# Toll-like Receptor Polymorphisms and Age-Related Macular Degeneration

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**Purpose.** Evidence from genetic-association studies in conjunction with the demonstration of complement deposition in the retina and choroid implicates noncellular pathways of innate immunity in the pathogenesis of age-related macular degeneration (AMD). The purpose of this study was to determine whether common variation in the 10 human toll-like receptors (TLRs) alters the risk of AMD.

**Methods.** Sixty-eight SNPs were iteratively genotyped across the TLR genes in a cohort of 577 subjects, with and without AMD. Two additional cohorts were used for replication studies. Standard genetic-association methods were used to analyze the results for association with disease and interaction with other loci.

**Results.** Coding SNPs in *TLR3* (rs3775291) and *TLR7* (rs179008) showed association with AMD in one group ($P = 0.01$ and $P = 0.02$, respectively) before correction for multiple testing. For both SNPs, the association with AMD arose due to an excess of heterozygotes compared with homozygotes for the major allele. The two coding SNPs were not associated with AMD in another case–control cohort or an extended-family cohort. Although an intronic SNP in *TLR4* was associated marginally with AMD ($P = 0.03$), it was not possible to replicate a previous association with the rare coding SNP D299G in this gene ($P = 0.6$).

**Conclusions.** Although borderline support for association between polymorphisms in TLR genes and AMD was reported for some cohorts, these initial observations of coding SNPs in *TLR3*, *TLR4*, and *TLR7* were not replicated. TLR variants are unlikely to have a major impact on overall AMD risk, and the common variants studied were not associated with AMD. (*Invest Ophthalmol Vis Sci.* 2008;49:1652–1659) DOI:10.1167/iovs.07-1378

Age-related macular degeneration (AMD) is a leading cause of vision loss in elderly individuals.[1] Although several polymorphisms have been reported to increase the risk of AMD, sequence variants within the genes encoding proteins of the alternative pathway of complement and a region on chromosome 10q26, spanning a hypothetical gene (*LOC387715*) and the promoter of a gene encoding a serine protease (*HTRA*1), have been consistently replicated.[2–12] More recently, the association between a coding variant in complement factor *C3* and AMD noted in a genome-wide scan of 600 subjects from the Age-Related Eye Disease Study (AREDS; http://www.ncbi.nlm.nih.gov/sites/entrez?db=gap) was replicated.[13] Six of the reported loci—*CFH*, *BF/C2*, *C3*, the human leukocyte antigen (HLA) locus, and Toll-like receptor 4 (*TLR4*)— encode proteins of innate immunity pathways that enable recognition of nonself, which is essential in mounting an immune response and tolerance.[14,15]

Toll-like receptors (TLRs) are pattern-recognition receptors that detect and bind pattern-associated molecular patterns such as lipopolysaccharide (*TLR4*), double-strand (ds)RNA (*TLR3*), and single-strand (ss)RNA (*TLR7*).[16–20] Further, *TLR4* has been implicated in transmembrane signaling in response to photoreceptor outer segment shedding.[21] To explore further the role of innate immunity in AMD pathogenesis, we surveyed all 10 human TLRs for association with AMD by evaluating both nonsynonymous SNP (nsSNPs) and gene-based SNPs designed to cover the genomic region of each gene.

## Methods

### Subjects

The research adhered to the Declaration of Helsinki and was approved by the institutional review boards of each institution. Three previously reported Caucasian cohorts were studied. The initial studies were performed in the Dallas cohort consisting of 577 research subjects (396 AMD cases and 181 non-AMD controls) ascertained by Edwards et al.,[2,22,23] by methods previously reported. Briefly, the Dallas cohort subjects represent highly affected cases (large drusen and sufficient total drusen area to fill a 700-$\mu$m circle or late AMD) and very normal control subjects with five or fewer hard drusen. AMD grading was performed on fundus photographs. Additional information from clinical examinations and medical records was used to supplement the

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**Table 1.** Allele and Genotype Association between AMD and the Initial 10 nsSNPs Genotyped across the 10 Human Loci Encoding TLRs in the Dallas Cohort

