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Analysis of single nucleotide polymorphisms in the *NOS2A* gene and interaction with smoking in age-related macular degeneration

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SUMMARY

Age-related macular degeneration (AMD) is a complex degenerative retinal disease influenced by both genetic and environmental risk factors. We assessed whether single nucleotide polymorphisms (SNPs) in the *NOS2A* gene increase risk and modulate the effect of smoking in AMD. 998 Caucasian subjects (712 AMD cases and 286 controls) were genotyped for 17 SNPs in *NOS2A*. Multivariable logistic regression models containing SNP genotypes, age, sex, smoking status and genotype/smoking interaction were constructed. SNP rs8072199 was significantly associated with AMD (OR = 1.3; 95% CI : 1.02, 1.65; $P = 0.035$). A significant interaction with smoking was detected at rs2248814 ($P = 0.037$). Stratified data by genotypes demonstrated that the association between AMD and smoking was stronger in carriers of AA genotypes (OR = 35.98; 95% CI: 3.19, 405.98) than in carriers of the AG genotype (OR=3.05; 95% CI: 1.36, 6.74) or GG genotype (OR=2.1; 95% CI: 0.91, 4.84). The results suggest a possible synergistic interaction of AA genotype with smoking, although the result bears replication in larger samples. Our data suggests that SNPs in the *NOS2A* gene are associated with increased risk for AMD and might modulate the effect of smoking on AMD.

Keywords

association; age-related macular degeneration; polymorphism; gene-environment interaction

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INTRODUCTION

Age-related macular degeneration (AMD) is a degenerative disorder that is characterized by loss of central vision due to the loss of the photoreceptors and retinal pigment epithelium (RPE) in the macula, an area in the central retina responsible for visual acuity (Jager et al., 2008). It is the main cause of irreversible visual loss in the elderly in developed countries (Resnikoff et al., 2004). AMD affects over 8 million Americans and its prevalence increases with age. The overall disease prevalence is also expected to increase to at least 50% by 2020, due to the increasing age of the American population (Friedman et al., 2004). AMD affects Caucasians more than other races

Linkage and association studies have reported genes that are risk factors for developing the disease; the most well-replicated of these are: complement factor H (*CFH*), complement factor B (*CFB*) and complement component 2 (*C2*), *ARMS2* (LOC387715)/HTRA1 complement component 3 (*C3)*, and apolipoprotein E (*APOE)* (Edwards & Malek, 2007; Swaroop et al., 2009). However, although these genes have been associated with AMD, some of the mechanisms by which they exert their actions as well as the role of possible unknown genes still remain to be elucidated.

In addition, several other non-modifiable risk factors such as age, female gender, and race; modifiable factors such as antioxidant intake, smoking, hypertension and obesity have also been associated with risk of AMD (Klein et al., 2004; Tomany et al., 2004). Of these, cigarette smoking is the strongest, most well-established risk factor for AMD. Most recently, it has been demonstrated in an animal model that mice exposed to smoking developed signs of degeneration in the RPE and Bruch's membrane, structures that are intimately involved in the AMD disease pathogenesis (Fujihara et al., 2008).

There have also been important environmental-gene interactions reported with smoking, e.g., cigarette smoking has demonstrated a synergistic interaction with genotypes of the *ARMS2* locus (Schmidt et al., 2006) and a joint effect with genotypes of the *APOE* gene (Schmidt et al., 2005); however, other groups have reported contradictory results (Conley et al., 2006; DeAngelis et al., 2007; Hughes et al., 2007). Although the pathophysiology of the disease is not clearly understood, it is well accepted that both oxidative stress and an abnormal inflammatory activation play important roles in the disease pathogenesis (Montezuma et al., 2007, Zarbin, 2004). The nitric oxide synthase (NOS) enzyme system is known to participate in both phenomena and it has been suggested that it plays an active role in advanced AMD stages (Campochiaro, 2000; She et al., 2007).

