Evaluation of the G protein coupled receptor-75 (GPR75) in age related macular degeneration

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Abstract

Background—A long term project was initiated to identify and to characterise genes that are expressed exclusively or preferentially in the retina as candidates for a genetic susceptibility to age related macular degeneration (AMD). A transcript represented by a cluster of five human expressed sequence tags (ESTs) derived exclusively from retinal cDNA libraries was identified.

Methods—Northern blot and RT-PCR analyses confirmed preferential retinal expression of the gene, which encodes a G protein coupled receptor, GPR75. Following isolation of the full length cDNA and determination of the genomic organisation, the coding sequence of GPR75 was screened for mutations in 535 AMD patients and 252 controls from Germany, the United States, and Italy. Employed methods included single stranded conformational polymorphism (SSCP) analysis, denaturing high performance liquid chromatography (DHPLC), and direct sequencing.

Results—Nine different sequence variations were identified in patients and control individuals. Three of these (–30A>C, 150G>A, and 346G>A) likely represent polymorphic variants. Each of six alterations (–4G>A, N78K, P99L, S108T, T135P, and Q234X) were found once in single AMD patients and were considered functional variants that could affect the protein function and potentially cause retinal pathology.

Conclusion—The presence of six potential pathogenic variants in a cohort of 535 AMD patients alone does not provide statistically significant evidence for the association of sequence variation in GPR75 with genetic predisposition to AMD. However, a possible connection between the variants and age related retinal pathology cannot be discarded. Functional studies are needed to clarify the role of GPR75 in retinal physiology.

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One challenge in molecular genetic research today involves the discovery and characterisation of genes that underlie common disorders with complex patterns of inheritance. In ophthalmic genetics, such a challenge is posed by age related macular degeneration (AMD), which represents the most common cause of visual impairment in the population over 65 years of age.1,2 The disorder is characterised by retinal drusen, abnormal pigmentation of the retinal pigment epithelium (RPE), and geographic atrophy and/or choroidal neovascularisation in association with advancing age.3 Numerous environmental contributors such as sun exposure,4 hypertension,5 cigarette smoking,6,7 and dietary factors8,9 have been suggested; however, genetic predisposition appears to be the most important risk factor.10,11 Elucidation of the genetic components will lead to a better understanding of the pathophysiology of AMD and, in turn, may result in the development of novel preventive therapies.

Application of conventional gene identification approaches, such as positional cloning or positional candidate gene analysis, to identify genetic factors conferring susceptibility to AMD has been hampered by the considerable clinical heterogeneity of the condition even in the same family, by the scarcity of families with several living affected members due to the late age at onset, and by the lack of penetrance in younger members of families in which older individuals may manifest the trait. Thus, most studies to date have been limited to screening AMD patients for genetic variation in genes implicated in monogenic retinopathies with similar phenotypes (for a review, see Yates and Moore12). Thus far, no associations have been found between AMD and the genes underlying several dominant Mendelian retinal disorders with either atrophic or disciform macular degeneration as a prominent feature: Sorsby fundus dystrophy,13 Best vitelliform macular dystrophy,14 and Doyne honeycomb retinal dystrophy/Malattia Leventinese.15 Screening of the photoreceptor specific ATP binding cassette transporter gene, ABCA4 (formerly ABCR), responsible for Stargardt disease,16 has revealed statistically significant association of at least two heterozygous mutations in ABCA4 with AMD.17,18 The extent of the contribution of these and other ABCA4 variants to AMD remains to be determined; however, AMD associated genetic variation in ABCA4 has been documented in a fraction of AMD patients, suggesting the contributions of other yet unidentified genes.19,20

We have initiated a long term project to characterise systematically a pool of genes that can be assessed for their role in predisposition to AMD.21 Reasoning that good candidates are those genes that are exclusively or preferentially expressed in the human retina, we searched the Unigene gene indexing database...
Oligonucleotide primers used for analysing the GPR75 gene

