Family-based exome sequencing identifies rare coding variants in age-related macular degeneration

Rinki Ratnapriya1,2,†, Ilhan E. Acar3,†, Maartje J. Geerlings3, Kari Branham4, Alan Kwong5, Nicole T.M. Saksens3, Marc Pauper3, Jordi Corominas3, Madeline Kwicklis1, David Zipprer1, Margaret R. Starostik1, Mohammad Othman4, Beverly Yashar4, Goncalo R. Abecasis5, Emily Y. Chew1, Deborah A. Ferrington6, Carel B. Hoyng3, Anand Swaroop1,‡ and Anneke I. den Hollander3,‡,*

1Neurobiology, Neurodegeneration and Repair Laboratory (NNRL), National Eye Institute, Bethesda, MD 20892, USA, 2Department of Ophthalmology, Baylor College of Medicine, Houston, TX 77030, USA, 3Department of Ophthalmology, Donders Institute for Brain, Cognition and Behaviour, Radboud University Medical Center, Nijmegen 6500, The Netherlands, 4Department of Ophthalmology and Visual Sciences, University of Michigan, Ann Arbor, MI 48105, USA, 5Center for Statistical Genetics, Department of Biostatistics, University of Michigan, Ann Arbor, MI 48109, USA and 6Department of Ophthalmology and Visual Neurosciences, University of Minnesota, Minneapolis, MN 55455, USA

*To whom correspondence should be addressed at: Department of Ophthalmology, Radboud University Medical Center, Philips van Leydenlaan 15, Route 409, Nijmegen 6525 EX, The Netherlands; Email: anneke.denhollander@radboudumc.nl

Abstract

Genome-wide association studies (GWAS) have identified 52 independent variants at 34 genetic loci that are associated with age-related macular degeneration (AMD), the most common cause of incurable vision loss in the elderly worldwide. However, causal genes at the majority of these loci remain unknown. In this study, we performed whole exome sequencing of 264 individuals from 63 multiplex families with AMD and analyzed the data for rare protein-altering variants in candidate target genes at AMD-associated loci. Rare coding variants were identified in the CFH, PUS7, RXFP2, PHF12 and TACC2 genes in three or more families. In addition, we detected rare coding variants in the C9, SPEF2 and BCAR1 genes, which were previously suggested as likely causative genes at respective AMD susceptibility loci. Identification of rare variants in the CFH and C9 genes in our study validated previous reports of rare variants in complement pathway genes in AMD. We then extended our exome-wide analysis and identified rare protein-altering variants in 13 genes outside the AMD-GWAS loci in three or more families. Two of these genes, SCN10A and KIR2DL4, are of interest because variants in these genes also showed association with AMD in case-control cohorts, albeit not at the level of genome-wide significance. Our study presents the first large-scale, exome-wide analysis of rare variants in AMD. Further independent replications and molecular investigation of candidate target genes, reported here, would assist in gaining novel insights into mechanisms underlying AMD pathogenesis.

†Authors contributed equally to this work.
‡Authors jointly supervised the project.

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Introduction

The genetic basis of common diseases continues to be a subject of significant interest in human genetics research in the post-genome era. Genome-wide association studies (GWAS) have been successful in uncovering hundreds of common genetic variants associated with multiple complex traits (1). Despite representing a major advance in deciphering genetic structure of complex diseases, GWAS have faced two major setbacks. First, the associated variants often reside in non-coding regions of the genome, representing a tag SNP (single nucleotide polymorphism) for the causal variant(s). Thus, the causal gene(s) and genetic variant(s) are not immediately evident for a majority of identified loci. Second, barring few exceptions, the associated variants identified by GWAS explain only a small proportion of the disease heritability (2). These observations have led to shifts in paradigms including possible involvement of rare variants in complex traits. In fact, both common and rare variants have been recognized to contribute to the genetic architecture of multifactorial diseases (3). Nevertheless, common variants have long been central to these investigations, primarily because of the identification of millions of SNPs across the genome that could be genotyped in a high-throughput manner (4,5). The advent of next-generation sequencing technologies and reduced cost of sequencing have prompted a new wave of studies for evaluating the impact of rare and low-frequency variants in complex diseases (6).

