

Genotypic Analysis of *ABCA4* Coding Sequence in Thai Patients with Stargardt Disease

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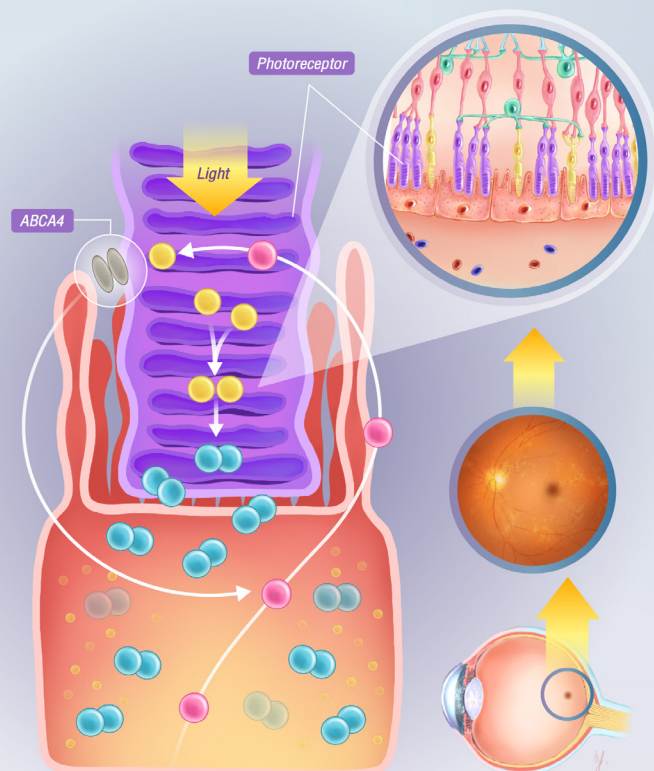
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Materials and Methods

DNA sequencing of all 50 exons of the *ABCA4* gene was performed in

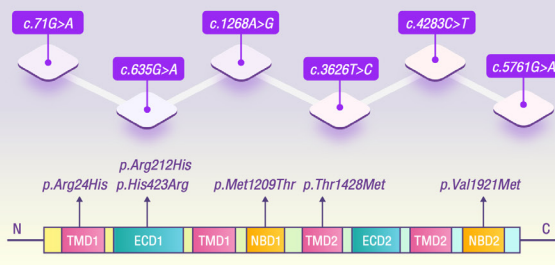
9 Thai patients with clinically diagnosed Stargardt disease.



Result

The most common variant was **c.1268A>G**

6 amino acid sequence variations in the *ABCA4* gene were found in 5 patients, including



Conclusion

In this cohort, only 56% of Thai Stargardt patients had



Missense mutations in the *ABCA4* gene



Mutations in the non-coding regions of the *ABCA4* or mutations in other genes

may be responsible for Stargardt phenotypes in the remaining patients.

Our findings are the first to reveal the mutational spectrum of *ABCA4* leading to Stargardt disease in the Thai population and demonstrate a potential for *ABCA4* screening as well as the importance of genetic variability in Thai patients with clinically suspected Stargardt disease.

Keywords

Stargardt disease

ABCA4 gene

DNA sequencing



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ABSTRACT

Objective: To study the mutational spectrum of the *ABCA4* gene in Thai patients with Stargardt disease.

Materials and Methods: DNA sequencing of all 50 exons of the *ABCA4* gene was performed in nine Thai patients with clinically diagnosed Stargardt disease.

Results: Amino acid sequence variations in the *ABCA4* gene were found in five patients. Six missense mutations, c.71G>A, c.635G>A, c.1268A>G, c.3626T>C, c.4283C>T, and c.5761G>A, previously associated with Stargardt disease, were identified in our cohort. The variant c.1268A>G was the most prevalent in our study.

Conclusion: In this cohort, only 56% of Thai Stargardt patients had missense mutations in the *ABCA4* gene. Mutations in the non-coding regions of the *ABCA4* or mutations in other genes may be responsible for Stargardt phenotypes in the remaining patients. Our findings are the first to reveal the mutational spectrum of *ABCA4* leading to Stargardt disease in the Thai population and demonstrate a potential for *ABCA4* screening as well as the importance of genetic variability in Thai patients with clinically suspected Stargardt disease.

