Original Article

Detection of *FZD4, LRP5* and *TSPAN12* Genes Variants in Malay Premature Babies with Retinopathy of Prematurity

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Abstract

Purpose: To determine the mutational analyses of familial exudative vitreoretinopathy (FEVR)-causing genes in Malay patients with retinopathy of prematurity (ROP) to obtain preliminary data for gene alterations in the Malay community.

Methods: A comparative cross-sectional study involving 86 Malay premature babies (ROP = 41 and non-ROP = 45) was performed from September 2012 to December 2014. Mutation analyses in (FEVR)-causing genes (*NDP, FZD4, LRP5, and TSPAN12*) were performed using DNA from premature babies using polymerase chain reaction (PCR) and direct sequencing. Sequencing results were confirmed with PCR-Restriction Fragment Length Polymorphism (RFLP).

Results: We found variants of FZD4, *LRP5, and TSPAN12* in this study. One patient from each group showed a non-synonymous alteration in *FZD4*, c.502C>T (p.P168S). A synonymous variant of *LRP5* [c.3357G>A (p.V1119V)] was found in 30 ROP and 28 non-ROP patients. Two variants of *TSPAN12*, c.765G>T (p.P255P) and c.*39C>T (3'UTR), were also recorded (29 and 21 in ROP, 33 and 26 in non-ROP, respectively). Gestational age and birth weight were found to be significantly associated with ROP (*P* value < 0.001 and 0.001, respectively).

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INTRODUCTION

Retinopathy of prematurity (ROP), previously called retrolental fibroplasia, is a vasoproliferative disease that is reported to be the leading cause of childhood

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Conclusion: Analysis of data obtained from the ROP Malay population will enhance our understanding of these FEVR-causing gene variants. The c.3357G>A (p.V1119V) variant of *LRP5*, and c.765G>T (p.P255P) and c.*39C>T variants of *TSPAN12* could be common polymorphisms in the Malay ethnic group; however, this requires further elucidation. Future studies using larger groups and higher numbers of advanced cases are necessary to evaluate the relationship between FEVR-causing gene variants and the risk of ROP susceptibility in Malaysian infants.

Keywords: Genetic Variants; Malay; Premature Infant; Retinopathy of Prematurity

blindness all over the world.[1-3] This disease causes abnormal growth of retinal blood vessels, and is associated with multiple factors, including short gestational age, low birth weight, and extended oxygen exposure.[4,5] In addition to environmental influences, genetic predisposition appears to be perhaps the strongest factor leading to ROP.[1,4-7] Recently, researchers have shown increased interest in the genetic factors involved in ROP. It has been proposed that multiple alterations in the nuclear-encoded genes are responsible for development of ROP. Nevertheless, the precise mechanism conferring the genetic susceptibility to ROP is still being investigated.[4-6]

ROP is considered to be part of familial exudative vitreoretinopathy (FEVR), a bilateral hereditary eye disorder, because of several clinical similarities between them.[1,5] FEVR is well-known as a heterogeneous genetic disorder involving several genes and different genetic variations, including point mutations, deletions, or insertions. The genetic heterogeneity of FEVR cases has been documented by identification of causative genes, which include Norrie disease protein (*NDP*), Frizzled-4 (*FZD4*), lipoprotein receptor-related protein 5 (*LRP5*), and Tetraspanin-12 (*TSPAN12*).[1,7] These genes all encode the ligand-receptor complex of Norrin/β-catenin signaling pathway, which is involved in the activation of vascularization in the retina.[5,8] Due to the phenotypic similarity between FEVR and ROP, alterations in genes linked to FEVR are also considered to be associated with the development of ROP.[5]

The Norrin-beta-catenin signaling pathway plays essential roles in controlling retinal vascular development. Protein components of FZD4, LRP5 and a newly discovered component, TSPAN12 are expressed by endothelial cells in the eye, and this contributes to the formation of the Fzd4/Lrp5 complex. Binding of the Norrin ligand to the complex activates the downstream β-catenin signaling pathway, which consequently initiates expression of genes responsible for vascularization process in the retina. The pathway is simplified and illustrated in Figure 1.[8,9]

Most reports on FEVR-causing gene mutations are from western countries[10-15] and Asian countries, especially from the Japan[1,5,16-18] and China[19-23]; however, there is no report of FEVR-causing gene mutations in Malaysian patients thus far. *FZD4* and *LRP5* variants were previously found to be in a range of 3% to 12% for overall ROP incidences.[1,7] In the current study, we performed a comprehensive FEVR-causing gene (NDP, FZD4, LRP5, and TSPAN12) sequence analysis in 41 Malay premature babies with ROP.

