Genetic aetiology of age related macular degeneration in population of Nepal: Hospital based comparative study

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Abstract

Background

Age related macular degeneration (AMD) is the leading cause of blindness after cataract among elderly in developing countries. AMD is the most common retinal disorder and one of the leading cause of blindness among elderly in Nepal. Various modifiable and non-modifiable risk factors have been identified for the development of AMD. Genetics of AMD has been extensively studied in the recent years and genetic susceptibility of AMD has been identified. The two major susceptibility genes for AMD are CFH (1q31) which codes for complement factor H, and ARMS2 (10q26) for which the gene product and function are poorly understood. The mutations in these genes increased the chance of AMD. Although many studies are conducted in the developed world, only limited studies are from low and middle income countries. To the best of our knowledge, there are no reported studies on genetics of AMD in Nepal. This study aimed to assess the genetic etiology of AMD among Nepalese population in a hospital setting.

Methodology: This study is a hospital based cross sectional study. Patients at the age 65 years and above with all types of AMD were enrolled for AMD cases. Control groups for the AMD cases included age matched subjects at the age 65 years and above and those without AMD and other retinal pathologies that hinders the grading of AMD. The number of cases and controls necessary for this study was estimated for chi-squared tests of association and linear regression

models. We estimated that recruiting 160 cases and 160 controls sufficient to identify associations between selected risk variants and AMD in the Nepalese population with a power of 95%. However, as there are no other baseline data from Nepal on genetic components, we enrolled additional 40 participants in each groups to address any unforeseen contingency such as poor image quality, problems in DNA extraction and genetic analysis etc. So, the total study participants included 200 cases of AMD and 200 cases of control in this study. Detailed history, ocular examination under mydriasis, fundus photography, macular OCT were taken from all study participants. 5 ml blood will be drawn to prepare DNA extraction and haplotype and diplotype analysis of DNA extraction were carried out to assess the genetic etiology.

Results/Conclusion: A total of 400 study participants were enrolled, which included 200 AMD cases and 200 control individuals aged 65 year and above. The mean age for AMD cases (200) was 72.86 years (SD 5.10) and the control group (200) was 72.58 (SD 5.33). The prevalence of AMD-associated loci in Nepal was ascertained. Nepali individuals show significant associations with key variants in the *CFH-CFHR5*, *ARMS2/HTRA1*, *C2/CFB*, and *C3* loci. Nepalis exhibit a particularly high frequency of the risk allele of *ARMS2/HTRA1*. At the *CFH-CFHR5* locus, the frequency of AMD risk at *CFH* rs1061170 is lower than what is observed in Europeans, but higher than what is observed in East Asians. The frequency of the protective allele *CFH* I62 (rs800292) is higher than what is observed in Europeans, while the incidence of the protective *CFHR3/CFHR1* deletion appears comparable, assuming that the rs12144939 variant is an effective tag for the *CFHR/CFHR1* deletion. Nepali haplotype structure in the *CFH-CFHR5* region appears similar to that observed in Europeans and other non-African populations. AMD haplotype associations largely mirror what is observed in well-studied European cohorts.

Key words: AMD, genetics, haplotype analysis, diplotype analysis, Nepal

Introduction

AMD is the most common cause of irreversible blindness in the developed world among elderly people.[1-5] AMD is the third leading cause of global blindness, contributing to 8.7% of the total blindness in the world.[6] Population-based studies showed slightly higher prevalence of AMD in developed world as compared to developing world.[3,4,7-9]

AMD was found as the leading causes of blindness in Nepal, contributing to 8.7% of the total blindness in one of the population-based study.[10] Among the retinal diseases, AMD was the most common retinal disease in a population based study in Nepal at the age 40 years and above.[11] Almost one third of the population had AMD in one or both eyes at the age 60 years and above in another population based study from Nepal.[12]

Wet AMD and geographic atrophy (10 to 15% of total AMD) are the leading cause of visual impairment and blindness in AMD.[13] Many modifiable and non-modifiable risk factors have been identified for the development of AMD.[14-18] Age and genetic predisposition are among the very important risk factors for AMD.

The genetic susceptibility of AMD has been identified. The two major susceptibility genes for AMD are CFH (1q31) which codes for complement factor H, and ARMS2 (10q26) for which the gene product and function are poorly understood. The CFH Y402H mutation reported 4.6 fold increased risk for AMD when heterozygous and 7.4 fold increased risk when homozygous. The ARMS2 A69S mutation confers a 2.7 fold increased risk for AMD when heterozygous and 8.2 fold increased risk when homozygous. When both of these genes are homozygous in the mutations, the risk for AMD increased to 50 fold.[19] Genetic testing are available for AMD and includes testing for many of the known risk allelles.

Rotterdam study showed the first-degree relatives of individuals affected with AMD have a four-fold higher risk of developing advanced AMD than those with unaffected relatives.[20] Swaroop and colleagues calculated that having a sibling with AMD increases an individual's risk 3-6 fold.[4] A genetic component is also demonstrated in twin studies that reported a higher degree of AMD concordance between monozygotic twins than dizygotic twins, in a ratio of 37% and 19% respectively.[21] A study conducted in India, showed the associations of neovascular AMD

for CFHY402H variant (rs1061170), ARMS2 (rs10490924), C2 (rs547154), ABCA1 (rs1883025) and an SNP near VEGFA (rs4711751). The study concluded that the major genetic determinants for neovascular AMD risk were similar to those with other ancestries while for early AMD suggest potential differences in the pathophysiology of AMD development.[22]

A study from China showed the gene variants in CFH, ARMS2, and HTRA1 contribute to AMD in this population.[23] Study conducted among the Asian population on AMD confirmed the association between rs13278062 at 8p21 and neovascular AMD in this populations.[24] AMD despite being of the leading cause of blindness in our population, there is lack of information on genetic risk of AMD in our Nepalese population. This study will help for the baseline information and further studies on genetics in Nepal.

