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Hypothetical *LOC387715* is a second major susceptibility gene for age-related macular degeneration, contributing independently of complement factor H to disease risk

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Age-related macular degeneration (AMD) is a multifactorial disease and a prevalent cause of visual impairment in developed countries. Risk factors include environmental components and genetic determinants. The complement factor H (CFH) has been the first major susceptibility gene for AMD identified within 1q32. Here, we focused on a second region of interest in 10q26 where a recent meta-analysis revealed strongest evidence for linkage to AMD at a genome-wide significance level. Within an interval of 22 Mb, we have analyzed 93 single nucleotide polymorphisms for allelic association with AMD in two independent case–control cohorts of German origin ($AMD_{combined}$ $n = 1166$; $controls_{combined}$ $n = 945$). Significant association was found across a 60 kb region of high linkage disequilibrium harboring two genes *PLEKHA1* and hypothetical *LOC387715*. The strongest association ($P = 10^{-34}$) centered over a frequent coding polymorphism, Ala69Ser, at *LOC387715*, strongly implicating this gene in the pathogenesis of AMD. Besides abundant expression in placenta, we demonstrate weak expression of *LOC387715* in the human retina. At present, however, there is no functional information on this gene, which appears to have evolved recently within the primate lineage. The joint contribution of the common risk allele at *LOC387715*, Ala69Ser, and at *CFH*, Tyr402His, was assessed in our case–control population, which suggests an additive model indicating an independent contribution of the two gene loci to disease risk. Our data show a disease odds ratio of 57.6 (95% CI: 37.2, 89.0) conferred by homozygosity for risk alleles at both CFH and LOC387715 when compared with the baseline non-risk genotype.

INTRODUCTION

Age-related macular degeneration (AMD) is a prevalent cause of visual loss in industrialized countries (1,2). Early signs of the disease are characterized by the presence of ophthalmoscopically visible soft drusen, areas of hyperpigmentation and depigmented areas, whereas later stages manifest as either choroidal neovascularization (CNV) or atrophy of photoreceptors and retinal pigment epithelium (RPE). Patients with early AMD [also referred to as age-related maculopathy (ARM)] rarely suffer from clinical symptoms but are at increased risk for developing sight-threatening pathology (3–5).

Besides personal risk factors such as age and smoking, genetic susceptibilities are known to contribute to AMD etiology (6,7). Consequently, defined allelic variants for a

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number of candidate genes have been implicated in disease development, including *ABCA4* (8), *APOE* (9), *FBLN5* (10), *ELOVL4* (11) and *TLR4* (12), although independent replication of single findings has been difficult to some extent. In addition, the individual contribution of these variants to overall AMD prevalence appears relatively minor and thus far has provided only limited information on disease pathogenesis. In an alternative approach, family-based genome-wide and candidate region linkage studies have been successful in identifying a number of chromosomal regions, likely harboring more common susceptibility loci for AMD (13–24). Remarkably, two of these regions, 1q31 and 10q26, have frequently been replicated, whereas others have been less consistent across the independent studies.

The 1q31 region was first recognized as an AMD locus by a classical genetic linkage approach in an extended pedigree segregating an autosomal dominant AMD-like phenotype (13). Subsequently, a Gln5345Arg missense mutation in the fibulin-6 (*FBLN6*) gene was identified as a probable disease-associated variant (25). Although the role of *FBLN6* in non-familial AMD awaits a more comprehensive assessment, recent findings have directed attention to the regulation of complement activation region ~11 Mb distal to the *FBLN6* locus (26–29). In these studies, the most significant association was found at a non-synonymous single nucleotide polymorphism (SNP) encoding a Tyr402His variant in the complement factor H (*CFH*), which increases the risk for AMD with an odds ratio of between 2.1 and 7.4 (26–30). As this variant may explain ~30–50% of AMD, *CFH* represents the first major susceptibility gene for this complex disorder, directing research to the role of inflammation and innate immunity in the etiology of AMD.

Family-based linkage scans have pointed to a second major AMD susceptibility locus at chromosomal region 10q26. These findings are strongly supported by at least five independent studies (16,17,19,21,22). In addition, a meta-analysis of six independent AMD genome screens found strongest evidence for an AMD locus between D10S1483 and 10qter on chromosome 10 at a genome-wide level of significance ($P = 0.00025$) (31). To identify the susceptibility gene within this region, we tested 93 SNPs with minor allele frequencies >0.2 across an ~22 Mb interval. Highly significant allelic association in a case–control design has refined the locus to a 60 kb region of strong linkage disequilibrium (LD) harboring two genes, *PLEKHA1* and hypothetical *LOC387715*. Refined LD mapping of the region and comprehensive association analyses of coding SNPs in our extended case–control cohort ($AMD_{combined}$ $n = 1166$; $controls_{combined}$ $n = 945$) strongly support a role of *LOC387715* as a second major AMD susceptibility gene.