| | | | Allele Association | | | | Genotype Association | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Gene** | **SNP** | **Location** | **Allele** | **AMD** | **Control** | ***P*** | **Genotype** | **AMD** | **Control** | ***P*** |
| *TLR1* | rs4833095 | 38622276 | C | 189 | 70 | 0.18 | CC | 22 | 6 | 0.37 |
| | Ser248Asn | 4p14 | T | 535 | 246 | | CT | 145 | 58 | |
| | | | | | | | TT | 195 | 94 | |
| *TLR3* | rs3775291 | 187379223 | C | 527 | 240 | 0.02 | CC | 171 | 92 | 0.01 |
| | Leu412Phe | 4q35 | T | 247 | 78 | | CT | 185 | 56 | |
| | | | | | | | TT | 31 | 11 | |
| *TLR4* | rs4986790 | 117554856 | G | 53 | 17 | 0.35 | GG | 1 | 0 | 0.63 |
| | Asp299Gly | 9q33.1 | A | 721 | 303 | | GA | 51 | 17 | |
| | | | | | | | AA | 335 | 143 | |
| *TLR4* | rs4986791 | 117555156 | T | 56 | 18 | 0.35 | TT | 2 | 0 | 0.74 |
| | Thr359Ile | 9q33.1 | C | 716 | 298 | | TC | 52 | 18 | |
| | | | | | | | CC | 332 | 140 | |
| *TLR5* | rs5744174 | 219656307 | C | 289 | 114 | 0.57 | CC | 28 | 4 | 0.08 |
| | Phe616Leu | 1q41-42 | T | 483 | 206 | | CT | 233 | 106 | |
| | | | | | | | TT | 125 | 50 | |
| *TLR5* | rs2072493 | 219656378 | G | 101 | 38 | 0.59 | GG | 5 | 3 | 0.56 |
| | Asn592Ser | 1q41-42 | A | 671 | 282 | | GA | 91 | 32 | |
| | | | | | | | AA | 290 | 125 | |
| *TLR5* | rs5744168 | 219656979 | T | 38 | 12 | 0.41 | TT | 1 | 0 | 0.73 |
| | Arg392Ter | 1q41-42 | C | 738 | 308 | | TC | 36 | 12 | |
| | | | | | | | CC | 351 | 148 | |
| *TLR6* | rs5743810 | 38652916 | C | 458 | 183 | 0.54 | CC | 142 | 50 | 0.33 |
| | Ser249Pro | 4p14 | T | 316 | 137 | | CT | 174 | 83 | |
| | | | | | | | TT | 71 | 27 | |
| *TLR7* | rs179008 | 12663316 | T | 113 | 25 | 0.007 | TT | 16 | 4 | 0.02 |
| | Gln11Leu | Xp22.3 | A | 383 | 161 | | TA | 81 | 17 | |
| | | | | | | | AA | 151 | 72 | |
| *TLR10* | rs11096957 | 38599057 | C | 281 | 103 | 0.19 | CC | 47 | 16 | 0.40 |
| | Asn241His | 4p14 | A | 493 | 217 | | CA | 187 | 71 | |
| | | | | | | | AA | 153 | 73 | |

The data from the X-linked SNPs (TLR7) are presented for women only, to enable calculation of genotypic association.

fundus grading. Control subjects did not have a family history of AMD. Subjects with eye diseases or treatments that might be confused with or obscure the diagnosis of AMD were excluded. The combined population of 396 cases consisted of 47% early, 16% pure geographic atrophy, and 37% exudative AMD subtypes. The post hoc power to detect genotypic association, determined by a log-additive genetic model with an odds ratio (OR) of 1.5, 2.0, and 3.0 for rs3775291 was 0.36, 0.96, and 0.99, respectively. The power for other SNPs was similar, with variations dependent on the minor allele frequency. The age, gender, and AMD subtype distributions are provided in Supplementary Table S1, online at http://www.iovs.org/cgi/content/full/49/4/1652/DC1. All 68 SNPs across the TLR loci were genotyped on this Dallas cohort.

To determine whether the initial observations in the Dallas cohort could be replicated, we genotyped the nonsynonymous single-nucleotide polymorphisms (nsSNPs) associated with AMD in *TLR3* (rs3775291) and *TLR7* (rs179008) in two additional cohorts. The Michigan cohort of 934 subjects consisted of 611 AMD cases (95 early, 138 pure geographic atrophy, and 378 exudative) and 323 control subjects without AMD ascertained according to criteria similar to those used for the Dallas cohort, as previously described.[11] The FARMS cohort consisted of 297 individuals in 34 extended families between 20 and 90 (average, 62.8) years of age. The subjects were recruited from the University of Wisconsin, and fundus photographs were graded with the quantitative scale incorporated into the Wisconsin Age-Related Maculopathy Grading Scheme, as previously reported.[24] The study sample comprised the 34 probands with advanced AMD, the average family size was 12 persons, and the average AMD score for affected individuals was 13.[25] In summary, the Dallas case–control cohort was used for the initial discovery studies and the Michigan case–control and FARMS family-based cohorts were used for replication studies of findings observed in the Dallas cohort.

## Genotyping

The SNPs used in this study first were selected based on their changing the predicted protein sequence, having a minor allele frequency (MAF) of 0.10 or greater, or having been reported to be associated with AMD. The primary experiment was the genotyping of the 10 nsSNPs identified by these criteria (Table 1). Additional SNPs were subsequently selected based on their expected coverage of linkage disequilibrium blocks spanning the 10 genes encoding Toll-like receptor (TLR) genes or proximity to SNPs already observed to be associated with AMD in this study.