Rational / Justification

AMD is the leading cause of blindness among elderly in Nepal. The genetic susceptibility of AMD has been identified. There are many studies reported from the developed world and many Asian countries. However, to the best of our knowledge, there are no information on genetics of AMD in Nepal. This study will help to assess the genetic etiology of AMD among Nepalese population. Knowing the genetics of AMD will help for further prevention and therapeutic management of AMD. This study will also provide the baseline information for further studies in this field.

Conceptual Framework

Two groups of study population will be assessed in the study. The first group will include those with cases of AMD at the age 65 years and above. The second group will include age matched cases of AMD at the age 65 years and above but no AMD and other retinal pathologies that hinders the grading of AMD.

After basic history, and examination, diagnosis of the eye will be derived. Fundus photographs and macular OCT will be recorded from all cases of AMD and control group enrolled in the

study. 5 ml of venous blood will be taken. DNA extraction will be prepared for further storage and analysis. Genetic analysis of DNA extract using haplotype and diplotype will be done using DNA extract both among the AMD cases and control group. Correlation will be done for the responsible genes involved in AMD cases and control group.

General Objective

To find out the genetic aetiology of AMD in Nepalese population

Specific Objective

1. To assess the demographic characteristics of people involved in the study
2. To assess the genes involved among cases of macular degeneration
3. To assess the genes involved among control group
4. To compare the genes responsible for the AMD cases and control group

Research Hypothesis

Two major AMD associated loci located on chromosomes 1 and 10 will associate with AMD cases in Nepal.

Research Method

This is a hospital based case- control study. The study was conducted at Tilganga Institute of Ophthalmology (TIO) Kathmandu, Nepal. Patients at the age 65 years and above with mild dry AMD, intermediate dry AMD, advanced dry AMD and wet AMD in one eye and or both eyes were enrolled for AMD cases. Control groups for the AMD cases were age matched subjects at the age 65 years and above and those without AMD and other retinal pathologies that hinders the grading of AMD.

The number of cases and controls necessary for this study was estimated for chi-squared tests of association and linear regression models. In the absence of previous study performed in the Nepali populations, estimates for chi-squared tests of association relied on the frequency of AMD-associated risk variants on chromosome 1 (*CFH-CFHR5*) and chromosome 10 (*ARMS2/HTRA1*) among cases and controls of European ancestry. These individuals were recruited to the Steele Center for Translational Medicine (SCTM), John A. Moran Eye Center, as part of a case-control study of the genetic etiology of AMD.[25] Estimates for linear models relied on the number of covariates were included in the models; these variables are age and gender. The expected proportion of variance explained by variables included in linear models was estimated from previous analyses performed with the SCTM case-control cohort (approximately 10-25%). The power of these analyses was set at 95%. The imposed significance level for associations was adjusted for multiple testing (the number of tests is equal to the number of variants considered for association, which was conservatively set at 20). Based on this, we estimate that recruiting 160 cases and 160 controls was sufficient to identify associations between selected risk variants and AMD in the Nepalese population with a power of 95%. However, as there are no other baseline data from Nepal on genetic components, we enrolled additional 40 participants in each groups to address any unforeseen

contingency such as poor image quality, problems in DNA extraction and genetic analysis etc. So, the total study participants included 200 cases of AMD and 200 cases of control in this study. AMD was classified using international ARM Epidemiological group.[26]

Study site and its Justification

The study will be conducted at Tilganga Institute of Ophthalmology (TIO). TIO is a tertiary care level hospital located at the capital city of Nepal. Large numbers of patients are treated at this institute with AMD. This will facilitate for the enrollment of adequate number of cases and control.

Research Design:

This study is a hospital based cross sectional study.

Description of Research design:

Detailed demographic information of the study subjects was taken as age, gender, occupation, education level, address and family history of AMD. Dietary history was taken as how frequently they consume green leafy vegetables. Consumption of smoking was taken in detail as present or past smokers. The number of cigarettes consumed per day and duration of smoking was recorded. Visual acuity of both presenting and best corrected visual acuity was taken. Anterior segment was evaluated in detail. Fundus was examined under mydriasis. Macula centered single fundus photographs (Zeiss Company), and Macular OCT (Zeiss Company Cirrus 6000) was recorded for each study participants. AMD was graded using International classification and grading system.[27] Briefly, AMD was classified as dry AMD and wet AMD. Dry AMD was further classified in to dry mild AMD, dry intermediate AMD and advanced dry AMD. Advanced dry AMD includes the geographic atrophy. Dry mild AMD and dry intermediate AMD were taken as early AMD. Late or advanced AMD includes the geographic atrophy and wet AMD. Height and weight was taken to assess the body mass index (BMI).

After the detailed history and examination, 5 milliliter of venous blood sample was drawn for the genetic analysis of AMD cases and control groups. Blood was immediately transferred in to EDTA vials and preserved in 2 to 4 degree centigrade. DNA extraction was prepared and kept in minus 20 degree centigrade. Using the same temperature, the DNA extract was transferred to

University of Utah, Moran Eye Center for analysis of genetic components involved in AMD cases and control. Briefly, All DNA samples were genotyped for approximately 20 single nucleotide polymorphisms (SNPs). These SNPs have previously been established to be associated with AMD to varying degrees in multiple ethnicities and represent previously-established AMD genetic loci, including *CFH-CFHR5*, *ARMS2/HTRA1*, *C2/CFB* and *C3*. All genotyping were done using the Taqman platform (Thermo Scientific).

