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# Analysis of Major Alleles Associated With Age-Related Macular Degeneration in Patients With Multifocal Choroiditis

## *Strong Association With Complement Factor H*

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**Objective:** To analyze the frequency of major age-related macular degeneration (AMD)-associated alleles in patients with multifocal choroiditis (MFC).

**Methods:** A cohort of 48 patients with MFC was compared with previously characterized cohorts of patients with advanced AMD (368 samples) and matched unaffected controls (368 samples). Allele and genotype frequencies of single nucleotide polymorphisms for the following AMD-associated alleles were evaluated: risk alleles in complement factor H (*CFH*) gene (Y402H and IVS14) and *LOC387715/HTRA1* gene on 10q26 (A69S) and protective alleles in *CFH* (IVS1, IVS6, and del-CFHR1-3) and complement factor B loci (H9L and R32Q).

**Results:** Frequencies of all major AMD-associated alleles in the *CFH* locus indicate a strong, statistically significant association of *CFH* gene single nucleotide polymorphisms and MFC. However, the same analysis for the single nucleotide polymorphisms in complement factor B and 10q26 loci matched the results in the control group.

**Conclusions:** Like AMD, the MFC phenotype is strongly associated with the major alleles/haplotypes in the *CFH* locus.

**Clinical Relevance:** We report compelling evidence of a strong association between *CFH* polymorphisms and MFC, which contributes to the understanding of MFC pathogenesis and suggests new potential therapeutic targets.

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MULTIFOCAL CHOROIDITIS (MFC) typically affects individuals younger than 50 years and leads to a dramatic decrease in their quality of life and productive capacity. It is typically seen as a chronic relapsing panuveitis with multiple chorioretinal lesions. Acute yellow-white lesions primarily involve the choroid and outer retina and may evolve to punched-out chorioretinal scars with pigmented borders.[1-4] Although 85% of cases are bilateral, the severity of the disease is commonly asymmetric.[3,4] Secondary vision loss occurs in up to 70% of cases, mainly as a result of complications in the macular area such as cystoid macular edema, epiretinal membrane, and choroidal neovascularization (CNV).[3,5,6] Reported frequencies of secondary CNV are variable in different series, but it is a major complication of MFC. Choroidal neovascularization may occur early in the course of the disease and is a main cause of visual acuity loss in patients with MFC.[3,5,7,8] An autoimmune inflammatory reaction is the proposed pathogenesis for MFC and concurrent panuveitis.[9] Histologic studies have suggested that subfoveal neovascularization secondary to both inflammatory and degenerative causes is associated with a local inflammatory response that varies with the underlying disease and the maturity of the neovascular membrane.[10] Early and aggressive immunosuppressive drug therapy reduces the risk of developing posterior pole complications and severe visual impairment.[3,8]

Age-related macular degeneration (AMD) is the leading cause of blindness and visual loss in the elderly in developed countries. It is a multifactorial disease with several established risk factors, including genetic and environmental components.[11-15] Genetic predisposing factors,[16-21] cellular

oxidative stress,[22,23] complement system pathways,[24,25] and local inflammatory processes[26,27] play a role in AMD pathogenesis.

Determination of contributing genetic factors in MFC might aid specific molecular diagnosis, suggest disease prognosis, and disclose novel potential targets for interventional strategies. Multifocal choroiditis and AMD seem to share fundamental pathophysiologic characteristics because immunologic mechanisms and chorioretinal inflammation play a central role in both conditions.[8-10,25-27] Although there is compelling evidence that polygenic risk factors and immune-mediated processes play a fundamental role in AMD pathogenesis,[24-27] disease-associated genotypes, to our knowledge, have never been described for MFC. Herein, we investigated the recently identified major risk and protective AMD-associated haplotype-tagging single nucleotide polymorphisms (htSNPs) in our cohort of patients with MFC.

## METHODS

Institutional review board approval was obtained for the study, and the principles outlined in the Declaration of Helsinki were followed. The MFC cohort consisted of 48 consecutive patients referred to a tertiary ophthalmologic center (Vitreous-Retina-Macula Consultants) during a 1-year period (from June 1, 2006, to May 31, 2007) enrolled after providing informed consent. Medical records were retrospectively reviewed. Most of the subjects (44) were whites of European-American descent, 2 were of Asian origin, and 2 were of Hispanic origin. The cohort included 46 unrelated patients and 2 siblings with a 3:1 female to male ratio (34 [71%] were female). The mean age at the time of enrollment in the study was 45 years, which does not coincide with the original time of MFC diagnosis, since most of the patients had been previously assessed by general ophthalmologists. At the time of enrollment, 77% of the cases (37 patients) showed secondary CNV of the so-called predominantly classic type.

