EXTENDED REPORT

ABCA4 mutations and discordant *ABCA4* alleles in patients and siblings with bull's-eye maculopathy

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Aim: To determine the frequency and nature of mutations in the gene *ABCA4* in a cohort of patients with bull's-eye maculopathy (BEM).

Methods: A panel of 49 subjects (comprising 40 probands/families, 7 sibling pairs and a set of three sibs) with BEM, not attributable to toxic causes, was ascertained. Blood samples from each patient were used to extract genomic DNA, with subsequent mutation screening of the entire coding sequence of *ABCA4*, using single-strand conformational polymorphism (SSCP) analysis and direct sequencing.

Results: Fourteen probands (35%) were found to have a potentially disease-causing *ABCA4* sequence variant on at least one allele. Three patients had a Gly1961Glu missense mutation, the most common variant in Stargardt disease (STGD), with 2 of these subjects having a macular dystrophy (MD) phenotype and a second *ABCA4* variant previously associated with STGD. The second most common STGD mutation, Ala1038Val, was seen in one patient with cone–rod dystrophy (CORD). Five novel *ABCA4* variants were detected. Two sibships were identified with a similar intra-familial phenotype but discordant *ABCA4* variants.

Conclusions: Variations in the *ABCA4* gene are common in BEM. Two sibships showed discordant *ABCA4* variants. One of these sibships illustrates that *ABCA4* variants can be identified in families that have another molecular cause for their disease, due to the high prevalence of *ABCA4* disease alleles in the population. The discordance evident in the second sibship may yet also be a chance finding in families with macular disease of another genetic cause, or it may represent a complex mode of inheritance determined/modified by the combination of *ABCA4* alleles.

The term bull's-eye maculopathy (BEM) was first introduced to describe the characteristic appearance of chloroquine retinopathy.[1] Bull's-eye lesions have since been reported in cone dystrophy (COD) and cone–rod dystrophy (CORD),[2] rod–cone dystrophy (RCD),[3] and several macular dystrophy phenotypes including benign concentric annular macular dystrophy,[4] fenestrated sheen macular dystrophy[5 6] and MCDR2.[7] The pathogenesis of BEM is poorly understood. The characteristic appearance in which there is annular retinal pigment epithelium (RPE) disturbance and central sparing may correspond to the pattern of lipofuscin accumulation in the RPE, which in healthy individuals is highest at the posterior pole but shows a depression at the fovea.[8 9] The initially spared centre in BEM usually becomes involved as the disease advances.

We have previously reported the nature and degree of phenotypic variation in a large panel of BEM patients.[10] Autofluorescence (AF) imaging findings were used to subclassify subjects into three distinct groups: (1) a ring of increased AF surrounding decreased foveal AF, (2) decreased foveal AF only and (3) a speckled AF pattern.[10 11] Patients were also classified as having macular dystrophy (MD), CORD, RCD or COD, on the basis of detailed electrophysiological testing.[10]

ABCA4 encodes a transmembrane rim protein located in the discs of rod and cone outer segments that is involved in ATP-dependent transport of retinoids from photoreceptor to RPE.[12–14] Failure of this transport results in deposition of a major lipofuscin fluorophore, A2E (*N*-retinylidene-*N*-retinylethanolamine), in the RPE.[14] It is proposed that this accumulation may be deleterious to the RPE, with consequent secondary photoreceptor degeneration.[15–17] Recessive mutations in *ABCA4* have been identified in Stargardt disease (STGD) and fundus flavimaculatus (FFM),[18] RCD[19] and CORD.[20] Since these phenotypes can be associated with BEM, our panel has been screened to determine both the frequency and nature of mutations in the gene *ABCA4* in a cohort of patients with BEM lacking other ophthalmoscopic features of STGD/FFM.