Keywords: Stargardt disease; *ABCA4* gene; DNA sequencing (Siriraj Med J 2024; 76: 705-712)

INTRODUCTION

Stargardt disease, also known as fundus flavimaculatus, is a prevalent form of familial macular degeneration. The disease is associated with mutations in the *ABCA4* (STGD1, MIM 248200), *ELOVL4* (STGD3, MIM 600110) and *PROM1* (STGD4, MIM 603786).¹⁻³ STGD1, the most prevalent subtype of Stargardt disease, has a prevalence between 1:8,000 and 1:10,000, and exhibits an autosomal recessive inheritance pattern with high genotypic and phenotypic heterogeneity.^{1,2} Patients with STGD1 generally carry homozygous or compound heterozygous mutations in the *ABCA4* gene.^{1,6} *ABCA4* encodes a 2,273-amino acid ATP binding cassette (ABC) transporter, primarily located in the outer segment discs of rod and cone photoreceptor cells. Its primary function is to transport N-retinylidene-phosphatidylethanolamine (N-Ret-PE) across the disc membranes from the lumen to the cytoplasm. This process is vital for preventing the accumulation of toxic bis-retinoid compounds that can lead to photoreceptor degeneration.³ Consequently, mutations in the *ABCA4* can lead to the accumulation of these harmful compounds, resulting in visual impairment in STGD1 patients.

Over 1,200 *ABCA4*-variants are associated with STGD1.^{1,4} Interestingly, data from several studies reveal a broad spectrum of *ABCA4* mutations across different ethnicities.⁵⁻⁸ In this study, DNA sequencing of all 50 *ABCA4* exons was performed using genomic DNA of nine Thai patients with Stargardt disease. To our knowledge, this is the first study reporting *ABCA4* variants in the Thai population with Stargardt disease.

MATERIALS AND METHODS

Participants

This study was approved by the Siriraj Institutional Review Board (SIRB), with protocol no 798/2560 (EC2), COA no Si 112/2018. Informed consent was obtained from all participants prior to their involvement in the study. Nine Thai patients with clinically confirmed Stargardt disease were recruited from the Ophthalmology Clinic at Siriraj Hospital, Bangkok, Thailand. The diagnosis was based on clinical characteristics and ocular electrophysiologic findings reported by *Fishman et al.*⁹ including pisciform flecks or pigmentary changes in the fundi of the patients. The disease was classified into four clinical stages for analysis. We recorded ophthalmic findings such as best corrected visual acuity (BCVA, logMAR), outcomes of fundoscopic examinations, Humphrey visual field (HVF), optical coherence tomography (OCT), fundus autofluorescence (FAF) and ocular electroretinography (ERG). Additionally, six milliliters of blood was drawn from each participant for mutational analysis.

Isolation of genomic DNA

Genomic DNA was extracted from blood samples using genomic DNA mini kit (Geneaid) according to the manufacturer's instruction. Genomic DNA samples were kept at -20°C.

Polymerase chain reaction (PCR) of *ABCA4* gene

The coding regions and adjacent intronic sequences of the *ABCA4* gene were amplified by PCR using primers (Supplementary Table 1) from a previously published

study.¹⁰ The PCR reaction consisted of 100 ng of genomic DNA template, 0.2 μ M primer mix and 12.5 μ l Taq 2X PCR master mix (New England BioLabs), bringing the total reaction volume to 25 μ l. The PCR protocol initiated with a denaturation step at 95°C for 5 minutes, followed by 35 cycles of 95°C for 30 seconds, annealing at 55°C for 30 seconds, and a final extension at 72°C for 30 seconds. It concluded with an elongated extension at 72°C for 10 minutes. After PCR-amplification, the products were purified using the GenepHlow™ PCR cleanup kit (Geneaid) in accordance with manufacturer's protocol.