<table>
<thead>
<tr>
<th>Amplicon</th>
<th>Primer name*</th>
<th>Sequence</th>
<th>Size (bp)</th>
<th>Restriction enzyme (sizes in bp)</th>
<th>$T_m$(°C)</th>
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<tbody>
<tr>
<td>1</td>
<td>91.R7</td>
<td>5'-TAATCCCTTCTTGTCTTCTGG-3'</td>
<td>348</td>
<td>Hae III (180+138)</td>
<td>59</td>
</tr>
<tr>
<td>2</td>
<td>91.S4</td>
<td>5'-AGGACAAGAAGACAGACAGAAG-3'</td>
<td>316</td>
<td>Mnl I (172+131)</td>
<td>56</td>
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<tr>
<td>3</td>
<td>91.R1</td>
<td>5'-ATCTCTATCCACACAGCAG-3'</td>
<td>327</td>
<td>Mnl I (257+182)</td>
<td>60</td>
</tr>
<tr>
<td>4</td>
<td>91.S5</td>
<td>5'-GTGCTGCTGGTTGGAGGAGGTGAGAT-3'</td>
<td>303</td>
<td>Nla III (201+147)</td>
<td>53</td>
</tr>
<tr>
<td>5</td>
<td>91.R2</td>
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<td>349</td>
<td>Acc I (257+182)</td>
<td>60</td>
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<tr>
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<td>91.S6</td>
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<td>337</td>
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<td>56</td>
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<tr>
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<td>303</td>
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<tr>
<td>8</td>
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<td>327</td>
<td>Nla III (201+147)</td>
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<tr>
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<td>337</td>
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<td>56</td>
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*PCR conditions were 94°C, 5 minutes; 30 cycles of 94°C, 30 seconds, TA, 30 seconds, 72°C, 30 seconds; 72°C, 5 minutes.

for clusters composed of ESTs derived entirely or predominantly from retinal cDNA libraries, and we determined their expression profile in vitro. We identified an EST cluster Hs.40763 that contains five retina derived ESTs and confirmed by northern blot and reverse transcription (RT) PCR analysis that it is expressed specifically in retina and brain. Assembly and subsequent analysis of the full length transcript confirmed by northern blot and reverse transcription (RT) PCR analysis that it is expressed specifically in retina and brain. Assembly and subsequent analysis of the full length transcript confirmed by northern blot and reverse transcription (RT) PCR analysis that it is expressed specifically in retina and brain.

Materials and methods
CDNA cloning and determination of gene structure
A cluster of five EST sequences derived from three different retinal cDNA clones (IMAGE clone nos 222124, 363848, 363252) was retrieved under accession number Hs.40763 as part of a search under the term “eye” in the Unigene Human Sequences Collection (http://www.ncbi.nlm.nih.gov/Unigene). A cDNA IMAGE clone 222124 was obtained from the UKHGMP Resource Centre (Hinxton, Cambridge, UK), and the complete DNA sequence of the insert was determined with the Thermosequenase radiolabelled terminator cycle sequencing kit (Amersham, Life Science). To extend the resulting sequence to the transcription start site, 5′-RACE was performed on total human retinal RNA with the 5′-RACE kit, Version 2 (Gibco BRL). First strand cDNA synthesis with primer 91.S8 (Table 1) was followed by two subsequent PCR reactions with nested primers 91.S4 (Table 1) and 91.S5 (5′-TGC TCC CCA AAA ATA CTC AG-3′). The resulting PCR products were separated electrophoretically on an agarose gel, excised, purified with the QIAxII Gel Extraction Kit (Qiagen), and sequenced directly. To obtain the genomic sequence, we screened the RPCI-1 PAC library (kindly provided by P de Jong, Roswell Park Cancer Institute, Buffalo, NY) with a radiolabelled 2057 bp cDNA insert from IMAGE clone 222124. Positive PAC clone dJ302H14 was partially sequenced and the genomic, and cDNA sequences were aligned with MacVector Sequence analysis program Version 4.1.4 (Kodak) to determine the exon-intron structure (Fig 1). We subjected the deduced protein sequence to homology searches with the BLASTP program (http://www.ncbi.nlm.nih.gov/BLAST/) and used the TMHMM program of the Husar package (http://www.genome.dkfz-heidelberg.de/) for prediction of the transmembrane domains.