Age-related macular degeneration (AMD; MIM, 603075) is a late-onset multifactorial, neurodegenerative disease, which affects the macular region of the retina, eventually resulting in the loss of central vision. AMD affects 8.7% of the worldwide population (7) with over 10 million individuals in the United States alone, posing a substantial healthcare burden (8). The clinical presentation of the disease is progressive and can be broadly divided into early, intermediate and late (advanced) stage (9). Early and intermediate stages are mostly asymptomatic and characterized by changes in the retinal pigment epithelium (RPE) and accumulation of extracellular aggregates, called drusen. Central vision loss characterizes the advanced stage, which can be further subdivided into two forms: geographic atrophy (GA) and choroidal neovascularization (CNV) (10). Therapeutic interventions are available for many patients with CNV, which represents a small proportion of all AMD cases (11).

AMD is a complex trait determined by a combination of advanced age, genetic variants and environmental factors such as smoking and nutrition (12,13). Genetic studies in AMD have contributed significantly to biological underpinnings of its pathogenesis (14,15). The most recent GWAS of 16,144 patients and 17,832 control individuals identified 52 independently associated variants at 34 loci that explain over 50% of the genomic heritability (16). These findings have highlighted the complement system, extracellular matrix remodeling and lipid metabolism in AMD pathogenesis. Rare variants are also suggested to contribute to the heritability in AMD (16,17). Targeted sequencing of candidate genes and whole exome sequencing (WES) have identified rare coding variants in the CHF, C3, CFI, C9, CFB and COL8A1 genes at GWAS loci (18–24). Additional studies have established an unequivocal role of the complement system in AMD pathology (25–30). At this stage, the contribution of rare variants outside of the GWAS loci remains largely unexplored due to power issues, as case-control designs require sequencing of large numbers of samples to achieve statistical significance.

Early investigations indicating the aggregation of AMD in families strongly indicated a strong genetic contribution (31–34), and such families can serve as a reference for identifying rare variants and causal genes. Linkage analysis in AMD families had initially led to the identification of the 1q31 and 10q26 loci (35–38), which were later identified as the top two association signals in GWAS and/or by candidate gene association at the CHF (39–43) and ARMS2-HTRA1 (44–46) loci, respectively. In addition, exome sequencing in large, multiplex families identified a rare variant in the FBN2 gene (47) in the early form of macular degeneration and confirmed the occurrence of rare variants in the CHF, CFI, C9 and C3 genes at multiple AMD loci (21,28,29,48–52). Thus, AMD families can provide new insights into AMD by identifying additional rare variants and pointing to candidate causal genes.

In this study, we performed WES of 264 individuals from 63 multiplex families. To determine causality of genes in loci previously identified by GWAS, we analyzed these families for rare protein-altering variants in coding regions at AMD-associated loci. In addition, we examined whole exome data for rare coding variants outside the AMD-associated loci to identify new AMD candidate genes.

Results

Whole exome sequencing in AMD families

We assembled a cohort of 226 affected and 38 unaffected individuals from 63 multiplex families with a history of AMD (Table 1). A majority of the cohort (51.5%) had advanced stages of the disease (GA, CNV or GA + CNV) with a mean age of 78.2 years. Individuals with early and intermediate stages had a mean age of 69.1 years and constituted 34% of the affected individuals (Table 1). We also included 38 unaffected members (age > 55 years) from 32 families with a mean age of 72.1 years. Exome capture and sequencing was performed at two different centers [at the National Eye Institute (NEI) for the NEI families and at ErasmusMC Rotterdam for the Radboudumc families] (Fig. 1). As the WES data were captured on different platforms, we extracted variants from the shared 58.55 Mb exome regions. WES data were analyzed following the guidelines of GATK best practices (Fig. 1), resulting in the identification of 107,348 coding variants (53). We identified 4957 variants after additional, multi-tiered filtering based on quality, allele depth, minor allele frequency (MAF) in matched controls and predicted effect of the variants (Fig. 1). The variants segregating in a family were considered only if the variant was shared among 80% or more affected family members and/or segregated in all but one affected individual. We excluded the variants that were present in available unaffected member(s) of the respective family.

Survey of rare coding variants in genes within GWAS loci

We first analyzed the families for rare (MAF < 1%) coding variants within 34 AMD-associated loci (Table 2). The locus regions were defined by genome-wide significant variants and those within linkage disequilibrium (LD) (r² > 0.5), with an additional 500 kb to both sides, as described in the most recent AMD-GWAS study (16). We identified 91 rare variants in 78 genes within the LD intervals of 29 known AMD loci and initially focused on loci where rare coding variants were identified in three or more families. Variants were detected in the CHF, PUS7, RXFP2 and PHF12 genes in three families and in the TACC2 gene in four families (Fig. 2A–D and F; Table 2). We also included genes that
Table 1. Overview of families analyzed

| Radboudumc cohort | NEI cohort |
|-------------------|------------|
| Number of families | 44         | 19         |
| Total number of individuals | 195       | 69         |
| Males, females     | 73,122     | 30,39      |
| Number of individuals with advanced AMD | 82        | 54         |
| Mean age for advanced AMD | 76.5      | 80.8       |
| Number of individuals with early or intermediate AMD | 82        | 8          |
| Mean age for early or intermediate AMD | 68.5      | 74.8       |
| Number of unaffected individuals | 31        | 7          |
| Mean age of unaffected individuals | 70.8      | 77.5       |