PATIENTS AND METHODS

Patients

A panel of 49 patients (40 families) with BEM, and in whom an acquired toxic aetiology could be excluded, was ascertained. The panel included 8 sibships; 7 sibling pairs and 1 set of three sibs. After informed consent was obtained, blood samples were taken from all individuals for DNA extraction and mutation screening of *ABCA4*. The protocol of the study adhered to the provisions of the Declaration of Helsinki and was approved by the Ethics Committee of Moorfields Eye Hospital.

Methods

Blood samples from each patient were used to extract total genomic DNA using a Nucleon® Biosciences kit. The entire coding sequence (50 exons), including exon–intron boundaries, of the *ABCA4* gene of each patient was screened using single-stranded conformational polymorphism (SSCP) analysis and direct sequencing. Direct sequencing of PCR products was undertaken on an ABI 3100 Genetic Analyser (Applied Biosystems, Foster City, CA), using previously published primer sequences and conditions, in both the PCR and sequencing reactions.[18]

Abbreviations: AF, autofluorescence; BEM, bull's-eye maculopathy; COD, cone dystrophy; CORD, cone–rod dystrophy; FFM, fundus flavimaculatus; MD, macular dystrophy; RCD, rod–cone dystrophy; RPE, retinal pigment epithelium; SSCP, single-strand conformational polymorphism; STGD, Stargardt disease

Subsequently, in the two sets of siblings with concordant phenotypes, but different *ABCA4* variants, genotyping was carried out with a CA repeat marker within the coding sequence of *ABCA4*. In the family from Uganda, the three siblings were genotyped (Cases 1A, 1B and 1C); blood samples were not available from their parents or other family members. In the British family, genotyping was undertaken in the 2 affected brothers (Case 10 and his brother), 2 of their unaffected siblings and both parents. Furthermore, segregation of the identified heterozygous *ABCA4* mutation (Ala1038Val) was investigated in this British pedigree.

Genotyping was carried out by utilising a dinucleotide repeat marker that was designed inhouse, HCAREP (HCAREPF: ttctgtcaaagaaccggaaga and HCAREPR: ctggcagtgctgtcagttgt), with the forward PCR primer being fluorescently labelled. PCR reactions were carried in a 25-μl reaction volume, containing 125 ng of DNA, 1× NH_4 buffer (Bioline^{TM}), 1 mM $MgCl_2$, 200 μM each dNTP, 2.50 pmol each of forward and reverse primer and 1U Bio*Taq*. The thermocycling profile used consisted of an initial denaturation of 4 min at 95℃, immediately followed by 35 cycles of 95℃ for 15 s, 61℃ for 30 s and 72℃ for 30 s, with a single final extension step of 72℃ for 5 min. PCR products were diluted and denatured in formamide and size-fractionated using an ABI 3100 Genetic Analyser. PCR products were automatically sized by the 3100 Data Collection Software version 1.0.1 program using ROX as the size standard and scored using the GeneMapper version 2.0 program.

RESULTS

Subjects were divided according to AF imaging findings and electrophysiological assessment (table 1). Patients in the panel were identified as having 1 of 3 AF imaging patterns: (1) a ring of increased AF surrounding decreased foveal AF, (2) decreased foveal AF only, and (3) a speckled AF pattern. Patients were also classified as having MD, CORD, RCD or COD, on the basis of detailed electrophysiological testing. All 3 AF patterns and all 4 electrophysiological phenotypes were found to be associated with *ABCA4* variants, with MD being the most common (64%) (table 1).

