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ORIGINAL ARTICLE

A novel nonsense mutation in Rhodopsin gene in two Indonesian Families with Autosomal Recessive Retinitis Pigmentosa

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ABSTRACT

Purpose: To report a novel, identical nonsense mutation in the rhodopsin (*RHO*) gene in two Indonesian families with autosomal recessive retinitis pigmentosa (arRP).

Methods: Mutation screening for the *RHO* gene was performed in 38 unrelated patients with retinitis pigmentosa (RP) by direct sequencing. Clinical features were also characterized, through complete ophthalmologic examination. Family members of RP patients testing positive for the *RHO* gene were subjected to genetic and clinical examination. To assess the founder effect in the two families, haplotype analysis also was performed.

Results: A novel homozygous nonsense mutation was detected in two patients by a G to A transition at nucleotide position 482 in exon 2 of the *RHO* gene, resulting in substitution of a tryptophan-to-stop at codon 161 (c.482G>A, p.W161X). Examination of family members of these 2 patients showed that the affected members were homozygous and unaffected carriers were heterozygous for the p.W161X mutation. Haplotype analysis revealed that members of the two families carried the same disease-associated variants in markers (*IVS1 RHO* and *D3S2322*). No p.W161X mutations were detected in 45 normal Indonesian subjects, nor were any mutations detected in exons 1–5 of the *RHO* gene in the remaining 36 RP patients.

Conclusion: We detected a novel, recessive nonsense mutation (p.W161X) in the *RHO* gene of two families through mutation screening of *RHO* in 38 Indonesian RP patients. Haplotype analysis suggested that p.W161X was the founder mutation.

KEYWORDS: Rhodopsin; autosomal recessive; retinitis pigmentosa; nonsense mutation

INTRODUCTION

Retinitis pigmentosa (RP) is the name given to a group of degenerative retinal disorders characterized by progressive degeneration of the retina. An estimated 1.5 million people are affected around the world, RP affects about 1 in every 4000 people.[1] RP is genetically heterogeneous, with different modes of inheritance. In the United States, the frequency of families by genetic type has been estimated to be 19% autosomal dominant RP (adRP), 19% autosomal recessive RP (arRP), 8% X-linked RP (XLRP), 46% isolates, and 8% undetermined.[1]

The clinical manifestations of RP include night blindness and loss of peripheral vision in early adulthood. This is followed by progressive constriction of the mid-peripheral and central visual fields and often

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culminates in legal or complete blindness. In addition to pigment deposition, progression of the disease is accompanied by retinal atrophy and vascular attenuation.[2] The disease is marked by great variability in expressivity and penetrance, even within families.[3]

The rhodopsin (*RHO*) gene is considered as the most commonly implicated gene in adRP pathogenesis. Approximately 30% of adRP families have *RHO* mutations,[2] whereas, to our knowledge, reports of *RHO* mutation in arRP are rare.[4–6] No RP mutations have thus far been reported in Indonesian people. Here we report a novel mutation of the *RHO* gene identified in two Indonesian families with arRP.

METHODS

The study was performed according to the tenets of the World Medical Association's Declaration of Helsinki regarding research involving human subjects. Ethical approval was obtained from both Juntendo University School of Medicine and Cicendo National Eye Hospital Ethics Committees. Informed signed consent was obtained from the affected and unaffected family members, and from control subjects.

Sample Collection and Clinical Ophthalmic Examination

Thirty-eight Indonesian unrelated RP patients from West Java Provinces of Indonesia were examined. The affected subjects, including 29 males and 9 females, were aged from 12 to 52 (average, 37) years at time of recruitment. Blood samples were obtained from a peripheral blood vessel. Forty-five normal Indonesian subjects were also recruited as control subjects. Upon detection of the mutation, family members of the proband were then recruited as well, where one parent of each proband, and the affected and unaffected family members from each family were included. Complete clinical examinations including fundus and visual field examinations, were given to all patients and control subjects. Electroretinography (RETI-port, Roland Consult, Brandenburg, Germany) and fundus photography were performed on selected patients.

