Association between the *SERPING1* gene and age-related macular degeneration: a two-stage case–control study

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Summary

Background—Age-related macular degeneration is the most prevalent form of visual impairment and blindness in developed countries. Genetic studies have made advancements in

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establishing the molecular cause of this disease, identifying mutations in the complement factor H (*CFH*) gene and a locus on chromosome 10 encompassing the *HTRA1/LOC387715/ARMS2* genes. Variants in complement 3 (*C3*) and an HLA locus containing both factor B and *C2* genes have also been implicated. We aimed to identify further genetic risk factors for this disease.

Methods—We used a case–control study design in a UK sample of patients with age-related macular degeneration (n=479) and controls (n=479) and undertook a low-density screen of 32 genes using 93 single nucleotide polymorphisms (SNPs). Genes were selected as candidates on the basis of potential functional relevance to age-related macular degeneration. Significant initial findings were confirmed by replication in an independent US cohort of 248 unrelated patients with disease and 252 controls, and by high-density genotyping around association signals.

Findings—The SNP variant rs2511989, located within intron six of the *SERPING1* gene, showed highly significant genotypic association with age-related macular degeneration (uncorrected p=4·0×10^{-5}, corrected p=0·00372). We detected no evidence for association between disease and the other 31 candidate genes. The odds ratio for age-related macular degeneration in rs2511989 G/A heterozygotes compared with wild type G/G homozygotes was 0·63 (95% CI 0·47–0·84). A similar comparison of the A/A homozygotes with the wild type yielded an odds ratio of 0·44 (0·31–0·64). We replicated the observed genotypic association in a US cohort (p=0·008). Furthermore, a secondary high-density genotyping study across the *SERPING1* gene region identified five additional SNP variants similarly associated with age-related macular degeneration (rs2244169, rs2511990, rs2509897, rs1005510, and rs2511988).

Interpretation—Genetic variation in *SERPING1* significantly alters susceptibility to age-related macular degeneration. *SERPING1* encodes the C1 inhibitor, which has a crucial role in inhibition of complement component 1 (*C1*) and might implicate the classic pathway of complement activation in this disease.

Introduction

Age-related macular degeneration is the most common cause of blindness in developed countries.1,2 In the population-based Rotterdam study,3 64% of people aged 80 years or older showed signs of this disease. The prevalence of late or advanced stage age-related macular degeneration causing central blindness rises to 11·8% after 80 years of age.2 Therefore, because of its high prevalence, general practitioners and a wide variety of hospital specialists will also have many patients who have this disease. As our ageing population expands, the economic burden of this disease continues to increase every year. In the UK, the yearly economic burden has been estimated to be as much as €101·1 million (£80·3 million).4 The total yearly costs of health-care usage are seven-times higher for patients with age-related macular degeneration than for controls. This difference is largely attributable to a substantial decrease in independence and increased need for assistance with daily living.5

Clinically, age-related macular degeneration can be phenotyped with a grading system, such as that used in the Age Related Eye Disease Study (AREDS).6 In this classification, the early stages of disease are defined on the basis of abnormal changes in retinal pigment epithelial (RPE) (geographic atrophy, depigmentation, and increased pigment), and drusen characteristics (size, hard versus soft, distinct or indistinct, and total area). However, abnormal changes in advanced stage age-related macular degeneration are classified by the presence or absence of features such as detachment of RPE, serous (or haemorrhagic) sensory retinal detachment, hard exudates, subretinal pigment epithelial haemorrhage, and subretinal fibrous tissue. In the subset of patients who develop neovascular tissue, a rapid loss of central vision often occurs within days or weeks as this tissue progresses to a fibrovascular scar in the macula.

If urgent treatment is started with intravitreal injections of inhibitors of vascular endothelial growth factor, then this process can be stabilised in approximately 90% of patients and reversed in approximately 30%.7,8 However, these interventions are expensive9,10 and not universally available. Therefore, many patients still progress to legal blindness when affected with this disease.