Genotyping on the Dallas cohort was performed in the Eugene McDermott Center for Human Growth and Development Genotyping Core Facility (UT Southwestern Medical Center, Dallas, TX) and the Mayo Clinic with genotyping assays (*Taq*Man; Applied Biosystems, Inc. [ABI], Foster City, CA). Selected SNPs associated with AMD were further genotyped (MassArray platform by Sequenom; San Diego, CA) to confirm the results of the ABI assay. The two platforms gave similar results. Detailed methods have been published.[2] Genotyping on the Michigan and FARMS cohorts was performed according to previously described methods.[11,24]

## DNA Sequencing

Resequencing of the *TLR7* and *TLR3* genes was performed on 16 subjects using dye termination chemistry (Big Dye Terminator with the model 3730xl sequencer; ABI). Regions of interest (TLR7, chrX: 12793123-12820401; TLR3, chr4:187225087-187245235) were amplified from genomic DNA (*Taq*Gold polymerase; ABI). PCR products were cleaned (Exosap-It enzyme; USB Corp., Cleveland, OH), to remove the unused primers and nucleotides. The cleaned products were mixed with 20 picomoles of the forward or reverse PCR primers for

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**Table 2.** Association between AMD and the Leu412Phe Polymorphism (rs3775291) in *TLR3* and the Gln11Leu Polymorphism (rs179008) in the X-linked Gene Encoding *TLR7* in the Dallas Cohort

| Gene | | Allele Counts and Distribution* | | Allele Association | | Genotype Counts and Distribution | | Genotype Association |
|---|---|---|---|---|---|---|---|---|
| | | AMD | Control | P | | All Cases | Control | P |
| *TLR3* | C | 527 (0.681) | 240 (0.755) | 0.02 | CC | 171 (0.442) | 92 (0.579) | 0.01 |
| | T | 247 (0.319) | 78 (0.245) | | CT | 185 (0.478) | 56 (0.352) | |
| | | | | | TT | 31 (0.080) | 11 (0.069) | |
| *TLR7* | A | 383 (0.772) | 161 (0.866) | 0.007 | AA | 151 (0.609) | 72 (0.774) | 0.02 |
| | T | 113 (0.228) | 25 (0.134) | | AT | 81 (0.327) | 17 (0.183) | |
| | | | | | TT | 16 (0.065) | 4 (0.043) | |

Counts and distribution (frequency) are shown for the alleles and genotypes observed.

* The C and T alleles code for Leu and Phe, respectively, in TLR3, whereas the A and T alleles code for Gln and Leu, respectively, in *TLR7.*

Sanger sequencing. Contiguous DNA sequences were compiled for each person, and the sequence variants identified (Mutation Surveyor ver 2.41; SoftGenetics, State College, PA, and Sequencher, ver 4.5, Build 1415; Gene Codes Corp., Ann Arbor, MI).

## Statistical Analyses

**Descriptive Testing and Genetic-Association Analyses.** Allele and genotype association and Hardy-Weinberg equilibrium (HWE) were performed with $\chi^2$ analyses. Analysis of genotypes showed that all SNPs were in HWE, with the possible exception of rs179008 ($P = 0.04$). The genotypes for rs179008 were confirmed (MassArray; Sequenom). Because allelic tests are dependent on the assumption of HWE (unlike genotypic tests that are not dependent on HWE), we primarily focused on genotype frequencies by using $\chi^2$ analyses. To determine whether SNPs associated with AMD are preferentially associated with a subtype of AMD (e.g., nonexudative versus exudative), $\chi^2$ analyses of differences between the two groups of cases were performed. The Fisher exact test was performed when genotype or allele cell counts were $<5$.

**Permutation Probabilities for Genotypic Association.** To evaluate the significance of the genetic association with AMD for the nsSNPs in both *TLR3* and *TLR7*, after adjustment for multiple testing of the 10 nsSNPs, permutation analysis was performed with 1000 simulations. For a given statistical test, the phenotype was permutated 1000 times. The probability from each statistical hypothesis was estimated and saved for each of the SNPs in the dataset. By examining the distribution of the minimum probability, the second smallest, the third smallest, and so on, from the 1000 permutations, we can estimate the overall experiment-wise probability for a particular hypothesis based on whether they have the best observed probability, the second best, and so forth—that is, if of the 1000 permutations, only 3 resulted in a probability smaller than our observed smallest probability, the experiment-wise probability would be estimated to be 3 in 1000 or 0.003.

