Copy Number Variations in Candidate Genes in Neovascular Age-Related Macular Degeneration

Melissa M. Liu,^{1,2} Elvira Agrón,³ Emily Chew,³ Catherine Meyerle,³ Frederick L. Ferris III,³ Chi-Chao Chan,¹ and Jingsheng Tuo¹

PURPOSE. The pathogenesis of age-related macular degeneration (AMD) is strongly influenced by genetic factors, and single nucleotide polymorphisms have been consistently linked to AMD. Copy number variation (CNV), or variation in the number of copies of a particular segment of DNA, may also contribute to AMD pathogenesis. This study evaluated CNVs in candidate genes that have been reported to be linked to AMD.

METHODS. Study participants were 131 patients with neovascular AMD and 103 elderly persons without AMD who were evaluated by retinal specialists at the National Eye Institute. DNA was collected from peripheral whole blood, and duplex RT-PCR based copy number (CN) assays were performed for the genes *CCR3*, *CFH*, *CX3CR1*, *ERCC6*, *HTRA1*, and *VEGF*. Quantitative CNs (CN = 0, 1, 2, or 3+) were determined.

RESULTS. Novel CNVs were discovered in *CCR3*, *CX3CR1*, and *ERCC6*. The unadjusted data suggested that CN = 3+ for *CX3CR1* might be mildly protective against AMD, but this trend did not persist after adjustment for age. AMD patients appeared to have an elevated mean *CFH* CN relative to controls (2.13 [95% confidence interval (CI), 2.05–2.21] vs. 2.01 [95% CI, 1.92–2.09 copies]; $P = 0.05$). No significant associations between CNV and AMD were observed for the remaining genes.

CONCLUSIONS. The methods described are suitable for quantitative characterization of CNV in candidate genes. The authors identified CNVs in AMD-associated genes but did not find strong evidence for a link with neovascular AMD. (*Invest Ophthalmol Vis Sci.* 2011;52:3129–3135) DOI:10.1167/iops.10-6735

Age-related macular degeneration (AMD) is the leading cause of blindness among the elderly in the developed world.¹ AMD is characterized by the formation of drusen in Bruch's membrane, the degeneration of photoreceptors and the underlying retinal pigment epithelium in the macula, geographic atrophy, and choroidal neovascularization. It is widely accepted that there is a complex involvement of both genetic and environmental factors in the pathogenesis of AMD. Studies have identified myriad AMD-associated genes in mechanistic pathways related to complement system activation, inflamma-

tion, microglial recruitment, DNA repair, extracellular matrix function, and neovascularization.²

Much of the previous work on genetic factors influencing AMD has focused on single nucleotide polymorphisms (SNPs). Although highly significant statistical associations have been discovered between various SNPs and AMD, they do not account for the entire genetic component of the disease. Moreover, it remains to be elucidated whether the identified SNPs are causally linked to aberrant gene function or whether they are simply genetic markers. Copy number variation (CNV) is another type of genetic structural variation that has been recognized as a substantial source of phenotypic variation in human populations.³

CNVs include deletions, duplications, and tandem repeats of segments of genomic DNA that are at least 1 kilobase long.³ Population-based studies have identified thousands of CNV loci throughout the human genome.^{4,5} There are a number of proposed mechanisms, including gene dosage, gene interruption, gene fusion, and positional effects, potentially linking CNV to aberrant gene function or regulation and subsequently disease.⁶ In fact, CNVs in specific genes have been linked to a variety of diseases, including systemic lupus erythematosus, Crohn's disease, and several neuropsychiatric disorders.⁷ CNVs in two glutathione S-transferases, *GSTM1* and *GSTT1*, have also been linked to cancer susceptibility, asthma, and cataract.^{8,9}

Although CNV contributes to human genetic diversity, its role in AMD pathogenesis has only been superficially evaluated. A recently reported genome-wide association study of CNV in AMD identified no significant CNV loci associated with AMD risk.¹⁰ It has been reported, however, that deletions in *CFH*-related proteins 1 and 3 (*CFHR1* and *CFHR3*) are protective against AMD.^{11–13} Nevertheless, the role of CNV is unknown for the most highly associated AMD genes. There is precedence for discovering that a CNV is causally linked to disease after having first identified an associated SNP in the same gene, as has been demonstrated for the α -synuclein gene and Parkinson's disease.^{14,15} There is also evidence for linkage disequilibrium between CNVs and SNPs in the human genome.¹⁶ To characterize the role of CNV as a potential contributor to the genetic component of AMD, this study performed quantitative copy number genotyping for highly associated AMD genes in patients with neovascular AMD and elderly controls. Novel CNVs were discovered in three genes, but no definitive associations between CNVs in the selected genes and AMD were observed.