Mutation Analysis

Genomic DNA was extracted from blood leukocytes using a DNA extraction kit (Pharmacia Biotech Inc. Piscataway, NJ) and exons 1–5 of the *RHO* gene were amplified by polymerase chain reaction (PCR) using primer pairs designed by Zhangl.[7]

PCR was performed in 20µl reaction volumes containing 20ng DNA using the QIAGEN® Multiplex PCR Kit (QIAGEN, GmbH Hilden, Germany), and in an automated thermo-cycler (GeneAmp, PCR system 9700, Applied Biosystems, Foster City CA, USA). The PCR conditions were an initial denaturation at 95°C for 15 min, followed by 94°C for 45 s, 60°C for 90 s and 72°C for 60 s for 35 cycles, and a final extension at 72°C for 10 min.

For direct sequencing, PCR products were purified with a kit (High Pure PCR Purification; Roche Diagnostics GmbH, Mannheim, Germany) and then the terminator reaction was performed with a DNA sequencing kit (BigDye® Terminator Cycle Sequencing, Ready Reaction v.1.1; Applied Biosystems). Sequencing was performed in an automated DNA Sequencer (model 3130; Applied Biosystems) using both forward and reverse primers.

Nucleotide sequences were compared with the published cDNA sequence of the *RHO* gene (GenBank accession number, NM_000539).

Founder Effect Detection

To examine the founder effect status in two families with the same *RHO* mutation, haplotype analysis was performed on family members using five microsatellite markers (Table 1). The primers used for markers *D3S1587* and *D3S621* were available from GenBank (http://www.ncbi.nlm.nih.gov/Genbank). The primers for *D3S3607*, $IVS1(CA)_m A(CA)_n$, and *D3S2322* were designed using Primer3 software (http://frondo.wi.mit.edu/primer3/) (Table 2). All forward primers were labeled with fluorescent dye. After the PCR process, the samples were run on an ABI 3130 genetic analyzer (Applied Biosystems), and genotyping was carried out using GeneMapper® software ver. 4.0 (Applied Biosystems).

RESULTS

Mutation Analysis

Sequencing within the coding region of the *RHO* gene revealed a single, identical, and homozygous mutation in two patients. This novel nonsense mutation was a G to A transition at nucleotide position 482 in exon 2 of the *RHO* gene (c.482G>A), resulting in substitution of a tryptophan to a stop codon (p.W161X). Examination of family members related to these two patients revealed that affected members were homozygous (Family 1: II-1 and II-2; Family 2: II-1 and II-6) and unaffected carriers were heterozygous (Family 1: I-1 and III-1; Family 2: I-2, II-3, and III-1) as shown in Figures 1 and 2. The p.W161X mutation was not found in any of the 45 normal controls.

Of the remaining 36 unrelated RP patients, no mutations were detected in exons 1–5 of the *RHO* gene.

Clinical Assessment

Fundus examinations in all patients revealed intraretinal bone spicule pigment deposits accompanying attenuation of the perifoveal retinal pigment epithelium (RPE). The arterioles were narrowed, and there were prominent intraretinal pigment deposits in the periphery. The symmetric involvement of both eyes was also evident.

The clinical features of the two patients (42 and 46 years old) with the homozygous p.W161X mutation were typical for retinitis pigmentosa. Both

TABLE 1 Microsatellite markers.

No	Marker	Accession	Product size (bp)	Position (Mega base)	Distance from *RHO* (cM)
1	D3S3607	Z53134	174	124.7	4.5
2	D3S1587	Z24044	219	128.2	1
3	IVS1(CA)$_m$A(CA)$_n$	NC_000003	378	129.2	0
4	D3S2322	L29740	197	130.1	0.9
5	D3S621	UNISTS:147033	208	144.4	15.2

Note: Marker No 2 (IVS1(Ca)$_m$A(CA)$_n$) is in Intron 1 of *RHO* gene (3q21-q24).

TABLE 2 Primers for haplotype analysis.

