Dissection of Chromosome 16p12 Linkage Peak Suggests a Possible Role for *CACNG3* Variants in Age-Related Macular Degeneration Susceptibility

Kylee L. Spencer,[1] Lana M. Olson,[1] Nathalie Schnetz-Boutaud,[1] Paul Gallins,[2] Gaofeng Wang,[2] William K. Scott,[2] Anita Agarwal,[3] Johanna Jakobsdottir,[4] Yvette Conley,[5] Daniel E. Weeks,[5] Michael B. Gorin,[6] Margaret A. Pericak-Vance,[2] and Jonathan L. Haines[1]

PURPOSE. Age-related macular degeneration (AMD) is a complex disorder of the retina, characterized by drusen, geographic atrophy, and choroidal neovascularization. Cigarette smoking and the genetic variants CFH Y402H, ARMS2 A69S, CFB R32Q, and C3 R102G have been strongly and consistently associated with AMD. Multiple linkage studies have found evidence suggestive of another AMD locus on chromosome 16p12 but the gene responsible has yet to be identified.

METHODS. In the initial phase of the study, single-nucleotide polymorphisms (SNPs) across chromosome 16 were examined for linkage and/or association in 575 Caucasian individuals from 148 multiplex and 77 singleton families. Additional variants were tested in an independent dataset of unrelated cases and controls. According to these results, in combination with gene expression data and biological knowledge, five genes were selected for further study: *CACNG3*, *HS3ST4*, *IL4R*, *Q7Z6F8*, and *ITGAM*.

RESULTS. After genotyping additional tagging SNPs across each gene, the strongest evidence for linkage and association was found within *CACNG3* (rs757200 nonparametric LOD* = 3.3, APL (association in the presence of linkage) $P = 0.06$, and rs2238498 MQLS (modified quasi-likelihood score) $P = 0.006$ in the families; rs2283550 $P = 1.3 \times 10^{-6}$, and rs4787924 $P = 0.002$ in the case–control dataset). After adjusting for known AMD risk factors, rs2283550 remained strongly associated ($P = 2.4 \times 10^{-4}$). Furthermore, the association signal at rs4787924 was replicated in an independent dataset ($P = 0.035$) and in a joint analysis of all the data ($P = 0.001$).

CONCLUSIONS. These results suggest that *CACNG3* is the best candidate for an AMD risk gene within the 16p12 linkage peak. More studies are needed to confirm this association and clarify the role of the gene in AMD pathogenesis. (*Invest Ophthalmol Vis Sci.* 2011;52:1748–1754) DOI:10.1167/iovs.09-5112

Age-related macular degeneration (AMD) is a late-onset disorder characterized by central field vision loss that may eventually lead to legal blindness. Genetic, environmental, and behavioral factors modulate susceptibility to AMD. The Y402H variant within the complement factor H (*CFH*) gene was the first widely agreed on AMD risk allele identified,[1–3] and candidate studies of other genes within the complement pathway soon led to the discovery of many other AMD-associated loci, including *C2/CFB*,[4–6] *C3*,[7–9] and *CFHR3/CFHR1*.[10,11] The *HTRA1/ARMS2* region on chromosome 10 contains several clearly associated alleles,[12–14] though a definitive causal allele has yet to be identified due to the extensive linkage disequilibrium (LD) throughout the region, and the mechanism by which one or more of these genes is involved in AMD pathogenesis remains unknown. Despite these discoveries, additional unidentified AMD susceptibility loci are likely, since the known genes do not explain all the genetic effects in AMD. Fine mapping under strong linkage peaks from genome-wide screens is one strategy being applied to narrow this search.

Chromosome 16p12 was first proposed to harbor an AMD susceptibility locus based on the results of a genome-wide linkage screening of 263 sib pairs from 102 pedigrees in the Beaver Dam Eye Study (BDES).[15] Strong linkage was observed across a 6-cM region centered at ~18 cM (26 Mb; *D16S679*; $P = 0.0086$). Interestingly, in this analysis AMD was treated as a quantitative trait by using the 15-level Revised Wisconsin Age-Related Maculopathy Coding Protocol.[15] This system assigns a severity score to each individual by considering (1) drusen size, type, and area; (2) pigmentary abnormalities; (3) geographic atrophy; and (4) signs of exudative AMD.

Using this same quantitative trait definition of AMD, linkage to D16S679 was replicated in the Family ARM Study (FARMS) of 349 sib pairs from 34 extended families ($P = 0.0046$).[16] In contrast to the BDES, a community-based study with the full range of AMD severity scores represented, FARMS probands were ascertained from a retinal clinic and typically had severe AMD. Linkage to the same marker in two independent populations using different ascertainment schemes, raises confidence that 16p12 harbors a true susceptibility locus. Furthermore, meta-analysis of the six largest AMD linkage screens confirmed this locus ($P = 0.02$ for the 30-cM bin ranging from

17 to 52 Mb).[17] Interestingly, there was some evidence of heterogeneity between studies (heterogeneity $P = 0.01$), suggesting that only a subset of families may be linked to this region.