For each genotyped variant, Nepalese allele frequencies were compared to those of Caucasian, East Asian and other ethnicities, using data from the University of Utah, as well as publicly-available data from the 1000 Genomes Project. In addition, allele frequencies of Nepalese AMD cases were compared to Nepalese controls in order to identify potentially causal genes in the Nepali population. Special proforma was designed to collect the data.

Study Population

Patients at the age 65 years and above with mild dry AMD, intermediate dry AMD, advanced dry AMD and wet AMD in one eye and or both eyes were enrolled for AMD cases. Control groups for the AMD cases were age matched subjects at the age 65 years and above and those without AMD and other retinal pathologies that hinders the grading of AMD.

Sampling unit

Individual each with AMD, and those without AMD at the age group 65 years and above.

Sample Size

The number of cases and controls necessary for this study was estimated for chi-squared tests of association and linear regression models. In the absence of previous study performed in the Nepali populations, estimates for chi-squared tests of association relied on the frequency of AMD-associated risk variants on chromosome 1 (*CFH-CFHR5*) and chromosome 10 (*ARMS2/HTRA1*) among cases and controls of European ancestry. These individuals were recruited to the Steele Center for Translational Medicine (SCTM), John A. Moran Eye Center, as part of a case-control study of the genetic etiology of AMD.[25] Estimates for linear models relied

on the number of covariates that were included in the models; these variables are age and gender. The expected proportion of variance explained by variables included in linear models was estimated from previous analyses performed with the SCTM case-control cohort (approximately 10-25%). The power of these analyses was set at 95%. The imposed significance level for associations was adjusted for multiple testing (the number of tests is equal to the number of variants considered for association, which was conservatively set at 20). Based on this, we estimate that recruiting 160 cases and 160 controls was sufficient to identify associations between selected risk variants and AMD in the Nepalese population with a power of 95%. However, as there are no other baseline data from Nepal on genetic components, we enrolled additional 40 participants in each groups to address any unforeseen contingency such as poor image quality, problems in DNA extraction and genetic analysis etc. So, the total study participants included 200 cases of AMD and 200 cases of control in this study.

Number of participants and justification:

We estimated that recruiting 160 cases and 160 controls were sufficient to identify associations between selected risk variants and AMD in the Nepalese population with a power of 95%. However, as there are no other baseline data from Nepal on genetic components, we enrolled additional 40 participants in each groups to address any unforeseen contingency such as poor image quality, problems in DNA extraction and genetic analysis etc. So, the total study participants included 200 cases of AMD and 200 cases of control in this study.

Sampling Technique:

All consecutive cases of AMD and age matched control cases presented at TIO were included during the study period.

Criteria for Sample selection:

Patients at the age 65 years and above with mild dry AMD, intermediate dry AMD, advanced dry AMD and wet AMD in one eye and or both eyes were form the AMD group for cases. Control groups for the AMD cases were age matched subjects at the age 65 years and above and those without AMD and other retinal pathologies that hinders grading of AMD.

Rationale of AMD genetic analysis:

In collaboration between Dr. Raba Thapa, PI at the Tilganga Institute of Ophthalmology and Dr. Gregory Hageman, director, at the Steele Center for Translational Medicine (SCTM), University of Utah, a cohort of 400 Nepali AMD case/control samples was collected, with the goal of assessing the frequencies and associations of known AMD-associated genetic variants.

Methods of genetic analysis of AMD

Blood samples were collected from 400 Nepali individuals. The cohort is composed of 200 AMD cases and 200 controls. DNA was extracted from whole blood in Nepal and shipped to the SCTM at ambient temperature. Samples were quantified, plated and genotyped at the SCTM using standard procedures. Eight SNPs were genotyped, representing the most highly-associated AMD loci in Caucasians: *CFH-CFHR5*, *ARMS2/HTRA1*, *C2* and C3. The variants chosen in the *CFH-CFHR5* region were rs800292 (*CFH* I62V), rs1061170 (*CFH* Y402H), rs1410996 (proxy for signal 1.1 from the International AMD Genomic Consortium AMD GWAS study) and rs12144939 (tag for a protective *CFHR3/CFHR1* deletion). Variants for *ARMS2/HTRA1* included rs10490924 (*ARMS2* A69S) and rs11200638 (*HTRA1* promoter variant). For *C2/CFB*, rs429608 was assessed, as it is a proxy for the lead variant from the IAMDGC GWAS. *C3* was represented by rs2230199 (*C3* C102G). All genotyping was done using the Taqman platform (Thermo Scientific). AMD associations were assessed using Haploview (https://www.broadinstitute.org/haploview/haploview). Phenotyping was performed at the SCTM using color and OCT images collected during study patient recruitment by Dr. Thapa.

AMD grades were assigned using the Rotterdam grading system. Statistical analyses were performed in Haploview.

Results

A total of 400 study participants were enrolled in the study. The mean age for AMD cases (200) was 72.86 years (SD5.10) and comparative group (200) was 72.58 (SD 5.33). The total mean age was 72.72 years (S.D 5.22). Table 1.

Table 1. Mean age of AMD cases and comparative group

Study group	N	Mean Age	Std. Deviation
Case (Present AMD)	200	72.865	5.1066
Comparative (Absent AMD)	200	72.580	5.3382
Total	400	72.723	5.2191

Majority of the study participants were from age group 65-70 years, which comprises of 40% of AMD cases and 44% of comparatives group. Males were more in both the cases (56.5%) and comparative group (52.5%). The study participants were from all the provinces. However, those from Bagmati provinces outnumbered among the other provinces. Majority of the study participants were from out of Kathmandu valley. Almost two fifth to half of the study participants were literate. Regarding the occupation, those hang no specific occupation (42.5%) were found in highest number in cases and household works (45.5%) in comparative group. Brahmin and Chhetri comprised the highest number (64.5%) among cases and 73% among the control group. Over four-fifths of the study participants were Hindus by religion. Table 2.

