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Specific correlation between the major chromosome 10q26 haplotype conferring risk for age-related macular degeneration and the expression of *HTRA1*

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Purpose: A region within chromosome 10q26 has a set of single nucleotide polymorphisms (SNPs) that define a haplotype that confers high risk for age-related macular degeneration (AMD). We used a bioinformatics approach to search for genes in this region that may be responsible for risk for AMD by assessing levels of gene expression in individuals carrying different haplotypes and by searching for open chromatin regions in the retinal pigment epithelium (RPE) that might include one or more of the SNPs.

Methods: We surveyed the PubMed and the 1000 Genomes databases to find all common (minor allele frequency > 0.01) SNPs in 10q26 strongly associated with AMD. We used the HaploReg and LDlink databases to find sets of SNPs with alleles in linkage disequilibrium and used the Genotype-Tissue Expression (GTEx) database to search for correlations between genotypes at individual SNPs and the relative level of expression of the genes. We also accessed Encyclopedia of DNA Elements (ENCODE) to find segments of open chromatin in the region with the AMD-associated SNPs. Predicted transcription factor binding motifs were identified using HOMER, PROMO, and RegulomeDB software programs.

Results: There are 34 polymorphisms within a 30-kb region that are in strong linkage disequilibrium (r^2>0.8) with the reference SNP rs10490924 previously associated with risk for AMD. The expression of three genes in this region, *PLEKHA1*, *ARMS2*, and *HTRA1* varies between people who have the low-AMD-risk haplotype compared with those with the high-AMD-risk haplotype. For *PLEKHA1*, 44 tissues have an expression pattern with the high-AMD-risk haplotype associated with low expression (rs10490924 effect size -0.43, $p = 3.8 \times 10^{-5}$ in ovary). With regard to *ARMS2*, the variation is most pronounced in testes: homozygotes with the high-AMD-risk haplotype express *ARMS2* at lower levels than homozygotes with the low-AMD-risk haplotype; expression in heterozygotes falls in between (rs10490924 effect size -0.79, $p = 7.5 \times 10^{-24}$). For *HTRA1*, the expression pattern is the opposite; the high-AMD-risk haplotype has higher levels of expression in 27 tissues (rs10490924 effect size 0.40, $p = 1.5 \times 10^{-7}$ in testes). None of the other 22 genes within one megabase of rs10490924, or any gene in the entire genome, have mRNA expression levels that correlate with the high-AMD-risk haplotype. More than 100 other SNPs in the 10q26 region affect the expression of *PLEKHA1* and *ARMS2* but not that of *HTRA1*; none of these SNPs affects the risk for AMD according to published genome-wide association studies (GWASs). Two of the AMD-risk SNPs (rs36212732 and rs36212733) affect transcription factor binding sites in proximity to a DNase I hypersensitive region (i.e., a region of open chromatin) in RPE cells.

Conclusions: SNPs in chromosome 10q26 that influence the expression of only *PLEKHA1* or *ARMS2* are not associated with risk for AMD, while most SNPs that influence the expression of *HTRA1* are associated with risk for AMD. Two of the AMD-risk SNPs affect transcription factor binding sites that may control expression of one of the linked genes in the RPE. These findings suggest that the variation in the risk for AMD associated with chromosome 10q26 is likely due to variation in *HTRA1* expression. Modulating *HTRA1* activity might be a potential therapy for AMD.

Age-related macular degeneration (AMD) is the leading cause of severe vision loss in older individuals. Single nucleotide polymorphisms (SNPs) in at least 34 loci influence the risk for AMD [1], and the mechanisms by which these variants influence AMD are still being elucidated. Although some AMD-risk SNPs change the coding region of genes, most reside outside coding regions, suggesting potential effects in regulating the expression of linked genes [2,3]. A set of closely linked SNPs on chromosome 10q26 is of special interest since this chromosome has more influence on the risk for AMD than any other AMD region [1,4-7]; however, which gene in the region confers the risk remains unclear [5,8-10]. Three genes are within 100 kilobase pairs (kb) of the SNPs associated with AMD. From the centromeric to telomeric ends of this region, the genes are *pleckstrin homology domain-containing family A member 1* (*PLEKHA1*; gene ID:

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Table 1. The 34 polymorphisms in linkage disequilibrium that form an AMD-associated haplotype block.

SNP	Chr 10 position (GPCh37)	REF/ALT Allele	r^2 LDlink/ HaploReg	D' LDlink/ HaploReg	AMD References
rs61871744	124,203,787	T/C	0.96/0.94	0.99/0.99	
rs59616332	124,208,562	ATAAAC/-	0.96/NA	0.99/NA	
rs11200630	124,209,684	T/C	0.99/0.98	1/0.99	
rs61871745	124,210,369	G/A	0.99/0.98	1/0.99	
rs11200632	124,211,536	A/G	0.99/0.99	1/1	
rs11200633	124,211,596	C/T	0.99/0.98	1/1	
rs61871746	124,212,913	T/C	1/1	1/1	
rs61871747	124,213,046	C/T	1/1	1/1	
rs370974631	124,213,143	AA/-	0.93/NA	1/NA	
rs200227426	124,213,671	C/A	0.96/NA	0.99/NA	
rs201396317	124,213,674	C/A	0.96/NA	0.99/NA	
rs199637836	124,213,677	C/A	0.96/NA	0.99/NA	
rs11200634	124,213,680	C/A	0.96/NA	0.99/NA	
rs75431719	124,213,688	C/A	0.96/NA	0.99/NA	
rs10490924	124,214,448	G/T	Reference SNP	Reference SNP	22, 23, 24, 25, 26, 27, 28, 36, and additional 140 refs
rs144224550	124,214,600	- /GT	1/NA	1/NA	
rs36212731	124,214,976	G/T	0.99/1	1/1	
rs36212732	124,215,198	A/G	1/1	1/1	
rs36212733	124,215,211	T/C	1/1	1/1	
rs3750848	124,215,315	T/G	1/1	1/1	29
rs3750847	124,215,421	C/T	1/1	1/1	30
rs3750846	124,215,565	T/C	1/1	1/1	1, 29
rs566108895	124,216,823	G/T	0.91/NA	0.99/NA	
rs3793917	124,219,275	C/G	0.99/0.98	1/0.99	29, 31, 32, 33
rs3763764	124,220,061	A/G	0.98/0.98	0.99/0.99	
rs11200638	124,220,544	G/A	0.98/0.9	0.99/0.97	17, 18, 19, 20, 21, 22, 23, and additional 100 refs
rs1049331	124,221,270	C/T	0.97/0.96	0.99/0.99	34, 37
rs2293870	124,221,276	G/C,T	NA/0.9	NA/0.95	34, 38, 39
rs2284665	124,226,630	G/T	0.96/0.94	0.99/0.98	35
rs60401382	124,227,624	C/T	0.84/0.82	0.98/0.97	
rs11200643	124,229,203	C/T	0.82/0.8	0.96/0.94	
rs58077526	124,230,024	A/C	0.91/0.89	0.96/0.95	
rs932275	124,231,464	G/A	0.93/0.89	0.97/0.96	29
rs2142308	124,234,037	G/C	0.92/0.89	0.97/0.96	

