An Intergenic Region between the tagSNP rs3793917 and rs11200638 in the *HTRA1* Gene Indicates Association with Age-Related Macular Degeneration

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Purpose. There is still a debate as to whether the *LOC387715* or *HTRA1* genes represent the key significant association identified with age-related macular degeneration (AMD) on the long arm of chromosome 10, region 26.

Methods. An Australian patient cohort was genotyped by using tagged single nucleotide polymorphisms (tSNPs) to identify a causal SNP within this region.

Results. Multiple tSNPs across the region showed association with AMD with the tSNP rs3793917 (odds ratio [OR], 3.45; 95% confidence interval [CI], 2.36–5.05, $P = 2.8 \times 10^{-13}$) having the highest association with AMD. This tSNP occurred in the intergenic region between the *LOC387715* and *HTRA1* genes. A second tSNP rs2672587 (OR, 2.92; 95% CI, 2.04–4.17; $P = 7.7 \times 10^{-11}$) located in intron 1 of the *HTRA1* gene had the second highest association with AMD. After logistic regression analysis, the only tSNP to survive covariate testing was rs3793917, which occurred in the same LD block as the *HTRA1* promoter SNP rs11200638 ($r^2 = 0.88$, $D' = 0.97$).

Conclusions. The findings indicate that the intergenic region between the tSNP rs3793917 and the SNP rs11200638 in the *HTRA1* gene is the most likely site explaining the significant association with AMD. (*Invest Ophthalmol Vis Sci.* 2010;51: 4932–4936) DOI:10.1167/iovs.09-5114

Several susceptibility genes appear to explain most of the variance in the eye disease age-related macular degeneration (AMD, OMIM 603075; http://www.ncbi.nlm.nih.gov/omim/ provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD). Previous linkage studies have indicated one of these major AMD susceptibility loci on the long arm of chromosome 10, region 26.[1–5] Genetic analysis within this region initially identified a three-gene cluster, including the pleckstrin homology domain-containing protein family A member 1 (*PLEKHA1*, OMIM 607772), *LOC387715* (also known as *ARMS2*, OMIM 611313), and *HTRA1* (high-temperature requirement factor A1; OMIM 602194), spanning a chromosomal region of approximately 140 kb.[6]

Investigations into the *PLEKHA1*, *LOC387715*, and *HTRA1* genes identified the single nucleotide polymorphism (SNP) rs10490924 (A69S) in *LOC387715* (*ARMS2*) to be highly associated with AMD, with an odds ratio (OR) of 8.21 (95% confidence interval [CI], 5.79–11.65).[6] In addition, the presence of an indel (372_81del443ins54) in the 3′UTR (untranslated region) of the *ARMS2* locus in AMD patients[7] has also been identified. Immunohistochemical studies of the LOC387715/ARMS2 protein initially indicated its presence in the outer membrane of mitochondria in transfected mammalian cells, suggesting a possible function for this gene in AMD through an oxidative damage mechanism.[7,8] However, more recent immunolocalization studies appear to suggest its presence in the cellular cytoplasm,[9] whereas another paper suggests ARMS2 to be an extracellular matrix protein that binds to other extracellular matrix proteins, such as fibulin-3 and -5, TIMP (tissue inhibitor of metalloproteinase), MMP (matrix metalloproteinase), and elastin, thereby making it an important part of matrix function.[10]

There is also evidence of a role of *HTRA1* in the etiology of AMD. Initial studies by two groups reported a promoter SNP rs11200638 (−512 bp) in this gene as being significantly associated with neovascular AMD in a comparison of individuals with homozygous risk alleles with those with wild-type homozygous alleles (OR, 10.0; 95% CI, 4.38–22.82)[11] and (OR, 6.56; 95% CI, 3.23–13.31).[12] Fine-mapping and haplotype studies involving both European-American and Asian individuals have since identified several variants in the *HTRA1* promoter (rs11200638; −625G>A) as well as in exon 1—rs2672598; −487T>C, rs1049331 (102C>T; Ala34Ala), and rs2293870 (108G>T; Gly36Gly)—to be associated with AMD.[13,14] In addition, studies in rhesus monkeys have shown that the risk allele (A) of rs11200638 leads to a doubling of promoter activity in the 293T human microvascular endothelial cell line.[15]

