Identification of a rare coding variant in complement 3 associated with age-related macular degeneration

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Macular degeneration is a common cause of blindness in the elderly. To identify rare coding variants associated with a large increase in risk of age-related macular degeneration (AMD), we sequenced 2,335 cases and 789 controls in 10 candidate loci (37 genes). To increase power, we augmented our control set with ancestry-matched exome-sequenced controls. An analysis of coding variation in 2,268 AMD cases and 2,268 ancestry-matched controls identified 2 large-effect rare variants: previously described p.Arg1210Cys encoded in the \(\text{CFH}\) gene (case frequency \(f_{\text{case}} = 0.51\%\); control frequency \(f_{\text{control}} = 0.02\%\>; odds ratio (OR) = 23.11) and newly identified p.Lys155Gln encoded in the \(\text{C3}\) gene (\(f_{\text{case}} = 1.06\%; f_{\text{control}} = 0.39\%\); OR = 2.68). The variants suggest decreased inhibition of C3 by complement factor H, resulting in increased activation of the alternative complement pathway, as a key component of disease biology.

Genetic and environmental factors contribute to AMD\(^1,2\), a major cause of vision loss in elderly individuals\(^3\). Pioneering discovery of association of AMD with complement factor H (encoded by \(\text{CFH}\)) was quickly followed by the identification of additional susceptibility loci that now include \(\text{C3}, \text{CFB}\) and \(\text{C2}\)\(^7,8\). Genome-wide association studies (GWAS) of AMD cases and controls have now identified multiple candidate regions (GWAS)\(^7,8\) and complement genes \(\text{C3}, \text{C2}-\text{CFB}, \text{CFI}\)\(^7\) & \(\text{CETP}\)\(^9\). Genome-wide association studies (GWAS) of AMD cases and controls have now identified common susceptibility variants at ~20 different loci\(^13,14\) and have begun to uncover specific cellular pathways involved in AMD biology.

Whereas common variants tag an associated genomic region, rare coding variants can provide more specific clues about the underlying disease mechanism\(^15\). For example, rare variant p.Arg1210Cys encoded in the \(\text{CFH}\) gene was recently associated with a large increase in AMD risk using targeted sequencing of rare \(\text{CFH}\) risk haplotypes\(^16\). The resulting altered protein has decreased binding to C3b, C3d, heparin and endothelial cells\(^17–19\). A reduction in the ability of CFH to inactivate C3, leading to increased cell killing activity of the complement pathway, could contribute to AMD, representing a much more specific and testable hypothesis about disease mechanism than provided by common \(\text{CFH}\) variants whose mechanistic consequences are unclear.

To systematically identify rare, large-effect variants, we carried out targeted sequencing of eight AMD risk loci identified in GWAS\(^20\) (near \(\text{CFH}, \text{ARMS2}, \text{C3}, \text{C2}-\text{CFB}, \text{CFI}, \text{CETP}, \text{LIPC}\) and \(\text{TIMP3}-\text{SYN3}\)) and two candidate regions (\(\text{LPL}\) and \(\text{ABCA1}\)\(^1\)) (Supplementary Table 1). We resequenced these regions in 3,124 individuals (2,335 cases and 789 controls) recruited in ophthalmology clinics at the University of Michigan and the University of Pennsylvania and in Age-Related Eye Disease Study (AREDS) participants\(^20,21\). We enriched genomic targets using a set of 150-bp probes designed by Agilent Technologies and generated sequence data on Illumina Genome Analyzer and HiSeq instruments. The 10 loci comprised 115,596 nucleotides of protein-coding sequence and totaled 2,757,914 nucleotides overall. We designed probes to capture 111,592 protein-coding nucleotides (96.5% of coding sequence) and 966,607 nucleotides overall (35.1% of the locus sequence), generating an average of 123,221,974 mapped reads per sample. Target enrichment of the 10 loci comprised 115,596 nucleotides of protein-coding sequence and totaled 2,757,914 nucleotides overall. We designed probes to capture 111,592 protein-coding nucleotides (96.5% of coding sequence) and 966,607 nucleotides overall (35.1% of the locus sequence), generating an average of 123,221,974 mapped reads per sample.