Over recent years, age-related macular degeneration has proven an excellent model for the study of complex genetic diseases. In 2005, researchers made a major advancement in the understanding of this disease by determining that variants in the complement factor H (*CFH*) gene altered susceptibility to age-related macular degeneration.11–13 This finding added to previous evidence suggesting that complement activation contributed to this disease.14 Subsequent additional studies implicating other genes, some of which are involved in the inflammatory process, have added to understanding the cause of this disorder.15–18 However, not all the genes that predispose to age-related macular degeneration have been identified.

The serpin peptidase inhibitor, clade G (C1 inhibitor), member 1 is encoded by the *SERPING1* gene (Genbank accession NM_000062) and is a member of a large family of serine proteases. It was chosen as one of 32 gene targets for investigation in this study since the protein encoded by this gene (C1 inhibitor) plays a crucial part in suppressing the activity of the first component of complement (*C1*). Inhibition of *C1* prevents activation of complement components 2 and 4 (*C2* and *C4*) and so has several downstream effects on the complement cascade. The C1 inhibitor also inhibits several other serine proteinasesincludingplasmin, kallikrein, andcoagulation factors XIa and XIIa.19 *SERPING1* contains eight exons, and the product is transcribed from the positive strand of chromosome 11q12.1—a region of the genome that has not previously been implicated in family studies of age-related macular degeneration.20 Mutations in *SERPING1* that result in either dysfunctional protein or subnormal concentrations of protein have been shown to cause hereditary angioedema.21,22 We tested *SERPING1* and other candidate genes for their association with age-related macular degeneration.

Methods

Study design and patients

We undertook a case–control study between Jan 1, 2008, and Aug 20, 2008. The primary UK sample for the candidate gene screen consisted of 479 patients with age-related macular degeneration (cases) and 479 unaffected controls for whom DNA stocks were available from an existing cohort. Of the 479 cases with disease, 233 were diagnosed as having choroidal neovascularisation (subretinal neovascular tissue) in at least one eye. All participants were white, aged older than 55 years, and ascertained through the Southampton Eye Unit (UK) or research clinics undertaken (by AL) in Guernsey (UK). Control patients were either spouses or partners of patients with disease or those who presented at eye clinics for an unrelated eye disease. An experienced retinal specialist examined all participants. Controls underwent a dilated retinal examination to exclude any clinical signs of age-related macular degeneration. Cases and controls were classified as having or not having disease on the basis of the AREDS classification system (table 1).6 Recruitment was approved by the Southampton and Southwest Hants local research ethics committee and followed the tenets of the Declaration of Helsinki. All participants provided informed written consent and underwent a detailed ophthalmic examination to confirm both positive and negative diagnoses.

Procedures

We obtained a 10 mL peripheral blood sample; DNA was extracted according to the salting-out method23 and stored at –20°C. 250 ng of each DNA sample was plated out in ten 96-well plates and dispatched to the genotyping service of the Wellcome Trust Clinical Research Facility (WTCRF) in Edinburgh, UK, where samples were genotyped using the Illumina GoldenGate assay (Illumina, San Diego, CA, USA).24

We selected candidate genes on the basis of putative functional relevance, interaction with known genes of age-related macular degeneration, or previously implicated biological pathways. Tagging single nucleotide polymorphisms (SNPs) with optimal design scores24 on the Illumina assay were identified for genotyping.

We used an independent cohort to replicate significant findings, which consisted of 248 unrelated patients with the clinical diagnosis of age-related macular degeneration who were enrolled at the University of Iowa Department of Ophthalmology and Visual Sciences, IA, USA, after providing written informed consent. All patients had been examined by fellowship-trained retina specialists and were diagnosed with disease with the same criteria as the UK cohort (table 1). We also screened a control group of 252 unrelated patients with no history of macular degeneration. The patients and controls were all enrolled during the same period and by the same clinic. All participating US patients with disease and controls described themselves as white. We extracted DNA from peripheral blood by a previously described protocol;25 the *SERPING1* rs2511989 SNP was genotyped with a TaqMan predesigned SNP genotyping assay (Applied Biosystems, Foster City, CA, USA).