SNPs from both the *LOC387715* and *HTRA1* genes have been reported to be in tight linkage disequilibrium (LD).[7,11,16–20] Thus, it has so far been difficult to tease apart the association of these two genes statistically and to fully resolve the extent of the involvement of the *LOC387715* and *HTRA1* genes in the etiology of AMD. However, regression analysis has indicated that the SNP rs10490924 (A69S) in the *LOC387715* (*ARMS2*) gene, either alone or as a variant in strong LD, could explain the bulk of the association between the 10q26 chromosomal region and AMD.[8]

To assess the association of variants within these genes in the 10q26 region with AMD, we undertook a tag SNP (t)SNP approach across the region encompassing the *PLEKHA1*, *LOC387715*, and *HTRA1* genes, to determine the regions most significantly associated with AMD.

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METHODS

Subjects

The cohort was collected either from outpatient clinics at the Royal Victorian Eye and Ear Hospital (RVEEH) or through private ophthalmology practices in Melbourne, Australia. Control subjects were collected from the same community, as part of the large, population-based epidemiologic eye study, the Melbourne Visual Impairment Project (VIP), or through aged-care nursing homes. All individuals in both study arms were Caucasian of Anglo-Celtic ethnic background. A total of 521 individuals—58 with early AMD, 295 with choroidal neovascularization, and 49 with geographic atrophy—with a mean age of 72.7 years, and 119 unrelated control subjects, with a mean age of 71.8 years, were recruited (Table 1).

All recruited individuals completed a standard risk-factor and disease history questionnaire. At the time of recruitment a clinical examination was performed, a fundus photograph obtained, and a blood sample collected for DNA analysis. Those with early AMD were included if they had drusen >125 μm in one or both eyes. Control individuals were included if they presented with a normal fundus (<10 hard drusen <63 μm) and no altered macular pigmentation in both eyes. Cases and controls were graded in line with the modified International Classification System for age-related maculopathy/age-related macular degeneration (ARM/AMD); for presence, type, size, location, and number of drusen and pigmentary abnormalities; and for size and centrality of the late features of AMD, by two independent graders (KZA, RHG).[21]

Written informed consent was obtained from all individuals, and ethics approval for the project was provided by the Human Research and Ethics Committee of the RVEEH, Melbourne. The study was conducted in accordance with the Declaration of Helsinki, the National Health and Medical Research Council of Australia's statement on ethical conduct in research involving human subjects, revised in 1999, and National Institutes of Health (NIH) guidelines.

tSNP Selection

The tSNPs selected for genotyping encompassed the *PLEKHA1*, *LOC387715*, and *HTRA1* genes and were derived through the use of the International HapMap Project (http://www.hapmap.org/). The region tagged extended from 2 kb upstream of the 5′UTR region of the *PLEKHA1* gene to 2 kb downstream of the 3′UTR region of the *HTRA1* gene. The tSNPs were selected by using the pairwise algorithm and a CEU (Caucasian) population. A strong LD tagging criterion of $r^2 > 0.8$ was used, and all SNPs had a minor allele frequency (MAF) of at least 0.1. The HapMap data were sourced from NCBI Build 35.

Single-Nucleotide Polymorphisms

The SNPs rs10490924 in the *LOC387715* gene and rs11200638 in the promoter of the *HTRA1* gene were selected for inclusion in the study for the purpose of replication.

Genotyping

Genotyping of SNPs was undertaken as previously described[22] (MassARRAY platform; SEQUENOM, San Diego, CA) through the Australian Genome Research Facility, Brisbane, Australia.