MATERIALS AND METHODS

This research adhered to the tenets of the Declaration of Helsinki. Each participant provided signed informed consent in accordance with protocols for human subject recruitment and clinical evaluation approved by the Institutional Review and Ethics Boards of the National Eye Institute.

From the ¹Laboratory of Immunology and the ³Division of Epidemiology and Clinical Applications, National Eye Institute, National Institutes of Health, Bethesda, Maryland; and the ²Johns Hopkins University School of Medicine, Baltimore, Maryland.

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Corresponding author: Jingsheng Tuo, 10/10N103, 10 Center Drive, Bethesda, MD 20892-1857; tuo@nei.nih.gov.

TABLE 1. Demographic and Clinical Information for Study Participants

	Control (<i>n</i> = 103)	Neovascular AMD (<i>n</i> = 131)	<i>P</i>
Age, y	69.6 ± 9.8	79.4 ± 8.3	<0.0001*
Female	61 (59.22)	63 (48.09)	0.09
Past smoker	52 (50.49)	67 (51.15)	0.92
Current smoker	3 (2.91)	10 (7.63)	0.12
Cataract	41 (39.81)	88 (67.18)	<0.0001*
Ocular inflammation	2 (1.94)	0 (0)	0.11
Glaucoma	5 (4.85)	8 (6.11)	0.68
Corneal disease	2 (1.94)	1 (0.76)	0.43
Optic nerve disease	2 (1.94)	3 (2.29)	0.85
Non-AMD retinal disease	0 (0)	10 (7.63)	0.0042*
Cardiovascular disease	45 (43.69)	93 (70.99)	<0.0001*
Alzheimer's disease	0 (0)	2 (1.53)	0.21

Data are presented as mean ± SD for age and as *n* (%) for all other entries. Non-AMD retinal diseases of patients in the study included central retinal vein occlusion (*n* = 3), epiretinal membrane (*n* = 2), diabetic retinopathy (*n* = 2), central retinal artery occlusion (*n* = 1), foveal macular dystrophy (*n* = 1), and macular hole with vitreomacular traction (*n* = 1).

**P* = 0.05.

Study Population

Study participants were 131 patients with neovascular AMD and 103 controls from the greater Washington, DC, area. All participants were self-identified as Caucasians of non-Hispanic descent (Table 1). All participants were clinically evaluated by retinal specialists at the National Eye Institute. AMD status was determined by independent grading of funduscopy photographs using the AREDS scoring criteria.¹⁷ All control subjects had either no drusen or fewer than 15 small (<63 μm) drusen, no significant extramacular drusen, and no evidence of AMD-related pigment abnormalities. Subjects with significant signs of any other retinal diseases involving the photoreceptors or outer retinal layers other than AMD—including high myopia, retinal dystrophies, central serous retinopathy, vein occlusion, diabetic retinopathy, and uveitis, or similar outer retinal diseases—were excluded from the control group. All patients with neovascular AMD had choroidal neovascularization characterized by edema, blood, or fibrovascular or fibrous tissue beneath the retinal pigment epithelium or sensory retina. The diagnosis of neovascular AMD was often confirmed by fluorescein angiography and optical coherence tomography. Patients were also assessed by indocyanine green angiography to exclude polypoidal choroidal vasculopathy. Subjects with non-AMD retinal diseases that developed before the age of 50 were excluded from the neovascular AMD group.

Genomic DNA Preparation

Peripheral whole blood was collected from all study participants for genomic DNA extraction (QIAamp DNA Blood Maxi Kit; Qiagen, Valencia, CA). DNA was quantified first by UV absorption (Beckman DU 640 Spectrophotometer; Beckman Coulter, Brea, CA) and then by RT-PCR amplification of RNase P RNA component H1 (*RNase P*), with standard DNA as a calibrator, using reagents (TaqMan *RNase P* Detection; Applied Biosystems, Foster City, CA). DNA concentrations were equalized before copy number genotyping of candidate genes.

