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# Molecular scanning of the ABCA4 gene in Spanish patients with retinitis pigmentosa and Stargardt disease: Identification of novel mutations

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PURPOSE. *Among the 500* ABCA4 *mutations identified so far in the retina-specific ABC transporter (*ABCA4*) gene, only 20 have been described in patients with autosomal recessive retinitis pigmentosa (arRP). In this study the gene was screened for mutations in a cohort of 25 patients of Spanish origin, to further assess* ABCA4 *involvement in retinal dystrophies.*
METHODS. *The 50 exons of the* ABCA4 *gene have been analyzed through denaturing high performance liquid chromatography (DHPLC) and direct sequencing of samples displaying altered elution profiles.*
RESULTS. *Four new and five known mutations were identified in our patients; except for one new deletion detected in a patient with Stargardt disease, all the remaining variations are single nucleotide substitutions resulting in missense mutations.*
CONCLUSIONS. *The results further underline the genetic heterogeneity of retinal disorders, suggesting that more than one gene could differentially contribute to at least some forms of retinal degeneration. Finding a high proportion of novel mutations merits the use of scanning methodologies to analyze the whole coding region of the* ABCA4 *gene. (Eur J Ophthalmol 2007; 17: 749-54)*

KEY WORDS. ABCA4 *gene, Autosomal recessive retinitis pigmentosa, DHPLC, Mutational scanning, Recessive Stargardt disease*

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## INTRODUCTION

Hereditary retinal degenerations are a group of genetically and phenotypically heterogeneous disorders caused by mutations in a wide variety of genes (see RetNet: http://www.sph.uth.tmc.edu/Retnet). Among these, *ABCA4* (MIM#601691; GDB:370748; GenBank U88667.1; NCBI:P78363) is a retina-specific gene encoding an integral membrane glycoprotein involved in retinoid transport (1). Based on genotype-phenotype correlations, it has been proposed that the severity of visual impairment inversely relates to residual *ABCA4* activity. According to this model, different combinations of molecular defects within the *ABCA4* gene would differentially modulate the severity of eye disease: two null alleles would be responsible for autosomal recessive retinitis pigmentosa (arRP) (MIM#601718), two moderately severe alleles or one null and one mild allele would be associated with Stargardt

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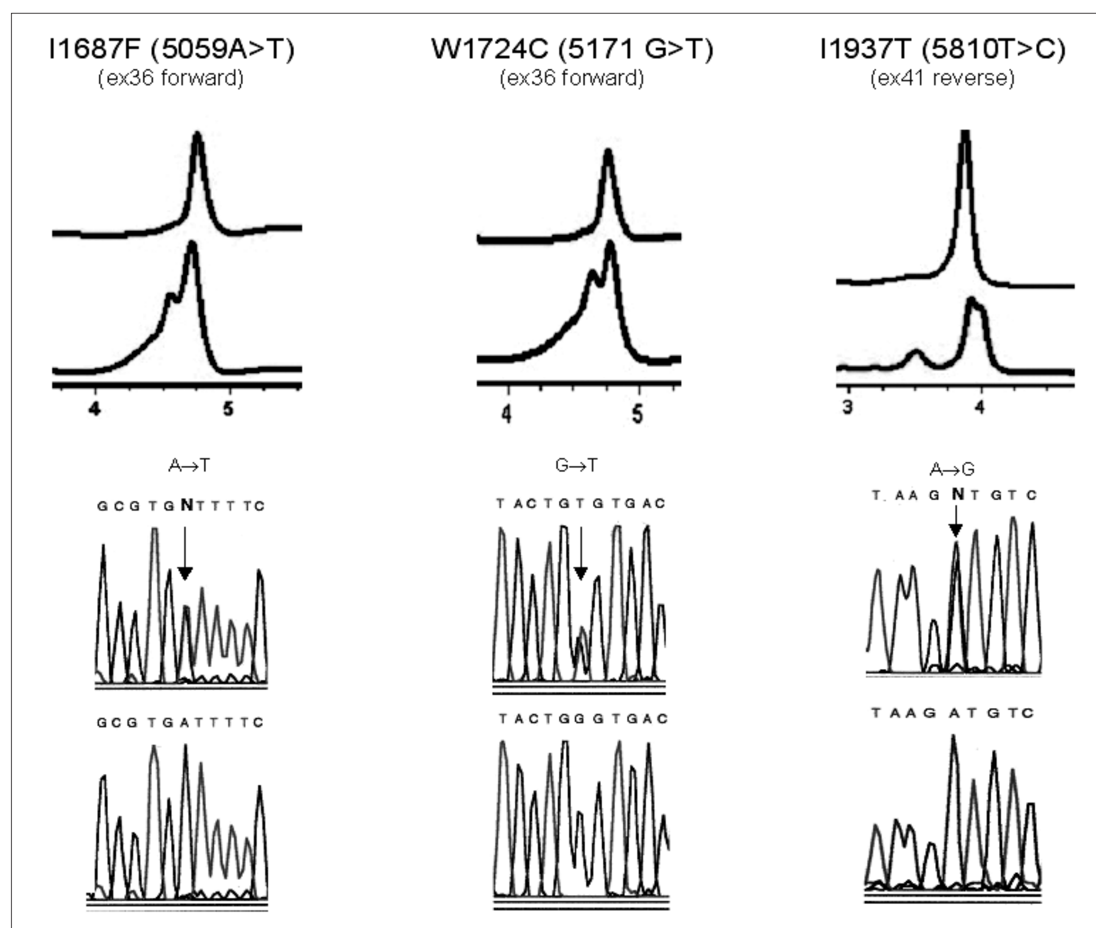


