Genetic and Functional Dissection of HTRA1 and LOC387715 in Age-Related Macular Degeneration

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Abstract

A common haplotype on 10q26 influences the risk of age-related macular degeneration (AMD) and encompasses two genes, LOC387715 and HTRA1. Recent data have suggested that loss of LOC387715, mediated by an insertion/deletion (in/del) that destabilizes its message, is causally related with the disorder. Here we show that loss of LOC387715 is insufficient to explain AMD susceptibility, since a nonsense mutation (R38X) in this gene that leads to loss of its message resides in a protective haplotype. At the same time, the common disease haplotype tagged by the in/del and rs11200638 has an effect on the transcriptional upregulation of the adjacent gene, HTRA1. These data implicate increased HTRA1 expression in the pathogenesis of AMD and highlight the importance of exploring multiple functional consequences of alleles in haplotypes that confer susceptibility to complex traits.

Introduction

Genome-wide association studies (GWAS) have catalyzed significant progress towards elucidating the molecular basis of complex traits [1]. However, a substantial gap remains between association of a trait with a genomic segment and the identification of the causative allele(s). A locus for AMD, ARMS2 on 10q26, illustrates this challenge.

ARMS2 is one of several regions in the genome shown by GWAS to confer susceptibility to the disorder. However, in contrast to the other confirmed AMD susceptibility loci (CFH (NM_000063) [2–8], C2/BF (NM_000063/NM_001710) and C3 (NM_000064) [9–11], which encompass a single gene; a region on 10q26 that contains three genes has been associated consistently with AMD susceptibility [12–15]. Recent data have refined this association to a haplotype that encompasses two genes, LOC387715 (NM_001099667), a primate-specific transcript with a proposed mitochondrial function [16] and HTRA1 (NM_002775), a multi-functional serine protease [17–18].

Initial reports describing the association between SNP rs11200638 in the 5’ end of HTRA1 and AMD focused on the possibility that the disease-associated allele of this SNP increased expression of HTRA1 [17–18]. However, the inability to verify this finding in heterologous expression systems led to the investigation of other alleles in the risk haplotype, and emphasis was placed on LOC387715 [16]. Consistent with this possibility, recent sequencing of the AMD-associated haplotype identified multiple SNPs, including an in/del, that are associated with an increased risk of AMD, and a decreased LOC387715 mRNA level with this AMD disease haplotype [19]. To delineate the causal genetic variations contained in the risk haplotype and to understand their functional roles in AMD susceptibility, we...
undertook genetic and functional investigations at the ARMS2 locus. We show that loss of LOC387715 is necessary, but not sufficient to explain AMD susceptibility and that a common disease haplotype including the in/del and rs11200638 also has an effect on the transcriptional upregulation of the adjacent gene, HTRA1. These data, which implicate increased HTRA1 expression in the pathogenesis of AMD, suggest that the AMD risk conferred by this region is potentially driven by multiple variants, and highlight the importance of exploring multiple functional consequences of alleles in haplotypes that confer susceptibility to complex traits.

### Results

Several studies have shown previously strong association of multiple single nucleotide polymorphisms (SNPs) in chromosome 10q26 encompassing PLEKHA1, LOC387715, and HTRA1 with advanced AMD [12–24]. From our previous study [20], we identified a common disease haplotype TAT tagged by rs10490924, rs11200638 and rs2293870 that is significantly associated with a risk of AMD ($P = 2.70 \times 10^{-6}$), as well as a haplotype GGG that is modestly, yet significantly, associated with protection from AMD ($P = 0.003$). Next, to define the extent of the haplotype structures and capture all variants in the region, we undertook a re-sequencing effort of a region spanning ~100 kb, starting in the 3'-UTR of PLEKHA and ending ~20kb downstream of the 3'-UTR of HTRA1 in six individuals with a homozygous risk haplotype and six individuals with a homozygous protective haplotype, followed by assessment of association of each discovered variant with AMD in 200 AMD cases and 200 normal controls (Table 1, Table S1, Table S2).