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bases of on-target sequence per individual (127.5× average depth when counting bases with quality of >20 in reads with mapping quality of >30 after duplicate read removal); 98.49% of sites with designed probes were covered at >10× depth. We applied variant calling tools and quality control filters similar to those used to analyze National Heart, Lung, and Blood Institute (NHBLI) Exome Sequencing Project (ESP) data22,23 (Supplementary Table 2). We identified an average of 1,714 non-reference sites in each sequenced individual. In total, we identified 31,527 single-nucleotide variants, of which 18,956 were not in dbSNP135. Discovered sites included 834 synonymous variants, 1,379 nonsynonymous variants and 43 nonsense variants, most of which were extremely rare (Supplementary Table 3). For 13 samples sequenced in duplicate, genotype concordance was 99.82% (when depth was >10×). For 908 samples previously examined with GWAS arrays20, sequencing-based genotypes were 98.99% concordant with array-based calls (again, when depth was >10×).

In an initial comparison of AMD cases and controls (Supplementary Table 4), no rare coding variants with frequency of <1% reached experiment-wide significance (P < 0.05/31,527 = 1.6 × 10^{-7} when including all discovered variants or P < 0.05/1,422 = 3.5 × 10^{-4} when considering only protein-altering variants), although several showed encouraging patterns of association. For example, the rare variant p.Arg1210Cys encoded in the CFH gene was observed in 23 of the 2,335 sequenced cases but in none of the 789 sequenced controls (exact test P = 0.0025). Common variants in several loci exhibited strong evidence of association, including in CFH (peak variant rs9427642: f_case = 12%; f_control = 27%; P value = 2.52 × 10^{-48}, ARMS2 (rs10490924: f_case = 33%; f_control = 18%; P value = 5.48 × 10^{-27}), C3 (rs2230199: f_case = 25%; f_control = 17%; P value = 3.94 × 10^{-7}) and C2-CFB (rs556679; f_case = 7%; f_control = 12%; P value = 1.32 × 10^{-10}).

A key requirement for establishing significance of rare disease-associated variants is the availability of sufficient numbers of control samples. To increase power, we sought to identify additional controls and focused on samples from NHBLI ESP23, which sequenced 15,336 samples in duplicate, genotype concordance was 99.82% (when depth was >10×). For 908 samples previously examined with GWAS arrays, sequencing-based genotypes were 98.99% concordant with array-based calls (again, when depth was >10×).

In this expanded analysis (Table 1), common variant signals at all loci increased in significance (in comparison to what is shown in Supplementary Table 4). In addition, two rare coding variants exhibited association with P < 0.01. The first variant was p.Arg1210Cys encoded in the CFH gene (observed in 1 control and 23 cases; OR = 23.11; exact P = 2.9 × 10^{-6}), providing strong support for the original report16.

### Table 1 Summary association results for 2,268 sequenced AMD cases and 2,268 sequenced controls

| SNP         | Chromosome | Position (bp) | Nearest gene | Consequence | Alleles (ref/alt) | Cases | Controls | OR     | P value | Conditional P value^a |
|-------------|------------|---------------|--------------|-------------|------------------|-------|----------|--------|---------|------------------------|
| rs1061170   | 1          | 196659237     | CFH          | p.His402Tyr | C/T              | 0.478 | 0.623    | 0.555  | 1.01 × 10^{-43} |
| rs438999    | 6          | 31928306      | SKIV2L       | p.Gln151Arg | A/G              | 0.058 | 0.098    | 0.566  | 1.26 × 10^{-12} |
| rs10490924  | 10         | 124214448     | ARMS2        | p.Ala69Ser  | G/T              | 0.329 | 0.197    | 1.990  | 1.32 × 10^{-10} |
| rs2230199   | 19         | 6718387       | C3           | p.Arg102Gly | G/C              | 0.253 | 0.206    | 1.300  | 1.58 × 10^{-7}  |