We next analyzed the expression of the eight genes where rare variants were identified in the retinal transcriptome and expression quantitative trait loci (eQTL) dataset that included both control and AMD donor samples (Fig. 3). Six of these genes (CFH, PUS7, PHF12, TACC2, SPEF2 and BCAR1) were expressed in the retina (counts per million, CPM > 0), while no retinal expression was detected for RXFP2 and C9 (Fig. 3A). Additionally, we evaluated gene expression changes during AMD progression by comparing the gene expression profile of the control retina with the profiles of the retina during early, intermediate and late stages of the disease. While we did not observe a significant difference [false discovery rate (FDR) < 10%] during disease progression, several candidates showed a trend of either increased or decreased expression (Fig. 3B). We note that expression of four of the candidates (PUS7, PHF12, SPEF2, BCAR1) is regulated in the retina through common eQTL variants (Fig. 3C).

Exome-wide analysis of rare pathogenic variants

We then extended our analysis in search of rare pathogenic variants in genes outside the GWAS loci. We selected genes with rare (MAF < 1%) variants that were detected in at least three families and applied an additional predictive causality filter to focus on the 1% most deleterious variants in the human genome (CADD score ≥ 20). We also removed the variants that did not pass the quality score filter in Exome Aggregation Consortium (ExAC) database. Our analyses resulted in the identification of 13 candidate genes with 38 rare variants segregating in three or more families (Table 3). As large or polymorphic genes harbor a large number of variants, we further prioritized the list based on the following criteria: (i) whether the gene was expressed in the human retina (CPM > 0) and is regulated by an eQTL, (ii) whether common variants around the candidate gene exhibited a suggestive association (P-value < 5 × 10^{-4}) for AMD and (iii) whether the candidate gene or a closely related gene was shown to have a function relevant to AMD pathology. Ten candidate genes demonstrated expression in the human retina with a few showing changes during AMD progression (Fig. 4A and B). Five of the genes also had an eQTL in the retina (Fig. 4C). One candidate, SCN10A, showed a modest association (3.8800182_C/T; P-value: 9.63 × 10^{-2}) in the AMD-GWAS (Fig. 4D). We identified three different rare variants in SCN10A, one nonsense variant (Q923X) and two non-synonymous variants (P893L and P332L) in three independent AMD families (Fig. 5A). A rare frameshift variant (S267fs) in KIR2DL4 is segregated in three families (Fig. 5B). Based on their role in mitochondrial biogenesis, ESRR and VPS13B, each with rare variants in three different families, could be presented as additional interesting candidates (Fig. 5C and D).
Given that segregation of rare variants in small families can be detected by chance, we performed simulation analysis to test the null case of no variant effect on disease by randomly assigning alleles to subjects in our study and then calculating the probability of observing segregation in three or more families. We applied the segregation criteria similar to the criteria that were used to identify variants in the AMD families and performed 100,000 gene-level simulations for two different variant sets: one with rare variants identified at the AMD loci and the other with exome-wide, rare variants after filtering by CADD score. We observed an average gene-level type I error rate of 7.7 × 10^{-7} for the AMD loci analysis and 1.87 × 10^{-7} for the CADD score filtered exome-wide analysis. The study-level type I error was 4.6 × 10^{-4} (AMD loci) and 2.2 × 10^{-4} (exome-wide). Given the pedigree structure and variants identified in our study, it is therefore unlikely to detect segregation of rare variants in three or more families by chance.