RESULTS

To identify the AMD susceptibility locus within the broad region of genome-wide significant linkage at the distal long arm of chromosome 10 (31), we obtained genotyping data from 93 SNPs across a 22 Mb region in 10q25.2-qter. An initial screening set consisting of 794 AMD cases and 612 controls from the Frankonian area of Northern Bavaria was examined for association (Supplemental Material, Table S1). Control genotypes were in Hardy–Weinberg equilibrium (HWE) ($P > 0.01$ for all SNPs). Initially four SNPs (rs4146894, rs2421022, rs2421016 and rs2292625) within the *PLEKHA1* locus showed a significant difference in allele frequency between cases and controls (Fig. 1 and Table 1) with the strongest association at rs4146894 (allele T: 59.5 versus 47.3%, $P_{uncorrected} = 1.5 \times 10^{-10}$, $P_{corrected} = 1.4 \times 10^{-8}$) and rs2421016 (allele T: 59.5 versus 47.0%, $P_{uncorrected} = 4.8 \times 10^{-11}$, $P_{corrected} = 4.5 \times 10^{-9}$). These two SNPs are in absolute LD ($\Delta = 0.99$ in controls). No other SNPs showed significant differences ($P > 0.05$) in allele frequencies between cases and controls.

To determine the extent of the association with the *PLEKHA1* locus, 13 additional SNPs (rs7893672, rs7916970, rs986960, rs1998345, rs2901307, rs9988734, rs10490924, rs3750847, rs3750846, rs2014307, rs4752700, rs2300431 and rs714816) over a 185 kb genomic region spanning adjacent genes *C10orf87*, *LOC387715* and *PRSS11* were genotyped in 63 individuals (24 cases and 39 controls randomly selected from our initial screening set). LD analysis revealed a region of linkage disequilibrium, which included the two gene loci *PLEKHA1* and *LOC387715* (Fig. 2). However, the LD did not extend to the adjacent genes with only weak linkage disequilibrium observed between SNPs at *PRSS11* and *PLEKHA1/LOC387715*, suggesting that the region of association was confined to ~60 kb containing *PLEKHA1/LOC387715*.

To search for additional potential risk-associated sequence changes, we resequenced 24 AMD cases and eight controls at the *PLEKHA1* and *LOC387715* loci, respectively (Supplementary Material, Table S2). The AMD cases were selected as heterozygous ($n = 13$) or homozygous ($n = 11$) for the *PLEKHA1* risk haplotype to maximize the power to detect variants occurring on this risk haplotype. In total, 15 distinct variants were detected in the two genes. Of these, one frequent coding SNP was found in exon 12 of *PLEKHA1* (rs1045216) (38/48 AMD alleles versus 1/16 control alleles). In exon 1 of *LOC387715*, a common coding SNP rs10490924 (27/48 versus 0/16) and two additional rare coding SNPs [rs10490923 (3/48 versus 1/16) and rs2736911 (3/48 versus 3/16)] were identified. The two latter SNPs occurred at only 6% in cases homozygous for the risk haplotype and therefore were considered too rare to genotype in our case–control cohort, because our study would have insufficient power to detect association. The two common coding SNPs, *PLEKHA1* rs1045216 and *LOC387715* rs10490924, were subsequently tested for association in the initial collection of cases and controls. At rs1045216, the G allele occurred at significantly higher frequency in cases than in controls (68.2 versus 58.5%, $P = 1.2 \times 10^{-7}$). At rs10490924, genotyping revealed a two-fold increase in the frequency of the T allele in cases when compared with controls (41.7 versus 19.6%, $P = 3.9 \times 10^{-34}$) (Table 1).

To confirm the association in the original cohort, a further independent collection of 373 German AMD cases and 335 controls from the Munich (Upper Bavaria) and Tuebingen (Swabia) area was genotyped for the six *PLEKHA1*- and *LOC387715*-associated SNPs (rs4146894, rs2421022, rs2421016, rs2292625, rs1045216 and rs10490924). Control

