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Association Assessment of Copy Number Polymorphism and Risk of Age-Related Macular Degeneration

Julie Sawitzke¹, Kate M. Im², Brittany Kostih², Michael Dean^{2,3}, and Bert Gold^{2,3}

¹ SAIC-Frederick, Frederick, MD 21702, USA

² Center for Cancer Research, Cancer Inflammation Program, Human Genetics Section, National Cancer Institute – Frederick, Frederick, MD 21702, USA

Abstract

Purpose—We previously identified a genetic copy number polymorphism (CNP147) that was statistically associated with age-related macular degeneration (AMD), and which resides downstream of the complement factor H (*CFH*) gene. Factor H protein is polymorphic at amino acid 402 in which the resulting histidine containing moiety has been established to impart significant risk of AMD. Here we present a method to precisely determine the exact copy number of CNP147 and examine in more detail the association with AMD.

Design—Case-control Study

Participants—421 AREDS (Age-related Eye Disease cohort Study) subjects of whom approximately 35% were diagnosed with neovascular disease, 19% with geographic atrophy, 16% with both, 30% with large drusen and 215 controls.

Methods—Using copy number assays available from Applied Biosystems Inc., we examined four loci spanning CNP147 and neighboring CNP148 in an AREDS matched case-control sample set. We analyzed these data by copy number while controlling for two high-risk *CFH* variants, rs1061170 (Y402H) and rs1410996. We phased the high risk *CFH* variants with CNP147 and analyzed haplotype frequencies in cases and controls. To further validate copy numbers, six Utah CEPH families (Centre D'étude du Polymorphisme Humaine) were typed for CNP147 and the segregation assessed.

Main Outcome Measures—Increased or decreased risk of AMD from genetic loci.

Results—Having fewer than 2 copies of CNP147 is associated with an estimated 43% reduction in odds of having AMD in this sample set (adjusted odds ratio=0.57, P=0.006). CNP148 variation is rare in Caucasians and it was not statistically significant. Common haplotypes reveal that the risk alleles for rs1061170 and rs1410996 most frequently segregate with higher copy numbers for CNP147; but not exclusively, and that one haplotype that carried a deletion of CNP147 was highly protective (odds ratio=0.25 P=1.3×10⁻¹³) when compared to the reference.

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³Corresponding Author: Bert Gold, Ph.D., FACMG, Staff Scientist, NCI-Frederick, Box B, Boyles Street, Fort Detrick, Frederick, Maryland 21702, golda@mail.nih.gov, VOICE: 301-846-5098, FAX: 301-846-70423.

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Conclusions—In this matched subset of AREDS subjects, after adjusting for two known risk variants in *CFH*, CNP147 deletion statistically associates with diminished risk for AMD.

Keywords

Copy number polymorphism; age-related macular degeneration; HapMap 3; TaqMan Copy Number Assays; qPCR

INTRODUCTION

Age-related macular degeneration (AMD) is a late onset retinal disease affecting older adults, which causes irreversible loss of central vision.¹ Aside from nutritional and environmental risk factors, regulation of the alternative complement pathway has been established as having a central role, and genetic variants coding for the proteins within this pathway are associated with risk of disease.^{2, 3}

The AREDS cohort (Age-related Eye Disease cohort Study) is a major clinical trial sponsored by the National Eye Institute and contains over 2000 participants. We obtained DNA samples from all the available patients, but only used a subset for this study so that we could match cases and controls on sex, race, and age. The AREDS cohort is predominantly composed of Caucasians.

