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 (r²>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 x 10⁻⁴ 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 x 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 x 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:
### 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              | G/T            | 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 |
| rs10490931      | 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.
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-\text{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                                    | 0.64    | -0.052      | 28.95                  | 89             |
| Brain - Cerebellar Hemisphere                  | 0.35    | -0.053      | 14.493                 | 241            |
| Esophagus - Mucosa                             | 0.47    | -0.067      | 105.377                | 87             |
| Pituitary                                      | 0.14    | -0.072      | 53.302                 | 218            |
| Esophagus - Muscularis                         | 0.12    | -0.074      | 42.25                  | 278            |
| Thyroid                                        | 0.067   | -0.081      | 110.21                 | 185            |
| Adipose - Visceral (Omentum)                   | 0.31    | -0.086      | 74.374                 | 124            |
| Skin -Not Sun Exposed (Suprapubic)             | 0.05    | -0.088      | 126.917                | 298            |
| Colon - Sigmoid                                | 0.12    | -0.089      | 36.749                 | 169            |
| Adipose - Subcutaneous                         | 0.15    | -0.089      | 114.907                | 183            |
| Colon - Transverse                             | 0.15    | -0.089      | 114.907                | 183            |
| 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            |
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 telomic) 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 × 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 expected because of their strong linkage disequilibrium (Appendix 2). The effect was most striking in ovarian tissue (effect size −0.43, p = 3.8 × 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 ARMS2, there is an intergenic region of about 5 kilobases between the genes to the middle of the HTRA1 gene.

The high-AMD-risk haplotype is associated with low expression of the ARMS2 gene: 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

**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 rs10490924, with each having a correlation coefficient r² ≥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., r² = 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.

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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

| 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 - Muscularalis                          | 0.00063 | −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            |
To test whether the high-AMD-risk haplotype defined by rs10490924-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 × 10^{-7}) and coronary artery (p = 5.5 × 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 has a distant 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 interchromosome 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      | 100.21                 | 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            |
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

| GeneID       | Gene Symbol | P-Value  | Effect Size | Tissue               |
|--------------|-------------|----------|-------------|---------------------|
| ENSG00000254636.1 | ARMS2        | 7.50E-24 | -0.79       | Testis              |
| ENSG000000107679.10 | PLEKHA1      | 1.40E-08 | -0.25       | Nerve - Tibial      |
| ENSG000000166033.7 | 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 PLEKHA1, 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.
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 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

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**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 | YY1, LHX2, LHX3, NKX6–1, ALX1, ALX3 | c-MYB |
| 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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