Rare variant hits (MAF < 1%; marginal and conditional P < 0.01 after conditioning on nearby common variants)

| SNP         | Chromosome | Position (bp) | Nearest gene | Consequence | Alleles (ref/alt) | Cases | Controls | OR     | P value | Conditional P value^a |
|-------------|------------|---------------|--------------|-------------|------------------|-------|----------|--------|---------|------------------------|
| rs121913059 | 1          | 196716375     | CFH          | p.Arg1210Cys| C/T              | 0.005 | 0.000    | 23.11  | 2.9 × 10^{-6}  | 6.0 × 10^{-4} (rs1061170) |
| rs147859257 | 19         | 6718146       | C3           | p.Lys155Gln | T/G              | 0.011 | 0.004    | 2.68   | 2.7 × 10^{-4}  | 2.8 × 10^{-5} (rs2230199) |

Samples in this expanded analysis include our sequenced AMD samples and genetically matched controls, sequenced by us or by the NHLBI ESP. The top coding variant in each locus is included in this table when P < 1 × 10^{-6}. Rare coding variants are included when the corresponding P value for conditional or marginal analysis was less than 1 × 10^{-4}. All P values were calculated using exact logistic regression. Ref, reference; alt, alternative; MAF, minor allele frequency.

^aFor rare variants, we re-evaluated statistical significance after adjusting for the top common variant in the locus to avoid shadow signals driven by linkage disequilibrium. The variant used for conditioning is named (in parentheses).
The second variant was p.Lys155Gln encoded in the C3 gene (observed in 18 controls and 48 cases; OR = 2.68; exact P = 2.7 × 10^-4; see Supplementary Fig. 1d). The evidence for association with p.Lys155Gln increased slightly (conditional OR = 2.91; exact P = 2.8 × 10^-5). Inspection of the raw read data showed that the variant was well supported and was unlikely to be an artifact of sequencing or alignment, a result further confirmed by Sanger sequencing (Supplementary Figs. 2–4). Finally, in an examination of our sequenced samples and available whole-genome sequences (Online Methods), we observed no additional variants in strong linkage disequilibrium with the mutation encoding p.Lys155Gln that might account for the association signal. Analysis with burden tests, which jointly evaluate evidence for association with rare variants at each gene, identified no additional variants in strong linkage disequilibrium with the variant, consistent with an OR of ~3 (Online Methods; Supplementary Table 5).

To confirm the signal corresponding to p.Lys155Gln, we genotyped additional samples totaling 4,526 cases and 3,787 controls and, again, observed strong association (f_control = 0.5%; f_case = 1.3%; follow-up P = 7.7 × 10^-7; combined P = 1.1 × 10^-9; Table 2). In addition, we genotyped 471 families with multiple AMD cases to identify 18 nuclear families where the mutation encoding p.Lys155Gln segregates. These families included 49 affected individuals, with at least 1 individual carrying an allele encoding p.Lys155Gln, and, adjusting for ascertainment, we estimated that 75% of the first-degree relatives of a p.Lys155Gln carrier who also had AMD would carry the variant, consistent with an OR of ~3 (Online Methods and Supplementary Table 5).

Further strong evidence for association of this variant with macular degeneration is provided in independent work by deCODE Genetics3 examining 1,143 Icelandic macular degeneration cases and 51,435 Icelandic controls (f_control = 0.55%; OR = 3.45; deCODE P = 1.1 × 10^-7; combined P = 1.6 × 10^-15). In 1,606 directly genotyped cases of macular degeneration from AREDS2 (ref. 32), the variant had a frequency of 1.77%, similar to our sequenced AMD cases (1.10%) and our follow-up AMD cases (1.30%) and notably higher than our sequenced controls (0.30%), our genotyped controls (0.50%), NHLBI ESP participants with primarily European ancestry (0.40%) and deCODE controls (0.55%). We found no evidence of the p.Lys155Gln variant in a small sample of individuals with atypical hemolytic uremic syndrome (aHUS; n = 53), a rare disorder whose genetic risk factors partially overlap with those of macular degeneration.

