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The interactions between *ARMS2*, *CFH*, *VEGF-A* and environmental factors on the risk of age-related macular degeneration

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Background: Age related macular degeneration (AMD) is a multifactorial disease caused by a combination of environmental and genetic factors. The prevalence of allele and genotypeof AMD-related genes is varied throughout the world due to racial and ethnic differences. Number of previous studies have shown that the polymorphisms in the *ARMS2*, *CFH* and *VEGF-A* genes are associated with AMD. In Mongolia, there is a lack of sufficient data on AMD development in its population and thus needs more studies on the topic. Therefore, it needs more studies about AMD development in the population. For this reason, we have investigated several specified polymorphisms in *CFH*, *VEGF-A* and *ARMS2* genes to reveal a relationship with AMD and determine the prevalence of alleles and genotypes of the genes in Mongolian population.

Methods: Totally 161 AMD patients and 223 controls were enrolled in this case-control study. The polymorphisms in *CFH*, *ARMS2* and *VEGF-A* were detected by using the methods of allele-specific polymerase chain reaction (ASPCR) and PCR based restriction fragment length polymorphism (RFLP). Statistical analysis were performed by STATA 13.0, SNPAlyze 9.0 and MDR 3.0.2.

Results: According to the study result, the characteristics of hypertension, constant-wearing sunglasses and anticoagulant medications in AMD group were significantly different from those in the control group. As for the dominant model, T allele of *ARMS2* rs10490924 (cOR=4.45; 95% CI, 2.44-8.13, p<0.001, aOR=5.08; 95% CI, 2.70-9.59, p<0.001) was more frequent among patients with AMD in comparison with the control group. Also, G/G genotype of *CFH* rs800292 (cOR=11.61; 95% CI, 3.41-39.51, p<0.001, aOR=12.49; 95% CI, 3.47-44.91, p<0.001) and G/G genotype of *CFH* rs1065489 (cOR=4.19; 95% CI, 2.53-6.93, p<0.001, aOR=4.67; 95% CI, 2.71-8.05, p<0.001) were significantly higher in AMD group after Bonferroni correction. This result suggests that people who carrying the risk genotypes of these polymorphisms had an increased risk for AMD development. As for the models of three or more SNP interactions, the participants with any combinations of risk genotypes have 6 to 106-fold higher risk for AMD development. This result suggests that there is some positive-additive interaction existing between the genetic variants of *ARMS2*, *CFH* and *VEGF-A* genes for AMD development. Our study also revealed that the participants with hypertension and carrying G/G for rs1065489 in *CFH* gene or non G/G for rs10490924 in *ARMS2* gene genotypes had 9 to 14 times higher risk for AMD development (cOR=9.05; 95% CI, 4.38-18.68, p<0.001, RERI=4.546; AP=0.502, S=2.298, cOR=13.98; 95% CI, 3.19-61.1, p<0.001, RERI=5.85; AP=0.419, S=1.821) with high level of significance. Moreover, it was found that the participants who avoided wearing sunglasses and had the G/G genotype of ARMS2 rs10490924 or G/G genotype of CFH rs800292 had an extremely higher risk for AMD development (p<.001).

Conclusions: In conclusion, it was observed that the combination of SNPs in *ARMS2*, *CFH* and *VEGF-A* genes increase the risk for AMD with 6 to 106-fold. Moreover, we found that the participants with hypertension and carrying the non G/G genotype of *ARMS2* rs10490924 or the G/G genotype of *CFH* rs800292 had an extremely higher risk of AMD development.

According to the report of 2020 from World Health Organization (WHO), 2.2 billion people with various cause-related vision loss had been diagnosed throughout the World among which majority of cases was linked into the people aged over 50 [1]. Approximately 81% of people with vision impairment may be prevented from vision loss [2].

Age related macular degeneration (AMD) is an important cause of central vision loss which is more common in older people aged over 60 [3]. So far, it has been reported that approximately 50 million people has diagnosed with AMD in the world [4,5]. Clinically, AMD is classified into 2 forms,

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including dry and wet forms. The dry form of AMD is dominantly spread throughout the world and accounts for 80-85% of the all AMD cases. Although the wet form of AMD (or neovascular AMD) is less common in the patients diagnosed with AMD, however, 90% of the cases can cause severe form of vision loss [6,7].

AMD is a multifactorial neurodegenerative disease relating with a combination of environmental and genetic factors, and a contribution of age effect [8,9]. Importantly, body mass index (BMI) and smoking have been reported to be the most crucial risk factors of environment for AMD development [10-12]. Also, obesity was investigated to be associated with the disease. Moreover, alcoholic drink usage [13], some diet habits including high glycemic, and red and processed meat, fried food, refined grains and high-fat dairy [14], ultraviolet ray of the sun [15], and some medical conditions such as having hypertension [16] and diabetes [17], and variability of iris color [18] have been revealed to be linked to the development of AMD.

In recent years, many genetic studies have reported that several gene mutations and variations are associated with the pathogenesis of AMD [19,20]. In a review, Olga Sergejeva et al reported that 36 genes including complement factor H (CFH), complement factor B (CFB), human high temperature requirement serine protease A1 (HTRA1) and age-related maculopathy susceptibility 2 (ARMS2) are more likely to be associated with the development of AMD [21].

The polymorphisms in *CFH* are reported to be associated with the AMD [22]. rs1061170, rs800292 and rs1065489 polymorphisms in *CFH* increases complement activation in eye, which may increase the susceptibility of AMD onset. Also, the polymorphism rs10490924 in *ARMS2* gene has been reported to be highly associated with pathogenesis of *AMD, which is* related to an indel mutation (del443ins54) [23]. Moreover, vascular endothelial growth factor (VEGF-A) gene was also reported to be most likely to associate with neovascular AMD development and its progression [24,25]. It was known that VEGF-A protein plays an important role in regulation of angiogenesis, vascular leakage and inflammation in the progression of neovascular AMD [26]. So far, several polymorphisms in VEGF-A such as rs833061, rs1413711 [27], rs2010963 [28], rs144854329, rs2146323 [29] and rs1570360 [30] have been investigated to discover the relationship with the progression of AMD.

In the world, the prevalence of allele and genotypic forms of the genes-related to AMD development is obviously different which can be explained by the involvement of genetic factors in racial and ethnic differences. Mongolia has a number of diagnosed cases of AMD, so it needs more studies on risk factors of AMD in the population, especially genetic variation studies. For this reason, we have investigated several specified polymorphisms in *CFH*, *VEGF-A* and *ARMS2* genes to reveal a relationship with AMD and determine the prevalence of alleles and genotypes of the genes in Mongolian population.