Statistical Analysis

Participant characteristics including sex and age at ascertainment or diagnosis with or without AMD and its subtypes were compared by using the χ^2 test or independent-samples *t*-test. Hardy-Weinberg equilibrium (HWE) was used to assess whether genotypes fell within a standard distribution. LD blocks were ascertained from Haploview v4.1 (http://www.broad.mit.edu/mpg/haploview/ provided in the public domain by The Broad Institute, Massachusetts Institute of Technology, Cambridge, MA), using the Gabriel algorithm[23] by entering genotype information through linkage format. Allele associations with AMD were investigated by using Unphased software,[24] and results are presented as OR with 95% CI. For conditional analyses, each SNP with a maximum significant $P < 2.97 \times 10^{14}$ was adjusted for each of the other SNPs among the targeted SNPs, to determine the dominance of the SNPs in the different models (SPSS 14.0; SPSS Inc, Chicago, IL).

RESULTS

Baseline data indicated that the only significant difference between cases and controls was that more participants with early AMD (58.2% vs. 40.3%, $P = 0.03$) and geographic atrophy (65.2% vs. 40.3%, $P = 0.004$) were likely to be smokers than were the control participants (Table 1).

Twenty-five tSNPs were genotyped, consisting of 6 tSNPs (rs7084349, rs10082476, rs10887148, rs10887149, rs7918867, and rs2292626) in the *PLEKHA1* gene, 3 (rs1882907, rs2014307, and rs3793917) positioned between the *PLEKHA1* and *HTRA1* genes but including the *LOC387715* gene, and 16 (rs2736914, rs2672591, rs7093894, rs4752699, rs2672590, rs2672588, rs2672587, rs4237540, rs2736917, rs2300431, rs2736919, rs11200651, rs763720, rs876790, rs2250804, and rs2268356) in the *HTRA1* gene (Table 2, Fig. 1). There was no evidence of a departure from HWE of any of the tSNPs in our study ($P > 0.05$; Table 2).

Initial univariate analysis indicated that 10 of 25 genotyped tSNPs in the three genes were significantly associated with AMD after the Bonferroni correction ($P_c = 0.0025$; Table 2). These tSNPs included rs7084349 and rs2292626 in the *PLEKHA1* gene; rs2014307 and rs3793917, lying intergenically between the *PLEKHA1* and *HTRA1* genes but including the *LOC387715* gene; and rs2672591, rs2672587, rs2736919, rs763720, rs2250804, and rs2268356 in the *HTRA1* gene (Table 2).

A total of seven separate LD groups were identified for the 25 tSNPs (Table 2, Fig. 1). The most significant tSNPs from each LD group were rs7084349 (OR, 1.45; 95% CI, 1.06–1.99; $P = 0.018$) in LD block 1, rs1882907 (OR, 1.68; 95% CI, 1.09–2.61; $P = 0.020$) in LD block 2, rs3793917 (OR, 3.45; 95% CI, 2.36–5.05; $P = 2.8 \times 10^{-13}$) in LD block 3, rs2672587 (OR, 2.92; 95% CI, 2.04–4.17; $P = 5.9 \times 10^{-11}$) in LD block 4, rs2736917 (OR, 1.47; 95% CI, 1.04–2.07; $P = 0.039$) in block 5, rs763720 (OR, 1.82; 95% CI, 1.26–2.62; $P = 6.02 \times 10^{-4}$) in LD block 6, and rs2250804 (OR, 1.84; 95% CI, 1.36–2.50; $P = 8.89 \times 10^{-5}$) in LD block 7 (Table 2). Even though rs2292626 (occurring between LD blocks 1 and 2) fell within the significance level with Bonferroni correction, in comparison to the

