Prospective Assessment of Genetic Effects on Progression to Different Stages of Age-Related Macular Degeneration Using Multistate Markov Models

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PURPOSE. Understanding the effect of genes on progression to different stages of age-related macular degeneration (AMD) may suggest stage-specific therapeutic targets and more precise prediction of the development of this disease.

METHODS. Progression events and time to each stage of AMD were derived from the longitudinal data of 2560 subjects without advanced AMD. SNPs in 12 AMD risk loci were genotyped. A multistate Markov model for progression from normal to intermediate drusen, then to large drusen, and eventually to neovascular disease (NV) or geographic atrophy (GA) was applied to estimate stage-specific hazard ratios for each SNP. The effects of these genetic factors were also estimated by a multivariate multistate Markov model adjusted for baseline age, sex, smoking, body mass index (BMI), education, antioxidant treatment, and the status of AMD in the fellow eye.

RESULTS. Controlling for demographic and behavioral factors and other SNPs, the TT genotype of rs10468017 in *LIPC* was associated with decreased risk of progression from large drusen to NV (HR = 0.57, $P = 0.04$) and tended to reduce the risk of progression from normal to intermediate drusen (HR = 0.72, $P = 0.07$). The SNP rs1883025 (T allele) in *ABCA1* was associated with decreased risk of progression from normal to intermediate drusen (HR per allele = 0.82 per allele, $P = 9.7 \times 10^{-3}$) and from intermediate drusen to large drusen (HR per allele = 0.77, $P = 5.2 \times 10^{-3}$). The genes *CFH*, *C3*, *CFB*, and *ARMS2/HTRA1* were associated with progression from intermediate drusen to large drusen and from large drusen to GA or NV.

CONCLUSIONS. Genes in different pathways influence progression to different stages of AMD. (*Invest Ophthalmol Vis Sci*. 2012;53:1548-1556) DOI:10.1167/iovs.11-8657

The presence of drusen in the macula is the hallmark of early and intermediate stages of AMD and may eventually lead to visual loss.^{1,2} The two advanced forms, geographic atrophy (GA) and neovascular disease (NV), can lead to irreversible central visual impairment.³ Demographic, behavioral, and genetic factors are related to progression from the early and intermediate stages to advanced stages of AMD, including age, smoking, higher body mass index (BMI), nutritional factors, and several genetic variants.^{4,5} Reasons for transitions among the earlier stages, however, have not been extensively explored.

AMD is known to be influenced primarily by genetic factors, although environmental and behavioral factors also play important roles.^{4,5} Multiple studies have shown that several genes in the alternative complement pathway (*CFH*, *C2*, *CFB*, *C3*, and *CFI*) are associated with AMD. This pathway and related mechanisms have been extensively studied in relation to AMD and is one of the major susceptibility factors.⁶⁻¹² The *ARMS2/HTRA1* region is also strongly associated with AMD,¹³⁻¹⁵ although its function is still not confirmed. More recently, genes in the high-density lipoprotein (HDL) cholesterol pathway (*LIPC*, *ABCA1*, and *CETP*), extracellular matrix pathway (*TIMP3*, *COL10A1*, and *COL8A1*), and angiogenesis pathway (*VEGFA*) have been shown to be associated with AMD in a genome-wide association study (GWAS).¹⁶⁻¹⁸

Our recent case-control studies confirmed the effects of previously reported genetic variants in the complement pathway and in the HDL pathway on the risk of advanced AMD.¹⁹⁻²¹ Our case-control study of the association between these variants and early, intermediate, and late AMD suggested that genes in the complement and the HDL pathways have specific influences on different stages of AMD.¹⁹ To our knowledge, no study has explored the effect of these recently reported genetic loci on AMD progression from normal to intermediate stages and then to advanced stages in a prospective study. To assess the effect of genetic and other prognostic factors on each stage of AMD and to apply genetic knowledge to assess its impact on the natural history of the disease, we studied the progression of AMD by combining the genetic, environmental, and demographic factors using Markov modeling.²² Multistate Markov modeling is designed for analyses that involve many disease stages. It can estimate the effects of each variable on the risk of transition from one disease stage to another. Multistate Markov models can be applied to estimate the probabilities of progressing from one stage to another, which could be of particular interest in predicting the risk and potential severity of AMD at an individual level. The purpose of the present study was to further expand our previous studies by the following: adding seven recently reported AMD genes to

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FIGURE 1. AMD progression through a five-state Markov model among subjects without advanced AMD at baseline. *Included 696 stage 2 subjects at baseline and 494 subjects who progressed from stage 1 to stage 2 during the study. †Includes 1151 stage-3 subjects at baseline and 376 subjects who progressed from stage 2 to stage 3 during the study.

progression analyses, incorporating transitions among five stages of AMD, and applying a new statistical model to evaluate the different effects of multiple genes on risk of progression to different stages of AMD.

