A variant of mitochondrial protein LOC387715/ARMS2, not HTRA1, is strongly associated with age-related macular degeneration


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Genetic variants at chromosomes 1q31-32 and 10q26 are strongly associated with susceptibility to age-related macular degeneration (AMD), a common blinding disease of the elderly. We demonstrate, by evaluating 45 tag SNPs spanning HTRA1, PLEKH1, and predicted gene LOC387715/ARMS2, that rs10490924 SNP alone, or a variant in strong linkage disequilibrium, can explain the bulk of association between the 10q26 chromosomal region and AMD. A previously suggested causal SNP, rs11200638, and other examined SNPs in the region are only indirectly associated with the disease. Contrary to previous reports, we show that rs11200638 SNP has no significant impact on HTRA1 promoter activity in three different cell lines, and HTRA1 mRNA expression exhibits no significant change between control and AMD retinas. However, SNP rs10490924 shows the strongest association with AMD (P = 5.3 x 10^-30), revealing an estimated relative risk of 2.66 for GT heterozygotes and 7.05 for TT homozygotes. The rs10490924 SNP results in nonsynonymous A69S alteration in the predicted protein LOC387715/ARMS2, which has a highly conserved ortholog in chimpanzee, but not in other vertebrate sequences. We demonstrate that LOC387715/ARMS2 mRNA is detected in the human retina and various cell lines and encodes a 12-kDa protein, which localizes to the mitochondrial outer membrane when expressed in mammalian cells. We propose that rs10490924 represents a major susceptibility variant for AMD at 10q26. A likely biological mechanism is that the A69S change in the LOC387715/ARMS2 protein affects its presumptive function in mitochondria.

Aging | genetic association | mitochondria | neurodegeneration | retinal disease

Age-related macular degeneration (AMD) is a common disorder that primarily affects the central region of the retina (macula) and is a leading cause of blindness in the elderly. Early symptoms of the disease are characterized by the presence of ophthalmoscopically visible soft drusen, with areas of hyper- or depigmentation, whereas later stages manifest as either choroidal neovascularization or atrophy of photoreceptors and retinal pigment epithelium (RPE) (1–3). Susceptibility to AMD is a multifactorial trait involving both genetic (4–6) and environmental factors (7, 8); however, its precise etiology remains elusive. A number of AMD-associated sequence variants exhibiting small effects have been reported in genes including ATP-binding transporter protein 4 (ABCA4) (9), apolipoprotein E (APOE) (10), excision-repair cross-complementing group 6 (ERCC6) (11), fibulin 5 (FBLN5) (12), elongation of very-long-chain fatty acids-like 4 (ELOVL4) (13), factor B/complement component 2 (BFC2) (14), toll-like receptor 4 (TLR4) (15), and vascular endothelial growth factor (VEGF) (16).

Genome-wide linkage studies have revealed disease susceptibility haplotypes of large effect at chromosomes 1q31-32 and 10q26 (6). In a remarkable example of the convergence of alternative approaches for gene mapping, independent research efforts identified the Y402H variant in complement factor H (CFH) on chromosome 1q32 as the first major AMD-susceptibility allele (7, 17–20). A second genomic region with similarly consistent linkage evidence is reported at chromosome 10q26 (6), where rs10490924 and nearby SNPs that span a 200-kb region of linkage disequilibrium show strong association to AMD (8, 21, 22). A consensus from multiple studies is that the 10q26 region harbors a second major genetic determinant of AMD susceptibility (8, 21–23). Markers showing evidence of association at 10q26 overlap with three genes, PLEKH1, LOC387715/ARMS2 (age-related maculopathy susceptibility 2), and HTRA1/PRSS11 (high-temperature requirement factor A1). PLEKH1 has a pleckstrin homology domain, whereas LOC387715/ARMS2 encodes a hypothetical protein of unknown function. It was initially proposed that polymorphisms in the region alter the risk of AMD by modulating the function of one of these two genes (8, 21, 22). More recently, two reports proposed a causal relationship between AMD susceptibility and rs11200638, another SNP in the same 200-kb region of 10q26, and suggested that this promoter variant affects the expression of a serine protease HTRA1/PRSS11 (24, 25). This interpretation contradicts with other reports (8, 21–23), which find the strongest association with rs10490924, T allele of rs10490924 maps to exon 1 of the hypothetical LOC387715/ARMS2 gene and changes putative amino acid 69 from alanine to serine.