METHODS

Subject Recruitment

A comparative cross-sectional study was conducted among Malay premature babies with birth weight ≤2000 g and gestational age at birth ≤36 weeks at the Hospital Universiti Sains Malaysia (HUSM) born between September 2012 to December 2014. The study adhered to the guidelines of the Declaration of Helsinki and was approved by the Universiti Sains Malaysia Ethics Committee. Informed consent was obtained from all parents or legal guardians prior to enrolment of the subjects for the study.

The premature babies were screened for ROP by the Pediatric Ophthalmology Team (and not the investigators) according to the ROP screening schedule. The first retinal examination of premature babies was performed depending on the risk factors for ROP, such as low birth weight (less than 1500 g), ventilator support for oxygen supplementation, sepsis, and intraventricular hemorrhage. For premature babies with risk factors for ROP (minimum of one risk factor), the first retinal examination was done at 32 weeks postmenstrual age (PMA). Whereas, for premature babies without risk factors for ROP, the first retinal examination was done at 34 weeks PMA.

If ROP was not detected during the first retinal examination (performed by the Pediatric Ophthalmology Team), the premature babies were re-examined every 2 weeks up to 38 weeks PMA. The next review was performed at 42 weeks PMA, and follow-ups were performed at the age of 6 months and 1-year-old. The premature babies were discharged from ROP screening if features of ROP were not detected at the age of 1 year. Upon confirmation of the absence of ROP in premature babies (at the age of 1 year), they were enrolled for the study as the non-ROP group and their blood samples were collected for genetic studies.

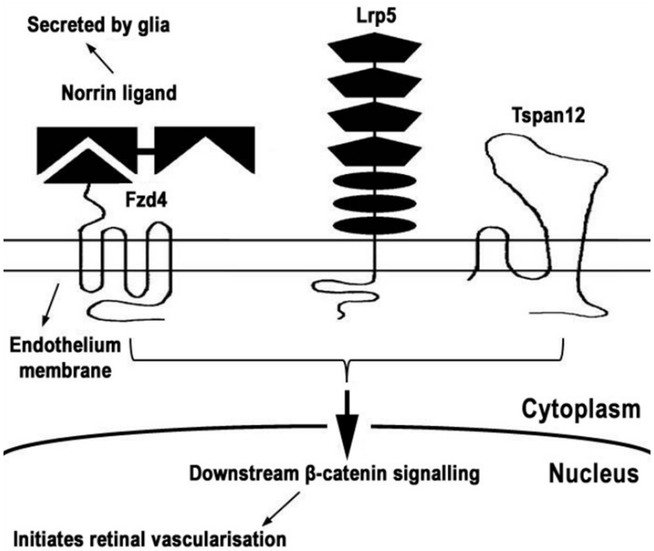

Figure 1. Norrin/-catenin signalling pathway. The binding of Norrin ligand to the receptor complex which consists of Fzd4, Lrp5 and Tspan12 protein components. The binding results in the activation of downstream -catenin signaling pathway which initiates the vascularisation process in the retina.[8,9]

At any time of retinal examination, if ROP features were detected, the premature babies were enrolled for the study as the ROP group and their blood samples were collected for genetic studies. The premature babies were enrolled through consecutive sampling and were divided into ROP and non-ROP groups. The classification of ROP is based on clinical features described by the International Classification of ROP (ICROP).[24] Diagnosis of ROP, at least in one eye, was required for enrollment of candidates in the ROP group. ROP was managed by the Pediatric Ophthalmology Team, and they assessed the development of ROP in premature babies based on its severity.

DNA Extraction

Whole blood samples were collected and extracted following the manufacturer's protocol using the QIAamp DNA blood mini kit (Qiagen, Hilden, Germany). DNA concentration and purity were determined using the NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), this was followed by agarose gel electrophoresis using 1% gels for determining quality of DNA samples.

Polymerase Chain Reaction for *NDP, FZD4, LRP5*, and *TSPAN12*

Entire *NDP*, *FZD4*, *LRP5*, and *TSPAN12* exons were amplified using sets of primers from previous reports.[1,12,23] Polymerase chain reaction (PCR) was performed in a 20 µl reaction volume using the 2 U Phusion high-fidelity DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA), 200 µM of each dNTP, 10 µM of each primer, 70 ng of DNA template, and 4 µl of 5 × Phusion HF buffer (Thermo Fisher Scientific, Waltham, MA, USA).