In addition, *in vitro* studies of glial cell cultures and murine lung epithelium cells demonstrated a direct interaction between smoking and the *NOS2A* gene that codes for the inducible form of nitric oxide synthase (iNOS). Cells exposed to smoke condensates demonstrated a reduction in iNOS protein expression and enzymatic activity (Hoyt et al., 2003; Mazzio et al., 2005) decreasing therefore the oxidative stress pathway activation. The *NOS2A* gene is an attractive AMD candidate gene given its interaction with smoking and its role in host defense, inflammation and neovascularization. The purpose of our study was to assess the main effect of single nucleotide polymorphisms (SNPs) in the *NOS2A* gene in AMD as well as a possible interaction with smoking.

MATERIALS AND METHODS

Subjects

Individuals were recruited from the Duke University Eye Center (DUEC), the Vanderbilt Eye Institute (VEI) and the Bascom Palmer Eye Institute (BPEI) at the University of Miami,

Miller School of Medicine under research protocols approved by the Institutional Review Boards at each institution. Written informed consent was obtained from all participants.

All study subjects were examined by a retinal specialist. The patients were examined by slit-lamp biomicroscopy and dilated fundus examination, including indirect ophthalmoscopy. In addition, fundus imaging was obtained in all patients. The pictures were graded using a modified grading system based on the Age-Related Eye Disease Study (AREDS) which has been previously described in detail (Schmidt et al., 2000). Briefly, the grading system was scored from 1 to 5. The 1 and 2 categories corresponded to controls. The rest corresponded to mild (grade 3) and advanced (grades 4 and 5) stages of AMD (For the complete grading list please see Supplemental Table 1).

In addition, a detailed smoking history was obtained by self-administered questionnaire. Participants were asked if they had smoked more than 100 cigarettes in their life time; an affirmative answer lead to the description of the amount of cigarettes per day, age they had started smoking, if they had quit, and when. From these measures, a binary measure of 'ever' smoking and pack-years of exposure were calculated.

We included in the study a total of 998 non-Hispanic Caucasian participants, (712 AMD cases [grades 3, 4 and 5] and 286 unrelated controls [grades 1 and 2]. The total population was assessed for a main SNP effect.

A subset of the population with environmental risk information, including smoking history, (705 non-Hispanic Caucasian participants) was included in the analysis of SNP/smoking interaction (466 AMD cases [grades 3, 4 and 5] and 239 unrelated controls [grades 1 and 2]). A full description of the participants by clinical findings and smoking status is presented in Table 1.

DNA Analysis and Genotyping

The whole blood obtained was processed for DNA extraction using a standard protocol (Puregene; Gentra Systems, Minneapolis, MN). The Tagger function of the Haploview program (Lewis & Zaykin, 2000) was employed to select 'tagSNPs' that capture the common (allele frequency ≥ 5%) variation among the phase II Hapmap SNPs. TagSNPs that tagged other variants with an $r^2 \geq$ of 0.67 (or were tags only for themselves) were selected for the study. In addition, two previously validated coding sequence SNPs (rs1060826 and rs16966563) were obtained form the NCBI data base and were also included in the analysis. We genotyped 17 SNPs in the 998 subjects included in the study using TaqMan assays (Applied Biosystems, Foster City, CA). Pairwise linkage disequilibrium (LD) (r^2) was assessed to evaluate for residual correlations in both cases and controls (presented in Supplemental Figures 1 and 2, respectively) for all pairwise marker combinations using the Haploview program. For quality control purposes, 2 different samples from the Fondation Jean Dausset-Centre d'etude de Polymorphisme Humain (CEPH) were plated in quadruplicate on each 384-well plate. Additionally, internal controls were replicated throughout the sample list to ensure efficiency. Laboratory personnel were blinded to sample phenotypes and replicate sample locations. Additionally, each plate met a quality control efficiency of 100% and a genotyping efficiency of ≥95%.

Statistical Analysis

Deviations from Hardy-Weinberg equilibrium (HWE) were evaluated using the Genetic Data Analysis program (GDA) (Barrett et al., 2005).

The association analysis was conducted using logistic regression as implemented in the Statistical Analysis Software version 9.1. (SAS Institute, Cary, NC, USA). First, each SNP

was examined for association controlling for confounding by age and sex. Then, in the subset for which smoking data were available, we fit models including genotype, age, sex, smoking status (ever/never) and a two-way interaction term between genotype and smoking.