To resolve the apparently contradictory reports, we undertook a detailed association analysis of SNPs at 10q26. Here, we show that the observed strong association of AMD susceptibility to rs10490924 cannot be explained by rs11200638 and that the region surrounding the rs11200638 variant does not bind to AP-2 transcription factor and has no significant effect on HTRA1 mRNA expression. Instead, the rs10490924 variant alters the coding sequence of an apparently primate-specific gene LOC387715/ARMS2, which we show can produce a protein that localizes to the mitochondria when expressed in mammalian cells. Taken together, our results suggest that changes in the activity or regulation of LOC387715/ARMS2 are likely responsible for the impact of rs10490924 on AMD disease susceptibility and that the association with rs11200638 is likely to be indirect.

Results

Association Analysis. To examine the association of rs10490924, rs11200638, and neighboring variants with AMD, we first geno-
SNP could be accounted for by rs10490924. These two degrees of freedom models also did not support the possibility that rs11200638 is the major determinant of disease susceptibility in the region.

Effect of rs11200638 on HTRA1 Expression. To follow-up our genetic analysis, we examined the impact of the previously proposed causal variant rs11200638 on HTRA1 expression and investigated the potential roles of LOC387715/ARMS2, the hypothetical gene whose coding sequence is altered by rs11200638. The SNP rs11200638 is located within a conserved genomic region upstream of human and mouse HTRA1 genes (24). To evaluate previous reports (24, 25) of the effects of SNP rs11200638 on HTRA1 promoter activity, we generated mammalian expression constructs carrying three different lengths of the WT HTRA1 promoter (WT-long, -medium, and -short) and the mutant sequence carrying the AMD-risk allele at the SNP rs11200638 (SNP-long and -medium). These constructs were transfected into HEK293, ARPE-19 (human RPE), and Y79 (human retinoblastoma) cells; in all three cell lines, WT and variant SNP promoter activities did not show statistically significant differences in the luciferase reporter expression, and the WT-short promoter (not including rs11200638 region) showed higher transcriptional activities than the others (Fig. 3 B–D).

Although the rs11200638 region includes several transcription factor binding sites as suggested by in silico analysis (Fig. 3E), Dewan et al. (24) focused on putative binding sites for transcription factors activating enhancer-binding protein-2α (AP-2α) and serum response factor. EMSA did not detect any supershift of the nucleotide sequence spanning rs11200638 variation with anti-AP-2α antibody (Fig. 3F, lane 5). Among the transcription factors examined, only stimulating protein 1 (SP-1) antibody produced a weakly shifted DNA–protein complex (Fig. 3F, lane 6). We also note that quantitative RT-PCR analysis provided suggestive evidence for a decrease in HTRA1 expression in AMD retinas (similar threshold levels after an average of 21.6 ± 0.6 RT-PCR cycles in control retinas versus 22.2 ± 0.3 cycles in AMD retinas; four independent retinas examined in quadruplicate for each). This finding contrasts
Immunoblot analysis reveals a predicted protein band of LOC387715/ARMS2 and faintly in the human retina and other cell lines, whereas expressed abundantly in JEG-3 (human placenta choriocarcinoma) effect on COX-IV, an inner membrane protein (Fig. 4 translocase of outer mitochondrial membrane 20, Tom20), with no loss of LOC387715/ARMS2 and outer membrane proteins (such as from the transfected COS-1 cells, with Proteinase K results in the results are obtained in the ARPE-19 and JEG-3 cells (data not