We also observed that only a subset of families was linked to 16p12 in our own initial analysis, in which a multipoint LOD score less than 1.0 rose to between 2.0 and 3.0, after using ordered subset analysis to consider important clinical covariates.[18] Linked families on average tended to have higher systolic blood pressure, higher intraocular pressure (IOP), and higher body mass index (BMI). By using a genome-wide linkage screen in the families and searching for areas of association that overlapped in both the family-based dataset and an independent case–control dataset, these preliminary analyses narrowed the region of interest to between 10 and 31 Mb, an interval containing hundreds of genes. Our goal was to further narrow the minimum candidate region and then to select candidate genes to test for association.

MATERIALS AND METHODS

Discovery Study Populations

Multiplex and singleton families, cases, and controls were ascertained through ophthalmology clinics at Vanderbilt and Duke University Medical Centers. All participants were of Caucasian descent. All patients and control subjects received an eye examination and had stereoscopic fundus photographs graded according to a modified version of the Age-Related Eye Disease Study (AREDS) grading system, as described elsewhere.[19,20] Briefly, grades 1 and 2 represent the controls. Grade 1 controls have no evidence of drusen or small (<63 μm), nonextensive (<15) drusen without pigmentary abnormalities, while grade 2 controls may show signs of either extensive small drusen or nonextensive intermediate drusen (63-124 μm) and/or pigmentary abnormalities. Grade 3 AMD cases have extensive intermediate drusen or large, soft drusen (≥125 μm), with or without drusenoid retinal pigment epithelial detachment. Grade 4 AMD cases exhibit geographic atrophy, and grade 5 individuals have exudative AMD, which includes nondrusenoid retinal pigment epithelial detachment, choroidal neovascularization, and subretinal hemorrhage or disciform scarring. Individuals were classified according to status in the more severely affected eye. Approval for the study was obtained from the appropriate institutional review boards at all participating institutions, all study participants gave informed consent, and the research adhered to the tenets of the Declaration of Helsinki.

Multistage Design

In the initial phase of the study, a genome-wide linkage screen was conducted in our family-based dataset, and supplemental SNPs were genotyped in both the families and unrelated cases and controls. Based on the linkage and association results from phase I, gene expression data, and literature review, five candidate genes were selected for follow-up in phase II of the study.

In phase I, 182 SNPs were genotyped in the family-based dataset across the length of chromosome 16 as part of a genome-wide screen (Human Linkage IVb panel; Illumina, Inc. San Diego, CA), according to the manufacturer's instructions. An additional 153 SNPs were genotyped (Taqman; Applied Biosystems, Inc. [ABI], Foster City, CA) in both the family-based and case–control datasets, based on even spacing and assay availability. At that time, our datasets included 541 individuals from 208 families and an independent group of 591 cases and 256 unrelated controls. In phase II, an additional 72 SNPs selected to tag common variation within the candidate genes were genotyped in both the family-based and case–control datasets. In phase II, our datasets grew to include 575 individuals from 225 families and an independent group of 721 cases and 377 controls (Table 1).

Selection of Candidate Genes for Phase II

Of the ~110 annotated genes in the region of interest, 29 had at least one interesting result (according to the arbitrarily chosen cut-offs of LOD > 2.0 or association $P < 0.01$), and only four had interesting results in both the family-based and case–control datasets: *CACNG3*, *HS3ST4*, *IL4R*, and *Q7Z6F8*. *ITGAM* was also selected as a candidate, as it lies within the region of linkage and serves as a subunit in the receptor for complement component 3, a gene that has been implicated in AMD.[7-9] Gene expression data were unhelpful in further narrowing this list, as all were either expressed in the eye or not assayed in EyeSAGE, a database of gene expression profiles of the human retina.[21]

Genotyping Quality Control

For all genotyping methods, duplicate samples within and across plates were checked for concordance. We verified that all SNPs were in Hardy-Weinberg equilibrium (HWE) in both the family-based and case–control datasets. HWE in the case–control dataset was examined both in the overall dataset and separately in the cases and controls. We used only founders to estimate allele frequencies in the family-based dataset, except when a family did not have any founders genotyped. For those families, one individual was selected at random to contribute to the allele frequency calculation. In the family-based dataset, three SNPs were excluded from analysis for failing HWE (threshold $= P < 0.0001$, the cutoff for a Bonferroni correction for testing 407 SNPs). In the case–control dataset, two SNPs failed HWE (threshold $= P < 0.0002$, the cutoff for a Bonferroni correction for testing 225 SNPs). One SNP was removed from both datasets because it was monomorphic. Over the course of the study, additional samples were added as they were ascertained. For each SNP, 95% of the samples tested had to produce a genotype for the SNP to be used in subsequent analyses.