Fourteen probands (35%) were found to have a potentially disease-causing *ABCA4* sequence variant on at least one allele (table 1). In 4 of these subjects (29%), both mutations were identified (Gly1961Glu/Cys1490Tyr, Gly1961Glu/Asn965Ser, IVS38-10T>C/Cys2150Tyr, Gln2238Stop/Gly1961Glu); with the second allele not being characterised in the remaining 10 patients. It is likely that IVS38-10T>C represents a non-disease-causing variant, although it is believed to label an ABCA4 disease-associated allele.[21] Sixteen sequence variants were identified in the panel, consisting of 13 missense variants, 2 nonsense mutations and 1 splice-site variant. Three patients had a Gly1961Glu missense mutation, the most common variant in STGD, with 2 of these subjects having a macular dystrophy phenotype and a second *ABCA4* variant previously associated with Stargardt disease (fig 1). The second most common STGD mutation, Ala1038Val, was seen in one patient with CORD. Five previously unreported *ABCA4* variants were detected; three missense mutations (Val552Ile; (GTA>ATA), Ala538Asp; (GCC>GAC), Arg508Cys; (CGG>TGC) and two truncating nonsense variants (Gln2238Stop; (CAG>TAG), and Leu661 ins1 ctG; (Stop (TAG) at codon 765).

Two sets of siblings with concordant phenotypes but different *ABCA4* variants were identified (fig 2). The first set of siblings originated from Uganda, comprising two sisters and a brother, with the brother (fig 3) and younger sister being heterozygous for the missense mutation Leu1201Arg, and the eldest sister harbouring a heterozygous 1bp insertion in codon 661 (fig 4). Their parents have been unavailable for clinical examination and molecular genetic testing; however, both mother and father are reported to be entirely asymptomatic with good vision at ages 61 and 80 years, respectively. Genotyping findings (fig 4) are consistent with the sequencing data that identified 2 siblings harbouring the Leu1201Arg mutation (on allele 4) and the third sibling having a different 1-bp insertion variant (on allele 6). Forensic markers have been used to demonstrate that these individuals are full siblings (data not shown, Ed Stone). This intra-familial discordance of *ABCA4* variants may be a chance finding in families with macular disease of another cause or may represent a complex mode of inheritance determined/modified by the combination of *ABCA4* alleles.

The second set of siblings with concordant phenotypes but different *ABCA4* variants are 2 British brothers, with the elder brother being heterozygous for the common substitution Ala1038Val, but the younger brother was not found to harbour an *ABCA4* variant (fig 5). Their parents were examined clinically and were found to have normal vision and fundus appearance. In contrast to the Ugandan family, the presence of parental segregation data and genotyping findings (fig 5) suggest that *ABCA4* is not the disease-causing gene in this family. The affected brothers share no alleles by descent, from the intronic CA repeat marker segregation analysis. Allele 3 identifies the proposed disease-causing maternal chromosome harbouring the mutation Ala1038Val, and only one affected son has inherited this maternal chromosome. X linked inheritance remains a possibility in this family.

DISCUSSION

We have demonstrated that variations in the *ABCA4* gene are common in a large carefully ascertained panel of patients with bull's-eye maculopathy, with a third of patients harbouring potentially disease-causing mutations. This finding also suggests that BEM is genetically heterogeneous, in keeping with other clinically defined inherited retinal disorders, including COD, CORD, RCD and ARMD.[2,28] While heterogeneity diminishes the value of a negative result following mutation screening, it does not lessen the value of a positive result.

Two sibships (two British brothers, and two Ugandan sisters and a brother) showed discordant *ABCA4* variants, despite a highly concordant intrafamilial phenotype. The British sibship illustrates that *ABCA4* variants can be identified in families that have another molecular cause for their disease. This is primarily due to the high prevalence of disease-associated alleles in the population (~1 in 50) and the polymorphic nature of the gene, thereby suggesting that caution should be exercised when counselling families. Furthermore, this family also demonstrates that the Ala1038Val variant, when not co-inherited with Leu541Pro, may not label a disease chromosome. In the Ugandan family, this discordance may be an epiphenomenon, in families with macular disease of another cause, or may represent a complex mode of inheritance determined by the combination of *ABCA4* alleles. It is possible that digenic inheritance may prove to explain the observations in this sibship, as is seen in an unusual form of retinitis pigmentosa, in which mutations of the *peripherin/RDS* gene and *ROM1* (rod outer segment protein 1) gene are present within the same family.[22] Individuals with a mutation of one gene but not the other are clinically unaffected. Affected individuals are double heterozygotes, with mutations of both *ROM1* and *peripherin/RDS*.