Initial denaturation was performed at 98°C for 30 s, followed by 30 cycles of denaturation, annealing at respective temperatures (available in references) and extension at 72°C. Final extension time was five minutes. Gel electrophoresis using a 3% agarose gel was performed to analyze amplified fragments, the sizes of which were determined using 50 bp DNA marker. Favorable PCR products were purified using QIAquick gel extraction kit (Qiagen, Hilden, Germany) and sequenced.

Direct Sequencing Analysis

Sequencing reactions were performed in both directions using forward and reverse primers. BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) was used to sequence PCR products using an ABI Prism 3700 DNA Analyzer automated sequencer (Applied Biosystems, Foster City, CA, USA). Electropherogram results were aligned using NCBI BLAST (http://www.ncbi.nlm.nih.gov/blast). The complementary DNA (cDNA) sequences for *FZD4* (NM_012193.3), *LRP5* (NM_002335.3), *TSPAN12* (NM_012338.3), and *NDP* (NM 000266.3) available in the database were used as references.

Validation of Variants Using PCR-RFLP

PCR-RFLP assays were used to validate the variants detected using DNA sequencing. Restriction enzymes used to cleave at detected single nucleotide polymorphism (SNP) sites were determined using several software applications available online, such as RFLP enzyme picker (http://www.insilicase.co.uk) and RestrictionMapper Version 3 (http://www.restrictionmapper.org). Variants were confirmed using FastDigest restriction enzymes (Thermo Fisher Scientific, Watham, MA, USA); c.502C>T confirmed using BseSI, c.3357G>A using XmiI, c.765G>T using MvaI, and c.*39C>T using BseNI.

Statistical Analysis

Statistical data were analyzed using the Statistical Package for Social Science (SPSS) version 21 (IBM Corp., Armonk, N.Y., USA). The association of clinical factors and detected gene variants with ROP and non-ROP were tested using Chi-square or Fisher Exact test. *P* value of < 0.05 was considered as statistically significant.

RESULTS

Clinical Features

A total of 86 premature babies were enrolled (ROP = 41 and non-ROP = 45) for this study. Out of 86 premature babies, 49 (57.0%) were male and 37 (43.0%) were female. The group of premature babies diagnosed with ROP consisted of 22 (53.7%) males and 19 (46.3%) females, whereas, the non-ROP group consisted of 27 (60.0%) males and 18 (40.0%) females. There was no significant difference in number of candidates from either gender between the ROP and non-ROP groups ($P = 0.553$) [Table 1].

The mean gestational age at birth for the ROP group was 27.2 weeks (range, 24 to 34 weeks) whereas that for the non-ROP group was 30.2 weeks (range, 26 to 34 weeks). Gestational age at birth ≤28 weeks was significantly associated with ROP ($P = <0.001$) [Table 1].

Premature babies in the ROP group had birth weight ranging from 570 to 1500 g with a mean of 968 g. Whereas, non-ROP premature babies had a mean of 1396 g with the birth weight range lies between 700 to 2000 g. Birth weight <1000 g was significantly associated with ROP ($P = 0.001$) [Table 1].

According to staging for ROP, the highest number of cases were from stage three ($n = 20$, 48.8%), followed by those from stage two ($n = 11$, 26.8%), and stage one ($n = 6$, 14.6%) [Table 1].

Mutational Analysis

A total of 86 premature babies were screened for genetic variants in FEVR-causing genes (*NDP*, *FZD4*, *LRP5* and *TSPAN12*). Gene variants for FZD4, *LRP5*, and *TSPAN12* were found upon screening. There was one heterozygous *FZD4* gene variant in one patient from each group (ROP and non-ROP group) [Table 2]. The *FZD4* c.502C>T (p.P168S, rs61735303) substitution was a partial non-synonymous change in exon 2 [Figure 2a].

One coding variant c.3357G>A (p.V1119V, rs556442) of the *LRP5* gene in exon 15 was found [Figure 2b]. This synonymous substitution was found in 30 (73.2%) ROP and 28 (62.2%) non-ROP samples [Table 2].

Two variants of *TSPAN12*, c.765G>T (p.P255P, rs41623) and c.*39C>T (rs41622), were identified in both groups (ROP, 70.7% and 51.2%, respectively; non-ROP, 73.3% and 57.8%, respectively) [Figure 2c and Table 2]. Of 86 subjects, 47 (54.7%) were found to show both TSPAN12 gene variants.