Linkage Disequilibrium

We examined the patterns of LD in the family-based and case–control datasets using Haploview (http://www.broad.mit.edu/mpg/haploview/ provided in the public domain by The Broad Institute, Massachusetts Institute of Technology, Cambridge, MA). We studied the overall case-control dataset, in addition to cases and controls separately. This

TABLE 1. Demographic Characteristics of the Datasets

	Family Dataset	Independent Case-Control Dataset	
Individuals	575 phenotyped (148 Mx, 77 Singleton families)	721 cases (grades 3, 4, 5)	377 controls (grades 1, 2)
Grade, %	1:61.8% 3:29.4%	3:27.7%	1:80.4%
	2:38.2% 4:14.2%	4:12.9%	2:19.6%
	5:56.4%	5:59.4%	
Mean age, y (SD)	66.8 (9.9) (unaffected)	76.4 (7.6)	69.1 (8.0)
	74.6 (9.2) (affected)		
Female, %	66.1	63.4	57.6
Ever smoked, %	56.9 (unaffected)	58.9	42.2
	50.0 (affected)		

analysis allowed us to (1) select a subset of SNPs for the multipoint linkage analysis that had pair-wise r^2 values no greater than 0.16, as SNPs in strong LD may bias LOD scores when parental genotypes are missing[22]; (2) study the pattern of LD in the overall dataset and also separately in cases and controls, as differing patterns may indicate a region of association; and (3) determine whether those SNPs showing interesting results (LOD > 2.0 or $P <$ 0.01) in subsequent analyses were independent or coming from a block of LD.

Statistical Analysis in the Family-Based Dataset

Two-point dominant and recessive LOD scores were calculated with Fastlink,[23,24] two-point nonparametric LOD scores were calculated with Allegro,[25,26] and multipoint nonparametric, dominant, and recessive LOD scores were estimated with Merlin.[27] We specified the following parameters for the parametric models, effectively creating an affecteds-only analysis, with a dominant model: disease allele frequency = 0.01, $f_0 = 0.0000$, $f_1 = 0.0001$, $f_2 = 0.0001$, where f_i is the penetrance of an individual with i susceptibility alleles; and a recessive model: disease allele frequency = 0.14, $f_0 = 0.0000$, $f_1 = 0.0000$, $f_2 = 0.0001$. Ordered subset analysis (OSA)[28] was used to examine the evidence for linkage in subsets of families determined by body mass index, intraocular pressure, and systolic and diastolic blood pressure. We tested each SNP for association in the families with association in the presence of linkage (APL)[29] and pedigree disequilibrium test (PDT),[30,31] as our dataset contains a mix of pedigree structures and it is unclear which method is optimal in this situation. APL is more powerful than PDT in many circumstances,[29] but APL is not valid in the 20 extended families that comprise nearly 10% of our dataset. In phase II of the study, we used also used the recently developed modified quasi-likelihood score (MQLS)[32] association test in the family-based dataset. Haplotype analysis was performed with a two-SNP sliding window in APL. The generalized estimating equation (GEE) method[33] was used to test for association after adjustment for age and sex.

Statistical Analysis in the Case–Control Dataset

In the case–control dataset we tested for association using the allele, genotype, and trend models implemented in PowerMarker.[34] We used Haploview to conduct haplotype analysis within candidate genes. Three SNPs (rs757200, rs2283550, and rs4787924) were selected for follow-up via logistic regression. We estimated odds ratios under additive, dominant, and recessive logistic regression models for the chromosome 16 SNPs, adjusting for age, smoking, CFH Y402H, ARMS2 A69S, CFB R32Q, and C3 R102G. We derived an ever/never smoking variable by coding those who responded that they had smoked at least 100 cigarettes as 1 and those who had not as 0. Age at examination was included as a continuous variable.

Analysis by Grade of AMD

All analyses were performed in the group of all AMD cases (grades 3, 4, and 5) compared with all AMD controls (grades 1 and 2). We also performed a subset of these analyses in the neovascular AMD cases only (grade 5, one eye or both eyes compared with grade 1 controls), to consider the possibility that the chromosome 16 susceptibility locus predisposes specifically to the neovascular form of AMD. These analyses included two-point LOD score calculations and single-SNP tests of association, but not the multipoint linkage, OSA, or haplotype analyses due to the reduced sample size.