### Table 2. Rare coding variants (MAF < 1%) in genes within GWAS loci identified by WES

| Locus (genes within LD ± 500 kb) | Gene | Family ID | Chr: Pos | rs Id | MAF_NFE | Variants | Exonic function | Protein change |
|---------------------------------|------|-----------|----------|-------|---------|----------|----------------|---------------|
| AMD580                          | CFH  | W11-2310  | 7:10514683 | rs139058270 | 0.006 | NM_001166058: exon1:c.5G > C | Non-synonymous | R2T |
| AMD479                          | CFH  | W10-0408  | 7:10514683 | rs139058270 | 0.006 | NM_001166058: exon1:c.5G > C | Non-synonymous | R175Q |
| KMT2E/SRPK2 (8)                 | PUS7 | AMD930    | 7:10514683 | rs139058270 | 0.006 | NM_001166058: exon1:c.5G > C | Non-synonymous | R303Q |
| TMEM9/VTN (38)                  | PUS7 | AMD479    | 7:10514683 | rs139058270 | 0.006 | NM_001166058: exon1:c.5G > C | Non-synonymous | C9R |
| B3GALTL (?                      | PUS7 | W08-0553  | 7:105148893| —      | —      | NM_001166058: exon1:c.5G > C | Non-synonymous | S32G |
| ARMS2/HRTA1 (15)                | RXFP2| W11-4656  | 13:32351535 | rs121918303 | 0.0072 | NM_001166058: exon1:c.5G > C | Non-synonymous | T222P |
| AMD580                          | RXFP2| W10-0408  | 13:32352714 | rs73163317 | 0.0078 | NM_001166058: exon1:c.67A > G | Non-synonymous | N260S |
| RXFP2                          | W07-0199 | 13:3267033 | rs139851290 | 0.0014 | —      | NM_001166058: exon1:c.5G > C | Non-synonymous | R508G |
| TMEM9/VTN (38)                  | PUS7 | W11-1525  | 17:27238315 | rs148347485 | 0.0083 | NM_001166058: exon10:c.2210A > G | Non-synonymous | N737S |
| AMD580                          | PUS7 | W11-1525  | 17:27238125 | —      | —      | NM_001166058: exon10:c.2210A > G | Non-synonymous | T173S |
| PUS7                           | PUS7 | W11-1525  | 17:27238125 | rs148347485 | 0.0083 | NM_001166058: exon10:c.2210A > G | Non-synonymous | N737S |
| PUS7                           | TACC2| W09-1832  | 10:123844894 | rs112188313 | 0.0068 | NM_001166058: exon10:c.2210A > G | Non-synonymous | R960K |
| TACC2                          | TACC2| W10-0408  | 10:123954596 | —      | —      | NM_001166058: exon10:c.2210A > G | Non-synonymous | T37M |
| TACC2                          | TACC2| W11-1525  | 10:123844894 | rs112188313 | 0.0068 | NM_001166058: exon10:c.2210A > G | Non-synonymous | R960K |
| TACC2                          | TACC2| AMD56    | 10:123809983 | rs202197379 | —      | NM_001166058: exon10:c.2210A > G | Non-synonymous | A22T |

### Discussion

GWAS and deep sequencing efforts have strongly supported a role for common and rare variants in AMD susceptibility. In
addition to conferring high risk for AMD, rare variant studies can provide important insights into the phenotypic characteristics of the carrier patients (55,56). The most recent GWAS examined both common and rare variants in a large cohort but was limited to the analyses of known rare variants that were present on the chip (16) and did not allow sequencing-based discovery of rare variants. A few sequencing studies attempted to harness the power of familial cases, but their search was restricted to known AMD loci (28,49). Here, we present the first, large-scale, genome-wide survey of rare coding variants in multiplex AMD families that identified several novel candidate genes/variants contributing to AMD susceptibility.

Within known AMD susceptibility regions, we identified rare variants at eight loci; two of these in CFH and C9 have previously been reported in case-control and familial studies (18,20). These findings further strengthen the role of the complement pathway in AMD and validate the presence of highly penetrant, functional rare variants as causal variants at these loci. Our study suggested...
Figure 3. (A) Mean expression of candidate genes within AMD-associated loci in human donor retina. (B) Heatmap showing the fold change differences observed in genes during early-, intermediate- and late-stage AMD compared to normal retina. None of the candidate reaches statistical significance of FDR ≤ 10%. (C) Violin plots showing the relationship between the variant and the gene of an observed eQTL. Fewer individuals with homozygous genotypes of PUS7 (G/G) and SPEF2 (T/T) resulted in single point in violin plots. P-values for the eVariants: (rs170690–3.97 × 10^{-5}), (7:105733118–1.39 × 10^{-5}), (rs117886541–1.12 × 10^{-5}), (rs78996920–3.85 × 10^{-10}), (rs12520223–1.75 × 10^{-8}), (rs150308036–3.15 × 10^{-7}), (rs8064132–2.04 × 10^{-5}).