Five novel *ABCA4* variants have been identified; three missense mutations and two truncating nonsense variants. The presence of the STGD-associated Gly1961Glu variant in three BEM patients may suggest that both disorders share a common molecular pathology, but that the macular appearances

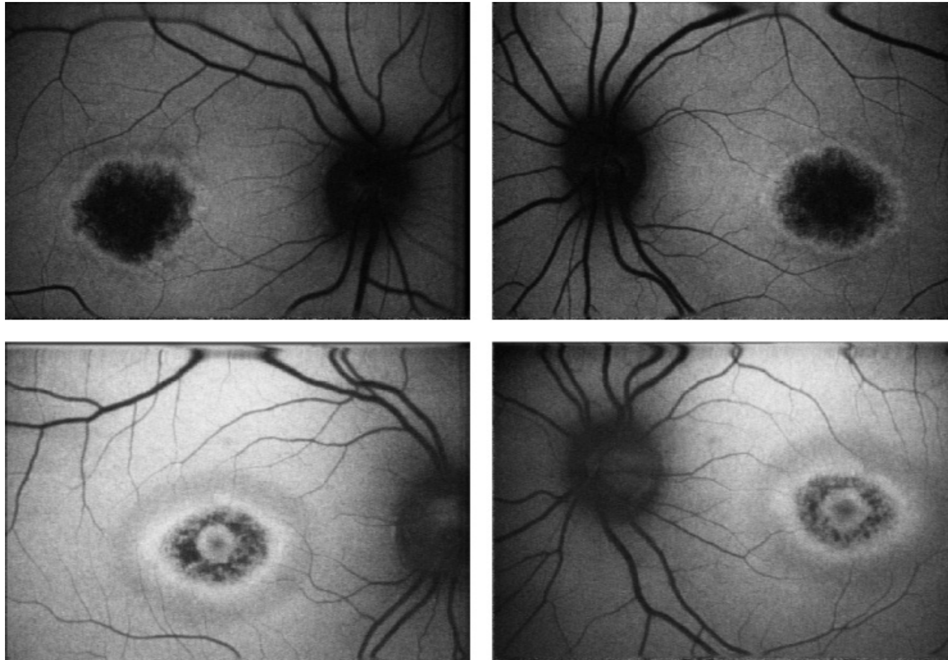

Figure 1 Cases 4 and 6 both have a MD phenotype and harbour the missense mutation Gly1961Glu, the commonest *ABCA4* variant identified in STGD, and an additional previously identified *ABCA4* change (Cys1490Tyr and Asn965Ser, respectively).

are modified by other genetic or environmental factors. The characterisation of these modifying factors represents an important challenge in retinal genetics, for, while our understanding of the underlying molecular basis of inherited retinal disorders has improved dramatically over the last decade, these insights have been accompanied by a growing realisation of the complexity of retinal disease. It is increasingly recognised that different mutations within the same gene may produce different clinical phenotypes (phenotypic heterogeneity). However, the same mutation in different individuals, even within the same family, may also produce different clinical consequences (clinical heterogeneity). Furthermore, mutations in different genes may cause the same retinal disease (genetic heterogeneity); with many different disease-causing mutations often identified in these genes (allelic heterogeneity).

ABCA4 encodes a transmembrane rim protein (an outwardly directed flippase of all-*trans* retinal), located in the discs of rod and cone outer segments, that is involved in ATP-dependent transport of retinoids from photoreceptor to RPE during the visual cycle.[12–14] Recessive mutations in *ABCA4* have been

