Retina

Analysis of Six Genetic Risk Factors Highly Associated with AMD in the Region Surrounding *ARMS2* and *HTRA1* on Chromosome 10, Region q26

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PURPOSE. To determine the relationship of six genetic variants (rs10490924, rs3750848, del443ins54, rs3793917, rs11200638, and rs932275) localized to the *ARMS2-HTRA1* region of chromosome 10, region q26, as risk factors for age-related macular degeneration (AMD), to define the haplotype structure of these six loci, and to confirm their genetic association with the disease.

METHODS. Caucasian patients ($n = 482$) were stratified into categories based on AREDS (Age-Related Eye Disease Study) grading criteria (groups 0 and 1 served as the control, groups 3 and 4 contained subjects with AMD, and group 2 was excluded from the analysis). The six genetic variants in the *ARMS2-HTRA1* region were genotyped and analyzed both independently and as a joint haplotype for association in subjects with disease ($n = 291$) compared with the control ($n = 191$).

RESULTS. The six high-risk alleles all showed a statistically significant association with AMD (the most significant SNP was rs10490924 [$P \le 3.31 \times 10^{-5}$, OR = 1.86]; the least significant SNP was rs932275 [$P \le 9.15 \times 10^{-5}$, OR = 1.78]). Multimarker analysis revealed that all six markers were in strong linkage disequilibrium with each other, and the two major haplotypes that captured >98% of the genetic variation in the region were both significantly associated with the disease: One increased the risk of AMD and contained only risk alleles ($P \le 2.20 \times 10^{-5}$), and the other haplotype decreased the risk of AMD and contained only wild-type alleles ($P \le 6.81 \times 10^{-5}$). Furthermore, 36 individuals comprising both cases and controls were identified outside of these two major haplotypes, with at least one discordant marker.

CONCLUSIONS. The results replicate the previously reported association between the high-risk alleles and AMD and independently confirm, for the first time, an association with AMD and the indel (del443ins54) polymorphism in a Caucasian population. Two major haplotypes that are associated with AMD and many minor novel haplotypes were identified. The novel haplotypes, identified from 36 cases and controls with discordant alleles spanning the *ARMS2–HTRA1* region provide unique opportunities to gauge the relative phenotypic contributions of each of these genetic risk factors. With the identification of more discordant patients in the future, it may be possible to resolve the ongoing controversy as to which of the risk alleles and genes (*ARMS2* vs. *HTRA1*) has the greatest impact on disease susceptibility. Future work should include the analysis of larger and more diverse populations, to further define the linkage structure of the region with a focus on phenotypic effects on AMD of the various haplotypes involving 10q26, as well as a functional analysis of the normal ARMS2 protein. (*Invest Ophthalmol Vis Sci.* 2010;51:2191–2196) DOI:10.1167/iovs.09-3798

A ge-related macular degeneration (AMD) is the most common cause of visual impairment in individuals older than 55 years in developed countries[1–4] and has caused more than 30 million people to become blind worldwide.[3] The major risk factors for AMD include age, smoking, and family history, with age being the strongest risk factor. Multiple population-based studies have confirmed the influence of age.[5–11] Pooled data from two population-based studies reveal an estimated prevalence of advanced AMD of ~0.2% in persons age 55 to 64 years, with a sharp increase to 13% in those older than 85 years.[12] Of importance, the number of individuals with AMD is expected to increase worldwide as the longevity of the population increases. Smoking has been shown to increase the risk of AMD twofold.[13,14] Of note, a smoking dose-effect that increases the risk of AMD with the increase in number of cigarettes smoked has also been reported.[15] Finally, familial studies have confirmed the existence of a genetic component in AMD.[16–19] In addition, twin studies have confirmed that genetic background accounts for 46% to 71% of the variation in the overall severity of AMD.[20]

Perhaps the most significant advancement in our understanding of the genetics of AMD came in early 2005 with the identification of a strong association between disease and variants associated with the complement factor H (*CFH*) gene.[21–24] This breakthrough association has now been validated in numerous studies worldwide and has been confirmed in multiple ethnic populations.[22,25–27]