METHODS

Ethics statement: Ethical approvals were obtained from the Medical Ethical Review Board of Mongolian National University of Medical Sciences (Approval numbers: №2018/3-11). According to the survey approval from the Ethics Committee, written consents from all adults of over 50 years old were obtained.

Study population and sampling: A case-control study was designed as hospital-based and conducted from 2018 to 2019. 161 patients with AMD were participated for the case group, who had been referred to “Bolor Melmii” Hospital of Ulaanbaatar, Mongolia. The control group consisted of healthy volunteers from Mongolia-Japan Hospital of Mongolian National University of Medical Science, Ulaanbaatar, Mongolia.

Study participants were sampled by using a non-probability sampling method. The population size for the case and control study was estimated in accordance with the population of over 50 years old in Ulaanbaatar, Mongolia. The population size in Ulaanbaatar was 107029 (Report from National Statistics Office of Mongolia, 2019). According to the population aged over 50 years, the calculated sample size in this study was 116 participants in case group and 223 participants in control group.

$$n_3 = \frac{\frac{1.96 \times 0.074 \times (1-0.074)}{0.05^2}}{(1007029 - 1 + \frac{1.96^2 \times 0.074 \times (1-0.074)}{0.05^2}} \times 1.1 \approx 116$$

Inclusion criteria: Several inclusion criteria were used to determine participation allowance for the case and control study groups. For the case group, the participants were allowed if they were matched with the following statements. 1) People aged over 50 years, 2) No cataract or early cataract was diagnosed, 3) Media in the eye should have no opacities, 4) Drusen are 63 µm and above for dry AMD 5) Visual acuity should be 0.1 and above, 6) Vision capacity should be 0.05 and above if having swelling and exudative form of AMD, 7) Participants should gave informed consent. For the control group, the participants were allowed if they were matched with the following statements. 1) People aged over 50 years,

2) No cataract or early cataract was diagnosed, 3) Media in the eye should have no opacities, 4) No AMD was diagnosed and 5) Participants should gave informed consent.

Exclusion criteria: The participants were excluded if they had a glaucoma, diabetic retinopathy, other maculopathy and retinopathy. Foreign national was not allowed to participate in the study. Also, those who did not give consent were excluded.

Questionnaires: A paper-based questionnaire written in Mongolian was adopted by the research team members. It was taken from each participants to clarify the demographics (age, gender, body mass index (BMI) and Living residence), occupational and educational status and determine the environmental risk factors for AMD development. The questions were either closed-ended or dichotomous and the explanations were given when necessary.

Genotyping: 3 ml of blood sample was collected from peripheral blood of study participants under strict infection control. Blood sample was centrifuged at a speed of 670 ×g for 20 min to isolate the white blood cells. The sample was transported in dry ice to the laboratory.

DNA was extracted by using a genomic DNA extraction kit according to the manufacturer's instruction (Cat. No. K-3032G, Bioneer, South Korea). The yield and purification of DNA for each sample were tested by Nano-drop spectrophotometer.

The polymorphisms in *CFH*, *ARMS2* and *VEGF-A* were detected by using the methods of allele-specific polymerase chain reaction (ASPCR) and PCR based restriction fragment length polymorphism (RFLP) which were performed as previously described [31-35]. The list of methods, primers, restriction enzymes and fragment length was summarized in Appendix 1. PCR reactions were performed with Accupower PreMix kit (Cat. No. K-2036, Bioneer, South Korea). The amplified DNA products and digested fragments were detected by agarose-gel electrophoresis and visualized by UV transilluminator.

Statistical analysis: Analyses were performed using STATA 13.0 (StataCorp, College Station, TX), Microsoft Excel (Microsoft Corporation, Redmond, WA) and SNPalyze software. Comparisons of qualitative variables were analyzed by Pearson's chi-square test ($\chi 2$) for 2x2, 2x3 or 2×4 contingency tables and the Fisher's exact test. For all univariate analysis, a p-value of 0.05 was considered statistically significant. A logistic regression model was used to evaluate the risk factors for AMD development. Crude (cOR) for the univariate model and adjusted odds ratios (aOR) for the multivariate model with a 95% confidence interval (CI) were calculated by logistic regression. SNPalyze 9.0 software was applied to detect and compare the frequency of haplotypes in each study groups. A basis of frequencies of allele and genotype of the polymorphisms, a suitable model was firstly chosen from 4 common models including dominance, recessive, over-dominance and co-dominance, and then the risk assessment was conducted for AMD development [36]. A calculation technique adopted by Noboyuki Horita et al was applied in this study [36]. The interaction of risk factors for AMD development was evaluated using relative excess risk due to interaction (RERI), synergy index (S) and proportion attributable of interaction (AP) variables developed by Knol MJ et al. [37] P values for multivariate model was corrected by Bonferronni correction. The statistical power was calculated by post-hoc test, to estimate the level of association.

RESULTS

161 patients with AMD and 223 healthy people were enrolled in our study groups. 95 patients with dry AMD and 66 patients with wet AMD were participated in case group. The baseline demographic characteristics for the study groups are summarized in Table 1. No significant differences were observed for age, gender, BMI, education level, occupational exposure to dust and smoking between the study groups. Interestingly, the characteristics of hypertension, constant-wearing sunglasses and anticoagulant medications in AMD group were significantly different from those in the control group.

To clarify the characteristic difference in the study groups, odd`s ratio and significance were calculated in univariate and multivariate model for above mentioned variables. For the anticoagulant users, an increased risk for AMD was found in univariate regression. According to the univariate and multivariate logistic regression analysis, significantly higher risk of AMD was observed for participants with hypertension (cOR=2.05; 95% CI, 1.31-3.19, p=0.001, aOR=2.01; 95% CI, 1.28-3.17, p=0.039). Also, it was observed that constant-wearing sunglasses were significantly lowering the risk (cOR=0.33; 95% CI, 0.19-0.59, p<0.001, aOR=0.34; 95% CI, 0.19-0.60, p<0.001) for AMD development (Table 2)

Distribution of the genetic polymorphisms among a population can be explained by estimating the Hardy-Weinberg equilibrium. According to the Hardy-Weinberg theory, the distribution of polymorphisms of *CFH*, *ARMS2* and *VEGF-A* genes was calculated and some explainable differences were found in our study groups. The prevalence data of alleles and genotypes of rs10490924 in *ARMS2* gene, rs1061170, rs1065489 and rs800292 in *CFH* gene, and rs833061 and rs2146323 in *VEGF-A* gene showed significant differences in AMD group as compared to control group.