TABLE 1. Characteristics of Participants from AMD Cases and Control Subjects

Characteristics	Control	Any AMD	*P**	Early	*P**	CNV†	*P**	GA	*P**
Total, $N = 521$	119	402		58		295		49	
Male, *n* (%)	53 (44.5)	147 (36.6)	0.12	17 (29.3)	0.05	109 (36.9)	0.15	21 (42.9)	0.84
Female, *n* (%)	66 (55.5)	255 (63.4)		41 (70.7)		186 (63.1)		28 (57.1)	
Smoker, *n* (%)	48 (40.3)	202 (53.4)	0.01	32 (58.2)	0.03	140 (50.5)	0.06	30 (65.2)	0.004
Age, mean y (SD)	71.8 (7.7)	72.7 (7.4)	0.23	70.3 (5.4)	0.19	73.5 (7.5)	0.03	70.8 (8.4)	0.44

* Based on χ^2 (categorical) or independent samples *t*-test.

TABLE 2. Tag SNP Analysis Covering the 120-kb Region of 10q26 Encompassing the *PLEKHA1, LOC387715,* and *HTRA1* Genes

					Allele Frequency		Allele Association					
									tSNPs		Non-tSNPs	
SNP Name	HW-P*	Position	LD Group	Risk Allele	Affected	Unaffected	OR (95% CI)	P†	*Rs3793917*	*Rs2672587*	rs10490924‡	rs11200638‡
PLEKHA1												
Rs7084349	0.91	124,135,130	1	G	0.71	0.62	1.45 (1.06–1.99)	0.018				
Rs10082476	0.99	124,154,644	1	A	0.81	0.77	1.30 (0.92–1.85)	0.151				
Rs10887148	0.99	124,155,206	1	C	0.87	0.84	1.25 (0.82–1.89)	0.290				
Rs10887149	0.92	124,156,994	—	G	0.76	0.69	1.35 (0.95–1.92)	0.074				
Rs7918867	0.71	124,159,656	—	G	0.89	0.88	1.09 (0.67–1.78)	0.702				
Rs2292626:1	0.96	124,176,704	—	T	0.61	0.52	1.42 (1.06–1.92)	0.022				
Chromosome 10 (including *LOC387715*)												
Rs1882907	0.82	124,198,389	2	T	0.90	0.85	1.68 (1.09–2.61)	0.020				
Rs10490924*	0.86	124,204,438	2	T	0.50	0.23	3.39 (2.39–4.82)	1.23×10^{-13}	*0.91*	2.7×10^{-4}	NA	0.61
Rs2014307	0.83	124,207,622	—	G	0.73	0.53	2.34 (1.72–3.17)	4.3×10^{-8}	0.09	0.003	0.11	0.08
Rs3793917:2	*0.32*	*124,209,265*	*3*	*G*	*0.46*	*0.20*	*3.45 (2.36–5.05)*	2.8×10^{-13}	*NA*	1.6×10^{-4}	0.11	*0.04*
HTRA1												
Rs11200638*	0.70	124,210,534	3	A	0.51	0.23	3.42 (2.47–4.74)	2.97×10^{-14}	*0.34*	1.9×10^{-1}	0.21	NA
Rs2736914	0.18	124,223,492	3	G	0.89	0.86	1.46 (0.91–2.35)	0.098				
Rs2672591	0.46	124,224,274	—	T	0.65	0.47	2.14 (1.56–2.94)	5.24×10^{-7}	0.20	0.02	0.23	0.81
Rs7093894	0.41	124,224,310	—	C	0.91	0.89	1.15 (0.70–1.89)	0.577				
Rs4752699	0.80	124,224,594	—	G	0.91	0.88	1.40 (0.87–2.24)	0.165				
Rs2672590	0.27	124,224,870	—	A	0.84	0.78	1.40 (0.98–2.01)	0.077				
Rs2672588	0.23	124,225,286	4	T	0.81	0.75	1.40 (0.99–1.98)	0.068				
Rs2672587:3	*0.59*	*124,225,345*	*4*	*G*	*0.46*	*0.23*	*2.92 (2.04–4.17)*	7.7×10^{-11}	*0.98*	*NA*	0.84	*0.87*
Rs4237540	0.95	124,227,488	—	A	0.66	0.57	1.44 (1.06–1.95)	0.018				
Rs2736917	0.18	124,228,973	5	A	0.83	0.77	1.47 (1.04–2.07)	0.039				
Rs2300431	0.74	124,232,807	5	G	0.79	0.76	1.21 (0.85–1.71)	0.294				
Rs2736919	0.80	124,233,447	—	G	0.44	0.31	1.77 (1.28–2.44)	3.09×10^{-4}	0.95	0.71	0.82	0.83
Rs11200651	0.86	124,235,992	6	A	0.84	0.82	1.11 (0.75–1.65)	0.589				
Rs763720:4	*0.76*	*124,252,434*	*6*	*A*	*0.31*	*0.20*	*1.82 (1.26–2.62)*	6.02×10^{-4}	*0.53*	*0.55*	*0.61*	*0.34*
Rs876790	0.85	124,253,525	7	A	0.80	0.78	1.12 (0.78–1.61)	0.537				
Rs2250804	0.69	124,254,868	7	G	0.46	0.31	1.84 (1.36–2.50)	8.89×10^{-5}	0.59	0.40	0.96	0.55
Rs2268356	0.91	124,255,316	—	G	0.59	0.47	1.61 (1.21–2.15)	1.6×10^{-3}	0.33	0.18	0.22	0.35