**Linkage Disequilibrium.** Linkage disequilibrium was assessed using both the $D'$ and $r^2$ measures implemented in the Haploview software package and locally written S-plus functions.[26]

**Haplotype Analyses.** To evaluate the association of haplotypes with the presence or absence of AMD, a global test of association was used. Since haplotypes are not observed directly, we accounted for their unknown phase with the score statistics implemented in the HaploStats software package.[27] Estimation of odds-ratios for haplotype effects were estimated with a generalized linear model, in which the covariate matrix contained the subject-specific haplotype posterior probabilities estimated from the EM-algorithm under an additive haplotype model using HaploStats. The test for haplotype association conditional on the nsSNP was based on a score test with empiric/simulated probability, in which the nsSNP was included as a covariate in the model.

**Genetic Model Analysis.** Analyses to determine the genetic model were performed using the Chaplin software package (ver. 1.2.2).[28,29]

**Meta-analysis.** A meta-analysis was performed using the probability generated on the Dallas, Michigan, and FARMS cohorts. Only women were used for X-linked SNPs. The test statistic was

$$\chi^2_{2k} = -2 \sum_{i=1}^{k} \ln(P\text{-value}_i),$$

which follows a $\chi^2$ distribution with degrees of freedom equal to 2k under the null hypothesis. $P$-value$_i$ is the $P$-value for the $i$th study.

**Conditional SNP Analyses.** To determine whether other SNPs in *TLR3* or *TLR7* would remain significantly associated with AMD after conditioning on the nsSNPs in each gene, conditional analyses were performed by using a sequential test of deviance for a logistic regression model. In fitting the model, the genotypes were coded as 0, 1, or 2 for the number of rare alleles.

**Modeling of Covariates and Assessing Interactions.** To adjust for traditional risk factors of AMD—namely, age, gender, and smoking status, coded as never smoked or ever smoked—we performed logistic regressions. The regression models included these risk factors in addition to the nsSNP genotypes coded as 0, 1, or 2 minor alleles. To assess SNP–SNP interactions and SNP–environmental interactions, generalized linear models that included terms for the two main effects (genotype and/or environmental effect) and a term for the interaction effect (SNP by environmental or SNP by SNP) were developed. Tests for significant interaction effects were completed by using a sequential analysis of deviance with a $\chi^2$ test. All models were fit by using S-plus.

# Results

## Genotyping of 10 nsSNPs across the TLR Genes

Initially, 10 nsSNPs changing the coding sequence of TLRs were genotyped in the Dallas cohort (Table 1). We were unable to replicate the previously reported association between the *TLR4* polymorphism D299G (rs4986790) and AMD in the Dallas cohort, possibly because of the low frequency of the minor allele in both cases (0.07) and controls (0.05).[11] However, nsSNPs in both *TLR3* and *TLR7* were associated with AMD (Table 2). The polymorphism Leu412Phe in *TLR3* was associated with AMD (allele, $P = 0.02$; genotype, $P = 0.01$; genotype permutation, $P = 0.11$), as was the polymorphism Gln11Leu in *TLR7* (allele, $P = 0.007$; genotype, $P = 0.02$; genotype permutation, $P = 0.13$). There was no significant difference in genotype frequencies between nonexudative and exudative cases for either SNP ($P > 0.12$ for both SNPs). These findings suggested that alterations in activation of TLR-dependent pathways by exogenous (viral) or endogenous RNA ligands might play a role in the development of AMD, but were not statistically significant after correction for multiple testing.

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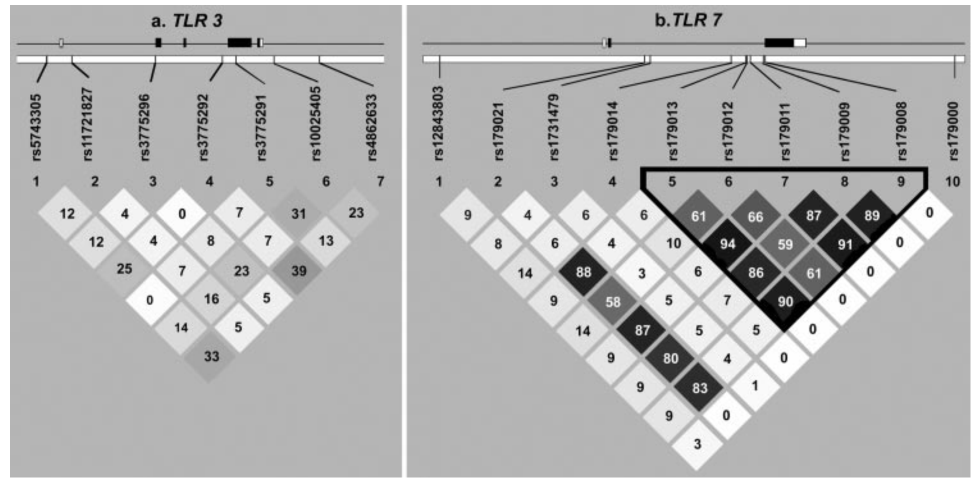



**FIGURE 1.** Linkage disequilibrium across TLR 3 (**A**) and TLR7 (**B**). *Top graphic*: location of noncoding (*white boxes*) and coding (*black boxes*) relative to the position of the SNPs along the genomic sequence (*middle graphic*). *Bottom graphic*: estimate of linkage disequilibrium ($r^2$) between the SNPs. Because TLR7 is X-linked, only female subjects were used for this locus.