METHODS

Study Population

Longitudinal records based on ocular examination and fundus photography of Caucasian subjects with DNA specimens in the Age-Related Eye Disease Study (AREDS) were analyzed. The participants were examined every 6 months to 1 year for up to 13 years. For this analysis, only subjects without advanced AMD at baseline were included in the analyses ($N = 2560$), to assess progression among patients who had

not yet developed advanced AMD in either eye. Clinical data at each visit were used to categorize both eyes of each subject into five AMD stages, according to the Clinical Age-Related Maculopathy Staging System (CARMS).²³ Eyes were assigned to stage 5 if there were any definitive signs for neovascular AMD such as hemorrhagic retinal detachment, hemorrhage under the retina or retinal pigment epithelium, or subretinal fibrosis. Eyes classified as stage 4 had geographic atrophy either in the center grid or anywhere within the grid and without any record of hemorrhage. Eyes with large drusen ($\geq 125 \mu\text{m}$) were assigned to stage 3 and eyes with intermediate drusen ($63\text{--}124 \mu\text{m}$) were assigned to stage 2, as long as there were no signs of advanced AMD. Eyes with either no drusen or only a few small drusen ($<63 \mu\text{m}$) were assigned to stage 1 as the starting stage and were considered normal for the purposes of the present analysis. The progression stage of a subject at each visit was determined by the worse eye (the eye with the most advanced stage of AMD). The baseline stage of a subject was the worse eye stage of his first visit. The AMD stage of the less severe eye at baseline was included in the multivariate analysis as a covariate. Similarly, the final stage for a subject was determined by the stage of his worse eye at the last visit, which may differ from that of the worse eye at baseline. The time of progression to a specific stage is the number of years since the baseline visit. Participants with less than 2 years' follow-up or with advanced AMD at baseline were excluded from analysis. Demographic and behavioral information, including age, sex, education, and smoking history, was obtained at the baseline visit from questionnaires. BMI was derived from height and weight measurements at the baseline visit. Antioxidant treatment was defined as "yes" for subjects in the antioxidants alone or the antioxidants-plus-zinc groups, and "no" for subjects in the placebo or the zinc groups. Antioxidant treatment groups were randomly assigned by the AREDS clinical trial. The research complied with the Declaration of Helsinki, and Institutional Review Board approval was obtained.

SNP Selection

We assessed variants in HDL cholesterol metabolism genes, including a functional variant (rs10468017) in the hepatic lipase (*LIPC*) gene on chromosome 15, region q22, which was associated with advanced AMD with genome-wide significance ($P = 1.34 \times 10^{-8}$) in our previous GWAS¹⁷; SNP rs1883025 in the ATP-binding cassette subfamily A member 1 (*ABCA1*) gene on 9q31; SNP rs3764261 in the cholesterol ester transfer protein (*CETP*) gene on 16q21, which had suggestive associations with advanced AMD in previous GWAS^{17,18} and which reached genome-wide significance in a larger GWAS cohort¹⁶; and haplotypes in the apolipoprotein E (*APOE*) gene, which have been reported to be associated with AMD in some studies.²⁴ We selected a recently reported SNP (rs9621532) in the tissue inhibitor metalloproteinase 3 (*TIMP3*) gene^{17,18} and a candidate SNP (rs13095226) in the collagen type VIII alpha1 (*COL8A1*) gene^{16,17} for genotyping. We also

TABLE 1. MAFs of SNPs in AMD Stage Categories at Baseline

SNP	Gene	Minor Allele	Normal (Stage 1)	Intermediate Drusen (Stage 2)	Large Drusen (Stage 3)
Subjects, n			713	696	1151
rs1061170	<i>CFH</i>	C	0.38	0.38	0.53
rs10490924	<i>ARMS2/HTRA1</i>	T	0.20	0.23	0.34
rs9332739	<i>C2</i>	C	0.05	0.04	0.03
rs641153	<i>CFB</i>	T	0.09	0.08	0.06
rs2230199	<i>C3</i>	G	0.21	0.22	0.27
rs10033900	<i>CFI</i>	T	0.49	0.48	0.51
rs10468017	<i>LIPC</i>	T	0.30	0.28	0.27
rs9621532	<i>TIMP3</i>	C	0.05	0.05	0.05
rs3764261	<i>CETP</i>	A	0.33	0.33	0.35
rs1883025	<i>ABCA1</i>	T	0.26	0.25	0.25
rs13095226	<i>COL8A1</i>	C	0.11	0.09	0.10
APOE*	<i>APOE</i>	E4	0.14	0.10	0.11

* APOE haplotypes were defined by alleles in rs7412 and rs429358.

TABLE 2. Characteristics of Subjects without Advanced AMD in Either Eye at Baseline

Variable	n	%
Sex		
Female	1458	57
Male	1102	43
Age at enrollment, y		
<65	629	25
65–74	1663	65
75+	268	10
Median age 68 y		
BMI		
<25	859	34
25–29	1064	43
30+	566	23
Smoking		
Never	1250	49
Past	1186	46
Current	124	5
Education		
≤High school	787	31
>High school	1771	69
Antioxidants		
No	1276	50
Yes	1284	50
Worse eye status at baseline		
None or small drusen	713	28
Intermediate drusen	696	27
Large drusen	1151	45
Fellow eye status at baseline		
None or small drusen	1317	51
Intermediate drusen	556	22
Large drusen	687	27
Years of follow-up		
<5	9	0
5–10	1172	46
>10	1379	54

genotyped a nonsynonymous SNP rs10490924 in the *ARMS2/HTRA1* region of 10q26, which is associated with advanced AMD.^{13–15,25} In addition, five known risk variants associated with advanced AMD in complement genes were genotyped, including SNP rs1061170 in exon 9 of the *CFH* gene on 1q31, which results in a substitution of histidine for tyrosine (Y402H) in the protein sequence of *CFH*; SNP rs9332739 in the complement component 2 (*C2*) gene, which results in an amino acid change *E318D* in exon 7; SNP rs641153 in the complement factor B (*CFB*) gene, which results in an amino acid change at *R32Q*; SNP

rs2230199 in the complement component 3 (*C3*) gene, which results in an amino acid change at *R102G* in exon 3; and SNP rs10033900 in complement factor I (*CFI*) gene on chromosome 4.^{6–12,15,25–28}

