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Genetic Polymorphisms Associated with Risk of Developing Age-Related Macular Degeneration in Patients with Chronic Aspirin Usage

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Abstract

Introduction: Age-related macular degeneration (AMD) is the leading cause of blindness in the elderly population. Aspirin usage has been reported to be associated with an increased risk of developing this disease. This study aims to determine whether the increased risk of AMD with chronic aspirin usage is related to genetic polymorphisms in the Vascular Endothelial Growth Factor A (VEGFA) and Catechol-O-Methyltransferase (COMT) genes.

Methods: This was a case-control study with a sample of 30 patients with AMD and a control group of 80 patients without visual impairment or macular disease. Patients were recruited from the Loyola University Medical Center Ophthalmology outpatient clinic. Patients underwent a one-time screening questionnaire and blood draw, with documentation of aspirin usage. After DNA sequencing, genotype frequencies were compared for three polymorphisms in the VEGFA gene and one polymorphism in the COMT gene between AMD patients and controls, stratified by aspirin usage.

Results: Among participants with chronic daily aspirin usage, those with the GG genotype of the VEGFA gene rs3025033 polymorphism were approximately 10 times more likely to have AMD than those with an AA genotype (p=.02) and 11 times more likely than those with an AG genotype (p=.02). Those with the AA genotype of the COMT gene rs4680 polymorphism were approximately 8 times more likely to have AMD than those with the GA genetic profile (p=.02). There were no significant differences in polymorphism genotypes between AMD patients and controls when not stratified by aspirin usage.

Conclusion: The polymorphism rs3025033 in the VEGFA gene and the polymorphism rs4680 in the COMT gene were found to be associated with AMD development only in chronic daily aspirin users. These polymorphisms may help predict patients who could develop AMD with chronic aspirin usage. Large-scale trials are warranted to further evaluate the impact of these polymorphisms.

Keywords: Age-related Macular Degeneration; Vascular Endothelial Growth Factor; Catechol-o-Methyltransferase; Aspirin; Polymorphism

List of abbreviations: AMD: Age-related Macular Degeneration; CNV: Choroidal Neovascularization; COMT: Catechol-O-Methyltransferase; SNP: Single Nucleotide Polymorphism; VEGFA: Vascular Endothelial Growth Factor A

Introduction

Age-related macular degeneration (AMD) is a multifactorial degenerative disease of the central portion of the retina, known as the macula, resulting in loss of central vision [1,2]. AMD significantly impacts functional status as well as quality of life. It is the leading cause of irreversible blindness in the elderly population with an estimated US $255 billion spent on treatment worldwide [2-6]. Although AMD is so common in the elderly population, the pathophysiology of this disease is not yet fully understood. Risk

factors for AMD include age (>50 years), smoking, and cardiovascular disease, and family history [2,7-12].

AMD is generally categorized into two types: exudative (neovascular, or wet), and non-exudative (non-neovascular, or dry). The exudative type is associated with a poorer prognosis for visual acuity [13]. It involves choroidal neovascularization (CNV), leading to leakage and fibrovascular scarring [1]. It has been shown that hypoxia results in the release of Vascular Endothelial Growth Factor A (VEGFA) and other inflammatory signals in the retina, triggering the growth of abnormal vessels under the macula, which may result in CNV [13,14]. Multiple studies have identified increased VEGFA levels in vitreous samples from patients with exudative AMD, and several studies have focused on the relation between single nucleotide polymorphisms (SNPs) of the VEGFA gene and AMD development [15,16]. There have been population-specific studies in a Northern European Caucasian population, Chinese populations, a Brazilian population, and a Turkish population [17-21]. This study focuses on an American population.

Long-term aspirin use has also been reported to be associated with a small but significant increase in the risk of wet AMD [22,23]. This may be due to increased CNV caused by aspirin, via a different mechanism than its cardioprotective effects [24,25]. A recent meta-analysis suggested that the association of aspirin with AMD development might be negligible, but this analysis was inconclusive and did not consider genetic variation for subgroup analyses. Previous work from our laboratory showed expression of VEGFA in retinal pigment epithelial cells challenged with aspirin and its metabolites, which might be the mechanism for increased CNV with aspirin use [26,27]. While the association between certain SNPs of the VEGFA gene and AMD has been shown in multiple populations, the modulatory effects of these SNPs in the presence of aspirin has not been explored thus far. Therefore, it is possible that these SNPs may confer an increased risk for the development of AMD in the presence of aspirin. In this study, we report the association of previously identified SNPs rs1413711, rs2146323, and rs3025033 in the VEGFA gene with AMD in the presence and absence of aspirin [21].