We next investigated the potential functional consequences of the p.Lys155Gln variant in silico. On the basis of protein crystallography, the model in Figure 1 shows that CFH variant p.Arg1210Cys (OR = 23.11), C3 variant p.Lys155Gln (OR = 2.91) and C3 variant p.Arg102Gly (OR = 1.31) all map near the surface where CFH and C3b interact, suggesting that they might affect binding of complement factor H to C3b. CFH inhibits C3b and limits the immune responses mediated by the alternative complement pathway. We hypothesize that p.Lys155Gln and p.Arg102Gly affect binding of the first macroglobular domain of C3 to CFH and thus interfere with inactivation of the alternative complement pathway, a hypothesis that must be confirmed with additional functional studies.

The first two Sushi domains from 2wii were docked to the binding site in C3b. The C-terminal Sushi domains were docked to the binding site in C3b. The two α-macroglobulin domains of C3b, MG-1 and MG-2, are shown in green and cyan, respectively. The locations of the p.Arg102Gly, p.Lys155Gln and p.Arg1210Cys alterations are marked in red.

**Table 2** Follow-up genotyping summary and meta-analysis summary

| Sample set                           | Controls |           | Cases |           | P value |
|--------------------------------------|----------|-----------|-------|-----------|---------|
|                                      | N        | MAF   | N     | MAF   |         |
| Discovery sample                     |          |        |       |        |         |
| Sequenced samples (N = 4,536)        | 2,268    | 0.004  | 2,268 | 0.011  | 2.7 × 10^-4 |
| Follow-up samples                    |          |        |       |        |         |
| Germany: University of Regensburg (N = 2,976) | 1,147 | 0.006  | 1,829 | 0.016  | 1.7 × 10^-3 |
| United States: Vanderbilt/Miami (N = 1,819) | 726    | 0.004  | 1,093 | 0.007  | 3.5 × 10^-1 |
| Netherlands: Rotterdam Study (N = 1,409) | 1,280 | 0.005  | 129   | 0.031  | 1.5 × 10^-4 |
| UK: Cambridge AMD Study (N = 1,279)  | 423      | 0.006  | 856   | 0.015  | 6.2 × 10^-2 |
| United States: University of California, Los Angeles/University of Pittsburgh (N = 830) | 211    | 0.004  | 619   | 0.017  | 8.3 × 10^-4 |
| deCODE study                        |          |        |       |        |         |
| deCODE discovery sample (N = 52,578) | 51,435   | 0.005  | 1,143 |         | 1.1 × 10^-7 |
| Meta-analysis                        |          |        |       |        |         |
| All follow-up samples (N = 8,313)    | 3,787    | 0.005  | 4,526 | 0.013  | 7.7 × 10^-7 |
| Discovery and all follow-up samples (N = 12,849) | 6,055 | 0.005  | 6,794 | 0.013  | 1.1 × 10^-9 |
| Discovery, all follow-up and deCODE samples (N = 65,427) | 57,490 | 0.005  | 7,937 |         | 1.6 × 10^-15 |

The table includes the number of cases and controls in each comparison, the corresponding allele frequency for the allele encoding p.Lys155Gln in each set of samples and the P value for a comparison of allele frequencies in cases and controls. Meta-analysis P values were calculated using Stouffer’s method.

*MAF values are unavailable for imputed cases from the deCODE study.

**Figure 1** C3 variants p.Arg1210Gly and p.Lys155Gln and CFH variant p.Arg1210Cys are in the interaction domains of the first α-macroglobular domains of C3b and CFH, respectively. A fragment of the crystal structure of the four Sushi domains of CFH (purple; one not shown for clarity) in a complex with complement fragment C3b (Protein Data Bank (PDB) 2wii) was used to explore the effect of disease-associated nonsynonymous changes. CFH residues 987–1230 were used to generate the structure with the first four Sushi domains from 2wii serving as a structural template (light purple, with cysteine residue side chains in yellow). The C-terminal Sushi domains were docked to the binding site in C3b. The two α-macroglobulin domains of C3b, MG-1 and MG-2, are shown in green and cyan, respectively. The locations of the p.Arg102Gly, p.Lys155Gln and p.Arg1210Cys alterations are marked in red.
exponentially. Interestingly, the three variants (p.Arg102Gly and p.Lys155Gln in C3 and p.Arg1210Cys in CFH) all involve the replacement of a positively charged residue.