The innate immune system, in its proper function, is aimed to attack and eliminate invading infectious agents in a non-specific manner.⁴ The alternative pathway of the complement system of immunity is pro-inflammatory and is continuously activated via a positive feedback loop of its most abundant molecule C3 (complement component 3). This pathway is indiscriminate and will damage host cells if it is not tightly controlled by regulatory proteins that inhibit the cascade.⁴ Complement factor H (the protein product of the *CFH* gene) is the main inhibitor of the alternative complement cascade on host cells, and its efficiency is dependent on how well it binds to cell surfaces.⁴ Mutations in the *CFH* gene, particularly Y402H (rs1061170), an amino acid changing variant, has been shown to reduce the binding properties of factor H resulting in less control of the cascade. Drusen observed in the earliest stages of AMD are deposits of cellular debris, and contain all alternative complement pathway proteins.⁵

The complement factor H gene (*CFH*) is located on the long arm of chromosome 1 adjacent to several *CFH* paralogs (partially or completely duplicated sequences that may or may not share the same function as the original gene). These similarities make it technically challenging to conduct genetic association studies of the variants in this region. Furthermore, recombination (crossover) during meiosis is inconsistent, making it difficult to determine haplotypes (alleles, variants, or numerous loci consistently inherited together on one chromosome during meiosis). Complex diseases such as AMD are rarely caused by one genetic variant, and analyzing haplotypes is an efficient way to collectively assess numerous linked variants to determine whether they are associated with a disease. An overview of the linkage disequilibrium (LD), an estimated measure of recombination events in a particular chromosomal region, between *CFH*, its paralogs, and CNPs, among a subset of AREDS is shown in Figure 1.

In 2005 we identified and characterized what we called “a large common deletion” downstream of the complement factor H gene (*CFH*) that encompasses two *CFH* paralogs, *CFH*-related 3 (*CFHR3*) and *CFH*-related 1 (*CFHR1*).⁶ This deletion is now called copy number polymorphism 147 (CNP147).⁶ In this and a subsequent study, we assessed this copy number polymorphism and found that individuals homozygous for this deletion are

highly protected from AMD compared to non-deleted.⁷ The detection method we developed and used to characterize this CNP was limited in that it gave only dichotomous results, either two copies or none. In this study, we revisited CNP147 using newer technology to determine more precisely the copy number in this region and whether it changed our risk estimates; and investigated the adjacent CNP148 to determine if it is associated with AMD. This newer technique permits the resolution of subjects carrying one copy from those carrying two.

We also sought to understand how the presence or absence of CNP147 (genes *CFHR3* and *CFHR1*), interact with or confound the risk previously seen for two *CFH* variants. Because they all bind to the same sites at the cellular surface, we hypothesize that subjects carrying higher copies of CNP147, and therefore more transcribed *CFH*-related proteins from paralogs *CFHR1* and *CFHR3*, may have higher risk of AMD due to these genes' protein products competing with factor H binding and interfering with its function.

METHODS

Case and controls

Individuals selected for this study were participants in the AREDS, which was initially conceived as a long-term multi-center, prospective study of the clinical course of AMD and age-related cataract. AREDS also included a clinical trial of high-dose vitamin and mineral supplements for AMD and a clinical trial of high-dose vitamin supplements for cataract.⁷ We obtained validated DNA samples of all the available AREDS participants from the National Eye Institute-AREDS Genetic Repository at Coriell Institute for Medical Research (Camden, NJ, USA). This sample set contains 421 cases and 215 controls; we matched two cases with one control on sex and race; and the difference in mean age between cases and controls was <2 years. This design was most suitable because of the small number of controls available to us since AREDS was designed as a prospective cohort study with an intervention, and only 215 disease-free patients were included as a control group. Because of this limit, a 2:1 match was favored over a 1:1 match for the simple fact that it gave us more statistical power, bringing our total sample to 636 patients.⁷ Of the 421 cases, 147 had neovascular disease (34.9%), 82 had geographic atrophy (19.5%), 66 had both neovascular disease and geographic atrophy (15.7%) and 126 had large drusen (29.9%). The National Institutes of Health Office of Human Subjects Research considered the use of de-identified DNA from the AREDS study exempt from needing Internal Review Board approval for this research, and certified this exemption as number 3366.