The SNP rs10490924 is the reference SNP used to obtain r^2 and D' values in both the HaploReg and LDlink websites. In the 1000 Genome project European population there are 34 polymorphisms, including rs10490924, in linkage disequilibrium using the inclusion criterion $r^2 \geq 0.8$ [11,12]. LDlink r^2 refers to the strength of the association of the alleles at the queried SNP with rs10490924. LDlink D' measures linkage disequilibrium normalized for allele frequency. NA = Not Available.

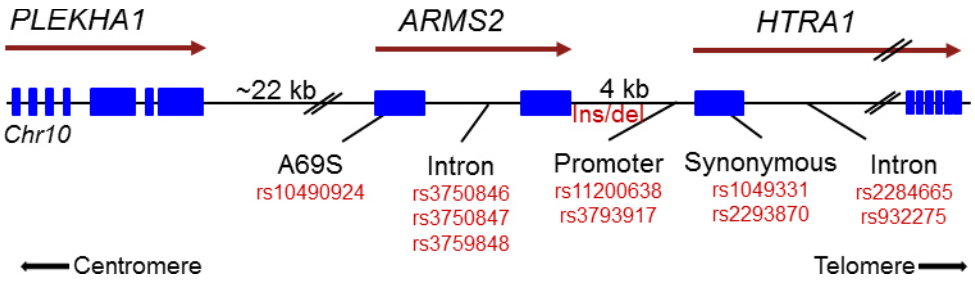

Figure 1. Diagram of the chromosome 10q26 region containing the *PLEKHA1*, *ARMS2*, and *HTRA1* genes, as well as the locations of the SNPs that have been associated with risk for AMD by published GWASs and genetic studies.

59338, OMIM: 607772), *age-related maculopathy-2* (*ARMS2*; gene ID: 387715, OMIM: 611313), and *high temperature requirement protein A1* (*HTRA1*; gene ID: 5654, OMIM: 602194). It is even conceivable that a more distant gene actually confers the risk for AMD.

We searched for associations between AMD-risk SNPs in the 10q26 region and the expression of closely linked genes. We also looked for open chromatin in the region in RPE cells and transcription factor binding sites in the open chromatin since such regions often correspond with regions that regulate transcription.

METHODS

Linkage disequilibrium analysis: Using the online tools HaploReg and LDlink, SNPs associated with AMD and other variants were placed in a haplotype block using Query SNP rs10490924 and the linkage disequilibrium inclusion criterion $r^2 \geq 0.8$ [11,12] in the 1000 Genome European population.

Transcriptome analysis: The GTEx database allows one to search for relationships between human genotypes and gene expression in specific tissues [13,14]. At the time of this analysis (January–October 2016), the GTEx project V6p (GtexPortal) contained genotypes from 449 human adult donors and whole genome transcription data from up to 53 tissues from an overlapping set of 544 donors. Transcript levels of *PLEKHA1, ARMS2*, and *HTRA1* were included. The steady-state mRNA level is measured as reads per kilobase of transcript per million mapped reads (RPKM). RPKM are normalized according to the number of sequencing reads and the read lengths. RPKM below 1 indicate levels of mRNA expression that are difficult to distinguish from background noise.

Association between genetic variants and mRNA expression: We searched for expression quantitative trait loci (eQTLs) in the chromosome 10q26 region by looking for correlations between SNP alleles and the expression of genes using the "Test your own eQTLs" option in the GTEx portal Version 6. The mRNA levels are expressed as rank normalized gene expression [15]. The effect size of an eQTL is the change in the value of the standardized gene expression level with each extra copy of the alternative (ALT) allele relative to the reference allele, conditional to all other adjustments (gender, probabilistic estimation of expression residuals factors, genotype principal components, and genotyping platforms). Because the gene expression levels of tissues have been transformed to a standard normal distribution (mean of 0 and standard deviation of 1), the effect size is also equivalent to the change in the population standard deviation; that is, an effect size of 0.2 means a change in 0.2 standard deviations from the baseline level with both reference alleles. The effect size provides the variation in the strength of expression with positive numbers indicating higher mRNA levels in samples from people with the minor allele compared to those with the major allele, and negative numbers indicating lower mRNA levels in samples with the minor allele. For the eQTL analysis, selected tissues were those for which high-quality mRNA results were available in at least 70 genotyped donors.

Search for open chromatin and transcription factor binding sites: We used the Encyclopedia of DNA Elements (ENCODE) to search for DNase I sensitive sites; this database has results from more than 125 cell types, including primary cultures of RPE. We used three online programs that predict transcription factor binding sites: HOMER, PROMO, and RegulomeDB. The consensus binding sequences are from JASPAR.

Statistical analysis: The GTEx consortium database has precalculated nominal eQTL p values for every human gene and all the informative SNPs analyzed. The p values for each SNP-gene pair were calculated using a two-tailed *t* test as described at that site (GtexPortal). We considered associations statistically significant when the p value was less than 0.05. In some cases, as stated in the text, we made adjustments for the multiple analyses.

Table 2. The rs10490924-T allele is associated with low levels (negative effect sizes) of *PLEKHA1* gene expression in most tissues.