Copy Number Genotyping

Copy number genotyping was performed using duplex RT-PCR-based copy number analysis (TaqMan Copy Number Assays; Applied Biosystems) for six AMD-associated genes (Table 2): chemokine (C-C) motif receptor 3 (*CCR3*), complement factor H (*CFH*), chemokine (C-X3-C) receptor 1 (*CX3CR1*), excision repair cross-complementing rodent repair deficiency, complementation group 6 (*ERCC6*), HtrA serine peptidase 1 (*HTRA1*), and vascular endothelial growth factor (*VEGF*) (Applied Biosystems). Standard curves were generated using twofold serial dilutions of genomic DNA to determine the RT-PCR amplification efficiency for each assay. Efficiency (*E*) was calculated using the equation $E = 10^{(-1/m)} - 1$, where *m* is the slope of the standard curve, plotting cycle threshold (Ct) against DNA quantity (log concentration). Copy number analysis (TaqMan Copy Number Assays; Applied Biosystems) was also performed for *GSTM1* and *GSTT1*, two genes for which CNVs have been well documented. Analyses were initially performed on a subset of 88 cases and 80 controls before determining whether further characterization using all study samples was merited. Unadjusted statistical analyses comparing CNV frequencies in cases and controls showed no significant differences for any of the genes except *CX3CR1*, which showed a borderline association. As a result, CNV genotyping was performed using all 131 patients and 103 controls for *CX3CR1* only. Copy number analysis (TaqMan Copy Number Assays; Applied Biosystems) was also performed for *AR* (androgen receptor), an X-linked gene, on a subset of 25 patients as well. For each 10-μL single-well reaction using 10 ng genomic DNA and 1× TaqMan Universal PCR Master Mix without AmpErase UNG, a 1× TaqMan Copy Number Assay, which contained forward primer, reverse primer, and FAM dye-labeled MGB probe specific for the gene of interest, was run simultaneously with a 1× TaqMan Copy Number Reference Assay, which contained forward primer, reverse primer, and a VIC dye-labeled TAMRA probe specific for *RNase P* according to the manufacturer's instructions. Primers and probes were selected for each gene of interest (ABI GeneAssist Copy Number Assay Workflow Builder; Applied Biosystems).¹⁸ When possible, assays targeting regions of previously reported CNV were selected.

PCR was performed in 96-well plates using a PCR system (7500 Real-Time PCR System; Applied Biosystems). Thermal cycling conditions were as described in the manufacturer's instructions. Data were

TABLE 2. Copy Number Assay Information

Gene	Assay ID*	Assay Location (NCBI build 37)	Assay Cytoband	Assay Gene Location
<i>CCR3</i>	Hs00751151_cn	chr3:46283769	3p21.31h	Exon 1
<i>CFH</i>	Hs00116895_cn	chr1:196621254	1q31.3c	Exon 1
<i>CX3CR1</i>	Hs02139194_cn	chr3:39305135	3p26.3c	Exon 5
<i>ERCC6</i>	Hs01920803_cn	chr10:50681068	10q11.23a	Intron 14-Exon 15
<i>HTRA1</i>	Hs03730183_cn	chr10:124264488	10q26.13b	Intron 3
<i>VEGF</i>	Hs00609007_cn	chr6:43738068	6p21.1d	Exon 1
<i>GSTM1</i>	Hs00273142_cn	chr1:110230461	1p13.3b	Exon 1
<i>GSTT1</i>	Hs00767125_cn	chr22:24376147	22q11.23b	Exon 5
<i>AR</i>	Custom design†	—	Xq11.2	Exon 1

*Additional assay-specific information may be found at <https://products.appliedbiosystems.com>.

†*AR* primers: forward, 5'-gctgtcattcagctactcctggat-3'; reverse, 5'-gcacctcgtgagtgacattggt-3'.