Patients underwent complete clinical ophthalmic examination, including visual acuity measurement, biomicroscopy, ocular tonometry, and indirect ophthalmoscopy. Color fundus retinography was performed in all patients. Other complementary imaging tests (fluorescein angiography, optical coherence tomography, and fundus autofluorescence imaging) were performed when indicated. Diagnosis of MFC was clinically confirmed on the basis of the presence of chorioretinal lesions (acute choroidal lesion or pigmented chorioretinal scar) associated with clinical signs of uveitis (inflammatory cells in anterior and/or posterior chambers, vasculitis, optic nerve head hyperemia and edema), as previously established.[1-3] None of the patients had any concurrent ocular or systemic diagnosis associated with host/cell tissue damage mechanism (uveitis of any other cause, AMD, and ocular or systemic autoimmune disease).

The cohort of patients with advanced AMD was composed of 368 subjects with end-stage disease; three-fourths had CNV and one-fourth had geographic atrophy. The control cohort included 368 disease-free individuals (no AMD or MFC) matched by age and ethnicity with the AMD cohort. The average (SD) age for each population was 71.3 (–8.9) years and 68.8 (–8.6) years, respectively. Both groups were the same as reported in previous studies, where the ascertainment procedures and clinical characterization are described in detail.[19,28] Genomic DNA was generated from peripheral blood leukocytes collected from study subjects by means of kits (QIAamp DNA Blood Maxi; Qiagen, Valencia, California).

The htSNPs for complement factor H (*CFH*) (OMIM 134370) and complement factor B (*CFB*) (OMIM 138470) genes were defined in previous studies.[19,28,29] The *LOC387715/HTRA1* gene high-risk AMD-associated A69S variant was described in previous studies.[20,30] The genotyped htSNPs included the following: in the *CFH* locus, (1) rs1061170 (Y402H), (2) rs1410996 (IVS14T>C), (3) rs529825 (IVS1C>T), and (4) rs3766404 (IVS6C>T); in the *CFB* locus, (1) rs4151667 (H9L), (2) rs641153 (R32Q); and in the *LOC387715/HTRA1* gene, rs10490924 (A69S).

Genotyping was performed by polymerase chain reaction–restriction fragment length polymorphism and/or by *Taq*Man assays (Applied Biosystems, Foster City, California). The technique used was identical to that previously described.[19] Briefly, 5 ng of DNA was subjected to 50 cycles on a 384-well thermocycler (ABI 9700, Applied Biosystems), and plates were read in a sequence detection system (7900 HT, Applied Biosystems). Further genotyping details are available on request. Statistical analyses were performed by standard $2 \times 2$ table and Fisher exact tests. Multiple-comparisons tests (eg, Bonferroni correction) were not applied because each htSNP was analyzed independently. However, because 1 to 5 single nucleotide polymorphisms (SNPs) were analyzed in each gene (**Table**), the application of Bonferroni correction would still result in statistical significance in all cases.

## RESULTS

Allele and genotype frequency of 8 htSNPs from 3 loci, all tagging major AMD-associated haplotypes, were characterized in a cohort of 48 patients with MFC and compared with the same data acquired previously on a cohort of 368 subjects with end-stage AMD and an AMD-matched control cohort of 368 individuals.[19,28] Four htSNPs and 1 deletion were analyzed in the *CFH* locus. The most analyzed htSNP in *CFH*, which tags the major high-risk haplotype in AMD (H1), is Y402H (rs1061170).[19] As in AMD, the Y402H allele was highly elevated in the MFC cohort (Table); the risk of (402H) allele frequency in the MFC cohort (55.3%) was even slightly higher than that in the AMD cohort (53.9%), whereas it was much lower (32.4%) in the control cohort ($P < .001$; $\chi^2 = 17.54$; odds ratio [OR], 2.46; 95% confidence interval [CI], 1.6-3.8). The same was true for the IVS14T>C (rs1410996) SNP, which is more frequent than Y402H and tags an additional high-risk allele in AMD. Again, the frequency of this allele was practically identical in the MFC and AMD groups (72.3% vs 71.5%), which is significantly higher than in the control group (52.8%; $P < .001$; $\chi^2 = 11.2$; OR, 2.2; 95% CI, 1.34-3.3).[19,28]