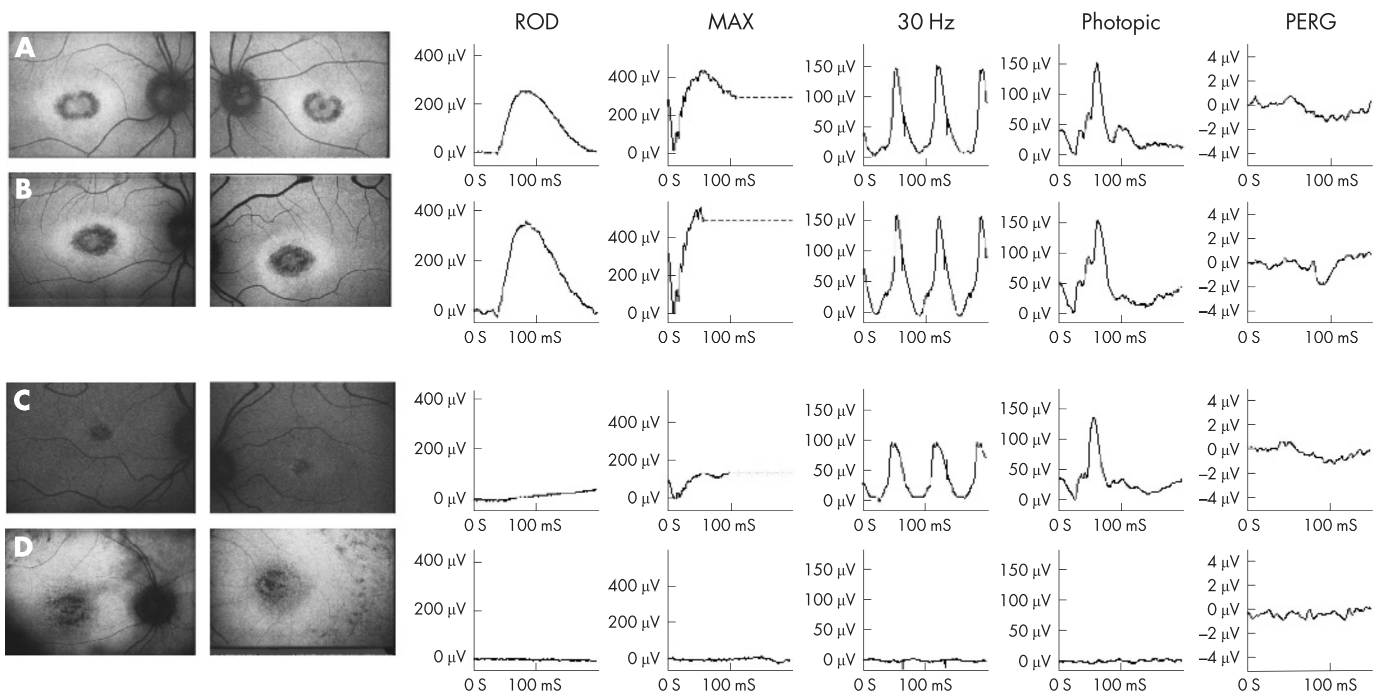

Figure 2 Two sets of siblings with concordant phenotypes but different *ABCA4* variants were identified. Above: A (Case 1A, 33 years old) and B (Case 1B, 24 years old): two sisters from Uganda with a group 1 AF pattern and MD phenotype with different *ABCA4* variants. Case 1A is heterozygous for 1 bp ins codon 661, while Case 1B and her affected brother (Case 1C, fig 3) are both heterozygous for Leu1201Arg. Below: C (Case 10, 15 years old) and D (brother of Case 10, 25 years old): Two British brothers, with a group 2 AF pattern and CORD phenotype. Case 10 harbours the common Ala1308Val mutation as a heterozygous change, while no *ABCA4* variants were identified in his brother.