By univariate and multivariate regression, it was found that G allele of *CFH*rs1065489 (cOR=4.19; 95% CI, 2.53-6.93, p<0.001, aOR=4.67; 95% CI, 2.71-8.05, p<0.001) and G allele of *CFH*rs800292 (cOR=11.61; 95% CI, 3.41-39.51, p<0.001, aOR=12.49; 95% CI, 3.47-44.91, p<0.001) were associated with AMD development according to the recessive model after Bonferroni correction. As for the dominant model, T allele of *ARMS2*rs10490924 (cOR=4.45; 95% CI, 2.44-8.13, p<0.001, aOR=5.08; 95% CI, 2.70-9.59, p<0.001) was associated with increased risk of AMD compared with the control

Table 1. Baseline characteristics of the groups.

Characteristics	Case (*n*=161)	Control (*n*=223)	*p* value
Age (years)	75.75±6.39	74.28±9.35	0.087[a]
Gender			
Female	96 (59.63)	131 (58.74)	0.862[b]
Male	65 (40.37)	92 (41.26)	
BMI (kg/m^2)	25.81±5.78	26.1±3.83	0.559[a]
Education			
Primary education	11 (6.83)	24 (10.76)	
Secondary education or college/senior	86 (53.42)	104 (46.64)	0.268[b]
Tertiary education	64 (39.75)	95 (42.6)	
Occupational condition/work outside			
Yes	41 (25.47)	65 (29.15)	0.426[b]
No	120 (74.53)	158 (70.85)	
Living residence			
Ger (traditional home)	81 (50.31)	124 (55.61)	0.305[b]
Apartment	80 (49.69)	99 (44.39)	
Anti-coagulant medication			
Yes	58 (36.02)	52 (23.32)	0.007[b]
No	103 (63.98)	171 (76.68)	
Wear sunglasses			
Yes	18 (11.18)	61 (27.35)	<0.001[b]
No	143 (88.82)	162 (72.65)	
Smoking			
Current smoker	34 (21.12)	46 (20.63)	
Ex-smoker	21 (13.04)	44 (19.73)	0.217[b]
Non-smoker	106 (65.84)	133 (59.64)	
Current smoker			
Cigarettes per day (number)	9.24±5.02	8.11±4.86	0.317[c]
Smoking duration (years)	37.47±18.55	30.54±10.37	0.056[c]
Pack years (number x years/20)	17.59±11.64	13.67±11.55	0.139[c]
Former smoker			
Cigarettes per day (number)	16.33±14.74	12.25±6.37	0.236[c]
Smoking duration (years)	27.3±18.85	23.55±9.95	0.399[c]
Pack years (number x years/20)	20.13±19.14	14.58±9.96	0.223[c]
Hypertension			
Yes	121 (75.16)	133 (59.64)	0.002[b]
No	40 (24.84)	90 (40.36)	

Note: The values are given as number (proportion) or mean ± standard deviation. p values were calculated by aStudent's t-test, bChi square (x2) test or cMann-Whitney U test. Abbreviations: BMI, Body mass index.

Table 2. Association between the risk factors and AMD.

Category	Case (*n*=161)	Control (*n*=223)	cOR	95%CI	*p* value	aOR	95%CI	*p* value
Wearing sunglasses								
Yes	18 (11.18)	61 (27.35)	0.33	0.19-0.59	<.001	0.34	0.19-0.60	<.001
No	143 (88.82)	162 (72.65)	1	-	-	1	-	-
Hypertension								
Yes	121 (75.16)	133 (59.64)	2.05	1.31-3.19	0.001	2.01	1.28-3.17	0.039
No	40 (24.84)	90 (40.36)	1	-	-	1	-	-
Use of anticoagulant or antiplatelet medication								
Yes	58 (36.02)	52 (23.32)	1.85	1.18-2.89	0.007	1.83	1.16-2.88	0.094
No	103 (63.98)	171 (76.68)	1	-	-	1	-	-

Note: The values were given as number (proportion). p value by two-tailed Chi square (x2) test for 2x4 contingency table. Odds ratio and confidence interval was calculated by logistic regression. Adjusted for age, gender, BMI, education level, occupational exposure of dust, smoking status and living residence; Abbreviations: cOR, Crude odds ratio; aOR, Adjusted odds ratio; CI, Confidence interval.

group after Bonferroni correction. This result suggests that people who carrying the risk genotypes above mentioned had an extremely higher risk for AMD development as compared to the participants without any of these genotypes. However, G/T genotype of *CFH*rs1065489 (cOR=0.30; 95% CI, 0.19-0.46, p<0.001, aOR=0.29; 95% CI, 0.18-0.47, p<0.001) had produced a protective effect for AMD development and it would be defined as an incomplete dominant model. Comparisons of all allele and genotype frequencies between the groups were shown in Table 3 and Table 4. We compared the frequencies of all alleles and genotypes between dry and wet AMD groups and control group but found no association.

Entropy-based gene-gene interaction was estimated in our study to reveal the functional relationship of the genes for AMD development and the gene-gene interaction network was depicted in Figure 1. The polymorphisms of rs10490924 (7.58%) in *ARMS2* gene, rs1065489 (6.82%), rs800292 (5.81%) and rs1061170 (2.13%) in *CFH* gene and rs2146323 (1.01%) in *VEGF-A* gene were found to contribute the high-independent effect (>1.0%) among all the genetic factors. High and moderate degrees of synergistic interaction were detected between *VEGF-A* gene polymorphisms such as rs833061, rs144854329, rs1413711, rs2146323 and rs1570360. In addition,

Table 3. Association between allele frequencies of SNPs and AMD.