Table 2. Demographic characteristics of the study participants

		Case (Present AMD)		Comparative (Absent AMD)		Total	
		Count	%	Count	%	Count	%
Age Group	65-70 Years	80	40.0%	88	44.0%	168	42.0%
	71-75 Years	52	26.0%	52	26.0%	104	26.0%
	76-80 Years	56	28.0%	48	24.0%	104	26.0%
	81-85 Years	10	5.0%	10	5.0%	20	5.0%
	86-90 Years	2	1.0%	2	1.0%	4	1.0%
	Total	200	100.0%	200	100.0%	400	100.0%
Gender:	Male	113	56.5%	105	52.5%	218	54.5%
	Female	87	43.5%	95	47.5%	182	45.5%
	Total	200	100.0%	200	100.0%	400	100.0%
Address	Koshi Province	17	8.5%	25	12.5%	42	10.5%
	Madhesh Province	13	6.5%	10	5.0%	23	5.8%
	Bagmati Province	144	72.0%	125	62.5%	269	67.3%
	Gandaki Province	12	6.0%	22	11.0%	34	8.5%
	Lumbini Province	10	5.0%	9	4.5%	19	4.8%
	Karnalli Province	3	1.5%	6	3.0%	9	2.3%
	Sudurpashchim Province	1	0.5%	3	1.5%	4	1.0%
	Total	200	100.0%	200	100.0%	400	100.0%
Inside_outside valley	Outside Kathmandu Valley	102	51.0%	134	67.0%	236	59.0%
	Inside Kathmandu Valley	98	49.0%	66	33.0%	164	41.0%
	Total	200	100.0%	200	100.0%	400	100.0%
Rural_Orban Municipality	1. Urban Municipality	150	75.0%	146	73.0%	296	74.0%
	2. Rural Municipality	50	25.0%	54	27.0%	104	26.0%
	Total	200	100.0%	200	100.0%	400	100.0%
Education:	1. Illiterate	87	43.5%	95	47.5%	182	45.5%
	2. Informal Education	56	28.0%	62	31.0%	118	29.5%
	3. Literate	57	28.5%	43	21.5%	100	25.0%
	Total	200	100.0%	200	100.0%	400	100.0%
Occupation:	1. Agriculture	28	14.0%	33	16.5%	61	15.3%
	2. Housewife/Household work	72	36.0%	91	45.5%	163	40.8%
	3. Business	3	1.5%	2	1.0%	5	1.3%

	4. Service	5	2.5%	3	1.5%	8	2.0%
	5. Labor	0	0.0%	0	0.0%	0	0.0%
	6. None	85	42.5%	63	31.5%	148	37.0%
	7. Student	0	0.0%	0	0.0%	0	0.0%
	96. Others specify…..	7	3.5%	8	4.0%	15	3.8%
	Total	200	100.0%	200	100.0%	400	100.0%
Others Occupation Specify ……	DRIVING	1	14.3%	1	12.5%	2	13.3%
	Ex SERVICE	3	42.9%	5	62.5%	8	53.3%
	PARISH PRIEST	1	14.3%	0	0.0%	1	6.7%
	SOCIAL WORK	2	28.6%	2	25.0%	4	26.7%
	Total	7	100.0%	8	100.0%	15	100.0%
Ethnicity	1. Brahmin & Chhetri	129	64.5%	146	73.0%	275	68.8%
	2. Janajati	57	28.5%	49	24.5%	106	26.5%
	3. Dalit	4	2.0%	2	1.0%	6	1.5%
	4. Madeshi	10	5.0%	2	1.0%	12	3.0%
	5. Muslim	0	0.0%	1	0.5%	1	0.3%
	96. Others Specify	0	0.0%	0	0.0%	0	0.0%
	Total	200	100.0%	200	100.0%	400	100.0%
Religion	1. Hinduism	167	83.5%	171	85.5%	338	84.5%
	2. Buddhism	24	12.0%	23	11.5%	47	11.8%
	3. Christinity	7	3.5%	2	1.0%	9	2.3%
	4. Islam	1	0.5%	1	0.5%	2	0.5%
	5. Kirat	1	0.5%	3	1.5%	4	1.0%
	96. Others Specify	0	0.0%	0	0.0%	0	0.0%
	Total	200	100.0%	200	100.0%	400	100.0%

The frequency of green leafy vegetables per day ranges from 0 to 14.
Those who consume >5 times/week were found in 80.5% in cases and 84.5% in comparative group.
Any vitamin consumption was found in 11% in cases and 9% in comparative group. Nonsmokers were Higher (57.5%) among the cases as compared to the comparative groups (74.5%).
Alcohol consumption was found in 34.5% in cases and 28.5% in control. (Table 3)

Table 3. Consumption of green leafy vegetables, vitamin consumption, smoking and alcohol use

Variable	***Category***	***Case (Present AMD)***	***Comparative (Absent AMD)***	***Total***

		Count	*%*	*Count*	*%*	*Count*	*%*
Frequency of vegetable consumption/day	0	1	0.5%	0	0.0%	1	0.3%
	1	0	0.0%	2	1.0%	2	0.5%
	2	5	2.5%	6	3.0%	11	2.8%
	3	6	3.0%	6	3.0%	12	3.0%
	4	12	6.0%	4	2.0%	16	4.0%
	5	15	7.5%	13	6.5%	28	7.0%
	6	7	3.5%	9	4.5%	16	4.0%
	7	12	6.0%	9	4.5%	21	5.3%
	8	28	14.0%	28	14.0%	56	14.0%
	9	11	5.5%	16	8.0%	27	6.8%
	10	36	18.0%	43	21.5%	79	19.8%
	11	15	7.5%	27	13.5%	42	10.5%
	12	6	3.0%	6	3.0%	12	3.0%
	13	6	3.0%	6	3.0%	12	3.0%
	14	40	20.0%	25	12.5%	65	16.3%
	Total	200	100.0%	200	100.0%	400	100.0%

