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Interleukin-8 promoter polymorphism –251A/T is a risk factor for age-related macular degeneration

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Abstract

Background/aims—To determine whether four expression-related cytokine polymorphisms are associated with age-related macular degeneration (AMD).

Methods—DNA from 478 cases with AMD and 555 normal controls was genotyped for the pro-inflammatory IL1β –511C/T, IL6 –174C/G, IL8 –251A/T and anti-inflammatory IL10 –1082G/A cytokine polymorphisms using the 5′ nuclease TaqMan® assay for allelic discrimination. Associations with AMD were analysed using allelic frequencies.

Results—The –251A allele of the IL8 promoter gene polymorphism was more prevalent in AMD patients than controls (p = 0.037, OR = 1.21, 95% CI = 1.01 to 1.44). Adjusting for age, sex, body mass index (BMI), current smoking and past smoking status did not alter the AMD association significantly (corrected p value = 0.043, OR = 1.23, 95% CI = 1.0 to 1.50).

Conclusion—The pro-inflammatory homozygous IL8 –251AA genotype is an important risk factor for AMD. This may have implications for future therapy with biological agents that could target this cytokine.

Increasingly evidence is emerging that variations in cytokine expression (and in the underlying genes which control their expression) may modulate susceptibility to age-related macular degeneration (AMD). Cytokines such as interleukin-6 (IL6) and interleukin-8 (IL8) are released by degenerating retinal pigment epithelial cells and associated with drusen in AMD.1 2 C-reactive protein (CRP) has been shown to be a biomarker for AMD.3 4 The main inflammatory cytokine stimulus for CRP is interleukin-6, and this has also been recently associated with AMD.4 5

Pro-inflammatory cytokines have also been shown to upregulate intercellular adhesion molecule-1 (ICAM1) at microglial sites,6 which along with locally released cytokines, may

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lead to the recruitment of inflammatory cells seen in AMD. IL8 is a primary mediator of angiogenesis, and this has been demonstrated in various carcinomas.7 8 Interleukin-10 (IL10) is a potent anti-inflammatory cytokine produced by T cells, macrophages, and retinal pigment epithelial cells.9 10 It strongly inhibits antigen-specific T cell proliferation and cytokine production, and downregulates both human leucocyte antigen (HLA) class 1 and class 2 antigen expression.11 12 IL10 is also important in the induction of antigen-specific anergy or tolerance.13 Genotypes associated with high IL10 expression have a protective role (possibly via inhibition of angiogenesis),14 15 while genotypes associated with high IL8 expression could be risk factors for stimulation of angiogenesis in AMD.

Based on this evidence for a possible role of cytokines in AMD, we chose four known expression-related cytokine polymorphisms in interleukin genes, pro-inflammatory IL1β –511C/T (rs16944), IL6 –174C/G (rs1800795), IL8 –251A/T (rs4073) and anti-inflammatory IL10 –1082G/A (rs1800896), to test the hypothesis that they may be associated with AMD.

Methods

The study was approved by the Southampton Local Research Ethics Committee (approval no. 347/02/t) and followed the tenets of the Declaration of Helsinki. Caucasian subjects over the age of 55 with a diagnosis of AMD and normal Caucasian controls over age 55 were recruited from general ophthalmology clinics at Southampton Eye Unit, UK. Patients for the study underwent a detailed ophthalmic examination to characterise AMD phenotypes. All cases and controls were also screened for the presence of cataract and glaucoma. Stereoscopic fundus photographs and fluorescein angiograms were recorded using a Topcon digital retinal camera (model TRC50IX). These photographs and angiograms were graded by a masked observer into dry and wet AMD groups, the choroidal neovascularisation (CNV) group was further categorised into occult, minimally classic and predominantly classic subgroups. General health was assessed, and care was taken to exclude patients who reported any infective illness in the preceding month. Information was also obtained about any family history of AMD, relevant past medical history, smoking history, ocular history, use of medications, vitamin or dietary supplementation. Height, weight and body mass index (BMI) measurements were recorded. A 10 ml peripheral blood sample was collected from these patients, and DNA was then extracted using the salting out method16 and stored at –20°C.

SNP Genotyping

All single nucleotide polymorphisms (SNPs) were genotyped using the 5′ nuclease assay for allelic discrimination. Primers and individual fluorogenic TaqMan® probes (consisting of an oligonucleotide labelled with both a fluorescent reporter dye, FAM, and a quencher dye, VIC) were designed using Primer Express software (version 2.0; sequences shown in table 13). These were then obtained from Applied Biosystems (Warrington, UK). All assays were performed using the supplied Q-PCR buffer (2.5 μl), PCR probe mix (0.125 μl) and 20 ng of genomic DNA in 2.375 μl of dH_2O to make a 5 μl reaction volume (384-well plate). The PCR thermocycling protocol consisted of 10 min at 95°C, followed by 40 cycles of 15 s at 92°C and 1 min at 60°C. Each genotyping plate contained eight wells without any DNA

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template (water controls) and randomly selected duplicate samples (10% of plate samples). Allelic level genotyping from fluorescence measurements was then obtained using the ABI PRISM 7900HT Sequence Detection System. SDS version 2.1 software was used to analyse real-time and endpoint fluorescence data.