Eyes from human donors were obtained from the Iowa Lions Eye Bank (Iowa City, IA, USA) and were dissected and frozen within 5 h after death. From this tissue we collected the peripheral neural retina and the combined RPE-choroid layers separately. RNA was isolated

from frozen tissues with the RNeasy kit (Qiagen, Valencia, CA, USA), and complementary DNA generation and PCR were done as described previously.26 We used the following primers for PCR analysis—*SERPING1* F1: 5´-ATT CTC CTA CCC AGC CCA CT-3´; and *SERPING1* R1: 5´-GGC GTC ACT GTT GTT GCT TA-3´. Primers were designed to amplify a 437 bp fragment. We used omission of reverse transcriptase as a negative control for these experiments.

Statistical analyses were done with a combination of SPSS (version 14.0) and SAS (version 8.02).

Role of the funding source

The sponsors of the study had no role in the study design, data collection, data analysis, data interpretation, or writing of the report. SE and AL had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

We successfully screened 93 SNPs across 31 genes in the UK sample. SNP genotype frequencies were tested in controls and conformed to Hardy-Weinberg equilibrium. Where multiple SNPs were genotyped across one candidate, variants were in very strong linkage dis-equilibrium (LD). 92 of the 93 tested SNPs showed no evidence for association (p>0·01 before correction for multiple testing) and were not carried forward for further testing. Table 2 provides a list of all genes examined. Further information detailing the individual SNPs tested is available from the Vision Research group, Clinical Neurosciences Division at the University of Southampton. However, the rs2511989 SNP within the *SERPING1* gene exhibited a very strong signal of association (p=5·4×10^{-6}), which withstood a highly conservative Bonferroni correction for multiple testing for all 93 SNPs tested (p=0·0005). The genotyping call rate at this SNP was 99·5%. Table 3 shows the uncorrected and corrected p values for allelic and genotypic counts, and the odds ratios for the A allele, the GA heterozygote, and AA homozygote. Two additional SNPs, rs3758919 and rs4926, were also genotyped within *SERPING1*. rs4926 represents the only HapMap verified non-synonymous SNP in this gene, and alters an aminoacid at position 480 (V480M) in the translated protein. Neither of these additionally typed SNPs provided any evidence of association with age-related macular degeneration (uncorrected p=0·94, uncorrected p=0·30, respectively).

Although samples from Guernsey had an equal proportion of cases and controls, and the genotypes from Southampton and Guernsey showed no evidence for heterogeneity at the rs2511989 SNP (p=0·6), we applied genomic control to allow for possible stratification because of mixed samples. With use of Devlin and Roeder's method,27 we computed a correction factor (λ=1·21) that had no significant effect on our findings when based on either the uncorrected p value (p=4·5×10^{-6}) or the corrected p value (p=0·0004) for the number of SNPs tested.

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Within both UK and US samples, the control groups were significantly younger than were the patients with age-related macular degeneration (table 1). This difference in age might reduce power by potentially diluting the control sample with (as yet undeveloped) cases. This discrepancy would therefore be expected to make the strength of findings more conservative since any observed association tests would be underestimated.

The rs2511989 variant is a non-coding SNP found in intron six of *SERPING1*. The rs2511989 A allele and the AA genotype occur less frequently in cases than in controls. This skewed distribution, whereby the (moderately) rarer allele occurs significantly more frequently in controls than in cases, suggests that it exerts some protective effect against macular degeneration—thus, the more commonly encountered G allele represents the risk allele for age-related macular degeneration at this locus.

In the UK patient samples only, we used the AREDS categorisation and applied the Jonckheere-Terpstra test for trend to examine for effects of carrying zero, one, or two alleles at the rs2511989 locus and observed no significant trend across the AREDS grades. However, our sample size was adequate only to detect quite large effects, and was not powered to detect more modest but nevertheless potentially important effects.

To replicate this finding, the rs2511989 SNP was genotyped in an independent US sample of patients with age-related macular degeneration and controls who were recruited from the University of Iowa, using the TaqMan method. We observed a very similar profile of allelic and genotypic counts at the rs2511989 SNP within this distinct sample (table 3). The level of significance was strongest in the UK sample for the allelic comparison (table 3). However, despite smaller numbers in the US sample, results were very similar to those for the UK sample after correction. The observed odds ratios for the AA genotype were very similar between studies, with a slightly narrower confidence interval in the UK samples than in the US samples due to greater sample size (table 3).