Gene	refSNP ID	Alleles	Location	AAC	Risk allele	RAF		cOR	95%CI	p value	Power
						Case *n*=322	Control *n*=446				
ARMS2	rs10490924	T/G	Exon	Ala/Ser	T	0.714	0.473	2.78	2.05-3.77	<.001	100%
CFH	rs1061170	T/C	Exon	Tyr/His	C	0.245	0.15	1.84	1.28-2.64	0.001	90.70%
CFH	rs1065489	G/T	Exon	Glu/Asp	G	0.634	0.513	1.64	1.22-2.20	0.001	91.80%
CFH	rs800292	A/G	Exon	Ile/Val	G	0.472	0.336	1.76	1.31-2.37	<.001	96.80%
VEGF-A	rs833061	T/C	Upstream	-	C	0.295	0.274	1.11	0.81-1.53	0.514	9.40%
VEGF-A	rs1413711	C/T	Intron	-	C	0.686	0.661	1.12	0.82-1.52	0.468	10.80%
VEGF-A	rs2010963	C/G	5` UTR	-	G	0.373	0.327	1.22	0.90-1.65	0.193	26.30%
VEGF-A	rs144854329	Del/Ins	Upstream	-	Del	0.702	0.688	1.07	0.78-1.46	0.688	6.10%
VEGF-A	rs2146323	C/A	Intron	-	C	0.702	0.668	1.17	0.86-1.59	0.322	16.70%
VEGF-A	rs1570360	G/A	Intron	-	G	0.826	0.785	1.3	0.90-1.88	0.157	28.80%

Note: The values were given as frequency. p value by two-tailed Chi square (x2) test for 2x4 contingency table. Odds ratio and confidence interval was calculated by logistic regression. Abbreviations: AAC, Amino acid change; RAF, Risk allele frequency; cOR, Crude odds ratio; aOR, Adjusted odds ratio; CI, Confidence interval.

Table 4. Comparison of genotype frequencies of SNPs in genetic models between the groups.

Gene	refSNP ID	Genetic model	Risk genotypes	cOR	95%CI	P value	Power	aOR	95%CI	P value	Power
ARMS2	rs10490924 T allele	**Do***	**T/T+T/G**	**4.45**	**2.44-8.13**	**<0.001**	**100%**	**5.08**	**2.70-9.59**	**<0.001**	**99.5%**
		Re	T/T	3.01	2.02-4.77	<0.001	100%	3.41	2.14-5.43	<0.001	99.3%
		In Do	T/G	0.84	0.56-1.28	0.421	12.4%	0.86	0.56-1.33	0.496	2.2%
CFH	rs1061170 C allele	Do	C/C+T/C	1.86	1.21-2.85	0.004	80.8%	1.97	1.25-3.10	0.003	51.1%
		Re*	**C/C**	**4.02**	**1.25-12.85**	**0.019**	**71.1%**	**4.59**	**1.36-15.53**	**0.014**	**41.3%**
		In Do	T/C	1.52	0.98-2.36	0.06	46.6%	1.58	0.99-2.51	0.054	17.7%
CFH	rs1065489 G allele	Do	G/G+G/T	0.86	0.45-1.64	0.645	6.9%	1.00	0.51-1.98	0.993	1.0%
		Re*	**G/G**	**4.19**	**2.53-6.93**	**<0.001**	**100%**	**4.67**	**2.71-8.05**	**<0.001**	**99.8%**
		In Do	**G/T**	**0.30**	**0.19-0.46**	**<0.001**	**100%**	**0.29**	**0.18-0.47**	**<0.001**	**99.7%**
CFH	rs800292 G allele	Do	G/G+A/G	2.17	1.34-3.50	0.002	90.2%	2.09	1.26-3.47	0.004	65.8%
		Re*	**G/G**	**11.61**	**3.41-39.51**	**<0.001**	**99.5%**	**12.49**	**3.47-44.91**	**<0.001**	**96.7%**
		In Do	A/G	1.12	0.73-1.72	0.609	7.3%	1.06	0.67-1.67	0.807	1.1%
VEGF-A	rs833061 C allele	Do	T/C+C/C	0.97	0.65-1.46	0.901	57.1%	0.91	0.59-1.40	0.678	26.8%
		Re*	**C/C**	**2.71**	**1.06-6.96**	**0.038**	**56.9%**	**2.81**	**1.04-7.55**	**0.041**	**26.7%**
		In Do	T/C	0.79	0.53-1.20	0.279	18.9%	0.75	0.48-1.16	0.192	4.2%
VEGF-A	rs1413711 C allele	Do	C/C+C/T	1.29	0.62-2.71	0.499	9.6%	1.58	0.72-3.50	0.255	1.5%
		Re*	C/C	1.13	0.75-1.70	0.556	8.6%	1.23	0.80-1.91	0.331	1.3%
		In Do	C/T	0.96	0.64-1.44	0.839	3.9%	0.93	0.61-1.43	0.744	0.5%
VEGF-A	rs2010963 G allele	Do	G/G+C/G	1.26	0.83-1.91	0.271	19.4%	1.32	0.85-2.04	0.215	4.3%
		Re*	G/G	1.44	0.75-2.77	0.276	19.9%	1.45	0.72-2.94	0.300	4.7%
		In Do	C/G	1.09	0.73-1.64	0.677	6.2%	1.14	0.74-1.74	0.554	0.8%
VEGF-A	rs144854329 Del allele	Do*	Del/ Del+Del/ Ins	1.31	0.64-2.67	0.458	10.8%	1.45	0.69-3.07	0.329	1.8%
		Re	Del/Del	1.02	0.68-1.53	0.928	3.1%	1.06	0.69-1.63	0.774	0.3%
		In Do	Del/Ins	1.07	0.71-1.62	0.728	5.3%	1.07	0.69-1.64	0.773	0.7%
VEGF-A	rs2146323 C allele	**Do***	**C/C+C/A**	**1.94**	**1.02-3.66**	**0.042**	**54.0%**	**2.15**	**1.10-4.22**	**0.025**	**21.6%**
		Re	C/C	0.98	0.65-1.47	0.918	3.2%	1.01	0.66-1.54	0.971	0.3%
		In Do	C/A	1.39	0.92-2.13	0.117	34.8%	1.43	0.91-2.23	0.117	10.9%
VEGF-A	rs1570360 G allele	Do	G/G+G/A	1.25	0.48-3.25	0.646	6.4%	1.27	0.47-3.45	0.636	0.8%
		Re*	G/G	1.38	0.89-2.13	0.142	31.0%	1.62	1.02-2.57	0.040	8.8%
		In Do	G/A	0.74	0.47-1.16	0.190	25.6%	0.62	0.39-1.01	0.055	6.5%

Note: The values were given as number (proportion). P value by two-tailed Chi square (x2) test for 2x4 contingency table. Odds ratio and confidence interval was calculated by logistic regression. Adjusted for the variables that age, gender, BMI, education level, occupational exposure of dust, smoking status, living residence, hypertension, use of anticoagulant or antiplatelet medication and wear sunglasses. p<.005 indicated a statistically significant difference after Bonferroni correction (shown in bold). *Suggested genetic model by four-model strategy calculation. Abbreviations: Do, Dominant; Re, Recessive; In Do, Incomplete dominant; cOR, Crude odds ratio; aOR, Adjusted odds ratio; CI, Confidence interval.

the interactions between other polymorphisms were detected as redundancy.