The same trend continued with the "protective" *CFH* htSNPs, IVS1C>T (rs529825) and IVS6C>T (rs3766404), which tag H2 and H4, respectively, the 2 previously identified major AMD-protective haplotypes in the *CFH* locus.[19] Again, the frequencies of the H2-tagging IVS1 T allele were practically identical between the MFC cohort (15.6%) and the AMD cohort (16.1%), which were both much lower than in the control cohort (26.1%). This difference was even more apparent with the H4-tagging SNP IVS6 T. The frequency of the minor allele was 17.8% in the control cohort, 8.3% in the AMD cohort, and only 3.1% in the MFC cohort, a highly significant difference ($P < .001$; $\chi^2 = 13.8$; OR, 0.18; 95% CI, 0.07-0.50). For comparison, the OR for the same difference in SNP frequency between AMD and controls was 0.48.[19] This observation was further confirmed by genotyping of the highly AMD-protective deletion in the *CFH* locus (delCFHR1-3), which lies on the H4 haplotype in 75% of cases.[19,28] No patient

**Table. Allele and Genotype Frequency of 8 htSNPs From 3 Loci, All Tagging Major AMD-Associated Haplotypes**

| htSNP[a] | MAF | | | Comparison | | |
|---|---|---|---|---|---|---|
| | MFC (n=96) | AMD (n=736) | Controls (n=736) | MFC-AMD | MFC-Controls | AMD-Controls |
| *CFH* Y402H (rs1061170) | 0.553 | 0.539 | 0.324 | OR=0.97[b]<br>*P*=.79 | OR=2.46<br>*P*<.001 | OR=2.43<br>*P*<.001 |
| *CFH* IVS14 (rs1410996) | 0.277 | 0.285 | 0.472 | OR=0.97[b]<br>*P*=.87 | OR=2.2<br>*P*<.001 | OR=2.3<br>*P*<.001 |
| *CFH* IVS1 (rs529825) | 0.156 | 0.161 | 0.261 | OR=1[b]<br>*P*=.9 | OR=0.52<br>*P*=.008 | OR=0.5<br>*P*<.001 |
| *CFH* IVS6 (rs3766404) | 0.031 | 0.083 | 0.178 | OR=0.5[b]<br>*P*=.08 | OR=0.18<br>*P*<.001 | OR=0.48<br>*P*<.001 |
| *CFHR* 1/3 del/del | 0 | 0.005 | 0.065 | ND[c] | ND[c] | OR=0.08<br>*P*<.001 |
| *CFB* R32Q (rs641153) | 0.083 | 0.033 | 0.104 | OR=2.67<br>*P*=.02 | OR=1[b]<br>*P*=.52 | OR=0.29<br>*P*=.001 |
| *CFB* H9L (rs4151667) | 0.052 | 0.017 | 0.044 | OR=0.3<br>*P*=.02 | OR=1.2[b]<br>*P*=.71 | OR=0.36<br>*P*=.001 |
| LOC/ARMS2 A69S (rs10490924) | 0.293 | 0.484 | 0.223 | OR=0.41<br>*P*<.001 | OR=0.9[b]<br>*P*=.13 | OR=3.3<br>*P*<.001 |

Abbreviations: AMD, age-related macular degeneration; *CFB*, complement factor B gene; *CFH*, complement factor H gene; *CFHR*, *CFH*-related 1 and 3 genes; htSNP, haplotype-tagging single nucleotide polymorphisms; MAF, minor allele frequency; MFC, multifocal choroiditis; NS, not significant after statistical analysis; OR, odds ratio calculated by 2×2 table.

[a] The htSNPs A69S (*LOC387715/HTRA1* gene) and Y402H and IVS14 (both in *CFH* gene) tag high-risk haplotype in AMD, whereas the other polymorphisms in *CFH* and *CFB* genes are associated with lower risk of AMD development. As in AMD, a strong association of *CFH* polymorphisms with MFC is shown.

[b] Not significant after statistical analysis.