Copy Number Variant Assays

A copy number variant (variant 4935 in the Database of Genomic Variants[35]) with the putative 3′ breakpoint lying within the first intron of the *CACNG3* gene was investigated in phase II of the study (Taqman Copy Number Assays; ABI) according to the manufacturer's instructions. Because the breakpoints are not known with certainty, six probes were designed to cover approximately evenly spaced intervals spanning the 5′ upstream region, first exon, and first intron of

CACNG3. All samples were run in quadruplicate, and RNaseP served as the control in the real-time PCR reaction.

Replication in the Pittsburgh/UCLA Dataset

We selected 10 tagging SNPs in and around *CACNG3* based on LD patterns in individuals of European descent (CEU) from the International HapMap Project[36] for genotyping in a replication cohort of 607 families with 1073 affected members and 106 unaffected members, plus an additional unrelated set of 177 cases and 120 controls from the University of Pittsburgh and University of California at Los Angeles (Taqman; ABI). The individuals in the Pittsburgh/UCLA cohort were all Caucasian and ascertained through recruitment of families from the retina clinic at the University of Pittsburgh and by mass mailings sent to patients suspected to have AMD from records of ICD-9 billing codes from multiple retina and ophthalmic practices across the United States.[37,38] All participants underwent fundus photography and/or extensive review of eye care records and were classified as affected or unaffected with AMD according to three decreasingly conservative definitions (A, B, and C), as described elsewhere.[39] For this study, only the most restrictive definition was used (A), so that affected individuals presented with extensive/coalescent drusen, pigmentary abnormalities, and/or signs of late-stage disease, including geographic atrophy and choroidal neovascularization. Unaffected individuals had to have fewer than 10 hard drusen, none greater than 50 μm in diameter, and no other retinal pigment epithelium changes. This analysis is comparable to the grades 3, 4, 5 versus 1, 2 analysis in the initial phase of the study.

GEE was used to test for association of SNPs after adjustment for age and sex and taking into account relatedness, under additive, dominant, and recessive models. We also performed a joint analysis of the SNPs in the combined discovery and replication samples, adjusting for the same covariates.

RESULTS

Phase I

Linkage. The peak multipoint LOD score of 2.2 occurred at 22.8 Mb under a dominant model (Fig. 1). Nonparametric and recessive LOD scores peaked at the same location (LOD = 1.6 and 1.4, respectively). Several two-point LOD scores in this region exceeded 1.0, the largest being 3.1 under a nonparametric model at 24.3 Mb in the *CACNG3* gene (data not shown). Using OSA to examine the evidence of linkage, after taking into account blood pressure, IOP, and BMI as covariates, did not significantly change the LOD score ($P >$ 0.10 for all, data not shown). This result implies that the evidence of linkage to this region of chromosome 16 did not increase after
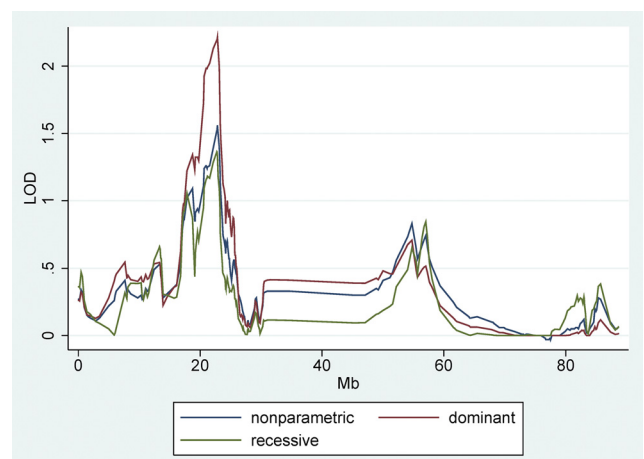

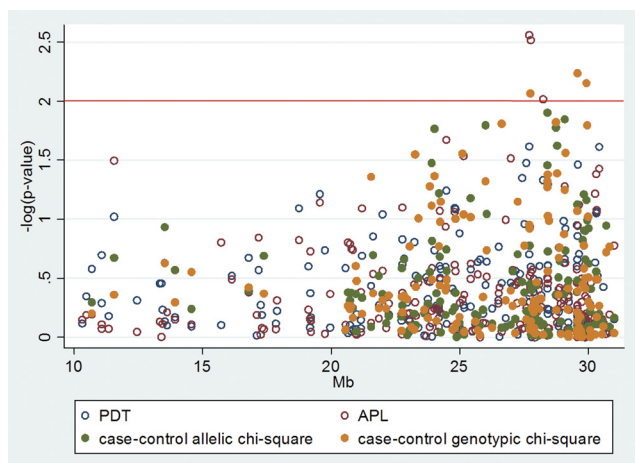**FIGURE 1.** Chromosome 16 multipoint linkage analysis.