the causal genes at other AMD loci. PUS7 encodes a key RNA-modifying enzyme. RXFP2 encodes a receptor for glycoprotein hormones that contains a unique low-density lipoprotein type A (LDLa) module (57); though this gene is not expressed in the adult human retina (54), its homolog in zebrafish is transcribed in the developing brain and retina (58). PHF12 encodes a member of the PHD zinc finger family of proteins involved in regulation of ribosomal biogenesis and senescence (59) and could contribute to AMD pathology through activation of senescence. Although present in only two families, the findings of rare variants in SPEF2 and BCAR1 are also noteworthy as both of these candidates were highlighted as most likely candidate at the AMD-GWAS loci based on a gene priority score (16). SPEF2 is highly expressed in the human retina and shows a slight increase in advanced stages of AMD. SPEF2 is a cilia-related protein and its absence causes male infertility and primary ciliary dyskinesia (60). This protein is shown to interact with IFT-related protein, IFT20 (61) that is required for opsin trafficking and photoreceptor outer segment development (62). BCAR1 encodes a Src family kinase substrate that is shown to be involved in early retinal development, and a mouse model of Bcar1 exhibits dramatic disruption of the ganglion cell layer (63). Recently, BCAR1 was also identified as a putative causal gene at the CTRB2/CTRB1 locus by co-localization of GWAS and single-cell eQTL data (64).

Our survey of candidate genes harboring rare variants outside AMD loci was aimed at identifying potential novel candidate genes underlying AMD pathology. Such candidates could have been missed in GWAS and other association studies if the gene confers the risk to AMD solely through rare variants, or if the effect size of the common risk variant is very low, warranting larger numbers of cases and controls to detect such associations. We identified 13 candidate genes that harbor rare variants in more than three families and show, by simulation studies, that the probability of these variants segregating by chance is very low. Thus, these genes represent reasonable candidates for AMD susceptibility. We propose SCN10A and KIR2DL4 as the most interesting AMD candidates. Common variants at the SCN10A gene have been associated with AMD in a recent AMD-GWAS
## Table 3. Candidates with rare coding variants (MAF &lt; 1%) segregating in three or more families identified in exome-wide analysis