In agreement with recent work [19], we discovered that both risk and protective haplotypes span 21.5kb in the ARMS2 region, starting from upstream of LOC387715 to rs58077526, in intron 1 of HTRA1 (Figure 1, Table 1, Table S1). We confirmed recent data [19], according to which 22 SNPs tagged a risk and a protective haplotype including previously reported SNPs rs10490924, rs3750848, an in/del, rs3793917, and rs11200638 (Figure 1 and Table 1). Moreover, the reported 54 base pair insertion and 443 base pair deletion (in/del) at the 3' end of LOC387715 resides exclusively on the disease risk haplotype that is strongly associated with a risk of AMD ($P = 1.90 \times 10^{-20}$) in a Utah

### Table 1. Association study for SNPs in 10q26 region in 200 cases and 200 controls in the Utah cohort.

| SNP          | Chromosome Position | Risk Allele | Case MAF | Control MAF | Trend p-Value |
|--------------|---------------------|-------------|----------|-------------|---------------|
| SNP-2 (New SNP) | 124198539-124198544 | del 6bp     | 0.40     | 0.27        | 5.96\times10^{-5} |
| rs11200630   | 124199674           | C           | 0.39     | 0.25        | 8.95\times10^{-6} |
| ENS SNP 6019764 | 124200359           | A           | 0.39     | 0.21        | 5.86\times10^{-6} |
| rs10490924   | 124204438           | T           | 0.41     | 0.23        | 4.90\times10^{-7} |
| SNP-3 (New SNP) | 124204546-124204547 | ins GT      | 0.49     | 0.37        | 3.75\times10^{-5} |
| rs3621732    | 124204966           | T           | 0.30     | 0.20        | 2.47\times10^{-3} |
| rs3621732    | 124205188           | G           | 0.42     | 0.26        | 6.64\times10^{-6} |
| rs3621733    | 124205201           | C           | 0.41     | 0.25        | 4.85\times10^{-7} |
| rs3750848    | 124205305           | G           | 0.41     | 0.23        | 1.23\times10^{-6} |
| rs3750847    | 124205411           | T           | 0.35     | 0.24        | 6.43\times10^{-4} |
| rs3750846    | 124205555           | C           | 0.45     | 0.24        | 5.87\times10^{-3} |
| SNP-4 (New SNP) | 124206332-124206333 | ins AT      | 0.69     | 0.61        | 0.02 |
| in/del/Wt    | 124206811-124207253 | ins 54bp, del 443bp | 0.40 | 0.22 | 2.66\times10^{-7} |
| SNP-5 (New SNP) | 124207752-124207753 | ins G      | 0.48     | 0.33        | 5.81\times10^{-5} |
| rs3037985    | 124207893           | del 7bp     | 0.68     | 0.59        | 0.03 |
| rs3793917    | 124209265           | G           | 0.40     | 0.22        | 3.22\times10^{-6} |
| rs3763764    | 124210051           | C           | 0.41     | 0.21        | 2.38\times10^{-7} |
| rs3763764    | 124210051           | G           | 0.46     | 0.30        | 4.20\times10^{-6} |
| rs11200638   | 124210534           | A           | 0.41     | 0.22        | 2.19\times10^{-7} |
| rs1049331    | 124211260           | T           | 0.43     | 0.29        | 2.68\times10^{-3} |
| rs2293870    | 124211266           | T, C        | 0.53     | 0.32        | 5.56\times10^{-7} |
| rs58077526 or rs61871752 | 124220014 | C     | 0.38     | 0.21        | 2.17\times10^{-6} |

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case-control cohort (705 cases, 650 controls) (Table 2 and Table 3), as well as an independent replication cohort of northern European ancestry (442 cases, 434 controls) (Table 2 and Table 3) and a third replication cohort of Han Chinese in China (138 cases, 591 controls) (Table 2 and Table 3) [19].

In contrast to the loss of function role of in/del in LOC387715, the T allele of SNP rs2736911, a non-synonymous coding SNP leading to a predicted premature stop (R38X) in LOC387715 is associated with a protective haplotype T-G-Wt-G ($P = 4.00 \times 10^{-24}$, Figure 2 and Table 2 and Table 4) defined by rs2736911, rs10490924, in/del/Wt and rs11200638. We replicated these findings twice and found that this haplotype is clearly not associated with AMD risk; rather it is (conservatively) neutral and possibly protective with regard to the disorder ($P = 0.001$ in the second replication cohort; $P = 0.007$ in third Han Chinese cohort, Table 4).

These findings present a paradox. Recent studies have shown the in/del to cause destabilization of LOC387715, suggesting that loss of function at that locus might confer risk to AMD. However, the introduction of the R38X mutation, which is also predicted to give rise to loss of LOC387715 message due to nonsense-mediated mRNA decay (NMD), is mildly protective. We considered two plausible alternatives; either that effect of R38X and the in/del have different effects on the transcript, or that loss of LOC387715 is insufficient to cause AMD. The first possibility is unlikely. Upon quantification of LOC387715 mRNA levels in seven placentas homozygous for the major disease haplotype, we found a 4.7-fold reduction in endogenous LOC387715 expression (Figure 3A), in agreement with recent published data [19]. However, we were also able to examine LOC387715 mRNA levels in five patients heterozygous for the R38X mutation, where we observed a 50% reduction.