Tissue	P-value	Effect Size	Expression (Mean RPKM)	Number samples
Brain - Nucleus accumbens (bg)	0.084	0.14	N/A	93
Stomach	0.45	0.038	20.678	170
Brain - Caudate (basal ganglia)	0.57	0.033	188.491	100
Brain - Hypothalamus	0.77	0.024	101.534	81
Heart - Left Ventricle	0.73	0.023	21.246	190
Brain - Frontal Cortex (BA9)	0.8	0.016	116.963	92
Brain - Putamen (basal ganglia)	0.82	0.016	193.492	82
Brain –Ant. cingulate cortex (BA24)	0.82	0.013	141.737	72
Heart - Atrial Appendage	0.93	0.0077	30.707	159
Small Intestine - Terminal Ileum	0.97	−0.0038	21.23	77
Testis	0.92	−0.0069	12.333	157
Liver	0.8	−0.024	52.72	97
Skin - Sun Exposed (Lower leg)	0.44	−0.032	62.651	302
Muscle - Skeletal	0.25	−0.041	6.046	361
Artery - Coronary	0.62	−0.043	145.955	118
Whole Blood	0.1	−0.046	1.153	338
Brain - Cerebellar Hemisphere	0.64	−0.052	28.95	89
Esophagus - Mucosa	0.35	−0.053	14.493	241
Pituitary	0.47	−0.067	105.377	87
Esophagus - Muscularis	0.14	−0.072	53.302	218
Thyroid	0.12	−0.074	42.25	278
Adipose - Visceral (Omentum)	0.067	−0.081	110.21	185
Skin -Not Sun Exposed (Suprapubic)	0.11	−0.084	60.731	196
Colon - Sigmoid	0.31	−0.086	74.374	124
Adipose - Subcutaneous	0.05	−0.088	126.917	298
Colon - Transverse	0.12	−0.089	36.749	169
Breast - Mammary Tissue	0.15	−0.089	114.907	183
Lung	0.0069	−0.11	36.291	278
Artery - Aorta	0.056	−0.11	268.567	197
Spleen	0.19	−0.14	26.428	89
Brain - Cortex	0.032	−0.15	118.249	96
Pancreas	0.051	−0.15	14.597	149
Artery - Tibial	0.0013	−0.18	139.083	285
Brain - Hippocampus	0.044	−0.18	129.512	81
Prostate	0.11	−0.18	37.874	87
Adrenal Gland	0.037	−0.21	36.181	126
Vagina	0.0068	−0.22	96.109	79
Uterus	0.083	−0.22	104.586	70
Nerve - Tibial	1.40E-08	−0.25	99.2	256
Brain - Cerebellum	0.003	−0.3	44.175	103

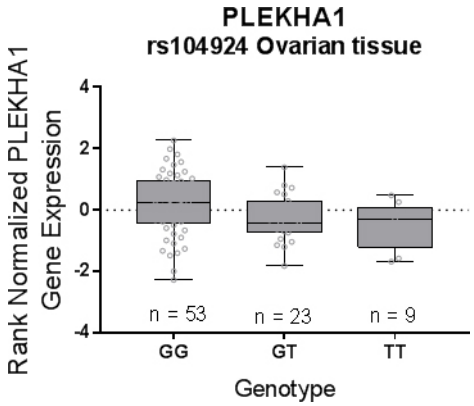

Figure 2. In the ovaries, women who are homozygous or heterozygous for the AMD-risk allele rs10490929-T have lower average *PLEKHA1* mRNA levels compared with homozygotes with the reference allele G. Error bars indicate the standard error of the mean. The sample size (n) in each group is indicated above each genotype.

RESULTS

SNPs that define the AMD-risk haplotype in 10q26: There are ten SNPs in a 17-kb region within 10q26 for which published data indicate strong associations of alleles with risk for AMD (Table 1 and Figure 1) [1,16-39]. One of the SNPs, rs10490929, changes the *ARMS2* coding sequence (G versus T, Ala69Ser). The other nine SNPs are in strong linkage disequilibrium with rs10490929, and each has a correlation coefficient r^2 ≥0.89 with rs10490924. Of these nine SNPs, rs3750846, rs3750847, and rs3750848 are in the only *ARMS2* intron, rs3793917 and rs11200638 are located in the *HTRA1* promoter region, rs1049331 and rs2293870 are synonymous variations in the *HTRA1* coding region, and rs2284665 and rs932275 are in the first *HTRA1* intron. We considered the set of alleles of these ten SNPs, all from published studies showing association with elevated risk for AMD, as a presumed haplotype that we refer to as the "high-AMD-risk" haplotype (Table 1). Although data from all ten SNPs are in the GTEx database, we used rs10490924 as the reference SNP because it is the most commonly evaluated SNP in studies of AMD [16-28]. The HaploReg and LDlink websites indicate that the haplotype defined by the ten SNPs includes an additional 24 polymorphisms (21 SNPs and three insertion-deletion polymorphisms) extending across a region of about 30 kb. Many of these 24 additional polymorphisms were not specifically evaluated in most previous genetic evaluations of AMD, but they likely have some association with risk for AMD because they have strong allelic correlations with rs10490924, with some having perfect correlations (i.e., $r^2 = 1$; Table 1). Of these 24 additional polymorphisms, 15 are included in the GTEx database. The present analysis of eQTLs (SNP-gene expression correlations) concentrated on the 25 SNPs in the GTEx database, comprising the ten that have been reported to be associated with risk for AMD and the 15 in strong linkage disequilibrium with those ten. We focused especially on the reference SNP rs10490924 because the strong linkage disequilibrium of the other SNPs with the reference SNP meant that results from any of them would approximately predict the results from the others. The present studies of the other SNPs in the haplotype confirmed that prediction.

The three genes in 10q26 closest to the region of the AMD-associated SNPs are (centromeric to telomeric) *PLEKHA1*, *ARMS2*, and *HTRA1*. There is an intergenic region of about 22 kilobases between *PLEKHA1* and *ARMS2* and an intergenic region of about 5 kilobases between *ARMS2* and *HTRA1* (Figure 1). The 30-kb AMD-risk haplotype stretches from the intergenic region between the *PLEKHA1* and *ARMS2* genes to the middle of the *HTRA1* gene.

The high-AMD-risk haplotype is associated with low expression of the PLEKHA1 gene: The *PLEKHA1* gene is about 60 kb in length and its termination codon, which is at the gene's telomeric boundary, is about 12 kb from the centromeric boundary of the AMD-risk haplotype. *PLEKHA1* is detectably expressed in all 53 tissues in the GTEx database (Table 2 and Appendix 1) with an average expression level of about 9.5 RPKM. In 32 tissues, the average *PLEKHA1* expression was lower in people with the high-AMD-risk haplotype defined by rs10490924-T, and the associated p values were below 0.05 in ten of those tissues (Table 2 and Appendix 2). Other SNPs in the AMD haplotype showed similar results, which was expected because of their strong linkage disequilibrium (Appendix 2). The effect was most striking in ovarian tissue (effect size −0.43, $p = 3.8 \times 10^{-5}$ for rs100490924; Figure 2). The effect appears to be semidominant with the expression level in heterozygotes falling between the TT and GG homozygotes. In the nine tissues that showed the opposite pattern (higher expression levels in people with the high-AMD-risk haplotype), the variation in mRNA levels among the genotypes was small and of no statistical significance, with all but one of the p values greater than 0.4. Thus, although the *PLEKHA1* gene is more than 22 kb away from rs10490924 and about 12 kb from SNP rs61871744 that defines the centromeric boundary of the AMD-risk haplotype, the expression of this gene in many tissues is influenced by sequences within the haplotype.