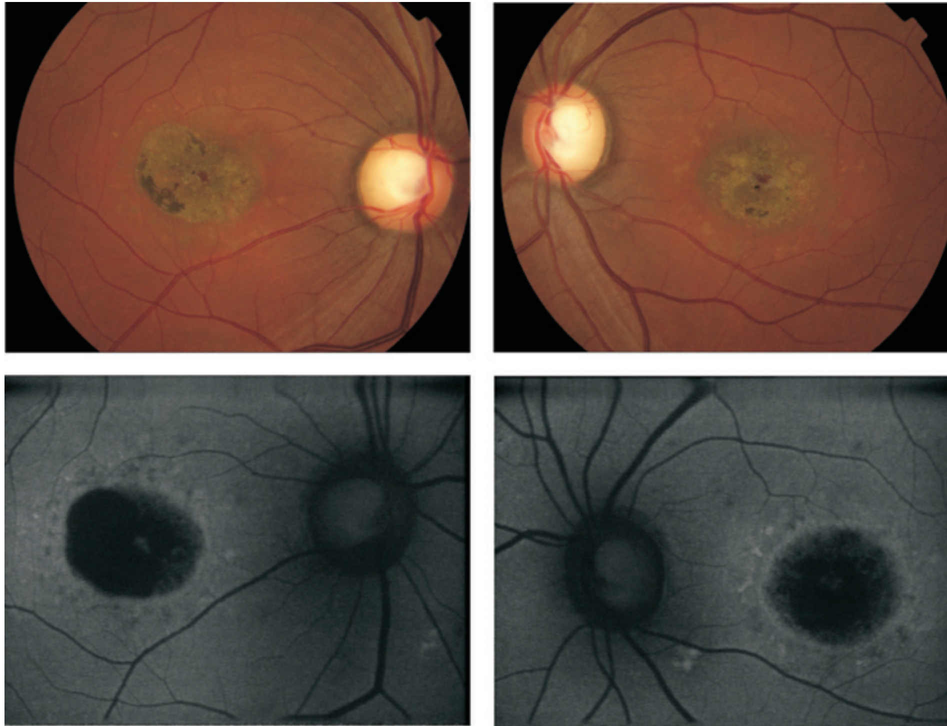

Figure 3 Case 1C (35 years old) with a group 1 AF pattern and MD phenotype, harbouring the Leu1201Arg missense mutation as a heterozygous change.

identified in STGD/FFM,[18] RCD[19] and CORD.[20] It is currently believed that: (1) homozygous null mutations cause the most severe phenotype of autosomal recessive retinitis pigmentosa (RP, RCD), (2) combinations of a null mutation with a moderate missense mutation result in autosomal recessive CORD and (3) combinations of null/mild missense or two moderate missense mutations cause STGD/FFM.[23]

The high allelic heterogeneity of *ABCA4* is clearly demonstrated by the fact that approximately 500 sequence variations in this gene have been reported. This highlights the potential difficulties in definitively assigning disease-causing status to sequence variants detected when screening such a large (50 exons) and polymorphic gene. Nonsense mutations that can be predicted to have a major effect on the encoded protein can be confidently predicted to be disease-causing. However, a major problem occurs with missense mutations, since sequence variants are common in controls (carrier frequency 1 in 50), and therefore establishing pathogenicity may be problematic. Large studies assessing whether particular sequence variants are statistically more frequently seen in STGD patients than controls are thereby likely to be helpful.[21] Direct evidence of pathogenicity can be established by functional analysis of the encoded mutant ABCA4 transporter protein, with either severely reduced ATPase activity associated with many variants, including Gly1961Glu,[24] or protein mislocalisation with retention of mutant ABCA4 in the photoreceptor inner segment,