Marker	Forward (5′ → 3′)	Reverse (5′ → 3′)
D3S3607	CATGCATTCCCTTTATGCAA	TTTTAGGTCAAAGGGCGTGT
D3S1587	TACAGTTCTATAAGGGCAGCC	AGGGAGACAGAGTGATGGATT
IVS1(CA)$_m$A(CA)$_n$	CCACATGTCCGGGTTATTTC	CTTGGGGAAGGAGAGAGCTT
D3S2322	TCCTGGAGGTGTACCTGCTT	CAGTGGATAATGGGAAATGGA
D3S621	ATACCCATGTTCACTGCACC	CACTTAGCACGTTTTCAAGG

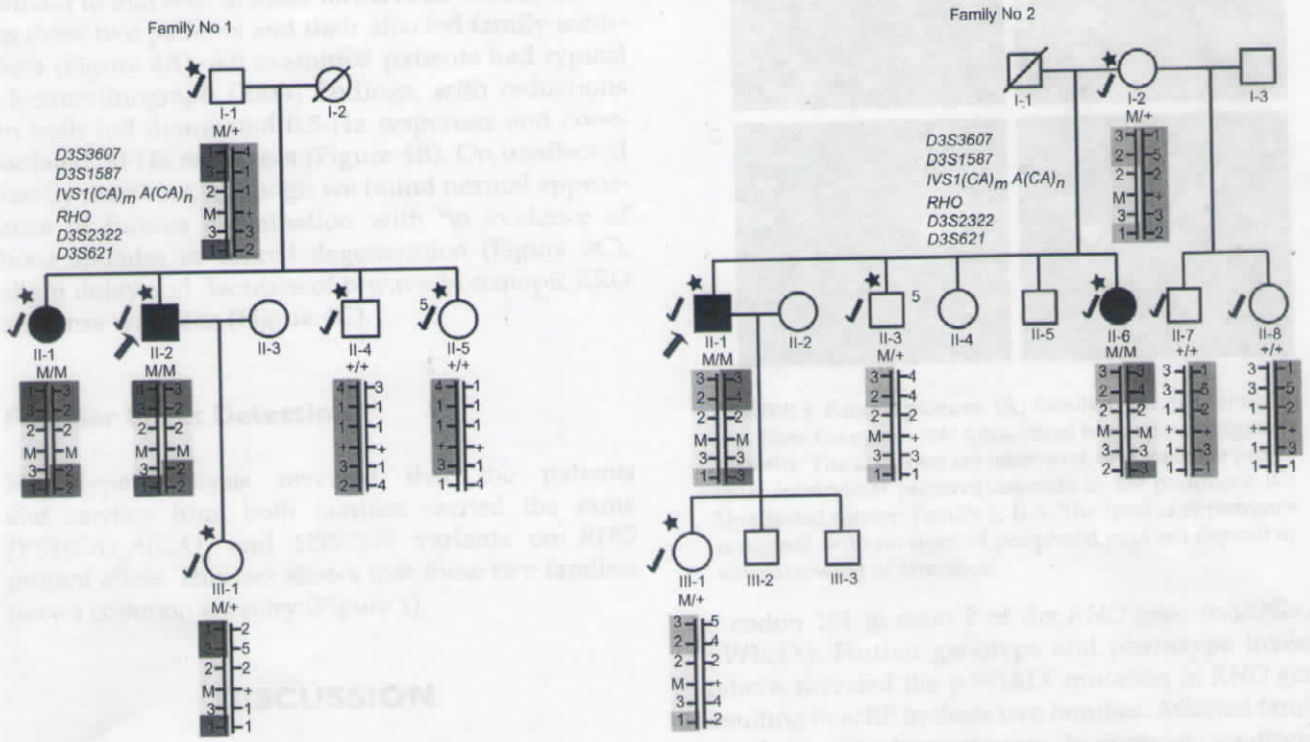

FIGURE 1 Pedigrees and haplotypes for five markers in Indonesian arRP families associated with the p.W161X mutation in the RHO gene. The solid and open symbols indicate affected and unaffected members, respectively. The arrow sign indicates the proband. The circle and square symbols show female and male, respectively. The slash sign indicates the deceased members. The "√" sign indicates individuals examined in this study and the star sign shows individuals that were clinically examined. The "+" sign indicates a normal allele and "M" indicates a mutant allele. The numbers (e.g. 2-, -4) show the variant type of each marker obtained by haplotyping analysis.