Catechol-O-Methyltransferase (COMT) is an enzyme which targets catecholamines involved in cardiovascular, sympathetic, and endocrine pathways. Variations in the levels of these signaling molecules are implicated in a broad spectrum of diseases [28-32]. COMT has been implicated in the development of oxidative stress in some human tissues, and oxidative stress is one of the pathways that has been shown to contribute to AMD development [33,34]. It is thought to lead to AMD development by inducing the release of cytokines from the retinal pigment endothelium [35]. It has been reported that polymorphisms in the COMT gene, particularly the genetic variant rs4680, may confer cardiovascular disease risk, and that this association may be modified by the treatment effects of aspirin [36]. However, the association between polymorphisms in COMT and development of AMD has not been explored, in the presence or absence of aspirin.

Recent studies have shown that treatment response in wet type AMD patients might be associated with genetic variations [37-42]. For instance, it has been shown that treatment with antioxidants and zinc can prevent AMD progression in patients with certain variants in CFH and ARMS2 genes, while patients with other variants are harmed by the same treatment [43-45]. Therefore, it is crucial that the effects of genetic variation in the presence of modifiable environmental risk factors be understood. As such, this study aims to determine the association between AMD and three SNPs related to VEGFA and one SNP related to COMT in patients with and without chronic aspirin usage.

Materials and Methods

Patients and Controls

A total of 30 patients with exudative or non-exudative AMD were selected for this study from the Loyola University Medical Center Ophthalmology outpatient clinic. The control group was composed of 80 patients from the same clinic without a diagnosis of AMD and without visual impairment or other macular diseases. A total of 110 subjects were recruited.

Patients who were younger than 18 years old or pregnant were excluded. Institutional review board approval was obtained prior to beginning the study, and all patients were educated about the research study and signed a consent form. The study was conducted as per the stipulations under the Declaration of Helsinki (2008).

All participants received a standard ophthalmologic examination, including visual acuity measurement, pupil exam, intraocular pressure, and visual field exam, slit lamp biomicroscopy, and dilated fundus exam. Each AMD patient had an optical coherence tomography image taken. For dry AMD, the severity was graded as early, intermediate, or advanced based on the age-related eye disease study (AREDS) report number 6 [46]. For wet AMD, classification was determined by the presence of a choroidal neovascular membrane on clinical exam and/or OCT imaging. For each patient, it was recorded whether they had daily aspirin usage with an 81mg or 325mg pill, or occasional usage but not daily usage, or no aspirin usage.

Sample Collection and DNA Extraction

Peripheral venous blood samples were obtained from all participants by intravenous access, and stored in citrated tubes with 0.5 ml of 3.2% sodium citrate solution. Genomic DNA was extracted from whole blood samples using the QIAamp DNA Mini and Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's specifications.

Amplicon Library Preparation and Sequencing

Sequencing ready libraries were generated using a 2-step polymerase chain reaction (PCR) using primers specific to genes of interest to identify single nucleotide polymorphisms (SNPs). The locations targeted were four SNPs: rs1413711, rs2146323, rs3025033 in the VEGFA gene, which were previously targeted by Bulgu, *et al.* [21] and rs4680 in the COMT gene. The primers used to amplify these regions are shown in Table 1.