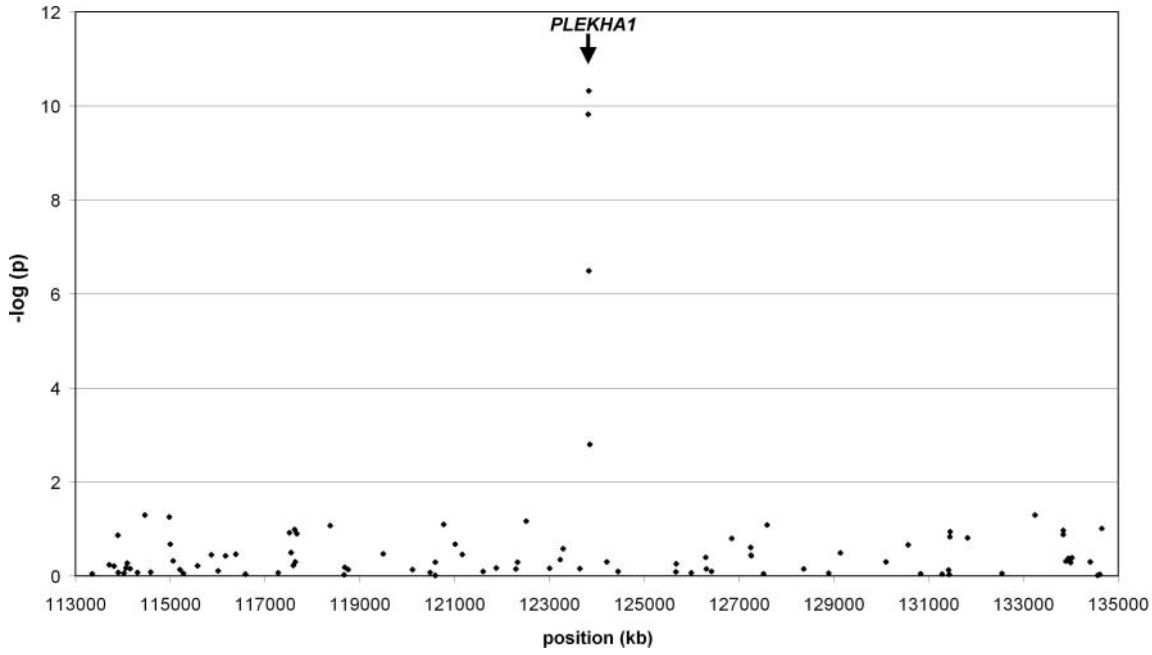

Figure 1. Case–control analysis for difference in allele frequency between 794 AMD cases and 612 controls is shown for 93 SNPs across a 22 Mb region on chromosome 10 between D10S1690 and 10qter. The position for each SNP is given on a kilobase pair scale according to dbSNP database assembly hg17, May 2004.

Table 1. *PLEKHA1* single SNP association in two independent German case–control cohorts

Marker	Gene	Risk allele	Original cohort				Replication cohort		
			AMD ($n = 794$)	Controls ($n = 612$)	P_U[a]	P_C[a]	AMD ($n = 373$)	Controls ($n = 335$)	P_U
rs4146894	*PLEKHA1*	T	0.595	0.473	1.5×10^{-10}	1.4×10^{-8}	0.633	0.497	3.5×10^{-7}
rs2421022	*PLEKHA1*	A	0.678	0.584	3.2×10^{-7}	0.00003	0.721	0.613	2.1×10^{-5}
rs2421016	*PLEKHA1*	T	0.595	0.470	4.8×10^{-11}	4.5×10^{-9}	0.633	0.499	4.7×10^{-7}
rs2292625	*PLEKHA1*	G	0.867	0.824	0.002	0.19	0.884	0.848	0.051
rs1045216	*PLEKHA1*	G	0.682	0.585	1.2×10^{-7}	—	0.721	0.618	4.6×10^{-5}
rs10490924	*LOC387715*	T	0.417	0.196	3.9×10^{-34}	—	0.460	0.215	2.0×10^{-21}

n, number of individuals.
[a]Significance values for the original cohort are given uncorrected (P_U) and with a Bonferroni correction (P_C) for multiple testing of 93 SNPs.

frequencies were similar to those in the original cohort. Association was replicated for all SNPs (Table 1); the most significant difference in allele frequency between cases and controls was observed for LOC387715 SNP rs10490924 (allele T: 46.0 versus 21.5%, $P = 2.0 \times 10^{-21}$).

To examine the contribution of specific SNPs or haplotypes from *PLEKHA1/LOC387715* to disease risk, a conditional modeling approach was used such that a test for association with each SNP or haplotype was carried out conditional on the presence of one or more other SNPs. Case and control genotypes were pooled across both German cohorts to maximize the power of the test. Conditional on the effect of rs10490924, none of the *PLEKHA1* SNPs contributed to disease risk ($P > 0.5$), i.e. the odds ratio for haplotypes containing the rs10490924 risk allele, was the same, irrespective of the allele at the other SNPs in the haplotype. However, conditional on an effect from any of the *PLEKHA1* SNPs, a haplotype effect with rs10490924 was highly significant ($P < 1 \times 10^{-40}$), indicating that the locus-specific association with individual *PLEKHA1* SNPs is not sufficient to explain the association. Thus, the disease risk due to variation at the *PLEKHA1/LOC387715* locus can best be described with a single SNP effect from rs10490924. Similar results were seen when each cohort was analyzed separately. LD analysis revealed that rs10490924 lies exclusively on a haplotype with rs2421016 and rs4146894 (the SNPs showing the strongest association at PLEKHA1) and with the PLEKHA1 coding SNP rs1045216 ($D' = 1$). In individuals who did not carry the LOC387715 rs10490924 risk allele (591 controls and 386 cases), there was no difference in the frequency of the PLEKHA1 risk allele (cases: 38.3%; controls: 37.2%; $P = 0.66$).

Disease risks for the two German cohorts, analyzed both separately and pooled, are shown in Table 2. Disease risks are similar in both populations; in the combined cohort, individuals heterozygous for the rs10490924 risk T allele