Genotyping

DNA was extracted from blood samples of participants. SNPs in the *CFH*, *ARMS2/HTRA1*, *C2*, *C3*, *CFB*, and *CFI* genes were genotyped (iPLEX assay; Sequenom) at the Broad Institute Center for Genotyping and Analysis. For SNPs not compatible with the assay, such as *APOE*, and other SNPs newly reported to be associated with advanced AMD, including *LIPC*, *CETP*, *ABCA1*, *TIMP3*, and *COL8A1* genes/regions, DNA samples were genotyped (TaqMan assay, using the PRISM 7900 Sequence Detection System; Applied Biosystems, Inc. [ABI], Foster City, CA) at the Tufts Medical Center core laboratory.

We implemented stringent quality control criteria for each SNP in our dataset. All the SNPs had a greater than 99% genotype call rate. The rates for missing SNPs genotyped on the genotyping platforms (iPLEX; Sequenom, and TaqMan; ABI) were similar. None of the SNPs were significant ($P < 10^{-3}$) in the Hardy-Weinberg equilibrium test or in the differential missing tests ($P < 10^{-3}$) between cases and controls. PLINK was used to perform all quality-control steps.²⁹

Statistical Analysis

The allele frequencies of each SNP in categories defined by baseline AMD stages or by progression status of each transition were estimated using PLINK. SNPs were coded by the number of risk alleles (0, 1, and 2), unless specific genotypes were combined (coded as 0, 1) due to low frequency or known dominant/recessive effect.¹⁹ Markov multi-state proportional hazards analysis was performed by the R package *mstate*,²² which is an extension of the Cox proportional hazards model and competing risks models.

We first set up a five-state univariate, continuous-time, homogeneous Markov model (Fig. 1) to test the effect of each gene on progression through four transitions: from normal to intermediate drusen (stages 1→2), from intermediate drusen to large drusen (stages 2→3), and from large drusen to GA (stages 3→4) or to NV (stages 3→5). Note that the two advanced AMD states (GA and NV) in this model are the absorbing stages. Once a patient has reached these stages, no further transition is allowed. As retrogression events are rare in AMD, the model was restricted to be unidirectional, and only transitions from the normal stage through the drusen stages to the advanced AMD stages were allowed. A person can contribute to the analysis of multiple types of transitions over the follow-up period. For example, a stage 1 subject at baseline could progress to stage 2 after 5 years, then progress to stage 3 after 2 additional years, and then remain

TABLE 3. MAFs of SNPs in Progressors and Nonprogressors at Each Transition

SNP	Gene	Transition Minor Allele	1 → 2		2 → 3		3 → 4		3 → 5	
			Non*	Pro*	Non*	Pro*	Non*	Pro*	Non*	Pro*
Subjects, n			219	494	814	376	949	280	949	298
rs1061170	<i>CFH</i>	C	0.37	0.39	0.36	0.43	0.44	0.62	0.44	0.62
rs10490924	<i>ARMS2/HTRA1</i>	T	0.17	0.21	0.20	0.28	0.28	0.41	0.28	0.41
rs9332739	<i>C2</i>	C	0.06	0.05	0.05	0.04	0.04	0.02	0.04	0.02
rs641153	<i>CFB</i>	T	0.11	0.09	0.09	0.07	0.08	0.05	0.08	0.04
rs2230199	<i>C3</i>	G	0.19	0.21	0.20	0.25	0.24	0.32	0.24	0.31
rs10033900	<i>CFI</i>	T	0.45	0.51	0.49	0.49	0.49	0.57	0.49	0.49
rs10468017	<i>LIPC</i>	T	0.34	0.29	0.28	0.28	0.29	0.24	0.29	0.26
rs9621532	<i>TIMP3</i>	C	0.06	0.05	0.05	0.06	0.06	0.04	0.06	0.04
rs3764261	<i>CETP</i>	A	0.31	0.34	0.33	0.35	0.34	0.33	0.34	0.39
rs1883025	<i>ABCA1</i>	T	0.32	0.24	0.26	0.21	0.24	0.24	0.24	0.25
rs13095226	<i>COL8A1</i>	C	0.09	0.11	0.11	0.07	0.08	0.14	0.08	0.12
<i>APOE</i> †	<i>APOE</i>	E4	0.14	0.13	0.12	0.10	0.12	0.09	0.12	0.10

* Nonprogressors (Non) groups and progressors (Pro) for each transition in the five-state Markov model.