<table>
<thead>
<tr>
<th>SNP</th>
<th>Allele</th>
<th>Risk allele frequency</th>
<th>lod, 1 df</th>
<th>P</th>
<th>+/+</th>
<th>+/-</th>
<th>-/-</th>
<th>( \lambda_{ab} )</th>
<th>RR1</th>
<th>RR2</th>
<th>Logistic regression, including covariates for the following</th>
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<td>rs10490924</td>
<td>T</td>
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<td>0.002</td>
<td>0.836</td>
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<td>0.7556</td>
<td>0.503</td>
<td>0.524</td>
<td>rs10490924 (rs2275799 T C)</td>
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<td>rs3750848</td>
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<td>1.57</td>
<td>0.116</td>
<td>0.858</td>
<td>0.603</td>
<td>1.182</td>
<td>0.275</td>
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<td>rs932275</td>
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<td>1.47</td>
<td>0.041</td>
<td>0.897</td>
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</tr>
<tr>
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<td>0.149</td>
<td>0.118</td>
<td>0.842</td>
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<td>0.238</td>
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<td>rs10490924 (rs2275799 T C)</td>
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For each SNP, the risk allele (−) is defined as the allele with increased frequency in affected individuals. Evidence for association, as evaluated by the LAMP program (26), is summarized through the risk allele frequency in the population (estimated using a parametric model that, in effect, weights cases and controls according to the estimated disease prevalence); lod (logarithm of odds) score (log_{10} likelihood ratio statistic comparing model with and without association); \( \lambda_{ab} \) value; and a series of estimated penetrances for nonrisk homozygotes (−/−), heterozygotes (+/−), and risk allele homozygotes (+/+) genotype relative risks RR1 and RR2 (which are computed by comparing estimated penetrances in heterozygotes and risk-allele homozygotes, respectively, and those for nonrisk homozygotes); and sibling recurrence risks \( \lambda_{ab} \). The \( \lambda_{ab} \) measure characterizes the overall contribution of a locus to disease susceptibility. It quantifies the increase in risk to siblings of affected individuals attributable to a specific locus (48). For example, \( \lambda_{ab} \) of 1.27 signifies that the SNP could account for 27% in risk of AMD for relatives of affected individuals. Association analysis using a simple \( \chi^2 \) statistic produced similar results. The last two columns summarize \( \chi^2 \) results of logistic regression analysis, including either rs10490924 or rs11200638 as covariates. As suggested in ref. 40, missing genotypes were imputed prior to the sequential analyses reported in the last two columns.

with the smaller original experiment suggesting an increase in HTRA1 expression in lymphocytes from AMD patients (\( P = 0.02 \)) (24, 25). Taken together, the data indicate no significant change in HTRA1 expression between AMD patients and controls.

Expression and Subcellular Localization of LOC387715/ARMS2. We investigated the possible role of LOC387715/ARMS2, the hypothetical gene whose coding sequence is altered by rs10490924. LOC387715/ARMS2 encodes a predicted human protein with a highly conserved ortholog in chimpanzee, but not in other mammals or vertebrates (Fig. 4 A). The T allele of SNP rs10490924 is predicted to result in a coding change (A969S) of the LOC387715/ ARMS2 protein. This alanine-to-serine substitution creates a new putative phosphorylation site and breaks a predicted ε-helix (Fig. 4 A).