In summary, our work and that described in the companion paper identify p.Lys155Gln as a rare C3 variant associated with ~2.91-fold increased risk of macular degeneration. Together with rare CFH variant p.Arg1210Cys and previously described common C3 variant p.Arg102Gly, p.Lys155Gln may reduce binding of CFH to C3b, inhibiting the ability of CFH to inactivate the alternative complement pathway. Clarifying the mechanistic impact of p.Lys155Gln is likely to be challenging, as illustrated by contradictory results from previous functional follow-up studies of AMD-associated loci34–36. But functional studies of complement activity suggest potential next steps33,37. Our work relied on targeted sequencing of GWAS-identified loci, genetic ancestry matching of our sequenced samples to additional sequenced controls analyzed with the same variant calling and filtering tools, focused analysis of regions deeply sequenced in both our project and previously sequenced controls, and avoidance of common calling artifacts near insertion–deletion polymorphisms. The use of publicly available samples to augment control sets may be useful in many targeted sequencing studies, but the strictness of matching and variant filtering required to prevent false positive findings due to population stratification and/or sequence analysis artifacts are areas deserving of further study. As the number of sequenced human genomes and exomes grows, we expect that the usefulness of the approach will grow, making it possible to match multiple controls to each case and to focus on progressively finer ancestry matches. Although our results emphasize that large sample sizes will be required for rare variant studies of complex human traits, they also show the promise of these studies for clarifying disease biology.

URLs. LASER software for estimation of genetic ancestry can be obtained from http://genome.sph.umich.edu/wiki/LASER. UMAKE and GotCloud tools for variant calling can be obtained from http://genome.sph.umich.edu/wiki/UMAKE and http://genome.sph.umich.edu/wiki/GotCloud, respectively. The QPLOT tool for assessing sequence quality can be obtained from http://genome.sph.umich.edu/wiki/QPLOT.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