The association between the 10q26 locus and AMD was originally identified through family linkage studies and fine mapping[28,29] and confirmed in several studies, including a genome-wide association study.[30,31] At least three potential

candidate genes reside in the 10q26 region. Currently, the identity of the gene that may induce disease susceptibility has been controversial, as there is strong linkage disequilibrium (LD) across the region.[32] The controversy has involved two nearby genes: *ARMS2* (age-related maculopathy susceptibility 2, also known as *LOC387715*)[28,29] and *HTRA1* (high-temperature requirement factor A1).[30,31] There are six risk alleles involving the region between *ARMS2* and *HTRA1* (rs10490924, rs3750848, del443ins54, rs3793917, rs11200638, and rs932275) that have been shown to be highly associated with AMD and are thought to reside on a single high-risk haplotype.[32] *HTRA1* is expressed in the human retina[33] and has a putative role in extracellular matrix homeostasis[34]; however, causal variants identified in the promoter region (rs11200638) of *HTRA1*[30,31] have not been reproducible in all studies.[32,35] *ARMS2* is also expressed in the retina, but only in primates, consistent with the fact that AMD occurs naturally only in primates.[36] Smoking has been reported to modify the susceptibility effect of *ARMS2*.[37] The G→C single-nucleotide polymorphism (SNP) (rs10490924), encoding a nonsynonymous A69S mutation in *ARMS2* has been reproducibly verified as a strong genetic risk factor for AMD.[29,32,35,37] Recently, the deletion allele of an insertion/deletion (indel) variant in the 3′ UTR region of the *ARMS2* gene has also been found to be highly associated with AMD versus its effect in controls (42.4% vs. 19.3%, $P = 4.1 \times 10-29$).[32] This indel polymorphism destabilizes the mRNA transcript of the *ARMS2* gene, leading to its rapid decay.[32] Although the functional properties of the normal ARMS2 protein are not yet known, the risk alleles rs10490924 and del443ins4 have been shown to alter the encoded protein and may represent the highly sought after functional variants relevant to AMD's etiology.

The purpose of our study was to determine the relationship between the six high-risk alleles (rs10490924, rs3750848, del443ins54, rs3793917, rs11200638, and rs932275) spanning the *ARMS2–HTRA1* region, to further define the haplotype structure of the two loci, and to confirm their genetic association with AMD.

METHODS

Subject Recruitment and Phenotype Classification

Our case–control sample consisted of 482 European-derived individuals; 291 had AMD and 191 were control subjects. All individuals were recruited from multiple retina practices throughout the Philadelphia area and underwent a clinical examination by a retina specialist.

Those with macular changes were classified based on AREDS (Age-Related Eye Disease Study) Report 6,[38] which we modified to allow grading of our subjects by funduscopic appearance during examination by a retina specialist. Patients were examined for the presence of drusen (appearance and size), pigmentary abnormalities, geographic atrophy (GA), and choroidal neovascularization (CNV). Eyes with only few small drusen were assigned grade 1; eyes with intermediate drusen were assigned grade 2; eyes with large confluent drusen or with pigmentary changes of the retinal pigment epithelium (RPE) were assigned grade 3; eyes with advanced changes such as GA or CNV were assigned grade 4; and eyes with none of the above were assigned a grade 0. The patients were graded based on the worse eye. When a CNV lesion was suspected, fluorescein angiography was performed to confirm its presence.

It has been shown that patients with a few small drusen have a nominal risk of AMD.[38] Our control subjects had grade 0 or 1 eyes and were ≥65 years old. Although patients with intermediate drusen have a small risk of advanced AMD, in a recent report, these drusen were identified as a risk factor.[38] We therefore excluded our grade 2 subjects from the analysis, as grouping them into controls or cases was less discrete. Our cases included subjects with either a grade 3 or 4 eyes.
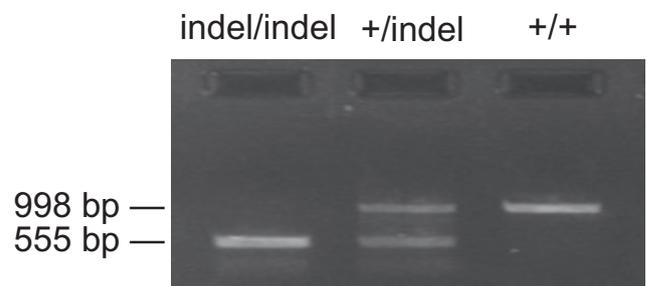

FIGURE 1. Genotyping indel with PCR-based assay. Three lanes of a PCR product are shown and labeled for an individual homozygous for the indel (indel/indel), an individual heterozygous for the indel (+/indel), and a wild-type individual (+/+). Product sizes in base pairs are also shown.