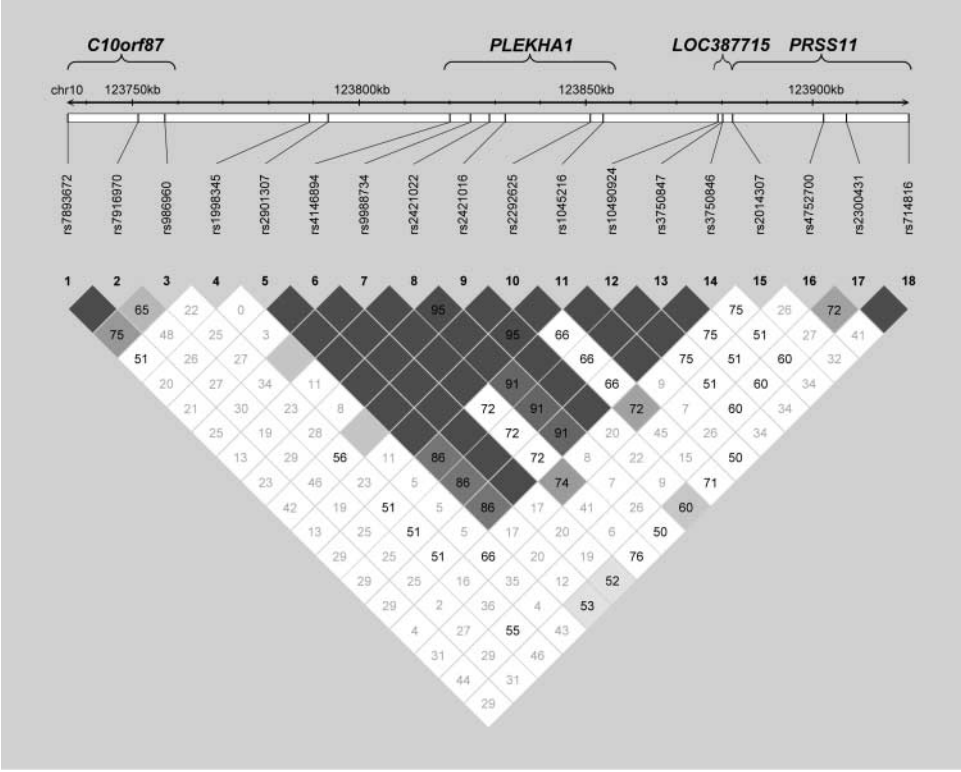

Figure 2. Haploview plot depicting the haplotype block structure of the AMD-associated PLEKHA1/LOC387715 region. Regions of LD were defined as pairwise values of $D' > 0.6$ and were estimated in 63 individuals (39 controls and 24 AMD cases). The relative chromosomal position of each SNP is given in the upper diagram.

Table 2. *LOC387715* rs10490924 genotype frequencies and disease-risk estimates

Cohort	Phenotype	GG	GT	TT	OR (GT versus GG)	95% CI	OR (TT versus GG)	95% CI
Original	Controls (594)	388	179	27				
	AMD (759)	268	349	142	2.82	(2.23, 3.58)	7.61	(4.90, 11.82)
Replication	Controls (328)	203	109	16				
	AMD (361)	117	156	88	2.48	(1.78, 3.47)	9.54	(5.35, 17.03)
Pooled	Controls (922)	591	288	43				
	AMD (1120)	385	505	230	2.69	(2.22, 3.27)	8.21	(5.79, 11.65)

confer an ~2.7-fold increased risk of developing AMD compared with GG homozygotes (OR = 2.69; 95% CI: 2.22, 3.27), whereas a 8.2-fold increased risk is associated with TT homozygosity (OR = 8.21; 95% CI: 5.79, 11.65). Non-overlapping odds ratio confidence intervals associated with GT and TT genotypes compared with the baseline GG genotype suggest a significantly higher degree of risk for individuals homozygous for the risk T allele.

Cases and controls were additionally genotyped for the Y402H polymorphism in the *CFH* gene, which has been shown earlier to be associated with AMD (26–29). A highly significant increase in the frequency of AMD risk C allele was observed in our cases when compared with controls in both the original Frankonian cohort (59.5 versus 38.2%, $P = 6.7 \times 10^{-29}$) (Supplementary Material, Table S1) and the German replication cohort (63.3 versus 49.9%, $P = 4.7 \times 10^{-7}$). Disease risks associated with this mutation are similar to those observed in the previous studies (Table 3).

Formal testing of genetic models in the pooled German data showed that a log-linear additive (but not dominant or recessive) model was an adequate fit for *LOC387715* rs10490924 genotype risks; a gene-dosage model did not provide a significantly better fit ($P = 0.59$). However, for *CFH* Y402H genotype risks, dominant, recessive and additive models were rejected in favor of a gene-dosage model ($P < 0.005$ for all nested models). The joint contribution of these two AMD susceptibility loci was assessed by logistic regression analysis, modeling case–control status on *LOC387715* and *CFH* genotypes. Risk due to *LOC387715* was modeled by the number of risk T alleles (0, 1 or 2) (corresponding to an additive model); independent genotype-specific risks (equivalent to a gene-dosage model) were calculated for *CFH*.

Table 3. *CFH* Y402H case–control analysis

		TT	CT	CC	OR (CT versus TT)	95% CI	OR (CC versus TT)	95% CI
Original (German)	Controls (611)	214	327	70				
	AMD (793)	137	368	288	1.76	(1.35, 2.28)	6.43	(4.58, 9.01)
Replication 1 (German)	Controls (335)	141	148	46				
	AMD (373)	62	162	149	2.49	(1.72, 3.61)	7.37	(4.72, 11.50)
Pooled	Controls (946)	355	475	116				
	AMD (1166)	199	530	437	1.99	(1.61, 2.46)	6.72	(5.14, 8.79)

Table 4. Two-locus genotype frequencies (%) and disease risks for *LOC387715* and *CFH* estimated in 1119 cases and 922 controls

rs10490924	CFH Y402H								
	Controls			Cases			OR (95% CI)		
	TT	TC	CC	TT	TC	CC	TT	TC	CC
GG	223 (24.2)	295 (32.0)	73 (7.9)	61 (5.5)	178 (15.9)	145 (13.0)	1	2.03 (1.61, 2.56)	7.10 (5.33, 9.46)
GT	103 (11.2)	149 (16.2)	36 (3.9)	84 (7.5)	225 (20.1)	196 (17.5)	2.85 (2.45, 3.31)	5.79 (4.36, 7.68)	20.24 (14.42, 28.31)
TT	19 (2.1)	21 (2.3)	3 (0.3)	46 (4.1)	105 (9.4)	79 (7.1)	8.11 (6.01, 10.94)	16.46 (11.14, 24.27)	57.58 (37.24, 89.00)