We observed no evidence of heterogeneity in the distribution of rs2511989 genotypes between the UK and US samples, with both showing the same trend of an excess of the rarer AA genotype within the control group (table 3). Combining genotype counts from the two samples yielded a p value for genotypic association of $6 \cdot 08 \times 10^{-7}$ for this variant with age-related macular degeneration (table 3).

Our modest preliminary scan of the *SERPING1* gene produced one highly associated SNP (rs2511989) and two non-associated SNPs (rs3758919 and rs4926). Although observation of non-associated SNPs within the immediate genomic vicinity of strongly associated SNPs is not unusual,[11] we would expect that other variants within the region should provide supporting evidence for the association. Therefore, we undertook a secondary scan of *SERPING1* with all common (minor allele frequency >0·05) tagging SNP variants across the 15 kilobase (kb) gene (n=3) and additional SNP variants both 5´ and 3´ of the gene (n=5). All additional SNPs were non-coding and had no reported functional relevance. DNA from the UK sample (n=958) was dispatched to KBioscience for genotyping with KASPar chemistry as previously described. For quality control purposes, we included the rs2511989 SNP to be re-genotyped alongside eight additional SNPs.

At the rs2511989 SNP, 26 samples were not assigned genotype calls with the KBioscience platform, which compares to five such no calls with the Illumina platform in the initial analysis. No sample failed on both platforms. The 927 samples successfully genotyped at the rs2511989 SNP on both platforms showed 99·9% concordance. All SNPs conformed to Hardy Weinberg equilibrium as tested in the control sample. We used the Cochran-Armitage test for trend[28] to examine association and plotted the negative natural log of the resultant p values (figure 1). Of the eight new SNPs genotyped in this secondary scan of the gene (red dots), five showed very strong significance, supporting the findings for rs2511989, and withstood a conservative Bonferroni correction for multiple testing of 93 SNPs in the original scan plus eight additional SNPs in the secondary *SERPING1* scan (dashed green line). One SNP, rs2509897, which lies 2·5 kb 5´of the transcriptional start site of the *SERPING1* gene and adjacent to a predicted promoter region, exhibited the strongest signal for association ($p=6\cdot17\times10^{-6}$). The region encompassing all 11 SNPs showed very strong LD with pairwise D´=1 between the leftmost rs2649663 and rightmost rs10896632 SNPs in the ^HapMap Ceu data.

We assessed the association between *SERPING1* and age-related macular degeneration in the presence of other genetic and environmental factors. We used stepwise logistic regression analysis of disease affection status against our most associated SNP genotypes from the *CFH* gene, Chr10q locus, and *SERPING1* (rs1061170, rs11200638, and rs2509897, respectively) and controlled for age, sex, and smoking status. The rs2509897 SNP entered the model and was significant for association in the presence of these confounding factors (p=0·001).

In RT-PCR experiments, amplification of a PCR product of the appropriate molecular weight was obtained from complementary DNA from both neural retina and RPE-choroid (figure 2). This finding is consistent with expression microarray data from the same tissues (R Mullins, unpublished data) and indicates that cells in the neural retina and RPE or choroid, or both, synthesise *SERPING1* mRNA locally.

Discussion

Of the 32 genes that we screened for association with age-related macular degeneration in a UK cohort, 31 showed no evidence of association with this disease. However, we identified a strong association signal between a genetic variant in *SERPING1* and age-related macular degeneration, and replicated this finding in an independent sample. Our results indicate that the slightly rarer rs2511989 AA genotype is present significantly more frequently in controls than in cases and provides a protective effect against development of age-related macular degeneration.