Each patient was asked to complete a general health survey, which provided baseline demographic information, including information regarding smoking, statin use, and family history of AMD. The study was approved by the Institutional Review Board of the University of Pennsylvania. All subjects provided signed informed consent before blood collection. The study adhered to the tenets of the Declaration of Helsinki.

Genotype Determination

We used PCR to validate the existence of the indel region in the 3′ UTR of *ARMS2*. The Primer3 Web-based application[39] was used to select a left (TCTGTGCAGCTGGTGAAATC) and right (TTTTGAACAGGGC-CAAGTCT) primer that flanked the indel to produce a 998-bp PCR product for the wild-type allele (no indel) and a 555-bp PCR product for the indel allele (Fig. 1). Primers were selected that were devoid of repeats and mapped to chr10:124206677-124207674 of the March 2006 (build 36.1; NCBI [National Center for Biotechnology Information], Bethesda, MD) human genome assembly. A PCR system (Gene-Amp 9700; Applied Biosystems, Inc. [ABI], Foster City, CA) was used to amplify 50-μL reactions with 100 ng of genomic DNA from the subjects, 0.4 μL of 100 mM dNTP mix, 1.25 U *Taq* polymerase, and 2 μL of 20 μM of the mixed 5′ and 3′ primers. Thermal cycling was initiated with a 2-minute incubation at 50°C, followed by a first denaturation step of 10 minutes at 95°C, and then 35 cycles of 15 seconds at 95°C and 1 minute at 60°C. PCR products were visualized by agarose gel electrophoresis. On a gel, a solitary larger band represents the wild-type alleles (no indel alleles), two bands represent a heterozygous state (one indel allele), and one smaller band represents the homozygous state (two indel alleles). The assay was validated by sequencing the PCR products that were purified from the gel and then mapping them back to the genome. The assay simplifies detection by obviating the need to resequence the indel directly, and it allowed us to quickly genotype our samples for the indel variant. The remaining five SNPs were genotyped with a commercially available genotyping assay (*Taq*man; ABI).

Statistical Association

PLINK[40] was used to perform all allelic association calculations (Fisher's exact test) and six-window haplotype association tests. LD calculations and visualizations were performed with the Haploview package.[41] Risk factor analysis of family, smoking, and statin histories was performed with a logistic regression, assuming a binomial probability distribution in the R language for statistical computing.[42]

RESULTS

We recruited 482 individuals (191 controls and 291 cases) of European-derived ancestry and classified them based on the presence of drusen (size and appearance), RPE changes, GA, and CNV (see the Methods section).

TABLE 1. Single-Marker Genotypic Association of AMD

Marker	Gene	Relative Position	Risk Allele	WT Allele	Frequency		P	OR
					Cases	Controls		
rs10490924	*ARMS2*	A69S	T	G	0.36	0.23	3.31×10^{-5}	1.86
rs3750848	*ARMS2*	Intron	G	T	0.36	0.23	4.61×10^{-5}	1.83
del443ins54	*ARMS2*	3′ UTR	1	2	0.36	0.23	3.46×10^{-5}	1.85
rs3793917	—	Intergenic	G	C	0.35	0.23	7.82×10^{-5}	1.80
rs11200638	*HTRA1*	Promoter	A	G	0.36	0.24	6.41×10^{-5}	1.80
rs932275	*HTRA1*	Intron	A	G	0.36	0.24	9.15×10^{-5}	1.78

1, no indel; 2, indel.

The risk allele frequencies at the six loci ranged from 35% to 36% in the cases, relative to 23% to 24% in the controls, a statistically significant difference at each locus (Table 1). By odds ratios, the most strongly associated marker was rs10490924, an SNP encoding a nonsynonymous alanine-to-serine substitution in the *ARMS2* proximal exon ($P \leq 3.31 \times 10^{-5}$, OR = 1.86). The next most strongly associated marker was the del443ins54 indel in the 3′ UTR of *ARMS2* ($P \leq 3.46 \times 10^{-5}$, OR = 1.85), followed by rs3750848 in the only intron of *ARMS2* ($P \leq 4.61 \times 10^{-5}$, OR = 1.83). The SNP, rs11200638, in the promoter of *HTRA1* was strongly associated ($P \leq 6.41 \times 10^{-5}$, OR = 1.80), as was rs3793917 in the intergenic region between *ARMS2* and *HTRA1* ($P \leq 7.82 \times 10^{-5}$, OR = 1.80) and rs932275 in an internal intron of *HTRA1* ($P \leq 9.15 \times 10^{-5}$, OR = 1.78).