Logistic regression results suggested a log-linear additive model for the effects of *CFH* and *LOC387715* as the best model to predict case–control status; the contribution of *LOC387715* was significant ($P < 1 \times 10^{-30}$) even after inclusion of the stronger effect of *CFH* in the model indicating that these two loci independently contribute to disease risk. A linear additive model on a logarithmic scale corresponds to a multiplicative model on the penetrance scale. Fitting an interaction model between *CFH* and *LOC387715*, there was no evidence of epistasis ($P = 0.79$). Two-locus genotype-specific risks and frequencies are shown in Table 4. Our data show a disease odds ratio of 57.6 (95% CI: 37.2, 89.0) for individuals homozygous for risk alleles at both loci when compared with the baseline wild-type (non-risk) genotype.

An extended collection of 848 cases (794 patients from the initial screening set in addition to 54 cases with early signs of AMD) was examined for clinical features of disease and smoking history, a well-established risk factor for AMD (32,33). Genotype and allele frequencies for *CFH* rs1061170 (T > C; Y402H) and *LOC387715* rs10490924 (G > T) were compared between sub-groups (Table 5). Frequencies of the *CFH* risk allele C were similar in AMD (59.9%) and ARM (59.0%) cases. Stratification of AMD by late manifestations revealed a higher frequency of allele C in patients with GA (64.6%) when compared with those without GA (56.6%, $P = 0.0025$); allele frequencies in both of these groups were significantly higher than that in controls (GA: $P = 3.3 \times 10^{-25}$; non-GA: $P = 3.1 \times 10^{-16}$). In contrast, the frequency of the *LOC387715* SNP risk allele T was significantly higher in cases with AMD when compared with those with ARM (43.9 versus 29.0%, $P = 5.1 \times 10^{-6}$). No significant differences were observed between patients with GA or those with CNV. Subsequently, cases with ARM were categorized into high or low risk of developing late manifestations, following established clinical criteria (3). A significantly higher frequency of *LOC387715* SNP allele T was observed in cases classified as high risk for AMD when compared with those with low risk (34.2 versus 22.7%, $P = 0.047$); the frequency in low risk cases was similar to that observed in controls (19.6%, $P = 0.48$). Further investigation of the association with *CFH* revealed that although frequencies of the risk allele C were similar in ARM and AMD cases, the allele frequency in cases classified high risk for AMD was significantly higher than that in those with low risk (66.5 versus 49.2%, $P = 0.0037$). No significant differences in risk allele frequency were detected between smokers and non-smokers for either *CFH* or *PLEKHA1*.

RT–PCR expression was analyzed for the *PLEKHA1* and *LOC387715* genes in six human tissues including retina from two unrelated donors, RPE/choroid, placenta, stomach, cerebellum and peripheral blood leukocytes. Ubiquitous and strong expression was detected for the PLEKHA1 transcript, while LOC387715 is expressed abundantly in placenta and weakly in retina (Fig. 3). None of the remaining tissues showed expression of LOC387715.

DISCUSSION

Estimates from 14 year incidences of AMD indicate that approximately one in 300 Northern Europeans per year will develop a late form of AMD (5). Extrapolation of these data to the 1.2 billion people currently living in developed countries would imply that approximately four million people are presently suffering from various degrees of visual impairment due to AMD pathology. Other estimates from US studies suggest even up to seven million AMD patients in developed countries (2). This makes this disease a highly prevalent health care problem with only a few insufficient options for preventive treatment at this stage (34). There is great promise that clarification of the genetic factors contributing to disease etiology will point to defined disease pathways disclosing novel targets for the development of long-sought interventional strategies.

Table 5. *CFH* and *LOC387715* genotypes stratified by smoking history and clinical features of disease

	CFH Y402H				*LOC387715* rs10490924			
	TT	TC	CC	Frequency (C)	GG	GT	TT	Frequency (T)
Disease category								
AMD ($n = 692$)	115	324	252	0.599	217	308	136	0.439
CNV only ($n = 417$)	82	197	137	0.566	131	186	78	0.433
GA only ($n = 154$)	19	71	64	0.646	54	67	28	0.413
CNV and GA ($n = 121$)	14	56	51	0.653	32	55	30	0.491
ARM ($n = 155$)[a]	29	69	57	0.590	71	61	11	0.290
High risk ($n = 85$)	11	35	39	0.665	32	36	8	0.342
Low risk ($n = 66$)	17	33	16	0.492	38	23	3	0.227
Smoking history								
Non-smoker ($n = 630$)	93	311	226	0.606	212	274	110	0.414
Smoking ($n = 212$)	50	79	82	0.576	75	92	36	0.404

n, total number of individuals with characteristic.
[a]Four ARM patients were unclassified as high or low risk for AMD.