RT-PCR analysis showed that LOC387715/ARMS2 mRNA is expressed abundantly in JEG-3 (human placenta choriocarcinoma) and faintly in the human retina and other cell lines, whereas HPRT (control) transcript is detected to a similar degree in all tissues/cell lines (Fig. 4 B). Using the human retinal RNA, we cloned the LOC387715/ARMS2 cDNA into an expression vector and expressed it in COS-1 (African green monkey kidney fibroblast) cells. Immunoblot analysis reveals a predicted protein band of \( \sim 16 \) kDa (12-kDa protein + 4-kDa Xpress epitope) using anti-Xpress and anti-LOC387715/ARMS2 antibodies (Fig. 4 C). Subcellular fractionation and containing patterns of MitoTracker and cytochrome c oxidase subunit IV (COX IV) demonstrate that the expressed LOC387715/ARMS2 protein colocalizes with mitochondrial markers, but not with other organelle markers for endoplasmic reticulum (ER), Golgi apparatus, and lysosomes (Fig. 4 D and Fig. 5). Similar results are obtained in the ARPE-19 and JEG-3 cells (data not shown). The treatment of mitochondrial protein fraction, prepared from the transfected COS-1 cells, with Proteinase K results in the loss of LOC387715/ARMS2 and outer membrane proteins (such as translocase of outer mitochondrial membrane 20, Tom20), with no effect on COX-IV, an inner membrane protein (Fig. 4 E).

Discussion

A strong association between AMD and variants at 10q26 has been confirmed by multiple studies (6, 8, 21, 22, 24, 25). Nevertheless, the identity of the causal variants in the region and the possible mechanism(s) through which these variants influence disease susceptibility have not been elucidated (8, 21–23). The AMD-associated variants at 10q26 overlap two known genes, PLEKH1, HTRA1/PRSS11, and a predicted gene LOC387715/ARMS2. Each of these can have a plausible biological relationship to macular degeneration (6). To clarify the genetic association and evaluate possible mechanism(s) of disease susceptibility, we genotyped a panel of 45 tag SNPs that have allowed us to comprehensively assess the impact of variants in the region on AMD. Because SNPs showing the strongest association alter the predicted coding sequence of LOC387715/ARMS2 and are upstream of HTRA1/ PRSS11, we carried out further experiments to investigate the biological function of LOC387715/ARMS2 and examine the previously proposed impact of rs11200638 on the expression of HTRA1/PRSS11. Our data enable a direct comparison of HTRA1 and LOC387715/ARMS2 SNPs and provide strong evidence for a single variant of large effect in the region. Specifically, after examining a set of SNPs that tags common variants in the region, we validate the strongest association with rs10490924, a SNP that affects the coding sequence of LOC387715/ARMS2 (\( P < 10^{-29} \)). Evidence for association is weaker at all other SNPs (\( P > 10^{-2} \)) and becomes nonsignificant after accounting for rs10490924 in a multiple-regression analysis. The rs11200638 SNP, associated with HTRA1 expression in a previous report and proposed to play a causal role in AMD susceptibility (24, 25), cannot explain the effect of rs10490924 (within the LOC387715/ARMS2-coding region). In contrast, rs10490924 can account for the observed association at rs11200638.

Given that rs10490924 alters the predicted coding sequence of LOC387715/ARMS2, we proceeded to evaluate the gene further. LOC387715/ARMS2 is listed as a hypothetical human gene with a highly conserved ortholog in chimpanzee, but not in sequences from other organisms. The two exons of LOC387715/ARMS2 encode a putative protein of 107 aa, which includes no remarkable motifs, except for nine predicted phosphorylation sites. Consistent with previous reports of LOC387715/ARMS2 expression in the retina and placenta (22), our RT-PCR analysis confirms the presence of LOC387715/ARMS2 transcripts in human retina and a variety of other tissues and cell lines. Furthermore, we can translate LOC387715/ARMS2 cDNA cloned from the human retina, demonstrating that LOC387715/ARMS2 encodes a bona-fide protein.
Localization of the LOC387715/ARMS2 protein to mitochondrial outer membrane in transfected mammalian cells suggests intriguing mechanisms through which A69S change may influence AMD susceptibility. Mitochondria are implicated in the pathogenesis of age-related neurodegenerative diseases, including Alzheimer’s disease, Parkinson’s disease, and amyotrophic lateral sclerosis (29). Mitochondrial dysfunction associated with aging can result in impairment of energy metabolism and homeostasis, generation of reactive oxygen species, accumulation of somatic mutations in mitochondrial DNA, and activation of the apoptotic pathway (29–34). Decreased number and size of mitochondria, loss of cristae, or reduced matrix density are observed in AMD retina compared with control, and mitochondrial DNA deletions and cytochrome c oxidase-deficient cones accumulate in the aging retina, particularly in the macular region (31, 35). Moreover, mutations in mitochondrial proteins (e.g., dynamin-like GTPase OPA1) are associated with optic neurodegenerative disorders (36). Photoreceptors and RPE contain high levels of polyunsaturated fatty acids and are exposed to intense light and near-arterial levels of oxygen, providing considerable risk for oxidative damage (33, 36–38). We therefore propose that the altered function of the putative mitochondrial protein LOC387715/ARMS2 by A69S substitution enhances the susceptibility to aging-associated degeneration of macular photoreceptors.