SNP x SNP interaction analysis was performed among all polymorphisms. The best interaction models identified MDR from 10-fold cross-validation for AMD were listed in Table 5. Significant associations were found in the frequencies of combination genotypes of rs10490924 in *ARMS2* gene and rs1065489*CFH* gene between the case and control groups. It was found that the participants carrying both of non G/G

of rs10490924 and G/G of rs1065489 (cOR=4.52; 95% CI, 2.93-6.99, p<0.001) genotypes have a higher risk for AMD development as compared to the participants without any risk genotypes. According to the models for three or more SNP interactions (Table 5), the participants who carrying any combinations of risk genotypes have 6 to 106-fold higher risk for AMD development in comparison with others. This result suggests that there is some positive-additive interaction existing between the genetic variants of *ARMS-2*, *CFH* and *VEGF-A* genes for AMD development risk.

In our study, stepwise analyses were applied to focus on revealing the relationship between the genetic polymorphisms and the risk factors associated with AMD development. According to the results, we have found some significant interactions between the polymorphisms and the risk factors. It was revealed that the participants with hypertension and carrying G/G for rs1065489 in *CFH* gene or non G/G for rs10490924 in *ARMS2* gene genotypes had 9 to 14 times higher risk for AMD development (cOR=9.05; 95% CI, 4.38-18.68, p<0.001, RERI=4.546; AP=0.502,

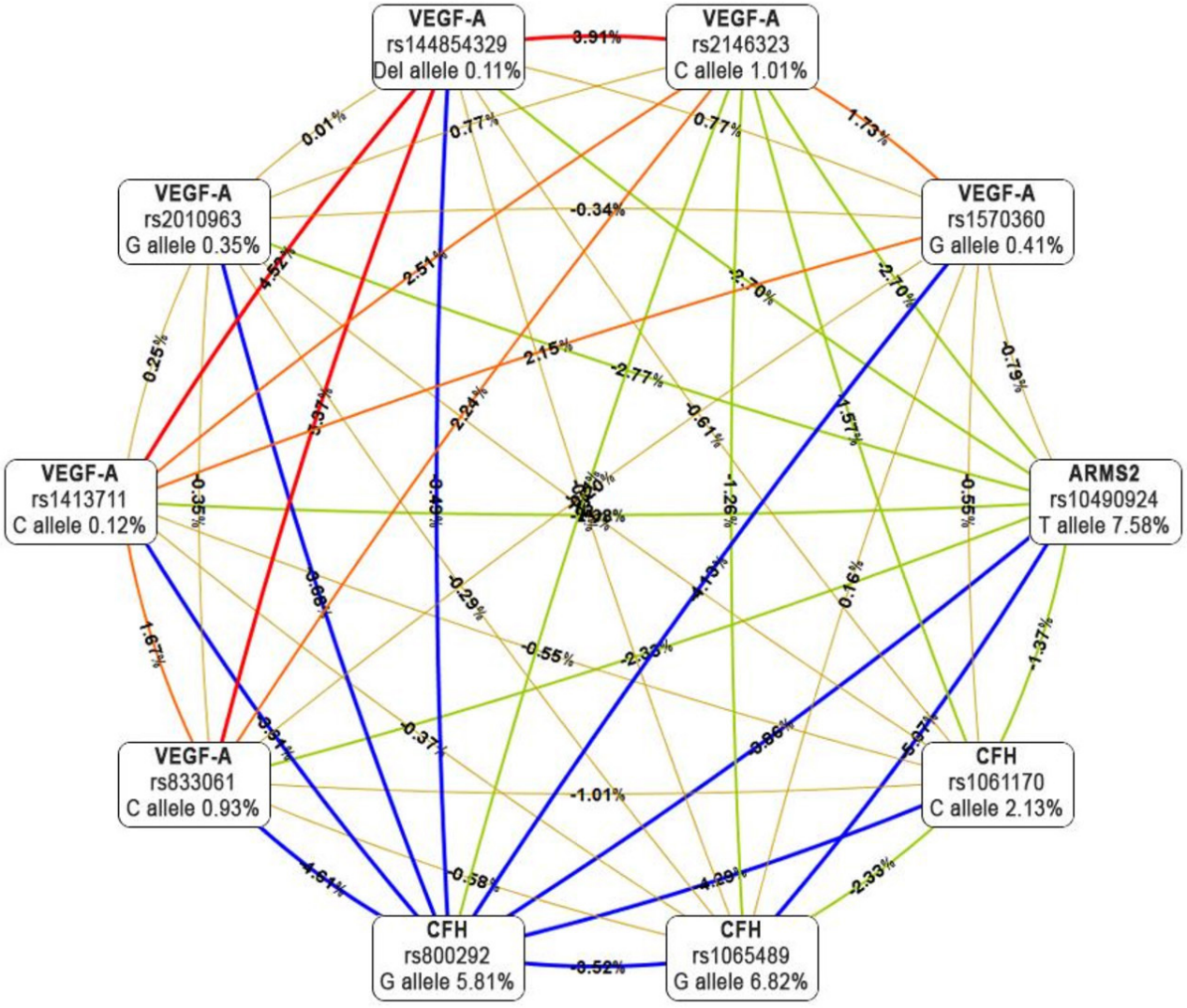

Figure 1. Entropy-based SNP-SNP interaction network of 10 polymorphisms of the genes in the case and control groups. The percent of the entropy for independent factors as well as their interactions are represented in the graph where positive percentage of entropy denotes more than additive or synergistic interaction while negative percentage denotes redundancy. The best multifactor dimensionality reduction (MDR) model for gene-gene interaction. Here, the red and orange color indicates more than additive or synergistic interaction and gold color denotes the mid-point, green color represents moderate redundancy while blue color denotes the highest.

Table 5. Best models of SNP-SNP interactions among all SNPs and AMD risk.