The high-AMD-risk haplotype is associated with low expression of ARMS2: The *ARMS2* transcriptional unit is about 2 kb and is completely contained within the 30-kb region containing the AMD-risk SNPs. In the GTEx transcriptome data set, *ARMS2* mRNA is not detected in 18 tissues and is at

a low level in most of the other tissues, averaging only about 0.2 RPKM and never above 1.0 RPKM except testes that have a level of 3.0 RPKM (Table 3 and Appendix 3). In the testes, homozygotes with the rs10490929-T allele (a marker of the high-AMD-risk haplotype) generally have lower *ARMS2* mRNA levels than homozygotes with the low-AMD-risk G allele (effect size −0.79, p = 7.5 × 10^{-24}; Figure 3). The effect appears to be semidominant with the expression level in heterozygotes falling between the TT and GG homozygotes. As expected because of strong linkage disequilibrium, another high-AMD-risk allele in the region (rs1120638-A), which is in the same haplotype as the rs10490924-T allele, is also associated with a low *ARMS2* mRNA level in the testes (effect size −0.76, p = 8.3 × 10^{-20}; Appendix 2). Eight other

Table 3. The rs10490924-T allele is associated with low levels (negative effect sizes) of *ARMS2* gene expression in most tissues.

Tissue	P-Value	Effect Size	Expression (Mean RPKM)	Number Samples
Brain - Anterior cingulate cortex (BA24)	0.95	0.012	N/A	72
Muscle - Skeletal	0.98	0.0017	0	361
Brain - Caudate (basal ganglia)	0.93	−0.015	0.057	100
Nerve - Tibial	0.71	−0.04	0.059	256
Liver	0.74	−0.048	0.099	97
Adipose - Subcutaneous	0.49	−0.056	0.113	298
Brain - Frontal Cortex (BA9)	0.76	−0.063	0.079	92
Breast - Mammary Tissue	0.51	−0.082	0.07	183
Colon - Transverse	0.18	−0.12	0.054	169
Stomach	0.1	−0.15	0.036	170
Thyroid	0.086	−0.16	0.051	278
Pituitary	0.33	−0.16	0.382	87
Brain - Putamen (basal ganglia)	0.37	−0.17	0.06	82
Heart - Left Ventricle	0.075	−0.18	0.055	190
Lung	0.098	−0.18	0.033	278
Prostate	0.35	−0.19	0.082	87
Adipose - Visceral (Omentum)	0.022	−0.2	0.116	185
Esophagus - Mucosa	0.069	−0.2	0.136	241
Brain - Cortex	0.24	−0.23	0.084	96
Brain - Hippocampus	0.23	−0.24	0.075	81
Vagina	0.088	−0.25	N/A	79
Heart - Atrial Appendage	0.042	−0.27	0.022	159
Artery - Tibial	0.00041	−0.35	0.099	285
Ovary	0.014	−0.35	0.472	85
Colon - Sigmoid	0.005	−0.4	0.152	124
Skin - Not Sun Exposed (Suprapubic)	0.0003	−0.41	0.115	196
Esophagus - Muscularis	0.000063	−0.45	0.136	218
Small Intestine - Terminal Ileum	0.0079	−0.45	0.044	77
Skin - Sun Exposed (Lower leg)	7.00E-07	−0.46	N/A	302
Artery - Aorta	0.00007	−0.47	0.109	197
Uterus	0.0049	−0.5	0.184	70
Brain - Nucleus accumbens (basal ganglia)	0.003	−0.54	0.05	93
Artery - Coronary	5.50E-06	−0.56	0.094	118
Brain - Hypothalamus	0.00019	−0.67	0.089	81
Testis	7.50E-24	−0.79	3.012	157

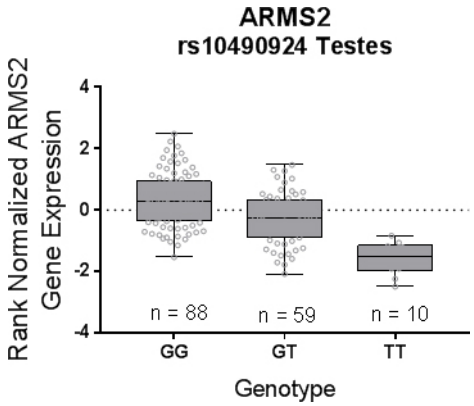

Figure 3. In the testes, men who are homozygous or heterozygous for the AMD-risk allele rs10490929-T have lower average *ARMS2* mRNA levels compared with homozygotes with the reference allele G. Error bars indicate the standard error of the mean. The sample size (n) in each group is indicated above each genotype.

AMD-associated variants in this region that we evaluated are associated with a similarly low expression of *ARMS2* in the testes (Appendix 2).

Among tissues other than the testes, 34 express detectable but low levels of *ARMS2* and thus provide poor statistical power (Table 3). In 32 of the 34 tissues, the *ARMS2* transcript levels were numerically lower in homozygotes with the rs10490929-T allele compared with heterozygotes or G-allele homozygotes, a trend that is concordant with the results from the testes (Table 3). The difference in expression between people with the high-AMD-risk T allele versus the low-AMD-risk G allele reached p values less than or equal to 0.05 in 14 of the tissues, such as sun-exposed skin ($p = 7.0 \times 10^{-7}$) and coronary artery ($p = 5.5 \times 10^{-6}$). Analysis of the other SNPs in the AMD-risk haplotype that we evaluated showed a similar pattern (Appendix 2).

The high-AMD-risk haplotype is associated with high expression of the HTRA1 gene: The SNP rs2142308, which forms the telomeric boundary of the AMD-risk haplotype, is inside intron 1 of *HTRA1*. *HTRA1* mRNA is ubiquitously expressed with RPKM values ranging from 1 (whole blood) to 269 (the aorta) and averages about 400-fold higher than *ARMS2* and 8.5-fold higher than *PLEKHA1* (81.2 RPKM versus 0.2 or 9.5 RPKM, respectively; Table 4 and Appendix 4). In 27 tissues, the average *HTRA1* expression was higher in people with the high-AMD-risk haplotype defined by rs10490924-T, and the effect was associated with p values of less than 0.05 in six of those tissues (Table 4 and Appendix 2). The effect was most striking in the testes (effect size 0.4, $p = 1.5 \times 10^{-7}$ for rs10490924; Figure 4 and Table 4). In one tissue (prostate), the opposite association was observed with an effect size of −0.23 (p = 0.02). Analysis of the other SNPs in the AMD-risk haplotype that we evaluated showed a similar pattern in HTRA1 mRNA levels in human tissues (Appendix 2). In one tissue (prostate) the opposite association was observed with an effect size of -0.23 (p = 0.02) with rs10490924.