R.K.W., J.R.H., E.Y.C., D.S., E.R.M., A.S. and G.R.A. conceived, designed and supervised the experiments. X.Z. and G.R.A. wrote the initial version of the manuscript. X.Z., D.E.L., C.W. and D.C.K. analyzed the data. D.E.L., D.C.K., R.S.F., L.F.F. and C.C.F. supervised data generation. C.W. developed statistical methodology. Y.V.S. analyzed protein structures. K.E.B. supervised sample and data collection. J.R.-G., G.J., Y.H., H.M.K. and D.L. contributed data and analysis tools. M.B., R.R. and A.B. assisted in laboratory experiments. M.O. and E.G. carried out experimental studies (genotyping and data analysis) for the Michigan and Regensburg samples, respectively. C.V.S. recruited the family members of sporadic AMD cases and controls and collected peripheral blood samples for the Regensburg study. L.M.O., M.A.P.-V. and J.L.H. provided results and analysis for the Vanderbilt/Miami samples. G.H.S.B., A.H., C.M.x.D. and C.C.W.K. provided results and analysis for samples from the Rotterdam Study, Erasmus Medical Center. V.C., A.T.M., H.S. and J.R.W.Y. provided results and analysis for the Cambridge AMDD Study samples. Y.I., Y.P.C., D.E.W. and M.B.G. provided results and analysis for the University of California, Los Angeles/University of Pittsburgh samples. D.J.M., I.K.K., L.A.F. and M.M.D. provided results and analysis for the Utah samples. M.P.I., J.B. and M.L.K. provided results and analysis and for the Oregon Health Sciences Center samples. S.C., A.I.R., R.H.G. and P.N.B. provided results and analysis for the University of Melbourne samples. H.L., H.O., M.M.Z. and K.Z. provided results and analysis for the University of California, San Diego samples. C.L. and E.G.P. provided results and analysis for a cohort of individuals with aHUS. B.H.F.W. was involved in the design and planning of the Southern Germany AMD Study. B.H.F.W. participated in study coordination and critically read the manuscript. All authors have critically commented on this manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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1. Priya, R.R., Chew, E.Y. & Swaroop, A. Genetic studies of age-related macular degeneration: lessons, challenges, and opportunities for disease management. Ophthalmology 119, 2526–2536 (2012).
2. Swaroop, A., Chew, E.Y., Rickman, C.B. & Abecasis, G.R. Unraveling a multifactorial late-onset disease: from genetic susceptibility to disease mechanisms for age-related macular degeneration. Annu. Rev. Genomics Hum. Genet. 10, 19–43 (2009).
3. Friedman, D.S. et al. Prevalence of age-related macular degeneration in the United States. Arch. Ophthalmol. 122, 564–572 (2004).
4. Haines, J.L. et al. Complement factor H variant increases the risk of age-related macular degeneration. Science 308, 419–421 (2005).
5. Edwards, A.O. et al. Complement factor H polymorphism and age-related macular degeneration. Science 308, 421–424 (2005).
6. Klein, R.J. et al. Complement factor H polymorphism in age-related macular degeneration. Science 308, 385–389 (2005).
7. Jakobssdotir, J. et al. Susceptibility genes for age-related maculopathy on chromosome 1qo26. Am. J. Hum. Genet. 77, 389–407 (2005).
8. Rivera, A. et al. Hypothetical LOC387715 is a second major susceptibility gene for age-related macular degeneration, contributing independently of complement factor H to disease risk. Hum. Mol. Genet. 14, 3227–3236 (2005).
9. Yates, J.R. et al. Complement C3 variant and the risk of age-related macular degeneration. N. Engl. J. Med. 357, 553–561 (2007).
10. Gold, B. et al. Variation in factor B (BF) and complement component 2 (C2) genes is associated with age-related macular degeneration. Nat. Genet. 38, 458–462 (2006).
11. Fagerness, J.A. et al. Variation near complement factor 1 is associated with risk of advanced AMD. Eur. J. Hum. Genet. 17, 100–104 (2009).
12. Maier, J.B. et al. Variation in complement factor 3 is associated with risk of age-related macular degeneration. Nat. Genet. 39, 1200–1201 (2007).
13. Fratich, L.G. et al. Seven new loci associated with age-related macular degeneration. Nat. Genet. 45, 433–439 (2013).
14. Arakawa, S. et al. Genome-wide association study identifies two susceptibility loci for exudative age-related macular degeneration in the Japanese population. Nat. Genet. 43, 1001–1004 (2011).
15. Nejentsev, S., Walker, N., Riches, D., Egholm, M. & Todd, J.A. Rare variants of IFIH1, a gene implicated in antiviral responses, protect against type 1 diabetes. Science 324, 387–389 (2009).
16. Raychaudhuri, S. et al. A rare penetrant mutation in CFH confers high risk of age-related macular degeneration. Nat. Genet. 43, 1232–1236 (2011).
17. Józsi, M. et al. Factor H and atypical hemolytic uremic syndrome: mutations in the C-terminus cause structural changes and defective recognition functions. J. Am. Soc. Nephrol. 17, 170–177 (2006).
18. Manuelian, T. et al. Mutations in factor H reduce binding affinity to C3b and heparin and surface attachment to endothelial cells in hemolytic uremic syndrome. J. Clin. Invest. 111, 1181–1190 (2003).

19. Ferreira, V.P. et al. The binding of factor H to a complex of physiological polyanions and C3b on cells is impaired in atypical hemolytic uremic syndrome. J. Immunol. 182, 7009–7018 (2009).

20. Chen, W. et al. Genetic variants near TIMP3 and high-density lipoprotein–associated loci influence susceptibility to age-related macular degeneration. Proc. Natl. Acad. Sci. USA 107, 7401–7406 (2010).

21. Age-Related Eye Disease Study Research Group. Risk factors associated with age-related macular degeneration. A case-control study in the age-related eye disease study: Age-Related Eye Disease Study Report Number 3. Ophthalmology 107, 2224–2232 (2000).

22. Tennenbaum, J.A. et al. Evolution and functional impact of rare coding variation from deep sequencing of human exomes. Science 337, 64–69 (2012).

23. Fu, W. et al. Analysis of 6,515 exomes reveals the recent origin of most human protein-coding variants. Nature 493, 216–220 (2013).

24. 1000 Genomes Project Consortium. An integrated map of genetic variation from 2,092 human genomes. Nature 491, 56–65 (2012).

25. Mathieson, I. & McVean, G. Differential confounding of rare and common variants in spatially structured populations. Nat. Genet. 44, 243–246 (2012).