Variable	***Category***	***Case (Present AMD)***		***Comparative (Absent AMD)***		***Total***	
		Count	***%***	***Count***	***%***	***Count***	***%***
Frequency of vegetable consumption/day	Upto 2	6	3.0%	8	4.0%	14	3.5%
	>2 to 5	33	16.5%	23	11.5%	56	14.0%
	>5	161	80.5%	169	84.5%	330	82.5%
	Total	200	100.0%	200	100.0%	400	100.0%

Variable	**Category**	**Case (Present AMD)**		**Comparative (Absent AMD)**		**Total**	
		Count	**%**	**Count**	**%**	**Count**	**%**
History of vitamin supplementation	Yes	22	11.0%	18	9.0%	40	10.0%
	No	178	89.0%	182	91.0%	360	90.0%
	Total	200	100.0%	200	100.0%	400	100.0%
Smoking History	Non-smokers	115	57.5%	149	74.5%	264	66.0%
	Present smokers	37	18.5%	25	12.5%	62	15.5%
	Past smokers	48	24.0%	26	13.0%	74	18.5%
	Total	200	100.0%	200	100.0%	400	100.0%
Alcohol History	Yes	69	34.5%	45	22.5%	114	28.5%
	No	131	65.5%	155	77.5%	286	71.5%
	Total	200	100.0%	200	100.0%	400	100.0%

Among the AMD cases enrolled in the study, majority were wet AMD (47.5%) followed by intermediate dry (334.5%)

RE was affected in 95% of cases and LE in 93.5% of cases.
Wet AMD was found in equal number (35.8%) in both the RE and LE. Table 4.

Table 4. Pattern of AMD among the enrolled study AMD cases

Worse AMD Type (Person)	Frequency	Percent
Mild Dry AMD	29	14.5
Intermediate Dry AMD	69	34.5
Geographic Atrophy	7	3.5
Wet AMD	95	47.5
Total	200	100.0

AMD	*Category*	*Count*	*%*
AMD OD	Yes	190	95.0%
	No	10	5.0%
	Total	200	100.0%
AMD OS	Yes	187	93.5%
	No	13	6.5%
	Total	200	100.0%

AMD	*Category*	*Count*	*%*
Types of AMD OD	1. Mild Dry AMD	46	24.2%
	2. Intermediate Dry AMD	69	36.3%
	3. Geographic Atrophy	7	3.7%
	4. Wet AMD	68	35.8%
	Total	190	100.0%
Types of AMD OS	1. Mild Dry AMD	35	18.7%
	2. Intermediate Dry AMD	77	41.2%
	3. Geographic Atrophy	8	4.3%

	4. Wet AMD	67	35.8%
	Total	187	100.0%

AMD	*Category*	*Count*	*%*
Types of AMD in RE and LE	1. Mild Dry AMD	81	21.5%
	2. Intermediate Dry AMD	146	38.7%
	3. Geographic Atrophy	15	4.0%
	4. Wet AMD	135	35.8%
	Total	377	100.0%

Systemic diseases:

Diabetes was found in 32 (16%) in cases, and 37 (18.5%) among the control group. Hypertension was found in 88 (44%) in cases, and 86 (43%) among the control group. Other cardiac diseases were found in 5 (2.5%) among the cases and 8(4%) among the control group.

Overall, 129 (64.5%) of cases and 137 (68.5%) of control group have no any concurrent systemic problems.

Results and Discussion on genetic analysis of AMD

Allele frequencies:

Allele frequencies in Nepali controls (AMD grades 0 and 1A) are shown in the table below, alongside various panels from the 1000 Genomes Project for comparison. The A allele at rs800292 represents the protective I62 isoform of *CFH*, while the C allele at rs1061170 encodes the AMD risk-associated 402H isoform. The T allele at rs12144939 is a tag for the protective

CFHR3/CFHR1 deletion. An important caveat is noted here: while this variant tags the deletion sufficiently in Caucasians, it is unclear whether it tags the deletion in the Nepali population. Moreover, frequencies for this variant in the 1000 Genomes Project groups come from a different *CFHR3/CFHR1* deletion tag, rs6677604. Therefore, comparisons of *CFHR3/CFHR1* deletion frequencies are imperfect. The A allele at rs1410996 is associated with protection and is the most highly associated variant in the chromosome 1 region. Dr. Hageman's team has previously shown this to be due to the fact that this allele is present on nearly all European haplotypes containing the protective allele at rs800292 or rs12144939. Effect alleles at the other loci (*ARMS2/HTRA1*, *C2/CFB* and *C3*) are all associated with AMD risk in European individuals. *ARMS2/HTRA1* is the primary locus responsible for genetic AMD risk in East Asian individuals. Table 5

Table 5. Effect allele frequencies of Nepali controls compared to 1000 Genomes Project ethnicity panels

			Effect Allele Frequency				
Locus	Variant	Effect Allele	Nepal Controls	1K Genomes South Asian	1K Genomes European	1K Genomes East Asian	1K Genomes African
CFH-CFHR5	rs800292	A	0.372	0.343	0.26	0.407	0.791
CFH-CFHR5	rs1061170	C	0.249	0.287	0.362	0.049	0.362
CFH-CFHR5	rs1410996	A	0.556	0.559	0.425	0.425	0.561
CFH-CFHR5	rs12144939	T	0.209	0.357	0.187	0.048	0.389
C2/CFB	rs429608	A	0.232	0.21	0.148	0.08	0.193
ARMS2/HTRA1	rs10490924	T	0.390	0.343	0.195	0.404	0.246
ARMS2/HTRA1	rs11200638	A	0.390	0.335	0.194	0.411	0.257
C3	rs2230199	G	0.045	0.103	0.221	0.003	0.033