### Table 2. Association of rs2736911, rs10490924, in/del/Wt, and rs11200638 in the three independent cohorts.

| SNP          | Case-control cohort | Utah cohort 705 vs 650 Allelic $p$-value | Hopkins cohort 442 vs 434 Allelic $p$-value | Han Chinese cohort 138 vs 591 Allelic $p$-value |
|--------------|---------------------|----------------------------------------|------------------------------------------|-----------------------------------------------|
| rs2736911    | Heterozygote (95% CI) | $0.611(0.475–0.768)$                     | $0.655(0.471–0.909)$                     | $0.411(0.236–0.715)$                           |
|              | Homozygote (95% CI)  | $0.497(0.223–1.107)$                    | $0.254(0.052–1.232)$                    | $2.48(0.410–15.031)$                           |
| rs10490924   | Allelic $p$-value    | $8.61 \times 10^{-26}$                 | $4.87 \times 10^{-24}$                 | $1.15 \times 10^{-13}$                        |
|              | Heterozygote (95% CI) | $2.069(1.645–2.603)$                     | $3.175(2.333–4.321)$                     | $2.664(1.284–5.527)$                           |
|              | Homozygote (95% CI)  | $7.191(4.518–11.444)$                  | $10.311(6.474–16.422)$               | $8.986(4.335–16.625)$                          |
| in/del/Wt    | Allelic $p$-value    | $1.90 \times 10^{-26}$                 | $8.35 \times 10^{-24}$                 | $6.03 \times 10^{-13}$                        |
|              | Heterozygote (95% CI) | $2.305(1.827–2.908)$                    | $3.195(2.339–4.364)$                    | $2.395(1.189–4.825)$                           |
|              | Homozygote (95% CI)  | $6.879(4.436–10.666)$                  | $7.998(5.255–12.173)$               | $7.983(3.965–16.072)$                          |
| rs11200638   | Allelic $p$-value    | $3.64 \times 10^{-26}$                 | $2.52 \times 10^{-24}$                 | $5.10 \times 10^{-15}$                        |
|              | Heterozygote (95% CI) | $2.413(1.922–3.031)$                    | $3.315(2.429–4.523)$                    | $4.087(1.602–10.425)$                         |
|              | Homozygote (95% CI)  | $6.851(4.396–10.676)$                  | $10.246(6.550–16.026)$               | $12.932(5.083–32.904)$                         |

Figure 1. Genes and the main SNPs in the AMD 10q26 region. The schematic diagram showing a 100 kb region subject to re-sequencing, the AMD-associated 21.5 kb region containing 22 SNPs in the same LD block with a disease haplotype. SNPs in bold were chosen for further studies. doi:10.1371/journal.pgen.1000836.g001
reduction (Figure 3A), suggesting that each of the R38X and in/del have the same mode of action.

Given these data, we considered the potential role of HTRA1, the only other locus encompassed by the major risk haplotype, in the pathogenesis of AMD. We have shown previously a three-fold increase expression of HTRA1 in the retinal pigmented epithelium (RPE) of patients with the risk haplotype [17], while others have also observed increased HTRA1 expression in AMD eyes in humans [25]. In addition, a functional SNP in the promoter region of HTRA1 is associated with increased HTRA1 expression in non-human primates with AMD-like phenotype [26–27]. We therefore expanded our analyses of mRNA levels in human placenta to determine the effect of a haplotype on endogenous expression. We found that, in addition to the observed decrease of LOC387715 message, the disease haplotype is also associated with a 2.7-fold increase in HTRA1 expression (Figure 3B). These data confirmed that the in/del or another component of the common risk haplotype affects LOC387715 mRNA stability. At the same time, it likely has an effect on HTRA1 expression. Although caution should be exercised when translating findings from one tissue to

| Table 3. Genotyping results of rs2736911, rs10490924, in/del/Wt, and rs11200638 in the three independent cohorts. |
|---------------------------------------------------------------|
| SNP | Case-control cohort | Utah case-control cohort | Hopkins case-control cohort | Han Chinese case-control cohort |
|     | Groups             | Case | Control | Case | Control | Case | Control |
|-----------------|---------------------|------|---------|------|---------|------|---------|
| Number          | Protective Allele T Freq. | 0.12 | 0.18 | 0.09 | 0.14 | 0.07 | 0.12 |
| rs2736911       | HWE                 | 1.00 | 0.45 | 0.50 | 0.71 | 0.11 | 0.29 |
| rs10490924      | Risk Allele T Freq. | 0.38 | 0.20 | 0.25 | 0.24 | 0.74 | 0.49 |
| in/del/Wt       | HWE                 | 0.05 | 0.34 | 0.66 | 0.80 | 0.07 | 0.99 |
| rs11200638      | Risk Allele A Freq. | 0.41 | 0.22 | 0.53 | 0.25 | 0.77 | 0.52 |
|                 | HWE                 | 0.38 | 0.22 | 0.10 | 1.00 | 0.05 | 0.28 |