We also genotyped a non-coding SNP situated about 2 kb 5´ of *SERPING1* (rs3758919) and a non-synonymous SNP at the 3´end of the gene (rs4926) in *SERPING1* as part of the preliminary candidate gene scan, but we observed no association with age-related macular

degeneration. Therefore, a secondary higher density genotyping experiment was undertaken across the *SERPING1* gene region. This secondary scan revealed five additional SNPs from across the region that also showed very strong association with the disease. The most significantly associated SNP, rs2509897, lies in the promoter region of the gene. However, in view of the very strong LD evident across *SERPING1*, further detailed functional studies are needed to identify which other genetic variants (SNPs, microsatellites, insertions, deletions) lie in close genetic proximity to our markers and to assess their possible contribution to expression and function. As with many other genes, multiple variants across this locus might affect disease status or severity.

RT-PCR of *SERPING1* mRNA (figure 2) shows expression of *SERPING1* mRNA in both retina and RPE-choroid layers of eyes from human donors. The expression of *SERPING1* in the tissues predominantly affected by age-related macular degeneration adds to the evidence that this protein might have a regulatory role for the complement system in ocular physiology.

The complement system is a powerful component of innate immunity which recognises and facilitates the elimination of pathogens and unwanted host material.[29] Variants within several genes that code for proteins involved in the complement cascade are recognised to either significantly increase the risk of age-related macular degeneration (*CFH*[11–13] and complement *C3*[18]) or decrease this risk (*C2* and factor B[16] and deletion of *CFH* related genes *CFHR1* and *CFHR3*[30]).

The protein product of *SERPING1* regulates the first component of complement (*C1*) by inhibition of the proteolytic activity of its subcomponents C1r and C1s.[19] It is a member of a large serine protease inhibitor (serpin) gene family and also inhibits several other serine proteinases including plasmin, kallikrein, and coagulation factors XIa and XIIa.[19] Mutation in *SERPING1* causes hereditary angioedema.[21] Inhibition of the proteolytic subcomponents of *C1* via genetic variation in *SERPING1* might implicate the classic pathway of complement activation in age-related macular degeneration.

In summary, our study shows a strong association between age-related macular degeneration and *SERPING1*, with supporting evidence from an independent replication and a secondary high-density scan of the gene. Further studies are now required to assess this association in independent populations of various ethnic origins. Furthermore, functional studies that are capable of discriminating between variants that cause disease and those which are co-inherited with a causal mutation, are now needed. Our findings add to the growing understanding of the genetics of age-related macular degeneration, which should ultimately lead to novel treatments for this common and devastating disease.