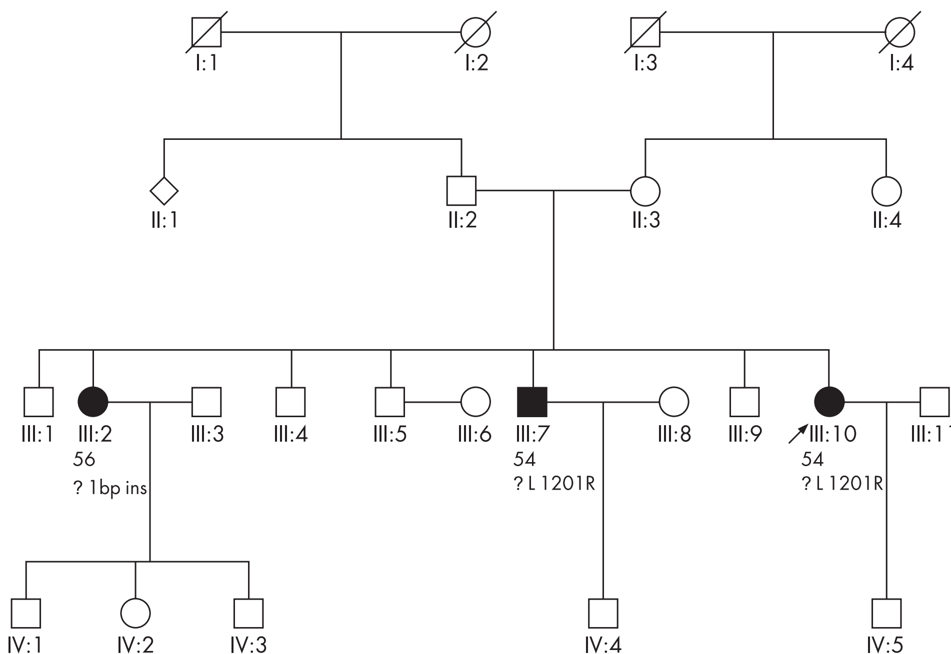

Figure 4 Four-generation pedigree of a Ugandan family with bull's-eye maculopathy. The alleles present for the dinucleotide repeat marker, HCAREP, and *ABCA4* mutations identified in the three siblings available for examination, are both shown.