† *APOE* haplotypes were defined by alleles in rs7412 and rs429358.

in stage 3 until the end of the study. This subject will be recognized as a progressor in transition (1→2) as well as in transition (2→3) and as a nonprogressor in transition (3→4) and transition (3→5). Individuals who progressed from stage 1 to 5 without intervening stages ($n = 2$) were excluded from the analysis. The risk of progression to the next stage of AMD can be described by four sets of hazard ratios (HRs) for transition from one specific stage to the other for each variable. The association of a vector of variables (Z) for the instantaneous risk of transition from stage g to stage h can be described by:

$$\lambda_{gb}(t|Z) = \lambda_{gb,0}(t) \exp(\beta_{gb}^T Z)$$

where $\lambda_{gh}(t | Z)$ indicates the hazard for transition from stage g to stage b at time t for an individual with baseline covariate vector Z . $\lambda_{gh}(t)$ is the baseline hazard for transition from state g to state b —that is, the hazard for a subject with 0 values for each covariate at baseline. β_{gb}^T is the vector of regression coefficients corresponding to the transition from state g into state b . The vector of HRs for this transition is given by $(\exp(\beta_{gb}^T))$. As the Markov model assumes that the future depends on the history only through the present, the cumulative transition hazard from stage g to stage b can be calculated by $A_{gb}(t | Z) = \int_0^t \lambda_{gb}(u | Z) du$. For a model with S states, the cumulative transition hazards can be represented by a $S \times S$ -matrix $\mathbf{A}(t)$. The cumulative hazard for an individual to remain in state g is the diagonal element $A_{gg}(t | Z) = -\sum_{b \neq g} A_{gb}(t | Z)$ of the transition hazards matrix $\mathbf{A}(t)$. Given $\mathbf{A}(t)$, the transition probability matrix $P(s, t)$ can be calculated by means of a product integral $P(s, t) = \Pi_{u \in (s, t)} (I + d\mathbf{A}(u))$, in which I indicates the identity matrix, and u indicates all event times in (s, t) .³⁰ The transition probability matrix $P(s, t)$ is the prime quantity of interest. It has elements $P_{gb}(s, t) = P(X(t) = b | X(s) = g)$, which denote the transition probability from state g to stage b over the time interval (s, t) .

Furthermore, we investigated the effects of these genetic variants using a five-state multivariate Markov model controlling for other known genetic factors; demographic and behavioral covariates, including age, sex, education ($\leq$ high school or $>$ high school); smoking (never, past, or current); BMI (<25 , $25-29.9$, and ≥ 30); the stage of the less severe eye at baseline; and randomized antioxidant treatment by AREDS. Since the purpose of our analysis is not to identify novel loci for AMD, we used a nominal significance level ($P \leq 0.05$) to evaluate the effects of the known AMD risk factors on each transition between different AMD stages. We then estimated 5- and 10-year probabilities of transition for representative profiles of patients with low, intermediate and high genetic risk for AMD to demonstrate schematically the prognosis of patients at these different stages using the five-state multivariate Markov model.

RESULTS

A total of 2560 Caucasian participants without advanced AMD in either eye at baseline were genotyped in this study. At baseline, there were 713 (28%) subjects with no drusen or only a few small drusen in both eyes, 696 (27%) subjects with intermediate drusen in the worse eye, and 1151 (45%) subjects with large drusen in the worse eye but not affected with advanced AMD in either eye. Table 1 shows the minor allele frequencies (MAFs) of each SNP in the group of subjects defined by baseline AMD status. The average follow-up time was 10.3 years (range, 2–13). The distribution of demographic and behavioral characteristics of participants is shown in Table 2. The median baseline age of participants was 68 years (range, 55–81). Almost all subjects were observed for more than 5 years, and approximately 50% were observed for more than 10 years; 57% of the subjects were women, 66% of subjects were either overweight or obese, 5% were current smokers, and 69% had more than a high school education.