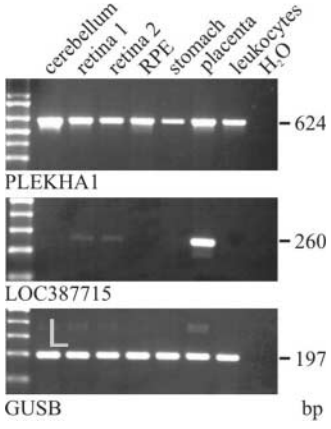

Figure 3. RT–PCR expression analysis of the *PLEKHA1* and *LOC387715* genes. In each case, forward and reverse primers were exon-spanning to avoid amplification products resulting from minor genomic contamination of the mRNA preparations. Expression of the housekeeping gene GUSB is shown as a control to test for first-strand cDNA integrity.

In a first breakthrough, a significant proportion of AMD cases was recently shown to be associated with defined polymorphisms in the *CFH* gene (11,26–30,35). In particular, the high-risk *CFH* variant Tyr402His encodes a *CFH* isoform with probable functional relevance for the basic pathogenic mechanisms in the development of AMD. Accordingly, the 402His isoform may ultimately affect complement inhibitory function of *CFH* at the interface of the RPE and the choriocapillaris. As a consequence, impaired *CFH* function possibly in connection with other modulating triggers such as infectious agents may lead to chronic imbalances in complement activation (36). Such a model of disease pathology is well supported by earlier findings connecting drusen formation, a hallmark of early AMD and significant risk factor for the development of late AMD complications, with immune-mediated processes (37,38). In the present study, we have replicated the earlier findings for *CFH* Tyr402His and confirm a highly significant association of *CFH* with AMD in our regionally well-defined case–control cohorts of German ancestry. This provides further evidence that *CFH* is a common susceptibility locus for AMD beyond ethnic boundaries.

The main objective of the present study is to identify the gene most likely responsible for the linkage signals in 10q26, which have been independently found in several family-based genome scans (16,17,19,21,22). We have focused on a 22 Mb region on distal 10q between D10S1760 and 10qter covering a region with strongest evidence for linkage at a genome-wide significance level (31). Our SNP selection strategy was aimed at maximizing coverage of the region by choosing 93 frequent SNPs from within known regions of LD ($\geq$50 kb), thus facilitating interrogation of $\sim$9 Mb of DNA. Apart from six SNPs within the *PLEKHA1*/*LOC387715* interval, no single SNP showed a significant difference in allele frequency between AMD cases and controls. A recent AMD-affected family linkage study similarly querying a large number of SNPs in the 10q26 region (35) also found a strong linkage peak over the region harboring *PLEKHA1*, *LOC387715* and *PRSS1*, although with insufficient power to distinguish between the three genes. In addition, they noted a weak signal over the *GRK5*/*RGS10* locus. Although our initial screen covered the latter region with two SNPs (rs2085185 and rs10886515), we found no indication of association in our initial set of case–control cohorts. A more comprehensive analysis with high-density SNP genotyping of the *GRK5*/*RGS10* region will be needed to further address the involvement of this locus with AMD.

In this study, we have focused our analysis on the further refinement of the association signal over the *PLEKHA1*/*LOC387715* region to establish a causal correlation of one of the genes with AMD. Conditional haplotype analysis in a large collection of cases and controls has identified the *LOC387715* coding SNP (rs10490924) rather than *PLEKHA1* variants as best describing the association signal at this region. From LD analysis across the *PLEKHA1*/*LOC387715* region including adjacent genes, only weak-to-moderate LD was observed with variants in the *PRSS11* gene. Furthermore, preliminary genotyping of *PRSS11* (rs714816) in 195 cases and 192 controls revealed a weaker association ($P = 0.0022$, data not shown) when compared

with the corresponding results in this data set for *LOC387715* ($P = 2.8 \times 10^{-14}$). It is therefore unlikely, although not entirely ruled out, that *PRSS11* harbors the true susceptibility variant(s). Resequencing of the region also identified two other rare LOC387715 coding variants (rs10490923 and rs2736911) and we cannot exclude the possibility that these represent additional rare causal variants. To further investigate this, large, well-powered association studies will be required.

At present, there is no biological evidence supporting the hypothetical *LOC387715* gene as a susceptibility locus for AMD. The two-exon gene is transcribed as a 818 bp mRNA (e.g. GenBank accession no. BC066349), which encodes a distinct 107 amino acid protein with no matches in public protein or protein motif databases. *LOC387715* appears to be a phylogenetically recent gene with conservation restricted to the primate lineage (human and chimpanzee share an amino acid identity of 97%). The *LOC387715* transcript is supported by one mRNA and 18 EST clones of which several are correctly spliced. Most clones are derived from placental cDNA libraries reflecting our expression data, which demonstrate a highly abundant expression in placental tissue. Additionally, we found weak expression in retinal tissues from two unrelated donors, but not in other tissues analyzed, strongly suggesting a tissue-restricted function of *LOC387715* in the retina. The complete absence of expression signals in the remaining tissues tested argues against an ectopic expression, for example, due to leaky promoter regulation. The weak expression in retina may be explained either by an overall low copy number of transcripts in the retinal cells or by cell-specific expression in a circumscribed retinal cell population. As a first step in defining the cellular function of *LOC387715*, it will be essential to identify the precise localization of the mRNA and protein expression within the retina.