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Figure 2. The haplotype block in D’ of rs2736911, rs10490924, in/del/Wt, and rs11200638 in the Utah case-control cohort. The risk haplotype C-T-in/del-A is strongly associated with advanced AMD (P = 4.05 × 10^{-28}); the non-risk haplotype T-G-Wt-G is significantly associated with protection of advanced AMD (P = 4.00 × 10^{-24}). In/del and Wt refers to presence or absence of 54 base pair insertion and 443 base pair deletion, while the letters G, T, A refer to nucleotides for the respective SNPs at rs2736911, rs10490924, and rs11200638.

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another, these data from placenta should serve as an indication for the effects of different haplotypes on the gene expression profiles in the eye.

To test this hypothesis further, we generated expression constructs that model the effect of the risk and protective haplotype on the \textit{HTRA1} promoter using a luciferase reporter assay on cultured human retinal pigment epithelial cells (Figure 4A and 4B). We observed a two-fold increase in luciferase expression in constructs that modeled the disease haplotype encompassing the in/del and the A allele of SNP rs11200638 (MT\((L^{+}\text{in/del})\), Figure 4A and 4B). We detected no increase in luciferase expression from constructs that contained a short disease haplotype including the A risk allele of SNP rs11200638 but did not contain the in/del (MT\((S)\), Figure 4A and 4B). These findings are consistent with Fritsche et al. and Kanda et al [16,19], who reported an inability to verify that the A allele of SNP rs11200638 by itself alters transcriptional activity in heterologous cell systems. We did not observe increased luciferase expression either when we placed the in/del or A allele of SNP rs11200638 on a construct containing a protective haplotype, suggesting that the in/del or A allele of SNP rs11200638 by itself is insufficient to drive \textit{HTRA1} expression (WT\((L^{+}\text{in/del})\), WT\((L-A)\), Figure 4B).

Because of the potentially ambiguous nature of \textit{in vitro} luciferase assays, we repeated these experiments in an \textit{in vivo} system, whereby the constructs containing the disease haplotype including the in/del and the protective haplotype described earlier (Figure 4A) were electroporated into the RPE of wild-type adult mice. Luciferase activity was assayed four days after transfection. Consistent with

\begin{table}
\centering
\begin{tabular}{|l|c|c|c|c|}
\hline
Case-control cohort & Haplotype & Case Freq. & Control Freq. & Chi Square \(P\) Value \\
\hline
\textbf{Utah case-control cohort} & C-G-Wt-G & 0.450 & 0.590 & 50.455 \(1.22 \times 10^{-12}\) \\
& C-T-in/del-A & 0.340 & 0.160 & 120.883 \(4.05 \times 10^{-26}\) \\
& T-G-Wt-G & 0.110 & 0.150 & 12.723 \(4.00 \times 10^{-4}\) \\
& C-G-Wt-A & 0.028 & 0.024 & 0.559 & 0.455 \\
& C-T-in/del/G & 0.014 & 0.018 & 0.602 & 0.438 \\
& T-G-Wt-A & 0.009 & 0.019 & 4.621 & 0.032 \\
& C-T-Wt-A & 0.003 & 0.009 & 3.576 & 0.059 \\
\hline
\textbf{Hopkins case-control cohort} & C-G-Wt-G & 0.370 & 0.580 & 76.762 \(1.93 \times 10^{-18}\) \\
& C-T-in/del-A & 0.500 & 0.210 & 167.657 \(2.40 \times 10^{-38}\) \\
& T-G-Wt-G & 0.076 & 0.120 & 10.773 & 0.001 \\
& C-G-Wt-A & 0.017 & 0.022 & 0.708 & 0.400 \\
& C-T-Wt-G & 0.010 & 0.021 & 3.752 & 0.053 \\
& C-G-in/del/G & 0.006 & 0.018 & 5.261 & 0.022 \\
& T-T-in/del-A & 0.014 & 0.007 & 2.085 & 0.149 \\
\hline
\textbf{Han Chinese case-control cohort} & C-G-Wt-G & 0.170 & 0.330 & 27.937 \(1.25 \times 10^{-7}\) \\
& C-T-in/del-A & 0.660 & 0.450 & 62.000 \(3.43 \times 10^{-15}\) \\
& T-G-Wt-G & 0.037 & 0.084 & 7.376 & 0.007 \\
& C-G-in/del-A & 0.040 & 0.028 & 0.992 & 0.319 \\
& C-T-Wt-G & 0.023 & 0.032 & 0.620 & 0.431 \\
& C-G-Wt-A & 0.016 & 0.031 & 1.874 & 0.171 \\
& C-T-Wt-A & 0.015 & 0.028 & 1.502 & 0.220 \\
& T-T-in/del-A & 0.017 & 0.018 & 0.006 & 0.940 \\
& C-G-in/del/G & 0.009 & 0.014 & 0.482 & 0.488 \\
& C-G-in/del-G & 0.001 & 0.014 & 3.488 & 0.062 \\
\hline
\end{tabular}
\caption{Haplotype structures generated by the Hapview program using rs2736911, rs10490924, in/del/Wt, and rs11200638.}
\end{table}
In the in vitro data, we observed a significant increase in normalized luciferase activity in a construct bearing the disease haplotype tagged by the in/del and A allele of SNP rs11200638 (P = 0.032) (Figure 4C), whereas no increased luciferase activity was observed in a construct with the in/del on a protective haplotype (P = 0.180).