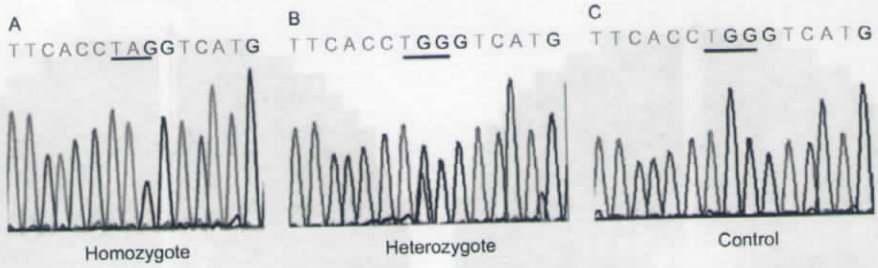

FIGURE 2 Nucleotide sequences of the region surrounding p.W161 of the RHO gene. (A) p.W161X mutation in a homozygous family member; Family 1, II-2. The transition of G to A in the second base of codon 161 shown by underline creates a stop codon. (B) p.W161X mutation in a heterozygous family member; Family 2, II-3. (C) p.W161 in a normal control.

patients had night blindness that was first noted at approximately 15 years of age. The visual acuity of the first proband (Figure 1; Family 1: II-2) was hand movement for the right eye and finger counting for the left eye. The best-corrected visual acuity of the second proband (Figure 1; Family 2: II-1) was 20/200 for the right eye and finger counting for the left eye. Both patients noted disturbance of visual acuity starting at around 25 years of age. Fundus examination disclosed bilateral pigmentary retinal degeneration, attenuation of the retinal arteries, and edematous macula (Figure 3A and 3B). A pattern of field loss similar to that seen in other forms of RP was observed in these two patients and their affected family members (Figure 4A). All examined patients had typical electroretinograph (ERG) findings, with reductions in both rod-dominated 0.5-Hz responses and cone-isolated 30-Hz responses (Figure 4B). On unaffected family members, although we found normal appearance of fundus examination with no evidence of bone spicules or retinal degeneration (Figure 3C), slight delay and decrease of b-wave in scotopic ERG response was seen (Figure 4C).

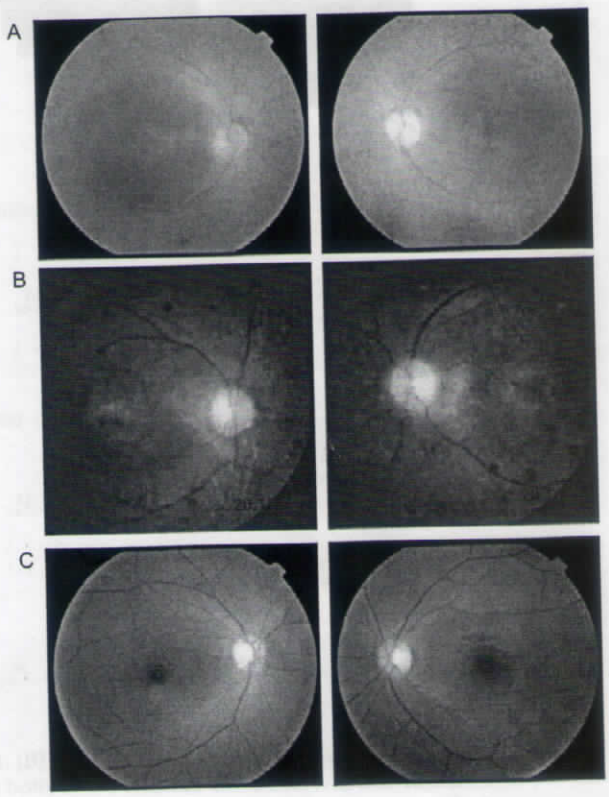

FIGURE 3 Fundus pictures. (A) Family 1, II-2. (B) Family 2, II-1. Note the symmetric intraretinal bone spicule pigment deposits. The arterioles are narrowed, and there are prominent intraretinal pigment deposits in the periphery. (C) Unaffected carrier: Family 2, II-3. The fundus appearance is normal, with no signs of peripheral pigment deposit or any narrowing of arterioles.

Founder Effect Detection

Haplotype analysis revealed that the patients and carriers from both families carried the same *IVS1(CA)$_m$A(CA)$_n$* and *D3S2322* variants on *RHO* mutant allele. This fact shows that these two families have a common ancestry (Figure 1).

DISCUSSION

Most reported *RHO* gene mutations have been found in adRP,[3,7–12] whereas to our knowledge, only a few reports have described mutations in arRP.[4–6] In the present study, we found a novel nonsense mutation in two Indonesian RP families that leads to a premature stop at codon 161 in exon 2 of the *RHO* gene (c.482G>A, p.W161X). Further genotype and phenotype investigations revealed the p.W161X mutation in *RHO* gene resulting in arRP in these two families. Affected family members were homozygous. In contrast, unaffected family members were heterozygous (Figures 1 and 2).