Multimarker analysis of the six markers showed that all the markers were in strong LD with each other, and haplotypes inferred from the six markers yielded three major haplotypes with a frequency greater than 1% (Fig. 2, Table 2). Two haplotypes captured >98% of the genetic variation for the six markers analyzed. The most common haplotype, GT1CGG (1 represents no indel), was found in 76% of the control subjects and represents a wild-type haplotype with wild-type alleles at each locus. The second most common haplotype, TG2GAA (2 represents indel), was found in 22% of the control subjects and represents a risk haplotype composed of risk alleles at each locus. The next most common haplotype, GT1CGA, was found in 2% of the subjects analyzed. Haplotype analysis showed that only the two major haplotypes (i.e., the risk haplotype and the wild-type haplotype) were significantly associated with AMD (Table 2). The risk haplotype (TG2GAA) significantly increased the risk of AMD in 35% of the cases versus 22% of the controls ($P \leq 2.20 \times 10^{-5}$), and the wild-type haplotype (GT1CGG) significantly decreased the risk of AMD in 63% of the cases versus 76% of the controls (6.81×10^{-5}).

There were 36 individuals who had neither complete risk nor wild-type haplotypes (Supplementary Table S1, http://www.iovs.org/cgi/content/full/51/4/2191/DC1), and 14 of these individuals were discordant at the three loci most likely to be functional: rs10490924 (the *ARMS2* A69S SNP), del443ins54 (the *ARMS2* indel), and rs11200638 (the *HTRA1* promoter SNP). Pair-wise comparisons of these three loci revealed that eight individuals had discordant genotypes at A69S and the indel. Seven of these eight individuals were cases, and four of them had more risk alleles at the indel locus than at A69S. There were 10 individuals with discordant genotypes at *ARMS2* A69S and the *HTRA1* promoter. Eight of these 10 individuals were cases, and half had more risk alleles at the *ARMS2* A69S. Also, there were 10 individuals who were discordant at the *ARMS2* indel and the *HTRA1* promoter. Seven of these 10 individuals were cases, and 4 of them had more risk alleles at the indel locus. Thus, analyses of the A69S SNP, the *ARMS2* indel, and the *HTRA1* promoter SNP did not favor any one genetic variant as a likely functional risk factor for AMD,

because their respective risk alleles were about equally distributed among the cases in each pair-wise comparison of the loci. Furthermore, a power analysis based on the empiric data (Supplementary Table S2, http://www.iovs.org/cgi/content/full/51/4/2191/DC1) suggests that the sample was underpowered to distinguish the causative disease locus (power = 0.12 at α = 0.05), and that a sample size of 6247 subjects would be necessary (power ≥ 80% at α = 0.05) to distinguish these alleles by case–control test for genetic association.

Finally, we analyzed the effect of smoking, family history, and statin use as risk factors for AMD (Table 3). We found family history to be a significantly associated risk factor. Of the patients, 31% reported a family history of AMD versus 17% of the control subjects ($P \leq 7.36 \times 10^{-4}$, OR = 3.12). The case patients were also less likely to be on a statin than were the
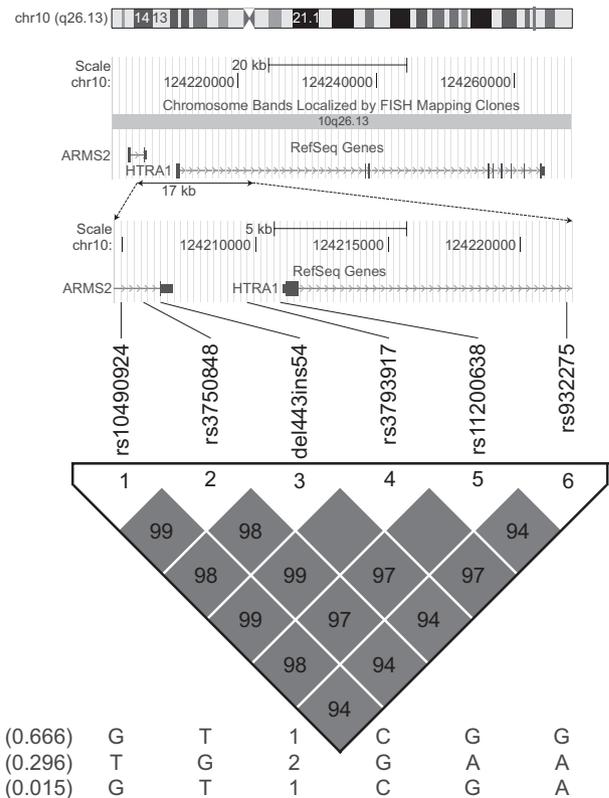