SNP x SNP interaction models	Bal.Acc.	Test.Acc.	CVC	cOR (95% CI)	X^2	*p* value
rs10490924	0.638	0.589	5/10	3.10 (2.02-4.77)	27.47	<.001
rs10490924 x rs1065489	0.682	0.635	7/10	4.52 (2.93-6.99)	48.69	<.001
rs10490924 x rs1065489 x rs800292	0.714	0.643	7/10	6.07 (3.88-9.49)	67.29	<.001
rs10490924 x rs1065489 x rs1413711 x rs2010963	0.753	0.621	6/10	9.08 (5.64-14.62)	92.51	<.001
rs10490924 x rs1065489 x rs800292 x rs1413711 x rs2010963	0.794	0.641	4/10	14.78 (8.79-24.84)	123.99	<.001
rs10490924 x rs1065489 x rs800292 x rs2010963 x rs144854329 x rs2146323	0.834	0.651	6/10	29.03 (15.75-53.50)	161.24	<.001
rs10490924 x rs1065489 x rs800292 x rs1413711 x rs2010963 x rs2146323 x rs1570360	0.865	0.598	6/10	68.85 (30.41-155.87)	192.19	<.001
rs10490924 x rs1061170 x rs1065489 x rs800292 x rs1413711 x rs2010963 x rs2146323 x rs1570360	0.889	0.612	6/10	68.55 (33.81-138.96)	219.45	<.001
rs10490924 x rs1061170 x rs1065489 x rs800292 x rs1413711 x rs2010963 x rs144854329 x rs2146323 x rs1570360	0.901	0.625	10/10	93.88 (43.68-201.78)	234.6	<.001
rs10490924 x rs1061170 x rs1065489 x rs800292 x rs833061 x rs1413711 x rs2010963 x rs144854329 x rs2146323 x rs1570360	0.904	0.631	10/10	106.31 (47.81-236.39)	238.23	<.001

Note: The best model speculated by MDR is composed of rs10490924, rs1061170, rs1065489, rs800292, rs833061, rs1413711, rs2010963, rs144854329, rs2146323 and rs1570360. p<.005 indicated a statistically significant difference after Bonferroni correction. Abbreviations: Bal.Acc, Balanced accuracy; Test.Acc, Testing accuracy; CVC, Cross validation consistency; cOR, Crude odds ratio; CI, Confidence interval; X2, Chi-square.

S=2.298, cOR=13.98; 95% CI, 3.19-61.1, p<0.001, RERI=5.85; AP=0.419, S=1.821) with high level of significance. Interestingly, it was also found that the participants who avoid wearing sunglasses and having with non G/G for rs10490924 in *ARMS2* gene or G/G for rs800292 in *CFH* gene genotypes had extremely higher risk for AMD development (p<0.001).

As a result, there are some haplotypes of *CFH* and *VEGF-A,* has found as risk or protective effect factors. The frequency of C-G-G (cOR=2.13; 95% CI, 1.41-3.24, p<0.001) haplotype of *CFH*, T-C-G-Del-C-G (cOR=1.74; 95% CI, 1.27-2.38, p=0.001) and C-T-C-Ins-A-G (cOR=2.03; 95% CI, 1.20-3.44, p=0.004) haplotypes of *VEGF-A*, were higher in AMD group. Contrariwise, we found that people who had T-T-A (cOR=0.39; 95% CI, 0.27-0.57, p<0.001) haplotype of *CFH*, T-T-G-Del-C-G (cOR=0.16; 95% CI, 0.04-0.69, p=0.003) and T-C-G-Del-A-G (cOR=0.27; 95% CI, 0.08-0.94, p=0.024) haplotypes of *VEGF-A*, significantly lower risk for AMD. We assessed the pairwise linkage disequilibrium for rs1061170, rs1065489, and rs800292 of *CFH* gene, rs833061, rs1413711, rs2010963, rs144854329, rs2146323 and rs1570360 of *VEGF-A* gene using the parameter of r^2. Linkage disequilibrium between rs833061 and rs1413711 (D` = 0.889, $r^2$ = 0.637), rs833061 and rs144854329 (D` = 0.931, r^2 = 0.774) observed with high D` value with the pairwise r^2 (Table 6, Table 7, Table 8 and Table 9).

DISCUSSION

AMD is now a well-recognized medical condition that can be a leading cause of hazy or no vision in the visual field center older aged population [38-40]. It was known that the development of AMD would be explained by a variety of causes including environmental factors, genetic factors, individual and health problems [8,41,42]. In recent years, the interactions of risk factors or causes for AMD development have been extensively investigated throughout the world to discover the authentic basis of triggering the disease among the population. Therefore, our study was focused on detecting the associations between the genetic, environmental and individual risk factors for development of AMD.

For this purpose, we have chosen several genetic factors such as single nucleotide polymorphisms in *ARMS2*, *CFH* and *VEGF-A* genes and individual factors such as wearing sunglasses and smoking, and medical conditions such as hypertension and using anti-coagulant medication to investigate the interactions and effects between a variety of risk factors on pathogenesis for AMD development in this study. In the current study, a total of 384 participants were allowed to participate in, in which 161 patients with AMD were in the case group and 223 healthy people were in control group.

In accordance with our study results, it has been detected that the individual and health condition-related risk factors were related to the pathogenesis for AMD development. As for the arterial hypertension, it was confirmed that it increases the risk of AMD in our study. The contribution of arterial hypertension in pathogenesis of AMD development was elucidated by damaging retinal vessels and vascular dysfunction [43,44]. Also, we have found that anticoagulant medication was a significant risk factor for AMD development. The role of anticoagulant medication in the pathogenesis of AMD would be related to a massive intraocular hemorrhage developed in the patients with exudative AMD [45,46]. There are many evidence that wearing sunglasses can increase the chance of not developing AMD due to protection from free radicals originated by sun exposure [47-49]. In our study, it was also demonstrated that constant-wearing sunglasses were significantly lowering the risk of AMD.

In the current time, there are many efforts on AMD development to figure out the pathogenesis and consider the prevention issues in further. Many studies have been conducted to find authentic causes or risk factors and then revealed some SNPs as genetic markers for AMD development. Sven Micklisch et al reported that rs10490924 in *ARMS2* gene were highly associated with AMD development, which can influence on a complement activator to become deficient [50]. Montserrat et al found that rs1061170, rs1065489 and rs800292 in *CFH* gene increased the risk of AMD [51]. In this study, the alleles and genotype prevalence of rs10490924 in *ARMS2* gene, rs1061170, rs1065489 and rs800292 in *CFH* gene, and rs833061 and rs2146323 in *VEGF-A* gene have been detected. There were some significant differences observed in AMD group as compared with the control group. Hence, it indicated that genetic risk factors might be an important role on AMD development.