| Gene      | Chr. Pos | rs Id       | No. of family | MAF_NFE | Exonic function | Nucleotide change | Amino acid change | CADD_phred |
|-----------|----------|-------------|---------------|---------|-----------------|------------------|------------------|------------|
| CTDSP2   | chr12: 58208216 | rs76940645 | 11            | 0       | Non-synonymous   | NM_005730        | I1067T           | 25.3       |
| TTN      | chr2: 179395813 | rs55865284 | 7             | 0.0001  | Non-synonymous   | NM_003319        | V26112M          | 21.1       |
|          | chr2: 179482937 | rs72672722 |              | 0.0026  | Non-synonymous   | NM_003319        | V6685I           | 21.6       |
|          | chr2: 179482994 | rs72677231 |              | 0.0035  | Non-synonymous   | NM_003319        | R6666C           | 22.2       |
|          | chr2: 179486037 | rs72677225 |              | 0.0065  | Non-synonymous   | NM_003319        | K6071N           | 22.3       |
|          | chr2: 179486223 | rs17354992 |              | 0.0079  | Non-synonymous   | NM_003319        | D604SN           | 22.2       |
|          | chr2: 179486345 | rs114331773| 0.0015        | Non-synonymous   | NM_003319        | E6004V           | 22.8       |
|          | chr2: 179537200 | rs202014478|              | 0.006   | Non-synonymous   | NM_003319        | V10321I          | 21.5       |
|          | chr2: 179549407 | rs72650031 |              | 0.0077  | Non-synonymous   | NM_003319        | P9631L           | 20.1       |
|          | chr2: 179582913 | rs72648981 |              | 0.003   | Non-synonymous   | NM_003319        | E7039K           | 22.7       |
|          | chr2: 179585312 | rs17452588 |              | 0.008   | Non-synonymous   | NM_003319        | S6482L           | 22.5       |
|          | chr2: 179588622 | rs201394117|              | 0.0007  | Non-synonymous   | NM_003319        | A5878T           | 23.6       |
| KIR3DL3  | chr19: 55237616 | rs14376380 | 4             | NA      | Non-synonymous   | NM_153443        | N56K             | NA         |
|          | chr19: 55246731 | rs602444   |              | NA      | Non-synonymous   | NM_153443        | H321Y            | NA         |
| DCHS2    | chr4: 155156598 | rs149584849| 4             | 0.002   | Non-synonymous   | NM_001293307     | P2614L           | 24.9       |
|          | chr4: 155411721 | rs19962086 |              | 0.0042  | Non-synonymous   | NM_00133378      | R263W            | 25.9       |
|          | chr4: 155411948 | rs184619033|              | 0.0074  | Non-synonymous   | NM_00133378      | R187L            | 24.2       |
| DNAH14   | chr21: 22540410 | rs17578819 | 4             | 0.0094  | Non-synonymous   | NM_0013373       | G1657E           | 21.8       |
|          | chr21: 225490915 | rs140066130|              | 0.0042  | Non-synonymous   | NM_0013373       | R2804C           | 34         |
|          | chr21: 225528175 | rs184094753|              | 0.0034  | Stopgain         | NM_0013373       | E3391X           | 58         |
| SCN10A   | chr3: 38768123 | —           | 3             | 0       | Stopgain         | NM_001293307     | Q923X           | 24.9       |
|          | chr3: 38768212 | rs138413438|              | 0.0014  | Non-synonymous   | NM_001293307     | P893L            | 24.7       |
|          | chr3: 38798606 | —           | 0             | 0       | Non-synonymous   | NM_001293307     | P332L            | 33         |
| ESRRB    | chr11: 64083290 | rs150848359| 3             | 0.0098  | Non-synonymous   | NM_001293306     | R375Q            | 22.4       |
|          | chr11: 64083293 | rs201971362|              | 0.0098  | Non-synonymous   | NM_001293306     | R376L            | 22.1       |
| ADPRHL1  | chr13: 114077172| rs154817729| 3             | 0.0084  | Non-synonymous   | NM_138430        | A344T            | 20.5       |
|          | chr13: 114107571 | rs138029763|              | 0.0076  | Non-synonymous   | NM_138430        | M61T             | 26.2       |
| KIR2DL4  | chr19: 55234674 | rs11371265 | 3             | NA      | frameshift_insertion | NM_001080722        | S2676s          | NA         |
|          | chr19: 55234674 | rs11371265 | 3             | NA      | frameshift_insertion | NM_001080722        | S2676s          | NA         |
| PLB1     | chr2: 28805359 | rs144737372| 3             | 0.0013  | Non-synonymous   | NM_00170585      | R563G            | 22.3       |
|          | chr2: 28854972 | rs74701215 | NA            | 0       | Non-synonymous   | NM_00170585      | P1312T           | 28.1       |
| TAF1C    | chr16: 84212875 | rs199796567| 3             | 0.0037  | Non-synonymous   | NM_001243158     | S429L            | 31         |
|          | chr16: 84213651 | —           | 0             | 0       | Non-synonymous   | NM_001243158     | Q002X            | 23.3       |
| UNC80    | chr2: 210683829 | rs200473652| 3             | 0.0019  | Non-synonymous   | NM_0032504       | Q602H            | 23.1       |
|          | chr2: 210707144 | rs78912192 | 0.0003        | Non-synonymous   | NM_0032504       | E1145A           | 27.9       |
|          | chr2: 210860221 | rs199783352| 0.0005        | Non-synonymous   | NM_182547        | E3203K           | 23.4       |
| VPS13B   | chr8: 100417957 | rs143205296| 3             | 0.0005  | Non-synonymous   | NM_0015243       | H520R            | 24.8       |
|          | chr8: 100443885 | rs61753722 | 0.0091        | Non-synonymous   | NM_0017890       | T1068I           | 24.2       |
Figure 4. (A) Mean expression of the candidate genes with rare variants (MAF < 1%) in three or more families that are located outside of AMD-associated loci. (B) Heatmap showing the fold change differences observed in genes during early-, intermediate- and late-stage AMD compared to normal retina. (C) Violin plots showing the relationship between the variant and the gene of an eQTL. Fewer individuals with homozygous genotypes of TTN (G/G) resulted in single point in the violin plot. P-values for the eVariants: (rs66773470–2.66 × 10^{−6}), (rs2278043–5.92 × 10^{−60}), (rs35615286–4 × 10^{−5}), (rs58805123–2.50 × 10^{−5}), (rs11889787–4.11 × 10^{−5}), (rs1800792–1.56 × 10^{−5}). (D) LocusZoom plot (80) showing the association signals around SCN10A in most recent GWAS analysis in AMD (16).