## Genotyping of 58 Gene-Based SNPs across the 10 Human TLR Genes

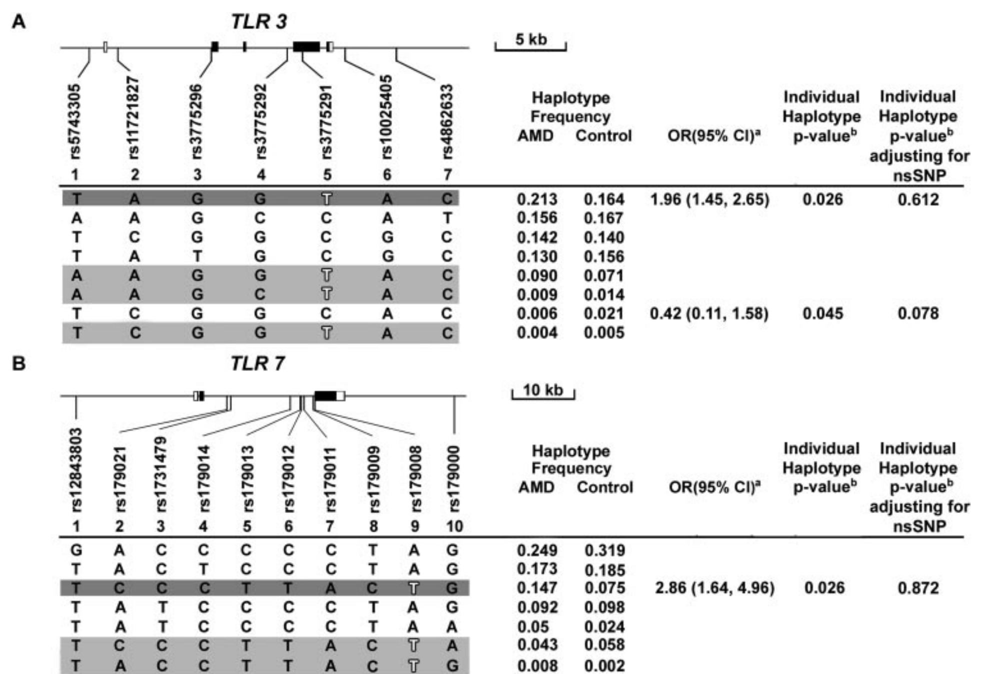
Given these initial findings, synonymous coding and intronic gene-based SNPs were genotyped to explore further the association between these genes and AMD. The gene-based SNPs were selected across each *TLR* locus based on minor allele frequency and the linkage disequilibrium structure of the locus to ensure coverage of each gene.[2] The allele and genotype counts for the additional 58 SNPs are presented in Supplementary Table S2, http://www.iovs.org/cgi/content/full/49/4/1652/DC1.

Two observations were made. First, a trend for association between the intronic SNP rs1927911 of *TLR4* (allele, $P = 0.10$; genotype, $P = 0.03$) and AMD was observed providing further support to the previous report of association between polymorphism in this gene and AMD (Supplementary Table S2).[11] Second, significant association between AMD and five additional noncoding SNPs in *TLR7* was observed in the Dallas cohort (Supplementary Table S2). This result suggests the presence of linkage disequilibrium between the TLR7 markers associated with AMD.

## Detailed Analysis of *TLR3* and *TLR7* Genotypes in the Dallas Cohort

To define the region of association and determine whether haplotypes in *TLR3* and *TLR7* contribute to the association with AMD, we evaluated linkage disequilibrium blocks and estimated haplotypes across both genes (Figs. 1, 2). There was modest linkage disequilibrium across *TLR3,* with no well-defined blocks by either $D'$ or $r^2$ criteria (Fig. 1). A global haplotype effect for all 7 SNPs across TLR3 was not observed ($P = 0.34$), although selected haplotypes were associated with AMD individually (Fig. 2). The association was no longer significant after conditioning on the nsSNP (Fig. 2).