In early studies, it was observed that the prevalence of AMD was different due to variations of ethnicity and races of the world population [52], especially it was related to the risky allele frequencies of rs10490924 in *ARMS2* gene, and rs1061170 and rs800292 in *CFH* gene [53-57]. Several studies reported that risky T allele of rs10490924 in *ARMS2* gene was more frequently spread in Asian population as compared to Caucasians. In our study, the risky T allele of rs10490924 in *ARMS2* gene was detected a highly frequented allele in the population of Mongolia (RAF=0.714, n=230) as comparison with those studies in South Korea (RAF=0.609, n=167) [53], Japan (RAF=0.609, 94) [54] and China (RAF=0.649, n=157) [56]. It indicated that the pathogenesis of AMD development in this geographic region might be more related to the risky T allele of rs10490924 in *ARMS2* gene. So far, the role of

Table 6. Possible additive interaction of the risk factors with ARMS-2, CFH and VEGF-A genes polymorphisms in AMD.

Risk factors			Case (n=161)	Control (n=223)	cOR	95%CI	P value	RERI	AP	S
Risk factor		Genotypes								
Wear sunglasses	No	**rs10490924 Non G/G**	**130**	**111**	**11.13**	**2.54-48.82**	**0.0014**	**6.086**	**0.547**	**2.506**
		rs1061170 Non T/T	**59**	**45**	**6.26**	**2.77-14.17**	**<0.001**	**1.445**	**0.231**	**1.378**
		rs1065489 G/G	53	25	13.43	5.75-31.37	<0.001	-3.984	-0.297	0.757
		rs800292 G/G	**20**	**2**	**37.5**	**7.92-177.51**	**<0.001**	**28.117**	**0.750**	**4.354**
		rs833061 C/C	**12**	**6**	**7.06**	**2.31-21.59**	**0.0006**	**1.566**	**0.222**	**1.348**
		rs2146323 Non A/A	129	133	7.76	0.96-62.92	0.055	2.331	0.300	1.526
Hypertension	Yes	**rs10490924 Non G/G**	**108**	**85**	**13.98**	**3.19-61.10**	**0.0005**	**5.850**	**0.419**	**1.821**
		rs1061170 Non T/T	**53**	**42**	**3.48**	**1.89-6.42**	**0.001**	**0.449**	**0.129**	**1.221**
		rs1065489 G/G	**47**	**16**	**9.05**	**4.38-18.68**	**<0.001**	**4.546**	**0.502**	**2.298**
		rs800292 G/G	17	3	14.57	4.02-52.83	<0.001	-14.528	-0.997	0.483
		rs833061 C/C	**10**	**4**	**5.88**	**1.73-19.95**	**0.0045**	**2.504**	**0.426**	**2.054**
		rs2146323 Non A/A	111	110	2.83	0.98-8.11	0.054	1.319	0.467	3.601
Anti-coagulant	Yes	rs10490924 Non G/G	51	42	9.11	3.92-21.16	<0.001	-1.562	-0.171	0.838
		rs1061170 Non T/T	**24**	**14**	**3.54**	**1.71-7.35**	**0.0007**	**0.838**	**0.236**	**1.491**
		rs1065489 G/G	**19**	**7**	**6.74**	**2.69-16.86**	**<0.001**	**0.734**	**0.109**	**1.147**
		rs800292 G/G	**9**	**0**	**35.38**	**2.04-614.83**	**0.014**	**26.528**	**0.750**	**4.380**
		rs833061 C/C	5	2	4.37	0.83-22.95	0.082	0.720	0.165	1.272
		rs2146323 Non A/A	**53**	**43**	**3.45**	**1.51-7.89**	**0.0033**	**1.075**	**0.311**	**1.781**

Note: The values were given as number. P value by two-tailed Chi square (x2) test for 2x4 contingency table. Odds ratio and confidence interval was calculated by logistic regression. p<.005 indicated a statistically significant difference after Bonferroni correction (shown in bold). Abbreviations: cOR, Crude odds ratio; CI, Confidence interval; RERI, Relative excess risk due to interaction; S, Synergy index; AP, Proportion attributable.

ARMS2 protein in the pathogenesis for AMD development is elucidated by weakening the function of mitochondria-rich retinal photoreceptor cell due to reduced level of the protein [58,59]. Also, Shughoury et al, reported that rs10490924 T allele predicted to result serine amino acid at 69th, creates a new phosphorylation site and breaks an alpha-helix. This structure and functional alteration may play a role in oxidative stress and damage to the retina [60]. However, the function of the protein for AMD development is still unclear. As for the risky C allele of rs1061170 and risky G allele of rs800292 in *CFH* gene, it was found more frequently in European population (35%) while it was comparably lower in Asian

Table 7. Estimations of *CFH* gene haplotype frequency and risk for AMD.

Haplotype	Case (n=322)	Control (n=446)	cOR (95%CI)	P value[a]	Power
T-G-A	115 (0.354)	150 (0.34)	1.09 (0.81-1.48)	0.583	6.0%
T-T-A	43 (0.134)	126 (0.281)	0.39 (0.27-0.57)	<0.001	99.9%
T-T-G	66 (0.206)	87 (0.195)	1.06 (0.74-1.52)	0.768	5.7%
C-G-G	61 (0.191)	44 (0.098)	2.13 (1.41-3.24)	<0.001	95.3%
T-G-G	19 (0.06)	15 (0.034)	1.80 (0.90-3.60)	0.116	40.8%
C-G-A	9 (0.029)	19 (0.042)	0.65 (0.29-1.45)	0.322	14.9%
C-T-G	5 (0.015)	5 (0.01)	1.39 (0.39-4.85)	0.746	9.8%
C-T-A	4 (0.011)	0 (0.00)	12.62 (0.68-235.18)	0.026	58.8%

Note: The values were given as number (frequency). Haplotypes presented in the order rs1061170, rs1065489 and rs800292. ap values based on 1000 permutations calculated by SNPAlyze 9.0. Abbreviations: cOR, Crude odd`s ratio; CI, Confidence interval.

population (5%) [51,61]. Tortajada A. et al, reported rs800292 G (Val62) allele may contribute to lack binding CFH to C3b and thereby leading to increase complement activation. CFH dysfunction affected by the risk alleles may lead to the development of localized chronic inflammation in the retina [62]. In the current study, the frequencies of the risky C allele of rs1061170 and risky G allele of rs800292 in *CFH* gene were also relatively lower in Mongolian population. It suggested that these alleles are rare but strong risk factors for AMD development in Mongolian population.