After Bonferroni correction, the significant *P* is expected to be (0.05/25) = 0.0025. The last three columns summarize *P*-value results of logistic regression analysis for the SNPs which are significant at a minimum of $P = 1.0 \times 10^{-5}$. Italicized SNPs represent the most significant tSNPs in each LD block after Bonferroni correction.

* Test of Hardy-Weinberg equilibrium in control subjects.

† Based on log likelihood ratio test in association with risk allele.

‡ SNPs rs10490924 and rs11200638 were not part of the tag SNP design, but were genotyped separately.

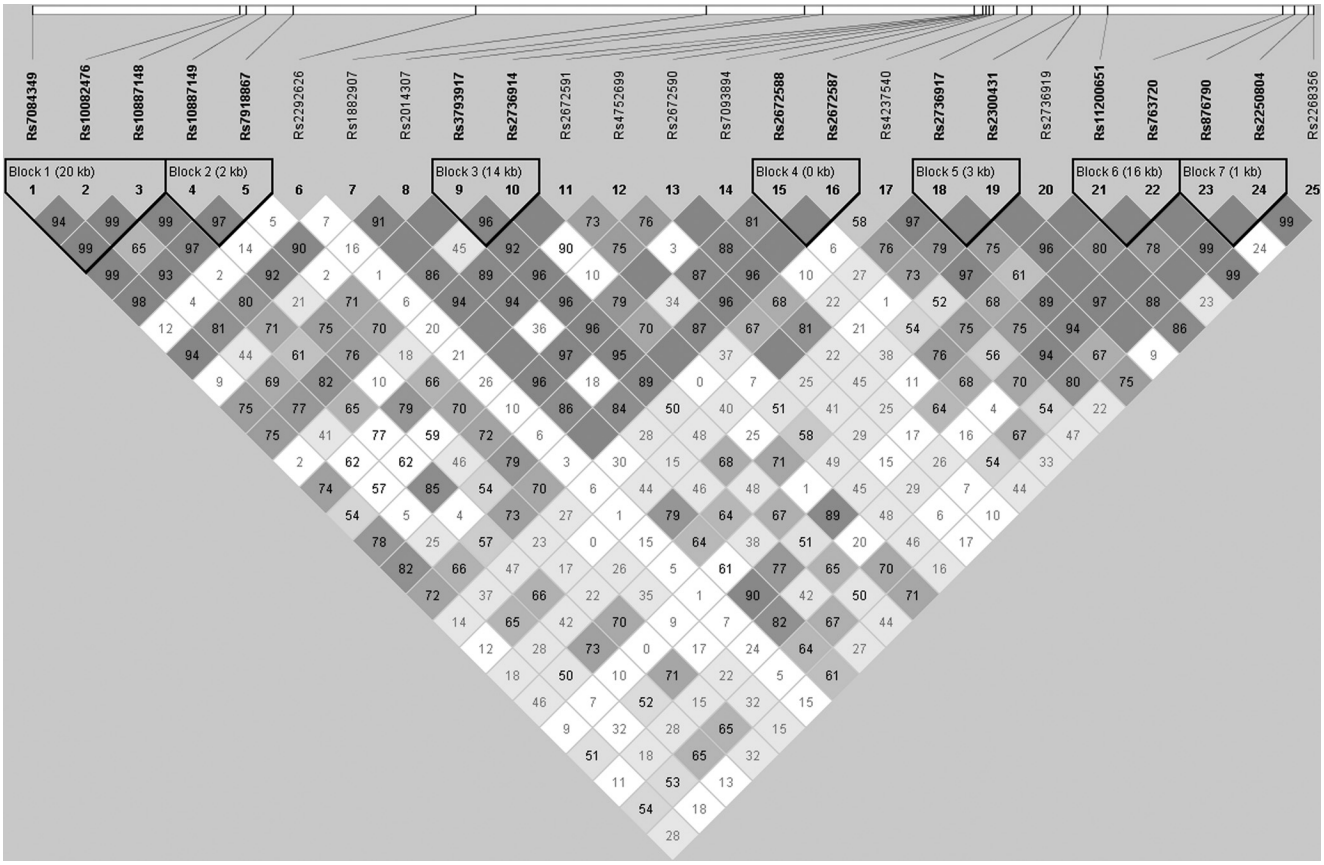

FIGURE 1. LD plot for 25 tSNPs, as well as rs10490924 and rs11200638 in the *PLEKHA1*, *LOC387714*, and *HTRA1* genes, indicating seven LD blocks. Genotypes were entered by linkage format into Haploview v4.1. The numbers inside the *diamonds* represent the D′ for pairwise analysis. The darker the square, the higher the LD between two variants. Bold polymorphisms are contained inside the LD blocks (defined by Gabriel et al.[23]).

other tSNPs in the other six LD blocks, it did not appear to be a significant contributor and so was not used in later logistic regression analyses. These findings indicated that variants in both the *LOC387715* and the *HTRA1* genes were associated with AMD.

An additional two SNPs (non-tSNPs), occurring in LD blocks 2 and 3, were also genotyped: the previously described SNP rs10490924 in the *LOC387715* gene (*ARMS2*) and rs11200638 in the promoter of the *HTRA1* gene. Of these two SNPs, the most significant association was found with SNP rs11200638 ($P = 2.97 \times 10^{-14}$), whereas for variant rs10490924, the association was slightly less significant ($P = 1.23 \times 10^{-13}$; Table 2).