Because *TLR7* is located on the X-chromosome, similar studies were performed separately on the women only. There was a high level of linkage disequilibrium between the 10 SNPs in *TLR7*, when using both the $D'$ and $r^2$ metrics, especially between the nsSNP rs179008 and four adjacent SNPs (Fig. 1). Four of these five SNPs in the block of highest linkage disequilibrium were associated with AMD, a region spanning 2.2 kb on the X-chromosome. There was no global haplotype effect



A TLR 3 (5 kb)

| 1 rs5743305 | 2 rs11721827 | 3 rs3775296 | 4 rs3775292 | 5 rs3775291 | 6 rs10025405 | 7 rs4862633 | Haplotype Frequency AMD | Haplotype Frequency Control | OR(95% CI)[a] | Individual Haplotype p-value[b] | Individual Haplotype p-value[b] adjusting for nsSNP |
|---|---|---|---|---|---|---|---|---|---|---|---|
| T | A | G | G | T | A | C | 0.213 | 0.164 | 1.96 (1.45, 2.65) | 0.026 | 0.612 |
| A | A | G | C | C | A | T | 0.156 | 0.167 | | | |
| T | C | G | G | C | G | C | 0.142 | 0.140 | | | |
| T | A | T | G | C | G | C | 0.130 | 0.156 | | | |
| A | A | G | G | T | A | C | 0.090 | 0.071 | | | |
| A | A | G | C | T | A | C | 0.009 | 0.014 | | | |
| T | C | G | G | C | A | C | 0.006 | 0.021 | 0.42 (0.11, 1.58) | 0.045 | 0.078 |
| T | C | G | G | T | A | C | 0.004 | 0.005 | | | |

B TLR 7 (10 kb)

| 1 rs12843803 | 2 rs179021 | 3 rs1731479 | 4 rs179014 | 5 rs179013 | 6 rs179012 | 7 rs179011 | 8 rs179009 | 9 rs179008 | 10 rs179000 | Haplotype Frequency AMD | Haplotype Frequency Control | OR(95% CI)[a] | Individual Haplotype p-value[b] | Individual Haplotype p-value[b] adjusting for nsSNP |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| G | A | C | C | C | C | C | T | A | G | 0.249 | 0.319 | | | |
| T | A | C | T | C | C | C | T | A | G | 0.173 | 0.185 | | | |
| T | C | C | C | T | T | A | C | T | G | 0.147 | 0.075 | 2.86 (1.64, 4.96) | 0.026 | 0.872 |
| T | A | T | C | C | C | C | T | A | G | 0.092 | 0.098 | | | |
| T | A | T | C | C | C | C | T | A | A | 0.05 | 0.024 | | | |
| T | C | C | C | T | T | A | C | T | A | 0.043 | 0.058 | | | |
| T | A | C | C | T | T | A | C | T | G | 0.008 | 0.002 | | | |

**FIGURE 2.** Analysis of estimated haplotypes at the (**A**) *TLR3* and (**B**) *TLR7* loci. The frequency of the five most common haplotypes and all haplotypes with the risk allele are shown. The odds ratio and significance testing, independently and conditional on the nsSNP in each gene, are shown for haplotypes with significant individual probability. Shaded haplotypes carry the risk allele (T both rs3775291 and rs179008) associated with AMD. Global tests for haplotype association with disease were not significant for either locus. Estimates of odds ratios are based on the generalized linear model, which in turn is based on the posterior probability of haplotypes in an additive genetic model. Test for haplotype association based on a score with empiric or simulated probability.

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when using haplotypes estimated from either all 10 SNPs ($P$ = 0.37) or a sliding window of 4 SNPs. These results demonstrated that individual haplotypes do not contribute to the association with AMD. The statistically significant association between four of the five SNPs in the block of high linkage disequilibrium and AMD supports the validity of the initial observation with the nsSNP rs179008 (Fig. 1).

The Akaike information criteria (AIC) for the recessive model were 997.1 for *TLR3* and 547.0 for *TLR7*. The AIC for the other three models (dominant, multiplicative, and general) was between 987.4 and 991.6 for *TLR3* and 539.2 and 541.1 for *TLR7*. The analysis suggested that the recessive model could be excluded, but we could not clearly distinguish between the other genetic models. These results support the use of log-additive modeling.[28,29] After conditioning on the nsSNPs in both *TLR3* (rs3775291) and *TLR7* (rs179008), no other SNP was significantly associated with AMD in either gene. These results demonstrate that the nsSNPs in both *TLR3* and *TLR7* explain the observed association with AMD.