EXPRESSION ANALYSES
RT-PCR was performed as described previously. Briefly, total RNA was extracted from human liver, lung, uterus, cerebellum, brain stem, prostate, retinal pigment epithelium, and retina tissue with the RNA Clean system (Hybaid). After treatment with the DNA-free kit (Ambion) to remove residual genomic DNA, 1–2 µl volumes of each preparation and 1–2 µl volumes of commercially obtained total RNA from heart, kidney, placenta, and thymus (Ambion) were reverse transcribed with the Superscript preamplification system (Gibco BRL), diluted 1:5, and subjected to PCR with oligonucleotide primers 91.S4 (Table 1) and 91.R13 (5′-GCT CCG GAC TGC GAG ATG-3′) resulting in a 357 bp product. To normalise for variations in the amount of input RNA and synthesised cDNA from each tissue, control PCR reactions were conducted on 2 µl
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INTERNATIONAL CONSORTIUM

A total of 198 German patients and 101 matched controls were screened for mutations with single stranded conformational polymorphism (SSCP) analysis. PCR amplification was carried out with Taq-Polymerase (Life Technologies) in a 25 µl volume with 1 × PCR buffer supplied by the manufacturer. To increase sensitivity of the SSCP analysis, the PCR products were digested with appropriate restriction enzymes to yield fragments <260 bp (Table 1). Radiolabelled PCR products were separated electrophoretically on a 6% non-denaturing polyacrylamide gel with or without 5% glycerol at 4°C. DNA fragments displaying mobility shifts were sequenced directly.

In all, 241 US and 96 Italian patients, and 112 US and 39 Italian controls were screened by DHPLC. PCR samples were separated on a DNASep column (Transgenomic, Omaha, NE, USA) on a Helix HPLC system (Varian Instruments, Walnut Creek, CA, USA) with the standard program supplied by the manufacturer (Varian).

Exon trapping

The effect of the 150G>A transition on RNA splicing was evaluated in an exon trapping system as described by Rivera et al.22 Briefly, a 333 bp exonic fragment encompassing this alteration was PCR amplified from the DNA of German AMD patient No 107 with oligonucleotide primers R1-EcoRI-F (5'-GAT GTA CTA GAC GAA AGC CTA TCA TCT TC-3') and S8-BamHI-R (5'-GGA TCT GGT AGG AAG GAA ACA AAG-3') were used for filter hybridisations in a 6% non-denaturing polyacrylamide gel with or without 5% glycerol at 4°C. DNA fragments displaying mobility shifts were sequenced directly.

Statistical analysis

To test statistically whether there is a relation between two categorical variables the Fisher’s exact test was used (http://www.matforsk.no/ola/fisher.htm). The sample size required to internationally accepted criteria; an approximately equal ratio of exudative to atrophic AMD is represented in the patient group. Most patients and controls have been studied in the VMD216 and ABCA4 genes.
Table 2  Sequence alterations in GPR75 detected in 535 AMD patients and 252 controls

<table>
<thead>
<tr>
<th>Amplicon</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
<th>AMD alleles (GER) (n=396)</th>
<th>Control alleles (GER) (n=202)</th>
<th>AMD alleles (USA) (n=482)</th>
<th>Control alleles (USA) (n=224)</th>
<th>AMD alleles (ITA) (n=192)</th>
<th>Control alleles (ITA) (n=78)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>−30A&gt;C</td>
<td>—</td>
<td>27 (6.8%)</td>
<td>14 (6.9%)</td>
<td>44 (9.1%)</td>
<td>13 (5.8%)</td>
<td>14 (7.3%)</td>
<td>5 (6.4%)</td>
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<tr>
<td>2</td>
<td>−4G&gt;A</td>
<td>0</td>
<td>0</td>
<td>1 (0.2%)</td>
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<td>0</td>
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<tr>
<td>3</td>
<td>150G&gt;A</td>
<td>A50A</td>
<td>1 (0.3%)</td>
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<td>2 (0.4%)</td>
<td>0</td>
<td>1 (0.5%)</td>
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<tr>
<td>2</td>
<td>234C&gt;G</td>
<td>N78K</td>
<td>1 (0.3%)</td>
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<tr>
<td>2</td>
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<td>2</td>
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<td>A116T</td>
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<td>11 (2.3%)</td>
<td>3 (1.3%)</td>
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<tr>
<td>3</td>
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<td>1 (0.3%)</td>
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</table>