Discussion

The AMD-associated region on 10q26 poses an interesting problem. Multiple lines of evidence support the causality of both LOC387715 and HTRA1 in conferring risk to AMD. At the same time, emerging data suggests that mutations in either gene alone might be insufficient to confer such risk. The association signal is best explained by a common disease haplotype including an in/del in the 3' UTR of LOC387715 and the A allele of SNP rs11200638. Although there is evidence that AMD patients exhibit loss of LOC387715 message, the fact that haploinsufficiency in LOC387715 alone (through the R38X mutation) does not confer risk to AMD, suggests that either that transcript is unrelated to the disorder, or that additional events within the risk haplotype must occur. Although we cannot formally reject the hypothesis that loss of LOC387715 is irrelevant to the disease, the spatiotemporal expression pattern of this gene and its exclusive emergence with the evolution of the macula in non-human primates, provide partial evidence for its role in AMD pathogenesis [14,16,19,26]. At the same time, the evidence for the involvement of HTRA1 upregulation is likewise compelling, including i) the upregulation of the transcript in the major risk haplotype, and ii) the observed increased HTRA1 expression in AMD eyes.

One can outline reasonable hypotheses to explain each aspect of the discrepant data. Alternatively, a single hypothesis of dual causality might explain all the observations. Specifically, we speculate that concomitant downregulation of LOC387715 and upregulation of HTRA1 can explain the disease. This theoretical binary model is consistent with the fact that i) the common AMD-associated haplotype affects both transcripts and ii) the protective haplotype containing the R38X LOC387715 allele is associated with normal HTRA1 expression (Figure 3B). Moreover, we suggest that if changes in either gene alone were sufficient to confer AMD susceptibility, one might have expected to discover rare alleles that recapitulate the effect of the in/del [such as rare loss of function mutations in LOC387715 or activating mutations in HTRA1], neither of which have been discovered to date by us or by other groups. The primate-specific nature of LOC387715 renders this binary model intractable in model organisms. Nonetheless, this model may be tested in the monkey using genetic manipulations.
Materials and Methods

Patients
This study was approved by the Institutional Review Boards of the University of Utah, University of California San Diego, Johns Hopkins University, and Sichuan Provincial People’s Hospital. Subjects gave informed consent prior to participation. Participants underwent a standard examination, which included visual acuity measurements, dilated slit lamp biomicroscopy, and stereoscopic color fundus photography. Grading was carried out with the classification established by AREDS [28]. Diagnosis of advanced AMD was based on the presence of GA or CNV (equivalent to AREDS category 4 or 5). Control subjects were >60 years of age, with no signs of AMD, defined as no drusen or RPE abnormalities in the Utah collection, and controls in the Hopkins cohort and the Chinese cohort were defined as being >60 years old, having fewer than 5 small drusen (<63 μm), and no RPE abnormalities. Patient characteristics of the case-control series are listed in Table 5.

Genotyping
SNPs were genotyped by SNaPshot on an ABI 3100XL analyzer (ABI, Foster City, CA, USA) according to the manufacturer’s instructions. PCR and SNaPshot primers are listed in Table S2. All SNPs had a genotyping success rate >98% and accuracy >99% as judged by random re-sequencing of 20% of samples in all three case-control series.