The genotype and haplotype analyses were repeated for the nsSNPs in *TLR3* and *TLR7*, including covariates known to alter the risk of AMD. For *TLR3*, only SNP rs3775291 was significantly ($P$ = 0.03) associated with AMD when age, gender, and smoking status were included in the model. For *TLR7*, rs179013 ($P$ = 0.03), rs179009 ($P$ = 0.03), and rs179008 ($P$ = 0.01) remained significantly associated with AMD when age and smoking status were included in the model. Even though smoking is a major risk factor for AMD, there was no interaction between any of the *TLR3* or *TLR7* SNPs and smoking categorized as ever smoked or never smoked.[30]

### DNA Sequencing of the *TLR3* and *TLR7* Loci

To determine whether additional DNA sequence variants in linkage disequilibrium with the SNPs in *TLR3* and *TLR7* that were associated with AMD would explain the observation, the *TLR3* and *TLR7* loci were sequenced in 16 individuals (Supplementary Table S3, http://www.iovs.org/cgi/content/full/49/4/1652/DC1). Four sequence variants in *TLR3* were exclusively found in either cases or controls—(6102G>A, 12739T>A, 13154G>A, and 19798A>G)—but none was near or within exons. A novel amino acid insertion (16141C>CT, p.F459FF) was observed in 5 controls, but in none of the 10 cases. Five sequence variants in TLR7 were exclusively found in either cases or controls (6562T>G>TG, 6949T>C, 16165dupAATAAT, 18270G>A, and 20279A>G, but all occurred in introns and were not in proximity to the intron–exon boundaries.

### Replication of nsSNP Association with AMD in the Michigan and FARMS Cohorts

Although multiple lines of evidence supported the involvement of *TLR* loci in AMD, replication is essential for validating association with disease in genetic-association studies. To this end, an attempt was made to replicate the two nsSNPs in *TLR3* and *TLR7* associated with AMD in another case–control population (Michigan cohort) and in 34 extended families (FARMS cohort). There was no evidence of association with AMD in the Michigan cohort in any case of AMD or exudative AMD (Table 3). In the combined Dallas and Michigan cohorts, there was a trend for association only between AMD and rs3775291(1 df test, additive model, $P$ = 0.09; general model, $P$ = 0.01). A meta-analysis using the log-additive model on the combined Dallas, Michigan, and FARMS cohorts gave a probability of 0.05 and 0.08 for the nsSNP in *TLR3* and *TLR7*, respectively. The FARMS cohort showed a trend toward association that was not statistically significant (Table 4). Because these results suggested a trend for association with AMD, exploratory subgroup analyses were performed.

Exploratory subgroup analyses of the combined Dallas and Michigan cohorts suggested a trend for a greater effect of the nsSNP in TLR3 and TLR7 in subjects with exudative AMD who carried the homozygous risk genotype for the Y402H variant in CFH; however, such effects were not consistent across the two cohorts. For example, CFH-Y402H interacted (with the ns SNP in *TLR3*; rs1061170*rs3775291) to a greater extent in exudative cases ($P$ = 0.009) compared with any AMD ($P$ = 0.04), but there was no interaction in the Michigan cohort. Further, the only significant interaction in the combined Dallas and Michigan cohorts was between the nsSNP in *TLR7* and CFH Y402H in exudative cases ($P$ = 0.008).

## Discussion

The TLRs are excellent candidates for altering the risk of AMD. They are pattern-recognition receptors that participate in the recognition of infectious agents and the clearance of potentially immunogenic or injurious self-molecules.[31,32] TLR3 and TLR7 recognize dsRNA and ssRNA, respectively.[18,20] Exogenous sources of dsRNA and ssRNA are viruses, and endogenous sources include phagocytosed nucleosomes, necrotic cells, chromatin, and small nuclear ribonucleoproteins that protect extracellular RNA from rapid degradation.[20,32–37]

Both the Leu412Phe (TLR3, rs3775291) and Gln11Leu (TLR7, rs179008) coding polymorphisms are located in the TLR receptor ectodomain, where the ligand-binding and dimerization necessary for TLR activation occur.[38] The structure of TLR3 has been solved and Leu412Phe is adjacent to a glycosylation site (Asn[413]) required for receptor activation and within the ligand-binding surface for dsRNA.[38,39] Ligand binding leads to altered conformation and dimerization of TLR receptors.[40–42] Thus, these amino acid polymorphisms could affect the activation of TLR3 and TLR7 through altered ligand binding or dimerization.

**Table 3.** Association between AMD and the Leu412Phe Polymorphism (rs3775291) in *TLR3* and the Gln11Leu Polymorphism (rs179008) in the X-linked Gene Encoding *TLR7* in the Michigan Cohort

| Gene | | Allele Counts and Distribution* | | Allele Association P | | Genotype Counts and Distribution | | Genotype Association P |
|---|---|---|---|---|---|---|---|---|
| | | AMD | Control | | | All Cases | Control | |
| *TLR3* | C | 835 (0.687) | 440 (0.694) | 0.747 | CC | 280 (0.461) | 158 (0.498) | 0.164 |
| | T | 381 (0.313) | 194 (0.306) | | CT | 275 (0.452) | 124 (0.391) | |
| | | | | | TT | 53 (0.087) | 35 (0.110) | |
| *TLR7* | A | 594 (0.775) | 252 (0.754) | 0.448 | AA | 238 (0.621) | 97 (0.581) | 0.645 |
| | T | 172 (0.225) | 82 (0.246) | | AT | 118 (0.308) | 58 (0.347) | |
| | | | | | TT | 27 (0.071) | 12 (0.072) | |

Counts and distribution (frequency) are shown for the alleles and genotypes observed.