DNA sequencing and data analyses

Sanger sequencing of the PCR products was performed with 5 μ M primers using 3730xl DNA analyzer (Applied Biosystems) at the ISO9001:2015 and ISO13485:2016-certified service sequencing lab (Bionics, Korea). Sequencing data was analyzed by aligning it with *ABCA4* reference sequence (NG_009073.1 and NM_00350) using ApE v2.0.61 (M. Wayne Davis). Missense variants identified through this process were then evaluated for potential deleterious effects using in silico prediction algorithms, specifically PolyPhen-2¹¹ and SIFT.¹² PolyPhen-2 is a web-based tool that predicts structural and functional consequences of amino acid substitution within human proteins. The tool briefly works by selecting a set of homologous sequences with a clustering algorithm then constructing and refining its multiple alignments. Sequence-based features and structural-based features of the substitution site are extracted and fed to a probabilistic classifier. The functional significance of an allele replacement is predicted from its individual features by a naïve Bayes classifier trained using supervised machine-learning. Sequences are predicted to be benign (Poly-Phen score ≤ 0.446), possibly damaging (Poly-Phen score > 0.446 and ≤ 0.908) or probably damaging (Poly-Phen score > 0.908). SIFT is a sequence homology-based tool that distinguishes tolerant from intolerant amino acid substitutions and predicts phenotypic effects from amino acid substitutions. The tool utilizes PSI-BLAST to search for similar sequences, then the closely related sequences that may have similar function to the query sequence are identified by PSI-BLAST and MOTIF. Alignment of the selected sequences is performed by PSI-BLAST and calculation of normalized probabilities for all possible substitutions from the alignment is done using Dirichlet mixture model. Positions with normalized probabilities less than 0.05 are predicted to be deleterious while those with normalized probabilities at least 0.05 are predicted to be tolerated. Classification of the identified variants was

done according to the joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology.¹³

RESULTS

ABCA4 mutational spectrum

Nine patients (3 males and 6 females) clinically diagnosed with Stargardt disease were recruited. The age of onset ranged from 5-58 years old (median 35 years old). Sequencing the 50 exons of the *ABCA4* gene revealed 11 point mutations, of which 6 were missense and 5 were synonymous (Fig 1 and Table 1).

There were 5 patients with at least one missense mutation (P1, P4, P5, P6 and P8). We could not identify missense mutations of the *ABCA4* exons in the other 4 patients and they carried only silent mutations (P2, P3, P7 and P9). Interestingly, all 6 missense mutations identified in our cohort were in heterozygous state. There were 3 patients being compound heterozygous for 2 missense mutations (P4, P6 and P8) and 1 patient for 3 missense mutations (P5). Whether there could be interactions between different heterozygous mutations in these patients needs further studies.

There were 3 patients carrying at least one probably damaging mutation predicted by PolyPhen and SIFT scores (P4, P6 and P8). However, they did not show different clinical phenotypes in terms of age of onset and visual acuity compared to the rest of the subjects.

Of note was that patients P2 and P3 were siblings and showed early onset of the disease compared to the others. However, there were no missense mutations found in their *ABCA4* exons, suggesting that mutations in other genes may be responsible for their phenotypes.

The details of each patient were described below.

Case series

Sanger sequencing of genomic DNA from patient P1, a 54-year-old female patient presenting with decreased visual acuity and recent logMAR BCVA of 0.94 OD and 0.92 OS, uncovered a heterozygous missense mutation in exon 10 (1268A>G). This mutation has been previously associated with STGD1.¹⁴ Additional three polymorphisms (141A>G, 4530G>A and 6069T>C, Table 2) were also identified. No family history of eye disease were found in this patient.

Patient P2, diagnosed with Stargardt disease, exhibited recent BCVA of 2 OD and 1.72 OS. Additionally, he displayed delayed development, attributed to juvenile type neuronal ceroid lipofuscinosis. Sanger sequencing of the *ABCA4* exons for this patient identified heterozygosity for three silent mutations, 141A>G, 5814A>G and 5844A>G.