TABLE 4. Univariate Five-State Markov Model for AMD Progression

Worse Eye Transition										
1 → 2			2 → 3			3 → 4			3 → 5	
SNP	Gene	Effective Allele/Genotype	HR*	P	HR*	P	HR*	P	HR*	P
rs1061170	CFH	C	1.01 (0.89, 1.16)	0.83	1.26 (1.09, 1.45)	1.3×10^{-3}	1.62 (1.37, 1.91)	8.4×10^{-9}	1.61 (1.37, 1.89)	5.3×10^{-9}
rs10490924	ARMS2/HTRA1	T	1.14 (0.98, 1.33)	0.09	1.4 (1.2, 1.64)	2.8×10^{-5}	1.51 (1.28, 1.78)	7.6×10^{-7}	1.52 (1.29, 1.78)	3.4×10^{-7}
rs9332739	C2	C†	0.9 (0.66, 1.23)	0.52	0.82 (0.55, 1.21)	0.31	0.56 (0.31, 1.03)	0.06	0.44 (0.23, 0.86)	0.02
rs641153	CFB	T†	0.91 (0.72, 1.16)	0.45	0.79 (0.59, 1.07)	0.13	0.64 (0.42, 0.97)	0.03	0.53 (0.34, 0.82)	4.3×10^{-3}
rs2230199	C3	G	1.11 (0.95, 1.29)	0.18	1.2 (1.01, 1.42)	0.03	1.36 (1.13, 1.63)	9.4×10^{-4}	1.3 (1.09, 1.55)	4.3×10^{-3}
rs10033900	CFI	T	1.13 (1, 1.28)	0.05	1.03 (0.89, 1.18)	0.70	1.29 (1.09, 1.52)	3.2×10^{-3}	0.95 (0.8, 1.11)	0.49
rs10468017	LIPC	TT†	0.71 (0.5, 1)	0.05	0.85 (0.56, 1.28)	0.44	0.81 (0.5, 1.31)	0.39	0.64 (0.38, 1.08)	0.10
rs9621532	TIMP3	C†	0.99 (0.73, 1.34)	0.94	1.06 (0.77, 1.47)	0.71	0.69 (0.44, 1.08)	0.11	0.71 (0.47, 1.09)	0.12
rs3764261	CEP	A	1.08 (0.94, 1.23)	0.28	1.03 (0.89, 1.2)	0.68	1 (0.84, 1.19)	0.99	1.27 (1.08, 1.49)	4.6×10^{-3}
rs1883025	ABCA1	T	0.82 (0.71, 0.95)	7.8×10^{-3}	0.79 (0.66, 0.95)	9.7×10^{-3}	1 (0.82, 1.21)	0.97	1.02 (0.85, 1.23)	0.81
rs13095226	COL8A1	C†	1.13 (0.91, 1.4)	0.29	0.67 (0.49, 0.9)	8.0×10^{-3}	1.55 (1.18, 2.04)	1.7×10^{-3}	1.36 (1.04, 1.79)	0.03
APOE†	APOE	E†	0.98 (0.8, 1.21)	0.87	0.91 (0.7, 1.17)	0.47	0.79 (0.58, 1.07)	0.12	0.88 (0.66, 1.17)	0.39

HR, hazard ratio for a specific transition.

* P value is bold for variables significantly ($P \leq 0.05$) associated with a specific transition.

† Genotypes are combined at the marked SNPs, which are coded 0 for subjects without the effective allele/genotype and 1 for subjects with the effective allele/genotype. APOE haplotypes were defined by alleles in rs7412 and rs429358. SNPs not marked are coded by the number of effective alleles (0, 1, or 2).

Figure 1 shows the number of subjects with transitions at each stage of the Markov model. Overall, 494 individuals progressed from normal to intermediate drusen, 376 progressed from intermediate to large drusen, 280 individuals progressed from large drusen to GA, and 298 progressed from large drusen to the NV stage. Note that the total sample analyzed for the 2→3 transition (1190) resulted from subjects in stage 2 at baseline (696) and the subjects who progressed to stage 2 (494) during the study. Table 3 shows the MAF of each SNP in participants who progressed or did not progress for each transition between two stages of AMD. Generally, alleles associated with increased risk of AMD are more common among progressors and in groups at higher AMD stages, whereas alleles associated with reduced risk of AMD are more frequently seen in nonprogressors and in groups at lower AMD stages.

Table 4 shows the HRs and *P* values for each SNP separately for each transition. *ABCA1* (HR = 0.82, $P = 7.8 \times 10^{-3}$), *CFI* (HR = 1.13, $P = 0.05$) and *LIPC* (HR = 0.71, $P = 0.05$) were significantly associated with the risk of progression to intermediate drusen. *ARMS2/HTRA1* (HR = 1.4, $P = 2.8 \times 10^{-5}$), *CFH* (HR = 1.26, $P = 1.3 \times 10^{-3}$), *ABCA1* (HR = 0.79, $P = 9.7 \times 10^{-3}$), *COL8A1* (HR = 0.67, $P = 8.0 \times 10^{-3}$), and *C3* (HR = 1.2, $P = 0.03$) were significant for progression from intermediate drusen to large drusen. The hazard of progression to GA was associated with *CFH* (HR = 1.62, $P = 8.4 \times 10^{-9}$), *ARMS2/HTRA1* (HR = 1.51, $P = 7.6 \times 10^{-7}$), *CFB* (HR = 0.64, $P = 0.03$), *C3* (HR = 1.36, $P = 9.4 \times 10^{-4}$), *CFI* (HR = 1.29, $P = 3.2 \times 10^{-3}$), and *COL8A1* (HR = 1.55, $P = 1.7 \times 10^{-3}$). For the risk of progression from large drusen to NV, *CFH* (HR = 1.61, $P = 5.3 \times 10^{-9}$), *ARMS2/HTRA1* (HR = 1.52, $P = 3.4 \times 10^{-7}$), *C2* (HR = 0.44, $P = 0.02$), *CFB* (HR = 0.53, $P = 4.3 \times 10^{-3}$), and *COL8A1* (HR = 0.67, $P = 8.0 \times 10^{-3}$) were significant.