FIGURE 2. Phase I association results within the linkage peak on chromosome 16p12. *Open symbols*: family-based results; *filled symbols*: case–control results. *Red line*: the -log(nominal *P*-value) for association tests, corresponding to *P* = 0.01. In phase I, 335 SNPs on chromosome 16 were tested in the family-based dataset and 153 SNPs were tested in the case–control dataset.

these covariates were considered in our expanded dataset; it does not imply that there is no linkage to the region. However, consistent with previous reports, the LOD score in subset of families with high BMI rose to ~2.3 at 22 Mb, although the difference was not statistically significant.

Association. Overall, PDT and APL gave similar results, with the most significant results (*P* < 0.01) clustering between 27.5 and 29 Mb (Fig. 2). Tests of allelic and genotypic association in the case–control dataset also produced a cluster of interesting results in this area, the strongest being a synonymous coding SNP in the apolipoprotein B48 receptor at 28.5 Mb (allelic association *P* = 0.013; Fig. 2).

We used a two-SNP sliding window in APL to test haplotypes for association in the family-based dataset. Haplotypes in the *IL4R* gene at 27.3 Mb and the *Q7Z6F8* gene at 27.8 Mb were associated with disease risk (*P* < 0.001 and *P* = 0.006, respectively, data not shown). In the case–control dataset we tested haplotype blocks defined by Haploview for association. We also used Haploview to test any two-SNP haplotype with *P* < 0.01 in the family-based dataset. A two-SNP haplotype in *IL4R* trended toward significance (*P* = 0.06) in the case–control dataset. The haplotype in the *Q7Z6F8* gene was not replicated in the case–control dataset (*P* = 0.35).

Several SNPs clustering between 23 and 30 Mb produced interesting results in the neovascular AMD analyses in both the family-based and case–control datasets (Supplementary Table S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.09-5112/-/DCSupplemental). These results agreed well with the analyses comparing all cases to all controls (grades 3, 4, 5 versus 1, 2) and suggest that the chromosome 16 AMD locus contributes to overall AMD rather than a particular subtype of the disease.

Selection of Candidate Genes for Phase II. In the phase I analyses, four genes contained variants producing LOD scores >2.0 or association at *P* < 0.01 in both the family-based and case–control datasets: *CACNG3*, *HS3ST4*, *IL4R*, and *Q7Z6F8*. Additional SNPs within these genes were selected for phase II genotyping and analysis. Because *ITGAM* contributes to formation of the receptor for complement component 3 (*C3*) and variants within C3 are strongly associated with AMD,[7–9] it was also selected as a candidate.

Phase II

Linkage. With additional multiplex families, the peak multipoint LOD score in the region decreased to 1.4 at 22.7 Mb under the dominant model (data not shown). The nonparametric and recessive multipoint linkage curves followed the same shape as the dominant model, but were weaker in magnitude. However, *CACNG3* showed even stronger two-point linkage to AMD (nonparametric two-point LOD = 3.3 at rs757200 compared to LOD = 3.1 in phase I). None of the other candidate genes yielded LOD scores greater than 2.0 in any analysis. None of the covariates (blood pressure, IOP, and BMI) used in the OSA analysis significantly increased the LOD score (data not shown), although there was a slight increase in the LOD score for families with higher average BMI (maximum LOD 1.5 at 21.6 Mb, *P* = 0.6).

Association Analysis for *CACNG3*. Of the five candidate genes, variants within *CACNG3* were most robustly and consistently associated with AMD (Fig. 3, Supplementary Table S2, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.09-5112/-/DCSupplemental). There was a high degree of LD across this gene (case–control dataset in Supplementary Fig. S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.09-5112/-/DCSupplemental, family-based dataset not shown). In the case–control dataset, an intronic SNP survived Bonferroni correction for the number of SNPs tested on chromosome 16 (rs2283550 *P* = 1.3 × 10^{-6} under an additive model), though we did not observe association of this SNP in the family-based dataset (APL, PDT, and MQLS *P* > 0.16). Interestingly, the minor allele was more common in the controls, indicating either a protective effect of the minor allele or a risk effect of the major allele. In the families, the most strongly associated SNP was rs2238498 (MQLS *P* = 0.006), although this SNP was not significantly associated in the case–control dataset (*P* > 0.15 in all tests). The most strongly linked SNP (rs757200, LOD* = 3.3) showed a trend for association in the family-based dataset (APL *P* = 0.06). Each of the two-SNP sliding window haplotypes containing rs757200 were also associated with AMD (rs9926669-rs757200 *P* = 0.05, rs757200-rs9921785 *P* = 0.02, data not shown). Limiting the analysis to the most severely affected individuals with neovascular AMD in both eyes compared with grade 1 controls, rs757200 was more strongly associated in the family-based dataset (APL *P* = 0.02, data not shown), despite the reduced sample size. However, rs757200