As multiple significant associations were evident from the tSNP and SNP analyses, we wanted to ascertain whether we could identify causality in any one SNP within this region. A logistic regression analysis using 8 tSNPs comprising rs2014307, rs3793917, rs2672591, rs2672587, rs2736919, rs763720, rs2250804, and rs2268356, as well as the non-tSNPs rs11200638 and rs10490924, which were used as covariate SNPs to adjust for rs3793917, rs2672587, rs10490924, and rs11200638 (significant with at minimum level of $P = 1.0 \times 10^{-4}$; Table 2, Fig. 1). After adjustment for the covariate, the tSNP most strongly independent was rs3793917, occurring between the 3-prime end of the *LOC387715* gene and the *HTRA1* gene, whereas the tSNP rs2672587 in the *HTRA1* gene had a much weaker independence and was therefore assumed not to be an independent major contributor to AMD.

SNP rs3793917 is in almost perfect LD (D′ = 0.97; Fig. 1) with rs11200638 of the *HTRA1* gene (both in block 3), being separated by 1269 bp, and lies upstream of the *HTRA1* gene. The SNP rs10490924 occurs in a different LD block (block 2), but also appears to be in high LD with rs3973917 (Fig. 1). When regression analysis was undertaken with either the tSNP rs3793917 or the SNPs rs10490924 or rs11200638 against the 10 significant tSNPs, these three SNPs could not be distinguished from each other to explain causality.