Statistical Analysis

Allelic and genotype distributions for each SNP were tested for conformity to Hardy–Weinberg equilibrium using control subjects. Alleles at each locus were said to be in Hardy–Weinberg equilibrium if the observed homozygote and heterozygote frequencies did not differ significantly ($p>0.05$) from expected frequencies. All p values were evaluated using Pearson's chi-squared statistic. The level of statistical significance in the study was set at $p<0.05$. All statistical analyses were performed using the SPSS statistical software package version 12.0.

Results

Baseline demographics of the unrelated Caucasian cohort recruited from Southampton eye unit (478 cases with AMD and 555 normal controls) are as described in table 2. AMD grading data from fundus photographs and angiograms were available for 283 (59.2%) AMD cases.

Genotype distributions were in Hardy–Weinberg equilibrium in the control groups. The IL8 –251AA genotype was found in a larger proportion of AMD cases (35% vs 27%). This genotype (see table 3) was significantly associated with AMD both before ($p = 0.037$, odds ratio (OR) = 1.21, 95% CI = 1.01 to 1.44) and after correcting for co-variables like age, sex, BMI, current smoking and past smoking status (corrected p value ($p_C = 0.043$, OR = 1.20, 95% CI = 1.0 to 1.50). Fluorescein angiographic data were available for 283 AMD patients. This subset of patients were then further categorised into CNV or dry AMD phenotypes (table 4). The CNV group included occult, minimally classic and predominantly classic CNV forms. However, no significant difference in allele frequencies was found on testing IL8 –251A risk allele frequencies in total CNV versus dry AMD and total CNV versus controls. Further analysis stratifying by CNV subtype showed a marginally significant difference in allele distribution between the minimally classic CNV group and normal controls, without correcting for multiple testing ($p = 0.04$, OR = 1.59, 95% CI = 0.92 to 2.71). Consistent with established data, we found evidence for a strong association between AMD status and smoking behaviour dichotomised as never smoked/ever smoked ($p = 2.0\times10^{-14}$). However, we found no significant association between IL8 genotype status and smoking behaviour (data not shown).

Discussion

Identifying genetic determinants associated with AMD17–20 provides new insights into disease pathogenesis and also assists in defining an "at risk" AMD population. This may allow preventive measures to be instituted. In this study, interleukin polymorphisms that functionally influence transcription levels and are associated with autoimmune diseases21

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22 were tested for association with AMD. To the best of our knowledge, none of the polymorphisms selected have been studied so far for association with AMD.

The IL8 –251A/T polymorphism has been previously associated with many inflammatory diseases and cancers.23 24 Our results show a similar association for AMD and the homozygous IL8 –251AA genotype ($p = 0.037$). This significance was preserved even after correcting for factors such as smoking ($P_C = 0.043$). This is of note as IL8 production has previously been associated with smoking, and the IL8 –251A/T genotype has been found to deter the initiation of smoking behaviour in a Japanese population.25 This pro-angiogenic genotype has also been shown to induce angiogenesis in rat cornea.26 Human recombinant IL8 implanted into rat cornea was found to induce vascular proliferation, and a similar proliferation and chemotaxis was also seen in human umbilical vein endothelial cells in this study. The IL8 –251AA genotype seems to act by influencing IL8 production. In a study of patients with respiratory bronchiolitis, the highest serum levels of IL8 were associated with the AA genotype.23 Alternatively, this polymorphism may also be in linkage disequilibrium with functional variants elsewhere in the IL8 loci or in neighbouring genes.

During the above analysis, the mean age of our control population was found to be lower compared with our cases (mean age 69.1 vs 78.4); however, controlling for age during regression analysis did not alter the described associations. All cases and controls in the study were aged 55 or older and therefore in the at-risk age group/range in which AMD is defined as occurring.27–29 Although the mean age of our controls is slightly lower, this would mean that any IL8 association found would represent an underestimate rather than an overestimate. True IL8 association with AMD may therefore be stronger than that indicated by this study.

In summary, our results demonstrate that the homozygous IL8 –251AA genotype (A allele) is a risk factor for AMD. Following on from this hypothesis-generating study, replication studies are needed to confirm these associations. In addition, where possible these should be complemented by functional studies to determine the contribution of this gene to the development of AMD and the angiogenic processes in AMD. This would help develop genetic screening tests to identify individuals at risk of developing AMD or those who may benefit from focused anti-inflammatory treatments.