**Fig. 1** - *DHPLC profiles (upper panels) and sequence analysis (lower panels) of the novel mutations.*

disease (STGD)/fundus flavimaculatus (FFM) (MIM# 248200) phenotype, while the combination of a null and a moderately severe allele would underlie cone-rod dystrophy (CRD) (MIM#601691) phenotype (2, 3).

About 400 mutations have been identified in the retina-specific ABC transporter gene associated with different types of macular degeneration. Among these mutations, only 20 have been described in patients with arRP. In the past, we developed a highly effective protocol for mutational scanning in the *ABCA4* gene, based on DHPLC coupled with direct sequencing of the observed variants (4). We proved that this approach is highly reliable (close to 100%) in detecting sequence alterations, provided optimized temperature setting is performed by the use of natural mutated or mutagenized controls for every region to be examined.

In the present study this strategy was applied to the complete molecular scanning of the *ABCA4* gene in a cohort of patients of Spanish origin with arRP and STGD.

## MATERIALS AND METHODS

### *Patients*

A cohort of 25 patients of Spanish origin, including 23 with arRP and 2 with STGD, was recruited by Hospital de la Santa Creu i Sant Pau, Barcelona, Spain. Informed consent was obtained from all patients as approved by the local Ethical Committee.

Ophthalmologic examination included measurements of visual acuity ophthalmoscopy, central and peripheral visual fields, electroretinography, and a color vision test.

Diagnosis of STGD was based on the following criteria: 1) a recessive mode of inheritance; 2) bilateral central loss of vision with a beaten-bronze appearance and/or the presence of orange-yellow flecks in the retina from the posterior pole to the midperiphery; 3) normal caliber of the retinal vessels and no pigmented bone-spicules in the retinal periphery; and 4) normal to subnormal electroretinograms (ERGs).

**TABLE I -** PHENOTYPES AND GENOTYPES OF PATIENTS BEARING THE IDENTIFIED MUTATIONS

| No. | Phenotype | Mutation no. | Exon | Reference |
|---|---|---|---|---|
| RP4 | arRP | V1433I (4297 G>A) | 29 | (8) |
| RP5 | arRP | **I1687F (5059 A>T)** | 36 | This study |
| | | **I1937T (5810T>C)** | 41 | |
| RP10 | arRP | L1970F (5908C>T) | 43 | (19) |
| RP18 | arRP | I156V (466A>G) | 5 | (20) |
| RP25 | arRP | G863A (G2588C) | 17 | (11) |
| STGD1 | STGD | **C671del** | 6 | (5) |
| STGD2 | STGD | P1486L (4457C>T) | 30a | (8) |
| | | **W1724C (5171G>T)** | 36 | This study |

Novel mutations in bold. cDNA numbering follows the rule that +1 is A of the ATG translation start codon. NCBI accession number P78363

The hallmarks of RP are night blindness, peripheral vision loss, and RPE hyperpigmentation in the form of bone spicules that alternate with atrophic regions, attenuation of the arterioles, and waxy pallor of the optic nerve head.
The patient cohort of arRP patients was selected from a larger cohort which had been screened for other genes; only patients found to be negative for two causative mutations in these genes were included in the present evaluation; one patient who was found to carry a p.L962del mutation in the Crumbs homolog-1 (*CRB1*) gene (MIM# 604210) was also included.