* The C and T alleles code for Leu and Phe, respectively, in *TLR3,* whereas the A and T alleles code for Gln and Leu, respectively, in *TLR7.*

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**Table 4.** Association between AMD and the Leu412Phe Polymorphism (rs3775291) in *TLR3* and the Gln11Leu Polymorphism (rs179008) in the X-linked Gene Encoding *TLR7* in the FARMS Cohort

| Gene | Referent Allele | Effect | AMD | | | Drusen Size | | | Drusen Type | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Model | β Coef | *P* | Model | β Coef | *P* | Model | β Coef | *P* |
| *TLR3* | C | Polygenic | Additive | −0.220 | 0.177 | Additive | −0.161 | 0.500 | Additive | 0.062 | 0.579 |
| | | | Dominant | −0.183 | 0.669 | Dominant | −0.419 | 0.511 | Dominant | −0.123 | 0.686 |
| | | | Recessive | −0.271 | 0.160 | Recessive | −0.141 | 0.614 | Recessive | 0.110 | 0.405 |
| *TLR7* | A | Polygenic | Additive | 0.481 | 0.098 | Additive | 0.580 | 0.187 | Additive | 0.067 | 0.742 |
| | | | Recessive | 0.481 | 0.098 | Recessive | 0.580 | 0.187 | Recessive | 0.067 | 0.742 |

Only women were analyzed for *TLR7*. β Coef, β coefficient.

TLR3 and TLR7 are expressed in tissues and cells involved in the pathogenesis of AMD, including the retinal pigment epithelium, macrophages, dendritic cells, and neural tissue.[43–51] Although the involvement of TLRs in AMD could involve inflammatory signaling triggered by molecules released by local or systemic pathogens, an equally attractive model would implicate TLR receptors in the binding of tissue-damage or danger-associated molecular patterns and the clearance of extracellular deposits.[52,53] The accumulation of extracellular deposits composed of autofluorescent byproducts of phototransduction, lipid peroxidation derivatives, extracellular matrix, cellular debris, and inflammatory proteins is a hallmark of AMD.[54–59]

Multiple lines of evidence, including animal models deficient in macrophage recruitment, a rare human disease with an AMD-like phenotype, and epidemiologic, and animal model evidence demonstrating that smoking and dietary fat intake contribute to the risk of AMD, suggest that increased deposition or defects in clearance of these extracellular deposits could contribute to AMD.[30,49,60–63] Because both adaptive and innate immunity have been implicated in the buildup of these deposits, the important role of TLR signaling in priming of dendritic cells and activation of macrophages could explain the association of TLR3 and TLR7 polymorphisms with AMD.[59,64,65] Altered binding of endogenous ligands or signal transduction through TLR pathways could overcome the immune privilege of the choroid and outer retina, as has been observed in other immune-privileged locations, or could result in defective clearance of extracellular deposits.[50,52,66]

This study describes the genotyping of 68 SNPs, with a comprehensive search for common coding and noncoding genetic variation that alter the risk of AMD. A well-characterized cohort of 577 subjects with extreme phenotypes was used for initial discovery analyses (Dallas cohort) and two additional cohorts were used for replication of initial observations in *TLR3* and *TLR7*. nsSNPs in TLR3 and TLR7 were marginally associated with AMD in the Dallas cohort. Because of previous publications suggesting that TLRs may be involved in the pathogenesis of AMD, we explored these results in greater detail.[11] Our initial observations could not be replicated in either the Michigan case–control or the FARMS family-based cohort and were not significant in the Dallas cohort after correction for multiple testing. In conclusion, there is no evidence of association of common amino acid or noncoding polymorphisms in TLR3, TLR4, and TLR7 with AMD. Although these observations do not exclude a role of TLRs in the pathogenesis of AMD, they suggest that the impact of genetic variation in *TLR* genes on overall AMD risk is modest.

Our observations highlight the importance of replication, genotype and subgroup analyses, and power in interpreting data from genetic-association studies of complex traits. Genetic-association studies can be affected by ascertainment biases, population substructure, and other factors. Replication is an essential step in the interpretation of all genetic-association results. Consideration should be given to using genotype observations for initial significance testing, because they are not dependent on the assumption of HWE as are allele tests for frequencies. Subgroup analysis should be used with caution and performed on the entire dataset without discarding information, to avoid spurious interpretations. Replication cohorts would ideally be as large as or larger than the original cohort, to ensure adequate power.

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