DISCUSSION

On the basis of haplotype and regression analyses, we identified the intergenic region occurring between the tSNP rs3793917 upstream of the *HTRA1* gene and the SNP rs11200638 in the *HTRA1* gene as being the region most likely associated with AMD. Variants within the *PLEKHA1* gene appeared at best to be weakly associated with AMD after regression analysis and this gene appears to have a minimal association with AMD. It can therefore be excluded as a candidate AMD gene in our cohort.

In this study, we did not identify one particular SNP that could explain causality on its own. This results is in contrast with a study[8] in which the SNP rs10490924 (A69S) was identified as the causal SNP. After undertaking an initial tSNP approach to ascertain the LD block structure of this region, we identified seven LD blocks (refer to Fig. 1). LD blocks 2 and 3 occurred between the *LOC387715 (AMRS2)* and *HTRA1* genes. The intergenic tSNP rs3793917 (occurring between the *LOC387715* gene and the *HTRA1* gene) was the most highly associated tSNP and the only tSNP demonstrating causality after multiple correction. The tSNP rs3793917 occurs approximately 1300 bp upstream of the *HTRA1* gene and in the same LD block (block 3) as the *HTRA1* gene. Based on this finding,

we conclude that the causal genetic variant most likely occurs in block 3, located intergenically to *LOC387715* and *HTRA1*, rather than in either gene itself.

After tSNP analysis we reassessed the tSNPs in conjunction with the SNPs rs10490924 in *LOC387715* and rs11200638 in *HTRA1*, previously associated with AMD in many other studies, and the result suggested that these SNPs are in high LD (>97%) with each other, as previously reported, as well as being in high LD with tSNPs in LD blocks 2 and 3. A previous tSNP analysis of the *PLEKHA1/LOC387715/HTRA1* region pinpointed rs11200638, rs2293870 (*HTRA1*), and rs10490924 (*LOC387715*) as the SNPs most significantly associated with AMD in this region.[13] The variant rs2293870 is located in exon 1 of the *HTRA1* gene. If it were to be placed in our tSNP analysis, it would fall directly into LD block 3, and thus in strong LD with SNP rs11200638 and tSNP rs3793917. This finding is in contrast to those reported by Kanda et al.,[8] who found that the SNP rs10490924 was the most likely causal SNP responsible for the association in this region, as opposed to SNP rs11200638 after regression analysis. However, Fritsche et al.[7] reported that the presence of an indel (372_81del443ins54) in the 3′UTR (untranslated region) of the *ARMS2* locus is the most likely site of association within this region (LD block 2). These findings tend to suggest the intergenic region between the *LOC387715* and *HTRA1* genes, but the region cannot be further delineated because of the high level of LD.

In the present study, we endeavored to gain a better understanding of the *PLEKHA1*, *LOC387715*, and *HTRA1* genes. The tSNP analysis that we used favored a site within LD group 3 as being the most likely for association with AMD. This site would position such a finding within the intergenic region between the 3-prime end of the *LOC387715* and the *HTRA1* genes. However, this finding is confounded by the lack of conclusive exclusion of the SNP rs10490924 in the *LOC387715* gene after regression analysis. Although some studies arrived at a similar conclusion,[12,13,18] another did not.[8] It is reasonable to suggest that there is unlikely to be a single SNP in this region of chromosome 10 that can explain causality, although by using a tSNP approach, we appear to have identified a region in LD block 3 that may contain the one or more variants responsible. However, it remains to be clarified whether this region contains elements that act on either the promoter of the *HTRA1* gene or neighboring genes to influence susceptibility to AMD. Further expression-based studies and changes influencing genetic architecture in this region are clearly warranted, to further resolve these issues and to identify which are the key components within the 6-kb region 10q26.

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