In the current time, genome-wide association studies (GWAS) revealed that more than 52 independent SNPs at 34 genetic loci have strong associations with AMD development [63]. Several studies reported that there are profoundly correlative effects between AMD-related genes [64-66]. Moreover, some studies had proved that no direct association between risk alleles of *CFH* and *ARMS2* was observed for the pathogenesis of AMD [23]. In a study, it was also reported that total genetic risk factors wasn't common for AMD development in individuals with aged 90 and over [67]. In our study, it was found that the participants who carrying any combinations of SNPs in *ARMS2*, *CFH* and *VEGF-A* genes have 6 to 106-fold higher risk for AMD development in comparison with the control groups. Therefore, our study suggests that direct or non-direct associations between AMD-related genes would be found and may enlighten the pathogenesis for AMD development.

So far, many studies have reported that the risk genes and other risk factors are combined to trigger the pathogenesis for AMD development. Tina Schick at el conducted an interaction analysis on age and polymorphisms for AMD and revealed strong age-related effects for rs570618 and rs3750846 [67]. Recently, it was also reported that a combination of risk alleles from *ARMS2* A69S and hypertension increases the risk of nAMD [68]. We have also found some significant interactions between the polymorphisms and the risk factors for AMD development. Our study revealed that the participants with hypertension and carrying G/G for rs1065489 in *CFH* gene or non-G/G for rs10490924 in *ARMS2* gene genotypes had 9 to 14 times higher risk for AMD development with

Table 8. Estimations of VEGF-A gene haplotype frequency and risk for AMD.

Haplotype	Case (n=322)	Control (n=446)	cOR (95%CI)	P value[a]	Power
T-C-G-Del-C-G	115 (0.357)	108 (0.242)	1.74 (1.27-2.38)	0.001	93.2%
C-T-C-Ins-A-G	36 (0.112)	26 (0.058)	2.03 (1.20-3.44)	0.004	76.5%
T-T-G-Del-C-G	2 (0.006)	17 (0.038)	0.16 (0.04-0.69)	0.003	83.6%
T-C-G-Del-A-G	3 (0.009)	15 (0.034)	0.27 (0.08-0.94)	0.024	62.8%
T-C-C-Ins-A-A	0 (0.00)	8 (0.018)	0.08 (0.00-1.39)	0.022	70.7%
T-C-C-Ins-C-G	0 (0.00)	8 (0.018)	0.08 (0.00-1.39)	0.026	70.7%
C-T-C-Del-A-A	0 (0.00)	6 (0.013)	0.11 (0.01-1.87)	0.042	54.4%
C-C-C-Ins-A-A	5 (0.016)	0 (0.00)	15.47 (0.85-280.77)	0.014	73.1%

Note: The values were given as number (frequency). Haplotypes presented in the order rs833061, rs1413711, rs2010963, rs144854329, rs2146323 and rs1570360. ap values based on 1000-fold permutations calculated by SNPAlyze 9.0. Abbreviations: cOR, Crude odd`s ratio; CI, Confidence interval.

Table 9. Pairwise linkage disequilibrium and statistical significance for the *CFH* and *VEGF-A* SNPs.

SNP1	SNP2	Allele counts	D'-value	95% CI	r-square	p-value
rs1061170	rs1065489	768	0.884	0.804-0.964	0.142	7.63E-26
rs1061170	rs800292	768	0.699	0.600-0.798	0.177	4.37E-31
rs1065489	rs800292	768	0.256	0.177-0.334	0.055	1.15E-10
rs833061	rs1413711	768	0.889	0.838-0.939	0.637	9.30E-109
rs833061	rs2010963	768	1.00	0.999-1.00	0.209	9.89E-37
rs833061	rs144854329	768	0.931	0.891-0.971	0.774	4.72E-132
rs833061	rs2146323	768	0.781	0.715-0.847	0.516	8.59E-89
rs833061	rs1570360	768	0.803	0.728-0.878	0.404	3.40E-70
rs1413711	rs2010963	768	0.801	0.715-0.887	0.166	7.27E-30
rs1413711	rs144854329	768	0.767	0.701-0.832	0.530	2.16E-90
rs1413711	rs2146323	768	0.633	0.561-0.706	0.382	5.62E-66
rs1413711	rs1570360	768	0.741	0.655-0.827	0.277	1.39E-47
rs2010963	rs144854329	768	0.979	0.939-1.00	0.224	5.17E-39
rs2010963	rs2146323	768	0.736	0.638-0.835	0.134	2.38E-24
rs2010963	rs1570360	768	0.967	0.903-1.00	0.122	5.41E-22
rs144854329	rs2146323	768	0.705	0.636-0.775	0.471	5.26E-81
rs144854329	rs1570360	768	0.838	0.768-0.908	0.393	9.66E-68
rs2146323	rs1570360	768	0.744	0.66-0.829	0.293	2.39E-50

Note: D` value, r square and p values were calculated by SNPAlyze 9.0. Abbreviations: 95% CI, 95% confidence interval;

high level of significance. Interestingly, it was also found that the participants who avoid from wearing sunglasses and having with non G/G for rs10490924 in *ARMS2* gene or G/G for rs800292 in *CFH* gene genotypes had extremely higher risk for AMD development. Hence, these results suggested that both of genetic and environmental risk factors play a significant role in the pathogenesis for AMD development.

In this study, we have proposed some strong evidence on understanding and augmenting the known pathogenesis in next step for AMD development. A risky T allele of rs10490924 in *ARMS2* gene is the most frequent allele for AMD diagnosed patients in this geographic region. Also, we found rs800292 G and rs1061170 C alleles have associated with increased risk of AMD. In addition, our study has found that there are direct and non-direct interactions between AMD-related genes, and both of genetic and environmental risk factors are related to the pathogenesis for AMD development. In further studies, it needs more investigations to reveal the authentic mechanisms for AMD development.

Conclusions: In conclusion, our findings indicated that risky T allele of rs10490924 in *ARMS2* gene for AMD development was detected a highly frequented allele in the population of Mongolia (RAF=0.714, n=230). Also, it was observed that the combination of SNPs in *ARMS2*, *CFH* and *VEGF-A* genes increase the risk for AMD with 6 to 106-fold. Moreover, it was found that the participants who have hypertension or avoid wearing sunglasses and having with non G/G for rs10490924 in *ARMS2* gene or G/G for rs800292 in *CFH* gene genotypes had extremely higher risk for AMD development.

APPENDIX 1. SUPPLEMENTARY TABLE 1.

To access the data, click or select the words "Appendix 1." The primers, restriction enzymes and length of fragments. Abbreviation: RFLP, Restriction fragment length polymorphism; AS-PCR, Allele-specific polymerase chain reaction; bp, base pair.

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