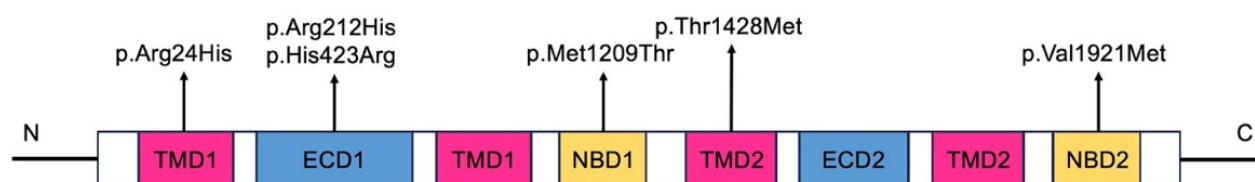


Fig 1. Distribution of *ABCA4* non-synonymous mutations found in this study. ECD1 - exocytosomal domain 1; ECD2 - exocytosomal domain 2; NBD1 - nucleotide binding domain 1; NBD2 - nucleotide binding domain 2; TMD1 - transmembrane domain 1; TMD2 - transmembrane domain 2.

TABLE 1. *ABCA4* variants in this study.

<i>ABCA4</i> mutations				Pathogenicity prediction		Allele frequency in patients	ACMG Variant interpretation	Reference
Reference SNP	Exon	Nucleotide change	Amino acid change	PolyPhen*	SIFT**			
rs4847281	2	c.141A>G	-	-	-	14/18	benign	(28)
rs62645958	2	c.71G>A	Arg24His	0.98	0.01	1/18	likely pathogenic	(29, 30)
rs6657239	6	c.635G>A	Arg212His	0.977	0.04	2/18	benign	(15, 16, 17, 18)
rs3112831	10	c.1268A>G	His423Arg	0	0.63	5/18	benign	(14, 20, 21, 22, 23, 31, 32)
rs76258939	25	c.3626T>C	Met1209Thr	0	1	1/18	benign	(27, 33)
rs1800549	29	c.4283C>T	Thr1428Met	0.01	0.09	2/18	benign	(4, 24, 25, 34)
-	30	c.4530G>A	-	-	-	1/18	benign	This study
rs61753032	41	c.5761G>A	Val1921Met	0.979	0	1/18	likely pathogenic	(25, 35)
rs4147857	41	c.5814A>G	-	-	-	2/18	benign	(28, 32)
rs2275029	42	c.5844A>G	-	-	-	2/18	benign	(28)
rs1762114	44	c.6069T>C	-	-	-	15/18	benign	(28)

*PolyPhen score: >0.908 probably damaging, ≤0.908 and >0.446 possibly damaging, ≤0.446 benign

**SIFT score: <0.05 deleterious, ≥0.05 tolerated

Abbreviations: ACMG, American College of Medical Genetics and Genomics; SNP, single nucleotide polymorphism

Furthermore, he was found to be a homozygote for the 6069T>C silent mutation. His sibling, patient P3, also diagnosed with Stargardt disease (recent BCVA of 2 OD and 0.02 OS) displayed normal development but carried the same mutations.

Patient P4, a 42-year-old female, had been experiencing progressive blurred vision for six years and had a recent BCVA of 1.5 OD and 1.42 OS. Mutational analysis revealed compound heterozygous missense mutations in exon 6 (635G>A) and exon 10 (1268A>G). Additionally, the patient was found to be homozygous for two single

nucleotide polymorphisms (SNPs, 141A>G and 6069T>C) (Table 2).

Sequence analysis of the complete *ABCA4* exons from the genomic DNA of a male patient (patient P5) diagnosed with Stargardt disease and with a recent BCVA of 1.52 in both eyes revealed three compound heterozygous missense mutations in exon 2 (141A>G), exon 25 (3626T>C) and exon 29 (4283C>T). Additionally, two synonymous mutations were identified in exon 2 (141A>G) and exon 44 (6069T>C).

TABLE 2. Participants' characteristics and type of *ABCA4* mutation found.