FIGURE 1. RT-PCR amplification efficiency of copy number assays. Standard curves for the RT-PCR amplification efficiency of each assay were generated by plotting Ct against DNA quantity (log concentration) for the gene of interest and the *RNase P* internal control. (A) *CCR3*; (B) *ERCC6*; (C) *CFH*; (D) *HTRA1*; (E) *CX3CR1*; (F) *VEGF*. Five twofold serial dilutions were performed in duplicate for each gene. Calculated efficiencies were close to 100% and were similar between each gene of interest and *RNase P*.

collected (Absolute Quantification Method, 7500 System SDS Software version 1.3.1; Applied Biosystems), and samples were assayed using triplicate wells for each gene of interest. Copy numbers were estimated (CopyCaller Software version 1.0; Applied Biosystems) using the $\Delta\Delta Ct$ relative quantification method. A maximum likelihood algorithm was used to estimate the mean ΔCt expected for copy number 1 (CN = 1) based on the probability density distribution across all samples, and this parameter was used in subsequent copy number calculations for each given gene. This analytical method was used to calculate the relative copy number of a target gene normalized to *RNase P*, a reference of known copy number (CN = 2), without the use of a calibrator sample for each target gene. The copy number assays were repeated on a subset of samples for each gene to determine reproducibility. In the event of a discrepancy in the copy number call between two duplicate assays, the call with the higher calculated statistical confidence was used for data analysis.

Statistical Analysis

Logistic regression, without adjustment for any confounding factors, and *t*-test were used to compare the frequencies of each copy number category (CN = 0, 1, 2, or 3+) and the mean number of copies of each gene, respectively, between patients with neovascular AMD and con-

trols. Age-adjusted logistic regression and analysis of covariance were also performed to evaluate the same parameters. All statistical analyses were performed using statistical software (SAS version 9.2; SAS Institute Inc., Cary, NC). Logistic regression compared the odds of AMD among the various copy number levels. Analysis of covariance performed comparisons of the mean copy numbers while taking into account both categorical and continuous covariates. All tests were performed as two-tailed analyses, and statistical significance was nominally set at $P = 0.05$, recognizing that there was no adjustment for multiple comparisons.

RESULTS

Evaluation of Quantitative Copy Number Determination Methods

Standard curves for the candidate gene-specific copy number assays (TaqMan; Applied Biosystems) showed that the RT-PCR amplification efficiencies were similar between each gene of interest and the internal control *RNase P*, a gene of known copy number (Fig. 1). Amplification efficiencies were calculated to be between 96% and 105% for all genes, and the

FIGURE 2. Representative amplification plots for quantitative copy number determination. Quantitative copy numbers were determined using differences in the amplification of the gene of interest (FAM-labeled) relative to the *RNase P* internal control of known copy number (VIC-labeled). Representative data show amplification plots for participants of CN = 1, CN = 2, and CN = 3 for *CX3CR1*.

differences between the slopes of the standard curves for each gene and *RNase P* were <7% for all assays. Consistent amplification of the internal control *RNase P* (VIC-labeled) confirmed that equal amounts of genomic DNA were being introduced in each assay, and differences in the amplification of the gene of interest (FAM-labeled) relative to *RNase P* were able to discriminate quantitative copy numbers (Fig. 2). Copy number assays, which included triplicate single-well analyses, were repeated on 7% of samples for each gene to determine reproducibility. The copy number calls were consistent in >97% of patients. Copy number assays for *AR* were also performed as an additional evaluation of the methodology for a subset of patients. With 100% accuracy, male and female subjects were determined to have CN = 1 and CN = 2, respectively (Fig. 3).

CNVs in Candidate Genes

Quantitative copy numbers (CN = 0, 1, 2, or 3+) were determined for *GSTM1*, *GSTT1*, and the six AMD-associated genes of interest in each sample (Table 3). CNVs were present in both AMD and control sample populations. For most genes, however, CN = 2 was the predominant copy number genotype. Excluding *GSTM1* and *GSTT1*, genes for which prevalent deletion polymorphisms have been reported, the rates of carriers with CN = 2 were 73.3% to 98.9% for controls and 82.3% to 100.0% for AMD across the six AMD-associated genes. Novel CNVs were observed within *CCR3*, *CX3CR1*, and *ERCC6* in both groups.

Associations between CNVs in Candidate Genes and AMD

The frequencies of each copy number category were compared relative to the CN = 2 category. CN = 2 was selected as the reference for statistical analysis because humans (diploids) are expected to have two copies of most genes. Comparisons of the CN frequencies between AMD patients and control subjects were performed using unadjusted and then age-adjusted logistic regression. The unadjusted association of CN = 3+ for *CX3CR1* was in the protection for AMD (odds ratio [OR] = 0.52; 95% confidence interval [CI], 0.27–1.0; $P = 0.05$), but this trend did not persist after adjustment for age

($P = 0.14$). There were no significant differences in the copy number category frequencies for any of the other genes.