We relied on the five AMD categories created and utilized in the original AREDS clinical trial to define our cases and controls.⁸ A brief description of these categories is as follows: category one was described as being completely free of AMD and all our controls were selected from this group. Category two subjects were ambiguous, meaning that they did not have any clear symptoms of early-stage AMD, but also could not be considered free from eye disease symptoms, and so they were identified as 'other'; these individuals did not qualify as an AMD case or a control by our definition and were excluded from our study. Category three subjects, having early-stage AMD, had many medium-sized drusen or one or more large drusen in one or both eyes. Category four patients had end-stage AMD in one eye; and subjects in category five had end-stage AMD in both eyes. All of our cases were selected from categories three, four, and five.

Taqman Copy Number Genotyping

We selected four Applied Biosystems Inc. copy number assays, two located in CNP147 (hs04211013cn and hs04197581cn) and two in CNP148 (hs03356469cn and

hs03345427cn), and genotyped the AREDS matched set described above. These polymerase chain reaction (PCR) based assays were performed on an ABI 7900HT in real-time absolute quantification mode using SDS 2.2 software. All assays were run along with the standard endogenous RNase P reference assay and were conducted in triplicate. Results were exported to ABI CopyCaller™ software for interpretation. We calibrated the detection settings under the assumption that most samples would have two copies. We discarded call results that were below 95% confidence and/or >2 Z score. Using these criteria we were able to unambiguously establish copy number calls for 393 out of 421 affected AREDS research subjects and 203 out of 215 controls.

Validation and quality control

To verify our calibration settings, each run included control samples of known copy number for that assay. We found these controls by downloading CEL files of Affymetrix SNP 6.0 GeneChip microarrays from the hapmap.org web page at:

http://hapmap.ncbi.nlm.nih.gov/downloads/raw_data/hapmap3_affy6.0 downloaded by KMI on July 14, 2010. These were run at Affymetrix in Sunnyvale, CA or at the Broad Institute of Harvard and MIT. These arrays provide probes for 946,000 copy number variants. We selected samples containing 2, 1, or 0 copies of CNP147 and CNP148, and obtained these DNA samples from the Coriell Repository. These controls were run in triplicate and analyzed along with our AREDS samples.

Additional verification was obtained using our known homozygous 0 copy samples. In our 2009 publication we used a PCR/gel assay to determine 0 or 2 copy status of each sample; these results did not allow us to distinguish between individuals with 1 and 2 copies, but we had definitive results for individuals who had zero copies. These deleted samples were crosschecked with our current results.

Copy numbers were further assessed for quality control by examining segregation patterns of six 3-generation Utah CEPH families. CEPH (Centre D'étude du Polymorphisme Humaine), is a non-profit research institute located in Paris, France, which stores, maintains, and distributes DNA from 61 large multi-generational families known as the CEPH reference panel. Examining the inheritance patterns of this copy number variant in these families provides a means of ensuring the sensitivity and specificity of our detection assays.

Statistical Analysis

Analyses of the four copy number assay results were compiled into 2×3 tables. We designated 2 copies as the reference group as it was the most common, then used Fisher's exact test to compare individuals with 1 copy to the reference and again to compare individuals with 0 copies to the reference. We also analyzed copy numbers for CNP147 in a logistic regression model and included *CFH* variants rs1061170 and rs1410996 as co-variables to get an adjusted test statistic. We included these SNPs in our analysis because they have previously been found to have a protective effect against AMD.⁷ In addition, Daly and his co-workers found that a surrogate for rs1410996 may mitigate the effect of the *CFHR* deletion and we wanted to account for this in our model.⁹ We used the haplotype estimation software PHASE version 2.11 (Matthew Stephens, University of Washington, Seattle) to estimate haplotypes for rs1061170, rs1410996, and CNP147. The four resulting common haplotypes were regressed as one categorical predictor, with the most common as the reference group. All the statistical associations and modeling was done using STATA 9 (StataCorp LP, College Station, TX).