Re-sequencing of haplotypes
To discover all variations in the AMD susceptibility locus at 10q26, we undertook a re-sequencing of 100kb in the region including genes PLEKH1, LOC387715 and HTRA1. We chose DNA samples from six individuals with a homozygous risk haplotype and six individuals with a homozygous protective haplotype for re-sequencing analysis. We designed PCR primer pair for amplification of 1kb amplicon; each amplicon had a 50bp overlap with next amplicons. The PCR amplicons were directly purified using a Qiagen kit (Qiagen, Valencia, CA, USA) and sequenced using forward and reverse primers using the BigDye Terminator v3.1 Cycle Sequencing Kit (ABI, Foster City, CA, USA) according to the manufacturer’s instructions. Then the sequencing results were annotated using the NCBI genomic DNA sequence information (http://www.ncbi.nlm.nih.gov, build 36).

Statistical analysis
All SNP genotyping results were screened for deviations from Hardy-Weinberg equilibrium with no SNP showing significant deviation (p>0.05). The chi-squared test for allelic trend for an SNP was performed with PEPI version 4.0 [29]. Odds ratios and 95% confidence intervals were calculated by conditional logistic regression with SPSS version 13.0. Linkage disequilibrium (LD) structure was examined with Haploview (version 4.0) [30]. Default settings were used, creating 95% confidence bounds on D’ to define pair-wise SNPs in strong LD [31]. Haploview was also used for allelic association tests.

Endogenous expression levels of HTRA1 and LOC387715
Total RNA was extracted from human placentas, and the first strand cDNA was generated by reverse transcript using reverse transcript kit (Invitrogen, Carlsbad, CA, USA). Real time PCR was performed for HTRA1 mRNA quantification using ABI human HTRA1 probe real time PCR kit (ABI, Foster city, CA, USA). RT-PCR was performed to qualify LOC387715 mRNA levels using primers 5’-atggcagctgcgtctccggt-3’ and 5’-tgcgagcctggtgtagagctgtag-3’ with ex taq polymerase (TaKaRa Bio USA; Mountain View, CA USA). G3PDH was used as the internal control. Human placenta tissues were harvested and genotyped. We measured RNA levels in seven individuals with a homozygous disease haplotype (C-T-in/del-A, Figure 3A and 3B), five individuals with a homozygous protective haplotype (C-G-Wt-G, Figure 3A and 3B), and five individuals with a homozygous non-risk haplotype with respect to in/del/Wt and a heterozygous CT allele at rs2736911 (C/T-G-Wt-G, Figure 3A and 3B). Significance was examined using SPSS’s independent sample t-test.

Heterologous Luciferase assays
A 442 bp DNA fragment containing the −1 to −4423 position from the HTRA1 translation start site including either the risk haplotype (MT/L-in/del), Figure 4A) or protective haplotype (WT/L-G, Figure 4A) was PCR amplified from genomic DNA of an individual with a homozygote risk haplotype or an individual with a protective haplotype using the following primers: forward: cggcgctcgccggctgctgggctgtgg; reverse: agatctcgagccggccgtgcctgccggctccg.

Table 5. Disease status, gender, and age of subjects in the study.

| Case-control cohort | Utah case-control cohort | Hopkins case-control cohort | Han Chinese case-control cohort |
|----------------------|--------------------------|-----------------------------|-------------------------------|
|                      | GA           | CNV       | Controls | GA         | CNV       | Controls | CNV     | Controls |
|----------------------|--------------|-----------|----------|-------------|-----------|----------|---------|----------|
| Sex-Number (%)       | Female       | 139 (60)  | 243 (50) | 400 (62)    | 80 (57)   | 163 (54) | 228 (53) | 66 (48)  | 272 (46) |
|                      | Male         | 93 (40)   | 230 (50) | 250 (38)    | 61 (43)   | 138 (46) | 206 (47) | 72 (52)  | 319 (54) |
| Average Age-years    | 84.09        | 82.85     | 75.30     | 82.51       | 79.51     | 78.10    | 68.10   | 66.50    |

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promoter driving luciferase reporter was obtained from Promega (Madison, WI, USA).