26. Li, J.Z. et al. Worldwide human relationships inferred from genome-wide patterns of variation. Science 319, 1100–1104 (2008).

27. Wellcome Trust Case Control Consortium. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. Nature 470, 661–678 (2007).

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ONLINE METHODS

Study samples. Macular degeneration cases and controls were recruited at ophthalmology clinics at the University of Michigan and the University of Pennsylvania and through the AREDS, as previously described. For replication, we contacted members of the International AMD Genetics Consortium; their samples are described in Fritsche et al. All participants provided informed consent allowing for the collection of genetic data, and all data contributors obtained approval from their local institutional review boards before generating genetic data. Our discovery sample, with ~2,350 sequenced cases and ~750 sequenced controls, provides 90% power to discover variants before generating genetic data. Our discovery sample, with ~2,350 sequenced contributors obtained approval from their local institutional review boards.

Sequence production and quality control. Illumina multiplexed libraries were constructed according to the manufacturer’s protocol with modifications: (i) DNA was fragmented using a Covaris E220 DNA Sonicator to range in size between 100 and 400 bp, (ii) Illumina adaptor-ligated library fragments were amplified in a 50–µl PCR runs for 18 cycles, and (iii) Solid-Phase Reversible Immobilization (SPRI) bead cleanup was used for enzymatic purification and final library size selection targeting 300–500-bp fragments. Samples were pooled in groups of 4–24 before hybridization. A custom targeted probe set of 150-bp probes was designed (Agilent Technologies) and captured 0.97 Mb of sequence. The concentration of each captured library pool was determined through quantitative PCR (Qapa Biosystems) to produce cluster counts appropriate for the Illumina Genome Analyzer Ix and HiSeq 2000 platforms. We generated approximately 1.7 Gb of sequence per sample, covering 80% of the targeted space at a depth of >20x. Reads were aligned to the NCBI37/hg19 reference sequence using Burrows-Wheeler Aligner (BWA). Where pre-existing genotype information was available, sample identity was confirmed by comparing sequence data with pre-existing array data.

Quality control and variant calling. Quality control steps for all BAM files included removal of duplicated reads; recalibration of base qualities; generation of diagnostic graphs and evaluation of sequencing quality (QPLLOT; see URLs); and checks for DNA contamination. After removing samples with high contamination, unexpected relatedness or high discordance rate, we retained 2,335 cases and 789 controls for an initial round of analysis. We calculated the sequencing depth using reads with mapping quality of >30 and bases with quality of >20. Across the 966,607-bp target region, we retained an average of 123,221,974 bases per individual (127.5× average coverage). Within targeted regions, 98.49% of the protein-coding exons had coverage of >10x.

We performed the variant calling step using UMAKE. Genotype calling and polymorphism discovery were attempted across the original target ±0.50kb. To remove low-quality variants, we excluded (i) sites with average depth of <0.5 or >500; (ii) sites with evidence of strand bias or cycle bias; (iii) sites within 5 bp of a 1000 Genomes Project indel; and (iv) sites with excess heterozygosity. These filters excluded 15,219 low-quality variants. The transition-transversion ratio (Ts/Tv) for the remaining 31,527 sites was 2.10. Concordance rates between sequencing-based genotypes in 13 duplicates were 99.82% when depth was >10x. Concordance with array-based genotypes was 98.99% when depth was >10x.

Overall, 59.8% of discovered variants were newly identified (compared to dbSNP135 and the 1000 Genomes Project). On average, each sample carried 40 synonymous variants, 34 nonsynonymous variants and 1 nonsense variant.

Initial analyses. We first performed single-variant association tests using Fisher’s exact test. This analysis confirmed strong association for common variants near the CFH, C2, ARMS2 and C3 genes. An initial examination of rare variants suggested that some signals were shadows of common variants with larger effects, so we focused on those signals where association remained significant after accounting for nearby common variants. Conditional signals were evaluated by exact logistic regression. Three coding variants had conditional exact P values < 0.01 (all also had marginal P values < 0.001).