The high-AMD-risk haplotype has no detectable effect on the expression of distant genes: To test whether the high-AMD-risk haplotype could influence the expression of genes even more distant than *PLEKHA1*, *ARMS2*, or *HTRA1*, we examined the transcription of the 22 genes located within 1 megabase of the *PLEKHA1-ARMS2-HTRA1* cluster in all 44 human tissues in the GTEx database. Of the 22 genes, 16 were detectably transcribed in at least one tissue. There was no statistically significant association between alleles at the reference SNP rs10490924 and the expression of any of these 16 genes in any of the 44 tissues. In fact, searching through the entire transcriptomes of all 44 tissues in the GTEx database with the criterion of a false discovery rate (FDR) of less than or equal to 0.5, none of the AMD-associated SNPs appeared to be eQTLs for any gene on any chromosome except *PLEKHA1*, *ARMS2*, and *HTRA1* (Table 5). Thus, the 10q26 high-AMD-risk haplotype appears to affect only *PLEKHA1*, *ARMS2*, and *HTRA1*, and it is unlikely that the high-AMD-risk haplotype has a distant eQTL or inter-chromosome effect on gene expression.

Most SNPs from 10q26 that influence the expression of PLEKHA1 and ARMS2 have not been associated with AMD: Many more SNPs in 10q26 influence the expression of *PLEKHA1* and *ARMS2* than *HTRA1* (Figure 5A). For example, the expression of *PLEKHA1* across 12 tissues is significantly influenced by more than 254 SNPs, with "significance" defined by the GTEx database significance criterion of an FDR of 5% or less. Of the 254 SNPs, 28 also affect the expression of *HTRA1*, while the remaining 226 SNPs affect the expression of *PLEKHA1* but not *HTRA1*. None of the 226 has been found to influence the risk for AMD. Only 25 of the 254 SNPs that influence the expression of *PLEKHA1* also modulate the risk for AMD, and all 25 are among those that modulate *HTRA1* expression. The expression of *ARMS2* in 13 tissues is influenced by 192 SNPs extending across 855 kb (Figure 5A and Appendix 2). Of the 192 SNPs, 35 also affect the expression of *HTRA1*, while the remaining 157 affect the expression of *ARMS2* but not *HTRA1*. None of the 157 has been found to influence the risk for AMD. Only 25 of the 192 SNPs that influence the expression of *ARMS2* also modulate the risk for AMD, and all 25 are among those that modulate *HTRA1* expression. Thus, more than 85% of the SNPs that influence the expression of *ARMS2* and *PLEKHA1* are not associated with risk for AMD. Ninety-nine of these SNPs have high minor allele frequencies (higher than 0.2),

Table 4. The rs10490924-T allele is associated with low levels (positive effect sizes) of *HTRA1* gene expression in most tissues.

Tissue	P-Value	Effect Size	Expression (Mean RPKM)	Number Samples
Testis	1.50E-07	0.4	12.333	157
Pituitary	0.016	0.28	105.377	87
Artery - Coronary	0.043	0.16	144.948	118
Artery - Aorta	0.017	0.13	268.567	197
Liver	0.12	0.12	52.72	97
Breast - Mammary Tissue	0.074	0.11	114.907	183
Whole Blood	0.029	0.1	1.153	338
Lung	0.032	0.098	36.291	278
Adipose - Subcutaneous	0.056	0.094	126.917	298
Small Intestine - Terminal Ileum	0.24	0.093	21.23	77
Vagina	0.12	0.09	96.109	79
Adrenal Gland	0.36	0.082	36.291	126
Nerve - Tibial	0.26	0.077	99.2	256
Colon - Transverse	0.074	0.066	36.749	169
Spleen	0.45	0.06	26.428	89
Brain - Hippocampus	0.63	0.051	129.512	81
Heart - Atrial Appendage	0.44	0.043	30.707	159
Thyroid	0.32	0.039	42.25	278
Esophagus - Mucosa	0.46	0.032	14.493	241
Muscle - Skeletal	0.34	0.026	6.046	361
Skin - Sun Exposed (Lower leg)	0.64	0.019	62.815	302
Artery - Tibial	0.78	0.01	139.083	285
Adipose - Visceral (Omentum)	0.86	0.0092	110.21	185
Heart - Left Ventricle	0.88	0.0084	21.246	190
Brain - Nucleus accumbens (bg)	0.97	0.0036	132.309	93
Pancreas	0.98	0.0024	14.597	149
Ovary	0.99	0.0012	211.834	85
Stomach	0.78	−0.012	20.678	170
Brain - Hypothalamus	0.83	−0.026	101.534	81
Brain - Cortex	0.68	−0.03	118.249	96
Brain - Caudate (basal ganglia)	0.68	−0.037	188.491	100
Brain - Anterior cingulate cortex (BA24)	0.29	−0.069	141.737	72
Esophagus - Muscularis	0.13	−0.081	53.302	218
Colon - Sigmoid	0.29	−0.089	74.374	124
Brain - Frontal Cortex (BA9)	0.21	−0.092	116.804	92
Brain - Cerebellum	0.18	−0.099	44.175	103
Skin - Not Sun Exposed (Suprapubic)	0.12	−0.1	60.731	196
Brain - Putamen (basal ganglia)	0.18	−0.1	193.492	82
Brain - Cerebellar Hemisphere	0.15	−0.12	28.95	89
Uterus	0.073	−0.21	104.586	70
Prostate	0.017	−0.23	37.874	87

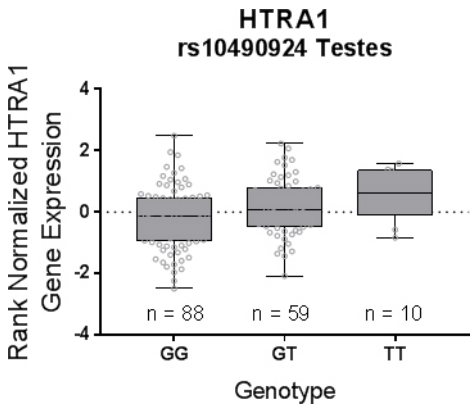

Figure 4. In the testes, men who are homozygous or heterozygous for the AMD-risk allele rs10490929-T have higher average higher *HTRA1* mRNA levels compared with homozygotes with the reference allele G. Error bars indicate the standard error of the mean. The sample size (n) in each group is indicated above each genotype.

and 35 have a high effect size (absolute value higher than 1; Appendix 2). Some of these SNPs were definitely included in published genome-wide association studies (GWASs). For example, 20 SNPs that affect *PLEKHA1* and *ARMS2* but not *HTRA1* are in the Illumina Human 610-Quad BeadChip used by Fritsch et al. who did not report an association with AMD with any of these SNPs [40]. It is likely that among the numerous, large GWASs conducted on patients with AMD, some of these SNPs would have been identified as influencing risk for AMD if they actually had an effect on risk for AMD.