Patient	Gender	Age of onset (years)	logMAR BCVA		Clinical stages	<i>ABCA4</i> mutations		
			OD	OS		Reference SNP	Nucleotide change	Amino acid change
P1	Female	36	0.94	0.92	3	rs3112831	c.1268A>G	His423Arg
						rs4847281	c.141A>G	-
						-	c.4530G>A	-
						rs1762114	c.6069T>C	-
P2	Male	5	2	1.72	1	rs4847281	c.141A>G	-
						rs4147857	c.5814A>G	-
						rs2275029	c.5844A>G	-
						rs1762114	c.6069T>C	-
P3	Male	5	0	0.02	1	rs4847281	c.141A>G	-
						rs4147857	c.5814A>G	-
						rs2275029	c.5844A>G	-
						rs1762114	c.6069T>C	-
P4	Female	35	1.5	1.42	1	rs6657239	c.635G>A	Arg212His
						rs3112831	c.1268A>G	His423Arg
						rs4847281	c.141A>G	-
						rs1762114	c.6069T>C	-
P5	Male	23	1.52	1.52	3	rs3112831	c.1268A>G	His423Arg
						rs76258939	c.3626T>C	Met1209Thr
						rs1800549	c.4283C>T	Thr1428Met
						rs4847281	c.141A>G	-
						rs1762114	c.6069T>C	-
P6	Female	50	0.46	0.44	2	rs62645958	c.71G>A	Arg24His
						rs3112831	c.1268A>G	His423Arg
						rs4847281	c.141A>G	-
						rs1762114	c.6069T>C	-
P7	Female	35	1.02	1.04	4	rs4847281	c.141A>G	-
						rs1762114	c.6069T>C	-
P8	Female	21	1.02	0.98	1	rs6657239	c.635G>A	Arg212His
						rs61753032	c.5761G>A	Val1921Met
						rs4847281	c.141A>G	-
P9	Female	58	1.14	2	4	rs4847281	c.141A>G	-
						rs1762114	c.6069T>C	-

Abbreviations: logMAR, logarithm of the minimum angle of resolution; BCVA, best corrected visual acuity; OD, right eye; OS, left eye

Patient P6, a female diagnosed with Stargardt disease, had a current BCVA of 0.46 OD and 0.44 OS. Sanger sequencing of her *ABCA4* genes revealed two heterozygous non-synonymous mutations: 71G>A and 1268A>G. These mutations result in an amino acid change from arginine to histidine (Arg24His) and from histidine to arginine (His423Arg), respectively (Table 2). Additionally, this patient was found to be homozygous for the synonymous mutations 141A>G and 6069T>C.

In patient P7, a 55-year-old female diagnosed with Stargardt disease, two benign variants, 141A>G and 6069T>C, were found in a homozygous state within *ABCA4* coding sequence. The onset of her disease was at age 45, and her recent BCVA was 1.02 OD and 1.04 OS. Sanger sequencing of all *ABCA4* exons in this patient did not reveal any pathogenic variants.

In patient P8, a 33-year-old female diagnosed with Stargardt disease, sequencing revealed two pathogenic heterozygous variants and one polymorphism. At the time of sequencing, her BCVA was 1.02 OD and 0.98 OS. The 635G>A mutation, resulting in an amino acid change from arginine to histidine (Arg212His), is associated with STGD1.¹⁵⁻¹⁸ Similarly, the pathogenic 5761 G>A variant, which causes a change of valine to methionine, was found in this patient. Additionally, she was homozygote for the 141A>G polymorphism.

Patient P9, a 63-year-old female, presented with a BCVA of 1.14 OD and 2 OS. Her clinical features were consistent with Stargardt disease. Genetic testing found her to be homozygous for two *ABCA4* polymorphisms, 141A>G and 6069T>C.