[c] Statistical analysis was impossible because the mutation did not occur in patients with MFC.

with MFC carried the homozygous deletion, which has been reported in approximately 6% of the white population and in approximately 1% of patients with AMD.[29]

Next, we analyzed the 2 major protective haplotypes from the *CFB*/complement component 2 (*C2*) locus (OMIM 217000), tagged by missense variants R32Q (rs641153) and H9L (rs4151667). Although relatively rare, each of these alleles has been shown to be 2 to 3 times more frequent in healthy elderly individuals than in patients with AMD.[28] The frequency of these htSNPs in the MFC cohort, however, was more similar to that of the control group than the AMD cohort. Specifically, the frequency of the protective 32Q allele was 8.3% in the MFC cohort (Table), 10.4% in the control cohort (difference not statistically significant), and 3.3% in the AMD cohort (a significant difference with both the controls and MFC). The difference between AMD and MFC was even more pronounced for the second allele (9L), which was detected in 1.7% of patients with AMD, 4.4% of controls, and 5.2% of patients with MFC (*P*=.02; OR, 0.3; 95% CI, 0.10-0.88). For this locus, the patients with MFC had as many or more AMD-protective alleles as did normal controls.

Finally, because CNV is a common complication in both end-stage AMD and MFC, we also determined the frequency of another major risk allele for AMD, the A69S variant in the *LOC387715/HTRA1* gene (*LOC387714*: OMIM 611313; *HTRA1*: OMIM 602194) on 10q26, which had been specifically associated with end-stage AMD (CNV and geographic atrophy) but not with early-stage AMD.[20] Like the *CFB* locus, the frequency of the AMD-associated 69S variant in the MFC cohort (29.3%) was much closer to that in the control cohort (22.3%) than in the AMD cohort (48.4%; *P*<.001; OR, 0.41).

The genetic results are summarized in the Table. Although our sample size had insufficient power to compare different subphenotypes among our patients with MFC, we did not observe a trend for a statistically significant difference in genotypes of patients with or without secondary CNV.

## COMMENT

An inflammatory mechanism is the proposed pathogenesis for MFC, in which a nongranulomatous choroiditis with a predominantly B-cell infiltrate is the principal histopathological finding. Acutely, the foci may show chorioretinal inflammation with consequential destruction of Bruch membrane, retinal pigment epithelium (RPE), and outer neurosensory retina.[9] Our results show that, in patients with MFC, the frequencies of *CFH* alleles are similar (almost identical) to those previously described for patients with AMD, which are strongly associated with an initial inflammatory component.[25,27] Therefore, our results identify a genotype that may lead to a host cell/tissue damage mechanism eventually manifested in the clinical findings characteristic of this entity and suggest that some disease mechanisms in MFC and AMD are similar, if not identical.

Age-related macular degeneration has been described as a complex disorder, derived from the interaction between multiple susceptibility gene loci modulated by environmental risk factors.[11,13,14,31-33] The anatomic and functional damage in AMD are secondary to degenerative and neovascular changes that affect the neurosensory retina and underlying choroid. Drusen deposition at the level of RPE is the hallmark lesion of early disease; local inflam-

mation and activation of the complement cascade have been implicated in their formation.[19,22-27] Complement system components such as complement pathway inhibitors, complement pathway activators, activation-specific complement fragments, and terminal pathway components, including the membrane attack complex, have been identified within drusenoid material, RPE cells, retinal basal membrane, and choriocapillaris in AMD.[25,34,35]

The major soluble inhibitor of the alternative complement pathway, CFH, is synthesized by RPE and has been implicated in inflammatory and oxidative damage of retinal cells.[19,27] Most of the AMD-associated polymorphisms in the *CFH* locus occur in functional domains of the encoded protein, including binding sites for C-reactive protein, heparin, C3b, and sialic acid.[19] The SNPs are likely to affect the function of the CFH protein through interaction with other proteins in the pathway. Different AMD-associated *CFH* gene SNPs and haplotypes that influence the disease severity and age at onset have been described.[19,29,36]