Linkage disequilibrium (LD) was estimated using Haploview using default parameters.¹⁰ AREDS genotyping data from the Illumina 100K scan was generously provided to us by Dr.

Emily Chew of the National Eye Institute. This data is available by request through dbGaP at the National Center for Biotechnology Information at NIH.

RESULTS

Figure 1 shows a map of the genetic region studied with two of the *CFH* coding single nucleotide polymorphisms (SNPs), I62V and Y402H, denoted as rs800292 and rs1061170, respectively and a SNP located in an intron of *CFH*, rs1410996. The figure also delineates the relationship of the *CFH* gene to the *CFHR1* and *CFHR3* genes comprising CNP147 and the location of the copy number probes used for our studies. The data for the colored triangle plots was compiled from an Illumina100K SNP Scan carried out on 205 of the 215 AMD affected research subjects for whom information was available. As mentioned in the Introduction, the linkage disequilibrium between *CFH* and *CFHR1* is very high (reflecting high co-inheritance between these gene loci), while the linkage disequilibrium between *CFH* and *CFHR4* is greatly reduced, reflecting their lack of co-inheritance. As is shown in the figure, *CFHR4* is present in a separate LD block (denoted Block 2) from *CFH*, *CFHR1*, and *CFHR3* (which are denoted in Block 1).

Counts of the copy numbers for CNP147 presence or absence are provided in Table 1. If CNP147 was absent in a research subject in both chromosomes, meaning that there is no detectable *CFHR1* or *CFHR3* gene present, we scored that subject as 0. As can be seen from Table 1, there were 18 zero copy individuals among the controls and 7 zero copy individuals among the AMD affected individuals in our matched AREDS cohort. In statistical terms this amounted to a very high level of protection from disease associated with a lack of *CFHR1* and *CFHR3* (OR 0.15, 95% CI 0.0–.37, $P=1.0 \times 10^{-5}$). In fact, the presence of CNP147 in the AREDS matched cohort showed it to be highly associated with AMD, evidencing protection in a multiplicative manner (r fold decrease in odds for 1 copy and r^2 fold decrease in odds for 0 copies). Even individuals deleted for 1 copy of *CFHR1* and *CFHR3* were significantly protected relative to the two copy reference, having an approximate two-fold reduction in odds of disease (odds ratio 0.39, 95% confidence interval=0.26–0.58, $P=3.1 \times 10^{-6}$).

In order to make certain that our assay was performing as expected we performed several quality controls on samples that had been typed using an earlier technology that could not resolve heterozygote (1 copy) individuals at CNP147.⁷ The three control samples we used from HapMap, GM18500, GM18505, and GM18861, were verified; and all but one of the 25 samples that were homozygous deleted from our previous publication were consistent with our current result.⁷ In addition, copy numbers in the CEPH families segregated correctly.

We used two assays for each of CNP147 and CNP148. The two assays amplifying CNP147 were 99% consistent with each other, confirming that they both amplified the same polymorphism. The two assays amplifying CNP148 were 99% consistent with each other, but different from our results for CNP147. CNP148 is very infrequent in Caucasians and has a minor allele frequency of less than 1% in our sample. It is not significantly associated with AMD disease (data not shown). Because the two assays in CNP147 yielded the same results, we used the data from one (hs04197581cn) as representative of CNP147. The most common copy number was two and we designated this group as the reference.

We also analyzed CNP147 by logistic regression (Table 2). After controlling for the amino acid changing polymorphism rs1061170 (*CFH* Y402H) and rs1410996 (another polymorphism in an intron), a statistically significant relationship remained between

CNP147 and AMD; the adjusted odds ratio was 0.57 (95% confidence interval= 0.38–0.85, $P = 0.006$).