Human RPE cells were split into 24-well plates and cotransfected 24 hours later with 1ng of the transfection control Renilla luciferase plasmid pRL-Rl (Promega, Madison, WI) and 200ng of one of the following plasmids: pGL3-basic, pGL3-control, pGL3-A, WT-L-G, WT[L(Cin/del), MT[L(Cin/del), WT(S)] and MT(S). Transfections (n = 6, three preps for each construct and 2 transfections for each prep) were done using the FuGene-6 protocol according to manufacturer’s specifications (Roche Applied Science, Mannheim, Germany). Forty-eight hours after transfection, cells were washed with PBS twice and luciferase activities measured with the Dual-Luciferase Assay Kit (Promega, Madison, WI). Fold induction was derived relative to normalized activities measured with the Dual-Luciferase Assay Kit.

In vivo transfection by electroporation

In vivo transfection was done by subretinal injection of reporter plasmids followed by electroporation as described [32]. Briefly, C57BL/6 mice (4–5 weeks old) were given a subretinal injection of 1ul of PBS containing 0.5 ug of pGL3 containing one of the firefly luciferase constructs described above and 0.25 ng of Renilla luciferase plasmid pRL-Rl (Promega, Madison, WI) for normalization. After injection, two steel electrodes separated by about 3.0 mm were placed on the posterior sclera and an ECM830 electroporator (BTX, San Diego, CA, USA) was used to deliver eight 50 ms electric pulses separated by 100 ms with a transfection efficiency of about 3.0 mm.

Analysis of the CMV injection group to give the relative luciferase activity ratio. The firefly luciferase value was divided by the Renilla luciferase value to give the normalized luciferase activity. The normalized luciferase activity of each test plasmid injection group was divided by the normalized luciferase activity of empty-pGL3 and pRL-CMV injection group to give the relative luciferase activity ratio. Statistical analysis was done using ANOVA and Bonferroni/Dunn test.

Supporting Information

Table S1 SNPs identified by re-sequencing.

Table S2 Primers for genotyping of SNPs.

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Author Contributions

Conceived and designed the experiments: ZY NK KZ. Performed the experiments: ZY JT JZ FL XS CZ KW LD HN DM RS FK DW MD PJ RY DG XLI YL CL YL BC RC. Contributed reagents/materials/analysis tools: ZY RC DJZ PC YF NK KZ. Wrote the paper: ZY MB DYL NK KZ.