We do not observe any significant difference in the expression, stability, or localization of the A69S variant LOC387715/ARMS2 protein in mammalian cells (data not shown). It is plausible that the A69S alteration modifies the function of LOC387715/ARMS2 protein by affecting its conformation and/or interaction. Additional analysis of the LOC387715/ARMS2 protein with Ala or Ser codon 69 and its function in vivo are needed to better understand its contribution to AMD pathogenesis.

In parallel to investigating LOC387715/ARMS2, we have examined the impact of rs11200638 on HTRA1 expression. Dewan et al. (24) and Yang et al. (25) previously reported that SNP rs11200638 alteration can increase HTRA1 promoter activity in HeLa (human cervical cancer) and ARPE-19 cells and results in higher expression of HTRA1 mRNA in the lymphocytes from AMD patients. Our HTRA1 promoter analysis experiments (reporter activity and gel-shift assays) with rs11200638 SNP haplotypes do not support these results. We do not observe any significant difference in the expression, stability, or localization of the A69S variant LOC387715/ARMS2 protein in mammalian cells (data not shown).
conclusions. In our real-time RT-PCR analysis, HTRA1 expression is not significantly different between chromosomal regions and AMD retinas. Together with the genetic association data, these studies show that rs11200638 SNP does not have a major contribution to HTRA1 gene regulation, and HTRA1 association to AMD may only be indirect.

In summary, in contrast to the CFH gene at 1q32, where multiple SNPs are required to explain the association signal (26), in this report we are able to identify a single SNP (rs10490924) that alone can account for the association of the 200-kb region at chromosome 10q26 with susceptibility to AMD. It is possible that other variants exist in the region, but their identification is likely to require very large sample sizes. Our data further illustrates the challenges of pinpointing causal alleles at susceptibility loci for complex diseases, even in regions where evidence for association is strong and replicated.

Materials and Methods
Genotyping and Data Analysis. We examined 535 affected individuals and 288 unrelated controls that were primarily ascertained and recruited at the Kellogg Eye Center (Ann Arbor, MI) as described in refs. 20 and 26. TaqMan assays (ordered from Applied Biosystems, Foster City, CA) were performed at the University of Michigan Sequencing Core Facility. For some SNPs (see Primers for 10q26 SNPs That Were PCR-Amplified and Sequenced in SI Text), PCR was used for amplification before sequencing. In a follow-up experiment, we genotyped a set of 20 overlapping markers (including rs10490924) by using an Illumina (San Diego, CA) Golden Gate experiment, we genotyped a set of 20 overlapping markers (including rs10490924) by using an Illumina (San Diego, CA) Golden Gate panel; a comparison with our original calls revealed an overall error rate of ~1.0%, which did not differ between cases and controls. The Illumina genotypes (with an overall completeness of 98.9%) support our conclusions, showing much stronger association for rs10490924 than for any other marker in the region and showing that rs10490924 can explain observed results for all other SNPs. However, we report the TaqMan data, despite the lower completeness, because it includes a larger number of SNPs in the region.

