of FA in neonates has been established since 2006.[16 17] In 2011, Lepore *et al*, published an atlas of fluorescein angiographic findings in eyes undergoing laser treatment for ROP. The authors concluded that FA clearly defined the zone I junction between vascularised and non-vascularised retina.[18] Recently, Velia *et al*,[19] investigated retinal development in premature infants using FA and revealed vascular changes in ROP eyes, such as loss of the normal dichotomous branching, vessel branching at the junction between vascular and avascular retina, arteriovenous shunts, and other abnormalities that were thought to be related to the immaturity of the vascular network. We observed similar findings such as irregular branching of large arterioles and circumferential vessel formation (figure 4C), and fluorescein leakage (figure 3E,F). Of significance, Velia *et al*[19] determined that dye leakage is the most significant sign of progression to severe ROP.

Blair *et al*[15] estimated the normal extent of peripheral retinal non-perfusion in normal children at various postnatal ages. In that study, the authors—using RetCam FA on 33 eyes from 31 normal children—estimated avascular retina using scleral indentation during FA to determine the distance of vascular termini to the ora serrata. None of these normal eyes had a distance greater than 1.5 disk diameters up to 13 years of age. The authors concluded that, conservatively, a distance of greater than 2 DD from the ora to the vascularised retinal margin should be considered abnormal. This data provided a useful practical standard to document the extent of peripheral retinal vascular development when screening infants with ROP using FA.

All neonates in our study had resolution of zone I and posterior zone II ROP documented by RetCam colour imaging at the time FA was performed. Previous studies have reported the favourable response of zone I and posterior zone II ROP to intravitreal injections of bevacizumab.[10 20] Our study focuses on the extent of normal retinal vessel growth in the peripheral retina in cases where zone I and posterior zone II ROP had been deemed to have responded favourably to a single injection of IVB as sole treatment.[10 14 21] It is often difficult to accurately determine the vascular–avascular junction in the peripheral retina using indirect ophthalmoscopy or colour RetCam images. We therefore chose to use FA, which allows accurate visualisation of the outer borders of the vascular retina.[18 19]