statistically detect a difference between our findings in AMD patients and controls at a given power was computed using an interactive web site at http://members.aol.com/johnp71/proppowr.html. The calculations incorporate a continuity correction to the usual sample size formula based on the normal approximation to the binomial distribution.29

Results
CHARACTERISATION OF THE HUMAN G PROTEIN COUPLED RECEPTOR-75 (GPR75)
The expression pattern in vitro of the transcript represented by Unigene cluster Hs. 40763 was determined by RT-PCR with a panel of first strand cDNA from 12 human tissues. Using oligonucleotide primers designed from IMAGE clone 222124, we amplified a 357 bp fragment from cDNA from retina, brain stem, and cerebellum (Fig 2A). No amplification products were detected in any other tissues, suggesting neuronal expression of the transcript with the major sites of expression in retina and brain.

Of the five EST sequences comprising the cluster, two derive from IMAGE clone 222124. Sequencing the full length of the clone insert resulted in a 2057 bp sequence containing an ATG start codon in a weak context (cgaacATGA)30 followed by an open reading frame of 1623 bp. An in-frame stop codon was localised 45 bp upstream of ATG. We were able to extend the sequence 19 bp with 5'-RACE. Alignment with genomic sequence revealed that the gene consists of two exons, at least 123 bp and 1732 bp in length, with the entire coding sequence contained within the larger second exon (acc nos AF303576, AF101472) (Fig 1). The two exons are separated by an intron of approximately 4.7 kb with the 5'-donor ( . . .CAGgtgaga . . .) and 3'-acceptor splice site ( . . .gcattttgttgtagG . . .) conforming to conserved sequences required for proper binding of the active spliceosome. The predicted protein product consists of 540 amino acids, has seven transmembrane domains and shows homology to the superfamilly of G protein coupled receptors (Fig 3).

We investigated the expression profile further by northern blot analysis. Probing with a 615 bp fragment PCR amplified from IMAGE clone 222124 revealed a 6.6 kb transcript in human cerebellum and retina (Fig 2B). Expression was not detected in lung, the RPE19 cell line, uterus, liver, heart, or peripheral blood leucocytes (Fig 2B). The same results were obtained with a 270 bp sequence amplified from PAC clone dJ302H14 probe and located approximately 2.3 kb downstream of the stop codon (TAA) (data not shown), demonstrating that the transcript contains a

![Figure 3 Schematic representation of the GPR75 protein showing the seven putative transmembrane domains and the locations of the amino acids affected by the five potentially disease associated variants (black boxes) and two likely polymorphisms (white boxes). Each circle represents a single amino acid.](http://bjo.bmj.com/)

www.bjophthalmol.com
large 3'-UTR which has not yet been characterised in its entirety.