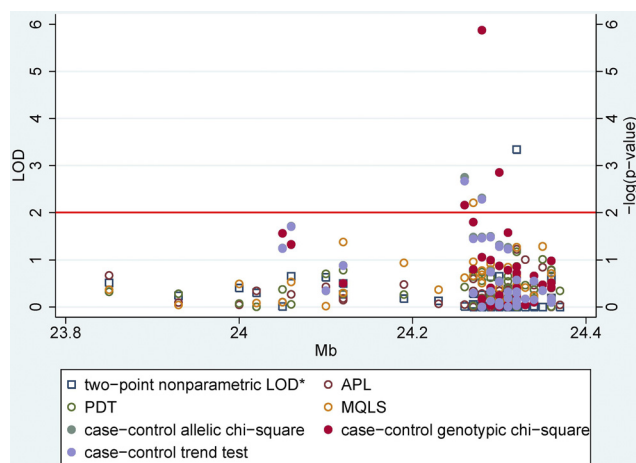**FIGURE 3.** *CACNG3* linkage and association analysis phase II results. Open symbols are family-based results, filled symbols are case–control results. MB = megabases. The -log(nominal *P*-value) is reported for association tests, with a red line drawn corresponding to *P* = 0.01. In all, 407 SNPs on chromosome 16 were tested in the family-based dataset and 225 SNPs were tested in case–control dataset.

was not associated in the case-control dataset ($P > 0.19$ under all genetic models, data not shown). Another SNP just upstream of *CACNG3*, rs4787924, was also associated in the case-control dataset ($P = 0.002$, $P = 0.007$, $P = 0.002$ in allelic, genotypic, and trend models, respectively; Supplementary Table S2, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.09-5112/-/DCSupplemental), although there was no evidence of association at this SNP in the family-based dataset (APL, PDT, and MQLS $P > 0.23$). rs4787924 is in modest LD with rs2283550 ($r^2 = 0.23$, Supplementary Fig. S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.09-5112/-/DCSupplemental), although the minor allele frequencies of the two SNPs are quite different (0.47 vs. 0.20 in the case-control dataset, respectively).

To adjust for environmental factors and other genetic loci known to be associated with AMD, we modeled the three most interesting *CACNG3* SNPs using logistic regression in the case-control dataset, adjusting only for age and sex in the first model, and then adjusting for age, sex, smoking, CFH Y402H, ARMS2 A69S, CFB R32Q, and C3 R102G in a second model (Table 2). rs2283550 was highly significant in both adjusted models (odds ratio [OR] = 0.19, 95% confidence interval [CI], 0.08-0.46, $P = 2.4 \times 10^{-4}$, in the fully adjusted model). rs757200, which was both linked and associated in the family-

TABLE 2. Logistic Regression Analyses of CACNG3 SNPs

Model	Factor	OR	95% CI	P
1	Age	1.13	1.11-1.15	<1.0E-08
	Sex	1.53	1.15-2.04	0.004
	rs2283550	0.18	0.09-0.36	1.9E-06
2	Age	1.13	1.11-1.15	<1.0E-08
	Sex	1.46	1.09-1.94	0.010
	rs757200	1.15	0.7-1.88	0.580
3	Age	1.13	1.10-1.15	<1.0E-08
	Sex	1.53	1.15-2.04	0.003
	rs4787924	1.25	1.03-1.51	0.027
4	Age	1.15	1.12-1.18	<1.0E-08
	Sex	1.56	1.05-2.31	0.028
	Smoking	2.17	1.47-3.19	8.4E-05
	rs2283550	0.19	0.08-0.46	2.4E-04
	CFH Y402H	2.45	1.85-3.24	<1.0E-08
	ARMS2 A69S	2.08	1.57-2.75	3.4E-07
	CFB R32Q	0.38	0.23-0.64	2.4E-04
	C3 R102G	1.51	1.1-2.06	0.010
5	Age	1.15	1.12-1.18	<1.0E-08
	Sex	1.48	1-2.19	0.048
	Smoking	2.14	1.46-3.13	9.4E-05
	rs757200	0.96	0.48-1.92	0.900
	CFH Y402H	2.45	1.86-3.24	<1.0E-08
	ARMS2 A69S	2.11	1.6-2.78	1.3E-07
	CFB R32Q	0.39	0.23-0.64	2.2E-04
	C3 R102G	1.39	1.02-1.88	0.036
6	Age	1.15	1.12-1.18	<1.0E-08
	Sex	1.57	1.06-2.33	0.023
	Smoking	2.14	1.46-3.14	<1.0E-08
	rs4787924	1.18	0.90-1.53	0.233
	CFH Y402H	2.50	1.89-3.31	<1.0E-08
	ARMS2 A69S	2.11	1.60-2.79	<1.0E-08
	CFB R32Q	0.37	0.22-0.61	<1.0E-08
	C3 R102G	1.38	1.02-1.88	0.038

