

ABCR Gene Analysis in Familial Exudative Age-Related Macular Degeneration

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PURPOSE. Identification of genetic factors in the pathogenesis of age-related macular degeneration (AMD) is of crucial importance in this common cause of blindness. Mutations in the Stargardt disease gene (*ABCR*) were previously reported in patients with atrophic forms of AMD. The purpose of this study was to analyze familial segregation of *ABCR* gene mutations in 52 unrelated multiplex cases of exudative AMD.

METHODS. A complete ophthalmological examination including visual acuity measurement, fundus examination, and fluorescein angiography (FA) was performed on each exudative AMD patient. The entire coding sequence of the *ABCR* gene was analyzed using a combination of single-strand conformation polymorphism and confirmatory sequencing of the exons showing an abnormal pattern of migration.

RESULTS. Six heterozygous missense changes were identified. A lack of familial segregation was observed in 4 of 6 codon changes (Arg943Gln, Val1433Ile, Pro1948Leu, and Ser2255Ile). Conversely, 2 codon changes cosegregated with the disease in 2 small families: Pro940Arg and Leu1970Phe.

CONCLUSIONS. The authors believe that segregation of the *ABCR* gene mutations with familial cases of AMD has not yet been shown. The analysis of familial segregation allowed the authors to exclude 4 of 6 codon changes as disease-causing mutations. Furthermore, it was shown here that the *ABCR* gene may be rarely involved in exudative AMD, with at best 2 of 52 familial cases (4%) related to this susceptibility factor. (*Invest Ophthalmol Vis Sci.* 2000;41:244-247)

Age-related macular degeneration (AMD) is the most common cause of irreversible vision loss in the elderly population in Europe and the United States.¹⁻³ Although the etiology of AMD remains unclear, it is generally considered to be a multifactorial disease. Concordance of AMD in monozygotic twins, familial aggregation of AMD, a protective effect of the $\epsilon 4$ allele of the apoE gene in exudative AMD, and, finally, linkage to chromosome 1q in a large family give strong support for the hypothesis of a genetically determined disease triggered by environmental stimuli.⁴⁻¹³

Mutations in the Stargardt disease gene (*ABCR*) have been previously reported in patients with Stargardt disease, fundus flavimaculatus, and retinitis pigmentosa (RP19) and in some cases of cone-rod dystrophy.¹⁴⁻²⁴ In 1997, Allikmets and colleagues identified 13 mutant alleles of the *ABCR* gene in 16% (26/167) of atrophic AMD patients,¹⁹ but the relevance of *ABCR* gene mutations in AMD has been questioned.²⁵⁻²⁷ Nev-

ertheless, the hypothesis of whether some *ABCR* alleles may increase the risk of AMD has to be considered even if a simple cause-and-effect relationship is unlikely. Our purpose was to analyze the segregation of mutations in familial cases of exudative AMD, but not to realize a case-control study as already performed.^{19,25-27}

METHODS

Patients

Fifty-two unrelated French patients referred to the Eye University Clinic of Créteil for unilateral or bilateral exudative AMD due to any type of choroidal neovascularization (well-defined, occult, or vascularized pigment epithelium detachment) were included in this study. Informed consent was obtained, as required by the French bioethical legislation, in agreement with the Declaration of Helsinki for research involving human subjects. Criteria for inclusion were age of 60 years or older, diagnosis of exudative AMD in one or both eyes, absence of association with other retinal disease, and positive familial history of AMD with at least one affected member alive. A complete ophthalmological examination (including visual acuity measurement, fundus examination, and fluorescein angiography (FA), using a Topcon camera (Topcon 50IA; Tokyo, Japan), was performed on each patient. The diagnosis of exudative AMD was made by one of the 3 physicians (EHS, GC, GS), according to the guidelines of the international classification.³