For *HTRA1*, only 41 SNPs influence its expression. Thirty-four of these 41 SNPs are concentrated in the 30-kb AMD haplotype region, including all 25 SNPs in the AMD-risk haplotype (Figure 5B). All AMD-risk SNPs that influence the expression of *PLEKHA1* or *ARMS2* also influence the expression of *HTRA1*.

Two AMD-risk SNPs alter predicted transcription factor binding sites: The ENCODE database of open chromatin regions includes data from the RPE, a monolayer of cells that plays a key role in the pathogenesis of AMD. Within the 30-kb AMD-risk region, there are three DNase I hypersensitive sites in RPE cells (Table 6). We evaluated whether any of the 34 polymorphisms in the AMD-risk haplotype, including the 25 in the GTEx database and the nine additional polymorphisms in the LDlink database, were within the DNase I hypersensitive sites. The site with the most DNase I hypersensitivity is a 170-bp segment extending from chromosome 10 positions 124,215,021-124,215,190 (Figure 5B). This site is 5.8 kb upstream of the transcription start site of *HTRA1* and within the intron of *ARMS2*. Using the transcription-factor-binding-motif-prediction software HOMER, PROMO, and RegulomeDB, we searched for consensus binding sequences for transcription factors in the open chromatin region that might include AMD-risk SNPs. The results implicated two AMD-risk SNPs, rs36212732 and rs36212733, which are 8 bp and 21 bp, respectively, away from the telomeric boundary of the hypersensitive site (Figure 6). In comparison with the low-AMD-risk haplotype, the high-AMD-risk haplotype loses sites for transcription factors YY1, LHX2, LHX3, NKX6–1, ALX1, and ALX3, and it creates a site for the transcription factor c-MYB (Figure 6). These transcription factors are expressed in human RPE cells [41,42].

The other two open chromatin sites in this region are less than one tenth as sensitive to DNase I (Table 6). One extends from 124,220,906-124,222,950 and contains two AMD-risk SNPs (rs1049331 and rs2293870), but these polymorphisms do not affect any potential transcription factor binding sites according to the HOMER, PROMO, and RegulomeDB programs. The other open chromatin site extends from 124,228,506-124,228,935 and has no AMD-associated SNPs within or nearby.

DISCUSSION

We used the GTEx genotype-tissue expression database to explore the effects of SNPs that influence the susceptibility for AMD on the expression of nearby genes in the 10q26 *ARMS2-HTRA1* region. The AMD-risk alleles at ten SNPs

Table 5. Single tissue significant cis-eQTLs for AMD-associated SNPs rs10490924.

Gencode ID	Gene Symbol	P-Value	Effect Size	Tissue
ENSG00000254636.1	ARMS2	7.50E-24	−0.79	Testis
ENSG00000107679.10	PLEKHA1	1.40E-08	−0.25	Nerve - Tibial
ENSG00000166033.7	HTRA1	1.50E-07	0.4	Testis
ENSG00000254636.1	ARMS2	7.00E-07	−0.46	Skin - Sun Exposed (Lower leg)

The table provides the results from a search of GTEx for the entire list of genes that are influenced by the reference SNP rs10490924 using the GTEx default criteria for significance. Note that only 3 genes appear, indicating that no other gene anywhere in chromosome 10q26 nor any gene in the GTEx database throughout the human genome is influenced by the reference SNP. Similar results appear for all of the 25 GTEx SNPs in the AMD-risk haplotype (data not shown), as expected since they are all in strong linkage disequilibrium.

in this region are in strong linkage disequilibrium, and there are 24 additional nearby SNPs or insertion-deletion polymorphisms that are similarly correlated, 15 of which are in the GTEx database. Based on results from 25 of these SNPs in the GTEx database, high-AMD-risk alleles are associated with lower levels of *ARMS2* and *PLEKHA1* mRNA and higher levels of *HTRA1* mRNA than low-AMD-risk alleles in many human tissues. The AMD-risk SNPS do not influence the level of expression of any other gene in this region or anywhere in the human genome with statistical significance after adjustment for the multiple comparisons. Thus, if the risk for AMD conferred by this region is due to variation in gene expression (rather than a change in the transcribed protein), then the risk is due to variation in the expression of one of these three genes.

The GTEx data additionally provide evidence that variations in *ARMS2* and *PLEKHA1* expression are less likely than *HTRA1* to influence risk for AMD. Hundreds of additional SNPs in this region influence the expression of *ARMS2* and *PLEKHA1* but not *HTRA1*. None of those additional SNPs has been associated with the risk for AMD in any published human genetics study although some of these SNPs were included in those studies. However, most of the SNPs (25/41)