Rhodopsin is the major protein of the rod outer segments and functions as the light absorbing protein, initiating a cascade of reactions that mediate vision

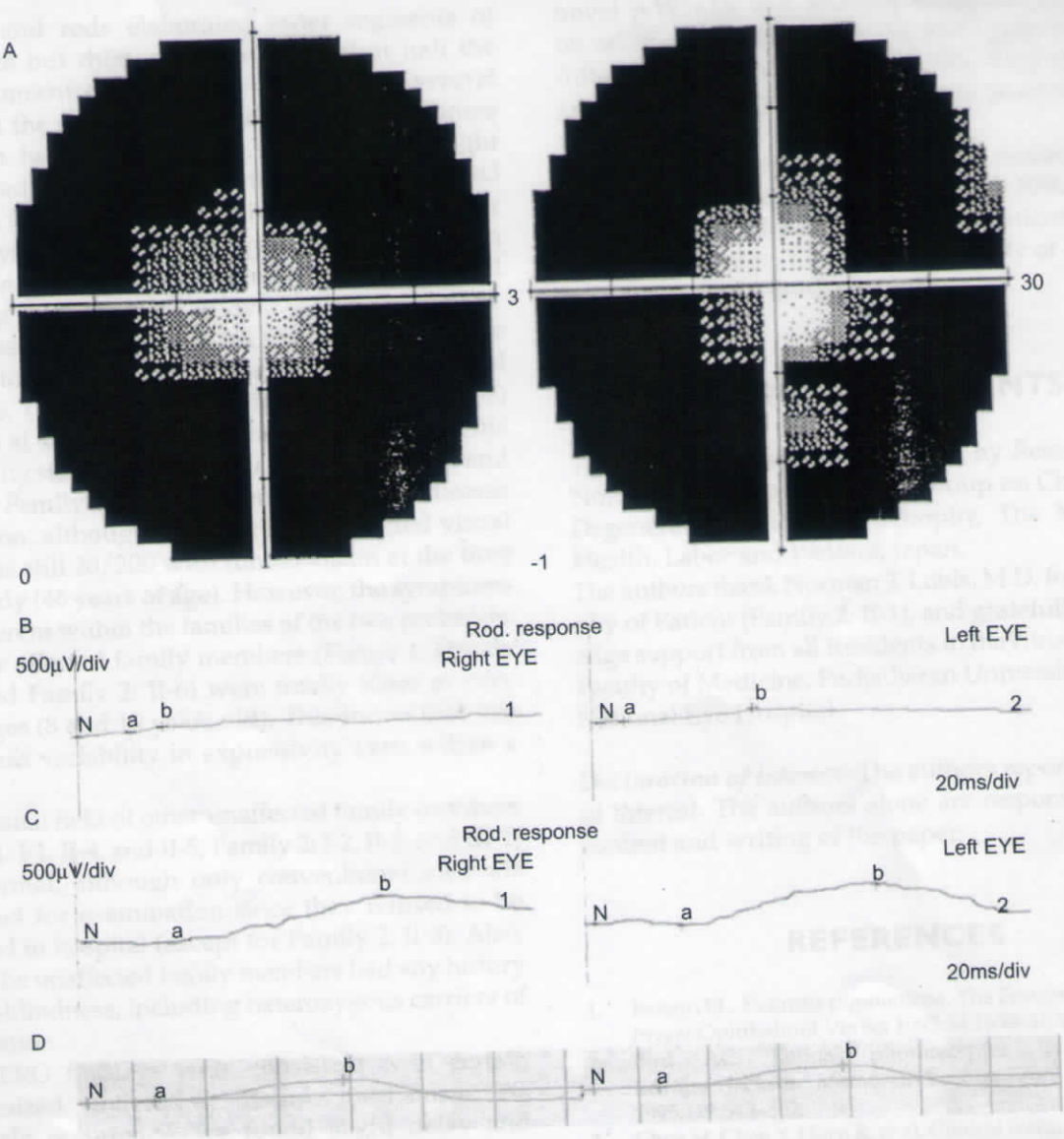

FIGURE 4 (A) Visual field examination for Family 2, II-1. (B) ERG examination for Family 2, II-1. Note the tunnel vision of the visual field and flat scotopic ERG in both eyes. A similar condition was also noted for the visual field and ERG of Family 1, II-2. (C) Rod response ERG for an unaffected family member (Family 2, II-3). Although the b-wave response was slightly delayed, the amplitude of the b-wave was still within the normal limit. (D) Normal reference for scotopic response (the same age group, from RETI-port ERG).