Results are shown in recessive models for rs2283550 and rs757200 and in an additive model for rs4787924. Models 1, 2, and 3 estimate the effect of the *CACNG3* SNPs, adjusting only for age and sex. Models 4, 5, and 6 also adjust for smoking and known AMD loci. Total number of participants in models 1, 2, and 3, ~1080; total number in models 4, 5, and 6, ~710 individuals. Smoking information was missing for majority of the participants (~85%) who were not included in the analyses in models 4, 5, and 6.

based dataset, was not associated with AMD in either adjusted model ($P > 0.5$ for both). rs4787924 was modestly associated after adjustment for age and sex under an additive model (OR = 1.25; 95% CI, 1.03-1.51; $P = 0.027$). Although not significantly associated with AMD in the fully adjusted model ($P = 0.23$), the effect size estimate for rs4787924 was similar (OR = 1.18; 95% CI, 0.90-1.53), despite the reduced sample size.

We performed a similar analysis to adjust for known risk factors in the combined discovery family and case-control datasets by using generalized estimating equations (GEEs) to account for relatedness (Table 3). rs2283550 and rs4787924 were both associated under an additive model when adjusted for age and sex ($P = 0.002$, 0.048, respectively). rs757200 was not associated ($P > 0.79$).

In the replication Pittsburgh/UCLA dataset, of the 10 SNPs within *CACNG3* that were genotyped, two SNPs (rs4787924 and rs2238498) were nominally associated with AMD, after adjustment for age and sex and taking into account relatedness (Table 3). Both of these SNPs had been associated with AMD in phase II of our study. When combining individuals from the discovery family-based and case-control datasets with the Pittsburgh/UCLA replication dataset in a joint analysis and using the same covariates, rs2283550 and rs4787924 were strongly associated with AMD ($P = 6.1 \times 10^{-5}$ in a recessive model and $P = 0.001$ in an additive model, respectively), and two other SNPs were nominally significant.

Copy Number Variant Analysis for *CACNG3*. Of the SNPs with some evidence of association, rs757200, rs2283550, and rs2238498 are intronic, and rs4787924 is upstream of *CACNG3*. While there are mechanisms by which intronic and intergenic variation can exert phenotypic consequences, it is possible that the linkage and association results at these SNPs are instead due to LD with an undiscovered variant. Because previous evidence for a copy number variant spanning the promoter and first exon of *CACNG3* with a frequency of 13 of 95 control samples[40] was reported by the Database of Genomic Variants, we investigated whether copy number variant (CNV) 4935 could be the functional variant associated with AMD in this region. This CNV was discovered by bacterial artificial chromosome (BAC) array comparative genomic hybridization (CGH), and therefore the precise breakpoints are unknown. The location of the BAC probe used (RP11-705C1) was chr16:23,996,676-24,177,670. We used CNV assays (Taqman; ABI) with six probes nearly evenly spaced across the promoter and first exon of *CACNG3* (probes spaced from chr16:24,168,379-24,285,083) to interrogate this region. After screening more than 1500 individuals, very few had a CNV for any probe, and none had a CNV at more than one probe in the region. This finding suggests that the breakpoints of this CNV do not lie within *CACNG3* and that a CNV within *CACNG3* is not responsible for the association signals in this region.

Association Analysis for *Q7Z6F8*, *HS3ST4*, *IL4R*, and *ITGAM*. None of the SNPs in *Q7Z6F8*, *IL4R*, *HS3ST4*, or *ITGAM* produced an LOD > 2.0 or association $P < 0.001$, in either the family-based or case-control dataset, regardless of the AMD subtype tested.

DISCUSSION

Chromosome 16p12, remains one of the most consistently linked regions with susceptibility to AMD. We used linkage and association analyses in phase I of our study to narrow the list of possibilities in this region to five candidate genes. Multiple variants in *CACNG3* have been linked and/or associated with disease in our family-based and independent case-control datasets. Two SNPs were nominally associated with AMD in the

TABLE 3. Association of CACNG3 SNPs in the Discovery Dataset, the Pittsburgh/UCLA Replication Dataset, and Joint Analysis of All Datasets