### *Mutational scanning*

Complete molecular scanning of all 50 exons and flanking intron sequences of the *ABCA4* gene was performed through DHPLC and direct sequencing of samples displaying an altered elution profile.
Conditions for PCR amplification, DHPLC, and sequencing of the *ABCA4* gene have already been reported (4). None of the four new mutations detected in the present study in arRP and STGD patients was present in 60 unaffected controls (120 chromosomes).

## RESULTS

DHPLC mutational scanning of the *ABCA4* gene in 23 arRP and 2 STGD patients of Spanish origin led to the identification of nine mutations, five known and four novel ones, in a total of seven patients, including five arRP and two STGD. No mutations were found in the remaining 18 arRP patients.
The newly detected mutations comprise I1687F, W1724C, I1937T, and a 671delC mutation; this last one had been previously identified by our group and cited in a technical report with no clinical comments (5) (Fig. 1 and Tab. I).
The 671delC and W1724C alterations were independently detected in two STGD patients while I1687F and I1937T were both found in an arRP compound heterozygous patient (Tab. I).

## DISCUSSION

In previous studies on arRP, all the reported 20 *ABCA4* mutations were identified by a combination of direct screening methods, such as microarrays and/or sequencing, and more cumbersome screening protocols such as DGGE and single-strand conformational polymorphism (SSCP).
Here we report the identification, by DHPLC scanning, of nine missense mutations associated with arRP and STGD phenotypes.
The 671delC mutation is predicted to generate a truncated protein containing only 240 amino acids, which is considerably shorter than the full-length protein containing 2173 residues. Therefore, it is likely a severe mutation resulting in a nonfunctional protein (or a null allele).
No additional mutation was detected in the *ABCA4* and other genes screened in this STGD patient.
The W1724C novel mutation was also identified in a STGD patient, compound heterozygous for P1486L (Patient STG2, Tab. I). He was a 46-year-old man with alterations that were associated with FFM, characterized by late-onset (third decade) and a slow progressive course.

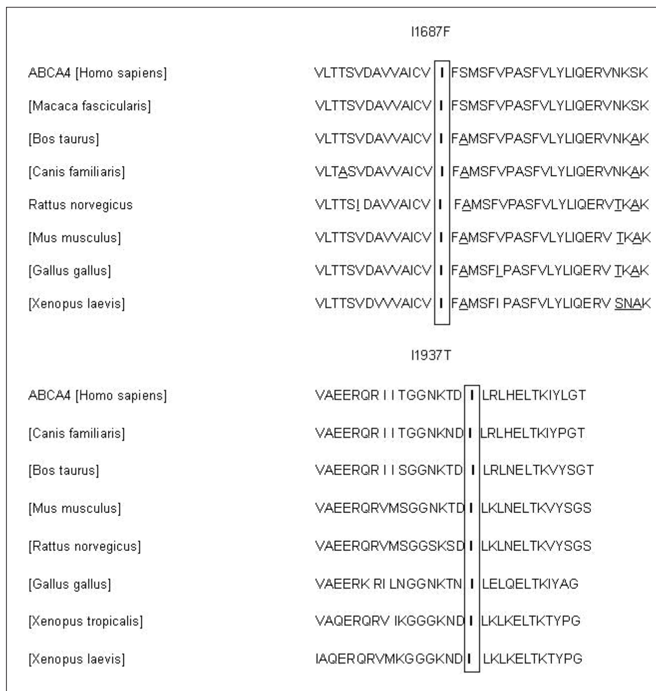


**Fig. 2 -** *Amino acidic alignment of the* **ABCA4** *protein portions encompassing the I1687 and I1937 positions in different species.*

According to a protein prediction model (6, 7) (NCBI accession number P78363), the W1724C mutation is located one base before the ninth alpha helix (H9) of the second transmembrane domain within a highly conserved DNA region, while P1486L is located in the second extracellular domain (ECD2) and had already been reported in a STGD patient (8).