Acknowledgments

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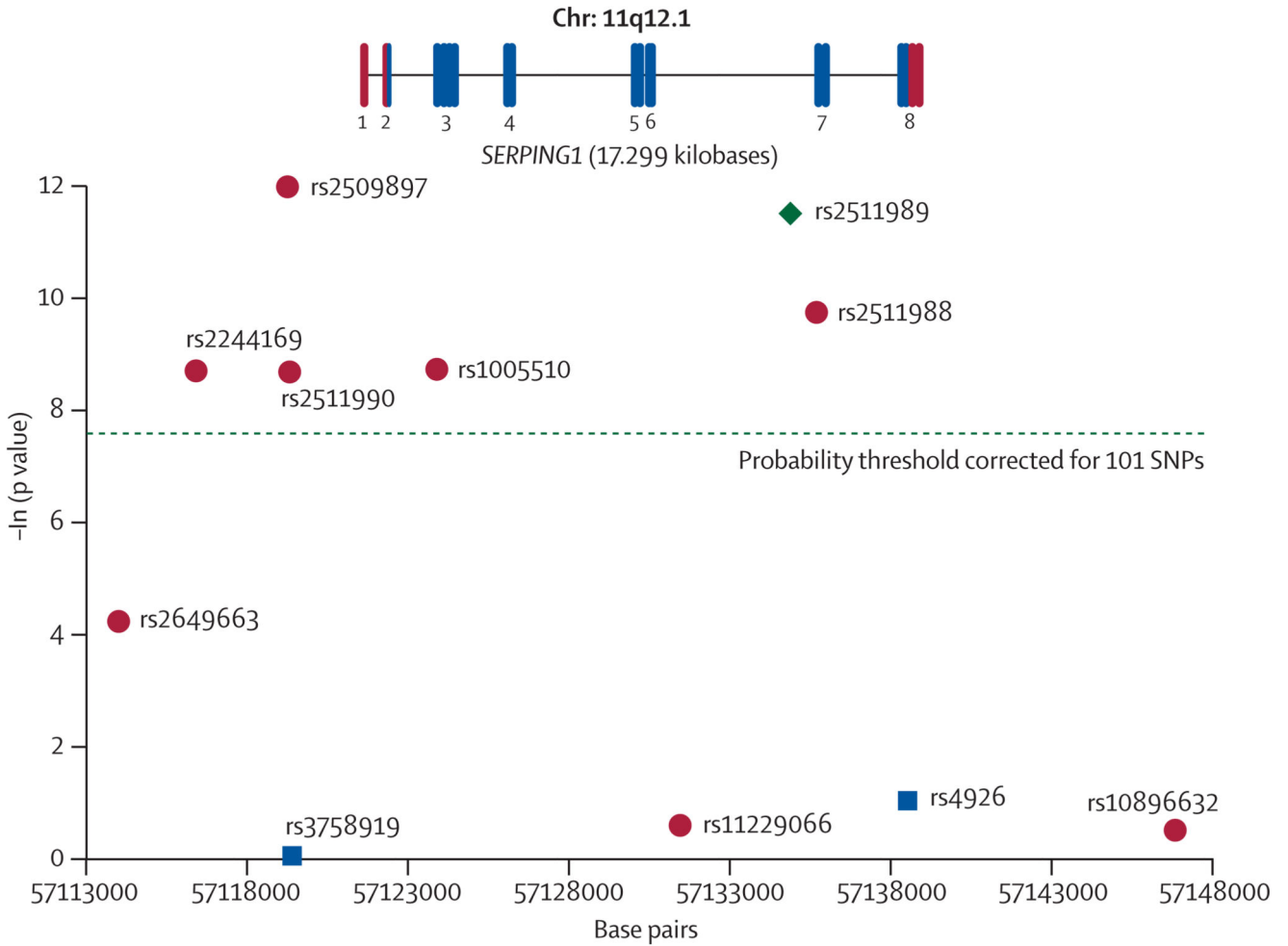
Figure 1. Association across *SERPING1* region with age-related macular degeneration
The x-axis shows the relative positions of the 11 single nucleotide polymorphisms (SNPs) genotyped in the UK sample using the University of California Santa Cruz, March, 2006, reference sequence (NCBI build 36.1).
The green diamond represents rs2511989, which was genotyped in both the original scan and the follow-up scan. The blue squares depict SNPs genotyped in the initial candidate gene scan. The red dots depict the SNPs that were typed in the follow-up scan only. The probability threshold for significance Bonferroni corrected for the 93 