A model was constructed coding for additive genotypic effects, and each term was tested for significant association with AMD using a Wald chi-square test and a nominal significance level of $p<0.05$. The strength of the association was evaluated by the odds ratio and 95% confidence intervals. For models with significant interaction terms ($p<0.05$), differential association of smoking by risk allele carrier status was assessed using a stratified analysis. No correction for multiple comparisons was utilized for the data analysis.

RESULTS

A multivariable logistic regression analysis adjusting for age and sex was performed on all 998 subjects [286 controls (grades 1, 2) and 712 AMD cases (grades 3, 4, 5)]. SNP rs8072199 was significantly associated with AMD under the additive model ($p= 0.035$) (Table 2). Models containing SNP/smoking interaction terms were created for all markers under the additive genetic model, for all 705 subjects with available smoking data [466 cases (grades 3, 4 and 5) and 239 controls (grades 1 and 2)]. No significant interactions were detected. However, when considering just grade 5 (273 cases) and grade 1 (169 controls), a SNP/smoking interaction was detected for rs2248814 ($p=0.039$); rs1060826 presented a borderline significant value ($p=0.061$). Due to high LD ($r^2 = 0.95$ in cases and 0.96 in controls) between these two SNPs and based on the exonic location of rs1060826 we chose both SNP genotypes to stratify the data and examine the modification of the effect of smoking by allele carrier status.

We also tested the previously reported interaction between the *ARMS2* SNP rs10490924 and smoking, previously reported in a subset the data set used in this paper (Schmidt et al., 2006). The ARMS2-smoking interaction was not statistically significant in the current data set ($p=0.10$), and the OR for the interaction term was weaker ($OR_i=1.7$) than observed for NOS2A and smoking ($OR_i=2.36$).

Consistent with a recessive model, the stratified analysis demonstrated that the strength of the association between smoking and AMD increased in homozygous carriers of the rs2248814 and rs1060826 risk allele A (OR=35.98, 95% CI [3.19, 405.98], $p=0.0038$ and OR=35.63, 95% CI [3.12, 406.67], $p=0.004$, respectively) than in carriers of the AG genotype (OR=3.05, 95% CI [1.38, 6.74] $p=0.0058$ and OR=2.83, 95% CI [1.27, 6.31] $p=0.011$, respectively) or GG genotype (OR=2.1, 95% CI [0.911, 4.84] $p=0.082$ and OR=2.13, 95% CI [0.94, 4.85] $p=0.072$, respectively) (Table 3).

DISCUSSION

In our study, we demonstrated that a SNP present in the *NOS2A* gene, rs8072199, conferred an increased risk for AMD when controlling for age and sex. Prior studies of *in vitro* glial cell cultures and murine lung epithelium cells exposed to smoke condensates demonstrated decreased oxidative stress activation by a reduction in inducible nitric oxide synthase (iNOS) protein expression and enzymatic activity (Hoyt et al., 2003; Mazzio et al., 2005). Therefore, we examined interactions between smoking and SNPs in *NOS2A* and detected a significant interaction with rs2248814 and a borderline interaction with rs1060826. To understand the nature of this interaction we conducted an analysis of the effect of smoking when stratifying the sample by rs2248814 and rs1060826 genotype. Individuals carrying the AA genotype in both SNPs were much more sensitive to the effect of smoking on AMD and having one copy of the major allele reduced this effect considerably. AA homozygous

individuals with AMD were 35 times as likely to have smoked as controls with the AA genotype. Individuals carrying one or no copies of the A allele were only 3 or 2 times as likely to have smoked as controls, respectively. Additionally, a similar trend was observed when the smoking effect was evaluated using pack-years of exposure, comparing 'heavier smokers' (above the median pack-years) or 'lighter smokers' (below the median) versus 'never smoked'. The smoking effect seemed to be stronger in the heavy smokers than in light smokers (data not shown). The pattern observed suggests a synergy between the effect of smoking and the AA genotype at rs2248814 and rs1060826. The fact that the SNP/smoking interaction was detected when comparing the grade 1 (controls) and grade 5 subjects (neovascular cases) is in agreement with previous work indicating that both smoking and iNOS induction are individually associated with the neovascularization process that occurs in advanced neovascular AMD (Ando et al., 2002; Suner et al., 2004).