Interestingly, all mutations detected in arRP patients in the present study are single nucleotide variations resulting in amino acid substitutions. This finding does not divert from the model which relates recessive RP to the presence of two null alleles but rather may help in understanding the genetic complexity underlying phenotypic variations better. Indeed, the identification of missense mutations in arRP patients had already been reported (9, 10) suggesting a refinement of the model based on the observation that some missense alleles might behave as true null allele at the functional level and may be responsible for severe impairment of the protein function (9). Accordingly, we could speculate that the missense mutations identified in the present study might also result in a severe phenotype, this hypothesis being supported by the following observations.

The two novel mutations I1687F and I1937T were both detected in a patient with arRP, an 84-year-old man who meets the standard definition of retinitis pigmentosa (Patient RP5, Tab. I). He had an onset of night blindness in his third decade and currently has only a small central island of vision and a nonrecordable electroretinogram.

I1937T is close to a L1940P mutation previously detected in a CRD-RP patient at the homozygous state (10), both mutations affecting the functionally relevant second nucleotide-binding domain (NBD 2) (6, 7). I1687F is located in the DNA region coding for the H8 second transmembrane domain (6, 7). Notably, both I1687F and I1937T mutations affect a phylogenetically highly conserved amino acid residue (Fig. 2). This, coupled with their location in critical protein domains, may support a somehow relevant role of these residues in protein function.

Concerning the already known mutations detected in our arRP patients, G863A was identified in Patient RP25 (Tab. I), displaying a moderate phenotype with slow progression of the disease. Indeed, G863A had initially been proposed as a mild defect (11) based on a genetic assumption, but a subsequent biochemical investigation in human embryonic kidney (293) cell lines showed that the protein yield and ATP-binding capacity of the 863 variant were both substantially impaired relative to wild-type (12). This might explain its involvement in arRP.

Concerning the L1970F mutation, this was identified in Patient RP10 (Tab. I), who presented the most severe phenotype among arRP phenotypes. Interestingly, L1970F affects a leucine residue which is adjacent to leucine 1971 involved in a previously reported L1971R mutation. This last one had been found to exhibit a reduced ATB-binding capacity, eliminating both basal and retinal stimulated ATP hydrolysis (12). It is reasonable to foresee that the proximity of the two leucine residues should produce the same biochemically relevant effect, accounting for the severity of patient phenotype.

The V1433I mutation, located in the extracellular loop between the H7 and H8 transmembrane domains, had been previously described in a patient with age-related macular degeneration (AMD) (MIM#153800) (8). In the present study it has been detected in a patient with early onset and severe arRP (Patient RP4, Tab. I). This patient had been previously analyzed for the whole coding region and exon boundaries of the *CRB1* gene by SSCP and direct sequencing and found to be a heterozygous carrier of a L962del mutation (13). Notably, the *CRB1* gene is involved in a form of Leber congenital amaurosis

(MIM#20400), also designated as RP12, a severe form of autosomal recessive RP characterized by a typical preservation of the para-arteriolar retinal pigmented epithelium (RPE) (14).

Thus we extended the molecular investigation to the proband family in order to assess whether the combination of the *ABCA4*, *V1433I*, and *CRB1* L962del mutations could account for the arRP phenotype. This study revealed the presence of both mutations also in a proband sister and a cousin who do not present any vision problem. This suggests that the *ABCA4* allele with the V1433I change is not pathogenic for the arRP disease while it could act as a modifier.

The remaining I156V mutation had been previously identified in a cone-rod patient (15). We have found this substitution, located in the first extracellular domain of the protein (ECD1), in Patient RP4. No additional mutation was found in this patient.

Based on the fact that the DHPLC scanning used in this study allows us to identify a high proportion of novel mutations (four out of nine), we highly recommend the use of fast methodologies allowing the screening of the complete coding regions of a specific gene.

When considering the possible pathogenicity of *ABCA4* variants in RP the probability of finding variants at random should be taken into account, since the prevalence of *ABCA4* alleles in the general population has been estimated at 1:10–1:20. Thus, one would expect to find one to two variants in 23 patients with RP as a random event. However, we found mutations in 5/23 patients with RP, including one compound heterozygote (a total of 13% of alleles), which is a significantly higher proportion than expected at random and also higher than previously reported in similar studies (16-18), reinforcing the hypothesis that genetic heterogeneity in ABCA4 locus underlies a substantial fraction of pathology in this group of retinal disorders.

Further studies are needed to elucidate the role that *ABCA4* and other genes as well as their synergic interrelations play in these disorders, based both on functional and genetic investigations.