There was no *NDP* variant found in this study. There was no significant difference for all gene variants between ROP and non-ROP groups.

DISCUSSION

ROP is a multifactorial disease characterized by abnormal retinal vessel growth that can lead to blindness in infants. As the ROP phenotype resembles the FEVR phenotype, the four genes encoding the ligand-receptor complex of the Norrin/β-catenin signaling pathway were considered to be causative genes for the progression of ROP.[1,5,8] The present study is the first to report mutational analyses of FEVR-causing genes in ROP patients from Malaysia. Previously, various types of mutations were found in the *FZD4* gene such as point mutations, deletions, and insertions. However, sequencing of the entire *FZD4* gene (translated region) revealed one type of heterozygous variant. The observed variant had a C

Table 1. Demographic data and staging of ROP

	Groups		*P*
	ROP (*n*=41)	**Non-ROP (*n*=45)**	
Gender (*n*, %)			
Male	22 (53.7%)	27 (60.0%)	0.553*
Female	19 (46.3%)	18 (40.0%)	
Gestational age at birth (week)			
Range	24-34	26-34	
Mean	27.2	30.2	
≤28 weeks (*n*, %)	33 (80.5%)	10 (22.2%)	<0.001*
>28 weeks (*n*, %)	8 (19.5%)	35 (77.8%)	
Birth weight (gram)			
Range	570-1500	700-2000	
Mean	968	1396	
<1000 grams (*n*, %)	22 (53.7%)	9 (20.0%)	0.001*
≥1000 grams (*n*, %)	19 (46.3%)	36 (80.0%)	
Staging of ROP (*n*, %)			
Stage 1	6 (14.6%)		
Stage 2	11 (26.8%)		
Stage 3	20 (48.8%)	NA	NA
Stage 4	2 (4.9%)		
Stage 5	2 (4.9%)		

*Chi-square test; $P<0.05$ significant. NA, not applicable; ROP, retinopathy of prematurity

Table 2. Genetic mutational analysis of FZD4, LRP5 and TSPAN12 genes

	Groups		*P*
	ROP (*n*=41)	Non-ROP (*n*=45)	
FZD4 variant (*n*, %): Location: Exon 2; cDNA: c.502C>T; Protein: p.P168S	1 (2.4%)	1 (2.2%)	1.000†
LRP5 variant (*n*, %): Location: Exon 15; cDNA: c.3357G>A; Protein: p.V1119V	30 (73.2%)	28 (62.2%)	0.279*
TSPAN12 variants (*n*, %) Location: Exon 8; cDNA: c.765G>T; Protein: p.P255P	29 (70.7%)	33 (73.3%)	0.788*
Location: Exon 8 (3'UTR); cDNA: c.*39C>T; Protein: NA	21 (51.2%)	26 (57.8%)	0.542*

*Chi-square test; †Fisher's Exact test, *P*<0.05 significant. ROP, retinopathy of prematurity; cDNA, complementary DNA

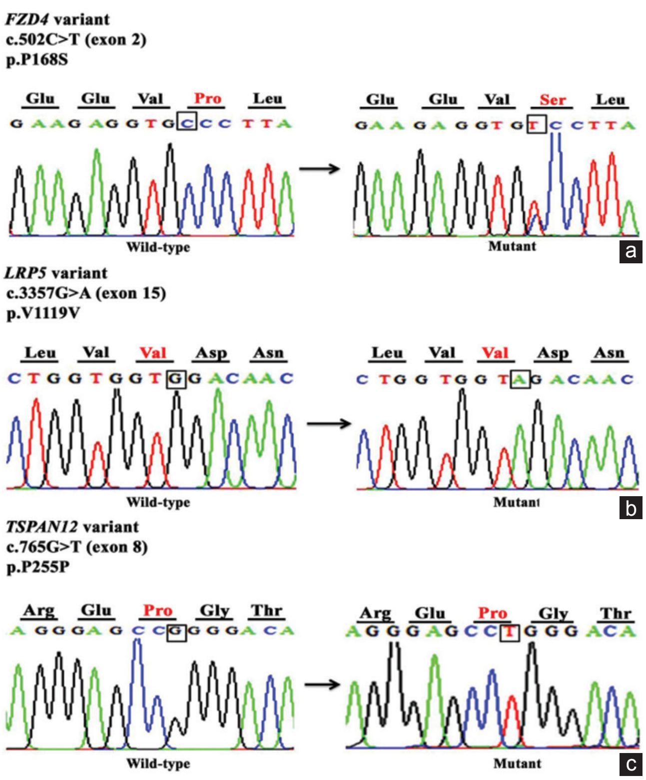

Figure 2. Chromatograms show the variants detected in multiple genes which mutant was compared to the wild-type A. Base and protein position of variant detected in the exon 2 of FZD4 gene, c.502C>T (p.P168S) B. Base and protein position of variant detected in the exon 2 of LRP5 gene, c.3357G>A (p.V1119V) C. Base and protein position of variant detected in the exon 8 of TSPAN12 gene, c.765G>T (p.P255P).