FIGURE 2. Linkage disequilibrium plot generated from 964 chromosomes genotyped across the *ARMS2–HTRA1* gene region. D′ values, a measure of LD, are illustrated for five SNPs and one indel polymorphism (1, no indel; 2, indel). Genome browser tracks of the RefSeq gene annotations are also shown, and estimated haplotypes with frequencies >1% are listed (Ref Seq is provided in the public domain by the National Center for Biotechnology Information, Bethesda MD, available at www.ncbi.nlm.nih.gov/locuslink/refseq/).

TABLE 2. Haplotype Genotypic Association with AMD

	Frequency		
Haplotype	Cases	Controls	P
TG2GAA	0.35	0.22	2.20×10^{-5}
GT1CGG	0.63	0.76	6.81×10^{-5}
GT1CGA	0.01	0.02	4.30×10^{-1}

1, no indel; 2, indel.

control patients (51% vs. 65%, $P \leq 2.09 \times 10^{-2}$, OR = 0.55). In our sample population, we did not find smoking to be associated with AMD, with 46% of the case group reporting a smoking history versus 53% of the control group ($P \leq 0.474$, OR = 0.83).

DISCUSSION

We have characterized the relationship between six genetic variants highly associated with AMD in the 17-kb region surrounding *ARMS2* and *HTRA1* on 10q26. The coverage of SNPs genotyped in the most recent phase (phase 3) of the HapMap project (www.HapMap.org) do not sufficiently cover the 17-kb region spanning the six risk loci, and this deficit motivated our analysis to define the LD structure of the six genetic loci with roles in AMD. Furthermore, and to the best of our knowledge, our significant findings represent the first replication of the indel with AMD in a Caucasian population. All six risk alleles were found in 35% to 36% of the AMD population versus 23% to 24% of the control population, and the most strongly associated locus was A69S ($P \leq 3.31 \times 10^{-5}$, OR = 1.86). Recent larger studies in a German population of Caucasian individuals (760 cases, 549 controls) report a larger effect for the A69S risk allele with AMD (42% in cases vs. 19% in controls; OR = 2.9; $P \leq 2.8 \times 10^{-29}$). The reduced effect of the risk allele in our study, when compared to the German study (OR = 1.86 vs. 2.9, respectively), may stem from the reduced power of our smaller sample size. Furthermore, due to different population migration histories, our European-derived subjects may represent a more pan-European population than the more geographically constrained German population. Of interest, the risk allele frequency in the HapMap CEU was similar to the risk allele frequency in our controls (21% vs. 23% respectively, $P \leq 0.5$, data not shown). Therefore, it may be that geographic constraints alter the susceptibility to AMD induced by the A69S risk allele. A phylogenetic analysis of genome-wide variation between these two different European-derived Caucasian populations may be used to visualize the relative geographic constraints of the two populations and may illustrate the effect of such constraints on the risk allele frequency.

Our results replicate the previously reported association between the *ARMS2* A69S variant and AMD and independently confirm the association of AMD with the indel polymorphism. We found exceptions, however, to the previously reported concordance between the various SNPs at the locus spanning *ARMS2* and *HTRA1*, and our results suggest an incomplete

linkage in the region. There were 36 individuals who demonstrated incomplete LD across the region. Of these, 8 had discordant genotypes at A69S and the indel, 10 had discordant genotypes at A69S and the *HTRA1* promoter, and 10 were discordant at the indel and the *HTRA1* promoter. These discordant patients represent novel haplotypes, which could prove extremely useful in determining which of the genes (*ARMS2* vs. *HTRA1*) has the biggest influence on the development of AMD—an area of controversy and uncertainty. However, given that AMD is a complex disease relying on multiple genetic and environmental risk factors, our sample consisted of too few discordant individuals to draw conclusions as to which SNP and gene is most causative of AMD. Nonetheless, finding additional minor novel haplotypes in more diverse populations will generate important hypotheses to be tested toward determining the causality of risk alleles in AMD.