SNP	Sequence (5'->3')
VEGF Rs1413711	
Primer Sense	5'-TGACAATATTCTCCCGGGACC-3'
Primer Antisense	5'-AGTGTGACCTTCAGAGGCCC-3'
Probe-1	5'-CTTCCAAGGCCAGGGGGCA-3'-FL
Probe-2	5'L640-AGGAGGGGCGGTTCTAGGCAGCA-3'
VEGF Rs2146323	
Primer Sense	5'-AAGCTTAGGGAAGTGCTTCAA-3'
Primer Antisense	5'-CTGCGCTGATAGACATCCAT-3'
Probe-1	5'-TGTAATGCCACTCTTTGGAGCTT-3'-FL
Probe-2	5'L640-GAATCAGGCAAGTCCTTCC-3'
VEGF Rs3025033	
Primer Sense	5'-AAGACTTTGTGGGGATTTCCTA-3'
Primer Antisense	5'-TTGGTTTCACATAGGGCCAA-3'
Probe-1	5'-AGGGAAGTCCTTGGAGTGTCTCCC-3'-FL
Probe-2	5'L640-CCCCAGCAATGTTCTTGTGGC-3'
COMT Rs4680	
Primer Sense	5'-TCGAGATCAACCCCGACTGT-3'
Primer Antisense	5'-AACGGGTCAGGCATGCA-3'
Probe-1	5'6FAM-CCTTGTCCTTCACGCCAGCGA-3'
Probe-2	5'VIC-ACCTTGTCCTTCATGCCAGCGAAAT-3'

Table 1: Primers and Probes for VEGF and COMT Genes
FL: Fluorescein; L640: LightCycler-Red 640; 6FAM: 6-Carboxyfluorescein; VIC: VIC Fluorescent Dye

The concentration of the products following the initial PCR reaction was measured using the Qubit Fluorometer (Invitrogen, Carlsbad, CA). The size distribution of the products and the efficiency of the primers were verified using the Agilent 2100 Bioanalzyer (Agilent, Santa Clara, CA). Sequencing adapters and dual-index barcodes were added to the amplified targets in a second, limited cycle PCR reaction (Illumina, San Diego, CA). The final PCR products were purified using the Agencourt AMPure XP system (Beckman Coulter, Brea, CA) and the Qubit Fluorometer and Bioanalyzer were used to measure the final concentration and size to ensure successful adapter incorporation. The final products were pooled together in equimolar concentrations to create a final library ready for sequencing. The samples were sequenced on the Illumina MiSeq bench-top sequencer rendering 250 bp paired-end reads.

Genetic Variant Identification

Low quality sequences were trimmed and adapters were removed using Cutadapt (v. 1.11). Trimmed reads were then aligned with BWA (v.0.7.5a-r405) to the human reference assembly from Ensembl, GRCh38. The aligned reads were sorted by coordinate and PCR duplicates were removed using samtools (v. 0.1.18). Read groups were assigned and the alignments were merged using Picard Tools (2.11.0). Mutations and structural variants were identified using the Bayesian variant caller, Freebayes [47] (v. 1.1.0-46-g8d2b3a0) resulting in a list of high confidence SNPs. The variants were visualized using the Integrative Genomics Viewer (Broad Institute, Cambridge, MA). The 3 sequenced regions within the VEGFA gene, as seen in the Integrative Genomics Viewer, are shown in Figure 1 [48,49].

Statistical Analysis

Binary logistic regression models were used to estimate the odds of AMD as a function of patient age, sex, use of aspirin, and race. In these models, expected frequencies were monitored, and a Firth correction was used when calculating the odds ratio for all race comparisons due to the presence of a zero-cell count among African Americans [50]. Regarding age, the linearity assumption between age and (the natural logarithm of) AMD was assessed as described by Hosmer and Lemeshow [51].