to T transition at nucleotide position 502, which is responsible for alteration in the codon (CCC) coding for proline into codon (TCC) coding for amino acid serine, at amino acid position 168 (P168S). It was detected in only one patient in each group (ROP and non-ROP group). Double peaks were observed at the location that suggested the variant was transmitted in a heterozygous manner from the mother or father.[16] This variant is possibly not pathogenic and does not contribute to the development of ROP in Malay infant patients. However, the non-synonymous or missense change might have knocked out the original function of the protein due to its occurrence at the functional site.[25]

Previously, Ells et al reported the same change in the Canadian population and the occurrence of P168S was identified together with P33S. Six ROP and one control patient were found to have the coupled variants, whereas a single non-coupled change of P168S was observed in another control patient.[26] These changes were classified as known polymorphisms with 1.42% allele frequency.[5] In an earlier report in the population of Michigan, United States by Drenser et al, the same coupled changes were found in four patients with ROP and five with FEVR, and both were referred to as missense mutations as they were not found in control patients.[27] Another study reported similar findings with a combination of changes in two of its Caucasian patients diagnosed with Coat's disease. In 2004, Toomes et al described the P168S alteration as a benign rare sequence variant where this missense change was found only once in 200 white control individuals (<0.3% frequency).[28]

Proline is known to be an atypical amino acid, which plays a unique role in providing rigidity in protein conformations. Several studies have suggested that the proline to serine missense substitution might be a causative factor for FEVR or ROP, as protein rigidity was found to be compromised. The effect is more prominent with the involvement of another variant, P33S as this mutation is found in the sequence responsible for translocation signaling of the FZD4 gene to plasma membrane.[27,29] Thus, both the variants were considered to play a role in the development of ROP as reported by Boonstra et al.[30] In a more recent study, Dailey et al revealed that the combination P33S/P168S change is the most prevalent *FZD4* variant and is statistically significant to be associated with ROP and FEVR. The *FZD4* coupling variants were considered to increase the incidence of retinal damaged causing ROP and intrauterine growth retardation, resulting in smaller newborns. The authors also suggested that these variants may be used as biomarkers for increased risk of development of ROP and preterm birth.[31]

The *LRP5* gene was previously reported to show various types of molecular abnormalities, such as missense or nonsense mutations, insertions, deletions, or splice site mutations in AD-FEVR and AR-FEVR patients. Kondo and colleagues analyzed the genetic variants of *LRP5* in 53 Japanese patients with advanced ROP in 2013. Ten different single-base substitutions were discovered in seven patients, mostly in the coding sequence of the gene.[5] We also identified a variant in exon 15 of the *LRP5* gene, V1119V, which was known as a synonymous change or nonsense mutation due to no changes observed in protein sequence. According to Kondo et al, the synonymous change was classified by the NLHBI Exome Sequencing Project, Seattle, WA as common polymorphism with known minor allele frequency greater than 10%.[5] In addition, Qin et al reported that it is a registered known polymorphism in the single nucleotide polymorphism database (dbSNP) and is categorized as a reference SNP.[32] *LRP5* is a receptor which functions along with *FZD4* in the Norrin/β-catenin signaling pathway. The coding sequences of *LRP5* are translated into a large number of amino acids, as much as 1615 individual proteins.[33] Several studies have reported the presence of the c.3357G>A (p.V1119V) variant in FEVR and advanced ROP among Japanese and Chinese patients.[7,20,32] It is not yet known whether this variant is involved in the progression of ROP.