in dim light. As p.W161 of *RHO* resides within transmembrane helix IV, the p.W161X is likely that only a limited amount of mRNA would be translated because this premature termination codon encodes terminal of the protein synthesis at the codon 161. Furthermore, these premature and truncated mRNAs, if synthesized and stable, are likely to be non-functional or might even be deleterious to cellular metabolism. However, it is likely considered that expression of this truncated protein is disrupted by nonsense-mediated mRNA dacay (NMD). The NMD is a translation-coupled mechanism that eliminates mRNA transcribed from alleles carrying nonsense mutations.[13–15] Therefore, we presume the mutated *RHO* mRNA to be degraded as a result of NMD. This would result in a lack of function or very limited function of the RHO protein. Therefore, the unaffected family members who carried only one p.W161X mutated allele have likely normal RHO protein translated from the copy of the wild-type gene.

In rhodopsin knockout mice,[16] retinas in mice lacking both opsin alleles (-/-) initially developed normally, except that rod outer segments failed to form, however, within months of birth, photoreceptor cells degenerated completely. Retinas from mice with a single copy of the opsin gene (+/-) developed

normally, and rods elaborated outer segments of normal size but rhodopsin was present at half the normal concentration. There also were several changes in the properties of the electrical responses to light in heterozygous rods. Sensitivity to light was reduced twofold, consistent with the decreased rhodopsin density.[16] These findings may be a similar basis in asymptomatic heterozygous carrier of human arRP in decreasing of light sensitivity, as reported previously[4,17] and present study.

As consequences, the p.W161X mutation would be expected to lead to very severe progressive clinical symptoms. The first proband (Figure 1; Family 1: II-2) was blind at 40 years old and the deterioration of his visual acuity started at school age. The second proband (Figure 1; Family 2: II-1) experienced a similar disease progression, although his right best corrected visual acuity was still 20/200 with tunnel vision at the time of the study (46 years of age). However, the symptoms were different within the families of the two probands. The other affected family members (Figure 1; Family 1: II-1 and Family 2: II-6) were totally blind at very young ages (8 and 10 years old). This shows that this disease has variability in expressivity even within a family.

The visual field of other unaffected family members (Family 1: I-1, II-4, and II-5; Family 2: I-2, II-3, and III-1) were normal, although only conventional methods were used for examination since they refused to be examined in hospital (except for Family 2, II-3). Also, none of the unaffected family members had any history of night-blindness, including heterozygous carriers of the mutation.

The ERG findings were consistent with certain hypothesized underlying disease mechanism. As previously reported,[4,17] we found slight delay and decrease of b-wave in the scotopic ERG responses in a heterozygous unaffected family member (Figure 4C). However, his visual field and fundus appearance (Figure 3C) were normal. Unfortunately, we could not get ERG findings of other heterozygous unaffected family members.

For the homozygous p.W161X mutation, we could not find any evidence of consanguinity between the parents of the proband in either of the families. The two families came from different geographical regions of Indonesia. However, haplotype analysis revealed that the two families are nevertheless distantly related. Although the *D3S2322*, which is located at 0.9 cM from the *RHO* gene, did not always link on both mutant alleles in all affected cases, at least one *RHO* mutant allele was closely linked with $IVS1(CA)_mA(CA)_n$ and *D3S2322* variants in both family (Figure 1). From these findings we concluded that these two families have a common ancestor, and the novel p.W161X is a founder mutation. Haplotypes on other markers far from the *RHO* gene were quite different between the two families. This result suggests that a long period of time has passed since the original p.W161X mutation event.

In summary, we found p.W161X mutation of the *RHO* gene in two arRP families through *RHO* mutation analysis of 38 Indonesian unrelated patients with RP. To our knowledge, this is the first study of rhodopsin in Indonesian RP families.

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