MUTATION ANALYSIS
SSCP or DHPLC analysis of the entire coding sequence of the GPR75 gene (Fig 1) was performed in 535 unrelated AMD patients and 252 age and ethnically matched controls from Germany, the United States, and Italy. This analysis identified nine different sequence alterations (Table 2 and Fig 3). Six of these were found in single AMD patients but not in control individuals. These include a guanine to adenine transition at position –4 of the UTR (–4G>A), three alterations leading to non-conservative amino acid substitutions (N78K, P99L, T135P), one alteration leading to a conservative amino acid substitution (S108T), and one nonsense mutation, a C to T transition creating a premature stop at codon 234 (Q234X). Analysis of DNA obtained from four unaffected sons (aged 36–48 years) of the German AMD patient (No 95) with the Q234X mutation revealed that one has inherited the mutation but, at age 47, had a normal ophthalmological examination including the retinas (data not shown). Three alterations were found in both AMD patients and control individuals. Two of these, an adenine to cytosine transition at position –30 in the 5'-UTR (–30A>C) and a guanine to adenine transition at nucleotide position 346 leading to a putative non-conservative amino acid substitution (A116T), were found in frequencies of 7.4% (117/1574 alleles) and 2.5% (40/1574 alleles), respectively. The frequencies of each of the variants were not significantly different between the AMD and the control group (–30A>C: p = 0.26; A116T: p = 0.19). A third change involving a G to A substitution at nucleotide position 150 (150G>A) was found in one German AMD patient, two US AMD patients, and one US control. This alteration does not affect the amino acid sequence but does introduce the dinucleotide AG into the DNA sequence downstream from a stretch of pyrimidine residues.

To determine whether the 150G>A alteration could create a cryptic acceptor splice site, we used an exon trapping system to present exonic sequence corresponding to the wild type and mutant alleles to the splicing machinery in COS7 cells. RT-PCR amplification with vector specific oligonucleotide primers SD6 and SA2 yielded the same 261 bp fragment from both the wild type and mutant clones (Fig 4A, B). Direct DNA sequencing confirmed that both fragments are composed of only vector sequence with both inserts spliced out as a result of vector related splice donor and acceptor sites. A second RT-PCR experiment was performed with vector primer SD6 and exonic primer 91.s4. The exonic primer precludes the amplification of the predominant splice product and should allow the detection of low abundance transcripts. The second experiment amplified a 505 bp fragment both from normal and from wild type clones (Fig 4A, C). This large fragment is completely unspliced and includes the full insert and flanking vector sequences. The predicted 138 bp fragment from the hypothetical cryptic splice site was not observed. In the positive control, differential splicing was observed between the mutant IVS20+5G>A and the wild type sequence in the ABCA4 gene (Fig 4A, D).

Discussion
In our programme to establish a comprehensive catalogue of retinal genes with a possible
predisposition to AMD, we identified a cluster of five EST sequences derived exclusively from retinal cDNA libraries. The in silico expression profile was expanded by in vitro studies demonstrating expression in retina and brain tissues. Analysis of the full length transcript and the respective genomic sequence revealed a two exon gene, named \textit{GPR75}, that codes for a member of the superfamily of G protein coupled receptors. During the course of our work \textit{GPR75} was cloned independently in the effort to identify the gene for Doyne honeycomb retinal dystrophy (DHRD) and Malattia Leventinese (ML) on chromosome 2p16.3. Mutation analysis in \textit{GPR75}, however, revealed that this gene is not underlying the DHRD/ML phenotype. During the course of our work a member of the superfamily of G protein coupled receptors. Furthermore, P99L and T135P protein activation in other G protein coupled receptors. 

The remaining five alterations, found in single AMD patients, are present in comparable frequencies in AMD patients and control individuals. The third variant (150G>A), found in three AMD patients and one control, was evaluated in an exon trapping system for its potential to introduce a cryptic splice site; however, no effect on splicing was observed. This still leaves uncertainty about the potential effect of this alteration on gene function. Another alteration (-4G>A) found in a single American AMD patient is also of uncertain significance. This variant occurs close to the putative ATG start codon but may not alter this codon (cgaaaATGA) substantially, which is already in weak context compared to the Kozak consensus sequence. The remaining five alterations, found in single AMD patients, potentially could have adverse effects on the protein (N78K, P99L, S108T, T135P, Q234X). All the amino acid substitutions affect residues that are in or close to the putative transmembrane domains, regions that are involved in either ligand binding and/or G protein activation in other G protein coupled receptors. Furthermore, P99L and T135P involve the removal and the addition, respectively, of proline residues, which are known to induce bends or folds into polypeptide chains and could therefore have serious consequences for the protein’s three dimensional structure. The stop mutation (Q234X) occurs in the large intracellular loop between the putative transmembrane domains V and VI and results in the deletion of a substantial portion of the protein, predictably most of this loop, two transmembrane domains, and the entire intracellular C terminal end of the receptor.