Two variants of *TSPAN12* in exon 8 were discovered in this study: a synonymous change c.765G>T (p.P255P, rs41623) and another–c.*39C>T in the 3'UTR. *TSPAN12* is one of the components of tetraspanin which is known to be involved in various membrane-associated activities, such as cell proliferation and activation of signaling pathways.[34] Its specific function is to allow the activation of the *FZD4*/*LRP5* receptor complex by interacting with Norrin or *LRP5*.[35] Complex formation of norrin/*FZD4*/*LRP5* might be affected due to any defects in *TSPAN12* which consequently affects vascularization. However, due to P255P which is a synonymous change, the relationship between the variant and ROP could not be further explained. P255P was also described by Kondo et al as non-pathogenic as it has a minor allele frequency of > 10%.[5]

Both changes were discovered in more than half of the population in this study which includes 47 (54.7%) out of 86 subjects. The P255P change was found in all subjects with the 3'UTR variant. As proposed, the 3'UTR is involved in the regulatory pathways of transcription.[36] The crucial function of the region is controlling gene expression as reported in previous studies involving SNPs at the 3'UTR.[37,38] In addition, recent studies have proposed that any changes at the region are associated with susceptibility to diseases.[39-43] Both variants were previously reported in Japanese and Indian populations as common single nucleotide polymorphisms and the details are available in dbSNP database (rs41622 and rs41623).[18,44]

In this study, we found no significant differences for all variants of *FZD4*, *LRP5*, and *TSPAN12* when comparing the prevalence between both groups. The insignificant associations observed in our study between the said genes and ROP possibly due to a limited number of advanced cases enrolled in this study. This is postulated based on the results of a previous study performed in Japanese population involving severe stages of ROP patients which showed high prevalence of *FZD4* or *LRP5* variants in advanced ROP patients.[5]

In this study, we did not detect any variants of the *NDP* gene in ROP patients. Possibly, this was due to the fact that ROP is a complex disease and is polygenetic; thus, the rate of detection of variants might be affected by the low frequency of NDP gene alterations in the population. In addition, samples obtained in the study were from the Malay ethnic group only and genotypic differences might be attributable to population stratification.[45] There might be differences in the genetic predisposition between Malay ethnicity and populations from Western countries or Eastern Asia (Japan, China, and Korea).[5,20,32] According to Ells et al, ROP has long been considered to be affected by racial factors.[26] Researchers questioned the contribution of ethnicity to the development of ROP in the 1950s, when there were significant differences in the number of ROP cases among different races in a particular country.[26]

Low gestational age leads to prematurity and low birth weight, which are two of the major causes of ROP.[46,47] In this study, gestational age ($P < 0.001$) and birth weight ($P = 0.001$) were found to be significantly associated with ROP [Table 1]. These findings are in agreement with previous reports from Pakistan, Iran, Egypt, and Serbia, which showed a correlation between gestational age and birth weight with ROP.[48-51] The degree of retinal immaturity depends solely on the degree of prematurity, which supports the significant probable link between gestational age and ROP.[52,53] Both, extreme prematurity (<28 weeks) and low birth weight (<1000 g) are proposed to have long term effects on visual functions that are believed to be significantly associated with ROP. A recent study suggested that babies with low birth weight tended to have narrower microvasculature development of the retinal arterioles.[54,55]

The major limitation of this study is the relatively small sample size and limited number of advanced/severe stage cases (stage four and five), which might influence the mutation rate detected. A previous study by Kondo and colleagues was used as a reference, wherein they reported that various types of mutations were detected in patients with advanced stages of ROP.[5] However, gestational age and birth weight were the two factors associated with ROP despite the limited number of advanced cases used for this study. It is necessary to perform further studies using a larger sample size to enable the screening of FEVR-causing gene mutations in Malaysian ROP patients. Further studies might elucidate whether these gene mutations are risk factors for the development and progression of ROP for Malay Kelantan premature babies.

In conclusion, four variants of FEVR-causing genes were detected in our study involving Malay patients with ROP. They were identified as c.502C>T (p.P168S) of the *FZD4* gene, c.3357G>A (p.V1119V) of the *LRP5* gene, c.765G>T (p.P255P) and c.*39C>T (3'UTR) of the *TSPAN12* gene. These variants were classified as known SNPs and their details are accessible in the dbSNP. Thus far, there is no compelling evidence to support that these polymorphisms play important roles in the development of ROP. However, it has been previously reported that patients with the non-synonymous variant of *FZD4* have a significantly increased risk of developing ROP and of preterm birth.[31] In addition to the molecular part, two of the major causes of ROP, low gestational age (prematurity) and low birth weight were found to be significantly associated with ROP. This is because degree of retinal immaturity depends on prematurity whereas low birth weight babies have narrower microvasculature development.

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Conflicts of Interest

There are no conflicts of interest.

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