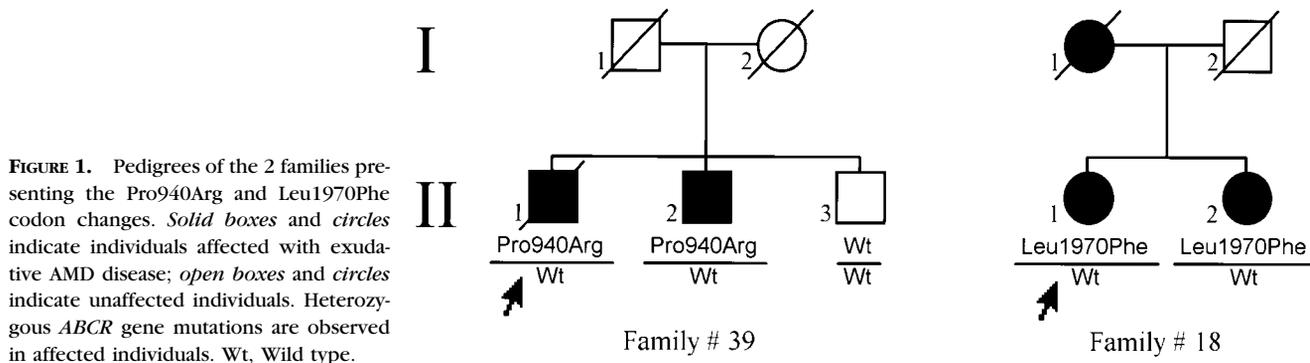
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ABCR Gene Analysis

We analyzed the entire coding sequence of the *ABCR* gene using a combination of single-strand conformation polymorphism (SSCP) and direct sequence analysis of each exon. For SSCP analysis, the entire coding region, and approximately 30 bp of intron sequence adjacent to each of the 50 exons, was analyzed, using the previously described primers.²⁸ The DNA (100 ng) from peripheral blood leukocytes was amplified using specific primers and 0.1 μ l of (α -33)d-CTP (10 mCi/ml) in a 25 μ l amplification mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTP, and 0.5 U *Taq* polymerase. The annealing temperature was adjusted for each exon. Amplified DNA (6 μ l) was mixed with an equal volume of formamide loading dye (95% formamide, 20 mM EDTA, 0.005% bromophenol blue, 0.05% xylene cyanol). The samples (5 μ l) were denatured for 10 minutes at 95°C, loaded onto a polyacrylamide gel (20 \times 45 \times 0.04 cm), and electrophoresed at 4 W for 18 hours at room temperature in 0.6 \times TBE running buffer. Gels were transferred onto Whatman paper, dried, and autoradiographed with Kodak X-OMAT film for 24 hours. Exons that displayed conformational polymorphism were sequenced on an automatic fluorometric DNA sequencer. The PCR fragments were excised from a low-melting-point gel (GTG NuSieve). Direct sequencing was performed on single-strand DNA, produced by asymmetrical PCR using the amplification primers as sequencing primers.

Familial Analysis

When examination of the siblings of our patients was not possible at our clinic, blood samples and the FA pictures were obtained. Segregation of nucleotide substitution identified in patients has been carefully studied in relatives of the AMD patients. Only alive members older than 55 years of age were analyzed. In a large family, linkage analysis has been performed using the MLINK and LINKMAP options of version 5.1 of the LINKAGE program.²⁹

Controls

The nucleotide substitutions observed in this screening were researched by SSCP analysis in a control population, obtained from 90 unrelated French individuals without any complaint of visual impairment. Our control group was not age- or sex-matched, and no ophthalmological examination was performed for these individuals.

RESULTS

Direct sequencing was performed for each abnormal SSCP profile. Six different heterozygous missense substitutions were observed in eight unrelated patients. The heterozygous codon changes observed were Pro940Arg, Arg943Gln (2 families), Pro1948Leu (2 families), Leu1970Phe, Val1433Ile, and Ser2255Ile.

The Arg943Gln, Pro1948Leu, and Ser2255Ile substitutions were observed in the control group: in 3 of 90, 3 of 90, and 1 of 90, respectively. Conversely, the Pro940Arg, Val1433Ile, and Leu1970Phe changes were not found in the control group.

No cosegregation of the base substitution with the disease was observed in the families harboring either Arg943Gln, Val1433Ile, Pro1948Leu, or Ser2255Ile changes. Indeed, the substitutions were present in some unaffected individuals and absent in some relatives affected with AMD. It is worth noting that the Pro1948Leu codon change seen in a patient belonging to a large family (13 siblings were alive and analyzed) did not segregate with the disease. Moreover, linkage analysis performed with polymorphic markers flanking the *ABCR* locus (D1S424, D1S2868, D1S236) allowed us to definitely exclude this gene as a disease-causing gene (data not shown). Conversely, a simulation test give a maximal lod score for this family equal to 2.45 at $\theta = 0$.

An association of the mutations with the disease was observed in the 2 small families harboring 2 mutations absent in controls: Pro940Arg and Leu1970Phe (Fig. 1). Clinical reports including age, age at onset of symptoms, visual acuity, fundus appearance, and FA features of all members of these 2 families are shown in Table 1.