TABLE 5. Multivariate Five-Stage Markov Model for AMD Progression

Variable	Worse Eye Transition							
	1 → 2		2 → 3		3 → 4		3 → 5	
	HR	<i>P</i> *	HR	<i>P</i> *	HR	<i>P</i> *	HR	<i>P</i> *
Sex								
Female	1.00		1.00		1.00		1.00	
Male	1.01	0.93	1.04	0.72	1.23	0.12	0.87	0.28
Age, y								
<65	1.00		1.00		1.00		1.00	
65–74	1.10	0.35	1.17	0.22	1.17	0.35	1.40	0.05
75+	1.88	3.2×10^{-3}	1.74	7.7×10^{-3}	1.37	0.14	2.31	3.8×10^{-5}
BMI								
<25	1.00		1.00		1.00		1.00	
25–29	0.91	0.37	1.10	0.45	1.33	0.06	1.00	0.97
30+	0.84	0.17	1.00	0.98	1.68	1.8×10^{-3}	1.22	0.21
Smoking								
Never	1.00		1.00		1.00		1.00	
Past	0.90	0.29	1.09	0.47	1.06	0.65	1.29	0.05
Current	0.89	0.60	0.85	0.64	2.18	1.6×10^{-3}	1.96	8.3×10^{-3}
Education								
≤High school	1.00		1.00		1.00		1.00	
>High school	0.93	0.53	0.81	0.07	0.81	0.11	0.93	0.59
Antioxidants								
No	1.00		1.00		1.00		1.00	
Yes	1.13	0.20	1.12	0.28	0.93	0.56	0.93	0.53
Fellow eye status								
None or small drusen	1.00		1.00		1.00		1.00	
Intermediate drusen	—	—	1.98	1.8×10^{-9}	2.13	0.03	2.14	5.0×10^{-3}
Large drusen	—	—	—	—	9.97	2.9×10^{-13}	4.52	3.0×10^{-9}
Genetic risk factors: gene (effective allele/genotype)								
rs1061170 <i>CFH</i> C	1.02	0.8	1.27	1.60×10^{-3}	1.33	1.10×10^{-3}	1.37	2.20×10^{-4}
rs10490924 <i>ARMS2/HTRA1</i> T	1.14	0.12	1.36	2.90×10^{-4}	1.24	0.02	1.27	5.30×10^{-3}
rs9332739 <i>C2</i> C†	0.82	0.23	0.68	0.07	0.82	0.52	0.6	0.14
rs641153 <i>CFB</i> T†	0.92	0.52	0.78	0.11	0.9	0.63	0.57	0.02
rs2230199 <i>C3</i> G	1.16	0.07	1.22	0.03	1.26	0.01	1.25	0.02
rs10033900 <i>CFI</i> T	1.09	0.18	1.06	0.47	1.19	0.05	0.87	0.1
rs10468017 <i>LIPC</i> TT†	0.72	0.07	0.77	0.23	0.78	0.34	0.57	0.04
rs9621532 <i>TIMP3</i> C†	0.94	0.71	1.11	0.56	0.84	0.45	0.79	0.3
rs3764261 <i>CETP</i> A	1.09	0.27	1.06	0.44	0.97	0.76	1.17	0.06
rs1883025 <i>ABCA1</i> T	0.82	9.70×10^{-3}	0.77	5.20×10^{-3}	0.96	0.69	0.97	0.79
rs13095226 <i>COL8A1</i> C†	1.13	0.31	0.68	0.02	1.23	0.16	1.2	0.21
APOE† <i>APOE</i> E4†	0.99	0.92	0.93	0.59	0.89	0.46	0.97	0.85

* Adjusted for age, sex, education (≤high school vs. >high school), smoking (never, past, current), BMI (<25, 25–29.9, 30+), antioxidant treatment, and the number of effective alleles for each genetic locus in this table. *P* value is bold for variables significantly ($P \leq 0.05$) associated with a specific transition.

† Genotypes are combined at the marked SNPs, which are coded 0 for subjects without the effective allele/genotype and 1 for subjects with the effective allele/genotype. APOE haplotypes were defined by alleles in rs7412 and rs429358. SNPs not marked are coded by the number of effective alleles (0, 1, and 2).

10^{-3}), *C3* (HR = 1.3, $P = 4.3 \times 10^{-3}$), *COL8A1* (HR = 1.36, $P = 0.03$), and *CETP* (HR = 1.27, $P = 4.6 \times 10^{-3}$) were significantly associated.

To evaluate whether the genetic effects on progression to each AMD stage are independent of other genetic, demographic, and behavioral factors, we used a multivariate five-state Markov model. In addition to all the genetic factors in Table 4, age, sex, BMI, smoking, education, antioxidant treatment, and fellow eye status were included. Table 5 shows the HRs and P values of the risk factors for each progression step estimated by this multivariate model. Three major loci at *CFH*, *ARMS2/HTRA1*, and *C3* significantly increased the hazard of progression from intermediate drusen to large drusen and from large drusen to both NV and GA. The risk of disease progression from intermediate drusen to large drusen (HR = 1.36, $P = 2.9 \times 10^{-4}$), from large drusen to GA (HR = 1.24, $P = 0.02$), and to NV (HR = 1.27, $P = 5.3 \times 10^{-3}$) was increased for patients with the T allele at rs10490924 in *ARMS2/HTRA1*. The allele C of rs1061170 in *CFH* was estimated to increase the risk of progression from intermediate drusen to large drusen (HR = 1.27, $P = 1.6 \times 10^{-3}$), from large drusen to GA (HR = 1.33, $P = 1.1 \times 10^{-3}$), and to NV (HR = 1.37, $P = 2.2 \times 10^{-4}$).