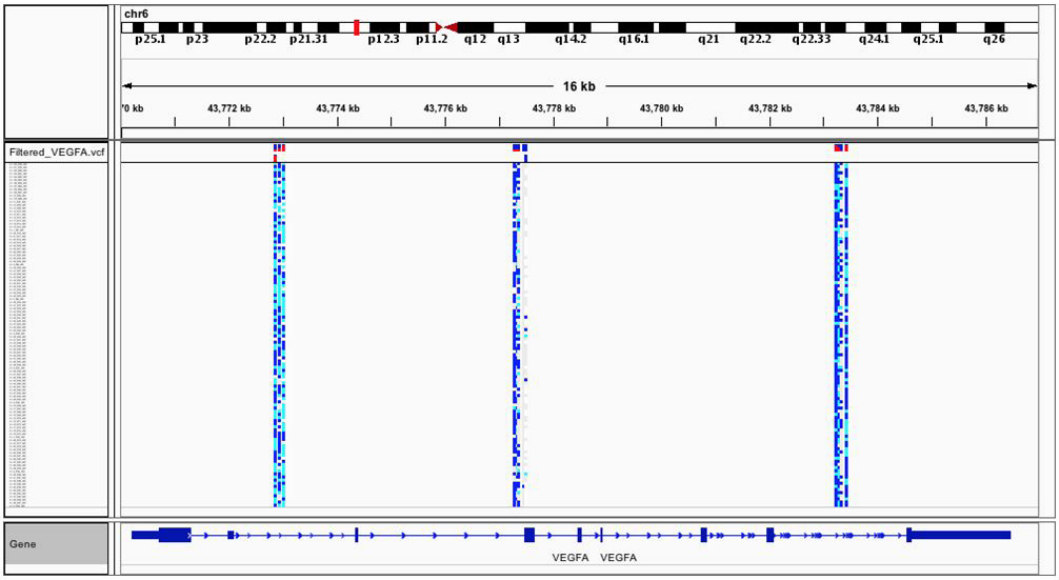

Figure 1: Sequenced Regions within VEGFA Gene in Integrative Genomics Viewer
As shown by the red line on the chromosome at the top of the image, the VEGFA gene lies within the p arm of chromosome 6. The VEGFA gene is shown in entirety at the bottom. Short vertical bars represent untranslated regions and tall vertical bars represent exons. The flat lines with arrows represent introns. From left to right, the 3 sequenced regions contain rs1413711, rs2146323, and rs3025033 respectively. These SNPs occur within introns.

A similar approach was used to estimate the odds of AMD as a function of patients' VEGFA and COMT genetic traits. In these models, expected frequencies were monitored and exact logistic regression models were used when such values were sparse as described by Agresti [52]. An *a-priori* decision was also made to stratify these genetic results by patients' use of aspirin (i.e., none or rarely versus daily). For these later comparisons, Pearson chi-square tests were used to test for an association between patients' stratified genetic traits and AMD status and, as before, Fisher's exact test was used to account for sparse expected frequencies. For two traits (i.e., VEGFA Rs3025033 and COMT Rs4680), an exact logistic regression model was used to estimate the odds ratio.

Finally, Pearson chi-square tests were used to test for an association between patients' genetic traits and wet versus dry AMD status among aspirin users. As before, expected frequencies were monitored and Fisher's exact test was used when these values were sparse. An exact logistic regression model was used to estimate the odds ratio for the COMT Rs4680 genetic trait. All analyses were completed using SAS version 9.4 (Cary, NC).

Results

Patient Characteristics

A total of 110 subjects were recruited for this study, of which 30 (27%) had AMD and 80 (73%) were in the control group. The demographic information, aspirin usage, type of AMD and severity of AMD for the subjects are shown in Table 2. The percentage of subjects using aspirin was exactly 50% in both the AMD group and control group by chance alone, as this information was not elicited until after subject selection. For every one-year increase in age, the odds of AMD increased by approximately 16% (*OR* = 1.16, 95% CI: 1.09 – 1.23; $p < .001$). Conversely, compared to females, males were only 0.30 (95% CI: 0.12 – 0.75) times as likely to have AMD – an approximate 70% decrease in the odds ($p = .01$). Further, compared to patients identifying as white, those identifying as African American were nominally less likely to have AMD, though the overall conclusions for race did not reach statistical significance (overall $p = .10$).

	Controls (n = 80)	AMD (n = 30)	Total (N = 110)	Odds Ratio (95% CI)	*p*
Mean Age (SD)	60.26 (14.81)	81.17 (9.26)	65.96 (16.41)	1.16 (1.09 – 1.23)	<.001
Sex					
Male	44 (55%)	8 (27%)	52 (47%)	0.30 (0.12 – 0.75)	.01
Female (ref)	36 (45%)	22 (73%)	58 (53%)		
Aspirin Usage					.98
None (ref)	20 (25%)	7 (23%)	27 (25%)		
Rarely	20 (25%)	8 (27%)	28 (25%)	1.14 (0.35 – 3.75)	.83
Daily	40 (50%)	15 (50%)	55 (50%)	1.07 (0.38 – 3.05)	.90