We have also examined a possible genetic interaction between the high-risk alleles at *CFH* and *LOC387715* for AMD predisposition. Logistic regression showed an association of *LOC387715* independent of the association of *CFH* with AMD. Homozygosity for risk alleles at both loci *CFH* and *LOC387715* is associated with a very high odds ratio of 57.6, which applies to about one in 200 individuals. The next lower category of risk (odds ratios between 16 and 20) could affect as many as one in 23 individuals. These considerations could make predictive DNA testing a tempting option. However, a strong cautionary note is advised, particularly because it is not clear, at present, how such risks may interact with other as yet unknown risk or protective factors. This is even more true as the biological consequences of the risk alleles at the two gene loci are not yet understood. Consequently, the knowledge of being carrier of risk alleles is currently not matched by adequate options for preventive strategies or possible treatment modalities.

To summarize, refined mapping of allelic association within 10q26 in a case–control design has narrowed the region of interest to a 60 kb interval of high LD harboring two genes *PLEKHA1* and *LOC387715*. The strongest association with AMD was found over the *LOC387715* gene conferring a 7.6-fold increased risk for individuals homozygous for a protein coding SNP, Ala69Ser. These findings have been fully replicated in an independent case–control cohort. Together, our large well-defined collection of German cases and controls has enabled robust estimates of disease risk, suggesting *LOC387715* as a second major susceptibility gene for AMD. Replication studies in other populations are needed to further corroborate our findings. Furthermore, we have replicated earlier reports of strong association of AMD with a coding variant, Tyr402His, in the CFH. Our results indicate an independent contribution of the effects of risk alleles at LOC387715, Ala69Ser, and CFH, Tyr402His, to the overall disease risk. Further independent studies will be needed to clarify the true population disease risk of these loci.

MATERIALS AND METHODS

Subjects and clinical assessment

The initial screening set consisted of 794 non-familial AMD patients and 612 unrelated control individuals exclusively recruited through the University Eye Clinic Würzburg between 2001 and 2004. Care was taken to ensure that both cases and controls originated from the lower Franconian region of Bavaria, Germany. Each patient underwent a single examination involving a general health interview protocol, visual acuity testing and a clinical ophthalmic examination. The latter included 30° confocal autofluorescence (AF) and infrared reflectance imaging (HRA, Heidelberg Engineering, Dossenheim), 50° digital fundus imaging (Zeiss, Funduskamera FF 450 plus) and fluorescein angiography upon suspicion of development of CNV. The subclassification of AMD cases was based on the convention of the international classification system (3) with minor modifications due to the application of 50° digital fundus images and the inclusion of HRA-AF and infrared reflectance images for the evaluation of geographic atrophy (GA) and reticular drusen.

Features such as drusen of different sizes and shapes, pigmentary changes or incipient atrophy of the RPE were classified as early AMD or ARM ($n = 102$). Blind to genotyping data, a refined classification of the ARM group was carried out according to a standardized classification (3) for 98 patients to enrich for those who likely have a low risk of progression to late AMD [i.e. soft distinct drusen only (63–125 μm); pigmentary abnormalities only without soft drusen] ($n = 52$) and those who have a high risk to evolve in late stage manifestations [i.e. soft indistinct drusen ($\geq$125 μm) or reticular drusen only; soft distinct drusen (63–125 μm) with pigmentary abnormalities; soft indistinct ($\geq$125 μm) or reticular drusen with pigmentary abnormalities] ($n = 46$). Subsequent to the initial screening efforts and for the purpose of stratification by clinical subgroups, the early AMD group was further extended by 54 patients recruited from the Frankonian region (low risk $n = 14$; high risk $n = 39$; unclassified $n = 1$). Patients with GA ($n = 154$) or CNV ($n = 417$) included those with features in one or in both eyes. One hundred and twenty-one patients had GA in one eye and development of CNV in the fellow eye. Subjects with early changes such as hard drusen and/or moderate pigmentary alterations in both eyes were excluded as these changes may refer to normal aging processes not necessarily linked to AMD. Furthermore, patients who presented with bilateral CNV or macular scars were not included in the study as the underlying phenotype may no longer be

discernible. All patients and controls were seen by one of the authors (C.N.K.) to avoid problems inherent to multiple observers and multicenter studies. Mean age at examination was 76.32 ± 6.90 years for 848 AMD patients (ranging from 52 to 94 years of age) and 76.21 ± 5.28 years for 612 controls (ranging from 65 to 97 years of age). Gender distribution for AMD cases was 35.7% male and 64.3% female, and gender distribution for the control sample was 38.4% male and 61.6% female. The control subjects were free of macular changes such as drusen, pigmentary alterations or diabetic maculopathy. Approximately 20% of controls were healthy spouses of AMD patients.

The German replication set consisted of 373 non-familial AMD cases (mean age 75.01 ± 7.51 years; 35.1% male, 64.9% female) and 335 unrelated controls (mean age 68.26 ± 8.14 years; 44.5% male, 55.5% female) which were examined by ophthalmoscopy and found to be free of macular disease. The study participants were recruited from the University Eye Clinics of Tübingen (area of Swabia) and Munich (area of Upper Bavaria) as part of a collaborative project with Lynkeus Biotech GmbH, Wuerzburg.

The study was approved by the Ethics Committee of the University of Wuerzburg and adhered to the tenets of the Declaration of Helsinki. All subjects, patients and controls, were informed about the nature of the study and signed a written consent prior to blood withdrawal.