While these results are intriguing, they should be interpreted cautiously. SNPs rs8072199 and rs2248814, significant for SNP main effect and SNP/smoking interaction, respectively, are in low linkage disequilibrium (r^2=0.07) and neither has a known functional effect on iNOS, so that the effect seen in this sample might be due to a third, untyped SNP located in the gene that is in moderate LD with both of these SNPs. The results presented here are from a single sample and do not withstand conservative corrections for multiple comparisons. In addition, while the point estimates of the OR for smoking are very different across genotype strata, the small samples sizes produce large 95% confidence intervals that overlap, indicating that the true value for the OR may not be different across genotypes. This indicates that futher examination in larger data sets is warranted. However, the results generate interesting hypotheses about a possible role of iNOS in AMD and a gene-smoking interaction in the disease which bear examination in other data sets.

Because we had previously reported an interaction between smoking and ARMS2 (Schmidt et al., 2006), we also performed a two way and a three way interaction analysis in our subset of individuals with smoking history data. The two way analysis that included SNPs in *NOS2A* and ARMS2 and the three way analysis including SNPs in the two genes and smoking did not detect any significant interactions (data not shown).

In AMD several mechanisms are thought to be responsible for disease pathogenesis including oxidative stress related processes and abnormal inflammatory pathway activation. The nitric oxide synthase (NOS) enzyme system is known to participate in both phenomena. The expression of *NOS2A* gene that codes for inducible nitric oxide synthase (iNOS) has been demonstrated in activated inflammatory cells in AMD as well as in RPE and Müller cells (Ando et al., 2002b) and in choroidal neovascularization membranes of AMD patients (Hattenbach et al., 2002): its blockade has demonstrated to improve the formation of aberrant choroidal vessel in late stage AMD (Ando et al., 2002). Prior work on RPE and photoreceptor cells exposed to NO have shown a decrease in proliferation (Goureau et al., 1993) and increase in apoptosis (Ju et al., 2001; Osborne & Wood, 2004) respectively, that could also play a role in the disease pathogenesis. Therefore, the fact that we found a main effect in a SNP in the NOS2A gene in our data set further implicates the gene in the disease.

Smoking has been traditionally identified as one of the strongest disease risk factors, increasing risk in a dose dependent fashion in smokers as compared to non smokers (Clemons et al., 2005; Khan et al., 2006; Tomany et al., 2004). Strong evidence exits for a possible causative role resulting in RPE dysfunction and death in an animal model of AMD (Fujihara et al., 2008). Smoking has been shown to decrease blood antioxidants levels and to favor an oxidative state (Moriarty et al., 2003), which then results in increased mitochondrial mediated apoptosis that could partially explain the cell death that ensues in AMD (Jiang et al., 2005). However, the fact that the known attributed risk conferred by

smoking can be so dramatically influenced by homozygous carriage of the risk allele constitutes a fascinating finding, with significant future implications not only in the research area but also in the public health arena.

Interestingly, the rs2248814 and rs1060826 AA genotypes are the same genotypes that have been previously reported to modify the smoking effect and confer an increased risk for Parkinson Disease (PD) (Hancock et al., 2006; Hancock et al., 2008). The main difference with the current study is that in PD smoking has a protective effect (Hague et al., 2004; Hancock et al., 2006; Levecque et al., 2003), partially attributed to the possible protective effect that nicotine may exert against neurotoxic insults (Fratiglioni & Wang, 2000; Preux et al., 2000) and a decreased iNOS induction and activity (Hoyt et al., 2003; Mazzio et al., 2005). Therefore, the presence of either rs2248814 or rs1060826 AA genotype in the *NOS2A* gene should remove or at least reduce the possible negative regulation of smoking on iNOS production and activity. In AMD, however, rs2248814/ rs1060826 AA genotype and smoking have a synergistic effect. These somewhat conflicting results may reflect varying degrees of linkage disequilibrium with one or more true functional variants, which remain to be identified. As previously stated, relatively few cases and controls carry the AA genotypes, and the wide confidence intervals on the estimated OR suggest that the true OR may not be significantly different. Therefore, these results should be regarded as hypothesis generating and further studies are necessary to determine if the presence of the AA genotype in either rs2248814 or rs1060826 is correlated with a modified protein expression or activity when cells are exposed to smoking that could account for the effects observed both in PD and AMD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Table 1

AGE, SEX, CLINICAL STATUS, AND CIGARETTE SMOKING HISTORY

(712 cases with age-related macular degeneration and 286 unaffected controls were included in the study.)