though not at the level of genome-wide significance, and a suggestive burden of rare variants in KIR2DL4 has been detected in a meta-analysis of WGS data in a case-control cohort composed of 3519 cases and 3754 controls (65). SCN10A is a voltage-gated sodium channel expressed in starburst amacrine cells and a subset of retinal ganglion cells (66), which is likely the reason for not observing its expression in the human retina. Gain-of-function mutations in this gene cause axonal degeneration leading to painful neuropathy (67). Several retinal diseases are caused by ion channel mutations including inherited macular degeneration (68). KIR2DL4, a member of the human killer cell Ig-like receptor (KIR) family, is expressed primarily in natural killer (NK) cells and plays an important role in innate immunity (69). NK cells interact with HLA class ligand through their KIR receptors and a certain combination of HLA-C, and KIR gene variants have been associated with AMD (70). ESRRA, an estrogen-related receptor is a key regulator of energy homeostasis and mitochondrial function. ESRRA-null mice show altered regulation of enzymes involved in lipid, eicosanoid and steroid synthesis (71). Dysfunction in lipid metabolism has been linked to AMD pathogenesis (72), making ESRRA an attractive candidate for further investigation. VPS13B is a transmembrane protein with a function in vesicle-mediated transport. Mutations in this gene cause Cohen syndrome, whose clinical features include non-progressive psychomotor retardation, microcephaly and retinal dystrophy (73).

Our study demonstrates that WES in extended families could prove valuable for identifying rare coding risk variants in potentially novel AMD genes. Similar results could be obtained from traditional case-control studies; however, that would require study designs involving much larger cohorts. A further incorporation of expression data from a large cohort of normal and AMD donors (54) also uncovered new insights. These candidates do not reach statistical threshold (FDR ≤ 10%) in the differential expression analysis because of the clinical and genetic heterogeneity among AMD and normal individuals and limited power of the study to detect such differences. Investigation of larger cohorts as well as other AMD-relevant tissues such as RPE and choroid will be useful as additional line of evidence in validating these candidates as AMD-causing genes.

In conclusion, our family-based exome sequencing studies identified rare coding variants for novel candidate genes at eight known GWAS loci and 13 additional candidates outside GWAS regions. Further independent replications and molecular investigations of the candidate genes and variants, reported here, could provide novel mechanisms and pathways underlying AMD pathogenesis.

Materials and Methods

Study samples

The study population consisted of 264 samples from 63 multiplex AMD families is collected at two different centers. The Radboudumc cohort (Fig. 1, Table 1) comprised of 44 families (195 subjects). All patients gave written consent and the local ethics committees on research involving human subjects approved the study. Family ascertainment and disease classification for NEI cohort have been described in detail elsewhere (16,37). Briefly, samples were ascertained from the clinical practice at the Kellogg Eye Center. Fundus photographs, fluorescein angiograms and eye examinations records were obtained for all probands and family members and were updated every 1–2 years. The recruitment and research protocols were reviewed and approved by the University of Michigan institutional review board, and informed consent was obtained from all study participants.
Figure 5. Segregation of the rare variants in SCN10A (A), KIR2DL4 (B), ESRRB (C) and VPS13B (D) in AMD families.

Fundus findings in each eye were classified on the basis of a standardized set of diagnostic criteria established by the International Age-Related Maculopathy Epidemiological Study (74). The Declaration of Helsinki principles was followed for all procedures. The NEI cohort (Fig. 1, Table 1) consisted of 19 families (69 subjects) that were collected at the University of Michigan. All patients signed informed consent, and the Institutional Review Boards of the University of Michigan and the National Eye Institute approved the study.

Whole exome sequencing
Genomic DNA was extracted from the peripheral blood using standard methods. Genomic DNA samples were quantified using the Promega QuantFluor® dsDNA system (Promega, Madison WI, USA), according to the manufacturer’s instructions. The NEI families were subjected to exome sequencing using standard library preparation protocol with Agilent SureSelect Human All exon 50 Mb kit (Agilent Technologies, Santa Clara, CA), following the manufacturer’s instructions (75). Captured libraries were
amplified and converted to clusters using Illumina Cluster Station, and single-end 101 bp sequencing was performed on Illumina GAIIx (Illumina, Inc., San Diego, CA). The Nimblegen SeqCap EZ Exome v2.0 4 Mb kit (Roche Nimblegen, Inc., Madison, WI) was used for performing WES in the Radboudumc families. Illumina HiSeq2000 sequencer was used to perform paired-end sequencing, using Illumina TruSeq V3 chemistry (Illumina, Inc., San Diego, CA).

Primary bioinformatics analysis

FastQC (available at http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to confirm quality of sequencing, after which adapter indexes were removed using Trimmmomatic (76). Mapping was performed on the hg19/GRCh37 human reference genome sequence build using BWA (77). Aligned reads were processed to mark duplicates using Picard (http://picard.sourceforge.net). The Genome Analysis Toolkit (GATK) recommendations for best practices were applied for variant calling, local realignment, base quality recalibration and variant recalibration (78). The annotation of variants was performed with ANNOVAR (79). Overlapping regions between the capture kits were identified using the mergeBed tools from the bedtools package (80). Alternate allele frequencies of the variants in non-Finnish European (NFE) populations were obtained from the Exome Aggregation Consortium, which constituted data from 33,370 individuals.