	Controls (n = 80)	AMD (n = 30)	Total (N = 110)	Odds Ratio (95% CI)	*p*
Race (Valid N = 100)					.10
Caucasian (ref)	37 (51%)	22 (81%)	59 (59%)		
African American	17 (23%)	0	17 (17%)	0.05 (0.003 – 0.90)	.04
Hispanic	13 (18%)	2 (7.4%)	15 (15%)	0.31 (0.07 – 1.38)	.12
Other	6 (8.2%)	3 (11%)	9 (9.0%)	0.90 (0.21 – 3.89)	.89

Table 2: Demographics of AMD Patient Group and Control Group

Association between VEGFA and COMT Genotypes and Alleles and Patient Phenotypes

Without accounting for aspirin usage, we found no significant differences between genotype frequencies in the AMD patient group and the control group for VEGFA genes rs1413711 ($p = .50$), rs2146323 ($p = .92$), rs3025033 ($p = .18$), or COMT gene rs4680 ($p = .20$) in our sample of data.

However, when stratified by patients' use of aspirin (i.e., none or rarely versus daily), there were significant associations between AMD development and the VEGFA rs3025033 and COMT rs4680 genes among daily aspirin users. In this population, 20% of AMD patients were of the GG genotype for the VEGFA rs3025033 gene versus none in the control cohort. That is, among daily aspirin users, those with a GG genotype were approximately 10 times more likely to have AMD than those with an AA genotype (exact $p = .02$) and they were 11 times more likely to have AMD than those with an AG genotype (exact $p = .02$). Further, among daily aspirin users, 40% of AMD patients were of the AA genotype for the COMT rs4680 gene versus 10% in the control cohort. An exact logistic regression model revealed that those with an AA genotype were approximately 8 times more likely to have AMD than those with a GA genetic profile (exact $p = .02$). See Table 3.

			AMD						*p*
			Control		AMD		Total		
			n	%	n	%	N	%	
No Aspirin or Aspirin Rarely	**VEGF Rs1413711**	**TT**	3	7.5%	5	33.3%	8	14.5%	.063
		TC	26	65.0%	7	46.7%	33	60.0%	
		CC	11	27.5%	3	20.0%	14	25.5%	
	VEGF Rs2146323	**CC**	18	45.0%	4	26.7%	22	40.0%	.33
		CA	21	52.5%	10	66.7%	31	56.4%	
		AA	1	2.5%	1	6.7%	2	3.6%	
	VEGF Rs3025033	**AA**	26	65.0%	9	60.0%	35	63.6%	.82
		AG	13	32.5%	6	40.0%	19	34.5%	
		GG	1	2.5%	0	0.0%	1	1.8%	
	COMT Rs4680	**GG**	17	42.5%	4	26.7%	21	38.2%	.51
		GA	13	32.5%	7	46.7%	20	36.4%	
		AA	10	25.0%	4	26.7%	14	25.5%	
Daily Aspirin	**VEGF Rs1413711**	**TT**	11	27.5%	2	13.3%	13	23.6%	.32
		TC	16	40.0%	5	33.3%	21	38.2%	
		CC	13	32.5%	8	53.3%	21	38.2%	
	VEGF Rs2146323	**CC**	22	55.0%	10	66.7%	32	58.2%	.75
		CA	13	32.5%	4	26.7%	17	30.9%	
		AA	5	12.5%	1	6.7%	6	10.9%	
	VEGF Rs3025033	**AA**	28	70.0%	9	60.0%	37	67.3%	.03
		AG	12	30.0%	3	20.0%	15	27.3%	
		GG	0	0.0%	3	20.0%	3	5.5%	
	COMT Rs4680	**GG**	7	17.5%	4	26.7%	11	20.0%	.01
		GA	29	72.5%	5	33.3%	34	61.8%	
		AA	4	10.0%	6	40.0%	10	18.2%	

Table 3: Genotype Frequencies of VEGF and COMT SNPs in AMD Patient Group and Control Group Stratified by Aspirin Usage

When the genotype frequencies were compared among daily aspirin users between the control group and only the wet AMD patients, excluding the dry AMD patients, the association between the COMT rs4680 gene and AMD development persisted. Among these daily aspirin users, 41.7% of wet AMD patients were of the AA genotype for the COMT rs4680 gene versus 10% in the control group. That is, those with an AA profile were approximately 11 times more likely to have wet AMD than those with a GA profile (exact p = .01).