Nepali control allele frequencies in the *CFH-CFHR5* region are similar to those from the 1000 Genomes South Asian panel, with the exception of the *CFHR3/CFHR1* tagging variant rs12144939, for which the Nepali samples show a decreased prevalence. Relative to European

individuals, which is the group that has been most widely-studied at this locus, the Nepali samples show an increased prevalence of protective alleles at rs800292 and the *CFHR3/CFHR1* deletion, as well as a decreased prevalence of risk at *CFH* rs1061170.

Nepali controls show an increased minor allele frequencies (MAF) at the *ARMS2/HTRA1* locus relative to Europeans and similar frequencies to those of East Asians. The MAF at rs429608 is relatively high, while the prevalence of the minor allele at rs2230199 is quite low. Table 6.

Single-marker Associations

AMD association analyses were performed comparing controls (grades 0 and 1A) to 1) all AMD (1B-4C), 2) early AMD (1B-3), and 3) late AMD (4A-4C).

Table 6. Single-marker association analysis of controls vs all AMD

0,1A vs 1B-4C	Variant	Assoc Allele	Case,Control Ratio Counts	Case,Control Freqs	Chi square	P value
CFH-CFHR5	rs800292	G	242:90, 285:169	0.729, 0.628	8.883	0.0029
CFH-CFHR5	rs1061170	C	134:198, 113:341	0.404, 0.249	21.301	3.93E-06
CFH-CFHR5	rs1410996	G	212:118, 200:250	0.642, 0.444	29.945	4.45E-08
CFH-CFHR5	rs12144939	G	300:32, 359:95	0.904, 0.791	18.032	2.17E-05
C2/CFB	rs429608	G	278:50, 335:101	0.848, 0.768	7.406	0.0065
ARMS2/HTRA1	rs10490924	T	234:98, 177:277	0.705, 0.390	76.248	2.50E-18
ARMS2/HTRA1	rs11200638	A	232:96, 174:272	0.707, 0.390	76.242	2.51E-18
C3	rs2230199	G	26:304, 20:424	0.079, 0.045	3.856	0.0496

Table 7. Single-marker association analysis of controls vs early AMD

0,1A vs 1B-3	Variant	Assoc Allele	Case,Control Ratio Counts	Case,Control Freqs	Chi square	P value
CFH-CFHR5	rs800292	G	72:36, 285:169	0.667, 0.628	0.57	0.4502
CFH-CFHR5	rs1061170	C	35:73, 113:341	0.324, 0.249	2.542	0.1109
CFH-CFHR5	rs1410996	G	64:44, 200:250	0.593, 0.444	7.669	0.0056
CFH-CFHR5	rs12144939	G	96:12, 359:95	0.889, 0.791	5.451	0.0196
C2/CFB	rs429608	G	89:17, 335:101	0.840, 0.768	2.543	0.1108
ARMS2/HTRA1	rs10490924	T	69:39, 177:277	0.639, 0.390	21.982	2.75E-06
ARMS2/HTRA1	rs11200638	A	68:38, 174:272	0.642, 0.390	21.981	2.75E-06
C3	rs2230199	G	13:95, 20:424	0.120, 0.045	8.769	0.0031

Table 8. Single-marker association analysis of controls vs late AMD

0,1A vs 4A-4C	Variant	Assoc Allele	Case,Control Ratio Counts	Case,Control Freqs	Chi square	P value
CFH-CFHR5	rs800292	G	170:54, 285:169	0.759, 0.628	11.693	6.00E-04
CFH-CFHR5	rs1061170	C	99:125, 113:341	0.442, 0.249	26.015	3.39E-07
CFH-CFHR5	rs1410996	G	148:74, 200:250	0.667, 0.444	29.403	5.88E-08
CFH-CFHR5	rs12144939	G	204:20, 359:95	0.911, 0.791	15.326	9.04E-05
C2/CFB	rs429608	G	189:33, 335:101	0.851, 0.768	6.249	0.0124
ARMS2/HTRA1	rs10490924	T	165:59, 177:277	0.737, 0.390	72.14	2.00E-17
ARMS2/HTRA1	rs11200638	A	164:58, 174:272	0.739, 0.390	72.061	2.09E-17
C3	rs2230199	G	13:209, 20:424	0.059, 0.045	0.574	0.4487

All variants show a significant association with all AMD (p-values are uncorrected for multiple testing). All effects are consistent in direction with what is observed in European cohorts. There is a particularly high frequency of risk alleles in Nepali AMD cases at the *ARMS2/HTRA1* locus, where the allele frequency is over 70%. This frequency is slightly higher than that observed in East Asia and much higher than that observed in Caucasians. Unlike East Asians, however, there remains a significant amount of risk derived from Chr1; the risk-associated C at *CFH* rs1061170 is present in over 40% of case chromosomes. Risk-associated *C2/CFB* and *C3* variants are also significant, but similar to Europeans, the degree of that significance is much lower than that of *CFH-CFHR5* and *ARMS2/HTRA1*.

Analyses done using only early AMD are underpowered here, as most of the affected individuals have a late AMD diagnosis. Analyses using only late AMD as cases resemble the analyses using all AMD as cases, but with slightly higher frequencies of risk alleles, as would be expected.