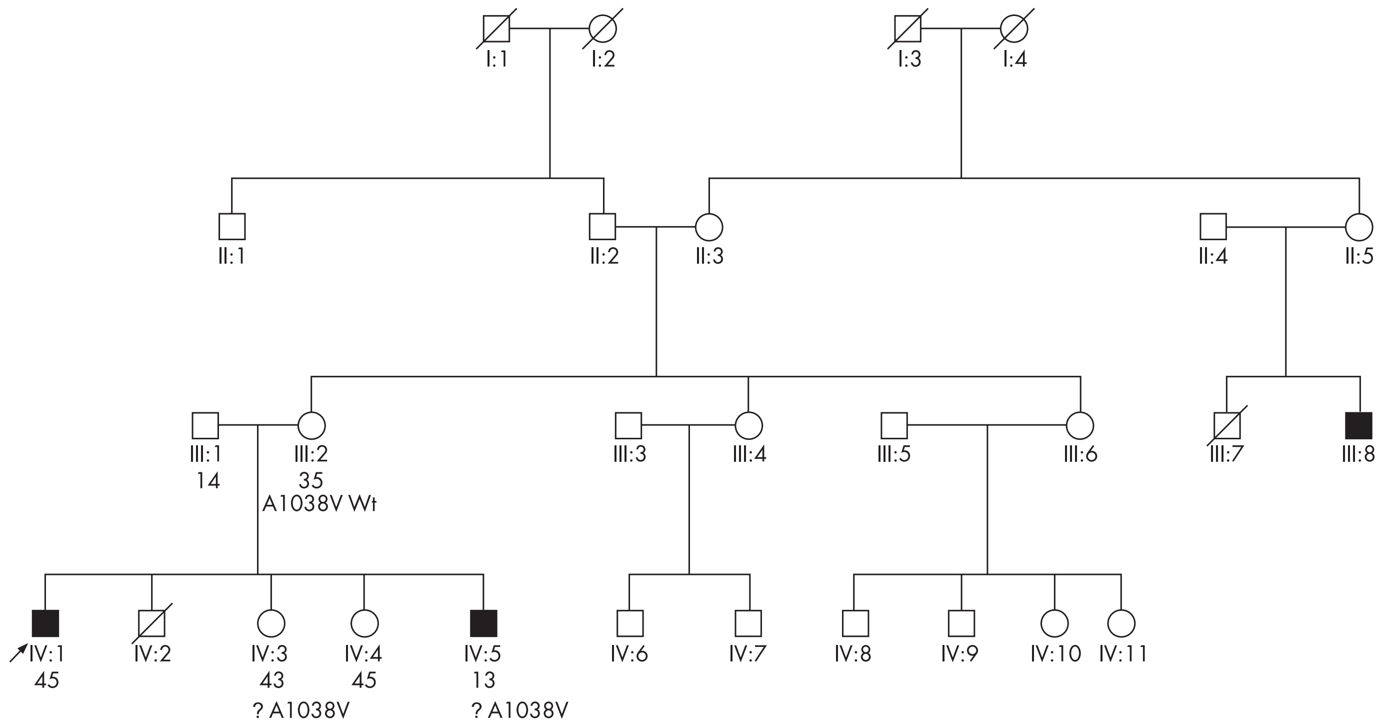

Figure 5 Four-generation pedigree of a British family with bull's-eye maculopathy. The alleles present for the dinucleotide repeat marker, HCAREP, and the segregation of the *ABCA4* mutation, Ala1038Val (A1038V), in the six subjects available for examination, are both shown.

Table 1 ABCA4 variants identified in BEM (Cases 1A to 1C are siblings)

Cases	Age	Visual acuity OD OS	Autofluorescence imaging pattern	Electrophysiological phenotype	*ABCA4* variants
1A	33	6/12 6/12	1	MD	Leu661 ins1 ctG
1B	24	6/36 6/36	1	MD	Leu1201Arg
1C	35	6/60 6/60	1	MD	Leu1201Arg
2	15	6/36 6/36	1	MD	Gly1961Glu Cys1490Tyr
3	39	6/6 6/6	1	RCD	Val552Ile
4	31	6/9 6/9	1	MD	Gly1961Glu Asn965Ser
5	13	6/24 6/24	1	COD	IVS38-10T>C Cys2150Tyr
6	42	6/60 3/60	1	MD	Gln2238Stop Gly1961Glu
7	43	6/12 6/12	2	CORD	Gly991Arg
8	15	6/6 6/9	2	CORD	Ala1038Val
9	66	3/60 3/60	2	CORD	Gly1961Glu
10	40	6/12 6/60	2	MD	Val1433Ile
11	66	6/60 6/60	2	MD	Pro940Arg
12	52	6/9 6/9	3	MD	Arg508Cys
13	41	6/36 6/36	3	MD	Thr1253Met
14	28	6/12 6/18	3	MD	Ala538Asp

identified in several ABCA4 mutants, including those harbouring the Ala1038Val substitution.[25] The availability of multiple independent families with the same mutation may also provide evidence in support of disease causation.

Failure of the ABCA4 transporter protein results in deposition of a major lipofuscin fluorophore, A2E, in the RPE.[14] It is proposed that this A2E accumulation may be deleterious to the RPE via the generation of DNA-damaging epoxides,[15–17] with consequent secondary photoreceptor degeneration. The antioxidants vitamins E and C have been shown to reduce A2E epoxidation, with a corresponding reduction in DNA damage and cell death.[15] Studies with the *abca4*$^{-/-}$ knock-out mouse have established two further potential strategies of reducing A2E-related toxicity by inhibiting the formation of such lipofuscin pigments; the first being to reduce light exposure (suggesting that wearing dark tinted spectacles may be beneficial),[26] and the second via the use of the pharmacological agent isotretinoin (13-*cis*-retinoic acid).[27]

We have shown that screening of *ABCA4* should be considered in patients with BEM, since a third of subjects are likely to harbour potentially disease-causing mutations in this increasingly well-characterised gene and its protein product. The identification of subjects with *ABCA4* mutations may prompt the clinician to consider counselling the patient regarding the potential benefits of avoidance of excessive light exposure. Furthermore, a molecular genetic diagnosis will allow pharmacological or gene-directed therapies, likely to become available in the near future, to be offered to appropriate patients.

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