Although the relatively small sample size in this study precludes the possibility of determining which of the variants associated with the *ARMS2* and *HTRA1* at the 10q26 locus is more likely to be responsible for the increased risk of AMD, our data suggest that >6000 samples would have to be analyzed to be able to discriminate (power $\geq$ 80% at $\alpha = 0.05$) among the genetic variants for potential causation. Although it may be possible to achieve such a large number of subjects via further recruitment and/or collaboration with other investigators who are studying the *ARMS2* gene in patient populations, careful characterization and functional testing of these loci, both in vitro and in vivo, may also be indicated to try to resolve the controversy of which genetic variant in which gene underlies the increased risk of AMD. Another option is to perform the association in another ethnic group (i.e., African Americans), which may distinguish *HTRA1* from *ARMS2*, based on a different LD block structure.

Susceptibility to AMD also depends on the complex interaction of many other susceptibility factors, including a positive family history, multiple environmental factors such as age and smoking,[13–15] and, less definitively, use of statins.[43] Of these risk factors, we found family history to be the most significant risk factor for AMD with 31% of the cases reporting a positive family history versus 17% of the controls ($P \leq 7.36 \times 10^{-4}$, OR = 3.12). We did not find an association between smoking history and AMD, with 46% of the case patients reporting a positive smoking history versus 53% of the control subjects ($P \leq 0.474$, OR = 0.83). This is unexpected, as smoking is thought to be a risk factor for the disease. However, as our study is more focused on the genetics underlying AMD, we may not have controlled for confounders for the smoking history of the subjects as well as other studies did. For instance, we grouped our patients into ever-smokers and never-smokers. It has been shown that smokers with a higher pack-year history have more risk of development of AMD, while smoking cessation appears to reduce the risk of disease development.[15] Many patients in our smoking group had less than a 20-pack-year history, whereas others quit tobacco use years ago, both of which may have neutralized the effect smoking has on AMD. It is also possible that our study was underpowered to detect an association between smoking and AMD.

TABLE 3. Effect of Family History, Smoking History, and Statin Use as Risk Factors for AMD

Risk Factor	Case n (Freq)	Control n (Freq)	P	OR
Family history vs. no family history	68 (0.31)	18 (0.17)	7.36×10^{-4}	3.12
Ever-smoker vs. never-smoker	117 (0.46)	77 (0.53)	0.474	0.83
Ever-statin vs. never-statin	121 (0.51)	90 (0.65)	2.09×10^{-2}	0.55

We found that the lack of statin use was a statistically significant risk factor for AMD, with a smaller portion of the case group having ever consumed a statin compared with those in the control group (51% vs. 65%, $P \leq 2.09 \times 10^{-2}$, OR = 0.55). The pathogenesis of AMD is thought to involve an inflammatory process, and it has been hypothesized that statin use has a protective role in AMD due to its anti-inflammatory property. However, this potential protective role has not been consistently demonstrated.[43] Although more of our control patients did take statins, larger and longer population-based studies are necessary to further evaluate the role of statins in the development of AMD.

To conclude, our results replicate the previously reported association between the high-risk alleles (rs10490924, rs3750848, rs3793917, rs11200638, and rs932275) and AMD and independently confirm for the first time an association of AMD and the indel (del443ins54) polymorphism in a Caucasian population. The novel haplotypes we identified from 36 individuals with discordant alleles spanning the *ARMS2–HTRA1* region provide unique opportunities to gauge the relative phenotypic contributions of each of these genetic risk factors. Particular focus on those individuals discordant at del443ins54 in the 3′ UTR of *ARMS2*, rs10490924 in the exon of *ARMS2*, and rs11200638 in the promoter region of *HTRA1* may be useful, as these markers are more likely to be functional, given their location. With additional discordant patients, it may be possible to further determine which of the risk alleles and genes (*ARMS2* vs. *HTRA1*) have the biggest impact on disease susceptibility, which has been an ongoing area of controversy. Future work should also focus on the function of the normal ARMS2 protein and analyze larger populations to further define the linkage structure of the region with a focus on phenotypic effects of the various haplotypes involving 10q26 on AMD.

Acknowledgments

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