Haplotype estimation for the two *CFH* variants (above) and CNP147 yielded six haplotype predictions as are shown in Table 3. In order from most frequent to least frequent, they are: C-A-N, T-A-N, T-G-N, T-G-D, C-A-D, and T-A-D, where the order of the haplotype read-out is rs106117-rs1410996-CNP147 deleted “D” or present “N”. The two rarest haplotypes (2.3% and 0.2%) were omitted from our analyses. Using the four common haplotypes we used a logistic regression model with the most common C-A-N haplotype as the reference. Haplotype T-A-N, which included the protective allele from rs1061170, had an odds ratio of 0.64 (95% CI 0.46–0.91); haplotype T-G-N, having protective alleles from both rs1061170 and rs1410996, was more protective with an odds ratio of 0.40 (CI=0.28–0.59); and lastly, T-G-D contains protective alleles from all three variants and had the most protective effect with an odds ratio of 0.25 (CI=0.17–0.36).

DISCUSSION

Our statistical results, combined with the fact that *CFH* paralogs *CFHR3* and *CFHR1* comprise CNP147, supports the hypothesis that this CNP contributes an independent, but related, risk of AMD.

The drusen in AMD contain almost all alternative complement pathway proteins, and the products of its activation and degradation, which indicates that there is a local, complement-mediated inflammation in the retina in AMD.¹¹ Complement factor H, the main inhibitor of this pathway, functions by binding to polyanions on cell surfaces where it interacts with C3. The factor H protein consists of 20 short consensus repeat (SCR) modules, and it is SCR7 and SCR20 specifically that bind factor H on cell surfaces thereby providing the necessary anchoring mechanism that mediates its function.¹² Crystallization studies showed that factor H variant Y402H (assay rs1061170) occupies a key position in SCR7 and is directly involved in cell surface binding.¹² A histidine at this position decreases the efficiency of SCR7, thereby leading to altered levels of factor H on the retinal/macular surface.¹²

Complement factor H related paralogs *CFHR1* and *CFHR3* proteins (CNP147) are involved in the terminal end of the alternative complement pathway, inhibiting the cascade by interacting with C5. Interestingly, and in contrast to the increased risk it has on AMD, having fewer copies of these factor H related proteins are associated with an increased risk of atypical hemolytic uremic syndrome (aHUS), a severe renal disease.¹³ This is proposed to occur because in aHUS the absence of *CFHR1* and *CFHR3* in plasma correlates with the presence of autoantibodies to factor H, thereby leaving host cells underprotected against spontaneous complement activity.^{11, 14} However, auto-antibodies to factor H have not been described in AMD as they have in aHUS.¹¹ Both *CFHR1* and *CFHR3* can compete with factor H for binding on cell surfaces, and their presence reduces factor H efficiency; yet they do not have complement inhibiting properties.¹¹ Therefore, as evidenced by our epidemiological results, an absence or diminution of *CFHR3* and *CFHR1* proteins may have a protective effect by allowing factor H to more efficiently inhibit the complement cascade. We observed a significant association between CNP147 and AMD disease, which exerts protection in a dose dependent manner. Our previous results for this variant were limited in that we could only detect 0 or 2 copies of CNP147.⁷ Here we present our findings that show that odds of disease decrease for individuals with one copy or zero copies compared to individuals with two copies. Even after adjusting for other highly associated variants in the *CFH* gene, there remains a statistical relationship between CNP147 and AMD. In our previous findings of Y402H in the *CFH* gene, we reported a significantly reduced risk of AMD due to this variant. However, in light of these new data on CNP147, our resulting

statistical analyses suggest that the reduction in odds of disease may be attributed to both *CFH* and CNP147.

In our haplotype estimations, risk alleles for rs1061170 and rs1410996 most frequently segregated with higher copy numbers for CNP147, but not exclusively. The high-risk C allele for Y402H segregated with the high-risk A allele in rs1410996 100% of the time; and how this variant affects *CFH*, if at all, remains to be determined through functional studies. The four common haplotypes, C-A-N, T-A-N, T-G-N, and T-G-D, showed a stepwise reduction in risk as each locus' risk allele was replaced with its protective allele. The magnitude of diminished risk is most striking for the haplotype containing the deletion of CNP147.