To determine whether there was a general trend toward increased or decreased copy number at the population level, the mean number of copies of each gene was also calculated (Table 4). Comparisons between AMD patients and controls were performed using *t*-test and age-adjusted analysis of covariance. The unadjusted data suggested a decrease in the mean number of copies of *CX3CR1* in AMD patients relative to controls (2.12 ± 0.41 vs. 2.23 ± 0.47 copies; $P = 0.05$). This difference was not observed after adjusting for age ($P = 0.10$). Although the unadjusted association of the mean *CFH* CN between AMD patients and controls was not statistically significant (2.10 ± 0.34 vs. 2.04 ± 0.37 copies; $P = 0.24$), the difference became nominally statistically significant (unadjusted for multiple comparisons) after adjusting for age (2.13 [95% CI, 2.05–2.21] vs. 2.01 [95% CI, 1.92–2.09] copies; $P = 0.05$).

DISCUSSION

This study investigated CNVs in candidate genes related to a variety of pathways implicated in AMD pathogenesis. Activation of the complement cascade plays a pivotal role in AMD pathogenesis, and genetic polymorphisms in a variety of complement pathway-related genes, including *CFH*, complement factor B (*CFB*), complement components 2 and 3 (*C2* and *C3*), *CFHR1*, and *CFHR3*, have been strongly linked to AMD.¹⁹ Two loss-of-function SNPs in *CX3CR1*, a chemokine receptor that plays a critical role in microglial migration, have also been associated with elevated AMD risk,^{20–22} providing further support for an immunologic basis to AMD pathogenesis. A polymorphism in *ERCC6*, which plays a role in DNA repair, has also been weakly linked to AMD.²³ Another important AMD susceptibility locus lies on chromosome 10q26 and includes two genes that are in strong linkage disequilibrium—age-related maculopathy susceptibility 2 (*ARMS2*) and *HTRA1*. *ARMS2* may play roles in mitochondrial and extracellular matrix function, and numerous polymorphisms in *ARMS2* have been linked to AMD.^{24–26} A well-characterized SNP in the promoter region of *HTRA1* has been associated specifically with neovascular AMD,^{27,28} potentially by facilitating choroidal neovascularization through enhanced degradation of extracellular matrix.² The chemokine receptor *CCR3*²⁹ and SNPs in *VEGF*³⁰ have also been linked to neovascular AMD. Epidemiologic and molecular studies that have linked each of these genes to AMD provide a strong mechanistic rationale for selecting them as candidate genes for evaluating additional types of genetic variations, including CNVs, which may modulate their associations with AMD.

FIGURE 3. Quantitative copy number genotyping for *AR*. Calculated *AR* copy number is plotted for each of 25 participants, with range bars indicating the minimum and maximum copy numbers calculated in the triplicate analysis. M, male; F, female.