Variant filtering and prioritization

We removed the variants that did not pass the variant quality score recalibration (VQSR) filter. We further retained variants based on read depth (≥ 5x), and at least 20% of the reads were attributed to the alternate allele. We applied a segregation filter where we retained variants that were shared among 80% or more affected family members or segregated in all but one affected individual. Variants were discarded if they were present in one or more unaffected family members. We generated a list of rare variants with MAF < 1% and low-frequency variants based on allele frequency data from ExAC non-Finnish Europeans (81). The AMD locus regions were defined by the genome-wide significant variants and the variants within linkage disequilibrium (LD) (r^2 > 0.5), adding a further 500 kb to both sides, as described in the most recent AMD-GWAS analysis (16). Finally, the variant-level data were collapsed into gene-level data by combining all variants observed in each gene across different families. For exome-wide analysis, we applied additional filters of predictive causality (CADD ≥ 20) and retained variants that passed variant quality score recalibration in the ExAC database. Common AMD-associated variants from the most recent GWAS (16) were visualized using LocusZoom (82).

Simulation analysis of rare variant segregation

Simulation analysis was performed to assess type I errors. We included all the variants that passed quality control, MAF and functional filters. We did not include the segregation filter in order to access the probability of genes/variants, segregating in three or more families. We started with our list of filtered variants with allele counts from the AMD loci analysis (2862 variant) and the exome-wide analysis (1299 variants) as our input. For each variant, we simulated the null case by randomly distributing its minor alleles across the 264 subjects in the study, with at most one allele per subject. Then, we applied the same segregation criteria we used in our real data analysis. Briefly, if any minor allele was assigned to a control subject, the variant was discarded; otherwise, we evaluated the segregation pattern of each family and counted properly segregating families as described above. We repeated this for all variants within each gene and then identified all segregating families. We performed 100,000 simulations for each gene in two different scenarios: (1) 2862 variants in 600 genes within the known AMD Loci and (2) 1299 variants with a CADD score ≥ 20 in 1176 genes exome-wide. The study-level type I error was calculated by multiplying the gene-level type I errors with the number of genes tested.

Expression and eQTL analysis of control and AMD retina

The transcriptome data of post-mortem human donor retinas from 453 donors at different stages of AMD and controls were analyzed for candidate gene prioritization. The donor retina that were graded for normal and disease status of AMD using Minnesota Grading System (MGS), with criteria similar to the Age-Related Eye Disease Study (AREDS). We analyzed 105 MGS1 (normal), 175 MGS2 (early AMD), 112 MGS3 (intermediate AMD) and 61 MGS4 (advanced AMD) after initial RNA-seq quality control described elsewhere. The mean age of donors was 80 years (range 55–107). The methods for RNA sequencing, gene expression quantitation, differential gene expression and eQTL analysis are described in detail elsewhere (54). Briefly, RSEM expected counts from 453 samples and 18,053 genes passing the expression-level filter (≥ 1 CPM in ≥ 10% of samples) were transformed into TMM-normalized CPM and then converted into log2 CPM with an offset of 1. Batch effects were estimated and corrected using the supervised surrogate variable analysis (supervised sva) method within the bioconductor sva package in R. The differential expression between MGS 1 controls and each disease stage was assessed using the limma package in R. Fold changes between these comparisons were presented as heatmaps and violin plots using in-house scripts in R. The eQTL analysis included 17,389 genes and 8,924,684 genotyped and imputed common variants. The mapping of cis-eQTLs [as defined by SNP-gene combination within ±1 Mb of the transcriptional start site (TSS) of each gene] was performed using QTLtools to identify genetic variants (eVariants) that control expression of genes (eGenes) at FDR ≤ 0.05.

Rare variant association analysis in AMD case-control studies

We analyzed two different cohorts for this analysis. The first cohort was analyzed using whole genome sequencing data in 4787 subjects consisting of 2394 cases and 2393 controls (24). All the cases had advanced AMD (GA or CNV). The second cohort was analyzed using whole exome sequencing in 1125 cases and 1361 controls (24). RAREMETAL was used to perform the meta-analysis of gene-based tests for rare variants (83).

Supplementary material

Supplementary material is available at HMG online.

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Conflict of interest statement: The authors declare no competing financial interests, except that G.R.A. is now employed by the Regeneron Pharmaceuticals.

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