SNP	Minor Allele	Discovery Dataset: 1143 Cases, 526 Controls					Pitt/UCLA: 1250 Cases, 226 Controls					Joint: 2393 Cases, 752 Controls				
		MAF Cases	MAF Controls	GEE Add	GEE Dom	GEE Rec	MAF Cases	MAF Controls	GEE Add	GEE Dom	GEE Rec	MAF Cases	MAF Controls	GEE Add	GEE Dom	GEE Rec
rs1976194	C	0.48	0.46	0.163	0.305	0.195	0.47	0.48	0.953	0.462	0.477	0.47	0.46	0.436	0.615	0.435
rs4787924	G	0.50	0.44	*0.048*	0.100	0.097	0.49	0.43	*0.035*	0.111	0.062	0.49	0.44	**0.001**	**0.007**	**0.002**
rs2238498	A	0.24	0.24	0.629	0.395	0.625	0.25	0.29	0.064	*0.050*	0.586	0.25	0.26	0.606	0.753	0.503
rs2214437	A	0.50	0.47	0.159	0.246	0.234	0.49	0.45	0.102	0.235	0.136	0.50	0.46	*0.018*	0.062	*0.040*
rs2283550	C	0.19	0.24	**0.002**	0.129	2.0×10^{-6}	0.19	0.18	0.854	0.621	0.613	0.19	0.22	*0.012*	0.196	6.1×10^{-5}
rs11640935	T	0.25	0.29	*0.039*	0.413	**0.001**	0.25	0.26	0.704	0.980	0.409	0.25	0.28	*0.035*	0.260	**0.003**
rs8052039	A	0.27	0.23	*0.046*	**0.009**	0.956	0.23	0.20	0.104	0.253	0.084	0.25	0.22	0.061	0.041	0.521
rs757200	T	0.24	0.30	0.886	0.797	0.881	0.30	0.28	0.498	0.364	0.991	0.30	0.29	0.759	0.689	0.996
rs2238521	A	0.25	0.25	0.889	0.726	0.227	0.24	0.27	0.091	0.170	0.137	0.24	0.26	0.238	0.194	0.773
rs8048828	G	0.44	0.44	0.218	*0.016*	0.393	0.43	0.41	0.413	0.368	0.667	0.43	0.43	0.991	0.477	0.367

GEE models were adjusted for age and sex, taking into account relatedness. Joint analysis included individuals from the family-based and case-control discovery datasets and the Pittsburgh/UCLA replication dataset. *P*-values ≤0.05 are shown in italic type; *P*-values ≤0.01 are shown in bold type. MAF, minor allele frequency; Add, additive; Dom, dominant; Rec, recessive.

Pittsburgh/UCLA replication dataset, both of which also showed evidence for association in the discovery dataset. In a joint analysis of the case–control, family-based, and replication datasets, four SNPs were nominally associated, and rs2283550 was strongly associated ($P < 0.0001$). Intriguingly, a fairly common CNV near the 5′ end of *CACNG3* has been described recently,[40] but we did not observe this CNV in our datasets.

CACNG3 codes for the gamma subunit of L-type voltage-dependent calcium channels. Though not an obvious candidate for AMD, this gene is highly expressed in the retina and neural tissues, compared to the rest of the body (National Eye Institute Serial Analysis of Gene Expression data). Retinal pigment epithelial cells require L-type Ca^{2+} channels to properly generate a light peak, and mutations in the gene responsible for Best's disease, another macular degenerative disorder, affect L-type channel activation kinetics and voltage dependence.[41] Furthermore, use of calcium channel blockers was weakly associated with AMD in the Beaver Dam Eye Study and the Women's Health Initiative Sight Examination Ancillary Study.[42,43] These data suggest a novel mechanism in AMD pathophysiology mediated through aberrant calcium signaling.

Interpretation of association results in light of multiple testing can be difficult, and statisticians disagree on the optimal way to correct for multiple comparisons. The most common method, the Bonferroni correction, assumes that each test is independent of all others. Clearly, this assumption is violated when SNPs in LD are tested, leading to an overly conservative correction. Rather than apply too stringent a correction and miss true positive results, we have chosen to report the nominal *P*-values, emphasizing that care be taken with their interpretation. We have called any LOD > 2.0 or $P < 0.01$ interesting or nominally significant, but all the *CACNG3* associations should be considered tentative, pending replication in independent datasets.

Even though we did not see strong evidence of association for *HS3ST4*, *IL4R*, *Q7Z6F8*, and *ITGAM*, we cannot rule out these genes in the involvement of AMD with certainty. We did not screen these genes for rare variants or CNVs, which may be associated with AMD. Furthermore, our study may have been underpowered to detect weak effects within these genes. For example, we estimated nearly 90% power needed to detect an association for a variant with minor allele frequency of 0.10 and an OR of 2.0 in a replication dataset of 1250 unrelated cases and 226 controls (equivalent in size to the Pittsburgh/UCLA dataset), but only ~40% power necessary to detect an association with an OR of 1.5. Given that many individuals are related in the Pittsburgh/UCLA dataset, we would expect our actual power to be less than that of these estimates, though how much less is difficult to quantify.

In conclusion, our linkage and association results, coupled with a plausible biological function related to AMD, make *CACNG3* a good candidate for the AMD locus on 16p12. Replication in independent datasets is necessary to confirm this effect.

Acknowledgments

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