Chromosome 1 Haplotype Analyses

Haplotype structure at the *CFH-CFHR5* locus is important in assessing AMD risk and/or protection. Nepali haplotypes were generated using Haploview and compared to those from other ethnic groups from the 1000 Genomes Project, generated using the LDhap tool (NIH). Table 9.

Table 9. Comparison of haplotypes in the *CFH-CFHR5* locus

Haplotype	rs800292	rs1061170	rs1410996	rs12144939	Nepal Control Freq	1K Genomes South Asian	1K Genomes EUR Freq	1K Genomes East Asian	1K Genomes African
GCGG	G	C	G	G	0.240	0.273	0.3539	0.0466	0.0522
ATAG	A	T	A	G	0.302	0.1861	0.2276	0.3214	0.1619
GTGG	G	T	G	G	0.183	0.1452	0.1988	0.4851	0.0325
GTAT	G	T	A	T	0.159	0.2229	0.1759	0.006	0.1172
GTAG	G	T	A	G	0.043	0.0143	0.0109	0.0556	0.0045
ATAT	A	T	A	T	0.051	0.1339	0.0109	0.0417	0.2617
ATGG	A	T	G	G	0.015	0.0102	0.0139	0.0417	0.053
ACGG	A	C	G	G	0.005	0.0123	0.008	0.002	0.2943
GCAG	G	C	A	G	0.002	0.0002	NP	NP	0.0008

Again, rs6677604, rather than rs12144939, is used as a proxy for the deletion in the 1000 Genomes sets. Given that caveat, *CFH-CFHR5* haplotype structure in the Nepali controls is similar to that generally observed in European individuals. The four most common haplotypes comprise approximately 88% of chromosomes (versus approximately 95% in EUR). The most common haplotype in the Nepali set is ATAG, which is composed the protective A at *CFH* rs800292, no risk at *CFH* rs1061170 and no *CFHR3/CFHR1* deletion. The second most common haplotype (GCGG) contains the risk-associated C at *CFH* rs1061170 and no genetic protection. The third most common haplotype (GTGG) contains no risk or protective alleles, followed by the GTAT haplotype which contains no risk allele and the *CFHR3/CFHR1* deletion.

Chromosome 1 haplotype AMD associations in Nepali individuals also largely reflect what is observed in European studies. As expected, the most common haplotype in AMD cases is the

GCGG (*CFH* risk with no protection) and this haplotype shows significant AMD risk. Common haplotypes containing the minor allele at either *CFH* rs800292 or rs12144939 (*e.g.* ATAG, GTAT) are protective against the development of AMD, similar to that observed in Europeans. The GTGG haplotype, which exists at nearly identical frequencies in European cases and controls, is skewed toward cases in the Nepali set. A possible reason for this is that the Nepali samples have a much greater percentage of their genetic AMD risk coming from *ARMS2/HTRA1*. Protective alleles from the *CFH-CFHR5* region have been shown to mitigate risk coming from *ARMS2/HTRA1*. Accordingly, AMD cases from populations showing extremely high frequencies of risk at *ARMS2/HTRA1* might be expected to have a higher frequency of non-protective alleles at *CFH-CFHR5*.

Table 10. *CFH-CFHR5* haplotype associations in controls (0,1A) vs all AMD (1B-4C)

Haplotype	rs800292	rs1061170	rs1410996	rs12144939	Freq.	Case, Control Ratio Counts	Case,Control Freqs	Chi Square	P Value
GCGG	G	C	G	G	0.306	132.3 : 199.7, 109.5 : 346.5	0.398, 0.240	22.64	1.95E-06
ATAG	A	T	A	G	0.272	75.5 : 256.5, 137.7 : 318.3	0.227, 0.302	5.422	0.0199
GTGG	G	T	G	G	0.2	74.9 : 257.1, 83.5 : 372.5	0.226, 0.183	2.155	0.1421
GTAT	G	T	A	T	0.121	23.9 : 308.1, 72.4 : 383.6	0.072, 0.159	13.468	2.00E-04
GTAG	G	T	A	G	0.039	10.8 : 321.2, 19.5 : 436.5	0.032, 0.043	0.553	0.4572
ATAT	A	T	A	T	0.039	8.1 : 323.9, 23.0 : 433.0	0.024, 0.051	3.481	0.0621
ATGG	A	T	G	G	0.016	4.9 : 327.1, 6.8 : 449.2	0.015, 0.015	0.002	0.9654
ACGG	A	C	G	G	0.005	1.6 : 330.4, 2.4 : 453.6	0.005, 0.005	0.01	0.9218
GCAG	G	C	A	G	0.001	0.1 : 331.9, 1.1 : 454.9	0.000, 0.002	0.528	0.4675

The Nepali cohort does contain one haplotype (ACGG) that has *CFH*-associated protection and risk on the same chromosome, although it is rare (0.5%). All haplotypes after the four most frequent are limited in power and larger sample sets will be required to more accurately assess their effect on AMD.

Conclusion

Nepali individuals show significant associations with key variants in the CFH-CFHR5, *ARMS2/HTRA1*, *C2/CFB*, and *C3* loci. Nepalis exhibit a particularly high frequency of risk from *ARMS2/HTRA1*. At the *CFH-CFHR5* locus, the frequency of genetic AMD risk at *CFH* rs1061170 is lower than what is observed in Europeans, but much higher than what is observed in East Asians. The frequency of protection from *CFH* I62 (rs800292) is higher than what is observed in Europeans, while the incidence of the *CFHR3/CFHR1* deletion appears comparable, assuming that the rs12144939 variant is an effective deletion tag for the *CFHR/CFHR1* deletion. Nepali haplotype structure in the *CFH-CFHR5* region appears similar to that observed in Europeans and other non-African populations. AMD haplotype associations largely mirror what is observed in well-studied European sets.