While final proof of causality is lacking, we cannot eliminate the possibility that some \textit{GPR75} mutations are involved in the observed retinal pathology in these rare instances. Consequently, a low fraction of AMD cases might occur from \textit{GPR75} mutations. The histories of the five patients with potential deleterious changes in the protein showed that four, German patients No 88 and No 95 and US patients No 119 and No 26554, have extensive soft drusen, retinal pigment epithelial detachments, and disciform macular degeneration. The US patient No 26037, who harbours a conservative amino acid substitution (S108T), has a different clinical picture with geographic atrophy and a positive family history of AMD. Interestingly, this patient is also heterozygous for the D2177N variant in the \textit{ABCA4} gene, although the concomitant appearance of these two variants has no clear implication on the issue of association or causality. The ages of onset of visual symptoms associated with macular degeneration in the five patients range from 63 to 85 years, and one heterozygous carrier, the son of patient No 95, had no retinal abnormalities by examination at age 47.

The results of this study are comparable to previous analyses of AMD patients in the \textit{VMD2} gene, that underlies juvenile onset vitelliform macular dystrophy (Best disease). Two different studies have found similar low frequencies (1–1.5%) of \textit{VMD2} mutations in AMD patients. It is unclear, however, whether specific \textit{VMD2} mutations lead to an AMD phenotype or whether these findings provide evidence for substantial clinical heterogeneity in Best disease with some milder cases misdiagnosed as AMD at later stages of the disease. Supporting the latter interpretation is the demonstration of identical \textit{VMD2} mutations in patients with Best disease and in individuals with adult vitelliform macular dystrophy, a phenotypically similar but later onset condition. Unlike \textit{VMD2}, however, mutations in \textit{GPR75} have not been associated previously with a retinal phenotype. As a consequence of the selection of genes based on their expression profile rather than an association with an hereditary retinal disorder, we have no information regarding the disease causing potential of \textit{GPR75}.

Support for an association between \textit{GPR75} variation and AMD may require screening of even larger cohorts of AMD patients and controls. If we consider the variants which are detected in our study with a frequency of less than 1% in all groups tested, we are left with nine sequence alterations in 535 AMD patients (frequency of 0.84%) and one variant in 252 controls (frequency of 0.2%). With a power of only 16% these data do not reach statistical significance (p = 0.12; one sided Fisher’s exact test). To reach a power of 80% (at α = 0.05), the testing of approximately 1899 patients and 894 controls would be required. On the other hand, to find association with our present sample size (535 patients and 252 controls) and with the given frequency of the identified variations in the control population (0.2%) (α = 0.05 with 80% power), the frequency of variants in the patient sample would need to be
10 times higher (2%) than in the control population—that is, the sample size analysed in the present study has ample power to detect an association that is 2% or greater. The ability to analyse large cohorts to achieve sufficient statistical power to accept or reject the hypothesis of an association of any disease allele with AMD has also been shown previously in the analysis of the ABCA4 gene (for a review, see Allikmets34).

Future investigations include identification of the ligand and the effector molecule(s) and the characterisation of the function of the GPR75 receptor in the human retina. In addition to clarification of any potential role of GPR75 in AMD, this information may also lead to the discovery of its role in other retinal phenotypes.

Sincere appreciation is extended to the individuals and families described herein and their attending physicians for their willing and continued participation in these studies. This work was supported in part by grants from the Deutsche Forschungsgemeinschaft (DFG), We 1259/11-1, the Bundesministerium für Bildung und Forschung (BMBF) under 01KW9921, The Foundation Fighting Blindness (Hunt Valley, MD, USA), Research to Prevent Blindness (New York, NY, USA), and The Ruth and Milton Steinbach Fund (New York, NY, USA). RAL is a senior scientific investigator of Research to Prevent Blindness, Inc.