Patients with allele G of rs2230199 in *C3* had increased risk of progression from intermediate drusen to large drusen (HR = 1.22, $P = 0.03$), from large drusen to GA (HR = 1.26, $P = 0.01$) and to NV (HR = 1.25, $P = 0.02$). *ARMS2/HTRA1* (HR = 1.14, $P = 0.12$) and *C3* (HR = 1.16, $P = 0.07$) also tend to increase risk for progression from normal to intermediate drusen, although the effect was not significant. Among all the genes included in the multivariate model, rs1883025 in *ABCA1* was the only one significantly associated with progression from normal to intermediate drusen (HR = 0.82, $P = 9.7 \times 10^{-3}$). The T allele of rs1883025 also decreased the risk of progression from intermediate drusen to large drusen (HR = 0.77, $P = 5.2 \times 10^{-3}$), but *ABCA1* was not significantly associated with the progression from large drusen to either GA or NV. *CFB*, *CFI*, and *LIPC* were significantly related to progression from the large drusen stage to one of the advanced AMD stages. The T allele of rs10033900 in *CFI* increased the risk of progression from large drusen to GA (HR = 1.19, $P = 0.05$). The TT genotype of rs10468017 in *LIPC* (HR = 0.57, $P = 0.04$) and T allele of rs641153 in *CFB* (HR = 0.57, $P = 0.02$) decreased the risk of progression from large drusen to NV.

FIGURE 2. Predicted probabilities of progression to the various AMD stages for patients with different genetic risk profiles using the five-state multivariate Markov model. Probabilities of being in each AMD stage for patients with low-, medium- or high-risk profiles are shown separately for 5-year (a, c, e) and 10-year (b, d, f) durations. Three examples for each risk profile are shown for patients, starting with worse eye of normal as stage 1 (a, b), intermediate drusen as stage 2 (c, d), and large drusen as stage 3 (e, f). Stage 4 is GA, stage 5 is NV. Patients were 65 to 74 years old, male, had a normal fellow eye, past smoking history, greater than high school education, and intermediate BMI levels (25–29) at baseline.

Demographic and behavioral factors also play important roles in AMD progression. Patients older than 75 years were predisposed to disease progression events across all the stages. Another important predictor for progression events was the fellow eye status. Patients with large drusen in both eyes had much higher risk of progression to GA (HR = 9.97, $P = 2.9 \times 10^{-3}$) and to NV (HR = 4.52, $P = 3.0 \times 10^{-9}$) than did patients with one normal eye and one eye with large drusen. Current smokers were twice as likely to progress from large drusen to one of the advanced stages, GA (HR = 2.18, $P = 1.6 \times 10^{-3}$) or NV (HR = 1.96, $P = 8.3 \times 10^{-3}$), than the nonsmoking patients. High BMI also significantly increased the risk of progression from large drusen to GA (HR = 1.68, $P = 1.8 \times 10^{-3}$).

This five-state multivariate Markov model could be used to estimate the probability of disease progression for patients with specific risk profiles. We set three example profiles for low, medium, and high genetic risk of AMD progression. For simplicity, all three patients were 65 to 74 years of age, male, had a normal fellow eye, past smoking history, greater than high school education, and intermediate BMI levels (25–29) at baseline. The high-risk individual's profile is homozygous on all genetic loci for the alleles that increase risk of AMD. The medium-risk profile is heterozygous on all risk genetic loci. The low-risk profile is homozygous on all genetic loci for the alleles that decrease risk of AMD. The worse eye status at baseline was set to be normal, intermediate drusen, or large drusen for the three profiles, and nine example scenarios were generated based on combining these variables. The probabilities of being in each AMD stage after 5 and 10 years were estimated for each example scenario, as defined by the risk profile and worse eye status at baseline (Fig. 2). Among individuals with normal status and high-risk genetic profile at baseline, there is about a 40% probability of developing large drusen in 10 years, a 4% probability of developing GA, and a 5% probability of developing NV (Fig. 2b). Among stage-2 individuals at baseline and high-risk genetic profile, there is approximately a 55% probability of developing large drusen in 10 years, a 10% probability of developing GA, and a 13% probability of developing NV (Fig. 2d). Among stage-3 individuals at baseline and high-risk genetic profile, there is approximately a 9% probability of developing GA, and a 15% probability of developing NV in 5 years (Fig. 2e), and a 17% probability of developing GA and a 26% probability of developing NV in 10 years (Fig. 2f). For low-risk genetic profile individuals at any baseline stage, there is a very low probability (<1%) of developing GA or NV in 10 years. The area under the receiver operating characteristic curve (AUC) for progression from nonadvanced stages (stages 1, 2, and 3) to advanced stages (stages 4 and 5) within 5 years was 0.883, and the AUC for progression from nonadvanced stages to advanced stages within 10 years was 0.895 (Fig. 3).