In this matched sample of 636 individuals, our data and results suggest that CNP147 contributes an independent risk for AMD disease. Subjects carrying fewer copies have an estimated 43% reduction in odds of having AMD than do persons carrying 2 copies. We anticipate with great interest any future studies of factor H function as well as functional studies of the CFHR proteins that may help clarify how their genes contribute to the etiology of AMD.

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Acronym glossary

ABI	Applied Biosystems Inc., recently a subsidiary of Life Technologies, Inc
aHUS	atypical hemolytic uremic syndrome
AMD	age-related macular degeneration
AREDS	Age-related Eye Disease Cohort Study
CEPH	Centre D'etude du Polymorphism Humaine
CFH	complement factor H gene
CFHRI	complement factor H related gene 1
CFHR3	complement factor H related gene 3
CI	confidence interval
CNP	copy number polymorphism
D'	The ratio between how often alleles are transmitted together versus their maximal likelihood of being transmitted together
LD	linkage disequilibrium
OR	odds ratio
SCR	short consensus repeat

SNP single nucleotide polymorphism

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Figure 1. Linkage Disequilibrium (LD) plot of the *CFH* region. Probes used for copy number detection and SNPs of interest are noted by black triangles in the upper portion of the figure. In a triangle plot, the color depth reflects the correlation between two SNPs being transmitted together on the same haplotype, whereas lack of color provides evidence that the SNPs are independently transmitted. D' , a measure of LD is provided in many red triangles. Darker triangles with larger values of D' demonstrates high LD. The yellow highlighted rectangle demonstrates high but imperfect LD between the Y402H SNP (rs1061170) and CNP147 Probe1 or Probe2.

Table 1

Results for CNP147 are presented below with 2 copies used as the reference.

copies	Controls	cases	odds ratio	confidence interval	P
2	109 (54%)	303 (77%)	1.00(ref)	-	-
1	76 (37%)	83 (21%)	0.39	0.26–0.58	3.1×10^{-6}
0	18 (9%)	7 (2%)	0.15	0.06–0.37	1.0×10^{-5}

Fisher's exact test was used to generate odds ratios for individuals carrying 1 copy as compared to the reference, and again for individuals carrying 0 copies as compared to the reference. P values are two-sided.

Table 2

Logistic regression analyses of variants associated with age-related macular degeneration.

Predictor	* Unadjusted OR, CI, P	** Adjusted OR, CI, P
rs1061170	0.44 (0.34–0.58) 1.3×10^{-9}	0.66 (0.47–0.95) 0.024
rs1410996	0.37 (0.27–0.49) 7.4×10^{-12}	0.64 (0.41–0.97) 0.038
CNP147	0.38 (0.28–0.53) 6.3×10^{-9}	0.57 (0.38–0.85) 0.006

Odds ratios (OR) and confidence intervals (CI) reflect the reduced odds of having age-related macular degeneration (AMD) associated with a one unit decrease in copy number.

* Unadjusted odds ratios, confidence intervals, and P values for each variant analyzed separately.

** Adjusted values when all three variants are included in one regression model.

Table 3
Common haplotypes analyzed by logistic regression with the most common used as the reference.

Haplotype	Controls	Cases	odds ratio	confidence interval	P
C-A-N	128	377	ref	-	-
T-A-N	76	145	0.64	0.46–0.91	0.013
T-G-N	70	83	0.40	0.28–0.59	2.1×10^{-6}
T-G-D	97	72	0.25	0.17–0.36	1.2×10^{-13}

1st position rs1061170 (T=protective allele)

2nd position rs1410996 (G=protective allele)

3rd position CNP147 (N=non-deleted, D=deleted)

Every individual research subject has two haplotypes on each chromosome; one from each parent. Each of these haplotypes is tabulated in this table.