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Table 1

Primer and probe sequences used for 5′ nuclease (TaqMan®) genotyping

Primer/probe	Sequence
IL1β-511 forward	5′ GGTCTCTACCTTGGGTGCTGTT 3′
IL1β-511 reverse	5′ TCCTCAGAGGCTCCTGCAAT 3′
IL1β-511 C probe	VIC—TGCCTC**G**GGAGCT—MGB
IL1β-511 T probe	FAM—TCTGCCTC**A**GGAGC—MGB
IL6 –174 forward	5′ GCTGCACTTTTCCCCCTAGTT 3′
IL6 –174 reverse	5′ GCTGATTGGAAACCTTATTAAGATTGT 3′
IL6 –174 G probe	VIC—CTTTAGCAT**C**GCAAGAC—MGB
IL6 –174 C probe	FAM—CTTTAGCAT**G**GCAAGAC—MGB
IL8 –251 forward	5′ GTCACATGGTCTATGATAAAGTTATCTAGAAATA 3′
IL8 –251 reverse	5′ TACATTTAAAATACTGAAGCTCCACAATTT 3′
IL8 –251 A probe	VIC—AAGCATACA**T**TTGAT AATT—MGB
IL8 –251 T probe	FAM—AAGCATACA**A**TTGATAATT—MGB
IL10 –1082 forward	5′ ACACACAAATCCAAGACAAACACTACTAA 3′
IL10 –1082 reverse	5′ GGAGGTCCCTTACTTTCCTCTTACC 3′
IL10 –1082 G probe	ATCCCTACTTCCCC**C**TCCCAAAGAA—MGB
IL10 –1082 A probe	CCCTACTTCCCC**T**TCCCAAAGAAGC—MGB

Forward and reverse sequences are primers. VIC and FAM probe sequences have positions of single nucleotide polymorphisms marked in bold. MGB, minor groove binding probe.

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Table 2

Basic available demographic characteristics of the study population

	AMD cases	Controls
	Total n = 478 (%)	**Total n = 555 (%)**
Age	n = 478	n = 555
Mean	78.8	69.0
Range	55–101	55–91
SD	7.9	9.7
Sex	n = 478	n = 555
Female	312 (65.4)	306 (55.1)
Male	166 (34.2)	249 (44.9)
BMI	n = 469	n = 551
Mean	26.3	26
Range	13.9 to 43.0	15.7 to 55.4
Smoking status	n = 478	n = 555
Ever smoked	301 (63.0)	323 (58.2)
Never smoked	177 (37.0)	232 (41.8)

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Table 3

IL genotype frequencies and their association with AMD cases and normal controls

	IL1β –511C/T		IL6 –174G/C		IL8 –251A/T*		IL10 –1082G/A	
	Case n = 475 (%)	**Control n = 548 (%)**	**Case n = 462 (%)**	**Control n = 553 (%)**	**Case n = 474 (%)**	**Control n = 540 (%)**	**Case n = 473 (%)**	**Control n = 551 (%)**
IL genotype	CC 218 (46)	251 (46)	GG 156 (34)	178 (32)	AA 166 (35)	147 (27)	GG 126 (26)	140 (25)
	CT 225 (47)	240 (43)	GC 223 (48)	270 (49)	AT 206 (43)	269 (50)	GA 230 (49)	270 (49)
	TT 32 (7)	57 (11)	CC 83 (18)	105 (19)	TT 102 (22)	124 (23)	AA 117 (25)	141 (26)
IL allele	C 661 (70)	742 (68)	G 535 (58)	626 (57)	A 538 (57)*	563 (52)	G 482 (51)	550 (49)
	T 289 (30)	354 (32)	C 389 (42)	480 (43)	T 410 (43)	517 (48)	A 464 (49)	552 (51)
p Value	0.36		0.56		0.037*		0.64	
OR (95% CI)	1.09 (0.91 to 1.32)		1.05 (0.88 to 1.26)		1.21 (1.01 to 1.44)		1.04 (0.88 to 1.24)	

*p Values by χ^2 test with 1 degree of freedom (df). P_C = 0.043, OR = 1.20, 95% CI = 1.0 to 1.50 (p Value adjusted for age, sex, BMI, current smoking and past smoking status). Sample size (n) data are less for some IL genotypes due to few failed genotyping reactions.

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Table 4

Risk for CNV associated with IL8 –251A/T genotypes and alleles in comparison with dry AMD and control groups

	All CNV	Dry AMD	Controls
	n = 150 (%)	n = 133 (%)	n = 540 (%)
IL8 –251A/T genotype			
AA	48 (32.0)	39 (29.3)	147 (27.0)
AT	68 (45.3)	67 (50.4)	269 (50.0)
TT	34 (22.7)	27 (20.3)	124 (23.0)
IL8 –251A/T allele			
A	164 (54.7)	145 (54.5)	563 (52.0)
T	136 (45.3)	121 (45.5)	517 (48.0)
‡Versus dry AMD	0.48, 1.0*		0.24, 1.10†
(p value, OR, 95% CI)	(0.72 to 1.40)		(0.84 to 1.44)
§Versus controls	0.21, 1.10		
(p value, OR, 95% CI)	(0.85 to 1.43)		

p Values by χ^2 test with 1 degree of freedom (df). Analysis was restricted to 283 patients in the AMD group where fluorescein angiographic data were available.

*Odds ratio for minimally classic CNV versus dry AMD was 1.59 (p = 0.04, 95% CI = 0.92 to 2.71).

†Allele association for dry AMD as compared with normal controls.

‡Allele association for CNV as compared with dry AMD and as compared with normal controls§.