TABLE 3. Copy Number Frequencies in AMD Patients and Controls

Gene	CN	Control <i>n</i> (%)	AMD <i>n</i> (%)	Unadjusted		Age-Adjusted	
				OR (95% CI)	<i>P</i>	OR (95% CI)	<i>P</i>
<i>CCR3</i>	0	1 (1.3)	1 (1.1)	0.93 (0.06–15.21)	0.96	0.35 (0.01–9.15)	0.53
	1	3 (3.8)	5 (5.7)	1.56 (0.36–6.75)	0.56	1.03 (0.17–6.32)	0.98
	2	70 (88.6)	75 (86.2)	1.0	—	1.0	—
	3+	5 (6.3)	6 (6.9)	1.12 (0.33–3.83)	0.86	1.81 (0.38–8.62)	0.45
	Total	79	87	—	—	—	—
<i>CFH</i>	0	0 (0)	0 (0)	—	—	—	—
	1	4 (5.1)	1 (1.1)	0.22 (0.02–2.05)	0.19	0.10 (0.01–1.40)	0.09
	2	68 (86.1)	76 (87.4)	1.0	—	1.0	—
	3+	7 (8.9)	10 (11.5)	1.28 (0.46–3.54)	0.64	2.10 (0.57–7.71)	0.26
	Total	79	87	—	—	—	—
<i>CX3CR1</i>	0	0 (0)	0 (0)	—	—	—	—
	1	2 (2.0)	4 (3.1)	1.36 (0.24–7.64)	0.72	1.41 (0.23–8.62)	0.71
	2	73 (73.0)	107 (82.3)	1.0	—	1.0	—
	3+	25 (25.0)	19 (14.6)	0.52 (0.27–1.01)	0.05*	0.55 (0.25–1.21)	0.14
	Total	100	130	—	—	—	—
<i>ERCC6</i>	0	0 (0)	0 (0)	—	—	—	—
	1	1 (1.3)	2 (2.3)	1.81 (0.16,20.40)	0.63	1.94 (0.08,44.87)	0.68
	2	78 (98.7)	86 (97.7)	1.0	—	1.0	—
	3+	0 (0)	0 (0)	—	—	—	—
	Total	79	88	—	—	—	—
<i>HTRA1</i>	0	0 (0)	0 (0)	—	—	—	—
	1	0 (0)	0 (0)	—	—	—	—
	2	74 (92.5)	82 (93.2)	1.0	—	1.0	—
	3+	6 (7.5)	6 (6.8)	0.90 (0.28–2.92)	0.86	1.98 (0.46–8.52)	0.36
	Total	80	88	—	—	—	—
<i>VEGF</i>	0	0 (0)	0 (0)	—	—	—	—
	1	0 (0)	0 (0)	—	—	—	—
	2	77 (96.3)	88 (100.0)	1.0	—	1.0	—
	3+	3 (3.8)	0 (0)	—	—	—	—
	Total	80	88	—	—	—	—
<i>GSTM1</i>	0	47 (58.8)	48 (54.5)	0.89 (0.46–1.72)	0.72	0.87 (0.40–1.91)	0.74
	1	0 (0)	2 (2.3)	—	—	—	—
	2	26 (32.5)	30 (34.1)	1.0	—	1.0	—
	3+	7 (8.8)	8 (9.1)	0.99 (0.32–3.10)	0.99	1.11 (0.29–4.33)	0.88
	Total	80	88	—	—	—	—
<i>GSTT1</i>	0	17 (21.3)	15 (17.4)	0.75 (0.31–1.83)	0.53	0.51 (0.18–1.45)	0.20
	1	39 (48.8)	38 (44.2)	0.83 (0.41–1.69)	0.61	0.82 (0.35–1.91)	0.64
	2	23 (28.8)	27 (31.4)	1.0	—	1.0	—
	3+	1 (1.3)	6 (7.0)	5.11 (0.57–45.57)	0.14	2.05 (0.18–23.94)	0.57
	Total	80	86	—	—	—	—

The frequencies of all CN categories were compared using unadjusted or age-adjusted logistic regression, with CN = 2 as the reference.

**P* = 0.05.

The duplex RT-PCR based copy number assay methods used in this study have been demonstrated to be a reliable and accurate strategy to precisely determine quantitative copy

numbers for specific candidate genes in both disease and control samples. Genomic DNA concentrations were rigorously quantified and controlled before copy number genotyp-

TABLE 4. Mean Copy Numbers in AMD Patients and Controls

Genes	Unadjusted			Age-Adjusted		
	Control	AMD	<i>P</i>	Control	AMD	<i>P</i>
<i>CCR3</i>	2.00 ± 0.39	1.99 ± 0.42	0.86	1.96 (1.87–2.06)	2.02 (1.93–2.11)	0.42
<i>CFH</i>	2.04 ± 0.37	2.10 ± 0.34	0.24	2.01 (1.92–2.09)	2.13 (2.05–2.21)	0.05*
<i>CX3CR1</i>	2.23 ± 0.47	2.12 ± 0.41	0.05*	2.23 (2.13–2.32)	2.12 (2.04–2.20)	0.10
<i>ERCC6</i>	1.99 ± 0.11	1.98 ± 0.15	0.63	1.99 (1.95–2.02)	1.98 (1.95–2.01)	0.75
<i>HTRA1</i>	2.08 ± 0.27	2.07 ± 0.25	0.86	2.05 (1.99–2.11)	2.09 (2.03–2.15)	0.36
<i>VEGF</i>	2.04 ± 0.19	2.00 ± 0.00	0.07	2.02 (1.99–2.05)	2.01 (1.98–2.04)	0.69
<i>GSTM1</i>	0.91 ± 1.13	0.98 ± 1.12	0.71	0.89 (0.62–1.16)	1.00 (0.74–1.26)	0.60
<i>GSTT1</i>	1.10 ± 0.74	1.28 ± 0.84	0.15	1.10 (0.90–1.29)	1.28 (1.10–1.47)	0.20

Data are presented as mean ± SD in the unadjusted results and as mean (95% CI) in the age-adjusted results. Unadjusted *P* values were calculated using the *t*-test, and age-adjusted *P* values were calculated using analysis of covariance.