Status	Mean Age (SD)	Sex		Grade		Ever Smoked		
		M	F			N	Y	Missing
Control (N=286)	66.9 (8.3)	126 (44.1%)	160 (55.9%)	1	209	88	81	40
				2	77	34	36	7
Case (N=712)	76.4 (7.6)	257 (36.1%)	455 (63.9%)	3	192	62	84	46
				4	90	19	28	43
				5	430	102	171	157

Table 2

RESULTS OF LOGISTIC REGRESSION ANALYSIS OF NOS2A AND AMD

(Association between single nucleotide polymorphisms (SNPs) in the *NOS2A* gene and age-related macular degeneration (AMD) was assessed in 712 AMD cases and 286 controls, adjusting for age and sex. Odds ratios (OR) and 95% confidence intervals (95% CI) indicate the strength of association between AMD and increasing numbers of minor alleles at each SNP. Significant ($p<0.05$) results are noted in bold.)

SNP	Basepair locationon on chromosome 17	Location in gene (amino acid change if exonic)	OR	95% CI	p-value
rs2255929	23112094	Intron 23	0.82	(0.65, 1.04)	0.099
rs1060826	23113994	Exon 22 (T919T)	1.16	(0.90, 1.49)	0.26
rs2297515	23117460	Intron 19	0.89	(0.64, 1.23)	0.48
rs2297516	23119857	Intron 17	0.82	(0.64, 1.04)	0.10
rs2297518	2312072	Exon16 (S608L)	1.08	(0.80, 1.46)	0.62
rs2248814	23124448	Intron 12	1.17	(0.91, 1.50)	0.22
rs2314810	23128237	Intron 11	1.03	(0.61, 1.76)	0.91
rs1137933	23130059	Exon 10 (D385D)	1.04	(0.78, 1.38)	0.81
rs4795067	23130802	Intron 9	1.04	(0.81, 1.34)	0.75
rs944725	23133698	Intron 6	0.99	(0.79, 1.26),	0.97
rs17722851	23134963	Intron 5	0.75	(0.52, 1.10)	0.14
rs3794764	23135555	Intron 5	0.90	(0.68, 1.20)	0.48
rs16966563	23140076	Exon 4 (P68P)	1.21	(0.56, 2.53)	0.62
rs8072199	**23140975**	**Intron 2**	**1.30**	**(1.02, 1.65)**	**0.035**
rs2072324	23141023	Intron 2	0.88	(0.67, 1.18)	0.41
rs3794766	23146048	Intron 2	1.24	(0.92, 1.66)	0.15
rs3730014	23149870	Exon 2 (A31A)	0.59	(0.19, 1.91)	0.38

Table 3

RESULTS FROM LOGISTIC REGRESSION MODELS OF NOS2A – CIGARETTE SMOKING INTERACTION AND AMD

(Association between cigarette smoking and age-related macular degeneration (AMD), stratified by genotypes at *NOS2A* single nucleotide polymorphisms rs2248814 and rs1060826 was determined adjusting for age and sex. Odds ratios (OR) and 95% confidence intervals (95% CI) are presented for the association of "ever" smoking with AMD in 263 grade 5 cases and 169 grade 1 controls. Statistically significant associations ($p<0.05$) between cigarette smoking and AMD are noted in bold.)

SNP	OR	95% CI	p-value	Cases	Controls
AA GENOTYPE					
rs2248814	**35.98**	**(3.19, 405.98)**	**0.0038**	**33**	**17**
rs1060826	**35.63**	**(3.12, 406.67)**	**0.004**	**33**	**17**
AG GENOTYPE					
rs2248814	**3.05**	**(1.38, 6.74)**	**0.0058**	**136**	**70**
rs1060826	**2.83**	**(1.27, 6.31)**	**0.011**	**132**	**68**
GG GENOTYPE					
rs2248814	2.10	(0.91, 4.84)	0.082	95	60
rs1060826	2.13	(0.94, 4.85)	0.072	98	60