Variants in CFH, as well as in other CFH-related proteins, have a role in the etiology of other immune-mediated diseases such as atypical hemolytic uremic syndrome or membranoproliferative glomerulonephritis, where some risk haplotypes overlap with those of AMD.[37,38] The first htSNP we analyzed in the *CFH* locus was Y402H, a common coding variant shown to confer elevated risk of soft drusen and of late-stage AMD in most studied populations; it is the major susceptibility marker for all forms of AMD, including bilateral early-onset cases.[19,39] The 402H allele was highly elevated in our cohort of patients with MFC, with a frequency almost identical to that previously found in AMD cohorts and significantly higher than in the control group. The complement system activity has been associated with CNV proliferation and other processes of inflammatory tissue response and tissue scarring,[40] although the precise impact of *CFH* Y402H polymorphisms on CNV phenotype is still unclear because variable genotypic/phenotypic correlations have been reported.[41] Unlike neovascular AMD, where lesion phenotype is highly variable, in MFC the neovascular lesion is virtually always classified as the predominantly classic type, or type II CNV, on the basis of fluorescein angiography of the retina. Interestingly, an association between lesion phenotype and *CFH* genotype has been previously demonstrated in AMD. The cohort with the AMD-risk C allele encoding the *CFH* 402H variant appeared to be highly correlated with the predominantly classic type of CNV.[42] Indeed, in our MFC cohort, 84.1% of patients had at least 1 C allele. At the time of enrollment in the study, 77% of cases (37 patients) had secondary CNV; all of them showed type II CNV.

An additional AMD-risk allele is tagged by the IVS14T>C, which was also found to have practically identical frequencies in MFC and AMD cohorts and significantly higher frequencies than in control patients. Inversely, the analyzed htSNPs IVS1C>T and IVS6C>T tag 2 previously reported major AMD-protective haplotypes.[19] Our results again showed similar genotype frequencies for both the MFC and AMD cohorts, significantly different from the control group. Actually, the patients in the MFC cohort possessed even fewer protective alleles than those affected with AMD, perhaps reflecting the early and severe choroidal inflammation commonly observed in MFC.[3-8]

The *CFH* and *CFH*-related genes closely reside within a locus on chromosome 1q32 and share extremely high sequence homology. Although the CFH-related proteins are expressed in serum, their function remains to be determined.[29] Genotyping the highly AMD-protective deletion in the *CFH* locus (delCFHR1-3) did not identify homozygous individuals in the MFC cohort, confirming that the frequency of protective *CFH* alleles in MFC is even lower than in patients with AMD.

Two haplotype-tagging polymorphisms, L9H and R32Q, in *CFB* have been associated with a "protective" effect in AMD, ie, they are found at a much higher frequency in disease-free individuals than in patients with AMD.[28] Complement factor B aids initiation of the alternative complement cascade, whereas C2 activates the classic pathway. Both are located in the same locus and are expressed in neural retina, RPE, and choroid. The CFB protein has been identified in drusen, Bruch membrane, and, less prominently, choroidal stroma.[28] Surprisingly, the *CFB* variants were not found to be protective in our MFC cohort because the frequency of the 2 haplotype-tagging SNPs was similar to that of the control group and statistically significantly different from that of the AMD group. The frequency of L9H was even higher in patients with MFC than in the disease-free control cohort, but, interestingly, they still developed MFC. In other words, *CFB/C2* alleles protect for AMD but have no protective effect for MFC.

Multifocal choroiditis and AMD share some determinative clinical features. Atrophy of photoreceptors and RPE or CNV is typical of the advanced manifestation in both entities.[3,8,10-12] The A69S is a common coding variant in a hypothetical *LOC387715/HTRA1* gene on chromosome 10q26. This SNP tags the second major AMD susceptibility locus and has been particularly associated with advanced AMD, unlike the variants in the *CFH* and *CFB/C2* complement loci that are associated with all stages of AMD.[19,20,28,30,43-45] The frequency of htSNP A69S found in the MFC cohort was much closer to that in the control group than in the AMD cohort. Consequently, although the major risk allele in the 10q26 locus is highly associated with advanced AMD of either neovascular or atrophic forms, it does not seem to play a significant role in the MFC cohort, where CNV is a major phenotype (present in 77% of MFC cases included in this study). Although it would be tempting to speculate on the possible reasons for this difference, the functional consequences of the genetic variation in the 10q locus remain obscure, which prohibits meaningful speculation at this time.

In summary, a primary biological function of the complement system is to mediate immunologic response to infection. Nonetheless, deregulated activation of the complement cascade exposed to different modulating and triggering factors leads to a chronic imbalance of the inflammatory process. The resultant bystander host-cell/tissue damage has been shown to play a prominent feature in immune-mediated diseases.[46]

The association of variants in a major regulator of the alternative complement cascade, CFH, with MFC establishes a genetic predisposition for an immune-mediated mechanism at the interface of RPE and choriocapillaris.

Indeed, MFC is characterized by a chronic recurrent choroiditis with panuveitis. The strong association of *CFH* polymorphisms with MFC provides the first evidence that aberrant regulation of the alternative complement pathway contributes also to the etiology of MFC.

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