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes according to established protocols. A total of 97 SNPs with minor allele frequencies >0.2 were first selected; however, only 93 SNPs provided suitable genotyping data and thus were included in the initial study. SNP data were taken from the public dbSNP database covering a genomic region of ~22 Mb at 10q25.2-qter (113 300 000–135 400 000 bp; assembly hg17, May 2004) including part of bin 10.5 (D10S1690–D10S1483) and the distal bin 10.6 (D10S1483-10qter) (31). One to four SNPs were selected for each block of linkage disequilibrium (LD) extending 50 kb in size ($n = 63$, setting of block computation method to pairwise D') (SNPbrowser Ver. 2.0, Applera Corp./Applied Biosystems). This resulted in an average distance between SNPs of ~220 kb. Genotyping of SNPs was achieved by primer extension of multiplex PCR products, with detection of the allele-specific extension products by the matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry method (Sequenom, San Diego, CA, USA). All SNPs showed high genotyping quality with an average call rate of 98%. To test for accuracy, 3.5% of all genotypings were performed in duplicate.

Sequence analysis

Genomic resequencing was done for the PLEKHA1 (NM_021622, NM_001001974) and the LOC387715 (XM_373477) genes and included the 5′- and 3′-untranslated regions, coding and non-coding exons as well as all ~20–30 bp of the exon/intron junctions. Primer sequences and conditions are available online (Supplementary Material, Table S2). Cycle sequencing products were analyzed on a Beckman CEQ 2000 sequencer with the corresponding Dye Terminator Cycle Sequencing and Quick Start Kit (Beckman Coulter GmbH, Krefeld, Germany).

Expression analysis

Reverse transcription (RT)–PCR was performed with total RNA from two unrelated human retinae, RPE/choroid (human donor eyes were obtained from the eye bank at the University Eye Clinic, Würzburg, Germany), placenta and peripheral blood leukocytes isolated with the RNeasy Mini Kit (Qiagen, Hilden, Germany) and the commercially available human total RNAs from cerebellum and stomach (Clontech, Heidelberg, Germany). First-strand cDNA served as template for subsequent PCR assays and was generated from 1.5 μg of total RNA by RT using the Superscript II polymerase (Life Technologies, Karlsruhe, Germany) according to the supplier's instructions. Dilutions of the resulting cDNAs were amplified in a reaction mix that included 1 × PCR buffer (10 × PCR buffer contains 160 mM $(NH_4)_2SO_4$, 670 mM Tris–HCl pH 8.8, 0.1% Tween-20), 1 μM of each dATP, dCTP, dGTP, dTTP, 200 nM of each PCR primer, 2 mM $MgCl_2$ and 1 U Superhot *Taq*-Polymerase (Genaxxon BioScience GmbH, Biberach, Germany). Amplification cycles consisted of a denaturation step at 94°C for 30 s, annealing at 58–64°C for 30 s (cycles 1–3: 64°C; cycles 4–6: 61°C; cycles 7–35: 58°C) and extension at 72°C for 30 s. The PLEKHA1 cDNA was amplified as a 624 bp fragment with exon-spanning primers PLEKHA1-ex5-6F (5′-CTA CTA AGC TAA GGC CAA AGG-3′) and PLEKHA1-ex7-10R (5′-GCA TCT CTT CAG GGC TAT CAG-3′), the *LOC387715* gene with exon-spanning primers LOC-ex1-F (5′-GAT GGC AAG TCT GTC CTC CT-3′) and LOC-ex2-R (5′-TTG CTG CAG TGT GGA TGA TAG-3′) as a 260 bp fragment. The integrity of the cDNAs was evaluated by amplification of a 197 bp fragment of the ß-glucuronidase (GUSB) gene with primers GUS-B3 (5′-ACT ATC GCC ATC AAC AAC ACA CTC ACC-3′) and GUS-B5 (5′-GTG ACG GTG ATG TCA TCG AT-3′).

Statistical methods

For the initial collection of cases and controls, 93 SNPs were assessed for HWE using a significant threshold of $P = 0.001$. Allele frequencies were compared between cases and controls using binomial proportions tests; significance values are reported both uncorrected (P_U) and corrected (P_C) for multiple testing (Bonferroni method). No correction was made in subsequent SNP genotyping or for the replication study. Haplotype analysis was carried out using COCAPHASE, a module of the UNPHASED program (39). Uncertain haplotypes were estimated using an expectation–maximization algorithm. A conditional analysis was carried out, which tests for equality of odds ratios for haplotypes identical at conditioning loci. Pairwise SNP LD coefficients D' and Δ (40,41) were calculated using Haploview (42). Single locus genetic models, fitted by logistic regression using Splusv6.0, were compared by analysis of deviance. Common genetic models (recessive, additive and dominant) were compared with the

fully saturated gene-dosage model such that if the simpler model holds, D_1–D_2 is distributed as chi-squared with df_1–df_2 degrees of freedom, where D_1 and D_2 are the residual deviances under the nested (simpler) and saturated models, respectively (43). Logistic regression was used to model two-locus genotype risks. Odds ratios for genotypic combinations across the two loci were obtained by multiplying the baseline contributions for each locus, and 95% confidence intervals were obtained directly from logistic regression parameter estimates.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. The authors declare no conflict of interest.

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