**P* = 0.05.

ing using two independent methods, UV absorbance and RT-PCR amplification of *RNase P*. This exactitude and same-well amplification of *RNase P* protected against artificial variations caused by differences in DNA loading or erroneous detection of the null genotype (CN = 0) caused by PCR failure or pipette error.

RT-PCR amplification efficiencies for each tested assay were calculated to be near 100%. Differences in the amplification efficiencies for each gene of interest and *RNase P* were <7%. This ensured the validity of the $\Delta\Delta C_t$ method, which was used to calculate quantitative copy numbers.³¹ Although the efficiencies for *GSTM1* and *GSTT1* were not determined in this study, copy number assays (TaqMan; Applied Biosystems) for both these genes have been evaluated in the literature.³² The efficiencies were reported to be consistently between 92% and 100% and approximately equal to that of *RNase P*.³² Other groups have also used these assays reliably for *GSTM1* and *GSTT1* copy number genotyping.^{9,33}

Quantitative copy numbers were determined using these methods with high reproducibility and accuracy. More than 97% of duplicated assays resulted in the same copy number call, and the number of copies of *AR*, known to exist with CN = 1 in men and CN = 2 in women, was correctly determined with 100% accuracy in a subset of samples. The copy number distributions determined for *GSTM1* and *GSTT1* in this study were in agreement with previously published results. The null deletion (CN = 0) was observed in 57.2% and 19.3% of all subjects for *GSTM1* and *GSTT1*, respectively. CNVs in these genes are well documented, and previous studies have found the frequencies of CN = 0 to be approximately 50% for *GSTM1* and 20% for *GSTT1* in European populations.^{32,33}

All these lines of evidence suggested that the methods used in this study were sufficient to quantitatively characterize CNVs in candidate genes. To our knowledge, local and direct investigation of CNV has not been documented for the six AMD-associated genes selected for this study. We report the discovery of three novel CNVs within *CCR3*, *CX3CR1*, and *ERCC6* in both control and AMD sample populations. CNVs at these sites have not been reported in the Database of Genomic Variants, a centralized summary of structural variations in the human genome.³⁴

After adjustment for age, an increase in *CFH* copy number appeared to be a risk factor for AMD. *CFH* is a regulator of the complement cascade, and it has been consistently demonstrated as a protective factor against AMD pathogenesis.¹⁹ The functional consequences of *CFH* copy number and its impact on AMD require further evaluation. There were trends in the unadjusted data that suggested *CX3CR1* might also be a gene of interest. However, the effect did not persist after adjustment for age. It has been previously reported that two loss-of-function SNPs in *CX3CR1*, V249I and T280M, are associated with increased AMD risk and that *CX3CR1* levels are lower in the maculae of AMD patients.^{21,35}

Current sample sizes might have offered insufficient power because of heavy stratification by copy number category. However, a significant increase in the sample size would not have changed the penetrance of *CFH* or *CX3CR1* CNVs at the onset of disease. Another limitation might have been the statistically significant difference between the ages of the AMD patient and control groups. As a result, statistical adjustments for age might have been incomplete. Nevertheless, further adjustments would dilute the significance of the associations, and they would not alter the conclusions of the study.

The levels of *CX3CR1* and *CFH* in the eyes of healthy persons with known gene copy numbers were not determined in this study. As a result, it is not possible to draw conclusions regarding the mechanistic impact of CNVs in either of these genes. Previous reports have demonstrated that increases in

gene copy number can lead to either increases or decreases in gene expression.³⁶ Functional studies may be able to directly determine whether CNV in *CX3CR1* or *CFH* can modulate AMD risk or contribute to AMD pathogenesis through gene dosage effects.

In summary, a duplex RT-PCR copy number genotyping approach was used to investigate quantitative CNVs in highly associated AMD candidate genes. Three novel CNVs were discovered in *CCR3*, *CX3CR1*, and *ERCC6*, but no definitive associations between CNVs in the selected genes and AMD were observed. Nevertheless, the rationale still remains for further evaluation of the role of CNV in AMD pathogenesis without a limitation on previously reported AMD-associated genes.

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