DISCUSSION

To our knowledge, this is the first study in which a multistate Markov model was used to evaluate 12 genetic variants, behavioral and demographic factors, and progression through four distinct stages of AMD. In this study, we found that several SNPs were associated with risk of progression through multiple stages of AMD, whereas others were associated only with a specific transition. A schematic diagram (Fig. 4) shows the genetic effects on different stages of AMD progression. This hypothetical model suggests that HDL genes play important roles in drusen initiation in the early stages of AMD, and then, as drusen accumulate between the RPE and Bruch's membrane, genes in the complement pathway are activated. This model is also supported by biochemical, histochemical, and ultrastructural studies.³¹ These new findings of transition-spe-

FIGURE 3. The ROC curve for progression from normal and nonadvanced stages (stages 1, 2, 3) to advanced stages (stages 4 and 5) of AMD within 5 and 10 years. ROC curves for 5- and 10-year progression from nonadvanced stages (stages 1, 2, and 3) to advanced stages (stages 4, 5) of AMD based on the model in Table 5. The area under the ROC curve (AUC) was 0.883 for 5-year progression from nonadvanced stages to advanced AMD and the AUC was 0.895 for 10-year progression from nonadvanced stages to advanced AMD (*dashed line*).

cific genetic effects for AMD progression may be helpful for designing treatment or prevention strategies at specific stages of the disease. The five-state model in this study could be used to predict risk of progression to each AMD stage at a given time for an individual with a given genetic and clinical profile. This model could also be useful for making treatment and follow-up decisions and counseling individual patients.

Recently, we reported the associations between genetic variants and different stages of AMD in a case-control study, in which stage of AMD was defined by the last known stage.¹⁹ The results herein expand on those results. The conventional case-control approach of association testing does not provide all the information for a disease with multiple stages, such as AMD. Genetic variants with a smaller effect on an intermediate disease stage may be missed by an association study if each phenotype category is considered separately. The allele frequencies in an intermediate disease stage group are influenced simultaneously by the number of patients who join this group (progress to this stage from a less severe stage) and the number of patients who leave this group (progress from this stage to a more severe stage). Considering only the allele frequencies between a specific stage of AMD and a control group does not capture the dynamic character of AMD progression. The Markov multistate model enables the evaluation of the effect of genetic and environmental factors associated with different stages and allows the estimation of the probability of each stage at a given time for an individual with a specific risk profile. The multistate model can also handle partial information due to censored observations and can be used to model disease progression to competing endpoints.

Only a few studies have investigated the possible role of genetic variants in drusen accumulation and early stages of AMD.^{19,32,33} In our recent study, genes in the HDL pathway were associated with early stages of AMD, whereas *ARMS2/HTRA1* and genes in the complement pathway were associated

FIGURE 4. The genetic effects on different stages of AMD progression. Genes that are significantly associated with the risk of a specific transition in the univariate or the multivariate Markov model are shown. *The SNP in the gene is significantly associated with risk of AMD progression in the multivariate Markov model.

with the advanced stages.¹⁹ The results of this multistate analysis for progression are consistent with our previous findings based on association studies and show that three major risk genes *CFH*, *ARMS2/HTRA1*, and *C3* increase the risk of progression from intermediate drusen to large drusen and from large drusen to GA and NV. In our previous case-control analysis, the T allele of rs1883025 in *ABCA1* was associated with a decreased risk of intermediate drusen, large drusen, GA, and NV.¹⁹ The Markov model suggests that the result of *ABCA1* in the case-control analysis may be driven by the effect of rs1883025 on decreasing risk of progression in the early stages of AMD (normal to intermediate drusen and from intermediate drusen to large drusen). The C allele of *COL8A1* appeared to increase the risk of progression to the advanced stages, which is significant in the univariate analysis. However, the C allele seems to be protective against the transition from intermediate to large drusen. Additional analyses in other longitudinal datasets of the effect of *COL8A1*, as well as other AMD genes, on the risk of progression to different AMD stages are needed.

In the multistate Markov model which included demographic, environmental, and ocular variables, our results suggest that BMI ≥ 30 significantly increases the risk of progression from large drusen to GA. It is possible that GA and high BMI are both pleiotropic phenotypes related to dysfunction of lipid metabolism.³¹ Our result also confirmed previous reports that smoking behavior, particularly current smoking, could strongly increase the risk of progression to advanced AMD.³⁴ Quitting smoking is probably the most effective lifestyle change for patients to reduce their risk of advanced AMD. It is known that eyes of patients with early or late AMD are often symmetrical.³⁵ The ocular status of the fellow eye of patients was highly predictive of the progression events that occur at each transition.

By combining demographic, behavioral, and ocular information together with genetic factors, we can use the multistate model to estimate the probability of disease progression. Individuals with a high-risk profile were more likely to be in the advanced stages than individuals with a low-risk profile. Our previous models for prediction of progression to advanced AMD used logistic regression and Cox proportional hazards regression.^{4,5} This risk prediction model adds new information and provides quantitative estimation of probabilities in five different AMD stages for a patient with a given risk profile. Its application in clinical practice may help clinicians to plan the frequency of eye examinations and treatment for patients according to risk profile.

CONCLUSIONS

Our results suggest that genes associated with AMD may be involved in transitions between different AMD stages during progression. Complement genes play important roles in the progression from intermediate drusen to large drusen and from large drusen to the advanced AMD stages. A novel association was also found between *ABCA1* in the HDL pathway that reduced the risk of progression from normal to intermediate drusen and from intermediate drusen to large drusen. Functional studies are needed to investigate the roles of the HDL pathway genes in the early stages of AMD. The multistate Markov model is a powerful tool for identifying novel genetic variants influencing progression in early stages of AMD and adds new insight into the mechanisms and etiology of this disease.

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