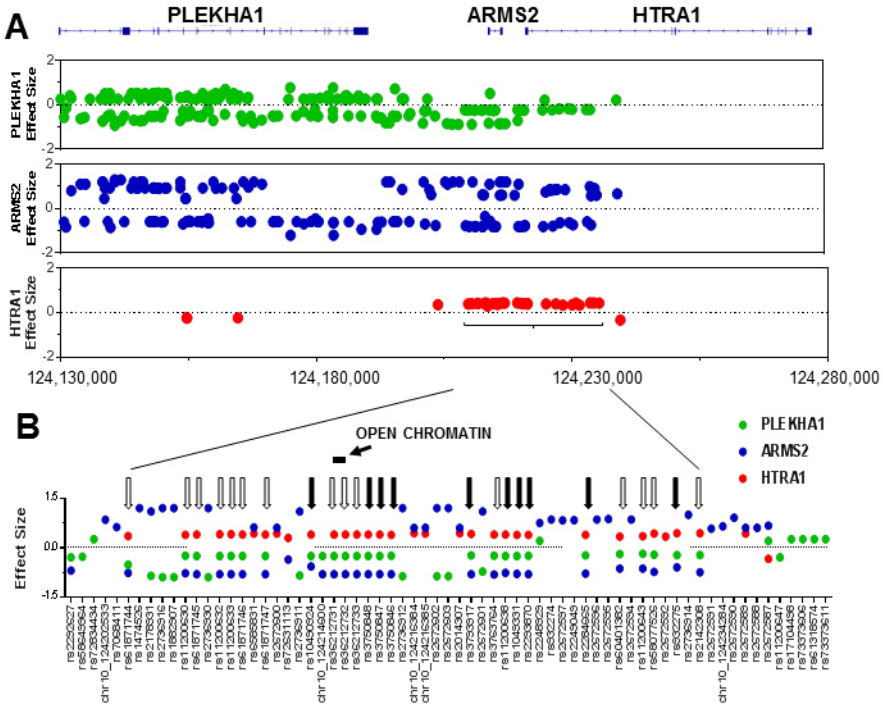

Figure 5. The locations of single nucleotide polymorphisms (SNPs) that influence the expression of *PLEKHA1*, *ARMS2*, or *HTRA1* (i.e., cis-expression quantitative trait loci (eQTLs)). **A**: The top of the figure shows the intron–exon structures of the *PLEKHA1*, *ARMS2*, and *HTRA1* genes. Under the schematic genome segment are three graphs with each graph showing the locations of SNPs that affect the mRNA expression (i.e., eQTLs) for each respective gene. The x-axis has the base pair locations, with the scale numbers at the bottom of the three graphs based on human genome reference GRCh37/hg19. The dots are colored so they correspond to panel B (green = *PLEKHA1*, blue = *ARMS2*, red = *HTRA1*). To be an eQTL on this graph, an SNP must meet the threshold for significance as calculated by the GTEx software (a false discovery rate of less than 0.5) in at least one tissue. The effect size (y-axis) is the maximum effect size across all tissues, with positive values meaning that the minor (alternative) allele is associated with higher expression. There are some eQTLs (75 for *PLEKHA1*, 44 for *ARMS2*, and four for *HTRA1*) off the ends of these graphs that are not included because of size limitations. **B**: Expanded view of the eQTLs in the region of the AMD-risk haplotype. The x-axis in this view is not linear. Black arrows point to the ten SNPs that have been reported in genome-wide association studies (GWASs) and genetic studies as associated with risk for AMD. Unfilled arrows are SNPs in strong linkage disequilibrium with the reported AMD-risk SNPs (linkage disequilibrium r^2 >0.8) and that therefore are highly likely to be associated with risk for AMD but have not been reported as such in GWASs, perhaps because they have not been included in those studies. Note that all AMD-risk SNPs are associated with high *HTRA1*, low *PLEKHA*1, and low *ARMS2* mRNA expression. Note also that there are 18 SNPs within the region of the AMD-risk haplotype in **B** and more than 100 that are away from it (shown in **A**) that affect the expression of *PLEKHA1* and *ARMS2* (denoted by SNPs with green or blue dots) but that have never been reported to influence risk for AMD. The location of the open chromatin region in the RPE with the highest DNase I sensitivity is indicated as a horizontal bar. Not shown is the ins/del polymorphism esv2663177 (del443/ins54) which is between rs3750846 and rs3793917.

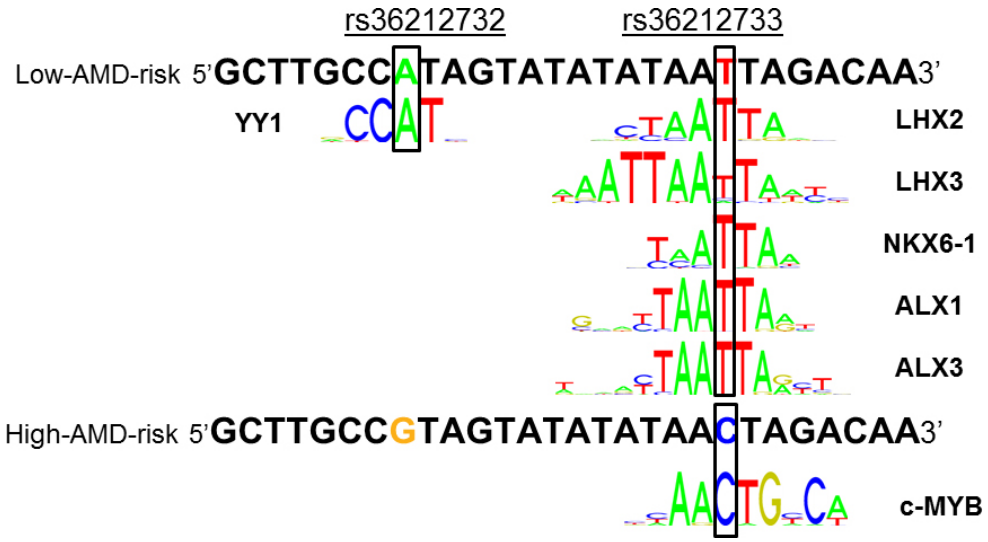

Figure 6. Changes in predicted transcription factor binding sites by SNPs associated with risk for AMD. The single nucleotide polymorphisms (SNPs) rs36212732 and rs36212733 are 8–21 bp away from the telomeric boundary of an open chromatin region with the high DNase I sensitivity in RPE cells. The low-AMD-risk haplotype has the base "A" at rs36212732 and the base "T" at rs36212733, while the high-AMD-risk allele has the bases "G" and "C" at the respective locations. In comparison with the low-AMD-risk haplotype, the high-AMD-risk haplotype has a predicted reduction in the binding of the transcription factor YY-1, LHX2, LHX3, NKX6–1, ALX1, and ALX3, and a predicted increase in the binding of the factor c-MYB, as predicted by the software HOMER, PROMO, and RegulomeDB. The consensus YY-1, LHX2, LHX3, NKX6–1, ALX1, ALX3, and c-MYB binding sequences are from the JASPAR website.

that influence *HTRA1* expression are associated with risk for AMD either from direct evidence from GWASs or because the SNPs are highly correlated with directly implicated SNPs. Two other items provide further support for disregarding *ARMS2* as an AMD-susceptibility gene: 1) *ARMS2* mRNA and protein are expressed at extremely low levels in eye tissues [9,18]; and 2) human genetics studies of AMD indicate that a specific SNP that creates a nonsense mutation (Arg38End) in *ARMS2* is associated with low risk for AMD [5,10,43-45]. In short, *HTRA1* is the most likely candidate gene for risk for AMD in this region, and if so, elevated expression of *HTRA1* likely increases risk for AMD.