DISCUSSION

This is the first study to investigate *ABCA4* variants in Thai patients with Stargardt disease. In our cohort, four patients (P4, P5, P6 and P8) carried compound heterozygous missense mutations in the *ABCA4* gene, one patient (P1) was heterozygous for a single *ABCA4* variant leading to amino acid change, while four patients (P2, P3, P7 and P9) presented with SNPs but no *ABCA4* mutant alleles. Out of the six missense mutations in our cohort, three (c.71G>A, c.635G>A and c.5761G>A) were classified as pathogenic or likely pathogenic, and the remaining three (c.1268A>G, c.3626T>C and c.4283C>T) were deemed benign or likely benign.¹³ Mutant variants were evenly distributed across the six functional domains of the *ABCA4* protein (Fig 1). Specifically, two mutations (c.635G>A and c.1268A>G) were found in the exocytosolic domain 1 (ECD1) of the protein. Within the transmembrane domain (TMD 1 and 2, two mutant variants were found (c.71G>A for TMD1 and c.4283C>T for TMD2). Two additional missense mutations were also detected in

nucleotide binding domain (NBD) 1 and 2 (c.3626T>C for NBD1 and c.5761G>A for NBD2). Thus, no *ABCA4* variant hot spots were found in our cohort, which is consistent with previous reports in other ethnicities.^{5,19}

The most prevalent disease-associated variant in our study was c.1268A>G (44.44%). Pathogenicity prediction tools, PolyPhen and SIFT, have classified this variant as benign or tolerated (Table 1). Despite an occurrence in the normal German population, it is the most frequently observed variant in Stargardt disease patients in the United States, where it is associated with abnormal electroretinogram findings.^{20,21} A study involving a Sicilian family indicated that the c.1268A>G variant could potentially delay the onset of Stargardt disease.²² Similarly, another study proposed its implication in late-onset forms of Stargardt disease.²³ Interestingly, in our study, the average age of onset of patients with this variant was higher, but not significantly, compared to patients without it (36 vs 29 years old, unpaired t-test, $p=0.6388$). Furthermore, when comparing patients of similar ages (P4 vs P7 and P6 vs P9), the fundoscopic findings for those carrying the c.1268A>G (P4 and P6) appeared to be more preserved than in those without the variant (P7 and P9) (Fig 2). This suggests that the c.1268A>G variant may influence the clinical phenotype, which aligns with findings from previous studies.

In this study, 55.55% of Stargardt disease patients were found to have identifiable *ABCA4* mutations, which is comparable to previous reports.^{8,17,24,25} The investigation was limited to the coding regions of *ABCA4*, meaning that patients without detected mutations might have unidentified *ABCA4* mutations in non-coding regions or mutations other genes associated with Stargardt disease. A more comprehensive whole genome sequencing approach might be required to identify disease-associated mutations in these patients. In addition, another limitation of this study is a low number of patients. Other studies investigating *ABCA4* variants in Stargardt disease and/or retinitis pigmentosa involved more patients (ranges from 40 to 345).^{5,17,19,26,27} Thus, it is likely that the *ABCA4* variants reported in this study, which solely comprises patients from the Ophthalmology Clinic at Siriraj Hospital who consented to genetic testing, may not fully represent the genetic diversity of Thai patients with Stargardt disease. Further research is warranted to explore genetic heterogeneity of Stargardt disease in the Thai population.