SNPs genotyped in the initial scan plus the eight additional SNPs genotyped in the follow-up is represented by a dashed green line. The y-axis shows the negative natural log of the p value for association using the Cochrane-Armitage test. The *SERPING1* gene is drawn to scale and shows all eight exons. Exon one is untranslated (red bars), whereas exons two and eight are partially translated. Exons three to seven are translated (blue bars).
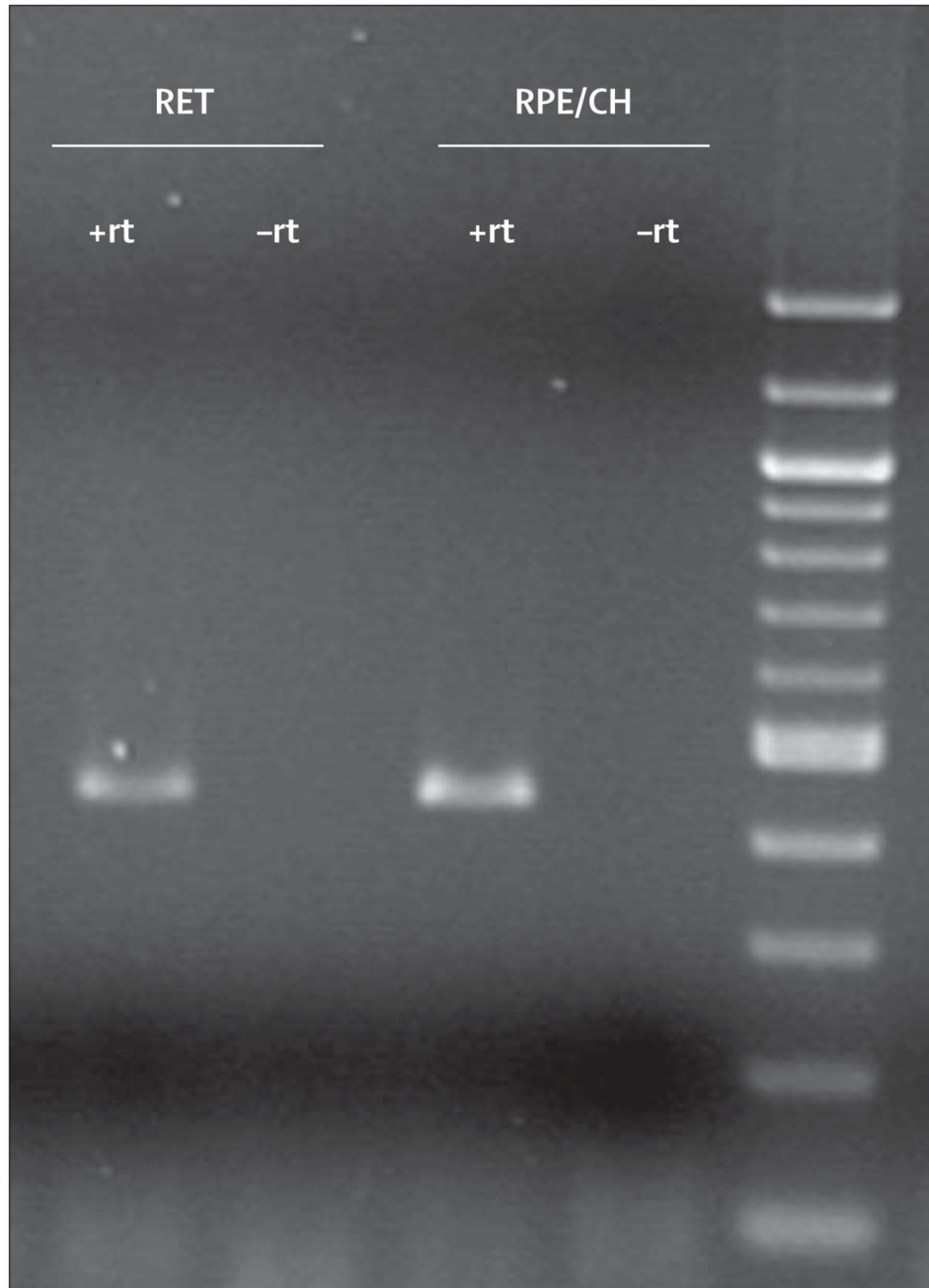
Figure 2. Expression analysis with RT-PCR showing amplification of complementary DNA for SERPING1 in both neural retina (RET) and RPE-choroidal tissues (RPE/CH)
No product was amplified from either tissue when the reverse transcription step was omitted (−rt).