References

1. Manolio TA, Brooks LD, Collins FS (2008) A HapMap harvest of insights into the genetics of common disease. J Clin Invest 118: 1590–1605.
2. Zareparsi S, Br anlam KE, Li M, Shah S, Klein RJ, et al. (2005) Strong association of the Y402H variant in complement factor H at 1q32 with age-related macular degeneration. Am J Hum Genet 77: 149–153.
3. Klein RJ, Zeiss C, Chew EY, Tsai JY, Sackler RS, et al. (2005) Complement factor H polymorphism in age-related macular degeneration. Science 308: 385–389.
4. Haines JL, Hauser MA, Schmidt S, Scott WK, Olson LM, et al. (2005) Complement factor H variant increases the risk of age-related macular degeneration. Science 308: 419–421.
5. Hageman GS, Anderson DH, Johnson LV, Hancox LS, Taiber AJ, et al. (2005) Variation in complement factor 3 is associated with risk of age-related macular degeneration. Nat Genet 38: 1049–1054.
6. Edwards AO, Ritter R, 3rd, Abel KJ, Manning A, Paulhuyns C, et al. (2005) Complement factor H polymorphism and age-related macular degeneration. Science 308: 421–424.
7. Li M, Atmaca-Sommez P, Ohman M, Bramazon KE, Khandan R, et al. (2006) CFH haplotypes without the Y402H coding variant show strong association with susceptibility to age-related macular degeneration. Nat Genet 38: 1055–1059.
8. Muller J, George S, Purcell S, Faghernaj J, Alsulhary D, et al. (2006) Common variation in three genes, including a noncoding variant in CFH, strongly influences risk of age-related macular degeneration. Nat Genet 38: 1055–1059.
9. Gold B, Merriam JE, Zernant J, Hancox LS, Taiber AJ, et al. (2006) Variation in factor B (BF) and complement component 2 (C2) genes is associated with age-related macular degeneration. Nat Genet 38: 458–462.
10. Yates JR, Sepp T, Matharu BK, Khan JG, Thurby DA, et al. (2007) Complement C3 variant and the risk of age-related macular degeneration. N Engl J Med 357: 553–561.
11. Muller JB, Faghernaj JA, Reynolds RG, Neale BM, Daly MJ, et al. (2007) Variation in complement factor 3 is associated with risk of age-related macular degeneration. Nature 449: 1209–1210.
12. Fisher SA, Abecasis GR, Yashar BM, Zareparsi S, Svarazoo A, et al. (2005) Meta-analysis of genome scans of age-related macular degeneration. Hum Mol Genet 14: 2257–2264.
13. Jakobshaa J, Conley VP, Weeks DE, Mah TS, Ferrell RE, et al. (2005) Susceptibility genes for age-related maculopathy on chromosome 10q26. Am J Hum Genet 77: 389–407.
14. Rivera A, Fisher SA, Fritsche LG, Krillciana CN, Lichtner P, et al. (2005) Hypothetical LOC387715 is a second major susceptibility gene for age-related macular degeneration, contributing independently of complement factor H to disease risk. Hum Mol Genet 14: 3227–3236.
15. Schmidt S, Hauser MA, Scott WK, Postol EA, Agarwal A, et al. (2006) Cigarette smoking strongly modifies the association of LOC387715 and age-related macular degeneration. Am J Hum Genet 78: 852–864.
16. Kandaa A, Chen W, Ohtman M, Branson KE, Brooks M, et al. (2007) A variant of mitochondrial protein LOC387715/ARMS2, not HTRA1, is strongly associated with age-related macular degeneration. PLoS Genet 3: 1002199.
17. Yang Z, Camp NJ, Sun H, Tong Z, Gibbs D, et al. (2006) A variant of the HTRA1 gene increases susceptibility to age-related macular degeneration. Science 314: 992–993.
18. Dewan A, Liu M, Hartman S, Zhang SS, Lin DT, et al. (2006) HTRA1 promoter polymorphism in wet age-related macular degeneration. Science 314: 989–992.
19. Fritsche LG, Loenhart T, Jansen A, Fisher SA, Rivera A, et al. (2008) Age-related macular degeneration is associated with an unstable ARMS2 (LOC387715) mRNA. Nat Genet 40: 892–896.
20. Gibbs D, Yang Z, Constantine R, Ma X, Camp NJ, et al. (2008) Further mapping of 10q26 supports strong association of HTRA1 polymorphisms with age-related macular degeneration. Vision Res 48: 683–689.
21. Jakobshaa J, Conley VP, Weeks DE, Ferrell RE, Gorin MB (2008) C2 and CFB genes in age-related maculopathy and joint action with CFH and LOC387715 genes. PLoS ONE 3: e1013. doi:10.1371/journal.pone.0002199.
22. Deangelis MM, Jr, F Adams S, Morrison MA, Harring AJ, et al. (2008) Alleles in the Htra serine peptidase 1 gene alter the risk of neovascular age-related macular degeneration. Ophthalmology 115: 1209–1215 e1207.
23. Hughes AE, Orr N, Patterson C, Esfandiary H, Hogg R, et al. (2007) Neovascular age-related macular degeneration. Science 314: 992–993.
24. Fritsche LG, Loenhart T, Jansen A, Fisher SA, Rivera A, et al. (2005) Hypothetical LOC387715 is a second major susceptibility gene for age-related macular degeneration, contributing independently of complement factor H to disease risk. Hum Mol Genet 14: 3227–3236.
25. Schmidt S, Hauser MA, Scott WK, Postol EA, Agarwal A, et al. (2006) Cigarette smoking strongly modifies the association of LOC387715 and age-related macular degeneration. Am J Hum Genet 78: 852–864.
25. Chan CC, Shen D, Zhou M, Ross RJ, Ding X, et al. (2007) Human HtrA1 in the archived eyes with age-related macular degeneration. Trans Am Ophthalmol Soc 105: 92–97; discussion 97–98.

26. Francis PJ, Appukuttan B, Simmons E, Landauer N, Stoddard J, et al. (2008) Rhesus monkeys and humans share common susceptibility genes for age-related macular disease. Hum Mol Genet 17: 2673–2680.

27. Singh KK, Krawczak M, Dawson WW, Schmidke J (2009) Association of HTRA1 and ARMS2 gene variation with drusen formation in rhesus macaques. Exp Eye Res 88: 479–482.

28. (1999) The Age-Related Eye Disease Study (AREDS): design implications. AREDS report no. 1. Control Clin Trials 20: 573–600.

29. Abramson JH, Gahlinger PM (2001) PEPI, ver. 4.0: Computer Programs for Epidemiologists. Salt Lake City: Sagebrush Press.

30. Barrett JC, Fry B, Maller J, Daly MJ (2005) Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 21: 263–265.

31. Gabriel SB, Schaffner SF, Nguyen H, Moore JM, Roy J, et al. (2002) The structure of haplotype blocks in the human genome. Science 296: 2225–2229.

32. Kachi S, Oshima Y, Esumi N, Kachi M, Rogers B, et al. (2005) Nonviral ocular gene transfer. Gene Ther 12: 843–851.