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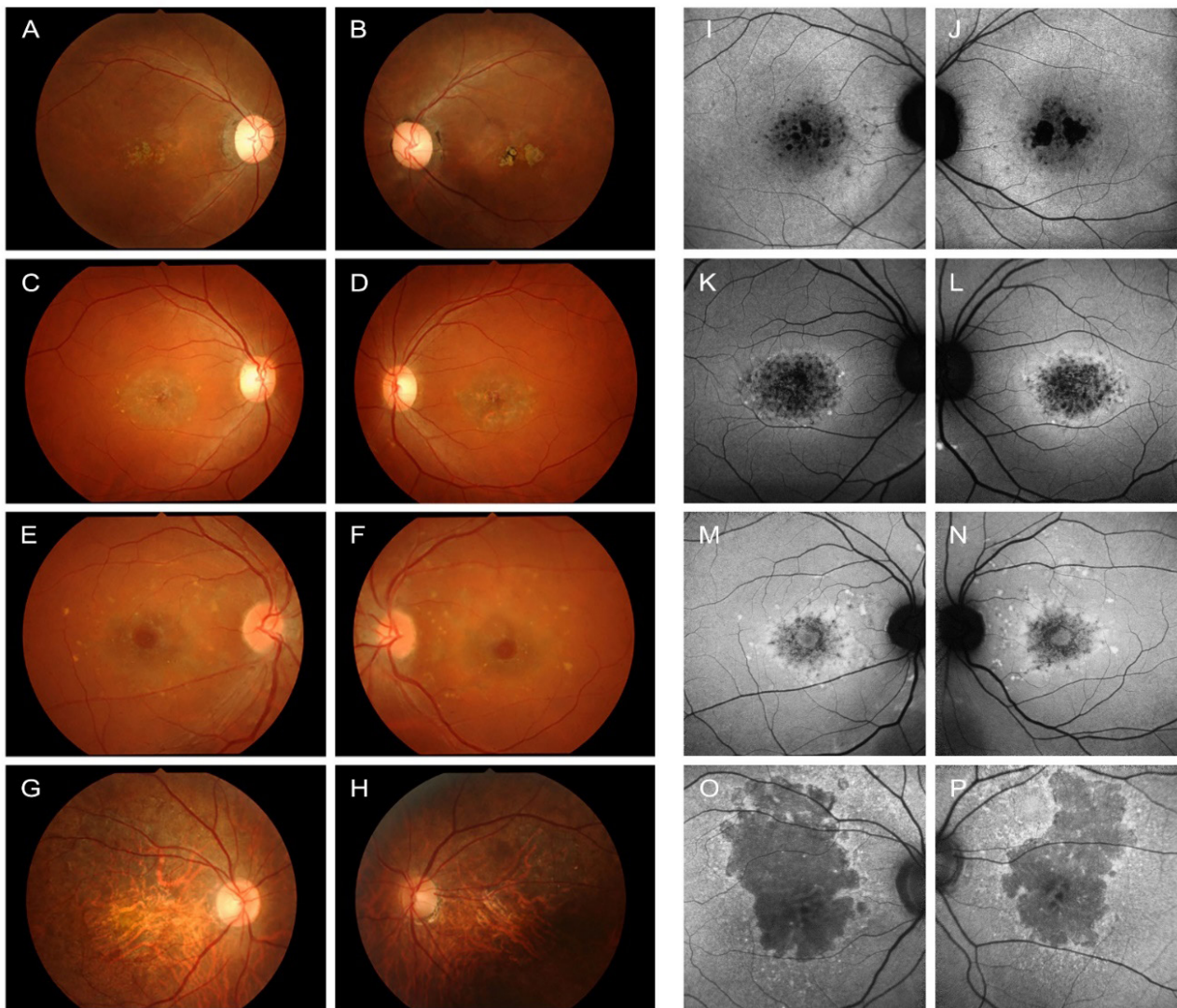


Fig 2. Fundus photographs (A-H) and corresponding fundus autofluorescence images (I-P) comparing Stargardt patients with and without the c.1268A>G variant at similar ages (patients P4, 37 years old vs P7, 35 years old and patients P6, 51 years old vs P9, 59 years old). Patient P4 (A, B, I and J) exhibited foveal retinal pigment epithelial (RPE) atrophy in both eyes, as well as hyperpigmented clumps of RPE in the left eye. The c.1268A>G variant was present in this patient. In contrast, patient P7 (C, D, K, and L) did not possess the variant and exhibited more severe RPE atrophy in both eyes. Patient P6 (E, F, M and N), who carried the variant, displayed fishtail-shaped flecks in the macular region with multiple small RPE atrophic foci better seen in by autofluorescence in both eyes. On the contrary, patient P9 (G, H, O and P) did not harbor the variant and demonstrated more extensive RPE atrophy with absorbed flecks in both eyes.

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Author Contributions

CV provided conceptual input, designed experiments, analyzed sequencing and clinical data and wrote the manuscript. RS, NC and NT designed and performed experiments. AS and DD performed experiments and analyzed sequencing data. SP collected and analyzed clinical data. SP and NP provided conceptual input, collected and analyzed clinical data and co-wrote the manuscript. All authors approved the manuscript.

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