Table 1

Grade of age-related macular degeneration according to the Age Related Eye Disease Study (AREDS) for both UK and US cohorts

	UK sample			US sample		
	n	Mean age (SD [years])	Male:female ratio	n	Mean age (SD [years])	Male:female ratio
Controls						
0	479	70·59 (9·35)	0·94	252	74·00 (9·04)	0·87
Cases						
2	79	71·35 (9·76)	0·72	6	61·17 (8·08)	0·00
3	119	80·61 (8·31)	0·59	48	76·92 (9·50)	0·71
4	281	78·46 (7·87)	0·60	194	82·85 (7·93)	0·67
All cases	479	77·85 (8·83)	0·61	248	81·18 (9·12)	0·65

Table 2

Genes tested in primary screen

	HUGO gene symbol	HUGO gene name	Location
1	ITGAM	Integrin, alpha M (complement component 3 receptor 3 subunit)	16p11.2
2	C1QTNF1	C1q and tumour necrosis factor related protein 1	17q25
3	CD59	CD59 molecule, complement regulatory protein	11p13
4	MASP1	Mannan-binding lectin serine peptidase 1 (C4/C2 activating component of Ra-reactive factor)	3q27-q28
5	MASP2	Mannan-binding lectin serine peptidase 2	1p36.3-p36.2
6	CR2	Complement component (3d/Epstein Barr virus) receptor 2	1q32
7	CR1	Complement component (3b/4b) receptor 1 (Knops blood group)	1q32
8	CRP	C-reactive protein, pentraxin-related	1q21-q23
9	VTN	Vitronectin	17q11
10	CD55	CD55 molecule, decay accelerating factor for complement (Cromer blood group)	1q32
11	CD46	CD46 molecule, complement regulatory protein	1q32
12	ITGB2	Integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)	21q22.3
13	CLU	Clusterin	8p21-p12
14	C4BPA	Complement component 4 binding protein, alpha	1q32
15	C4BPB	Complement component 4 binding protein, beta	1q32
16	ELN	Elastin (supravalvular aortic stenosis, Williams-Beuren syndrome)	7q11.1-q21.1
17	C8A	Complement component 8, alpha polypeptide	1p32.2
18	C8B	Complement component 8, beta polypeptide	1p36.2-p22.1
19	C9	Complement component 9	5p14-p12
20	C6	Complement component 6	5p13.1
21	C7	Complement component 7	5p13.1
22	SERPING1	Serpin peptidase inhibitor, clade G (C1 inhibitor), member 1, (angioedema, hereditary)	11q12-q13.1
23	APP	Amyloid beta (A4) precursor protein (peptidase nexin-II, Alzheimer's disease)	21q21.2
24	SERPINA3	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3	14q32.1
25	RFX5	Regulatory factor X, 5 (influences HLA class II expression)	1q21
26	FGA	Fibrinogen alpha chain	4q28
27	FGB	Fibrinogen beta chain	4q28
28	FGG	Fibrinogen gamma chain	4q28
29	PSEN1	Presenilin 1 (Alzheimer's disease 3)	14q24.3
30	PSEN2	Presenilin 2 (Alzheimer's disease 4)	1q31-q42
31	CYP46A1	Cytochrome P450, family 46, subfamily A, polypeptide 1	14q32.1
32	C1QTNF5	C1q and tumour necrosis factor related protein 5	11q23.3

Table 3

Allelic and genotypic tests of rs2511989 from primary screen of UK sample (n=953), US sample (n=500), and the two cohorts combined

	UK cohort					US cohort				Combined			
	Case	Control	OR (95% CI)	Uncorrected p value	Corrected p value*	Case	Control	OR (95% CI)	p value	Case	Control	OR (95% CI)	p value
Allelic				5.4×10^{-6}	0.0005				0.0037				7.49×10^{-8}
G	597 (63%)	500 (52%)	..	..	..	322 (65%)	282 (56%)	..	..	919 (64%)	782 (54%)	..	..
A	355 (37%)	454 (48%)	0.65 (0.55–0.79)[†]	..	..	174 (35%)	222 (44%)	0.69 (0.53–0.89)[†]	..	529 (37%)	676 (46%)	0.67 (0.57–0.77)[†]	..
Genotypic				4.0×10^{-5}	0.0037				0.0080				6.08×10^{-7}
GG	191 (40%)	132 (28%)	..	..	..	100 (40%)	79 (31%)	..	..	291 (40%)	211 (29%)	..	..
GA	215 (45%)	236 (50%)	0.63 (0.47–0.84)[‡]	..	..	122 (49%)	124 (49%)	0.77 (0.53–1.14)[‡]	..	337 (47%)	360 (49%)	0.68 (0.54–0.86)[‡]	..
AA	70 (15%)	109 (23%)	0.44 (0.31–0.64)[‡]	..	..	26 (11%)	49 (19%)	0.42 (0.24–0.73)[‡]	..	96 (13%)	158 (22%)	0.44 (0.32–0.60)[‡]	..

*
Corrected for 93 single nucleotide polymorphisms.

[†]
Relative to G allele.

[‡]
Relative to wildtype (GG) homozyte.