DISCUSSION

We report here the results of *ABCR* gene screening in familial cases of exudative AMD and the segregation analysis of missense substitutions identified in patients.

Since the initial publication of Allikmets and colleagues,¹⁹ there have been several rebuttals,²⁵⁻²⁷ criticizing the initial results and demonstrating contradictory statistical data, in case-control studies. In these studies, the Arg943Gln and Ser2255Ile codon changes have been reported as polymorphisms. Here, the lack of detection of Arg943Gln, Pro1948Leu, and Ser2255Ile in controls was not particularly meaningful and did not permit us to establish statistical significance.

The use of familial cases is an important strategy in understanding the potential contribution of gene alterations with

TABLE 1. Clinical Data of Individuals from the Pedigrees with Pro940Arg and Leu1970Phe Codon Changes

Individual	Age, y	Age at Onset of Symptoms, y	Visual Acuity		Fundus Appearance and FA	Genotype
			OD	OS		
II.1, #39	77	75	CF	20/60	Well defined new vessels and PED	Pro940Arg/Wt
II.2, #39	75	70	20/100	20/200	Well defined new vessels	Pro940Arg/Wt
II.3, #39	74	None	20/20	20/20	Normal examination	Wt/Wt
II.1, #18	65	63	20/60	20/25	Occult new vessels	Leu1970Phe/Wt
II.2, #18	61	None	20/20	20/25	Large confluent drusen	Leu1970Phe/Wt

CF, count fingers; #, the number of the family.

respect to a genetically heterogeneous disorder. Certainly, if AMD is caused by specific alterations in the *ABCR* gene and if these alterations are felt to be sufficient to cause the disorder, then segregation within AMD families is a crucial prerequisite test. Analysis of the familial segregation showed 2 different sets of cases. First, a lack of segregation was observed, for the Arg943Gln, Val1433Ile, Pro1948Leu, and Ser2255Ile changes. Unaffected siblings carrying one of these codon changes were observed. It cannot be ruled out that some of these unaffected individuals might display an AMD some years later, because the risk of this multifactorial disease increases with age. However, for each of these 4 *ABCR* gene substitutions there were some siblings affected with AMD who did not carry the changes. The lack of association of an *ABCR* variant that is present in an AMD proband compared with a sibling does not totally exclude the possibility that the 2 individuals have different genetic etiologies for AMD. However, based on our findings, we support the hypothesis that the 4 missense substitutions were not disease-causing in these familial cases and could be considered as polymorphisms. It is worth noting that Val1433Ile, not observed in controls, was excluded only using segregation analysis.

Second, for Pro940Arg and Leu1970Phe we observed possible segregation with AMD. However, the families were too small to draw any definite conclusions about causality because only one generation could be analyzed. It is possible that the siblings harboring these changes were affected with AMD only by chance, regarding the high frequency of the disease. On the other hand, we can also entertain the idea that these 2 *ABCR* gene missense substitutions confer an increased risk for AMD. The Pro940Arg and the Leu1970Phe codon changes occur in codons adjacent to the first and second ATP-binding sites, respectively. However, in the absence of a functional assay for the ABCR protein, we cannot state explicitly how the mutated alleles could affect the protein. The Leu1970Phe mutation has been observed in compound heterozygous Stargardt patients so that this mutation is presumed to induce important ABCR protein alterations.¹⁸ This mutation was also previously identified in one case of AMD but not observed in the general population ($n = 220$).¹⁹ To the best of our knowledge, the Pro940Arg mutation has not been reported previously.

We selected familial cases to increase our chances of showing meaningful *ABCR* gene mutations, but at best 2 of 52 familial cases (4%) could be related to this potential susceptibility factor. Because the sensitivity of SSCP is between 80% and 90%, it is possible that a few additional mutations may have escaped detection by our SSCP screening.^{30,31} Nevertheless, it seems clear that the *ABCR* gene is

involved to only a small degree in the familial cases of exudative AMD.

In the "ABCR controversy," the main point of discussion concerns the statistical comparison of codon change frequencies between AMD and control groups. Interestingly, the analysis of segregation of *ABCR* gene mutations in familial cases of AMD appears to be an alternative way. Indeed, it allowed us to exclude 4 of 6 missense changes as disease-causing mutations. Furthermore, in contrast with atrophic forms of AMD,¹⁹ the exudative forms of AMD seem rarely to be associated with *ABCR* gene mutations. This could explain in part the contradictory data about *ABCR* variant frequencies in AMD.

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