The present analysis was based heavily on the idea that the level of expression of a gene on 10q26 determines risk for AMD. An alternative explanation is that there is a change in the primary structure of an encoded protein. Only one such polymorphism is known among the three candidates. It involves the reference SNP rs10490924, which is a missense polymorphism (Ala69Ser) that affects the *ARMS2* coding sequence. The Ala69 *ARMS2* allele corresponds to low risk for AMD and high *ARMS2* expression while the Ser69 allele corresponds to high risk for AMD and low *ARMS2* expression. Evidence against this polymorphism as the basis for risk for AMD is as follows. If expression of Ala69-*ARMS2* protects against AMD, one would expect that loss of *ARMS2* expression would always be associated with elevated risk for AMD. However, a separate *ARMS2* variant, the nonsense change Arg38End, would be expected to produce no functional *ARMS2*, yet the variant has been found to confer low risk for AMD. The possibility remains that the Ser69 allele

Table 6. DNase I hypersensitive clusters at chromosome 10q26.13 in HRPEpiC* and changes in transcription factor binding sites from low-AMD-risk reference alleles to high-AMD-risk alternate alleles.

Position	Genomic size	Signal	SNPs in the AMD haplotype	Transcription factor motif in reference allele	Transcription factor motif in alternate allele
chr10:124215021–124215190	170	163	rs36212731 rs36212732 rs36212733	YY1, LHX2, LHX3, NKX6–1, ALX1, ALX3	c-MYB
chr10:124220906–124222950	2045	12	rs1049331 rs2293870		
chr10:124228506–124228935	430	13			

* HRPEpiC: Human Retinal Pigment Epithelial Cells

promotes the development of AMD because of some toxic effect of the Ser69-*ARMS2* protein. This mechanism remains a possibility, but we feel it is unlikely because the Ser69 variant is expressed at low levels across all evaluated tissues. In particular, a recent report showed that the Ser69-*ARMS2* protein could not be detected in monocytes from patients carrying the homozygous risk for AMD rs10490924-T variant [46].

Support for *HTRA1* as the risk for AMD factor comes from reports of two- to threefold higher *HTRA1* expression in eyes with AMD [10,17,47-51], although other groups report no effect [28,45,52-55]. Some support for low *HTRA1* expression protecting against AMD comes from patients who lack *HTRA1* due to recessive, null mutations. Such patients have cerebral arteries with small lumens and thick walls with reduplicated elastic laminas. No AMD has been observed in such patients [56].

There are weaknesses in the present analysis. Although the GTEx database includes six tissues with a strong correlation between high-AMD-risk SNP alleles and higher *HTRA1* expression level and 21 others have a trend in the same direction, one tissue (prostate) showed a correlation in the opposite direction. It would be ideal to have ocular tissues for evaluation, but unfortunately, no data from ocular tissues are available in the GTEx database. It is still unclear to what extent systemic factors influence one's risk for AMD [57]. It is conceivable that variation in the systemic expression of *HTRA1*, not its local ocular expression, is responsible for increasing the risk for AMD.

A potential mechanism for the variation in the expression *HTRA1* due to the AMD-risk haplotype involves an open chromatin (DNase I-sensitive) region in the RPE that we found in the ENCODE database. This region is 8–21 bp away from the AMD-risk SNPs rs36212732 and rs36212733. Specifically, the change from the low-AMD-risk allele to the high-AMD-risk allele switches transcription factor binding from YY-1, LHX2, LHX3, ALX1, ALX3, or NKX6–1 to c-MYB (Figure 6).

A recently published evaluation of the *ARMS2-HTRA1* region provides additional evidence for the importance of the open chromatin region and the SNPs near it that potentially affect transcription factor binding sites [58]. Based on 33,000 AMD cases and controls, the interval most likely responsible for risk for AMD was narrowed down to a 7136-bp segment bounded by SNPs rs11200630 and esv2663177. This interval is within the AMD haplotype we defined and contains 13 of the 25 SNPs. This interval includes the open chromatin region we uncovered, as well as the SNPs rs36212732 and rs36212733 that affect transcription factor binding sites. It is conceivable that variation in these two SNPs is the fundamental cause for risk for AMD in 10q26 and that the changes in transcription factor affinity mediated by the SNP alleles cause the variation in the expression of the *HTRA1* gene located 5.8 kb away.

There are other possible mechanisms for the variation in the expression *HTRA1* or other genes due to the AMD-risk haplotype, three of which are the following. 1) AMD-risk SNPs, such as rs10490924, are in strong genetic linkage disequilibrium with the insertion/deletion polymorphism del443ins54 in the 3′ untranslated region of *ARMS* mRNA [29]. It is possible that the del443ins54 polymorphism introduces a conformational change in chromatin thus affecting the expression of genes in 10q26. However, the recently reported minimal region responsible for risk for AMD does not include this polymorphism, making it unlikely that it modulates risk for AMD [58]. 2) The reported pattern of DNA methylation in the promoter region of *ARMS2* correlates with the risk for AMD allele rs10490924-T [59]. However, the variation in methylation would more likely affect the expression of *ARMS2* rather than *HTRA1* or *PLEKHA1*. 3) It is possible that transcriptional activity of the *ARMS2* or *PLEKHA1* genes might interfere with transcription of the nearby *HTRA1*. Chimeric transcripts starting from *PLEKHA1* and ending in *ARMS2* were recently reported [5]. The reduction in *ARMS2* and *PLEKHA1* transcription by the high-AMD-risk variants might consequently allow more *HTRA1* mRNA to be transcribed. This indirect effect on *HTRA1* gene expression may explain why fewer tissues with increased *HTRA1* gene expression reached statistical significance compared to *ARMS2* in the GTEx database analysis.

APPENDIX 1. *PLEKHA1* MRNA LEVEL IS EXPRESSED IN ALL TISSUES EVALUATED IN THE GTEX PROJECT.

To access the data, click or select the words "Appendix 1."

APPENDIX 2. MAXIMUM EFFECT SIZES FOR *PLEKHA1*, *ARMS2*, AND *HTRA1* CIS-EQTLS.

To access the data, click or select the words "Appendix 2." In the column corresponding to each gene, the number in a box is the maximum eQTL effect seen across all tissues in the GTEx database. We excluded eQTLs that are not statistically significant (false discovery rate > .05). In the rare instances where one tissue has an effect in the opposite direction from the majority of tissues, only the maximum effect from the majority of tissues is included.

APPENDIX 3. *ARMS2* MRNA LEVEL IS HIGHEST IN TESTIS COMPARED TO OTHER TISSUES IN THE GTEX PROJECT.

To access the data, click or select the words "Appendix 3." Many tissues have expression levels below 1 RPKM which are difficult to distinguish from background noise and may indicate no substantive expression

APPENDIX 4. *HTRA1* MRNA LEVEL IS EXPRESSED IN ALL TISSUES EVALUATED IN THE GTEX PROJECT.

To access the data, click or select the words "Appendix 4."

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