Discussion

In this study, we examined the association between AMD and three SNPs related to VEGFA and one SNP related to COMT in patients with and without chronic aspirin usage. This was the first study to look at the association between AMD development and a SNP related to the COMT gene. We found that in daily aspirin users, the derived alleles of the VEGFA rs3025033 and COMT rs4680 SNPs were significantly associated with AMD development. This association was stronger when looking specifically at wet AMD patients, where the derived AA allele of the COMT rs4680 SNP was associated with an 11 times greater likelihood of AMD development compared with the GA allele. Notably, there was no significant association found in patients without daily aspirin usage, and there was no significant association when looking at the whole population without stratifying by aspirin usage.

Multiple studies have investigated the link between SNPs in the VEGFA gene and AMD development, with the goal of eventually predicting response to anti-VEGF treatment. There have been few significant results in population-specific studies, as shown in Table 4.

Study	Population	rs1413711	rs2146323	rs3025033
Churchill *et al* [17]	Northern European	CC (derived)	No association	--
Haines *et al* [53]	American	--	No association	--
Lin *et al* [18]	Chinese	No association	--	--
Fang *et al* [54]	Caucasian	No association	No association	--
Immonen *et al* [37]	European	--	No association	No association
Qu *et al* [19]	Chinese	No association	--	--
Almeida *et al* [20]	Brazilian	TT (derived)	--	--
Lu *et al* [15]	European	CC (derived)	--	--
Bulgu *et al* [21]	Turkish	AA (derived)	AA (derived)	No association

Table 4: Alleles Conferring Increased Risk of AMD in Population-Specific Studies

Like many of these studies, our population showed no significant results without considering aspirin usage, but the association with VEGFA SNP rs3025033 was uncovered when looking only at daily aspirin users. Likewise, the association between COMT SNP rs4680 and AMD development was uncovered in daily aspirin users. This suggests that these SNPs may serve as enabling mutations for the effect of aspirin increasing the risk of AMD development. The analysis by Zhu *et al*. which found a negligible effect of aspirin on AMD development may have shown more significant results if the effect was analyzed only in patients with derived alleles in the SNPs identified in this study [26].

Notably, in this study we ran a 2-step PCR to simultaneously isolate the four SNPs: rs1413711, rs2146323, rs3025033 in the VEGFA gene, and rs4680 in the COMT gene. We had a significant yield from all primers without interference. To our knowledge, this is the first time these four primer sets have been used simultaneously and this indicates that they could be used together for future diagnostic testing without concern for adverse interactions.

Future studies could continue to look at the association between AMD development and the alleles identified in this study or other alleles in chronic aspirin users. The results of this study show that the effects of environmental factors on AMD development are modified by specific genetic polymorphisms. This effect could be studied with other known risk factors for AMD development such as smoking, sun exposure, obesity, and hypertension. It could also be studied with other platelet inhibitors than aspirin to see if the risk is due to the platelet inhibition or a different property of aspirin. Additionally, we suggest that the effect of aspirin use on VEGFA or COMT RNA expression could be studied, rather than looking at the association with DNA markers. We have focused in this study on whether specific SNPs in VEGFA or COMT enable aspirin to increase the risk of AMD development. However, it may be that aspirin conversely increases RNA transcription of genes related to VEGFA or COMT that could be detected in patients with AMD using RT-PCR or a microarray. Future studies elucidating this relationship could guide treatment options for patients at risk of developing AMD.

Conclusion

We found that SNP rs3025033 in the VEGFA gene and SNP rs4680 in the COMT gene are associated with AMD development in chronic daily aspirin users. This association is especially pronounced between COMT SNP rs4680 and the wet AMD type. These polymorphisms may help predict patients who could develop AMD with chronic aspirin usage. Large-scale trials are warranted to further evaluate the impact of these polymorphisms.

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