References

1. Vingerling JR, Dielemans I, Hofman A, et al. The prevalence of age related maculopathy in the Rotterdam Study. Ophthalmology. 1995; 102(2):205–210
2. Mitchell P, Smith W, Attebo K, Wang JJ. Prevalence of age-related maculopathy in Australia. The Blue Mountains Eye Study. Ophthalmology. 1995;102(10):1450–1460.
3. Klein R, Klein BE, Linton KL. Prevalence of age-related maculopathy. The Beaver Dam Eye Study. Ophthalmology. 1992;99:933–943.
4. Klein R, Cruickshanks KJ, Nash SD, et al. The prevalence of agerelated macular degeneration and associated risk factors: the Beaver Dam Offspring Study. Arch Ophthalmol. 2010;128(6):750–758.

5. Cruickshanks KJ, Hamman RF, Klein R, et al. The prevalence of age related maculopathy by geographic region and ethnicity. The Colorado Wisconsin Study of age-related maculopathy. Arch Ophthalmol. 1997;115(2):242–250.
6. Resnikoff S, Pascolini D, Etya'ale D, et al. Global data on visual impairment in the year 2002. Bull World Health Organ. 2004;82(11): 844–851.
7. Raman R, Pal SS, Ganesan S, Gella L, Vaitheeswaran K, Sharma T. The prevalence and risk factors for age-related macular degeneration in rural-urban India, Sankara Nethralaya Rural-Urban Age-related Macular Degeneration Study, Report No. 1. Eye. 2016;30(5):688–697.
8. Kawasaki R, Wang JJ, Amirul FM, et al. Is bilateral age-related macular degeneration less common in Asians than Caucasians? Ophthalmic Epidemiol. 2011;18(6):253–258.
9. Gupta SK, Murthy GV, Morrison N, et al. Prevalence of early and late age-related macular degeneration in a rural population in northern India: the INDEYE Feasibility Study. Invest Ophthalmol Vis Sci. 2007;48(3):1007–1011.
10. Sapkota YD, Pokharel GP, Nirmalan PK, Dulal S, Maharjan IM, Prakash K. Prevalence of blindness and cataract surgery in Gandaki Zone, Nepal. Br J Ophthalmol. 2006;90(4):411–416.
11. Thapa SS, Thapa R, Paudyal I, et al. Prevalence and pattern of vitreoretinal disorders in Nepal: the Bhaktapur Glaucoma Study. BMC Ophthalmol. 2013;13:9
12. Thapa R, Bajimaya S, Paudyal G, Khanal S, Tan S, Thapa SS, van Rens GHMB. Prevalence of risk factors for age related macular degeneration in Nepal: The Bhaktapur Retina Study. *Clinical Ophthalmology* 2017, 11:963-972.
13. Bressler NM. Early detection and treatment of neovascular age related macular degeneration. J Am Board Fam Pract. 2002;15(2):142–152.
14. Klein R, Klein B, Tomany SC, Cruickshanks KJ. The association of cardiovascular disease with the long-term incidence of age-related maculopathy. Ophthalmology. 2003;110(6):1273–1280.
15. Yasuda M, Kiyohara Y, Hata Y, et al. Nine-year incidence and risk factors for age-related macular degeneration in a defined Japanese population the Hisayama Study. Ophthalmology. 2009;116(11): 2135–2140.
16. Van Newkirk MR, Nanjan MB, Wang JJ, Mitchell P, Taylor HR, Mc Carty CA. The

prevalence of age-related maculopathy: the visual impairment project. Ophthalmology. 2000;107(8):1593–1600.

17. Chaine G, Hullo A, Sahel J, et al. Case-control study for the risk factors for age related macular degeneration. Br J Ophthalmol. 1998; 82:996–1002.
18. Chakravarthy U, Mc Kay GJ, de Jong PT, et al. ARMS2 increases the risk of early and late age-related macular degeneration in the European Eye Study. Ophthalmology. 2013;120(2):342–348
19. Ratnapriya R, Chew EY. Age-related macular degeneration-clinical review and genetics update. Clin Genet. 2013;84(2):160-166.
20. Klaver CC, Wolfs RC, Assink JJ, et al. Genetic risk of age-related maculopathy. Population-based familial aggregation study. Arch. Ophthalmol. 1998; 116(12):1646–1651.
21. Hammond CJ, Webster AR, Snieder H, et al. Genetic influence on early age-related maculopathy: A twin study. Ophthalmology. 2002; 109(4):730–736.
22. Rajendran A, Dhoble P, Sundaresan P, et al. Genetic risk factors for late age-related macular degeneration in India. Br J Ophthalmol 2018;102:1213–1217.
23. Tian J, Yu W, Qin X et al. Association of genetic polymorphisms and age-related macular degeneration in Chinese population. Invest Ophthalmol Vis Sci. 2012;53:4262–4269
24. Nakata I, Yamashiro K, Akagi-Kurashige Y et al. Association of Genetic Variants on 8p21 and 4q12 with Age-Related Macular Degeneration in Asian Populations. Invest Ophthalmol Vis Sci. 2012;53:6576–6581
25. Pappas, C.M., Zouache, M.A., Matthews, S., Faust, C.D., Hageman, J.L., Williams, B.L., Richards, B.T. and Hageman, G.S. 2021. Protective chromosome 1q32 haplotypes mitigate risk for age-related macular degeneration associated with the CFH-CFHR5 and ARMS2/HTRA1 loci. *Human genomics* 15(1), p. 60.
26. Bird AC, Bressler NM, Bressler SB, et al. An international classification and grading system for age-related maculopathy age-related macular degeneration. The International ARM Epidemiological Study Group. Surv Ophthalmol. 1995;39(5):367–374.

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