Genotypes were checked for quality by examining call rates per panel; a comparison with our original calls revealed an overall error rate of ~1.0%, which did not differ between cases and controls. The Illumina genotypes (with an overall completeness of 98.9%) support our conclusions, showing much stronger association for rs10490924 than for any other marker in the region and showing that rs10490924 can explain observed results for all other SNPs. However, we report the TaqMan data, despite the lower completeness, because it includes a larger number of SNPs in the region.

RT-PCR Analysis. Human retina tissues were procured from National Disease Research Interchange (Philadelphia, PA). Total RNA from retinas of four adults, each with AMD (ages 60–93 yr) or without any maculopathy (ages 64–100 yr) was reverse transcribed per standard protocols (41). Quantitative PCRs were performed in triplicate with Platinum Taq polymerase (Invitrogen, Carlsbad,
CA) by using the iCycler iQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA). SYBR green I (Invitrogen) was used for detection, and results were analyzed by the ΔΔCt method using HPRT for normalization. All primers are listed in Primer and Oligonucleotide Probe Sequences in SI Text.

**Plasmid Construction and Mutagenesis.** Three regions of the HTRA1 promoter (-3,652 to +57, -775 to +57, and -425 to +57) (GenBank accession no. AF157623) were subcloned into pGL3-basic vector (Promega, Madison, WI). The full-length LOC387715/ARMS2 (XM.001131263) cDNA was amplified from human retinal RNA by RT-PCR and cloned into pcDNA4 His/Max C vector (Invitrogen). The QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to generate all mutants of the HTRA1 promoter and LOC387715/ARMS2 expression construct.

**EMSA.** Nuclear extracts from bovine retina were used for EMSA per standard protocols (41). In supershift experiments, antibodies against AP-2 and SP-1 (Santa Cruz Biotechnology, Santa Cruz, CA), and NRL (a retina-pineal specific transcription factor) (42) were added after the incubation of 32P-labeled oligonucleotides with retinal nuclear extract.

**Antibody Generation.** Rabbit anti-LOC387715/ARMS2 polyclonal antibody was raised against the linear peptide sequences 34GEGASDKQKRSKL59 and 87QRRFQQPQHHLTHE100, derived from the predicted human LOC387715/ARMS2 protein (XP.001131263).

**Transfections, Protein Analysis, and Immunocytochemistry.** Cells were cultured according to standard procedures and transfected at 80% confluency with plasmid DNA by using FuGENE6 (Roche Applied Science, Indianapolis, IN). For luciferase assays, each plasmid containing pGL3-HTRA1 WT or SNP (0.5 μg per well) was cotransfected with cytomegalovirus-β-galactosidase (0.1 μg per well) plasmid to normalize for the amount of DNA and transfection efficiency, and the reporter activity was measured by a kit from Promega. Transfections were done in triplicate and repeated three times. Cell extracts were subjected to immunoblotting using mouse monoclonal anti-Xpress antibody (Invitrogen), rabbit anti-cytochrome c oxidase IV (COX IV) (Abcam, Cambridge, MA), or rabbit anti-Tom 20 antibody (Santa Cruz Biotechnology), according to the standard protocols (43).

Fractionation of COS-1 cell extracts was performed as described in ref. 44. In some experiments, the mitochondrial fraction was treated with Proteinase K for 3 min at 26°C. Immunostaining was performed as described in ref. 45 by using anti-Xpress antibody, MitoTracker and LysoTracker (Molecular Probes, Eugene, OR), rabbit anti-cytochrome c oxidase IV (COX IV) and rabbit anti-Giantin (Abcam), and rabbit anti-protein disulfide isomerase antibody (PDI) (StressGen Biotechnologies, Victoria, Canada).

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