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## REFERENCES

1. Allikmets R, Singh N, Sun H, et al. A photoreceptor cell-specific ATP-binding transporter gene (ABCR) is mutated in recessive Stargardt macular dystrophy. Nat Genet 1997; 15: 236-46.
2. Maugeri A, van Driel MA, van de Pol DJ, et al. The 2588G–>C mutation in the ABCR gene is a mild frequent founder mutation in the Western European population and allows the classification of ABCR mutations in patients with Stargardt disease. Am J Hum Genet 1999; 64: 1024-35.
3. Shroyer NF, Lewis RA, Allikmets R, et al. The rod photoreceptor ATP-binding cassette transporter gene, ABCR, and retinal disease: from monogenic to multifactorial. Vision Res 1999; 39: 2537-44.
4. Stenirri S, Fermo I, Battistella S, et al. Denaturing HPLC profiling of the ABCA4 gene for reliable detection of allelic variations. Clin Chem 2004; 50: 1336-43.
5. Cremonesi L, Stenirri S, Fermo I, et al. Denaturing HPLC analysis of DNA deletions and insertions. Hum Mutat 2003; 22: 98-102.
6. Bungert S, Molday LL, Molday RS. Membrane topology of the ATP binding cassette transporter ABCR and its relationship to ABC1 and related ABCA transporters: identification of N-linked glycosylation sites. J Biol Chem 2001; 276: 23539-46.
7. Peelman F, Labeur C, Vanloo B, et al. Characterization of the ABCA transporter subfamily: identification of prokaryotic and eukaryotic members, phylogeny and topology. J Mol Biol 2003; 325: 259-74.

8. Webster AR, Heon E, Lotery AJ, et al. An analysis of allelic variation in the ABCA4 gene. Invest Ophthalmol Vis Sci 2001; 42: 1179-89.
9. Shroyer NF, Lewis RA, Yatsenko AN, Lupski JR. Null missense ABCR (ABCA4) mutations in a family with Stargardt disease and retinitis pigmentosa. Invest Ophthalmol Vis Sci 2001; 42: 2757-61.
10. Paloma E, Coco R, Martinez-Mir A, Vilageliu L, Balcells S, Gonzalez-Duarte R. Analysis of ABCA4 in mixed Spanish families segregating different retinal dystrophies. Hum Mutat 2002; 20: 476.
11. Maugeri A, Flothmann K, Hemmrich N, et al. The ABCA4 2588G>C Stargardt mutation: single origin and increasing frequency from South-West to North-East Europe. Eur J Hum Genet 2002; 10: 197-203.
12. Sun H, Smallwood PM, Nathans J. Biochemical defects in ABCR protein variants associated with human retinopathies. Nat Genet 2000; 26: 242-6.
13. Bernal S, Calaf M, Garcia-Hoyos M, et al. Study of the involvement of the RGR, CRPB1, and CRB1 genes in the pathogenesis of autosomal recessive retinitis pigmentosa. J Med Genet 2003; 40: e89.
14. den Hollander AI, Davis J, van der Velde-Visser SD, et al. CRB1 mutation spectrum in inherited retinal dystrophies. Hum Mutat 2004; 24: 355-69.
15. Ducroq D, Rozet JM, Gerber S, et al. The ABCA4 gene in autosomal recessive cone-rod dystrophies. Am J Hum Genet 2002; 71: 1480-2.
16. Fukui T, Yamamoto S, Nakano K, et al. ABCA4 gene mutations in Japanese patients with Stargardt disease and retinitis pigmentosa. Invest Ophthalmol Vis Sci 2002; 43: 2819-24.
17. Ozgul RK, Durukan H, Turan A, Oner C, Ogus A, Farber DB. Molecular analysis of the ABCA4 gene in Turkish patients with Stargardt disease and retinitis pigmentosa. Hum Mutat 2004; 23: 523.
18. Klevering BJ, Yzer S, Rohrschneider K, et al. Microarray-based mutation analysis of the ABCA4 (ABCR) gene in autosomal recessive cone-rod dystrophy and retinitis pigmentosa. Eur J Hum Genet 2004; 12: 1024-32.
19. Rozet JM, Gerber S, Souied E, et al. Spectrum of ABCR gene mutations in autosomal recessive macular dystrophies. Eur J Hum Genet 1998; 6: 291-5.
20. Papaioannou M, Ocaka L, Bessant D, et al. An analysis of ABCR mutations in British patients with recessive retinal dystrophies. Invest Ophthalmol Vis Sci 2000; 41: 16-9.