Augmenting our sample. We sought ancestry-matched controls among samples sequenced in ESP. First, we used genome-wide reads to infer sample ancestries on a worldwide population map. Briefly, we first generated a genetic ancestry PCA space using genotyped reference samples (such as those from the Human Genome Diversity Panel). Then, we generated a series of sample-specific genetic ancestry PCA data that were calibrated to the exact sequencing depth and coverage pattern of each sample and included the reference samples together with a single sequenced sample. Finally, we transformed sample-specific PCA coordinates onto the original map using Procrustes analysis. This procedure generates a metric (Procrustes similarity) that summarizes the similarity of reference sample placements using array genotypes to placements using sequencing data, and we only considered samples where this metric was >0.95 as candidates for matching. Second, we used a procedure inspired by propensity score matching to pair cases and controls. For matching, we used a greedy algorithm to match cases and controls, allowing matches when the respective propensity scores differed by <0.001. An alternative matching algorithm that matched cases and controls mapping close together in principal-component space according to the Euclidean distance between them gave similar results (association at p.Lys155Gln had OR = 2.68; exact P = 4.5 × 10−5 using Fisher’s exact test).

To avoid artifacts from variant calling, we applied very stringent filters to both the AMD study and ESP study call sets. For both studies, we examined only sites with call rates of >90% and Phred-scaled variant quality scores of >30 that passed all study-specific quality control filters, had depth of >10x for >90% of the samples in the AMD or ESP call sets and were >5 bp from a 1000 Genomes Project indel. Primers used to confirm the presence of the mutation encoding p.Lys155Gln by Sanger sequencing are given in Supplementary Table 6.

Analyses using the combined AMD and ESP data set. As in our initial analysis, we first applied Fisher’s exact test for association with all variants. Next, we examined variants with frequency of <1% for which signal remained significant after adjusting for common variants. This analysis highlighted p.Arg1210Cys encoded by CFH and p.Lys155Gln encoded by C3 (Fig. 1).

Linkage disequilibrium analysis. To search for variants that might explain the signal encoding p.Lys155Gln, we evaluated linkage disequilibrium between the variant encoding p.Lys155Gln and all variants within 1 Mb, both within the samples sequenced for this experiment and also in preliminary whole-genome sequence data for 600 individuals (300 macular degeneration cases and 300 controls; A.S., D.S. and G.R.A., unpublished data). This analysis did not find variants in strong linkage disequilibrium in the nearby region. The variant was only present in one 1000 Genomes Project sample, which did not allow for reliable estimates of linkage disequilibrium.

Segregation analysis. In a segregation analysis, one identifies probands who carry p.Lys155Gln and then evaluates the probability that they transmit the variant to affected relatives (under the null hypothesis, we would expect to find the variant in 50% of the first-degree relatives of a carrier). We genotyped 471 pedigrees with multiple affected individuals. In each pedigree where p.Lys155Gln was found in more than one affected individual, we selected the nuclear family with the largest number of affected individuals. We recorded the number of affected individuals (N) and the number of carriers of p.Lys155Gln (C). Then, to average over possible choices of proband, we assigned each family specifically a weight of C/N (this is the probability that a randomly selected proband in the family carries p.Lys155Gln) and then scored the number of affected first-degree relatives (N – 1) and carriers among those (C – 1). The estimated fraction of carriers among affected first-degree relatives of a proband was then calculated by summing C/N × (C – 1) and C/N × (N – 1) over families and taking the ratio of the two quantities.
38. Li, H. & Durbin, R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 25, 1754–1760 (2009).
39. McKenna, A. et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res. 20, 1297–1303 (2010).
40. Jun, G. et al. Detecting and estimating contamination of human DNA samples in sequencing and array-based genotype data. Am. J. Hum. Genet. 91, 839–848 (2012).
41. Cox, D.R. & Shell, E.J. Analysis of Binary Data 2nd edn. (CRC Press, New York, 1989).
42. Hirji, K.F., Mehta, C.R. & Patel, N.R. Computing distributions for exact logistic-regression. J. Am. Stat. Assoc. 82, 1110–1117 (1987).
43. Rosenbaum, P.R. & Rubin, D.B. The central role of the propensity score in observational studies for causal effects. Biometrika 70, 41–55 (1983).